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Mutation Profile of SOX9, DAX1, and SRY Genes in Senegalese Patients with Disorders of Sex Development

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Abstract: The genes *SRY, SOX9* and *DAX1*, key genes in human sex determination, due to their associated sex-reversal phenotypes (Figure 1) upon mutation (*SRY, SOX9*) or overexpression (*SOX9, DAX1*). The objective of this study was to characterize mutations occurring in *SRY*, *DAX1* and *SOX9* genes known to be implicated in the sexual determinism to better understand disorders of sex development (DSD) in our population. 87 DSD patients were identified and for which we carried out a clinical review and karyotype. Moreover, molecular analysis of the *SRY*, *SOX9,* and *DAX1* genes (PCR amplification, Sanger sequencing and expression by qPCR) have been done. Based on the results, it was found in patients 46, XX an absence of female internal genitals or the presence of testicles depending on the patients while for patients 46, XY no testicles were found. Among the 87 patients, the genomic DNA of 29 of them was extracted and used for molecular analysis, which identified 4 patients 46, XX SRY positive and 2 patients 46, XY SRY negative. In addition, the sequencing of the *SRY*, *SOX9* and *DAX1* genes revealed different mutations, including two new for *SRY* (c.265 G>A, p.89Glu>Lys in 2 patients 46, XY and c.171 G>C, p.57Gln>His in a patient 46, XX) and one variant (c.349 G>C, p.117Gln>Glu) in coding sequence for *SOX9* in a 46, XY SRY positive patient while for *DAX1*, any new mutation was found. These results highlight the differences in the expression of the *SRY*, *SOX9* and *DAX1* genes in the determination of sex in humans and increase the spectrum of mutations of genes in cascade. The complexity of gene interactions that lead to the development of the bipotential gonad to a testicle or ovary is increasingly recognized, but there is still much to be done.

Keywords: DSD, AHC, *SRY*, *DAX1*, *SOX9*

1. Introduction

Sex determinism is the set of mechanisms leading an undifferentiated fetal gonad to engage a male or female developmental pathway leading to testicles or ovaries [1]. Moreover, for several years, cytogenetic studies of patients with sex reversal (and also studies conducted in mice) have made it possible to identify different genes involved in sexual determinism [2–7]. One of the most important stages of differentiation is initiated by the SRY (Sex Determining Region Y, Yp11.31) gene which is expressed in undifferentiated gonadal cells between the 5th and 7th week after conception, and leads to the formation of testes in humans [2, 8, 9]. In absence of *SRY*, the gonad spontaneously evolves into the ovary. This gene allows a cascade of reactions with the intervention of several architect genes including *SF1, WT1,*

DAX1, SOX9, and *WNT4* involved in gonadal differentiation [10-12]. The DAX1 gene (Dosage sensitive sex reversal (DSS), Adrenal hypoplasia congenital (AHC) critical region on the X chromosome, gene 1) coding for NR0B1 (Nuclear Receptor Subfamily 0 group B member 1) triggers the formation of ovaries when activated on both X chromosomes [10, 13]. These two main genes (*SRY* and *DAX1*) compete for the control of a third gene *SOX9* (*SRY-box transcription factor 9*), located in chromosome 17 (Wright, Snopek, et Koopman 1993; Vincent R. Harley 2002; Vidal, Chaboissier, et Schedl, 2002). These three genes *SRY, SOX9* and *DAX1* are key genes in human sex determination, due to their associated male-to-female sex reversal phenotypes upon mutation (*SRY, SOX9*) or overexpression (*SOX9, DAX1*) [11, 17-19]. Therefore an imbalance in these processes during early embryonic development, due to genetic or environmental factors, induces a wide range of syndromes (i.e. Disorder of Sex Development, DSDs), ranging from complete gonadal dysgenesis (sexual reversion), gonadoblastoma to the less severe phenotype of cryptorchidism (undescended testis) [4, 20, 21]. Although it has been reported that expression of less or more copy numbers of these genes can induce a sex reversal [19, 22-24]. In this study we investigated the mutations profile of *SRY, SOX9,* and *DAX1* genes in different phenotypes of DSD, in 87 Senegalese patients diagnosed in our laboratory).

2. Methodology

2.1. Study Population

After informed consent from 87 patients received at the Clinical Cytology - Cytogenetic - Reproductive Biology Laboratory of the Aristide Le Dantec Hospital (HALD), we collected clinical and paraclinical data (echography, hormonal tests). Then patients with normal and ambiguous karyotypes relative to the clinic were included (29 patients) in this study (Table 1). These patients had different phenotypes ranging from ambiguous female or male to different degrees of DSD (and vice versa). We have also include male (1) and female (2) controls who are married and have had children. Patients with syndromic karyotypes (45X0, 47XXY, 46XX/46XY…) were excluded.

Table 1. *Clinical and paraclinical table of patients with a mutation.*

Patients	declared sex	Age consultation	karyotype	XES	SRY2	SRY1	Clinique	Echography
TH	M		46, XY	+	+	+		
TF	F		46, XX	-	-	-		
P14	M	7mois	46, XY	+	+	+	No palpated testicles in the bursa / normal penis size and appearance	
P21	F	2ans	46, XX	-	-	-	Presence of labia majora surrounding a kind of penoclitoris with a naked glans, there is no vagina, presence of a hypospadias orifice through which the patient urinates, no testicles palpated	OGI female type, empty adrenal box
P53	M	1an6mois	46, XY	+	+	+	Scrotum not developed/ genital orifice absent, micropenis and urinary orifice, hypospadias, no palpated testicles	
P54	M	5ans	46, XY	-	-	-	Micropenis and scrotum not developed/ no testicles palpated	
69	F	16ans	46, XY	+	+	+	Male morphotype, hoarse voice/ no mammary development (Tanner stage I). Pubic hair+++/ micropenis with naked glans/ 1 mass palpated on the right side of the pubis suggesting a testicle, presence of an orifice resembling a vagina through which urine the patient. Absence of menarches.	
71	M	17mois	46XY	+	+	+	Genital tuber having the appearance of penis with naked glans hypospadias, no palpated testicles, incomplete development of the bursa	
73	F	67jours	46, XX	+	+	+	Genital tubercle penis clitoris. genital lip-like bumps, presence of a small orifice (urinate)	No pelvic testicles or inguinal canals. No uterus found. No abnormalities in the abdomen.
75	F	20ans	46, XX	+	+	+	pilosity+++/clitoral hypertrophy, palpated 2 masses/presence of 2 orifices	Absence of female internal genitalia, testis presence
78	M	3ans	46, XY	-	-	-	well-developed penis and hypospadias, well pigmented and	absence de visualisation des testicules dans le trajet

Patients	declared sex	Age consultation	karyotype	XES	SRY2	SRY1	Clinique	Echography
							pleated scrotum, no testicle, early development pubic pilosity.	inguino-scrotal et en intra abdominal
84	F	17ans	46XX	+	+	+	Stage S4 of Tanner absent axillary pilosity, pubic pilosity stage P2 of Tanner. External genitalia (OGE) female type with palpated 2 masses in symmetrical bilateral pubis, vaginal slit with small clitoris	ultrasound appearance in favor of a feminizing testis syndrome with two ectopic testicles in inguinal position
91	M	36ans	46, XY	+	+	+	morphologically normal testicles, cryptozoospermia on spermogram	
95	F	19ans	46 XX	+	+	+	soft abdomen without palpated mass, female type OGE, normal clitoris, present vaginal orifice, pubic pilosity + (P2 of tanner)	
103	M	6mois	46XY	+	+	+	Posterior hypospadias, vulviform aspect of the scrotum, micropenis/ clitoris, testis in the bursa	
107	M	17mois	46XY	+	+	+	normal looking penis, one testicle palpated in the bursa, hypotrophies	OGE macrobiopsy: structures corresponding to a rudimentary uterus with its tube

2.2. Mutation Screening of SRY, DAX1 and SOX9 Genes

Genomic DNA was extracted from whole blood using the Quick-DNA Miniprep (Zymo Research) extraction kit following the manufactures recommendations. Subsequently, the extracted DNA quality was verified by electrophoresis on 1.5% agarose gel and quantified with Nanodrop 3300. PCR amplification of the SRY gene was performed on a Profiles PCR system thermocycler using the primer pairs listed in Table 2. A final 25 µl volume mix PCR was prepared with 12.5 µl of OneTaq® Quick-Load® 2X Master Mix with Standard Buffer 0.5 µl of each primer diluted to 1/10 for 100mM and 9.5 µl of milli_Q water to which we add 2 µl of the patient's DNA. The PCR program used for each amplified primer pair is detailed in Table 2; [19, 25–28]. PCR products were checked by electrophoretic migration on 2.5% agarose gel from 5 µl of the amplicons and in TAE 50X buffer diluted 1:10 with 5 µl of safeview dye. The size of the amplified gene was estimated using a 1500 bp Quick-Load® purple 50bp DNA Ladder size marker. DAX1 and SOX9 genes were also amplified. 15ul of the PCR product obtained for each patient was sent for sequencing to Eurofins Genomics Anzinger Str. 7a 85560 Ebersberg based in Germany.

Table 2. *Oligonucleotide sequences and program to amplify SRY, SOX9, DAX1 genes.*

gene	Primers symbol	primer sequences 5' 3'	Favourable PCR conditions	Product size (bp)
SRY 1	SRY1 F SRY1 R	CATGAACGCATTCATCGTGTGGTC CTGCGGGAAGCAAACTGCAATTCTT	94°C-2 min (94°C-1min, 65°C-1min, 72°C-1min) ×35 cycles, 72°C-10 min	254
SRY 2	SRY2 F SRY2 R	GAATATTCCCGCTCTCCGGAG ACCTGTTGTCCAGTTGCACT	94°C-2 min (94°C-1min, 58°C-1min, 72°C-1min) ×35 cycles, 72°C-10 min	418
XES	XES10 XES11	GGTGTTGAGGGCGGAGAAATGC GTAGCCATTGTTACCCGATTGTC	94°C-5 min (94°C-1min, 62°C-2min, 72°C-2min) ×30 cycles, 70°C-10 min	779
SOX9 A	9A F 9A R	CGGGTGGCTCTAAGGTG TTGTGCAAGTGCGGGTA	95°C-5 min (95°C-1min, 55°C-1min, 72°C-1min) ×35 cycles, 70°C-10 min	823
SOX9 B	9B F 9B R	AGAGGAAGCCGAGTGGT AGGCGGGACGGAGATAG	95°C-5 min (95°C-1min, 56°C-1min, 72°C-1min) ×35 cycles, 70°C-10 min	416
SOX9 C	9C F 9C R	ACCGACCACCAGAACTCC TGTATAAATCCCCTCAAAATG	95°C-5 min (95°C-30sec, 52°C-1min, 72°C-30sec) ×35 cycles, 70°C-10 min	698
DAX1 1	X1 F X1 R	CCGCGCCCTTGCCCAGACC GCCGCCTGCGCTTGATTTGT	94°C-12 min (94°C-1min, 66°C-1min, 72°C-1,5min) ×35 cycles, 70°C-10 min	786
DAX1 2	X2 F X2 R	CGCGCAGAGGCCAGGGGGTAAAG CCCCGACACTCTCCTGATCACTG	94°C-12 min (94°C-1min, 59°C-1min, 72°C-2min) ×35 cycles, 70°C-10 min	750
DAX1 3	X3 F X3 R	TTGGGTCTTGTTTAATTGGGATGAA CCATGAAATTGCTACACTTGTGAAAA	94°C-12 min (94°C-1min, 59°C-1min, 72°C-1,5 min) ×35 cycles, 70°C-10 min	644

For identify mutations, raw sequencing data were submitted to Mutation Surveyor® Version 5.1 and DNA Baser Assembler v5 comparing the submitted chromatograms with the said reference sequence of SRY (NT_011896_2654396_F_Synthesis_897.scf; NT_011896_2654396_R_Synthesis_897.scf), SOX9 (NT_010783_70066683_F_Synthesis_113182.scf; NT_010783_70066683_R_Synthesis_113085.scf) and DAX1

(NT_167197_30322039_F_Synthesis_5281.scf; NT_167197_30322039_R_Synthesis_5281.scf). The detected mutations were submitted to public databases such as NCBI (National Center for Biotechnology Information) and Ensemble Genome Browser 105 to verify whether the mutation was already described or novel using dbSNP and ClinVar. For each mutation we collected the location, nature and effect on the coding sequence (Table 3). Protein sequences of the SRY gene, coding portion were submitted to ScanProsite to search for matches by specific pattern.

Table 3. *Position and Mutation Types of SRY, SOX9 and DAX1 Genes.*

Patients (p)	Chr pos	variants	cDNA	mRNA	amino acid	Molecular consequence	dbSNP	Clinical consequence and prediction of new SNPs
SRY variants								
P14 et P91	Y:2655380	903G>GA	c.265	c.403	p.89Glu>Lys	Non Synonyme	New	Benign
P69, P73	Y:2655014	1269T>A	c.*16	c.769		variante 3'UTR	rs1361161627	
P73	Y:2655474	809G>GC	c.171	c.309	p.57Gln>His	Non Synonyme	New	Damaging
P69	Y:2655014_ Y:2655015	1268_1269insAA	c.*15_*16	c.768_769		variante 3'UTR	New	
SOX9 Variants								
84-9C	17:70120561	113284C>CT	c.*33	c.1935		3'UTR Variant	New	
P21 et P103-9C	17:70120551	113294T>TG	c.*23	c.1925		3'UTR Variant	dbSNP:1042667	benign
P103-9A	17:70117881	115964G>GC	c.349	c.721	p.117Gln>Glu	Non Synonyme	dbSNP: 1555629037	pathogen
DAX1 Variants								
P121, 71, 75, 78, 95, CAB-X1	X:30326983	1013G>A	c.498	c.513	p.166Arg>Arg	Synonyme	dbSNP:2269345	benign
P107-X2	X:30326452	1544G>A	c.1029	c.1044	p.343Leu>Leu	Synonyme	dbSNP:112775648	Probably benign, benign

2.3. Genes Expression of SOX9 and DAX1 Using qPCR Analysis

The copy number variation of 750-bp DAX1 exon 1 and SOX9 exons 1 (823bp) and 2 (413bp) was detected for 15 patients and 2 controls by qPCR using SYBR®Green dye and StepOnePlus™ Real-Time PCR software. We used GAPDH as an internal reference gene. For the gene amplification conditions we referred to the conditions used by A. K. Saxena et al. [24] for SOX9 and GAPDH and for DAX1 [27]. PCR amplification was applied to a 25 ml volume reaction containing 10x Tris (pH 8.4), 50 mM KCl, 25mM MgCl 10pM of oligonucleotide primers. The primer pairs used are shown in Table 4.

Table 4. *Primers used for expression of SOX9 and DAX1 genes.*

gene	Primers symbol	primer sequences 5' 3'	references
SOX9 A	FA	CGGGTGGCTCTAAGGTG	[24]
	RA	TTGTGCAAGTGCGGGTA	
SOX9 B	FB	AGAGGAAGCCGAGTGGT	[24]
	RB	GGCGGGACGGAGATAG	
GAPDH	F	TGAAGGTCGGAGTCAACGGATTTG	[24]
	R	CATGTGGGCCATGAGGTCCACCAC	
DAX1 (X2)	F	CGCGCAGAGGCCAGGGGGTAAAG	[27]
	R	CCCCGACACTCTCCTGATCACTG	

3. Results

3.1. Classification and Research of the SRY Gene

Of the 29 patients, 13 are karyotype 46, XX (6 positives in SRY and 7 negatives) and 16 are karyotype 46, XY (11 positives in SRY and 5 negatives) Table 1. Sequencing of the SRY gene (Figure 1) showed a new variant *c.265 G>A, p.Glu89Lys* in 2 patients male (P14 and P91) with a normal 46, XY karyotype. A second variant already found in the literature *IVS615 +16T>A* (rs1361161627), a variant of the untranslated region (3'UTR) found in 2 patients female (P69 and P73), one of which has a 46, XY karyotype with micropenis and testes and the other one has a 46, XX karyotype with penis clitoris and absence of testes and uterus. In addition, in patient P69 (46, XY) an insertion IVS615 +15_ IVS615 +16insAA was found. Finally, a third novel variant *c.171 G>C, p.Gln57His* in patient P73 with karyotype 46, XX (See table 1 clinical of patients) was also found. All the variant sequences of the coding part of the SRY gene found and submitted to ScanProsite show that they are located in the HMG domain (High mobility group box

domain) (Figure 2).

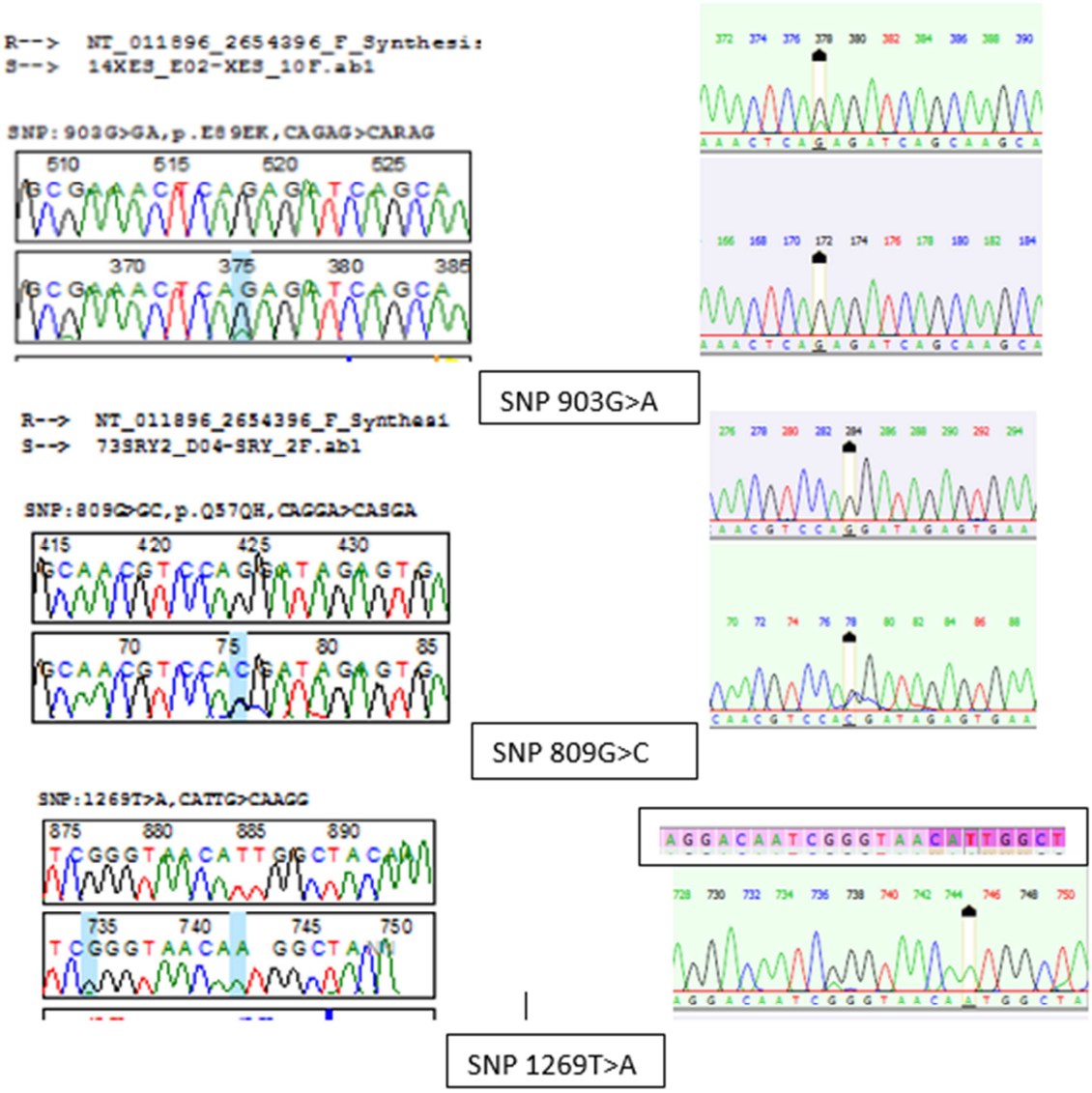

Figure 1. *DNA sequencing to identify mutation for SRY (SNP Mutation Surveyor compare to DNA Baser).*

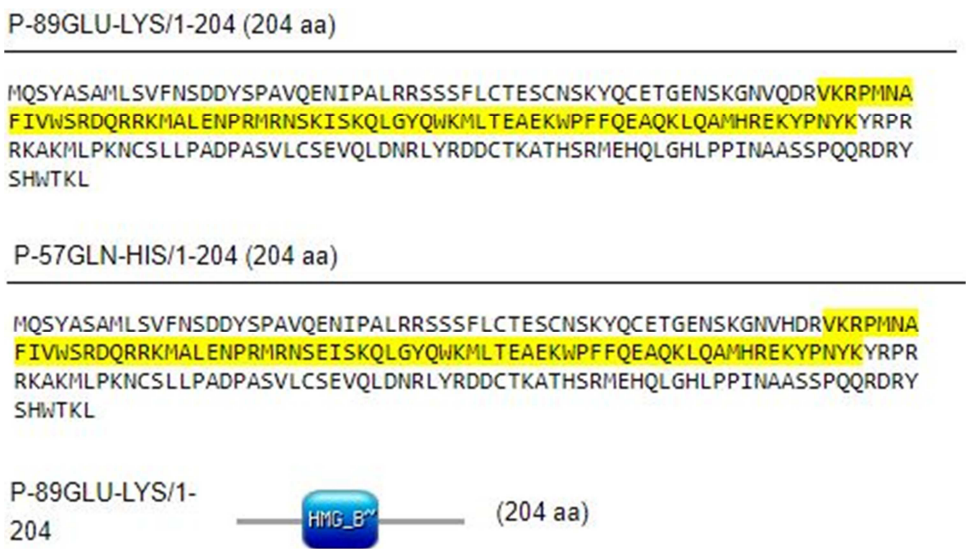

Figure 2. *HMG boxes A and B DNA-binding domains profile.*

3.2. Detection of SOX9 and DAX1 Variants

For SOX9, a mutation *c.349 G>C, p.Gln117Glu* was found in one patient on the coding part of exon 1. For exon 3, two variants of the 3'UTR (Figure 2) were found in 3 patients, one *113294T>G* in patients (P21 with karyotype 46,-XX SRY- and P103 with karyotype 46, XYSRY+) and the other *113284C>T* which is a new variant in patient P84 with karyotype 46, XXSRY+. There was no mutation found on exon 2 of SOX9. Concerning DAX1, two variants of the coding sequence (Synonym) were found in exon 1 only (figure 3), the first variant c.513 G>A, p.Arg166Arg in 5 patients (table 3) and the other in one patient c.1044 G>A, p.Leu343Leu (figure 3). In summary, for DAX1, no variants changing the amino acid or shifting the reading frame were found.

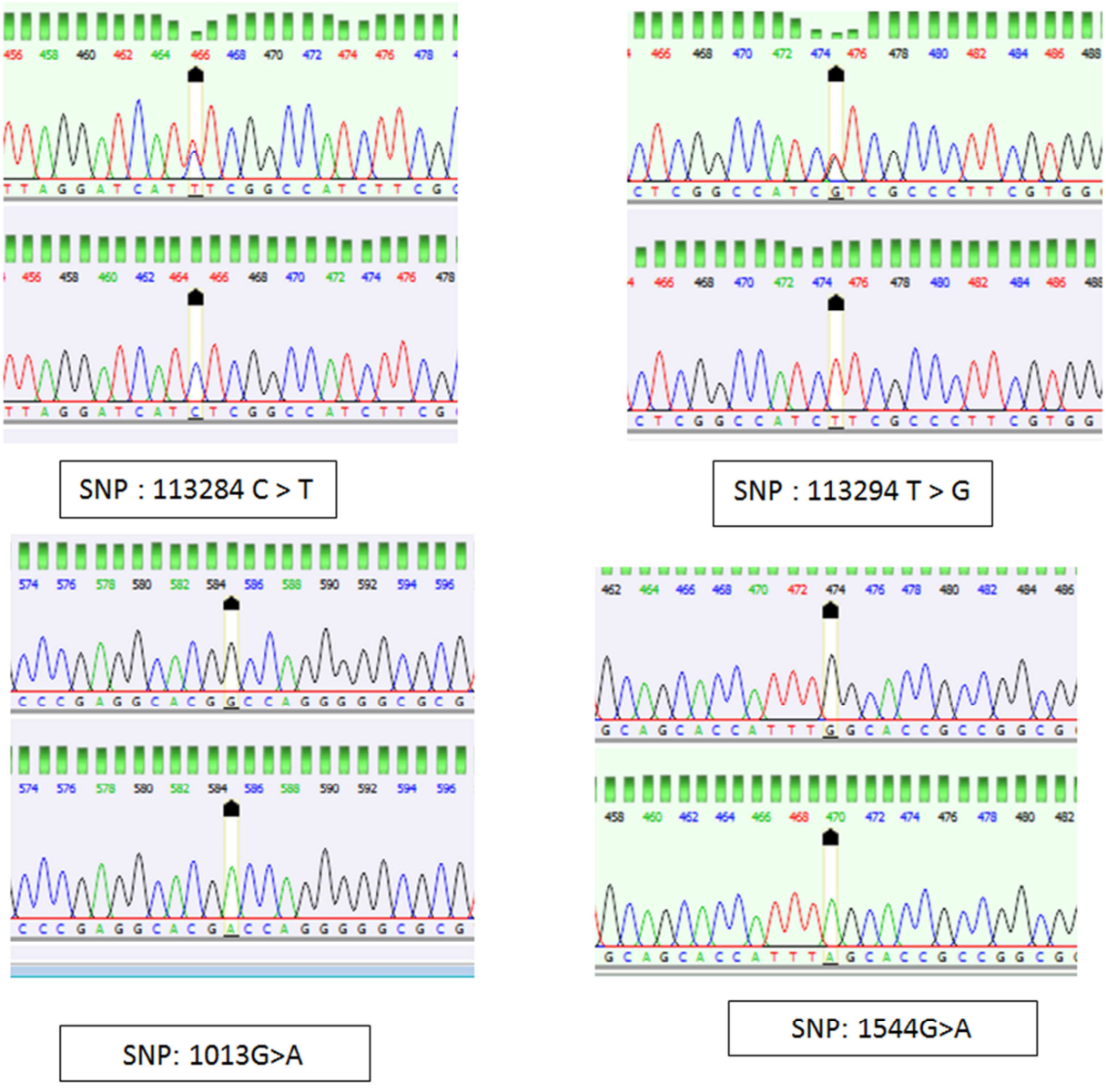

Figure 3. *DNA sequencing to identify mutations for SOX9 and for DAX1.*

3.3. DAX1 AND SOX9 Expression

Compared to our controls, gene expression for SOX9 and DAX1 is normal for most of our patients. Except four patients (P53, P54, P69 and P75) where SOX9 exon 2 is overexpressed. In patient P53 and P69 there is also an overexpression of DAX1 despite the presence of the SRY gene. In contrast, in patient P14 (46XYSRY+) SOX9 exon 2 is poorly expressed (Figure 4) and not present in patients P87 (46XYSRY+) and P98 (46XXSRY-).

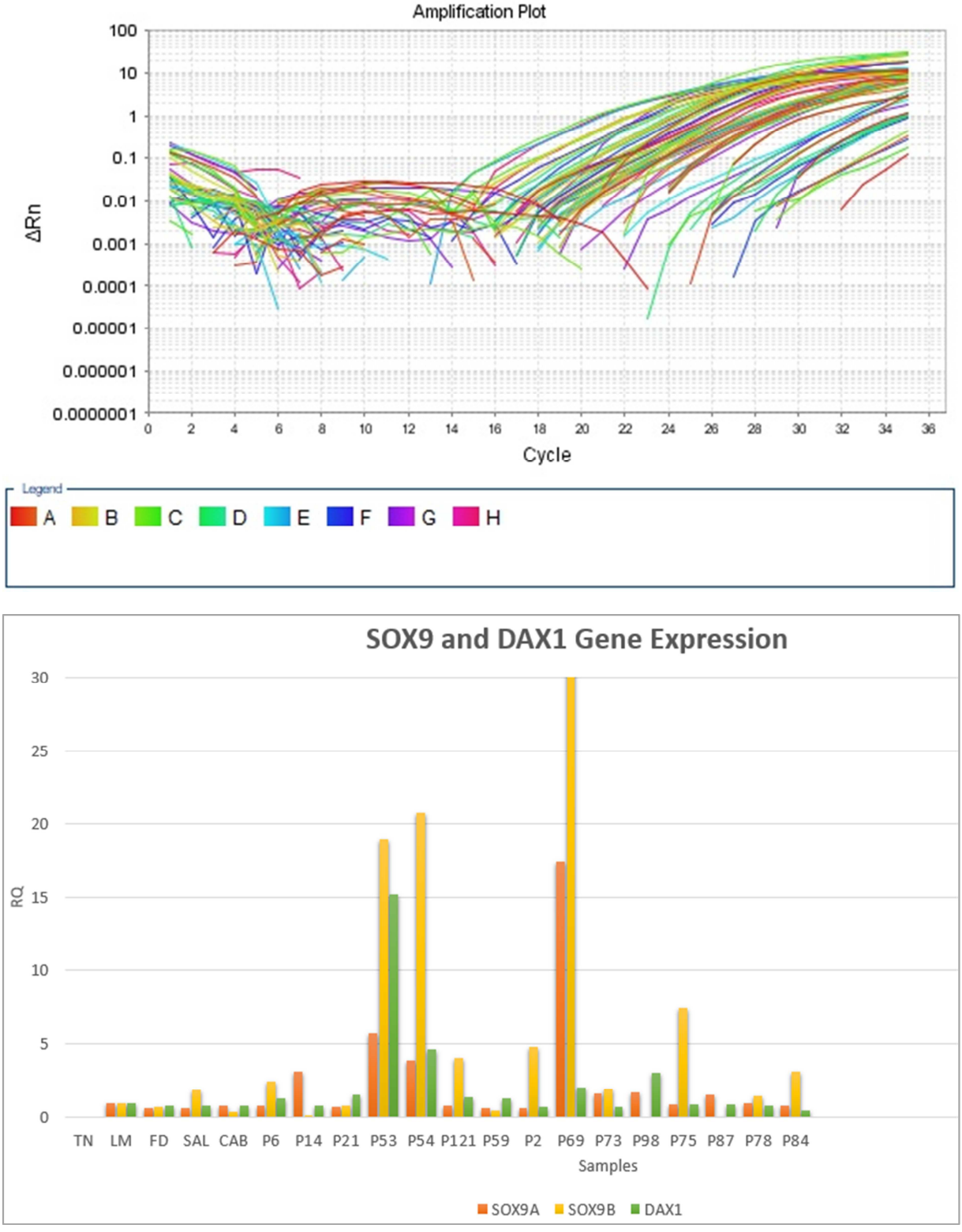

Figure 4. *Amplification plot (to left) and genes expressions of SOX9 and DAX1 (to right, FD female control; LM male control).*

4. Discussion

During the development of the XX and XY embryo, the gonads develop similarly way in both sexes, male and female (bipotential) with the presence of primitive sex cords as well in the cortical region than in the medullary region, until SRY expression is triggered [8, 29], it's the undifferentiated stage. At this stage the genital tract is also undifferentiated, two types of genital tract sketches correspond to two pairs of ducts: the Müller's ducts (drafts of the future female genital tract) and the Wolff's ducts (drafts of the male genital tract).

These genital tract outlines, regardless of the sex of the embryo, are both present, have the same structure and disposal [30]. Several genes have been identified as playing a crucial role in the formation of genital crests, in particular WT1 (Wilms' tumor gene 1) (Hossain and Saunders, 2001) [31] and SF1 (steroidogenic factor 1) or (NR5A1) [32, 33]. Therefore, adequate regulation is necessary for the differentiation of the gonads and other anatomical structures related to the sex of the individual [34]. In most cases of DSD, mutations in the *SRY* and *SOX9* genes are responsible for Gonadal Dysgenesis in approximately 20% of cases, mutations causing sex reversion also in 30% of cases are related to the DAX1 (or NR0B1), SF1 (NR5A1), SOX9, WNT4 (Wilms' tumor-4), DHH (desert hedgehog) [35, 36]. For the remaining 50% the genetic cause is uncertain [37].

Concerning–the SRY gene, the majority of mutations reside in the HMG domain, which is involved in the binding and bending of DNA [38, 39]. In addition, new mutations localized in this area or in proximity to it have been reported *(p.Glu89Lys and p.Gln57His*), which can reduce nuclear imports of SRY. The 46, XX sex reversal 1 phenotype found is characterized by the inconsistency between gonadal sex and chromosomal sex and the presence of SRY DNA in 46, XX patients shows a translocation of the Y chromosome or a small fragment, including this SRY locus, on the X chromosome or on an autosome [40]. For two patients 46, XY whose *SOX9* and *DAX1* gene expression appears to be normal, clinical presentation and biological data support complete gonadal dysgenesis (P2 and P59) related to SRY gene loss [41]. Patient 46, XY (P54, micropenis and scrotum not developed) with strong SOX9 expression despite the absence of the SRY gene suggests partial gonadal dysgenesis [42]. In this case SOX9 could also play a role in the repression of genes involved in ovarian development such as *WNT4, FOXL2* and the transcription factor DMRT1 [41]. In general, mutations in *SOX9* (belonging to a family of twenty SRY-related HMG box (SOX) proteins, most of which contribute to cell type specification and differentiation in discrete lineages) cause campomelic dysplasia (rs1555629037 in two patients). It is a severe skeletal malformation syndrome often associated with XY sex reversal, and milder skeletal dysplasia's, namely acampomelic campomelic dysplasia and the Pierre Robin sequence [43]. During sex differentiation, low-level DAX1 expression precedes SRY expression peak by at least 10 days and persists in Sertoli cells throughout the sex determination period. In Dosage Sensitive Sex Inverse, the antitesticular properties of DAX1 overexpression could act before the peak effects of SRY and continue during the SOX9 expression period [11]. Overexpression of DAX1 in two men 46, XY may explain the clinic of these patients (micropenis, no testicles) as overexpression of DAX1 may alter other developmental pathways and interfere with gonad development and ovarian follicle maintenance, thus generating a dysgenesis gonad [44]. We also characterized two variants (rs2269345 in 6 patients) and (rs112775648 in one patient) both related to phenotypes 46, XY sex reversal 2 and adrenal hypoplasia congenital X-linked (AHC). In the case of AHC, usually the testes may be present with confusion of the testicular cords resulting in disorders of gonadal function but mitigating [19].

5. Conclusion

In the present study we were able to characterize several mutations in the SRY, SOX9 and DAX1 genes and correlate them with several phenotypes but also to demonstrate differences in expression for the SOX9 and DAX1 genes in Senegalese patients for the first time. This study provides important insight into the variants of SRY, SOX9 and DAX1 from DSD individuals. In recent years have seen considerable progress in the knowledge of the genetic causes of DSD with normal karyotype in particular. Being a developing country and due to the development of technology, we owe a great awareness of DSD as a public health problem. All this leads us to go in a more accurate diagnostic plan by using new technologies such as comparative genome hybridization or high resolution genome sequencing.

Statement of Ethics

After examination according to the rules decreed by the National Ethics Committee for Health Research of Senegal and by the procedures established by the University Cheikh Anta DIOP of Dakar (UCAD) for any research involving human participants, the ethical approval of this study was obtained (Ref: Protocol 053/202l/CER/UCAD).

Consent to Participate Statement

This project is a human experiment involving sick people and members of their families. All ethical considerations are taken into account from data collection to publication of results. The data was collected after informed consent of the participants who received all the necessary information regarding the content of the project. A consent form was developed and validated and subsequently submitted to the Comity National of Ethics for the research in health (CNERS) of Senegal at the Cheikh Anta Diop University in Dakar.

Conflict of Interest Statement

The authors declare that they have no competing interests.

Author Contributions

Fatou Diop GUEYE designed the study, performed the laboratory work and wrote the manuscript. Arame NDIAYE and Adji Dieynaba Diallo Supervised the laboratory work and performed the statistical analyses. Mame Venus GUEYE, for clinical examination. Ndiaga DIOP, Rokhaya NDIAYE, reading and editing document. Oumar FAYE and Mama SY Supervised this study. All co-authors revised the manuscript.

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