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What Impact on Phenotype for Patients with Karyotype 46, XX DSD SRY Positive at CHU Dantec in Senegal: About 5 Cases?

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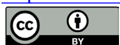

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Abstract

Background: In disorders of sexual differentiation, sexual development may not conform to the chromosomal structure, thus forming different types of abnormalities. Among these abnormalities is syndrome 46, XX DSD where most patients are female phenotype with clitoral hypertrophy that can go to complete masculinization especially in the presence of the SRY gene. **Objective:** The goal of this work is to demonstrate a relationship between the genotype and the phenotype in five patients karyotype 46, XX with the presence of the SRY gene. **Methodology:** The study involves five patients referred to the laboratory under suspicion of sexual development anomalies. The diagnosis took place through hormonal and echography examinations, a classic cytogenetic study (Barr chromatin and karyotype) and an amplification of the SRY gene located on the Y chromosome. The resulting PCR products were sent for sequencing. **Results:** Based on the results of clinical and paraclinical tests carried out it was found clitoral hypertrophy, the presence of clitoris penis for some, presence of normal penis for others. In addition, echography revealed a lack of female internal genitalia (P2, P3), and a presence of testicles (P3, P4, P5). Genetic analysis (chromosomal and molecular) showed a karyotype 46, XX SRY (+) for all patients. New mutations were found c.246 T > A, p.82 Asn82Lys and c.171 G > C, p.57 Gln57His. **Conclusion:** In our study, we were able to correlate each DSD with karyotype 46, XX to a pathology such as

46, XX DSD testicular, 46, XX DSD with clitoral hypertrophy and ovotestis 46, XX. The next step will undoubtedly be the integration of other molecular techniques (genotyping, FISH, CGH or even the CGH array) to further genetic exploration.

Keywords

46, XX DSD, SRY, Phenotype, Hypertrophy

1. Introduction

Sexual identity is an important element in the personality, in the perception of our entourage but also and especially, in the relationships that we develop throughout our life with others. This sexual identity started from embryonic life through a succession of events, each stage of which may be the site of abnormalities leading to Disorders of Sex Development (DSD) [1]. These conditions affect 1/5500 children at birth and were the subject of a new nomenclature as well as an international consensus in Chicago in 2005 [2]. DSDs are defined by consensus as congenital disorders in which chromosomal, gonadal or anatomical sex development is atypical [2] [3], that is, a malformation of the internal and/or external genitals. This consensus proposes to classify the various anomalies according to the karyotype as a prefix (46, XX DSD formerly female pseudo hermaphroditism, 46, XY DSDformerly male pseudohermaphroditism and chromosomal DSD) [1] [2] [4] and type of impairment (gonadal or adrenal). Chromosomal DSDs include abnormalities in the number of sex chromosomes; 46, XY 45, X0 (Mixed gonadal dysgenesis), 46, XX/46XY (Ovotestis) as well as the 45, X (Turner and variants), 47, XXY (Klinefelter and variants) which were not in the old classification [4].

In the case of gonadal involvement, we distinguish the ovotestis (ex-hermaphroditism true), gonadal dysgenesis and the 46, XX DSD testicular. These occur in about 1 in 20,000 to 25,000 male newborns [5] and can be classified clinically into three groups. The first group consists of individuals with male development without anomaly of the external genitals (phenotypically normal) nor reproductive tract and is most frequently encountered; the second group is composed of individuals with an ambiguity of the external genitals and the third group describes true hermaphrodites with ovotesticular development represented by the presence of testicular and ovarian tissues [6] [7] [8]. The diagnosis of his patients is most often in adulthood because they have infertility due to several factors (azoospermia, very small testicles or absence of müllerian structures among others; Ahmad *et al.* 2012). In most of these cases, the Y chromosome is translocated on the X chromosome following recombination in the distal parts of the short arms of the X and Y chromosomes during paternal meiosis with the presence of the SRY gene (Sex determining Region Y [9]. However, in 10% of cases of 46, XX DSD testicular or ovotestis, the diagnosis is made in neonatal period most often with hypospadias, testicles not lowered or varying degrees of inade-

quate virilization in the observed external genitals.

In 90% of gonadal disorders 46, XX DSD, the SRY gene (Sex determining Region of the Y chromosome) is responsible [10] while knowing that, there is a panel of genes that can be responsible (NR0B1, NR5A1, WT1, SOX9…) [11]. Some of these genes play a leading role in gonadic ebauches while other areinvolved in differentiation of Sertoli (testicular or granulosa (ovary). Indeed the SRY gene plays a major role in the differentiation of undifferentiated gonads and controls the synthesis of a SRY protein constituting the testicular determining factor TDF "Testis Determining Factor" [12].

In our present study we looked at several patients referred to the laboratory of Clinical Cytology, Molecular Biology, Reproductive Biology and Development of the Aristide le Dantec Hospital University for a sexual development abnormality according to different diagnostic hypotheses (gonadal dysgenesis, 46, XX DSD SRY+, 46, XY DSD. We tried through the clinic and genetics to characterize the type of gonadal involvement 46, XX DSD for these patients to compare clinical phenotypes to genetic data.

2. Methodology

2.1. Statement of Ethics and Consent to Participate Statement

After examination according to the rules decreed by the National Ethics Committee for Health Research of Senegal and in accordance with 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).

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.

2.2. Patients Presentation and Clinical Data Associated

Out of 87 patients received in our structure and diagnosed with DSD, only 5 patients 46, XXSRY+ were included after completion of the karyotype and SRY test (see 1.2.2 and 1.2.3). After informed consent for all patients, we collected clinical data and echography in 5 patients registered to the civil state at the birth of the female sex (**Figure 1**) except for the only P1 patient who was reported as male. This patient had different phenotypes nursing from female ambiguous to male in different degrees. Among all patients, only hormonal data from the P4 patient were able to be collected with the FSH = 5.57 mui/mL (ref for male < 12.4 mUl/mL) and testosterone = 9.79 ng/mL (ref for male 2.80 - 8 ng/mL). In all cases a clinical diagnostic(by consulting the clinician to define the genital phenotype) approach was conducted and summarized in the table below (**Table 1**).

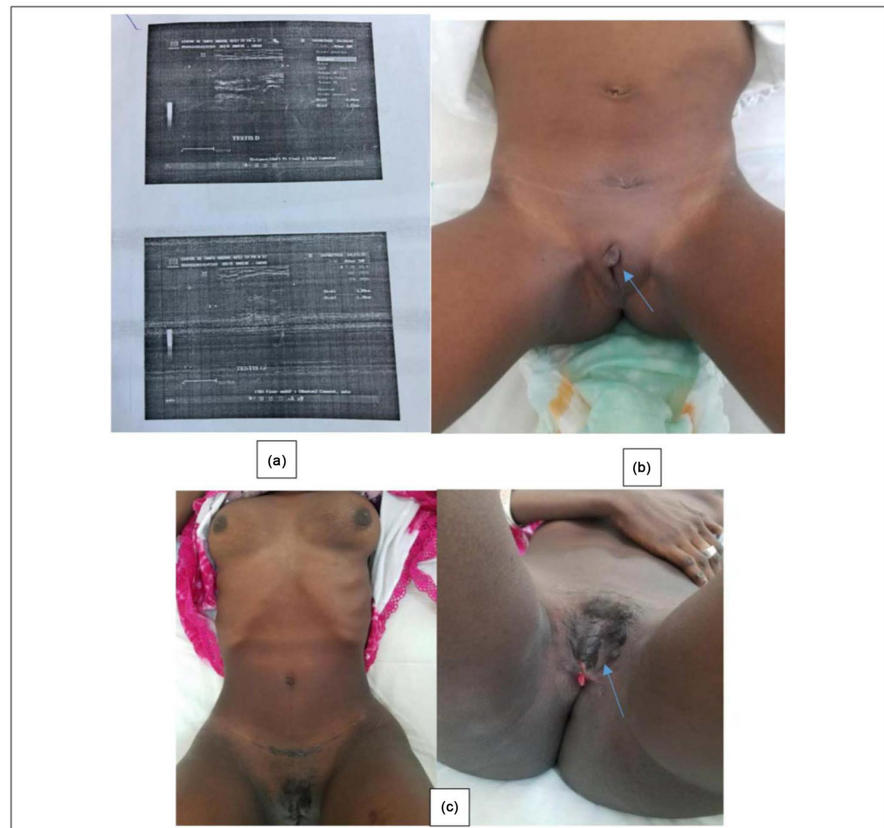

Figure 1. Patients declared to civil state at birth of the female sex.

For the Clinic we searched for each patient:

- The penis clitoris organ on its central face, we note the presence or not of erectile body;
- The gonads are sought;
- The labio-scrotal folds are described by their colour, appearance and implantation in relation to the base of the shaft;
- The urethral meatus will be sought and its position well defined.

2.3. Genetic Studies

2.3.1. Barr Chromatin

We took a sample of the jugal mucosa membrane for three patients. Then we performed the Barr chromatin test using the GUARD coloration technique [13] [14] [15] [16]. This technique brings its best compared to others as it allows us to clarify the etiology but also the mechanism of these chromosomal anomalies. After coloring, the slides have been observed under a microscope where sexual chromatin appears as a dark blue corpuscle located at the inner surface of the internal nuclear membrane. The percentage is obtained by the ratio of nuclei with Barr chromatin out of a total of 200 nuclei observed at least. If this rate is between 0 and 5%, the chromatin sex is male; between 15% and 30%, the chromatin sex is female and when the rate is between 6% and 14%, the chromatin sex is said to be intermediate.

Table 1. Clinical information and ultrasound exploration of patients of which 4 reported at birth of female (F) and one male (P1).

Patients (P)	First Age of consultation	morphotype	External genitalia (OGE)	Abdomino-pelvic echography
P1	2 years	………………………	OGE male type, no palpated testis	………………………..
P2	67 days	Absence of craniofacial dysmorphia, abdomen slightly increased in volume, presence of umbilical hernia.	OGE: 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
P3	20 years	mammary development, flexible abdomen without palpated masses, pilosity+++	OGE: clitoral hypertrophy, palpated 2 masses (one at the pubic level on the right and one at the inguinal level on the left), presence of 2 orifices	Absence of female internal genitalia, testis presence
P4	17years	morphotype android, thorax: mammary development stage S4 of Tanner absent axillary pilosity, pubic pilosity stage P2 of Tanner, Flexible abdomen without palpated masses.	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
P5	12 days	………………………	clitoral hypertrophy, urethral meatus, vagina in place and even labia majora	Left uterus and ovary visualized normal size and echostructure. Visualization of a right testicle in the inguinal position

2.3.2. Karyotype

A heparin tube blood sample was taken to perform the GTG-band karyotype. For this, we carried out a cell culture (72 h at 37˚C with 5% CO_2 from 0.5 ml of blood following a protocol previously described [16] [17]. Subsequently, cell pellets were obtained, spread on a blade, denatured by enzymatic action (Trypsin) and buffered to saline phosphate (PBS) to obtain the G (GTG) bands. The resulting metaphases were observed on a LEICA CW4000 Cytogenetics imaging station to get the karyotype. The study of karyotype allows us to define the genetic sex of the individual but also to detect possible chromosomal abnormalities that may be linked to rearrangements.

2.3.3. SRY Gene Amplification

For this part, we search for SRY gene located on Y chromosome using EDTA tube sampling for only 4 patients while cell culture pellet was used for the fifth patient. Add to these samples, two controls (one male and one female married and had children each) were included in our study. On these samples, genomic DNA was extracted using *Quick*-DNA Miniprep kit (Zymo Research) following the manufacturer's protocol. Following this, DNA extracted was respectively

checked and quantify by electrophoresis using 1.5% agarose gel and Thermo Scientific™ NanoDrop™ 3300 Fluorospectrometer. PCR amplification was performed on a Profiles PCR system thermocycler for both the SRY and ATL1 (control) genes using the primer pairs listed in **Table 2**. ATL1 (300 bp) is the sequence of the FMR1 gene located on the long arm of the X chromosome [18]. It is used as internal control in this study because the use of polymorphism ATL1 allows further separation of X chromosomes into related groups with growth rates apparently different enough to repeat the expansion [19]. To amplify the SRY gene and effectively attest his in the patients considered (in the presence of positive control), three different primer pairs were used as indicated in **Table 2**. First, the XES-10/11 primer pair was used to amplify the entire SRY gene (779 bp), confirmed by a second PCR. Then two additional primer pairs, targeting smaller fragments of the SRY gene (SRY-F1/R1: 254 bp and SRY-F2/R2: 417 bp) were used in case first primer pair does not work to or if smear is present. To perform the PCRs, we prepared a final mix of 25 μL including 12.5 μL of OneTaq® Quick-Load® 2X Master Mix with Standard Buffer 0.5 μL of each primer (diluted 1/10 for 100 mM) and 9.5 μL of milliQ water to which we add 2 μL of the patient's DNA. The PCR programs used for each primer pair have been detailed in **Table 2** [18] [20] [21]. PCR products were finally checked by electrophoretic migration on 2.5% agarose gel and sent for Sanger sequencing at Eurofins Genomics.

2.3.4. Sequences Verification and Mutations Detection

The sequences obtained were checked and corrected using BioEdit Sequence Alignment Editor software v7.1.9 (Hall, 1999). Moreover, multiple alignment was performed using Seaview v5.4.1 (Gouy *et al.*, 2010) and then translated into proteins to compare our patients sequences to the reference sequence (NT_011896_2654396) and male/female controls included in this study.

After the sequences verification, we searched for variants from the raw sequencing data obtained using Mutation Surveyor® V5.1 software which compared submitted chromatograms to the reference sequence of SRY gene (NT_011896_2654396). The mutations detected by software were subsequently submitted to public databases such as NCBI (National Center for Biotechnology Information) and Ensemble Genome Browser 105 to verify if the mutation was already known or not using dbSNP and ClinVar. For each mutation we detected, his location, nature and effect on the coding sequence were determined.

2.3.5. Pathogenicity Prediction of Missense Mutations

In order to determine whether non synonymous mutations in the SRY gene are deleterious or benign, protein sequences (Q05066) were submitted to Polyphen 2 [22], UMD-Predictor [23] and Provean [24]. The UMD-Predictor system is an innovative bioinformatics solution to predict the pathogenicity of any SNP from any human transcript. It relies on an original combinatorial approach that

Table 2. Oligonucleotide sequences and program used to amplify the SRY gene.

gene	Primers name	primer sequences 5' → 3'	PCR conditions	Product size (bp)
SRY 1	SRY-1 F	CATGAACGCATTCATCGTGTGGTC	94˚C-2 min (94˚C-1 min, 65˚C-1 min, 72˚C-1 min)	254
	SRY-1 R	CTGCGGGAAGCAAACTGCAATTCTT	×35 cycles, 72˚C-10 min	
SRY 2	SRY-2 F	GAATATTCCCGCTCTCCGGAG	94˚C-2 min (94˚C-1 min, 58˚C-1 min, 72˚C-1 min)	418
	SRY-2 R	ACCTGTTGTCCAGTTGCACT	×35 cycles, 72˚C-10 min	
XES	XES10	GGTGTTGAGGGCGGAGAAATGC	94˚C-5 min (94˚C-1 min, 62˚C-2 min, 72˚C-2 min)	779
	XES11	GTAGCCATTGTTACCCGATTGTC	×30 cycles, 70˚C-10 min	
ATL1	ATL1-F	CCCTGATGAAGA ACT TGTATC TC	94˚C-2 min (94˚C-1 min, 58˚C-1 min, 72˚C-1 min)	300
	ATL1-R	GAAATTACACAC ATAGGTGGCACT	×35 cycles, 72˚C-10 min	

consistently outperformed other predictors [23]. For PROVEAN (Protein Variation Effect Analyzer), its primary function is to provide a prediction for a protein sequence originated from any organisms. The tool accepts a protein sequence and amino acid variations as input, performs a BLAST search to identify homologous sequences (supporting sequences), and generates PROVEAN scores. To provide binary predictions, the cutoff for PROVEAN scores was set to −2.5 for high balanced accuracy [24]. As regards PolyPhen-2 (Polymorphism Phenotyping v2), it's an automatic tool for prediction of the possible impact of an amino acid substitution on the structure and function of a human protein. The prediction is based on a number of sequence, phylogenetic, and structural features characterizing the substitution. For a given amino acid substitution in a protein, PolyPhen-2 extracts various sequence and structure-based features of the substitution site and feeds them to a probabilistic classifier. Any mutation with a score of 0.9 is classified as potentially harmful mutation [22].

3. Results

3.1. Cytogenetic Study

Among the 5 patients studied, Barr chromatin analyses showed in 03 patients (P1, P3 and P4) a male chromatin sex (between 0% and 5%) while for the remaining two patients (P2 and P5) the bar chromatin could not be realized. The karyotypes performed showed a karyotype of type 46, XX for all patients corresponding to the 46, XXDSD category (**Figure 2**).

3.2. Molecular Study

The SRY gene was found in all patients studied (**Figure 2**). However, the size of the fragments of the SRY gene found differed from one patient to another. Indeed, the entire coding fragment of the SRY gene (779 bp) was found in patients P2, P3 and P4, whereas a shorter SRY fragment (254 bp) was found in patients P1 and P5.

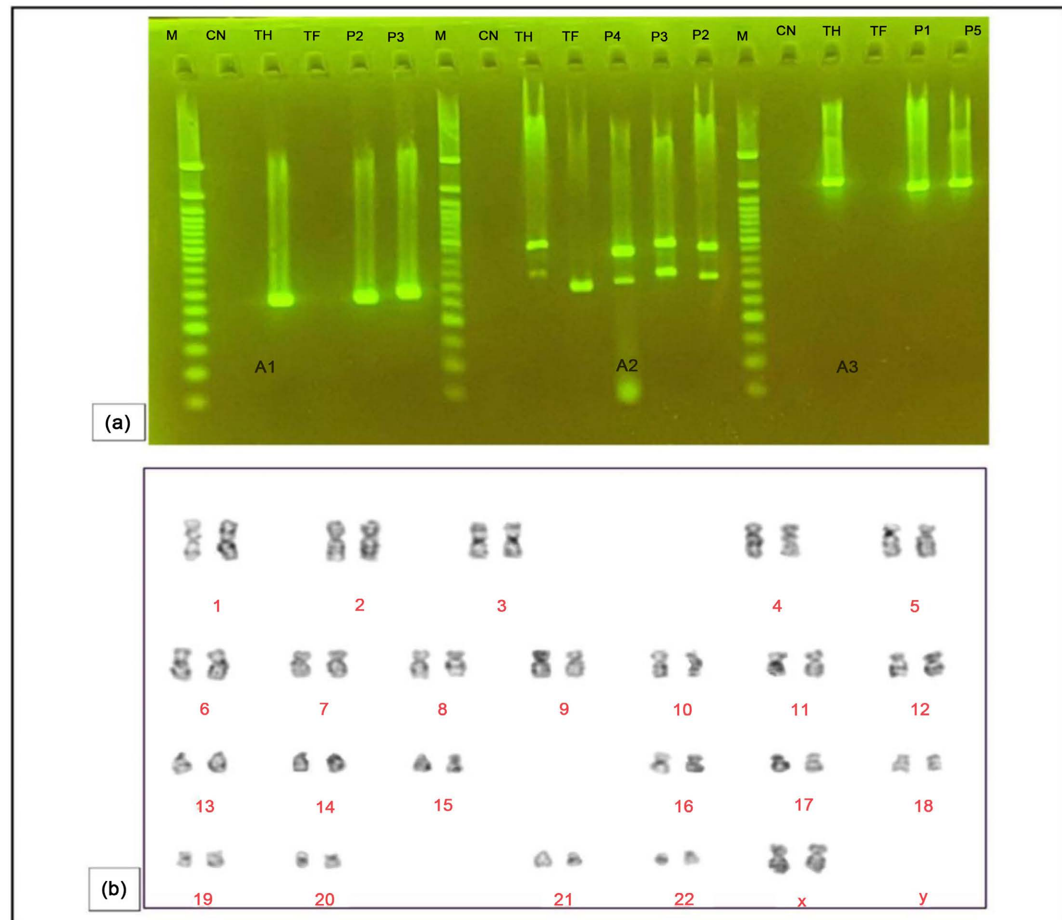

Figure 2. (a) Photograph of the gel corresponding to the SRY gene amplification for the five patients. (b) Example Karyotype at 46, XX in GTG.

3.3. Mutations and Pathogenicity Analysis

For all our patients, only three mutations have been recorded in P1 and P2 (Table 3) while in patients P3, P4 and P5 sequences are back to normal. Obtained are recorded below.

The two variants detected in the coding part of the SRY gene (showed in Figure 3) have not yet been found in the databases(DbSNP, Clinvar, Ensembl…) consulted and are therefore considered as new. The mutations c.246 T > A and c.171 G > C induce a change of the amino acid p.82Asn > Lys and p.57Gln > His respectively. These variants are certainly deleterious according to the pathogenicity analysis (Table 4). On the other hand, the mutation 1269 T > A located in the 3' untranslated region has already been found in the literature (rs1361161627).

4. Discussion

In Senegal, so far few genetic studies on sexual development abnormalities are available and yet these are frequently encountered in our health structures [15] [25]. As stated at the beginning, gonadal developmental disorders, 46, XX DSD include a group of disorders whose degree of severity and consequences are

Table 3. Position and mutation types of SRY gene.

Patients (P)	Chr position sur (GRCh37)	Variants	cDNA	mRNA	amino acid	SnRatio	Score	Molecular consequence	dbSNP
P1	Y:2655399	884 T > TA	c.246	c.384	p.82Asn > Lys	3.15	22.85	non-synonymous	novel
P2	Y:2655474	809 G > GC	c.171	c.309	p.57Gln > His	1.54	8.91	non-synonymous	novel
	Y:2655014	1269 T > A	c.*16	c.769	…………	4.29	53.53	variante 3'UTR	rs1361161627

Table 4. Predicting pathogenic character of missense mutations with SRY gene.

SNP	Polyphen 2 (Score, 0 - 1)	UMD-Predictor (0 - 100)	Provean (cutoff = −2.5)
c.246 T > A, p.82Asn > Lys	Probably Damaging (1)	pathogen (88)	deleterious (−5.807)
c.171 G > C, p.57Gln > His	Probably Damaging (1)	Probable polymorphism (55)	deleterious (−3.345)

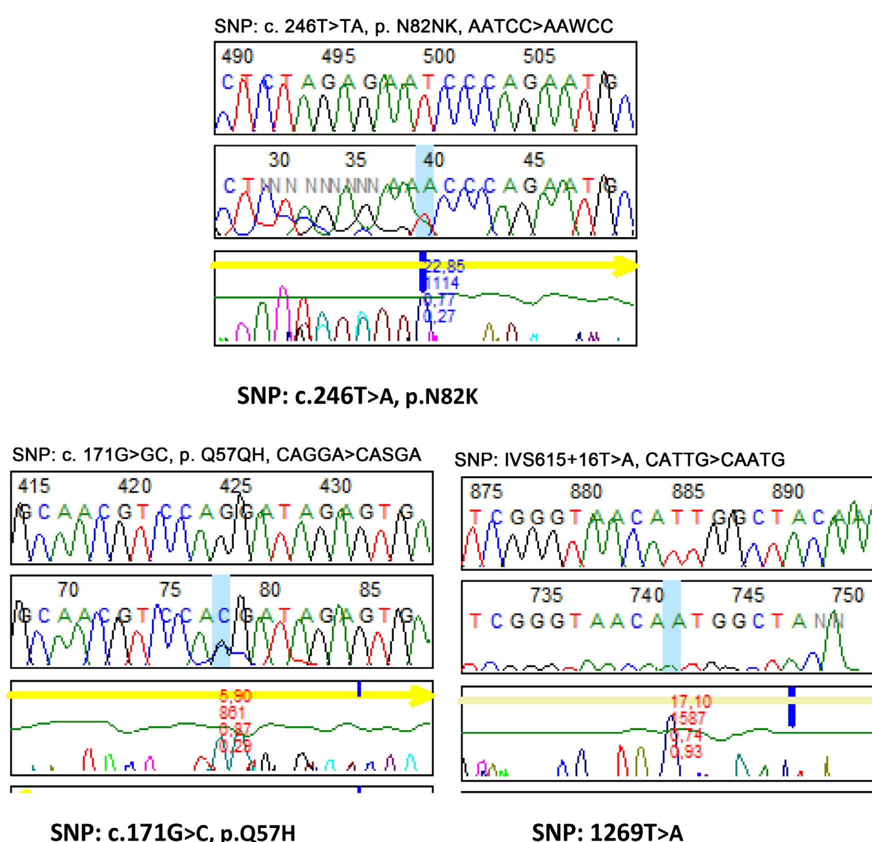

Figure 3. The SRY gene variants found in P1 and P2 patients.

variable [26]. They cause masculinization or virilization of the fetus 46, XX. Individuals with 46, XX (SRY+) rarely have atypical genitals and are less likely than individuals with 46. XX (SRY) to have gynecomastia [27]. This is the case of P2 patient with atypical OGE (penis, clitoris). This presence of the SRY gene protein, TDF (Testis Determining Factor) in all 5 patients (P1, P2, P3, P4 and P5) of karyotype 46, XX, suggests the translocation of the Y chromosome or a small fragment, including this SRY locus, on the X chromosome, which is due to

recombination during paternal meiosis [5]. When SRY was discovered in 1990, researchers had to demonstrate that the gene encodes a DNA-binding protein which would fit well with the prediction that the sex-determining gene would be a principal regulator that would activate many downstream pathways leading to testicular development (P3, P4, P5) [12]. Indeed the masculinization of a fetus 46, XX concerns the OGE ranging from a simple clitoral hypertrophy as observed in patients P3 and P5 up to the appearance of a complete male phenotype [28] with or without palpable gonad as we can see for patient P1. The latter can be referred to the male syndrome still called Chapel syndrome which according to the clinical picture already defined at the top [29] having a variant $c.246\ T > A$, $p.82Asn > Lys$ with an uncertain significance. The 46, XX DSD testicular syndrome which is the classic form *i.e.* 46, XX normal male (P1) has been reported [5] [29]. Most often the diagnosis is made during puberty or adult due to infertility [30] because there is no warning sign in advance except cryptorchidia [31]. Patients with ambiguous genitalia (clitoral hypertrophy and penis/clitoris) and SRY-positive (P2 and P3) are usually detected at birth. The variant $c.171G > C$, $p.57Gln > His$ (probable polymorphism) near the HMG (high mobility group) box is found in the patient with the phenotype clitoral hypertrophy with presence of testis. Speaking of ovotesticular DSDs which concern only about 10% of individuals 46, XX DSD [5] [9], only 15% are positive for SRY. These ovotesticular disorders of sexual development formerly known as true hermaphroditism can be defined as the presence of testicular and ovarian tissue in an individual [28] [32] as our P5 (testis on one side and ovary on the other). Most often, about 20% of affected individuals are diagnosed before the age of 5 years and have most of the female OGI (uterus, hemi-uterus or rudimentary uterus) [5]. The variability of phenotypes observed in XX individuals carrying SRY can be explained by a more or less important inactivation of the region of 1'X carrying SRY [33] [34]. However, in the absence of the SRY gene, other causes such as SOX9 duplication, mutations in the RSPO1 or NR5A1 genes may also be responsible for ovotesticular 46, XX DSD [35] [36]. A final and rather peculiar observation concerns a patient who, on echography, reveals a feminizing testis syndrome with two ectopic testicles in an inguinal position and who is SRY positive. In fact, the dysfunction of the androgen receptors causes sexual development disorders even in the presence of the SRY gene, which according to [37] in individuals 46, XY. In these patients, there is testosterone secretion but it's ineffective, which results in the total or partial absence of the male type of external genital and the location of the testicles is internal (**Figure 1(a)**). At puberty the breasts develop but with primary amenorrhea as is our patient. However according to [38] individuals 46, XX may be carriers. Sequencing of the SRY gene for this patient came back normal.

5. Conclusion

The identification of sex developmental disorders follows a well-defined strategy

ranging from clinical orientation to molecular cytogenetic techniques. This combination allows for better clinico-genetic analysis and matching. The complexity of the gene interactions that drive the development of the bipotential gonad to a testis or ovary is increasingly recognized. In this study, we have tried to distinguish the phenotypes observed (type 46, XXDSD with positive SRY) from the genotype in patients seen at CHU LE Dantec and which constitute a real public health problem. This is confirmed by the studies of DiNapoli and Capel (2008) where only 10% of sex reversal cases are linked to mutations in SRY, indicating that other genes must also play an essential role in the pathway of sex determination [39] hence the importance of prenatal diagnosis. However, conventional cytogenetics alone cannot meet all the challenges of nature and in our context, the next step will undoubtedly be to highlight chromosomal micro rearrangements responsible for the various anomalies of sexual development without forgetting the multidisciplinary management. The sequencing of the DAX1 and SOX9 genes is in progress.

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Author Contributions

Fatou Diop GUEYE and Arame NDIAYE: data acquisition and redaction of draft; Mame Venus GUEYE: clinical data acquired; Macoura Gadji, Adji Dieynaba DIALLO and Ndiaga DIOP: draft reading and corrections; Oumar FAYE and Mama SY DIALLO: scientific supervision of work. All co-authors revised the manuscript.

Data Availability Statement

All data acquired in the frame of this study will be available upon request to Fatou Diop Gueye:gfatoudiop@gmail.com.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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