www.sphinxsai.com

ChemTech

International Journal of ChemTech Research
CODEN (USA): IJCRGG ISSN : 0974-4290
Vol.6, No.8, pp 4015-4021, September 2014

ICSET-2014 [6th – 7th May 2014]
International Conference on Science, Engineering and Technology

Analysis of exon – 1 mutation in NR5A1 gene of Ambiguous Genitalia patients using SSCP analysis

Chelna Galada, Namrata Pai, Rajiv S, Nishu S and Abilash V.G.*

Division of Biomolecules and Genetics, School of Bio Sciences and Technology, VIT University, Vellore, India.

***Corres.author: abilash.vg@vit.ac.in, Phone No: +91 416 22023276, +91 9840364805**

Abstract: Ambiguous genitalia, a disorder of sex development (DSD), are a group of conditions where normal development of the reproductive organs and genitals are not seen. The term DSD was given to congenital conditions that are atypical showing differences in development of chromosomal, gonadal,or anatomical sex. Ambiguous genitalia are a mixture of male and female sexual characters found in a person. In the present studywe have considered the gene, Nuclear receptor subfamily 5, group A, member 1 (NR5A1). This gene is responsible for the production of transcription factor, steroidogenic factor-1 (SF-1), which plays an important role in the development of gonads and the adrenal glands. A mutation on this gene is associated with ambiguous genitalia. The objective of this study is to find the mutational analyses in ambiguous genitalia patients. The DNA was extracted from blood samples of 12 patients and analysed using bio-photometer. PCR technique was used to amplify the NR5A1 region and confirmed using SSCP. Further confirmations will be done by sequencing analysis.
Keywords: Disorders of sexual development, NR5A1 gene, SF1 factor, ambiguous genitalia, SSCP.

Introduction and Experimental

Ambiguous genitalia, a disorder of sex development (DSDs), are a group of conditions where normal development of the reproductive organs and genitals is not seen. A mixture of male and female sexual characters is found in such patients. The term DSD was given to congenital conditions that are atypical showing differences in development of chromosomal, gonadal, or anatomical sex[1,2]. The disruption of the development of proper female or male tissue in fetus leads to ambiguous genitalia. Nuclear receptor subfamily 5, group A, member 1 (NR5A1) gene also known as steroidogenic factor-1 (SF-1) belongs to the nuclear receptor superfamily and is involved in the functions associated with the steroidogenic tissues that are adrenal function and gonadal development, sex determination and differentiation. The NR5A1 gene locus is found on chromosome 9q33[3,4]. It is a transcription factor that coordinately regulates the expression of steroidogenic P-450 enzymes[5]. The expression pattern of NR5A1 (SF-1) is constrained to the adrenal cortex and gonads, spleen, pituitary gonadotropes and ventro-medial hypothalamic (VMH) nucleus in the brain[12]. NR5A1 (SF-1) has a known role as a global regulator of steroidogenesis in the adrenal cortex and gonads.The essential role of NR5A1 (SF-1) as a master steroidogenic gene was described by its forced expression in embryonic and mesenchymal stem cells experiments which gave the results that it was sufficient to activate steroidogenic genes and to initiate steroid expression[6]. The regulation of SF-1gene activity is generally regulated by Adrenocorticotropin (ACTH) and gonadotropin(LH and FSH), and

intracellular cAMP/PKA signal pathway which is a major signaling pathway that transmits the signal from extracellular stimuli to the nucleus[4]. Phosphorylation and acetylation increases SF-1 activity whereas sumoylation suppresses SF-1 activity[4]. Dax-1, a negative regulator only specific to SF-1 suppresses its activity. Mutations associated with SF-1 gene are generally missense mutations, nonsense mutations caused by nucleotide deletions and duplications, one nucleotide polymorphism and a 3 Mb deletion spanning SF-1.Studies on NR5A1(SF-1) gene and ambiguous genitalia have reported that a heterozygous frame shift mutation results in ambiguous genitalia. Several case studies have supported this fact. Adrenal function however was normal, only gonadal development was seen to be affected in these cases. These mutations seem to have been raised from de novo events. Heterozygote mutations of NR5A1 are associated with disorders of sex development, premature ovarian failure or male infertility[3]. Adrenal insufficiency has been confirmed only in few cases due to mutation on NR5A1[6]. The two key genes SOX9 (SRY box-9), and anti-mullerian Hormone (AMH) which are involved in early development of sex determination and differentiation is positively regulated byNR5A1 gene[1,7,8]. NR5A1 also amplify the expression of many factorsinvolved in cholesterol mobilization and steroid hormone biosynthesis including HMG-CoA synthase, steroidogenic acute regulatory protein (StAR)[1,9]. The NR5A1 (SF-1) gene plays a role in infertility along with other genes; this gene is studied in mice model rather than *Homo sapiens*, because XY knockout mice do not contain gonads and adrenal glands. Any mutation (i.e; mis-sense) in DNA binding domain of Sf-1 can result an XY mice to exhibit streak gonads and completely developed mullerian structures[5]. Leydig cell possibly helps in regulating the production of steroid hormones in knockout mice[6]. As the earlier studies of these patients had notshown any variations in the SRY and AR gene, the present study looks into the NR5A1gene which is associated with ambiguous genitalia to check for any mutational variations using PCR and SSCP and further confirm with sequence analysis.

Molecular Genetics of Sf-1/NR5A1 gene

SF-1 gene is located on chromosome 9 between 127,243,514 base pair to 127,269,768 base pair. It is an autosomal gene found at the 9q33 region. It is a 33182 bp long gene having 7 exons.

Mechanism of Action of NR5A1 gene

NR5A1gene is a member of nuclear receptor family that regulates expression of steroid hydroxylases[5]. This gene helps in regulating the transcription of number of genes which are involved in sexual differentiation, steroidgenesis and reproduction. It gives instruction for production of transcription factor, Steridogenic factor-1 (SF1). SF-1 is a protein which binds to a particular region of DNA to control the activities of specific genes that are related to gonad development and adrenal glands (located in the upper portion of each kidney).

Role of NR5A1 in Disease

Incase of mutation it may lead to a syndrome along with gonadal dysgenesis. The mutation in Sf-1 gene leads to Swyer syndrome. In this syndrome, complete or pure gonadal dysgenesis patients are found. The formation of male sexual differentiation gets affected in such cases, which leads to the development of female appearance despite having the chromosome pattern typical of males. Other disorder which may be due to a mutation in this gene is partial gonadal dysgenesis. The individuals might have external genitalia which cannot evaluate the difference in an individual's genitalia or abnormality in secondary reproductive organs and adrenal glands. Adrenal gland abnormality may rise due to deficiency of hormones which further might result in various health problems. The phenotype in genetic females would be expected to consist of adrenal failure, delayed puberty with absence of breast development, and primary amenorrhoea, with raised gonadotrophins[6].

Materials and Methods

Patients and Controls

In this research we collected 12 ambiguous genitalia patients and 5 control samples along with their family history of three generations with the help of qualified Gynecologist from Institute of Obstetrics and Gynaecology (IOG), Government Hospital for Women and Children, Egmore, Chennai, Tamil Nadu, India. The data was taken on the base criteria depicted in the Table1.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.

Patients and Controls

In this research we collected 12 ambiguous genitalia patients and 5 control samples along with their family history of three generations with the help of qualified Gynecologist from Institute of Obstetrics and Gynaecology (IOG), Government Hospital for Women and Children, Egmore, Chennai, Tamil Nadu, India.The data was taken on the base criteria depicted in the Table1. 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.

Genotyping of NR5A1 gene

Sample blood from 12 patients and 5controls were collected in a vacutainer with EDTA. Molecular analysis was done in all the patients to analyse any mutation in exon-1 of NR5A1 gene which may lead to ambiguous genitalia.

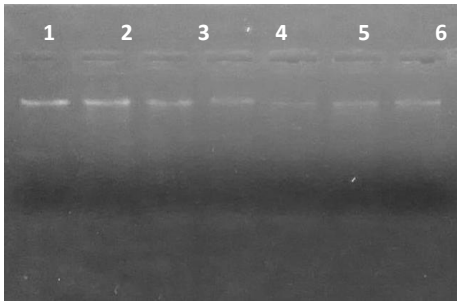
Table 1: clinical features of patients with ambiguous genitalia

S.NO	CLINICAL FEATURES	1	2	3	4	5	6	7	8	9	10	11	12
1	AGE AT REPORTING	1yr	1yr	1yr	2yrs	2yrs	3yrs	3yrs	9yrs	11yrs	30yrs	6yrs	2yrs
2	EXTERNAL GENITALIA	Male type	Female type	Male type	Male type	Male type	Male type	Male type	Female type	Male type	Male type	Male type	Male type
3	TESTES	Palpable	Descended	Palpable	Undescended	Undescended	Descended	Right undescended	Inguinal	Palpable	Descended	Inguinal	Undescended
4	HYPOSPADIAS	Present	Absent	Absent	Absent	Present	Present	Present	Absent	Present	Present	Absent	Absent
5	AXILLARY AND PUBIC HAIRS	-	-	-	-	-	-	-	-	-	Sparse	-	-
6	CLITOROMEGALY	Absent	Enlarged clitoris	Absent	Absent	Absent	Absent	Absent	Enlarged clitoris	Absent	Absent	Absent	Absent
7	WOLFFIAN DERIVATIVES	Absent	Absent	Present	Absent	Present	Present	Present	Present	Present	Present	Present	Absent
8	MULLERIAN DERIVATIVES	Present	Present	Absent	Present	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Present
9	SCROTUM	Normal	Normal	Bifid	Normal	Normal	Normal	Normal	Absent	Bifid	Normal	Absent	Rigid
10	PENIS	Rudimentary	Absent	Micropenis	Rudimentary	Rudimentary	Micropenis	Rudimentary	Absent	Micropenis	Rudimentary	Micropenis	Micropenis
11	URETHERAL OPENING	Absent	Present	Absent	Absent	Absent	Absent	Absent	Present	Present	Present	Absent	Absent
12	FALLOPIAN TUBES	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
13	KARYOTYPE	46,XY	46,XY	46,XY	46,XY	46,XY	46,XY	46,XY	46,XY	46,XY	46,XY	46,XY	46,XY
14	SRY GENE	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
15	AR GENE	Normal	Normal	Normal	Normal	Normal	normal	Normal	Normal	Normal	Normal	Normal	Normal

Extraction of DNA and Qualitative Analysis

DNA was extracted from 2ml EDTA blood and it was qualitatively analysed on 1% agarose gel using electrophoresis[10]. 10 ng/µl concentration of DNA dilutions were made by using Tris Ethylene diamine tetra acetic acid (TE) buffer, pH 8.0. The DNA was first qualitatively analysed on 1% gel and later checked for DNA amplified product by visualizing the gel under UV illumination (Fig1).

Fig1: Agarose gel electrophoresis of the qualitative analysis of DNA extraction.



PCR Amplification

The qualitatively analysed DNA samples of patiens and controls were subjected to PCR amplification using forward(5'-GCAGAGTCACGTGGGGGCAGAG-3')andreverse(5'-GAAGGAGGCTGGCCATT AG AG-3') primers of exon-1 of NR5A1 that were run on 2% agarose gel electrophoresis containing 2.5µg of ethidium bromide (EtBr). 5µl of PCR master mix was taken for each sample that contains dNTPs, Taq polymerase and dye. 0.5µl forward primer, 0.5µl reverse primer, 10µl of milliQ water and 4µl of sample DNA were added to the mix.

Table 2: PCR components and volumes per sample

PCR Components	Volume in µl
PCR master mix	5µl
Forward Primer	0.5 µl
Reverse Primer	0.5µl
Milli Q Water	10µl
DNA	4 µl
Total	20 µl/sample

PCR Conditions

The PCR was inured according to the requirements for each stage of Polymerase chain reaction. The denaturation temperature was set to 95℃ for 30 seconds, the annealing temperature was standardized at 56℃ for 40 seconds, and the elongation temperature was set to72℃ for 30 seconds.

Table 4. PCR Conditions

Reaction	Temperature	Duration
Primary Denaturation	95℃	5 minutes
Denaturation	95℃	30 seconds
Annealing	56℃	40 seconds
Extension	72℃	30 seconds
Final Extension	72℃	5 minutes
On Hold	4℃	∞

SSCP Analyses

Single Strand Conformation Polymorphism (SSCP) of the PCR product was carried out to screen the samples for confirmation of any mutations in Exon-1 of NR5A1 gene. 8µl of PCR products were used for SSCP which was performed by the modified and standardized protocol of Orita et al[11].

Results

Clinical analysis

The study was carried out with 17 patients of whom 5 were controls. The 12 patients were ambiguous genitalia.Out of these 10 had male external genitalia and 2 had female external genitalia showing a karyotype 46, XY. SRY gene was present and AR was normal in all. All three kinds of testes; descended, undescended and palpable testes were found among these patients. Wolffian and mullarian derivatives were either present or absent. Normal penis was not seen in these patients. Table 2 shows the percentages of clinical features of these 12 patients.

Molecular analysis

The study was done to analyse any mutation in Exon 1 of the NR5A1 gene of samples collected from 12 ambiguous genitalia patients from Tamil Nadu. Exon 1 of NR5A1 gene having a

product size of 286bp was amplified by PCR. This was subjected to electrophoresis on 2% agarose gel to confirm the amplification (Fig 2). SSCP was carried out to confirm for any polymorphism in exon 1 of NR5A1 gene. No polymorphism was seen in the 12 patients (Fig 3).

Discussion

Ambiguous genitalia are birth defects where the outer genitals develop partially or fail to develop completely. When the process involved in the development of the fetal tissue to become "male" or "female" is disrupted, ambiguous genitalia can develop. Easy identification of the infant as male or female becomes difficult. The extent of the ambiguity varies. In very rare instances, the physical appearance may be fully developed as the opposite of the genetic sex. For example, genetic male may have developed the appearance of a normal female. The NR5A1 gene is involved in the sexual differentiation of the fetus.

Table 2: Percentage details of the 12 patients under study with ambiguous genitalia

Sno	Clinical Features	Percentage
1	**External genitalia**	
	Female type	20
	Male type	80
2	**Hypospadias present**	50
3	**Testes**	
	Palpable	25
	Undescended	33.33
	Descended	25
	Inguinal	16.67
4	**Enlarged Clitoris**	16.67
5	**Wolffian Derivative present**	66.67
6	**Mullarian Derivative present**	33.33
7	**Scrotum**	
	Bifid	16.67
	Normal	58.33
	Absent	16.67
	Rigid	8.33
8	**Penis**	
	Rudimentary	41.67
	Micropenis	41.67
	Absent	14.67
9	**46,XY karyotype**	100
10	**SRY gene positive**	100
11	**AR gene normal**	100

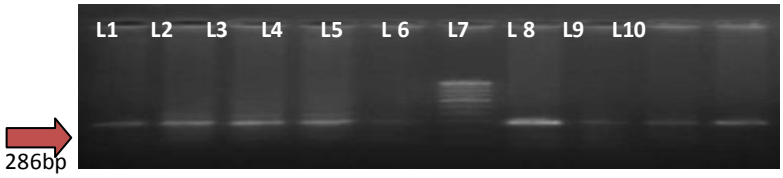


Fig 2: 2% Agarose gel of the PCR amplified product of Exon 1 of NR5A1 gene. The lane L6 is the 100bp ladder. L1 is the control sample. Lanes L2-L5 and L7-L10 are ambiguous genitalia patient samples

Fig3: Polyacrylamide gel showing the stained bands after the SSCP procedure. L1 is the control and L2 - L7 are the patient samples showing no polymorphism.

It is a transcriptional activator essential for sexual differentiation and formation of the primary steroidogenic tissues. It also regulates the AMH/Muellerian inhibiting substance gene as well as the AHCH (also known as Dax1) and STAR (steroidogenic acute regulatory protein) genes. Mutations in NR5A1 can be the primary cause of intersex genitals, absence of puberty or infertility. Missense, in-frame and frameshift mutations of NR5A1 are primarily involved in 46,XY disorders of sex development, 46,XX gonadal dysgenesis and 46,XX primary ovarian insufficiency. Individuals of either karyotype may not enter puberty, although expression of the phenotype, penetrance, fertility, and modes of inheritance can vary. Some mutations are dominant, some are recessive. Mutations on NR5A1 gene can cause 46, XY sex reversal, premature ovarian failure, spermatogenic failure.[1]

In the study the aim was to find mutations on NR5A1 gene of exon-1 that may lead to ambiguous genitalia. The DNA samples of 12 patients were isolated along with 5 control samples and run on agarose gel. The Samples were further subjected to PCR amplification and SSCP (single strand conformation Polymorphism).

Conclusion

The samples under study did not show any mutational variations when they were run on agarose gel after conducting PCR. The PCR products that were analysed using SSCP also indicated no polymorphism in the gene. Further conclusions can be made only after the analysis of all the 7 exons of NR5A1 gene and also increase the number of samples under study and later confirm by sequence analysis.

In general it may be concluded that ambiguous genitalia in a 46,XY new born is due to either abnormal formation of the early foetal testes, low production of testosterone, deficient 5-α- reductase activity, or the inability to respond to androgen causing androgen sensitivity.[13,14] This underlies a need for careful clinical investigation of patients presenting with ambiguous genitalia and abnormal level of sex hormones.[14]

Acknowledgement

The authors would like to give hearty thanks to VIT University management, for providing facilities to carry the work successfully.

References

1. Slimane Allali, Anu Bashamboo, et al., Mutation Analysis of NR5A1 Encoding Steroidogenic Factor 1 in 77 Patients with 46, XY Disorders of Sex Development (DSD) including Hypospadias, PLoS ONE, October 2011, 10.
2. Hughes I.A., Houk C., Ahmed S.F., Lee P.A., LWPES Consensus Group. ESPE Consensus Group Consensus statement on management of intersex disorders, Arch Dis Child, 2006, 91,554–563.
3. Bruno Ferraz-de-Souza, Lin Lin, John C. Achermann, Steroidogenic factor-1 (SF-1, NR5A1) and human disease, Molecular and Cellular Endocrinology, 2011, 336, 198-205.
4. Chia-Yih Wang, Wei-Yi Chen, Pao-Yen Lai, Bon-chu Chung, Distinct functions of steroidogenic factor-1 (NR5A1) in the nucleus and the centrosome, Molecular and Cellular Endocrinology, 2013, 371, 148-153.

5. Enzo Lalli, Isabelle Mus-Veteau et al., Beyond steroidogenesis: Novel target genes for SF-1 disco -vered by genomics, Molecular and Cellular Endocrinology, 2013, 371,154–159.
6. Ferraz-de-Souza B., Lin L., Achermann J.C.,et al., Steroidogenic factor-1 (SF-1, *NR5A1*) and human disease, Molecular and Cellular Endocrinology, 2011, 336,198–205.
7. Sekido R., Lovell-Badge R., et al, Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer, Nature, 2008, 453, 930–934.
8. De Santa Barbara P., Bonneaud N., Desclozeaux M., Moniot B., Sudbeck P., et al., Direct interaction of SRY-related protein SOX9 and steroidogenic factor1 regulates transcription of the human anti-Mu¨llerian hormone gene, Molecular Cell Biology, 1998, 18, 6653–6665.
9. Lin L.,Achermann J.C.,Steroidogenic factor-1(SF-1,Ad4BP, NR5A1)and disordersoftestis development, Sex Dev., 2008, 2, 200–209.
10. Gill P., Lygo J.E., Fowler S.J., llerrett D.J., An evaluation of DNA fingerprinting for forensic purposes, Electrophoresis, 1987, 8, 38-44.
11. Orita M., et al., Detection of Polymorphisms of Human DNA by Gel Electrophoresis as SSCPs, Proceedings of the National Academy of Sciences of the United States of America, 1989, 86, 2766-70.
12. Ikeda Y., Lala D.S., Luo X., Kim E., Moisan M.P., Parker K.L, Characterization of the mouse FTZ-F1 gene, which encodes a key regulator of steroid hydroxylase gene expression, Molecular Endocrinology, 1993,7,852–860.
13. Olaf Hiort, The differential role of androgens in early human sex development, HiortBMC Medicine, 2013,11,152.
14. Claude J. Migeon, Amy B. Wisniewski, John P. Gearhart, Heino F.L. Meyer-Bahlburg, John A. Rock, Terry R. Brown, Samuel J. Casella, Alexander Maret, Ka Ming Ngai, John Money and Gary D. Berkovitz, Ambiguous genitalia with perineoscrotal hypospadias in 46,XY individuals. Long-Term Medical, Surgical, and Psychosexual Outcome. Pedriatics, September 2002, 3, 110.

*****