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Novel candidate genes for 46,XY gonadal dysgenesis identified by a customized 1 M array-CGH platform☆

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ABSTRACT

Half of all patients with a disorder of sex development (DSD) do not receive a specific molecular diagnosis. Comparative genomic hybridization (CGH) can detect copy number changes causing gene haploinsufficiency or over-expression that can lead to impaired gonadal development and gonadal DSD. The purpose of this study was to identify novel candidate genes for 46,XY gonadal dysgenesis (GD) using a customized 1 M array-CGH platform with whole-genome coverage and probe enrichment targeting 78 genes involved in sex development. Fourteen patients with 46,XY gonadal DSD were enrolled in the study. Nine individuals were analyzed by array CGH. All patients were included in a follow up sequencing study of candidate genes. Three novel candidate regions for 46,XY GD were identified in two patients. An interstitial duplication of the *SUPT3H* gene and a deletion of *C2ORF80* were detected in a pair of affected siblings. Sequence analysis of these genes in all patients revealed no additional mutations. A large duplication highlighting *PIP5K1B*, *PRKACG* and *FAM189A2* as candidates for 46,XY GD, were also detected. All five genes are expressed in testicular tissues, and one is shown to cause gonadal DSD in mice. However detailed functional information is lacking for these genes.

1. Introduction

During embryonic development, the gonad is initially formed as bipotential. At gestational week 7–8 sex determination occurs, when the somatic and primordial germ cells of the gonad differentiate into ovarian or testicular cell types depending on the predominant activation of the *RSPO1*/*WNT4* or *SOX9*/*FGF9* signaling pathways [1,2]. The *SRY* gene on the Y chromosome initiates the differentiation towards testicular development by promoting *SOX9* expression [3,4]. The testes secrete androgens that lead to the formation of male internal and external genitalia, and antimüllerian hormone (AMH) that causes regression of Müllerian structures [5,6]. In absence of androgen signaling the external genitalia develop into the female pattern [7].

Errors along this pathway will lead to different disorders of sex development (DSD). One form of gonadal DSD is gonadal dysgenesis (GD) where testes or ovaries have failed to develop and in the complete form, only streak gonads are present. Affected patients present as females with normal female internal and external genitalia regardless of chromosomal sex. Dysgenetic gonads in 46,XY subjects are at high risk of malignant transformation, thus prophylactic gonadectomy needs to be considered [8–10].

There are several genes described that can cause 46,XY GD when mutated. The most frequent genetic defects are *SRY* mutations/deletions found in approximately 10–15% of all cases [11]. Mutations of *SOX9*, *NR5A1* and *WT-1* are reported in GD [12–14], often in association with other symptoms such as campomelic dysplasia, adrenal disturbances, and kidney defects, respectively. *NR5A1* mutations can present with a wide spectrum of DSD, from hypospadias to complete GD [15]. Mutations of other genes, e.g. *DHH* and *TSPYL1* have been reported in a few cases [16,17]. In addition, copy

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number changes affecting *NR0B1*, *WNT4* and *DMRT1* can cause GD, either in isolated form, or with associated features depending on the extent of the duplication/deletion [18–21].

Despite advances in the understanding of the genetic basis of sex development and increased diagnostic possibilities, only approximately half of all DSD patients receive a molecular diagnosis [22,23]. It is therefore likely that several yet unknown factors are involved in normal gonadal development. A common mechanism among factors causing gonadal DSD is a dosage effect, where impaired or abnormal gonadal development is caused by gene haploinsufficiency due to inactivating mutations or gene deletions, or by gene over-expression due to gene duplications. Genes found to affect gonadal development are located not only on the sex chromosomes, like *SRY* and *NR0B1*, but also on autosomes [24]. Therefore, array-comparative genomic hybridization (CGH) represents a useful and powerful technique to identify submicroscopic genomic imbalances that could cause GD, on a genome wide scale. In addition to genes already known to be involved in gonadal development, identified gains or losses could also include novel genes, thus unraveling new factors involved in gonadal development.

The purpose of this study was to identify new potential candidate regions involved in gonadal development by detecting submicroscopic genetic imbalances in patients with complete 46,XY GD. Using a customized 1 M array-CGH platform with whole genome coverage and 2.2 kb average probe spacing, we expected to find very small copy number alterations. The high resolution is important as even small, single or partial gene dosage alterations can cause gonadal DSD [25,26]. Our platform is enriched with probes targeting 78 genes involved in gonadal development. This allows for rapid screening of known candidate genes at increased resolution. This array-CGH platform has the highest resolution compared to other platforms for detection of copy number changes that have previously been applied to patients with XY GD. In addition to single reports [18,27], also three studies with larger groups of patients presenting with various forms of DSD have been described [23,28,29], resulting in the identification of known and potentially causative aberrations in approximately a third of the patients (36%, 22% and 21.5% respectively).

Using a customized 1 M array-CGH platform, we investigated a selected group of patients with complete GD for novel copy number changes potentially causing gene haploinsufficiency or over-expression. Candidate genes identified by array-CGH, where the patient has a likely haploinsufficiency, were further investigated by sequencing in the entire group to identify possible loss of function mutations.

2. Materials and methods

2.1. Patients

14 patients with 46,XY gonadal DSD were selected for inclusion in the study among the DSD patients referred to the clinical genetic laboratory of Karolinska University Hospital, Stockholm, Sweden. The group consisted of 13 patients with 46,XY GD, including a pair of affected siblings. We also investigated one patient with 46,XY ovotesticular DSD. Of these 14 patients, DNA samples of good quality for array-CGH analysis were available from nine unrelated patients with 46,XY GD. All 14 patients were included for the follow up study of candidate genes. The regional Ethics Committee at the Karolinska Institutet, Sweden, approved the study and all participants gave written informed consent.

The clinical diagnosis of GD was based on a XY karyotype, female external genitalia, internal Müllerian structures (uterus) and hypergonadotropic hypogonadism. In addition to GD, one patient also presented with neuropsychiatric problems, cleft palate and juvenile chronic arthritis. The other patients had isolated complete GD. The patient with 46,XY ovotesticular DSD presented with an XY karyotype, female external genitalia as well as a small phallus, uterus, one gonad resembling a testicle, and one gonad with both testicular and ovarian tissue.

Sex chromosome mosaicism was excluded using fluorescent *in situ* hybridization (FISH) with DNA probes from chromosome X and Y on peripheral blood smears and when available, on touch preparations from gonadal tissue. Sequence analysis of *SRY*, *NR5A1*, *WT1*, and MLPA analysis targeting the 9p24 region, *NR5A1*, *SRY*, *SOX9*, *WNT4*, *DHH*, *WT1*, *NR0B1* and other DSD genes [25,30,31] were performed in all patients.

2.2. DNA extraction

DNA was extracted from peripheral blood lymphocytes. All samples were analyzed for concentration and quality using a Nanodrop 1000 spectrophotometer. Some samples were further purified using the QiAmp DNA minikit (QIAGEN), to achieve acceptable values for array-CGH analysis.

2.3. Array-CGH

Nine unrelated patients with 46,XY GD were analyzed using our customized 1 M oligomarkers array-CGH platform developed at Oxford Gene Technology (OGT). In addition to whole genome coverage, the platform is enriched with probes targeting 78 genes implicated in sex development (Supplementary Table 1).

Preparation of labeled DNA and subsequent hybridization were performed according to the "Agilent oligonucleotide array-based CGH for genomic DNA analysis" protocol (v6.2). In short, 2.5 μg of patient or control DNA were labeled with Cy5-dUTP or Cy3-dUTP respectively, using the ENZO labeling kit (AH diagnostics) with Klenow Exo-DNA polymerase. Labeled DNA was cleaned using the QiaQuick PCR purification kit (QIAGEN). Patient and control DNAs were pooled, 50 μg of Human Cot-1 DNA (Invitrogen) were added together with blocking agent and 2× hybridization buffer (Agilent Technologies) prior to hybridization for 48 h at 65 °C in the hybridization oven (Agilent G2545A). A commercial DNA sample with pooled human genomic DNA from 10 male controls (Promega) was used as reference DNA.

Data was analyzed using the Cytosure interpret software v3.4.3 (OGT). Circular Binary Segmentation analysis to detect copy number changes was performed using the following parameters; minimum probe count: 5, threshold for gains: 0.35, threshold for losses: 0.65, chromosome average method: median segment.

By comparing data with the online Database of Genomic Variation (DGV) [32,33], common copy number variants (CNV) found in normal controls were excluded from further investigation. However, aberrations only partially overlapping with rare reported CNVs were not excluded. Small intronic variations and aberrations not affecting genes were excluded after verification that they were not located just upstream or downstream a known gene causing DSD as a positional effect should then be considered.

2.4. Database search

For all identified candidate genes the following databases were searched for information. Data, when available, is presented in the discussion section.

NCBI (http://www.ncbi.nlm.nih.gov/) including PubMed, UCSC (http://genome.ucsc.edu/) [34], GeneCards (http://www.genecards.org/), The Human Protein Atlas (http://www.proteinatlas.org/) [35], Gene expression profiles during sex determination by Dr. Serge Nef (http://nef.unige.ch/microarrays.php) [36], DECIPHER (Database of

Table 1
Array-CGH results.

Pat. no.	Chr. band	Start	End	Size (kb)	Probe count	Del/dup	Gene(s)	Inheritance
1	–	–	–	–	–	–	–	–
2	2p23.1	31452728	31707641	255	85	Dup	**XDH**, **SRD5A2**[a]	Paternal
3	6p21.1	44996834	45215062	217	104	Dup	**SUPT3H**[b]	Maternal[c]
	2q34	208743127	208765512	22	10	Del	**C2ORF80**	Maternal[c]
4	–	–	–	–	–	–	–	–
5	12q21.31	79153498	79256353	103	36	Del	**OTOGL**	N.D.
6	9q21.11	70736778	71197088	454	175	Dup	**PIP5K1B**, **PRKACG**, **FXN**, **TJP2**, **FAM189A2**[b]	N.D.
7[d]	–	–	–	–	–	–	–	–
8	–	–	–	–	–	–	–	–
9	–	–	–	–	–	–	–	–

All coordinates given in NCBI36/hg18 build Chr. Band., chromosomal band; N.D., not determined.
[a] Previously reported as normal variant.
[b] Partially overlapping with CNV.
[c] Also detected in affected sister with 46,XY GD.
[d] Presenting with neuropsychiatric problems, cleft-palate, juvenile chronic arthritis in addition to 46,XY GD.

Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources) (http://decipher.sanger.ac.uk/) [37].

2.5. MLPA (multiplex ligation-dependent probe amplification)

All copy number variations that remained after exclusion criteria were confirmed by MLPA [38]. Probes were designed according to the recommendations by Stern et al. [39] using two probes per aberration. Probes were combined in several probe sets, all including four control probes (RELN2, PCLN16, RB1 and CREBBP) and a sex chromosome specific probe (Supplementary Table 2).

MLPA reactions were performed according to the EK1 reagent kit (MRC-Holland) recommendations using 200 ng of DNA and the in-house designed probe set. The commercial DNA sample used for array-CGH and a sample from one healthy 46,XX female were used as controls.

PCR products were separated by capillary electrophoresis on an ABI 3100 genetic analyzer and trace data analyzed using GeneMapper v3.7 software (Applied Biosystems).

Trace data were exported and analyzed in an Excel 2007 spreadsheet. Each sample's peak heights were normalized to the average peak height of the reference probes and subsequently normalized to the average peak height of the control samples. The analysis was accepted if the ratio for reference probes was between 0.8 and 1.2. Threshold values for deletions and duplications were set at 0.75 and 1.25 respectively.

2.6. RNA extraction and RT-PCR analysis

Total RNA was extracted from EBV-transformed lymphocytes using the RNeasy kit (QIAGEN) including the DNAse digestion step. First strand synthesis was performed with the First-Strand cDNA synthesis kit (Amersham Biosciences) with 1 μg of total RNA and random hexamer primers in a final reaction volume of 15 μl. The DNA/RNA strand was subjected to PCR reaction with DyNAzyme EXT polymerase (Fischer Scientific) using different primer pairs (Supplementary Table 3) designed with Primer 3 software (v0.4.0) [40].

RT-PCR products were cleaned with Exonuclease 1 and Shrimp Alkaline Phosphatase (Thermo Scientific) and sequenced using the ABI BigDye Terminator v3.1 kit (Applied Biosystems) according to the manufacturer's instructions. Fragments were separated on a 3730 DNA Analyzer (Applied Biosystems).

2.7. Sequencing of C2ORF80 and SUPT3H

The 9 exons of the *C2ORF80* gene and the 13 exons of the *SUPT3H* gene, including intron/exon boundaries, were amplified by PCR using DyNAzyme EXT polymerase. Primers were designed using Primer 3 software [40] (Supplementary Table 4).

PCR products were cleaned and sequenced as described above. Electropherograms were analyzed against the reference sequence NM_001099334.2 for *C2ORF80*, NM_181356.2 and NM_003599.3 for *SUPT3H* using the SeqScape v2.5 program (Applied Biosystems).

3. Results

3.1. Array-CGH

DNA samples from nine unrelated patients with 46,XY GD were analyzed by array-CGH. A total of 455 aberrations were detected, with an average of 51 changes per patient (range 27–78) with size ranging from 3 kb to 1266 kb. Of these, 450 were excluded because they constituted common CNVs, intergenic changes or intronic variations. Four of the nine analyzed patients had novel copy number alterations, with a total of five rearrangements (Table 1). Four rearrangements constitute candidate regions for gonadal dysgenesis (Fig. 1). The fifth is a previously described duplication of the *SRD5A2* gene that is inherited from the patient's father, and is considered a benign variant [30].

3.2. MLPA

Novel copy number alterations were confirmed by MLPA. When available, parental samples were analyzed for inheritance pattern. The findings in patient 3 were also confirmed by MLPA to be present in her affected sister, and been inherited from the mother.

3.3. SUPT3H expression and duplication characterization

The duplication within the *SUPT3H* gene was detected in the pair of siblings. By MLPA, we established that it extends from exon 5 to 12. Due to the large intron size, MLPA was not considered appropriate for further characterization. We hypothesized an interstitial duplication and verified this by RT-PCR (Fig. 2). *SUPT3H* is present in two isoforms. Using isoform specific primers we verified the expression of both in control EBV-immortalized cell lines (data not shown). Subsequently we attempted to amplify the mRNA that an interstitial duplication of exon 5–12 would generate using a forward primer within exon 11 and a reverse primer within exon 5. Only an allele with the interstitial duplication would result in a product. As hypothesized, fragments were obtained from EBV-immortalized lymphocytes from the patient and her carrier mother, but not from the father or the male control. The fragments were sequenced, and confirmed to originate from a *SUPT3H* mRNA

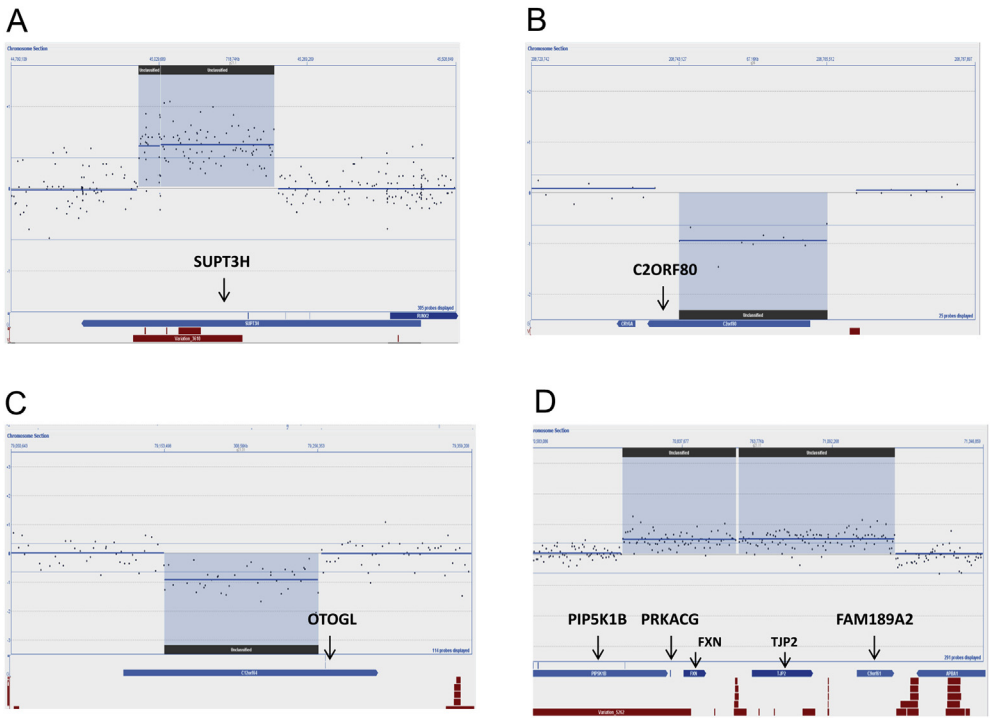

Fig. 1. Novel candidate regions. Images from Cytosure software v3.4.3 using the NCBI36/hg18 assembly. Black dots dispersed along blue horizontal lines represents oligonucleotide markers. Detected duplicated/deleted regions are marked by blue background and offset of horizontal blue line. Blue horizontal arrows on bottom of each image represent genes. Red regions below genes indicate reported CNVs in DGV. A, Interstitial duplication of the *SUPT3H* gene on 6p21.1; B, Deletion of *C2ORF80* on 2q34; C, Deletion of *OTOGL* on 12q21.31; D, Dup9q21.11.

where exon 11 is followed by exon 12 and subsequently exon 5, without intronic sequences.

3.4. Sequencing of C2ORF80 and SUPT3H

Sequencing of the *C2ORF80* and *SUPT3H* genes in 14 patients with 46,XY gonadal DSD revealed no mutations.

4. Discussion

Using a customized 1 M array-CGH platform with whole genome coverage, we have analyzed nine unrelated patients with complete 46,XY GD for submicroscopic genomic imbalances. Some samples had previously been analyzed using a BAC array-CGH platform where a *NR0B1* duplication and a 9p24 deletion were identified, and these patients were not included in this study [18,31]. All patients had also undergone MLPA analysis for detection of dosage imbalances in several known gonadal DSD genes. Therefore it was not surprising that no rearrangements affecting already known genes causing GD were detected. Of the identified dosage alterations, most represented common CNVs, intergenic changes or small intronic variations. However, to distinguish between a benign CNV and a pathogenic alteration in patients with DSD is more difficult than in patients with dominant/de novo disorders as the effect of gene dosage alteration can act in a sex-chromosome dependent way. For example *NR0B1* duplications cause 46,XY GD but have no effect on XX subjects [41]. Thus rare CNVs in the Database of Genomic Variation (DGV) must be interpreted with caution and the sex of the control material, if available, should be taken into account. In addition, CNVs affecting known gonadal genes like *NR0B1* (variation_3265), *SOX9* (variation_5028), *WT1* (variation _4753) are found in the DGV.

4.1. SUPT3H duplication on 6p21.1

A duplication within the *SUPT3H* gene (suppressor of Ty 3 homologue *Saccharomyces cerevisiae*) was detected in the affected pair of siblings. Also, a deletion affecting the *C2ORF80* gene, discussed below, was identified in both sisters. Both changes are inherited from the healthy mother. The inheritance does not exclude these regions from follow-up as gene dosage alterations causing GD in XY subjects do not necessarily affect development of female gonads. It is possible that one of these changes is a benign variant, or that the causative mechanism in this pair of siblings is by an interacting or additive effect. However, at the moment no interaction with common pathways, are known between SUPT3H and C2ORF80.

Expression data from the UCSC genome browser indicates high *SUPT3H* expression in testicular cells (Leydig, insterstitial and germ cells), and the Human Protein Atlas shows moderate expression of *SUP3TH* in cells in seminiferous ducts.

The detected duplication is 217 kb long and spans from exon 5 to 12 of the *SUPT3H* gene (Fig. 1A). The two CNVs partially overlapping this region are smaller and only reported in one out of 270 controls, without information on sex chromosome complement (variation_3610 and variation_2627). Therefore, the duplication was not excluded from further study. Using RT-PCR we have shown the duplication to be interstitial, placing exons in the order 1–12 followed by 5–13. There are two major isoforms of *SUPT3H* and the duplication affects both isoforms. The theoretical consequence on protein translation is that the two wild type proteins of 317 and 328 amino acids (aa) would be replaced by two proteins of 308 and 329 aa, where the last normal 14 aa are substituted by 4 aa (VFFR). The SUPT3H protein is well conserved in mammals, including the C-terminal region. It is therefore very difficult to predict if the detected terminal change affects protein function or stability.

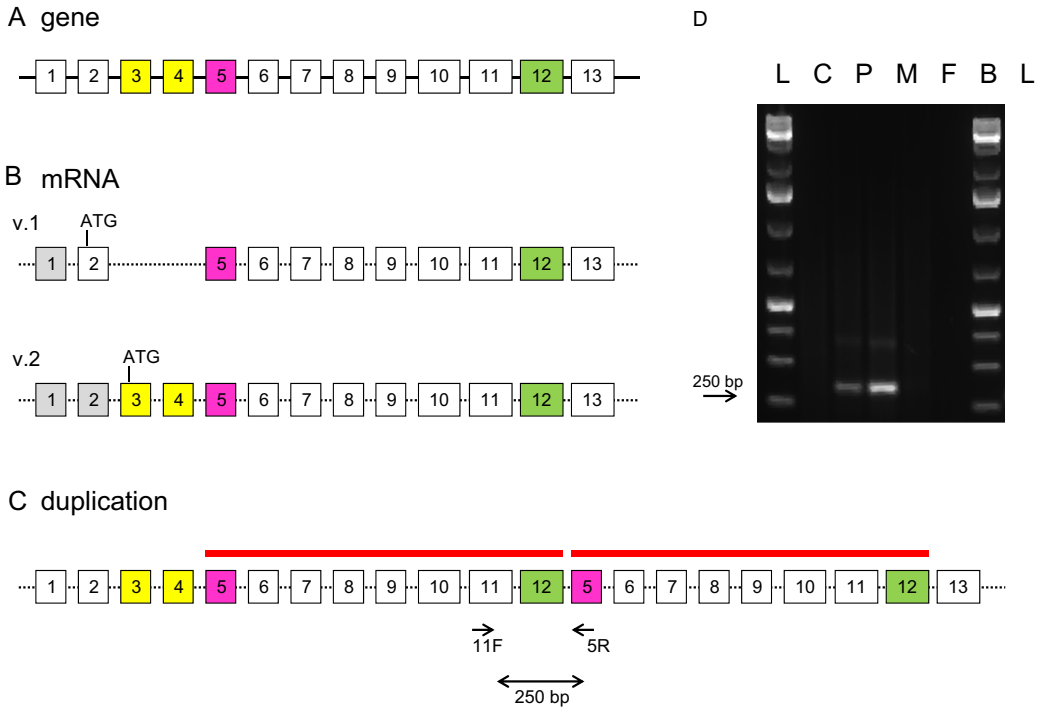

Fig. 2. *SUPT3H* expression and duplication characterization. A, *SUPT3H* gene, exon organization. Yellow exons are not present in mRNA variant 1. Exon 5 and exon 12 have been colored in pink and green respectively, to help to understand the duplication structure; B, *SUPT3H* mRNA variants. The exons in gray are not coding. ATG indicates the initiation of translation; C, Duplication structure; D, RT-PCR results. C, male control; P, patient; M, mother; F, father; B, blank; L, GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific, Malmö, Sweden).

The SUPT3H protein is the human homolog of the yeast transcription factor Spt3 that is a part of the SAGA transcription complex [42]. Mutations in this gene have been shown to cause defects in mating and sporulation with a variable phenotype [43]. The transcriptional control of Spt3 is believed to be conserved from yeast to human [44] where the SAGA like complex STAGA, including SUPT3H, is conserved in humans and is a transcriptional co-activator [45].

As the indentified interstitial duplication would most likely, if causative, act through a haploinsufficiency mechanism, we sequenced the *SUPT3H* gene in all patients included in the study to identify possible inactivating mutation leading to haploinsufficiency. No mutations were found. However, due to the small group size this negative result must be interpreted with caution. Considering the likelihood that no mutation of any new gene will be more common than *SRY* mutations which have a frequency of 10–15% [11], this is not enough to exclude this genes as a candidate gene for XY GD. In conclusion, we consider *SUPT3H* to be an interesting candidate gene for 46,XY GD that should be studied further.

4.2. C2ORF80 deletion on 2q34

A 22 kb deletion of almost the entire *C2ORF80* gene (chromosome 2 open reading frame 80) was detected in the same pair of siblings, also maternally inherited. The deletion spans the first eight of a total of nine exons, thus leading to loss of one allele (Fig. 1B). The gene is not characterized. C2ORF80 is a 193 aa long protein and data from the Human Protein Atlas shows moderate expression in testicular cell types (Leydig cells and cells in seminiferous ducts) and ovarian stroma cells, among others. The protein does not contain any known functional domain, and does not present homology with any known protein. It is highly conserved in other species down to frog and fish, with a 47% sequence homology with *Danio rerio*.

A patient described in the Decipher database carries a deletion of the same gene, together with three other changes. This patient has a 46,XY karyotype and cryptochidism, along with intellectual disability, macrocephaly, muscular hypotonia, megalocornea and tall stature. The deletion is inherited from the healthy mother and has also been seen in an additional patient with a 46,XX karyotype in the same DECIPHER contributing centre [46]. This, together with the fact that both sisters share the deletion, lead us to consider *C2ORF80* a candidate gene, and we sequenced *C2ORF80* in all the other DSD patients included in the study. No mutations were detected. However, these results are not sufficient to exclude *C2ORF80* as a candidate gene for XY GD, and further characterization of C2ORF80 is thus necessary.

4.3. OTOGL deletion on 12q21.31

An almost complete gene deletion spanning exon 9 to 41 of the 58 exons of the *OTOGL* gene was detected in patient 5 (Fig. 1C). *OTOGL* mutations have been found causing moderate sensorineural hearing loss in both fertile females and males (OMIM nr 614925) [47,48]. This contradicts *OTOGL* as a candidate gene for XY gonadal dysgenesis.

4.4. Dup9q21.11

In patient 6, a 454 kb duplication within 9q21.11, involving the five genes *PIP5K1B*, *PRKACG*, *FXN*, *TJP2* and *FAM189A2*, was identified (Fig. 1D). Regarding the *PIP5K1B* (Phosphatidylinositol-4-Phosphate 5-kinase, Type1, Beta) gene, only the last four exons are duplicated. The array-CGH method does not give positional data so we can only make supposition about the position of the

duplication. If it is in tandem, it is likely that the patient still has two functional gene copies (although a positional effect cannot be excluded). It is also possible that the duplication disrupts one gene copy, thus leading to haploinsufficiency. The *PIP5K1B* gene encodes a lipid kinase that produces a signaling phospholipid [49]. It is highly conserved in the mammalian clade, and has a 67% sequence homology with *D. rerio*. The PIP5K1B enzyme function is not completely understood, but the produced bioactive lipid is an active signaling molecule involved in, cytoskeleton reorganization, cell survival and apoptosis, epithelial cell morphogenesis and more [49]. It is also suggested to have a possible role in nuclear events [49]. In the Human Protein Atlas nuclear staining for PIP5K1B in three cell lines is shown. Furthermore a moderate level of expression in Leydig cells is described. A study of murine tissues shows a very high expression of *Pip5Ka* (corresponding to human *PIP5K1B*) in testis [50]. (There is a historic confusion of nomenclature between human and murine PIP5K isozymes where alpha corresponded to beta and vice versa. In NCBI this is now addressed and corrected.) Also, interesting mouse knock-out models have been described for *Pip5Ka* and *Pip5Kb* (corresponding to human *PIP5K1A*) and also for a double knock-out. Double deficient mice were completely sterile due to decreased sperm number, and had a 25% reduction in testes weight compared to WT mice. The *Pip5Ka* only knockout mice were subfertile due to reduced motility and abnormal morphology of sperm. Also, both Pip5Ka and Pip5Kb were found to be widely distributed in other testicular cell types such as germ, Sertoli, and Leydig cells [49]. Although it is not certain if the *PIP5K1B* gene function is disrupted in our patient this gene should be considered an interesting candidate gene for testicular development and function.

The *PRKACG* gene (homo sapiens protein kinase, cAMP-dependent, catalytic, gamma) encodes the gamma form of the catalytic subunit of cAMP-dependent kinase, also called protein kinase A. The gamma gene is thought to be a retrotransposon derived from the alpha form. In contrast to the widely expressed alpha and beta forms, the gamma form is highly tissue-specific. Early studies have only detected expression in human testis, however ovarian and brain tissues were not included in the analysis [51]. A later study pinpoints the expression to germ cells in human and primate testis [52]. According to the Human Protein Atlas there is strong expression in testis seminiferous duct cells and ovarian follicle cells among a few others.

Activation of protein kinase A by cAMP leads to activation of the catalytic subunit which phosphorylates a large number of cytosolic and nuclear proteins. The gamma subunit is 351 aa long and has 79% identity to the *D. rerio* Prkacab protein. The different functions of the three catalytic subunits are not yet understood, but due to the tissue specific expression profile, a localized specific function for the gamma form in testicular tissue has been hypothesized [51]. We consider this an interesting candidate gene for XY GD.

The Frataxin (*FXN*) gene encodes a mitochondrial protein that regulates iron transport and respiration. *FXN* gene mutations lead to Friedriech ataxia, a neurological disease (OMIM nr 606829 and 229300) [48]. We believe it is a less likely candidate for GD.

The fourth gene in the duplicated region is *TJP2*. This gene is included in a previously described duplication on chromosome 9q21.11 associated with autosomal dominant deafness-51 [53] and is carried by both fertile males and females, thus excluding *TJP2* as a candidate for gonadal dysgenesis.

The last gene encompassed by the duplication is the *FAM189A2* gene (Homo sapiens family with sequence similarity 189, member A2). The Human Protein Atlas shows a wide expression pattern with strong staining in epididymis and fallopian tube with moderate expression in testicular cell types. The gene encodes a 450 aa membrane protein that has 87% identity in mouse (*Mus musculus*) and 58% in *D. rerio*. No function is so far described, but the protein is distantly related to the CD20-like protein family. The *FAM189A2* gene needs to be characterized further, also as a candidate gene for XY GD.

Finally proving causality for identified variants is challenging. *In vitro* functional characterizations comparing mutant and wild type proteins are important and should ideally be undertaken. However, this requires substantial experimental efforts for each identified variant as well as detailed mechanistic knowledge of each affected pathway. Animal models are helpful but even more laborious, costly and time consuming. Species differences may also preclude accurate conclusions from such experiments. We believe that an important way forward is through collaboration between groups working with these rare patients, by sharing of data and knowledge. The finding of recurrent and related genetic variants in larger groups patients with carefully documented phenotypes should enable the identification of additional critical molecules and pathways involved in each subtype of DSD.

4.5. Conclusion

With our platform we have identified five novel candidate genes for gonadal dysgenesis in two out of nine patients (22%). This is at an expected level when comparing with previous studies for CNV detection in gonadal DSD [23,28,29], and constitutes a valuable contribution to the diagnostic arsenal for these patients where today only half receive a molecular diagnosis. Array-CGH is a powerful technique with high potential resolution, capable of detecting diagnostic genomic imbalances as well as novel candidate genes in patients with gonadal DSD. We therefore recommend that all patients with 46,XY GD without established molecular diagnoses should undergo testing using a high-resolution array-CGH platform.

Conflict of interest

None to declare for any author.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmg.2013.09.003.

References

[1] C.R. Cederroth, J.L. Pitetti, M.D. Papaioannou, S. Nef, Genetic programs that regulate testicular and ovarian development, Mol. Cell Endocrinol. 265–266 (2007) 3–9.

[2] D.M. Maatouk, L. DiNapoli, A. Alvers, K.L. Parker, M.M. Taketo, B. Capel, Stabilization of beta-catenin in XY gonads causes male-to-female sex-reversal, Hum. Mol. Genet. 17 (2008) 2949–2955.

[3] J. Kent, S.C. Wheatley, J.E. Andrews, A.H. Sinclair, P. Koopman, A male-specific role for SOX9 in vertebrate sex determination, Development 122 (1996) 2813–2822.

[4] S. Morais da Silva, A. Hacker, V. Harley, P. Goodfellow, A. Swain, R. Lovell-Badge, Sox9 expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds, Nat. Genet. 14 (1996) 62–68.

[5] O. Soder, Sexual dimorphism of gonadal development, Best Pract. Res. Clin. Endocrinol. Metab. 21 (2007) 381–391.

[6] O. Hiort, The differential role of androgens in early human sex development, BMC Med. 11 (2013) 152.

[7] D.T. MacLaughlin, P.K. Donahoe, Sex determination and differentiation, N. Engl. J. Med. 350 (2004) 367–378.

[8] L.H. Looijenga, R. Hersmus, J.W. Oosterhuis, M. Cools, S.L. Drop, K.P. Wolffenbuttel, Tumor risk in disorders of sex development (DSD), Best Pract. Res. Clin. Endocrinol. Metab. 21 (2007) 480–495.

[9] M. Cools, K.P. Wolffenbuttel, S.L. Drop, J.W. Oosterhuis, L.H. Looijenga, Gonadal development and tumor formation at the crossroads of male and female sex determination, Sex Dev. 5 (2011) 167–180.

[10] A.M. Kersemaekers, F. Honecker, H. Stoop, M. Cools, M. Molier, K. Wolffenbuttel, C. Bokemeyer, Y. Li, Y.F. Lau, J.W. Oosterhuis, L.H. Looijenga, Identification of germ cells at risk for neoplastic transformation in gonadoblastoma: an immunohistochemical study for OCT3/4 and TSPY, Hum. Pathol. 36 (2005) 512–521.

[11] F.J. Cameron, A.H. Sinclair, Mutations in SRY and SOX9: testis-determining genes, Hum. Mutat. 9 (1997) 388–395.

[12] T. Wagner, J. Wirth, J. Meyer, B. Zabel, M. Held, J. Zimmer, J. Pasantes, F.D. Bricarelli, J. Keutel, E. Hustert, U. Wolf, N. Tommerup, W. Schempp, G. Scherer, Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9, Cell 79 (1994) 1111–1120.

[13] J.C. Achermann, M. Ito, P.C. Hindmarsh, J.L. Jameson, A mutation in the gene encoding steroidogenic factor-1 causes XY sex reversal and adrenal failure in humans, Nat. Genet. 22 (1999) 125–126.

[14] K.M. Call, T. Glaser, C.Y. Ito, A.J. Buckler, J. Pelletier, D.A. Haber, E.A. Rose, A. Kral, H. Yeger, W.H. Lewis, et al., Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus, Cell 60 (1990) 509–520.

[15] S. Allali, J.B. Muller, R. Brauner, D. Lourenco, R. Boudjenah, V. Karageorgou, C. Trivin, H. Lottmann, S. Lortat-Jacob, C. Nihoul-Fekete, O. De Dreuzy, K. McElreavey, A. Bashamboo, Mutation analysis of NR5A1 encoding steroidogenic factor 1 in 77 patients with 46, XY disorders of sex development (DSD) including hypospadias, PLoS One 6 (2011) e24117.

[16] P. Canto, D. Soderlund, E. Reyes, J.P. Mendez, Mutations in the desert hedgehog (DHH) gene in patients with 46, XY complete pure gonadal dysgenesis, J. Clin. Endocrinol. Metab. 89 (2004) 4480–4483.

[17] E.G. Puffenberger, D. Hu-Lince, J.M. Parod, D.W. Craig, S.E. Dobrin, A.R. Conway, E.A. Donarum, K.A. Strauss, T. Dunckley, J.F. Cardenas, K.R. Melmed, C.A. Wright, W. Liang, P. Stafford, C.R. Flynn, D.H. Morton, D.A. Stephan, Mapping of sudden infant death with dysgenesis of the testes syndrome (SIDDT) by a SNP genome scan and identification of TSPYL loss of function, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 11689–11694.

[18] M. Barbaro, M. Oscarson, J. Schoumans, J. Staaf, S.A. Ivarsson, A. Wedell, Isolated 46, XY gonadal dysgenesis in two sisters caused by a Xp21.2 interstitial duplication containing the DAX1 gene, J. Clin. Endocrinol. Metab. 92 (2007) 3305–3313.

[19] T. Ogata, N. Matsuo, Sex determining gene on the X chromosome short arm: dosage sensitive sex reversal, Acta Paediatr. Jpn. 38 (1996) 390–398.

[20] B.K. Jordan, M. Mohammed, S.T. Ching, E. Delot, X.N. Chen, P. Dewing, A. Swain, P.N. Rao, B.R. Elejalde, E. Vilain, Up-regulation of WNT-4 signaling and dosage-sensitive sex reversal in humans, Am. J. Hum. Genet. 68 (2001) 1102–1109.

[21] V. Calvari, V. Bertini, A. De Grandi, G. Peverali, O. Zuffardi, M. Ferguson-Smith, J. Knudtzon, G. Camerino, G. Borsani, S. Guioli, A new submicroscopic deletion that refines the 9p region for sex reversal, Genomics 65 (2000) 203–212.

[22] I.A. Hughes, C. Houk, S.F. Ahmed, P.A. Lee, Consensus statement on management of intersex disorders, Arch. Dis. Child 91 (2006) 554–563.

[23] S. White, T. Ohnesorg, A. Notini, K. Roeszler, J. Hewitt, H. Daggag, C. Smith, E. Turbitt, S. Gustin, J. van den Bergen, D. Miles, P. Western, V. Arboleda, V. Schumacher, L. Gordon, K. Bell, H. Bengtsson, T. Speed, J. Hutson, G. Warne, V. Harley, P. Koopman, E. Vilain, A. Sinclair, Copy number variation in patients with disorders of sex development due to 46, XY gonadal dysgenesis, PLoS One 6 (2011) e17793.

[24] R. Sekido, R. Lovell-Badge, Genetic control of testis development, Sex Dev. (2012).

[25] M. Barbaro, M. Cools, L.H. Looijenga, S.L. Drop, A. Wedell, Partial deletion of the NR5A1 (SF1) gene detected by synthetic probe MLPA in a patient with XY gonadal disorder of sex development, Sex Dev. 5 (2011) 181–187.

[26] S. Ledig, O. Hiort, L. Wunsch, P. Wieacker, Partial deletion of DMRT1 causes 46, XY ovotesticular disorder of sexual development, Eur. J. Endocrinol. 167 (2012) 119–124.

[27] G. Vinci, S. Chantot-Bastaraud, B. El Houate, S. Lortat-Jacob, R. Brauner, K. McElreavey, Association of deletion 9p, 46, XY gonadal dysgenesis and autistic spectrum disorder, Mol. Hum. Reprod. 13 (2007) 685–689.

[28] S. Ledig, O. Hiort, G. Scherer, M. Hoffmann, G. Wolff, S. Morlot, A. Kuechler, P. Wieacker, Array-CGH analysis in patients with syndromic and non-syndromic XY gonadal dysgenesis: evaluation of array CGH as diagnostic tool and search for new candidate loci, Hum. Reprod. 25 (2010) 2637–2646.

[29] M. Tannour-Louet, S. Han, S.T. Corbett, J.F. Louet, S. Yatsenko, L. Meyers, C.A. Shaw, S.H. Kang, S.W. Cheung, D.J. Lamb, Identification of de novo copy number variants associated with human disorders of sexual development, PLoS One 5 (2010) e15392.

[30] M. Barbaro, A. Cicognani, A. Balsamo, A. Löfgren, L. Baldazzi, A. Wedell, M. Oscarson, Gene dosage imbalances in patients with 46, XY gonadal DSD detected by an in-house-designed synthetic probe set for multiplex ligation-dependent probe amplification analysis, Clin. Genet. 73 (2008) 453–464.

[31] M. Barbaro, A. Balsamo, B.M. Anderlid, A.G. Myhre, M. Gennari, A. Nicoletti, M.C. Pittalis, M. Oscarson, A. Wedell, Characterization of deletions at 9p affecting the candidate regions for sex reversal and deletion 9p syndrome by MLPA, Eur. J. Hum. Genet. 17 (2009) 1439–1447.

[32] J. Zhang, L. Feuk, G.E. Duggan, R. Khaja, S.W. Scherer, Development of bioinformatics resources for display and analysis of copy number and other structural variants in the human genome, Cytogenet. Genome Res. 115 (2006) 205–214.

[33] A.J. Iafrate, L. Feuk, M.N. Rivera, M.L. Listewnik, P.K. Donahoe, Y. Qi, S.W. Scherer, C. Lee, Detection of large-scale variation in the human genome, Nat. Genet. 36 (2004) 949–951.

[34] T.R. Dreszer, D. Karolchik, A.S. Zweig, A.S. Hinrichs, B.J. Raney, R.M. Kuhn, L.R. Meyer, M. Wong, C.A. Sloan, K.R. Rosenbloom, G. Roe, B. Rhead, A. Pohl, V.S. Malladi, C.H. Li, K. Learned, V. Kirkup, F. Hsu, R.A. Harte, L. Guruvadoo, M. Goldman, B.M. Giardine, P.A. Fujita, M. Diekhans, M.S. Cline, H. Clawson, G.P. Barber, D. Haussler, W. James Kent, The UCSC Genome Browser database: extensions and updates 2011, Nucleic Acids Res. 40 (2012) D918–D923.

[35] M. Uhlen, P. Oksvold, L. Fagerberg, E. Lundberg, K. Jonasson, M. Forsberg, M. Zwahlen, C. Kampf, K. Wester, S. Hober, H. Wernerus, L. Bjorling, F. Ponten, Towards a knowledge-based human protein atlas, Nat. Biotechnol. 28 (2010) 1248–1250.

[36] S. Nef, O. Schaad, N.R. Stallings, C.R. Cederroth, J.L. Pitetti, G. Schaer, S. Malki, M. Dubois-Dauphin, B. Boizet-Bonhoure, P. Descombes, K.L. Parker, J.D. Vassalli, Gene expression during sex determination reveals a robust female genetic program at the onset of ovarian development, Dev. Biol. 287 (2005) 361–377.

[37] H.V. Firth, S.M. Richards, A.P. Bevan, S. Clayton, M. Corpas, D. Rajan, S. Van Vooren, Y. Moreau, R.M. Pettett, N.P. Carter, DECIPHER: database of chromosomal imbalance and phenotype in humans using Ensembl resources, Am. J. Hum. Genet. 84 (2009) 524–533.

[38] J.P. Schouten, C.J. McElgunn, R. Waaijer, D. Zwijnenburg, F. Diepvens, G. Pals, Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification, Nucleic Acids Res. 30 (2002) e57.

[39] R.F. Stern, R.G. Roberts, K. Mann, S.C. Yau, J. Berg, C.M. Ogilvie, Multiplex ligation-dependent probe amplification using a completely synthetic probe set, Biotechniques 37 (2004) 399–405.

[40] S. Rozen, H. Skaletsky, Primer3 on the WWW for general users and for biologist programmers, Methods Mol. Biol. 132 (2000) 365–386.

[41] M. Barbaro, J. Cook, K. Lagerstedt-Robinson, A. Wedell, Multigeneration inheritance through fertile XX carriers of an NR0B1 (DAX1) locus duplication in a kindred of females with isolated XY gonadal dysgenesis, Int. J. Endocrinol. 2012 (2012) 504904.

[42] D.M. Eisenmann, K.M. Arndt, S.L. Ricupero, J.W. Rooney, F. Winston, SPT3 interacts with TFIID to allow normal transcription in *Saccharomyces cerevisiae*, Genes Dev. 6 (1992) 1319–1331.

[43] J.N. Hirschhorn, F. Winston, SPT3 is required for normal levels of a-factor and alpha-factor expression in *Saccharomyces cerevisiae*, Mol. Cell Biol. 8 (1988) 822–827.

[44] J. Yu, J.M. Madison, S. Mundlos, F. Winston, B.R. Olsen, Characterization of a human homologue of the *Saccharomyces cerevisiae* transcription factor spt3 (SUPT3H), Genomics 53 (1998) 90–96.

[45] E. Martinez, V.B. Palhan, A. Tjernberg, E.S. Lymar, A.M. Gamper, T.K. Kundu, B.T. Chait, R.G. Roeder, Human STAGA complex is a chromatin-acetylating transcription coactivator that interacts with pre-mRNA splicing and DNA damage-binding factors in vivo, Mol. Cell Biol. 21 (2001) 6782–6795.

[46] N. DiDonato, DECIPHER information regarding patient 254783 in personal e-mail correspondence, 2013.

[47] K.O. Yariz, D. Duman, C.Z. Seco, J. Dallman, M. Huang, T.A. Peters, A. Sirmaci, N. Lu, M. Schraders, I. Skromne, J. Oostrik, O. Diaz-Horta, J.I. Young, S. Tokgoz-Yilmaz, O. Konukseven, H. Shahin, L. Hetterschijt, M. Kanaan, A.M. Oonk, Y.J. Edwards, H. Li, S. Atalay, S. Blanton, A.A. Desmidt, X.Z. Liu, R.J. Pennings, Z. Lu, Z.Y. Chen, H. Kremer, M. Tekin, Mutations in OTOGL, encoding the inner ear protein otogelin-like, cause moderate sensorineural hearing loss, Am. J. Hum. Genet. 91 (2012) 872–882.

[48] Online Mendelian Inheritance in Man, OMIM®, Johns Hopkins University, Baltimore, MD, MIM Number: {233300}: {11/02/2011}, World Wide Web URL: http://omim.org/.

[49] H. Hasegawa, J. Noguchi, M. Yamashita, R. Okada, R. Sugimoto, M. Furuya, T. Unoki, Y. Funakoshi, T. Baba, Y. Kanaho, Phosphatidylinositol 4-phosphate 5-kinase is indispensable for mouse spermatogenesis, Biol. Reprod. 86 (2012), 136, 131–112.

[50] H. Ishihara, Y. Shibasaki, N. Kizuki, H. Katagiri, Y. Yazaki, T. Asano, Y. Oka, Cloning of cDNAs encoding two isoforms of 68-kDa type I phosphatidylinositol-4-phosphate 5-kinase, J. Biol. Chem. 271 (1996) 23611–23614.

[51] S.J. Beebe, O. Oyen, M. Sandberg, A. Froysa, V. Hansson, T. Jahnsen, Molecular cloning of a tissue-specific protein kinase (C gamma) from human testis-representing a third isoform for the catalytic subunit of cAMP-dependent protein kinase, Mol. Endocrinol. 4 (1990) 465–475.

[52] N. Reinton, T.B. Haugen, S. Orstavik, B.S. Skalhegg, V. Hansson, T. Jahnsen, K. Tasken, The gene encoding the C gamma catalytic subunit of cAMP-dependent protein kinase is a transcribed retroposon, Genomics 49 (1998) 290–297.

[53] T. Walsh, S.B. Pierce, D.R. Lenz, Z. Brownstein, O. Dagan-Rosenfeld, H. Shahin, W. Roeb, S. McCarthy, A.S. Nord, C.R. Gordon, Z. Ben-Neriah, J. Sebat, M. Kanaan, M.K. Lee, M. Frydman, M.C. King, K.B. Avraham, Genomic duplication and overexpression of TJP2/ZO-2 leads to altered expression of apoptosis genes in progressive nonsyndromic hearing loss DFNA51, Am. J. Hum. Genet. 87 (2010) 101–109.