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ORIGINAL RESEARCH

Genetic Analysis Reveals Complete Androgen Insensitivity Syndrome in Female Children Surgically Treated for Inguinal Hernia

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ABSTRACT

Background: Complete androgen insensitivity syndrome (CAIS) is a congenital condition caused by genetic defects in the androgen receptor (*AR*) gene located on the X chromosome, which lead to a phenotypical female individual with a 46, XY karyotype. Early diagnosis of CAIS is essential for proper clinical management, allows assessment of familial risk and contributes to healthcare decisions. However, diagnosis of CAIS can be overlooked in girls with inguinal hernia, resulting in inappropriate management. *Methods:* Five female patients from three unrelated families presented to our genetic clinic with primary amenorrhea. Each patient had been diagnosed with inguinal hernia in childhood and had undergone hernia repair without further investigation into what was contained in the hernial sac. We carried out physical examination, cytogenetic studies, hormonal evaluation, and molecular analysis to establish a comprehensive diagnosis. Family history and pedigree were collated to identify at-risk family members. *Results:* All patients presented with female external genitalia. Cytogenetic studies revealed a 46, XY karyotype and hormonal analysis suggested a diagnosis of CAIS. Sequencing of the *AR* gene in all patients and suspected family members revealed pathogenic variants in the *AR* gene and confirmed the molecular diagnosis of CAIS. *Conclusions:* We report the delayed diagnosis of CAIS in female Indonesian patients with a history of inguinal hernia in childhood. An early diagnosis of CAIS is essential for appropriate clinical management, as well as assessing familial risk. Increasing awareness among clinicians is paramount, and we encourage a CAIS diagnosis to be considered in any patient presenting with female appearance and inguinal hernia.

Keywords: androgen insensitivity syndrome; inguinal hernia; hernia repair; genetic testing

INTRODUCTION

Androgen Insensitivity Syndrome (AIS; testicular feminization; OMIM #300068) is an X-linked genetic disorder presenting with various degree of under virilization in 46, XY patients [1]. Clinical presentation in AIS patients depends on the severity of androgen resistance and patients are mainly classified as three different categories: complete (CAIS), partial (PAIS), and mild (MAIS) form. Phenotypes in PAIS have been described as ranging from a predominantly male phenotype with micropenis, severe hypospadias, and/or cryptorchidism to predominantly female phenotype with mild virilization while MAIS is characterized by gynecomastia at the onset of puberty and infertility [1]. The prevalence of CAIS is reported to be between two in 100,000 to five in 100,000 live births while the incidence of MAIS and PAIS varies [2].

As the most severe manifestation of androgen insensitivity, CAIS presents with a 46, XY karyotype

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and external female genitalia, with testes located in inguinal or abdominal region. If the condition is not diagnosed at birth, patients can present with unilateral or bilateral inguinal hernia in childhood, or primary amenorrhea during puberty with breast development and pubertal growth spurts at the appropriate age [1]. CAIS is caused by variants in the *Androgen Receptor* gene (*AR* OMIM #313700), which results in complete resistance to the biological effects of androgens in 46, XY individuals with normal testis development [1]. The *AR* gene located at Xq11-12, has eight exons and encodes a 919 amino acid protein comprising of four major functional domains: the N-terminal domain (NTD, encoded by exon 1), the central DNA-binding domain (DBD, encoded by exons 2 and 3), a C-terminal ligand-binding domain (LBD, encoded by exons 4–8) and a hinge region connecting the LBD to the DBD [3]. To date, over 800 different pathogenic variants have been identified in *AR* gene [4].

The association between CAIS and inguinal hernia in pre-pubertal girls has been known for over 60 years [5]. Clinicians treating inguinal hernias during childhood may have the first opportunity to diagnose CAIS, allowing early genetic counseling and comprehensive management. However, a lack of awareness of the possibility of CAIS can lead to mis-diagnosis and delayed management. For example, hernia repairs in girls without further investigation, such as karyotyping or evaluation of the gonad/testis in the inguinal sac, is an example of this. Here, we report the late CAIS diagnosis and clinical management of five Indonesian patients from three unrelated families who were phenotypically female children that underwent hernia repair without further investigation.

MATERIALS AND METHODS

Patients with primary amenorrhea were referred to our center. This study was approved by Institute Research Board in our institute. All of the patients (or parent/guardian) have given written informed consent prior to their participation in this study.

Clinical data including medical history, age of initial diagnosis, gender assigned, family history, and consanguinity were obtained. All patients had a physical examination in order to rule out other syndromes associated with genital anomalies. Clinical evaluation by expert clinicians included a detail description of labioscrotal folds, genital tubercle, number and location of the perineal openings and gonads. A blood sample was obtained for karyotyping, hormonal analysis, and DNA extraction. G-banding cytogenetic analysis of peripheral blood lymphocytes was performed for all patients in our laboratory. Serum concentrations of follicle stimulating hormone (FSH), luteinizing hormone (LH), and total testosterone (T) were measured by commercially available immunoassays. Genomic DNA was extracted from peripheral EDTA-blood samples using the salting out method [6].

TABLE 1. Primers sequences used for PCR reaction amplification of *AR* gene.

Exon		Sequence (5′ –> 3′)
1.1	F	GAAGTAGGTGGAAGATTCAGCC
1.1	R	TGGTGTAACCTCCCTTGAAAG
1.2	F	CGAATGCAAAGGTTCTCTGCT
1.2	R	CGAAAGGCGACATTTCTGGAA
2	F	GCCTATTTCTGCCATTCAGTGAC
2	R	GGGCCCTGAAAGGTTAGTGTC
3	F	TGTTTGGTGCCATACTCTGTC
3	R	CCTGTGTCTAGAGCATGGCT
4	F	AATTCAAGTCTCTCTTCCTTCCC
4	R	CTCATGCTCCCACTTCCCTTT
5	F	CCGTCAGTACCCAGACTGACCA
5	R	TCTGGCCAAGCTGCTGTATTTT
6	F	GGCAATCAGAGACATTCCCTC
6	R	CCTCTCTGAATCTCTGTGCCC
7	F	CTTTGTCTAATGCTCCTTCGTG
7	R	CCCTCCATCGTTTGCTTAC
8	F	ACCTCCTTGTCAACCCTGTTT
8	R	AAGGCACTGCAGAGGAGTAGT

Three patients (family two and three) were sequenced by Sanger sequencing methods and two patients (family one) were analyzed by Massively Parallel Sequencing (MPS). The MPS targeted DSD gene panel was used as previously described [7]. For Sanger Sequencing, all eight exons of the *AR* gene were amplified by polymerase chain reaction (PCR) using the primers listed in Table 1. The amplifications were carried out in a total volume of 20 μl, containing 1 μl genomic DNA (approximately 50–100 ng/ml), 0.5 μl of each forward and reverse primers (0.25 mM), 4 μl HF buffer, 0.4 μl dNTPs, 0.2 μl Phusion (NEB, Ipswich, Massachusets, USA), and 13.4 μl sterilized distilled water. The PCR was performed using an initial denaturation step at 98 °C for 30 seconds, followed by 35 cycles of denaturation at 98 °C for 10 seconds, annealing at 64 °C for 15 seconds, and extension at 72 °C for 30 seconds, and a final extension at 72 °C for 5 min (GeneAmp 9700; Applied Biosystems; Thermo Fisher Scientific, Inc). PCR products were examined by 1% agarose gel electrophoresis for the presence and sizes of amplicons, the DNA was visualized using GelRed (Biotium, CA, USA). Sequencing reactions were performed at the Australian Genomics Research Facility (AGRF). DNA variant numbering is based on GenBank reference DNA sequence NM_000044.3, with the A of the ATG initiation codon designated +1. Predicted protein annotations are based on NP_000035.2.

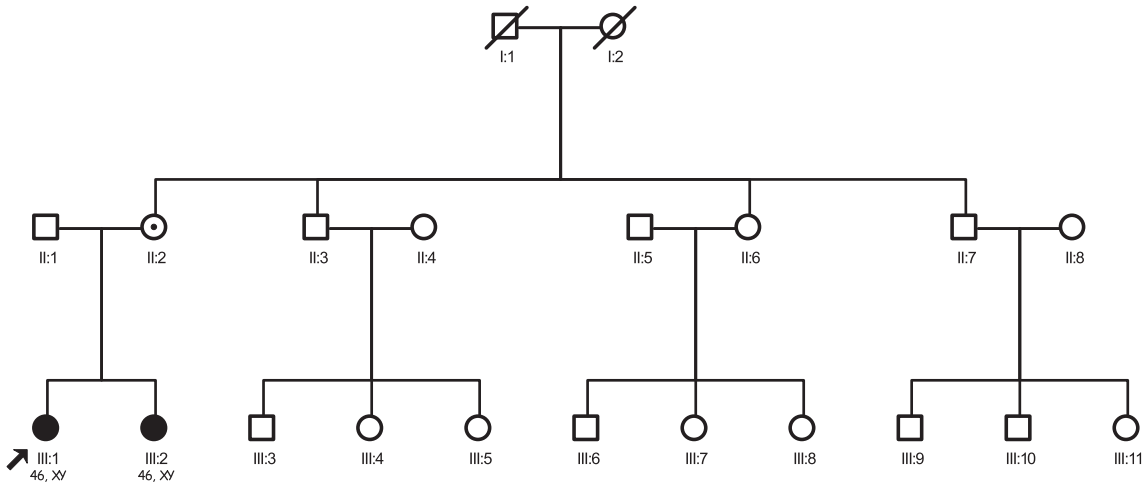

FIGURE 1. Pedigree of family 1 (case 1 and 2). The arrow denotes the proband (III:1) and her affected sister (III:2). The mother (II:2) is a heterozygous carrier.

RESULTS

Case Presentations

Family 1. In family 1 (see Figure 1), the proband (F1.III:1) was a 20-year-old female, who was referred to our clinic for primary amenorrhea. Physical examination revealed normal breast development, absence of axillary hair, female external genitalia with scarce pubic hair. Bilateral inguinal solid palpable masses resembling gonads were identified. Abdominal and pelvic ultrasonography showed the absence of a uterus and ovaries, and the presence of isoechoic oval structure in both inguinal regions consistent with testicles. The serum hormone concentrations were as follows: testosterone: >1,500 ng/dL (normal: 8.40–48.1); LH: 19.01 IU/L (normal: 1.7–11); FSH: 13.64 IU/L (normal: 1.25–8.9).

Her 12-years-old sister (F1.III:2) presented with unilateral inguinal hernia at 2 years of age, and surgery for a right inguinal mass by a general surgeon in a rural area was undertaken when she was 11 years old. No histological analysis or follow up was performed. Based on the pedigree construction, we then invited her for further evaluation. Physical examination revealed female external genitalia, a scar on the right inguinal area and she was in the pre-pubertal age. The chromosomal analysis of (F1.III:2) revealed a 46, XY karyotype. Serum concentrations of testosterone: 431.7 ng/dL (normal: <2.5–6.12); LH: 4.93 IU/L (normal: 0.7–2.0); FSH: 9.33 IU/L (normal: 0.38–3.6) were significantly higher than normal range.

MPS targeted DSD gene panel analysis [7] on the siblings revealed a hemizygous missense variant c.2343G > A in exon 6 (see Figure 2) of the *AR* gene. The younger sister (F1.III:2) harbored the same variant as the proband. It resulted in an amino acid

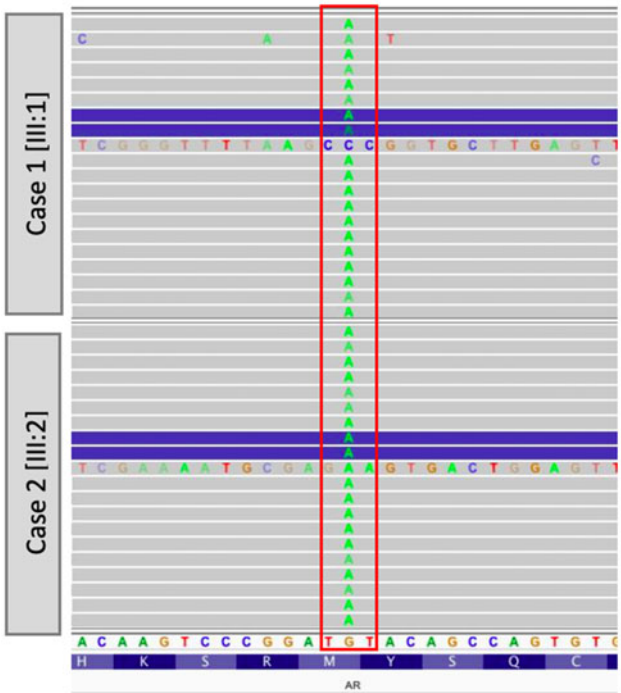

FIGURE 2. Integrative Genomics Viewer (IGV) of *AR* gene variant in c.2343G > A in siblings from family 1 (F1.III:1 and F1.III:2).

substitution of methionine to isoleucine at codon 781, (c.2343G > A; p. Met781Ile) which falls within the ligand binding domain of the *AR* gene. Sanger sequencing showed the mother was heterozygous for the change (F1.II:2) (see Figure 3 and Table 2), confirming maternal inheritance. These data are consistent with a previous report describing this variant which possesses a loss of receptor function leads to CAIS [8].

Family 2. The third case was a 25-year-old female who was referred to our gender team with primary

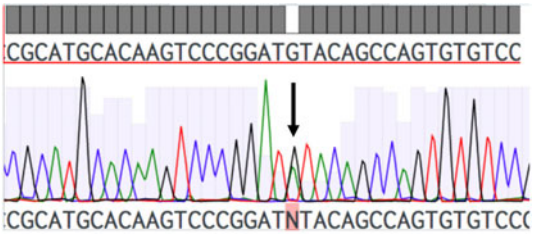

FIGURE 3. Partial sequence of the *AR* gene confirmed the presence of the variant in the mother of Family 1 (F1.II:2) indicating a heterozygous G to A change at nucleotide 2343 (arrow).

amenorrhea. She had no family history of primary amenorrhea or infertility (see Figure 4). At 8 months of age, the patient had presented with bilateral palpable masses in the groin that swelled during crying and was diagnosed with bilateral inguinal hernia and underwent a hernia repair. The clinician did not perform any further examination and the patient had no other major complaints until she reached puberty. At puberty, the patient presented with normal female external genitalia, normal breast development (Tanner 5) and an absence of axillary and pubic hair were observed during the physical examination. An ultrasound revealed the absence of a uterus and no gonads were clearly visualized. Hormonal evaluation revealed FSH: 14.9 IU/L (normal: 0.95–11.95); LH: 27.5 IU/L (normal: 0.57–12.07) and testosterone: 586.32 ng/dL (normal: 142.39–932.14). The chromosomal analysis revealed a 46, XY karyotype.

Sanger sequencing analysis of the proband (F2.III:4) confirmed a hemizygous G to A change in the exon 3 of the *AR* gene, which leads to the replacement of arginine with histidine at amino acid position 616, the change falls in the second zinc finger DNA-binding domain (c.1847G > A; p.Arg616His). Sanger sequencing showed the mother (F2.II:6) was negative for the change (see Figure 5 and Table 2), suggesting this variant was *de novo*. This variant was previously reported as a pathogenic mutation associated with CAIS [9–11].

Family 3. A 29-year-old married female (F3.III:8) was referred to our hospital with primary amenorrhea. There was a history of prior medical consultation with no clear diagnosis. She had a history of inguinal hernia repair in childhood, but without pathology analysis and no further evaluation. A swelling was palpated during the procedure, presumed to be a bowel structure, and placed back into the abdomen through the inguinal canal that was then closed. Subsequently, the patient sought medical consultation again when she reached pubertal age because of primary amenorrhea. Ultrasound revealed the absence of female internal organs and an unidentified ovary-like structure. When she was 28-year old, she was admitted to the emergency department complaining of abdominal pain in the right region which came on suddenly during morning physical exercise. The clinician diagnosed her with right cyst torsion. Subsequently, emergency surgery had been performed and the specimen which was suspected of being the cyst torsion structure was taken for histology. Pathology examination identified a *rete testis* structure.

On further investigation, physical examination revealed breasts were Tanner stage 5, with absence of axillary and pubic hair. She has a short vagina with absence of clitoromegaly. No uterus and ovaries were identified on pelvic ultrasound along with the presence of bilateral gonads in the inguinal canals. Hormone investigation revealed increased levels of testosterone: 784 ng/dL (normal: 100–900) and LH: 14.89 IU/L (normal: 3.9–12) while FSH: 2.23 IU/L (normal: 1.5–8) was within normal range. Her karyotype was 46, XY. Pedigree construction revealed she had a 20-year-old cousin (F3.III:11) with primary amenorrhea who passed away with presumptive diagnosis of ovary cancer prior to chromosomal analysis. She also had an aunt (F3.II:18) and 17-year-old cousin (F3.III:23) who had primary amenorrhea (see Figure 6). Hence, a diagnosis of familial CAIS was considered for this patient. Molecular analysis of the *AR* gene was conducted for the patient and her cousin. We identified a hemizygous variant c.2117A > G in exon 4 (see Figure 7 and Table 2). In both, the substitution was inherited from the heterozygous mother. This variant leads to a p.Asn706Ser change in the ligand-binding domain which has been previously reported in CAIS and PAIS cases [4].

DISCUSSION

These cases illustrate the complexities associated with complete androgen insensitivity syndrome (CAIS). In particular, we find that female infants diagnosed with inguinal hernia should have further investigation into a possible diagnosis of CAIS, something previously suggested [12]. We also show how a timely diagnosis of CAIS may result in subsequent testing within family. In this report, all of the presenting patients had had inguinal hernias with palpable gonads in childhood but were only diagnosed with CAIS during puberty. Unfortunately, the surgeons who performed the hernia repair did not carry out further investigation of the inguinal hernia, such as ultrasound to evaluate the content of the hernial sac and the Mullerian structures, histological confirmation of the gonads, immunohistochemistry, karyotyping, hormonal analysis, or

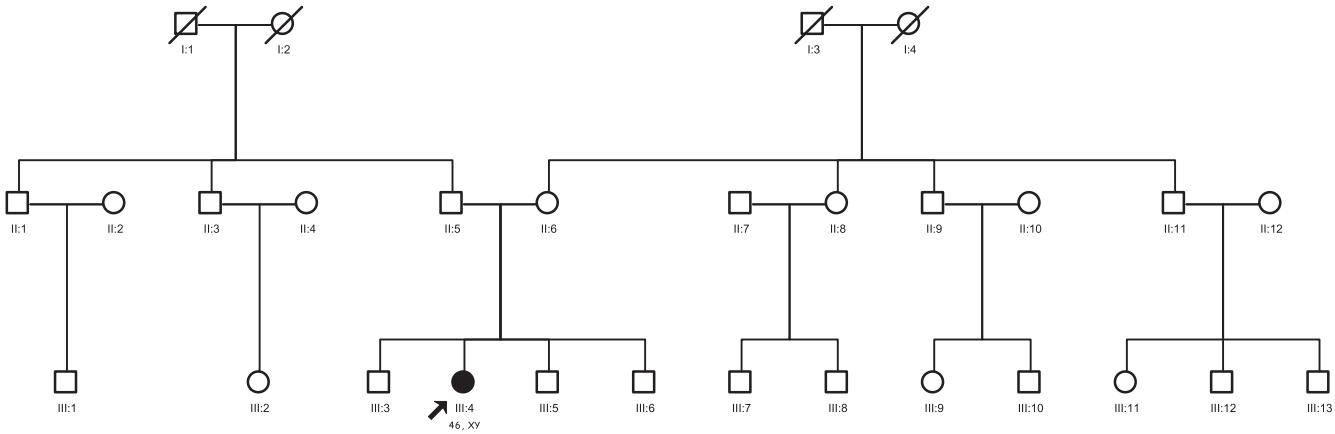

FIGURE 4. Pedigree of Family 2 (case 3). The arrow denotes the proband (III:4).

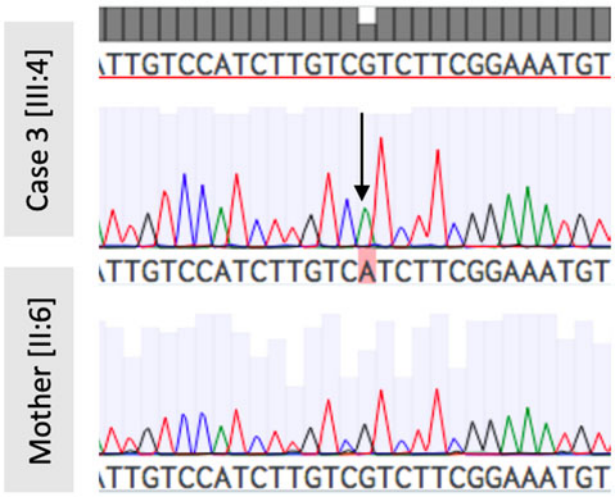

FIGURE 5. Partial sequence of the *AR* gene from affected individual from family 2 (F2.III:4) and her mother (F2.II:6). The mother showed a normal sequence while the patient showed a pathogenic variant c.1847G > A (arrow).

molecular analysis. General surgeons who are not familiar with the occurrence of CAIS may overlook gonadal features during hernia repair. When performing hernia repair in girls with inguinal hernia, clinicians need to identify atypical gonadal features macroscopically so that further investigation could be carried out [13]. However, the reluctance to do further investigations, may result from a lack of awareness about this condition because of its relatively low prevalence [14,15]. Indeed, in females with a bilateral inguinal hernia the incidence of CAIS is estimated to be 1%–2% [16].

Hormone levels such as gonadotrophin and androgens are evaluated when AIS is suspected. Patients with AIS classically show an elevated LH and testosterone serum level representing the hormone-resistant state in AIS. This demonstrates the impaired negative feedback of anterior pituitary hormone secretion [17]. However, patient III.2 in family 1, had an elevated FSH serum concentration. This may occur due to damage to the seminiferous tubules since the testes are located in the inguinal canal, which would result in impaired inhibin B feedback, causing higher levels of FSH [18]. As inguinal hernia is the most common presentation of CAIS in childhood, the diagnosis of CAIS and indeed other 46, XY disorders of sex development should be considered in all female infants with inguinal swelling containing gonads [15].

Approximately two-thirds of CAIS cases are inherited in an X-linked manner, however, *de novo* variants in the germ or egg cells can cause this disease without any family history [19]. A carrier mother who has an altered copy of the *AR* gene on one of their two X chromosomes will present with a normal phenotype due to X chromosome compensation. She is therefore at risk of passing the faulty gene to her affected 46, XY offspring, who would be affected, or to her 46, XX daughters, who would in turn be carriers. AR variants can also arise *de novo* – in the mother's egg cell before the fetus is conceived or during early fetal development. The pathogenic variants in all but case 2 were inherited from the heterozygous mother, while in case 2 a *de novo* variant was found. In the familial cases, our genetic findings have facilitated genetic counseling in patients and families. A molecular diagnosis also provides an increased understanding of the pathophysiology, impaired reproductive function, and supports medical management such as gonadectomy and hormonal therapy.

An early diagnosis including molecular testing of CAIS is also important to rule out the risk of gonadal malignancy. Increased risk of malignant transformation of the gonads is well documented in phenotypic female patients with Y chromosomes. The malignancy in CAIS arise from seminoma of the testis or various non-seminomatous tumors, with the risk of malignancy around 1% [20]. Given the possibility of malignant

TABLE 2. Summary of androgen receptor gene mutations in Indonesian patients with complete androgen insensitivity syndrome.

Family	Base change	AA change	Location	Type of mutation	Heterozygote carriers	Other members affected	Previous study	Clinvar
1	c.2343G > A	p. Met781Ile	Exon 6	Missense	Mother	Sister	Jakubiczka *et al*. 1997	Pathogenic
2	c.1847G > A	p.Arg616His	Exon 3	Missense	De novo variant	None	Mowstowicz *et al*. 1993 Brown *et al*. 1993 Hiort *et al*. 1996	Pathogenic
3	c.2117A > G	p.Asn706Ser	Exon 4	Missense	Mother	1 Maternal aunt	Gottlieb *et al*. 2012	Pathogenic

AA: Amino acid.

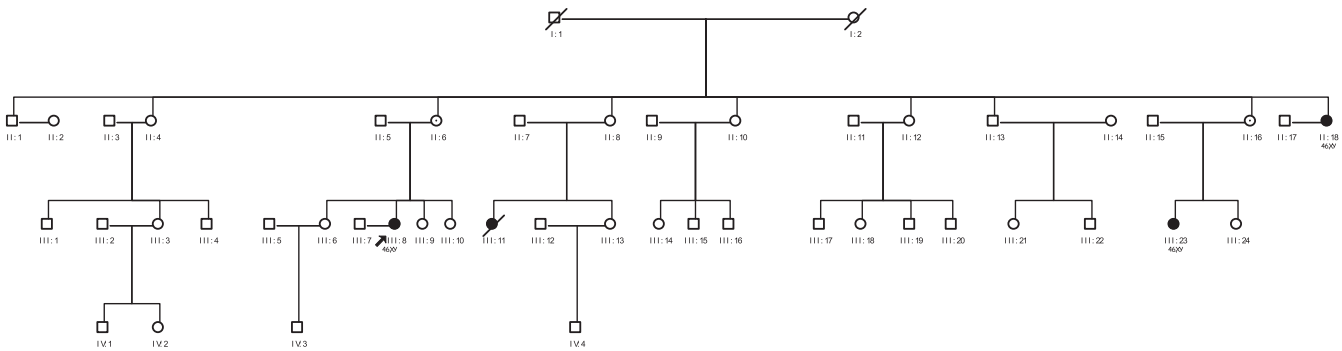

FIGURE 6. Pedigree of family 3 (case 4 and 5). Affected individuals were shown as filled symbols and the arrow pointed to the proband.

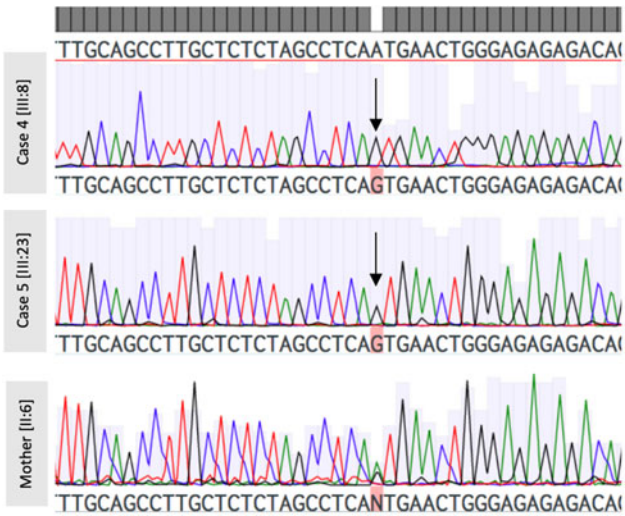

FIGURE 7. Partial sequence of the *AR* gene from affected individuals of family 3 (F3.III:8, F3.III:23). The mother of case 4 (F3.II:6) is heterozygous for the pathogenic variant c.2117A > G.

transformation, gonadectomy is recommended soon after puberty has been achieved [1]. However, some patients are reluctant to undergo gonadectomy, preferring to keep their gonads [21] as conversion of testosterone produced by testes to endogenous estrogen (via aromatization) is important for growth and spontaneous female puberty [22]. Therefore, in these cases, the clinicians need to assess the risk of germ cell malignancies in these patients, and to monitor for any changes. A testicular biopsy followed by immunohistochemistry analysis using OCT3/4 staining is one way of evaluating this risk [20].

In summary, an inguinal hernia during childhood can be a clear early sign of CAIS. In cases of inguinal hernia, we urge clinicians to carry out appropriate examination to establish the definitive diagnosis and guide comprehensive management. In Indonesia, especially in rural areas, a karyotype should be requested as a first-tier test [23]. A thorough medical history, a cautious physical examination to discover the location and structure of the gonads should be considered. In addition, imaging to evaluate the hernia sac and other internal structures, hormonal assays, immunohistochemistry testing, cytogenetic analysis, and molecular studies such as *SRY* and *AR* gene sequencing are all suggested to establish a definitive diagnosis [1]. This comprehensive diagnostic procedure for CAIS in patients presenting with inguinal hernia will reduce mis-diagnosis and when combined with increased awareness among medical professionals, will ultimately lead to improved clinical management and health outcomes for patients and their families.

DECLARATION OF INTEREST

The authors whose names are listed certify that they have no conflict of interest. All of the patients (or parent/guardian) have given written informed consent prior to their participation in this study.

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ETHICAL CLEARANCE
No. 24/EC/FK-RSDK/I/2017

Komisi Etik Penelitian Kesehatan Fakultas Kedokteran Universitas Diponegoro-RSUP. Dr. Kariadi Semarang, setelah membaca dan menelaah Usulan Penelitian dengan judul :

CLINICAL, CYTOGENETIC AND MOLECULAR ANALYSIS OF 46, XY DSD WITH GONADAL DYSGENESIS AND 46, XX TESTICULAR DSD PATIENTS

Peneliti Utama : ***dr. Nurin Aisyiyah Listyasari***

Pembimbing : 1. Prof. dr. Sultana MH Faradz, PhD
2. dr. Achmad Zulfa Juniarto, Sp.And, PhD

Penelitian : Dilaksanakan di Pusat Riset Biomedik (CEBIOR) FK UNDIP Semarang

Setuju untuk dilaksanakan, dengan memperhatikan prinsip-prinsip yang dinyatakan dalam Deklarasi Helsinki 1975, yang diamended di Seoul 2008 dan Pedoman Nasional Etik Penelitian Kesehatan (PNEPK) Departemen Kesehatan RI 2011

Penelitian harus melampirkan 2 kopi lembar Informed Consent yang telah disetujui dan ditanda tangani oleh peserta penelitian pada laporan penelitian.

Peneliti diwajibkan menyerahkan :
- Laporan kemajuan penelitian (*clinical trial*)
- Laporan kejadian efek samping jika ada
- Laporan ke KEPK jika penelitian sudah selesai & dilampiri Abstrak Penelitian

Semarang, 19 JAN 2017

Komisi Etik Penelitian Kesehatan
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