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

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BACKGROUND: XY gonadal dysgenesis (XY-GD) is a heterogeneous disorder characterized by failure of testicular development despite a normal male karyotype. Non-syndromic and syndromic forms can be delineated. Currently, only a minority of cases can be explained by gene mutations.

METHODS: The aim of this study was to detect microdeletions and duplications by using high-resolution Agilent oligonucleotide arrays in a cohort of 87 patients with syndromic or non-syndromic 46,XY-GD.

RESULTS: In 26 patients, we identified gains or losses in regions including genes involved in XY-GD (DMRT1, SOX9, DAX1) or in regions, which have not been described as polymorphic copy number variants (CNVs).

CONCLUSIONS: This study shows that array comparative genomic hybridization (CGH) analysis is a useful tool for the molecular diagnosis of XY-GD as well as for the identification of potential candidate genes involved in male sexual development.

Key words: XY gonadal dysgenesis / array CGH / diagnostic / candidate loci

Introduction

Testis development is the result of a tightly regulated signalling network. Since testis development can be impaired at different stages, XY gonadal dysgenesis (XY-GD) is a very heterogeneous disorder, with a frequency in the range of 1 in 3000 births (Camerino et al., 2006). Clinically, a complete form can be distinguished from a partial form of XY-GD. The complete form is characterized by female external genitalia and hypergonadotropic hypogonadism (in adults) due to streak gonads consisting only of fibrous stroma but without hormonally active tissue and gametes. Clinical features of complete XY-GD are primary amenorrhea and absence of breast development. Müllerian derivatives like the uterus, Fallopian tubes and vagina are not impaired because of the deficient production of Anti-Müllerian hormone (AMH) due to missing Sertoli cells. There is no virilization of the external and internal genitalia, since the androgen-producing Leydig cells are lacking. In partial XY-GD, residuals of endocrine tissues can be present. Therefore, virilization is possible because of androgen production from residual Leydig cells, which induce the Wolffian ducts to differentiate into the epididymis, vasa deferentia and seminal vesicles. Since AMH production by Sertoli cells is partially impaired, a mixture of Wolffian and Mullerian ducts coexists.

Furthermore, XY-GD can occur as an isolated (non-syndromic) form or in combination with various other extragonadal malformations in syndromic forms.

Chromosome aberrations in patients with gonadal dysgenesis have contributed to the identification of genes being implicated in testis development, for example sex-determining region Y (SRY), dosage-sensitive sex reversal adrenal hypoplasia congenita critical region on...
the X chromosome gene 1 (DAX1), Wilms tumour 1 (WT1), double-sex and mab-3 related transcription factors 1–3 (DMRT1/2/3) and wingless-type MMTV integration site family, member 4 (WNT4).

The gene most commonly affected in patients with non-syndromic XY-GD is SRY in Yp, which triggers the differentiation of Sertoli cells. In about 20–30% of the patients with non-syndromic XY-GD, deletions or nucleotide mutations of the SRY gene have been reported (Cameron and Sinclair, 1997).

Additional genes involved in male gonadal differentiation are steroidogenic factor 1 (SF1), DAX1, SRY-box 9 (SOX9), desert hedgehog (DHH) or testis-specific Y-encoded-like protein 1 gene (TSPYL1). Mutations of these genes are usually associated with additional anomalies, but can also result in isolated XY-GD.

SF1 together with WT1 plays an essential role in early gonadal development and later in AMH expression in Sertoli cells. Mutations of SF1 result in gonadal dysgenesis with or without adrenal failure (Achermann et al., 1999; Correa et al., 2004). Microdeletions including SF1 have been found by array-CGH analysis in patients with XY-GD (Schaubitz et al., 2007; van Silfhout et al., 2009).

WT1 is essential for the development of the urogenital tract, where it regulates expression of SRY, and later it plays a pivotal role together with SF1 in the production of AMH in Sertoli cells (Nachtigal et al., 1998; Hossain and Saunders, 2001). Heterozygous missense mutations and splice site mutations in WT1 are associated with Deny-Drash syndrome (DDS; MIM#194080) and Frasier syndrome (FS; MIM#136680), which are characterized by genital malformations, progressive glomerulopathy and development of genitourinary tumours.

Distal monosomy of 9p including DMRT1–3 genes has been described in some XY-GD patients, and a case with bilateral ovotestes has also been reported (Muroya et al., 2000; Ünnap et al., 2004). Among the three DMRT genes, DMRT1 is the strongest candidate gene. However, nucleotide mutations in DMRT1 seem to be a very rare cause of XY-GD, since mutational analysis of a large cohort of patients revealed only one possible missense mutation (Raymond et al., 1999).

X chromosomal DAX1 antagonizes the action of SRY, WT1 and SF1 and hence AMH production. Therefore, duplications of the region including DAX1 cause 46,XY-GD or ambiguous genitalia (Bardoni et al., 1994). Mutations and deletions of DAX1 are responsible for adrenal hypoplasia and hypogonadotropic hypogonadism (Muscatelli et al., 1994). Moreover, a small deletion of approximately 250 kb upstream of DAX1 has recently been found by array CGH in a patient with 46,XY pure GD (Smyk et al., 2007). The authors suggest that the deletion removes regulatory elements.

The transcription factor SOX9 is necessary for cartilage formation and testis differentiation. Mutations of SOX9 located in 17q24 lead to campomelic dysplasia (CD, MIM#114290), in which about two-thirds of 46,XY individuals also have a partial or complete form of XY-GD (Wagner et al., 1994). In 10% of the CD cases, an atypical form without bowing of the long bones is found and is named acampomelic campomelic dysplasia (ACD). Most of the cases of (A)CD are caused by heterozygous mutations of the SOX9 gene leading to haploinsufficiency, but there are also reports of deletions and chromosomal rearrangements, which affect regions up- and downstream of SOX9 in (A)CD patients, suggesting the existence of cis-acting regulatory elements of the SOX9 gene in these regions (Pfeifer et al., 1999; Pop et al., 2004; Lecointre et al., 2009).

WNT4 in 1p belongs to the wingless-type MMTV integration site (WNT)-family encoding cystein-rich glycoproteins, which act as extracellular signalling proteins. The expression of DAX1 in Sertoli and Leydig cells is increased by WNT4. Thus, a duplication of WNT4 in a chromosomal male has been associated with XY-GD (Jordan et al., 2001). However, a duplication of 1p identified in a patient with syndromic XY-GD does not affect the WNT4 gene (Wieacker and Volleth, 2007).

Homozygosity and heterozygosity for missense and frame shift mutations in DHH in 12q have been found in patients with complete and partial XY-GD (Umehara et al., 2000; Canto et al., 2005). Patients typically exhibit a polyneuropathy (Umehara et al., 2000). Experiments in mice suggest that DHH plays an essential role in the development of testicular tubules, Leydig cell differentiation and spermatogenesis (Bitgood et al., 1996; Clark et al., 2000).

A homozygous frameshift mutation in autosomal gene TSPYL1 has been linked to a syndrome, which occurred in an Amish family and is characterized by sudden infant death with dysgenesis of the testes (SIDDT, MIM#608800) (Puffenberger et al., 2004).

Recently, compound heterozygosity for two missense mutations in the polycomb gene chromobox homologue 2 (CBX2) has been documented as being causative for complete XY-GD. Surprisingly, the girl shows normal ovarian tissue and primordial follicles (Biaison-Lauber et al., 2009).

Despite these findings, the cause of gonadal dysgenesis is still unclear for the majority of XY-GD patients. We therefore attempted to identify microdeletions and duplications affecting the genes mentioned above and novel candidate genes by performing high-resolution array CGH in a large cohort of patients with XY-GD.

Methods

Patients

This study was approved by the institutional review board. We analysed a cohort of 87 patients with XY-GD. Of the investigated patients, 43 patients have a complete form of XY-GD with female external and internal genitalia and no sign of virilization. Another 33 patients have a partial form of XY-GD with different degrees of virilization and/or different degrees of regression of the Müllerian derivatives. In eight cases of partial 46,XY gonadal dysgenesis, male gender has been assigned. In the remaining 11 cases, it is not clear if they have a mixed or a pure 46,XY gonadal dysgenesis. Among the 87 patients, 71 have a non-syndromic GD and 16 patients have a syndromic form. Associated traits encompass mental retardation, facial dysmorphism, hearing loss, thrombocytopenia and malformations of the skeleton, kidneys, heart, limbs, diaphragm, eye, adrenal gland and skin. In addition, three patients had a positive family history, but only in one case DNA from the affected sister was available. For a more detailed description of the patients, in whom we identified non-described CNVs, see Table I. In 86 patients, chromosome analysis on blood cells revealed a normal male karyotype, while in patient 9, a structural aberration was suspected. Furthermore, only patients without a mutation in the SRY gene were included in the study. In some of the syndromic cases, according to the associated symptoms, sequential analysis of genes, such as WT1, SF1, SOX9, WNT4, ATRX and DHH, have been performed without identifying any mutation. In only three cases (patients 9, 14 and 40), material from the parents was available.
| PAT | Female/ Male | Chromosomal region | Del/ dup | Start<sup>a</sup> | End<sup>a</sup> | Size | Gene(s) | Phenotype |
|-----|-------------|---------------------|---------|------------------|----------------|------|---------|-----------|
| 8   | Female      | 9p24.3-p23          | Del     | 152931           | 10776275       | 10.6 Mb | DOCK8, KANK1, DMRT1, VLDLR, GLIS3, SLC1A1, JAK2 | Syndromic form of pXYGD, clitoris hypertrophy, dygerminoma, mild MR |
| 14* | Female      | 9p24.3-p23          | Del     | 229226           | 9933540        | 9.7 Mb  | DOCK8, KANK1, DMRT1, VLDLR, GLIS3, SLC1A1, JAK2 | Syndromic form of pXYGD, hydrops, facial dysmorphism, cleft palate, camptodactyly, malformation of the feet, multicystic and dysplastic kidneys |
|     |             | 16p13.3-p13.13      | Dup     | 45 058           | 11454770       | 11.41 Mb | HBA1, HBA2, AXIN1, LMF1, STR5, GNPTG, CLCN7, IGFL5, PKD1, TSC2, ABCA3, MEFV, CREBBP, GLIS2, ALG1, ABAT, PIMM2, CIITA | |
| 35  | Female      | 9p24.3              | Del     | 270055           | 1091607        | 821.6 kb | DOCK8, KANK1, DMRT1, DMRT3, DMRT2 | cXYGD |
| 44  | Female      | 9p24.3              | Del     | 752747           | 855979         | 103.2 kb | DMRT1 | cXYGD |
| 12  | Female      | 17q24.2-q24.3       | Del     | 63746339         | 67022162       | 3.276 Mb | AMZ2, SLC16A6, ARSG, WIPI1, PRKAR1A, FAM20A, ABCA8, ABCA9, ABCA6, ABCA10, ABCA3, MAP2K6, KCNJ16, KCNJ2 | Syndromic form of 46,XYGD, ACD, kyphoskoliosis |
| 9   | Female      | Xp22.2-p21.1        | Dup     | 15528787         | 31759751       | 16.23 Mb | API52, NHS, CDKL5, RS1, PHKA2, PDHA1, RP56KA3, SMS, PHEX, SAT1, POLA1, ARX, IL1RAPL1, DAX1, GK, DMD | Syndromic form of cXYGD with uterus, IUGR, facial dysmorphism, disturbance of pulmonary adaption, muscular hypertonia, hearing defect, MR, developmental delay, short stature, macrocephaly |
| 52  | Female      | Xp21.2              | Dup     | 30170062         | 30890999       | 729 kb  | MAGEB4, MAGEB1, DAX1, CXorf21, GK, MAP3K7IP3 | cXYGD, testicular residues, clitoris hypertrophy |
| 47  | Female      | Yq11.223            | Loss    | 22780666         | 23283889       | 503.2 kb | TTTY5, RBMY1F, RBMY1F2P, RBMY1J, TTTY6B, PRY2, PRY | cXYGD |
| 85* | Female      | 1p36.32             | Dup     | 2694355          | 3167922        | 473.6 kb | ACTRT2 FLJ42 875, PRDM16 | pXYGD, hirsutism |
|     |             | 9q31.2              | Dup     | 107403144        | 107575411      | 172.3 kb | FKTN, TAL2, TMEM38B | |
| 42* | Female      | 2p16.1              | Dup     | 54990975         | 55087598       | 96.62 kb | EML6, RTN4 | cXYGD |
|     |             | 4q12               | Dup     | 57359930         | 57572181       | 212.3 kb | SPINK2, REST, C4orf14, POLR2B | |
| 68  | Female      | 3q26.31             | Del     | 174296433        | 174506577      | 210.1 kb | SPATA16 | |
| 23  | Female      | 4q35.2              | Del     | 187504814        | 187761728      | 256.9 kb | MTNR1A, FAT1 | pXYGD, no uterus, clitoris hypertrophy |
| 20* | Female      | 5p15.33             | Del     | 1212658          | 1645795        | 433.1 kb | SLC6A19, SLC1A8, TERT, CLPTM1L, SLC6A3, LPCAT1, SDHAP3 | pXYGD, clitoris hypertrophy |
|     |             | 8q24.3              | Dup     | 142274733        | 146092035      | 3.817 Mb | JRK, SLRP1, CYP11B1, CYP11B2, FAM83H, PLEC1, SLC39A4, RECQL4 | pXYGD |
| 46  | Female      | 6p22.2              | Dup     | 24433564         | 24609197       | 175.6 kb | DDCC2, KAA1G, MRS2, GYPD1, ALDHS1A | cXYGD |

Continued
For array-CGH analysis, genomic DNA was extracted from peripheral blood, lymphoblastoid cell lines or fibroblast cultures of the patients by standard methods. The patients’ DNA was analysed by using the commercially available Human Genome CGH Microarray Kit 105A (Agilent Technologies, Santa Clara, USA) comprising 99 000 60-mer oligonucleotide probes with a median probe spacing of 21.7 kb. Labelling and hybridization of patients’ DNA and of 10 pooled male control DNAs was performed according to the manufacturer’s protocol ‘Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis’ (version 4.0, June 2006, Agilent Technologies, Santa Clara, USA). Briefly, 1 μg of patients’ DNA and the pooled control DNA were double digested with AluI and RsaI (Promega, Madison, USA) and subsequently differentially labelled with Cy5-dUTP and Cy3-dUTP (Agilent Technologies, Santa Clara, USA), respectively. After purification of the labelled DNAs by filtration (Microcon YM-30, Millipore, Billerica, USA), patient and control DNA were pooled and hybridized with 25 μg of human Cot1 DNA (Kreatech, Amsterdam, Netherlands) for 40 h at 65°C in the hybridization oven (Agilent Technologies, Santa Clara, USA). After post-hybridization washes, the arrays were scanned using a Microarray Scanner (G2565BA, Agilent Technologies, Santa Clara, USA), and the spot intensities were measured by ‘Feature Extraction Software’ (version 9.5.31, Agilent Technologies, Santa Clara, USA). Further analyses and visualization were performed with ‘CGH Analytics’ (version 3.5.14 by utilizing the following parameters: aberration algorithm.

**Table I** Continued

| PAT | Female/male | Chromosomal region | Del/ Dup | Start* | End* | Size | Gene(s) | Phenotype |
|-----|-------------|--------------------|-------|-------|------|------|---------|-----------|
| 26  | Female      | 9p21.3             | Del   | 20992666 | 21192369 | 199.7 kb | PTPLAD2, IFNB1, IFNW1, IFNA21, IFNA4, IFNA7 | cXYGD     |
| 67  | Male        | 9q21.32            | Dup   | 85466180  | 85658965  | 192.8 kb | UBQLN1, GKAP1, KIF27 | pXYGD, hypoplastic uterus, scrotal hypospadias, bifid scrotum |
| 95  | Female      | 10q11.23           | Dup   | 50764788  | 51264692  | 499.9 kb | PARG, AGAP8, AGAP7, MSMB, NCOA4 | XYGd, uterus, normal breast development |
| 74  | Female      | 10q21.3            | Dup   | 67103959  | 67499226  | 395.3 kb | CTNNAL3 | pXYGD, clitoris hypertrophy, pubertal virilization |
| 18  | Female      | 12q24.11           | Dup   | 108043263 | 108142585 | 99.3 kb | ACACB | pXYGD, ambiguous genitalia, inguinal gonads, vaginaanlage present, uterus sonographically not detectable |
| 57  | Female      | 12q24.13-q24.21    | Del   | 112762082 | 113002746 | 240.7 kb | RBM19 | pXYGD, no uterus, external genitalia partially virilized, Prader III |
| 64  | Female      | 13q21.1-q21.2      | Dup   | 57009787  | 57565953  | 649.8 kb | PCDH17 | pXYGD, inguinal dysgenetic tests, hypoplastic uterus, hypospadias |
| 81  | Female      | 15q24.3-q25.1      | Dup   | 75908049  | 76174598  | 266.6 kb | LOC645752, LOC91 450, TBCID2B, SH2D7 | cXYGD |
| 65  | Female      | 17q12              | Del   | 29356051  | 29446047  | 90 kb | ACCN1 | Syndromic form of pXYGD, no Mullerian derivatives, inguinal tests, clitoris hypertrophy, adrenal insufficiency (no SF1-mutation), thrombocytopenia, duplication of left pyelon |
| 6   | Male        | 20p13              | Dup   | 940665    | 1109400   | 168.7 kb | PSMF1, C20orf46 | cXYGD |
| 40* | Female      | 21q21.1            | (mat) | 15913926  | 16060366  | 146.4 kb | USP25 | cXYGD |
| 21q22.11 | (mat)       | 31014008           | 31163085 | 149.1 kb | KRTAP21-2, KRTAP21-1, KRTAP8-1, KRTAP7-1 | cXYGD |
| 17  | Female      | Xp11.22            | Del   | 49848727  | 50356530  | 507.8 kb | AKAP4, CCNB3, DGKK, SHROOM4 | cXYGD, sister also affected |

cXYGD, complete 46,XY gonadal dysgenesis; pXYGD, partial 46,XY gonadal dysgenesis; MR, mental retardation; ACD, acampomelic campomelic dysplasia; IUGR, intrauterine growth retardation.

In case of large chromosomal aberrations only disease-associated genes are mentioned. Bold marks represent the gene which is responsible for XY DSD.

*According to hg18.

*Patient carries a second CNV.
Array CGH in patients with XY gonadal dysgenesis

ADM-2, threshold 6.0, fuzzy zero, centralization, moving average window 1 Mb, four-point aberration filter, from Agilent Technologies, Santa Clara, USA). Aberrant signals including four or more adjacent probes were considered as genomic aberrations and were further evaluated by real-time quantitative PCR (RT-qPCR), if they were not listed as a frequent variant in the Database of Genomic Variants (http://projects.tcag.ca/variation/). However, information of the Database of Genomic Variants should be interpreted with caution, especially in the context of disorders of sexual development (DSD). The influence of a given genetic imbalance on sexual development depends on the chromosomal sex, since deletion 9p in females has no impact but in the context of a male karyotype, it causes XY-GD. Unfortunately, in the Database of Genomic Variants information about the sex of the control persons tested is rarely available. Coordinates of CNVs are based on NCBI build 36. In this study, we consider only gains and losses of at least 100 kb.

RT-qPCR

All microdeletions and -duplications detected by array CGH with a size of at least 100 kb and which were not or only rarely listed in the Database of Genomic Variants were confirmed by RT-qPCR with SYBR Green detection (SensitMixPlus SYBR Kit, Quantace, London, UK) using two non-polymorphic markers evenly distributed within the deleted/duplicated region. One exception is dup16p13.3-p13.13, which was assessed by fluorescent in situ hybridization (FISH) analysis (results not shown). RT-qPCR primers were designed using Primer3 Software online (http://frodo.wi.mit.edu/primer3/) with the following criteria: amplicon size 60–120 bp, GC content of 20–80%, and melting temperature (Tm) of 59–61°C. Primer sequences are available on request. The primers were checked with MFOLD (http://mfold.rna.albany.edu/?id=5) and SNPcheck (http://www.ncbi.nlm.nih.gov/SNPcheck/SNPcheck.html#). Real-time detection was performed using the LightCycler 480 (Roche, Basel, Switzerland). Absolute quantification of target amplicons in the patients DNA was performed by interpolation of the crossing point (Cp) against the corresponding standard curve obtained by amplification of a sex-matched control DNA pool. We used 10 ng of genomic DNA from the patients as well as from the pooled normal control DNA. In this manner, values of 10 ng indicate a diploid situation, whereas values of 5 or 15 ng indicate deletion or duplication, respectively. Exceptions are X-chromosomal located gains, which will result in values of 20 ng.

The RT-qPCRs were performed in triplicate for each reaction.

Results

A total of 679 genomic rearrangements were identified by performing array CGH in the genomic DNA samples of 71 non-syndromic and 16 syndromic XY-GD patients, totalling 87 patients. This averages 7.8 CNVs per patient. Of these rearrangements, 648 were excluded because they have been described as known polymorphisms in the Database of Genomic Variants (http://projects.tcag.ca/variation). In 5 of the 16 patients with syndromic XY-GD, 6 non-described CNVs could be detected: 4 of these include genes involved in testis differentiation and 2 are possibly disease-relevant rearrangements. In 21 of the 71 patients with non-syndromic gonadal dysgenesis, 25 non-described CNVs were detected: 4 of these comprise genes responsible for testis differentiation and 21 (in 17 patients) include possible new candidate genes. In 10 of these 21 cases, the affected genes may play a role in male fertility. All deletions and duplications detected by array CGH and not listed in the Database of Genomic Variants were confirmed by RT–PCR analyses (Fig. 1). Table I summarizes the chromosomal rearrangements detected in the 5 syndromic and 21 non-syndromic XY-GD patients.

Discussion

By array CGH, the cause of gonadal dysgenesis could be definitively detected in 25% (4/16) of syndromic and in 5.6% (4/71) of non-syndromic XY-GD patients. We first discuss chromosomal rearrangements involving genes known to cause XY-GD and then discuss rearrangements possibly involved in gonadal dysgenesis.

Known causes of XY gonadal dysgenesis

DMRT1 deletions

Deletions of DMRT1 were the most frequent aberrations (patients 8, 14, 35 and 44). So far, three genes (DMRT1–3) in this region have been identified as possible candidate genes for XY-GD. In patient 8, a female with a partial form of XY-GD in combination with a learning disability, we detected a 10.6 Mb deletion including the DMRT1–3 genes but also other genes. A smaller deletion affecting nearly the same genes was found in a 2 months old girl (patient 14) with a syndromic form of 46,XY-GD including hydrops, facial dysmorphism, cleft palate, camptodactyly, deformities of the feet, multicystic and dysplastic kidneys. In addition to the partial monosomy 9p, the girl carries a large gain of approximately 11.4 Mb on chromosomal band 16p13.3-p13.13. Since no such chromosomal rearrangement could be identified in the parents of this girl by FISH analysis, it can be expected that the aberration has arisen de novo. The more severe phenotype of this girl in contrast to patient 8 can be explained by this additional aberration. In another female patient (patient 35) with a non-syndromic form of XY-GD, we identified a smaller deletion of about 820 kb encompassing the DMRT1–3 genes as well as dedicator of cytokinesis 8 (DOCK8) gene and KIN motif and ankyrin repeat domains 1 (KANK1) gene. Interestingly, in this patient heterozygous partial deletion of DOCK8 and total deletion of KANK1 is not associated with mental retardation (autosomal dominant 2, MIM#611432) and cerebral palsy spastic quadriplegic (MIM#612900) in contrast to reports in the literature (Lerer et al., 2005; Griggs et al., 2008). However, Lerer et al. (2005) showed that the deletion encompassing KANK1 was also found in the healthy father of the affected children, suggesting that the disorder only occurs in individuals who inherited the deletion from their father. In a fourth patient with gonadal dysgenesis (patient 44), we detected the smallest aberration affecting only exons 1 and 2 of DMRT1 (Fig. 2). To the best of our knowledge, we report here on the smallest deletion disturbing the DMRT1 gene in a patient with XY-GD, suggesting that a mutation of DMRT1 alone is sufficient for gonadal dysgenesis, although targeted deletion of Dmrt1 in male mice results only in hypoplastic testis, but causes no sex reversal (Raymond et al., 2000). As the parents of patient 44 could not be studied, it is not known if the partial DMRT1 deletion occurred de novo.

Deletions upstream of SOX9 gene

In patient 12, we detected a 3.3 Mb deletion upstream of SOX9. Haploinsufficiency of SOX9 causes campomelic dysplasia (CD) with approximately 66% of XY patients also showing XY-GD. In the ACD form of CD, the campomelia is absent. While most of the
CD cases carry intragenic loss-of-function mutations, also deletions, inversions and balanced translocations located up and downstream of SOX9 have been observed in (A)CD patients. It is thought that these chromosomal rearrangements affect enhancers of SOX9 (Gordon et al., 2009). The deletion we describe here maps from 67 kb to 3.8 Mb upstream of the SOX9 transcription start site and affects several genes. Two of them, KCNJ2 and PRKAR1A, are associated with diseases. Dominant-negative mutations in potassium inwardly rectifying channel J2 (KCNJ2) gene are associated with Andersen syndrome (¼ long QT syndrome 7; LQT7; MIM#170390) and short QT syndrome 3 (SQT3, MIM#609622), while loss-of-function mutations of cAMP-dependent protein kinase, regulatory (PRKAR1A) gene cause Carney complex (MIM#160980). Patient 12 has complete XY-GD and ACD with kyphoscoliosis, but currently no signs of Carney complex. Andersen syndrome and SQT3 phenotype are not expected because the patient carries a deletion and not a dominant-negative mutation of KCNJ2.

DAX1 duplications

Large duplications of the DAX1 region are a rare cause of XY-GD and are usually associated with extragenital anomalies. Small duplications affecting DAX1 and only few flanking genes have been reported in patients with pure XY-GD (Barbaro et al., 2007; Barbaro et al., 2008). In patient 9 affected by a syndromic XY-GD with intrauterine growth retardation, facial dysmorphism, short stature, macrocephaly, developmental delay, mental retardation, muscular hypotonia and hearing defect, we detected a large 16.23 Mb duplication. The duplication was inherited from the patient’s mother. In patient 52 with a non-syndromic gonadal dysgenesis we found a smaller 729 kb duplication including, according to array-CGH results, DAX1, and melanoma antigen family B 1 (MAGEB1) and melanoma antigen family B 4 (MAGEB4) gene as well as genes encoding chromosome X open-reading frame 21 (CXorf21), glycerol kinase (GK) and mitogen-activated protein kinase kinase kinase 7 (MAP3K7IP3). Duplication of the genes melanoma antigen family B 2 and 3 (MAGEB2 and MAGEB3), which belong together with DAX1 and MAGEB1 and 4 to the minimal common region in humans responsible for dosage-sensitive sex reversal (DSS), has not been excluded by further analyses. Therefore, it is possible that the duplication affects also these genes.

Azoospermia factor deletion

In patient 47, a female with a complete form of XY-GD, we identified a small deletion of about 503 kb affecting the azoospermia factor (AZFb and AZFc) region, which have been associated with spermatogenic failure (Vogt et al., 1996). It has been suggested that such Y chromosomal microdeletions predispose to the complete loss of the Y chromosome due to increased Y chromosome instability, leading subsequently to the development of a 45,X cell line and therefore to development of 45.X/46.XY mosaicism (Patsalis et al., 2005). Patients with a mosaic 45.X/46.XY karyotype exhibit a wide range of phenotypic manifestations, ranging from females with or without Turner stigmata and streak gonads through individuals with mixed gonadal dysgenesis and ambiguous genitalia to males with normal male genitalia depending on the proportion of 46,XY and 45,X cells in the gonads (Röpke et al., 2004). Although we found in our patient no indication of 45.X/46.XY mosaicism in blood cells, it is possible that such mosaicism is confined to the gonads. Interestingly, in two other patients who...
were excluded from this study since they had a suspicion of 45,X/46,XY mosaicism, we identified by array CGH additional Y chromosome microdeletions involving AZFb and AZFb + c regions.

**Novel candidate regions for XY-GD**

In the following we discuss rearrangements, which have not been described in patients with XY-GD so far. Only imbalances containing genes involved in spermatogenesis or testis development will be considered.

**Dup1p36.32**

In patient 85 with a 473.6 kb gain in 1p36.32, three genes are duplicated. Actin-related protein T2 (ACTRT2) gene and FLJ42 875 gene are fully duplicated, while only exons 1–3 of both known isoforms of PD domain-containing protein 16 (PRDM16) gene are duplicated. ACTRT2 is, together with the X-chromosomal-encoded actin-related protein T1 (ACTR1) protein, a component of the cytoskeletal structure of mammalian sperm heads called calyx. The expression of these two genes is restricted exclusively to the testis. However, expression of both genes appears very late in spermatid differentiation, speaking against a major role in testis differentiation (Heid et al., 2002). Therefore, it is questionable whether an amplification of ACTRT2 is causative for XY-GD. In the same patient, we identified a gain in chromosomal region 9q31.2. An association between these three duplicated genes and the phenotype of the patient is not obvious.

**Dup4q12**

In patient 42 we identified a gain including serine protease inhibitor, Kazal-type 2 (SPINK2) gene, RE1-silencing transcription factor (REST) gene, chromosome 4 open reading frame 14 (C4orf14) gene and polymerase II, RNA, subunit B (POL2RB) gene. SPINK2 encodes an acrosin-trypsin inhibitor, which inhibits the function of acrosin. A reduced acrosin activity has been linked to infertility. It is conceivable that an increase of SPINK2 activity due to the duplication causes a lowering of the acrosin concentration. Therefore, this duplication cannot be excluded as being causative. The same patient also has duplication in chromosomal region 2p16.1. To date, no correlation between the genes affected and the patient’s phenotype can be found.

**Dup4q35.2**

In patient 23 with a partial form of XY-GD, a duplication at 4q35.2 including melatonin receptor 1A (MTNR1A) gene and FAT tumour-suppressor homologue 1 (FAT1) gene was detected. The neurohormone melatonin, which is synthesized during the night by the pineal gland, has two known biological functions: first regulation of mammalian circadian rhythm and second regulation of the reproductive activity in photoperiod-dependent seasonal breeders. Furthermore, it has
been shown that melatonin subtype 1A receptors are expressed in Leydig cells and that melatonin exerts an inhibitory effect on the testosterone secretion via melatonin subtype 1A receptors in the hamster testis (Frungieri et al., 2005). Because in this patient duplication is present, a causative effect for gonadal dysgenesis seems to be unlikely.

**DelSp15.33**

In patient 20, who has a partial XY-GD, the telomerase reverse transcriptase (TERT) gene encoding the rate-limiting catalytic subunit of the telomerase is deleted. Mutations in the TERT gene are causative for autosomal dominant dyskeratosis congenital (MIM#127550). Activity for telomerase is high in very proliferative cells like germ line cells and the protein is strongly expressed in the adult testis. Consistent with these findings Tert-deficient mice show decreased fertility due to smaller litter sizes and testicular atrophy (Farazi et al., 2006). Therefore, a causative effect cannot be excluded. In the same patient, we found a large gain at 8q24.3.

**Dup9q21.32**

Patient 67 affected by hypoplastic uterus, scrotal hypospadias and a bifid scrotum, has a duplication of approximately 192.8 kb on 9q21.3. One of the duplicated genes is G kinase-anchor protein 1 (GKAP1). The mouse homologue (Gkap42) is exclusively expressed in the testes, especially in spermatocytes and early round spermatids and less in spermatogonia, late spermatids as well as Sertoli and Leydig cells (Yuasa et al., 2000). According to these expression data a correlation between GKAP1 and the phenotype of the patient is possible.

**Dup10q11.23**

One of the major proteins of the human semen is encoded by beta microsominoprotein (MSMB) gene in 10q11.23. This gene is duplicated together with four other genes in patient 95 who is affected by XY-GD with normal breast development. An elevated MSMB concentration has been reported in the seminal plasma of subfertile patients (Anahi Franchi et al., 2008). Also affected by the gain in chromosomal region 10q11.23 detected in patient 95 is the nuclear receptor coactivator 4 (NCOA4) gene. NCOA4 acts as a ligand-dependent androgen receptor (AR) associated protein that specifically enhances the transcriptional activity of AR in the presence of dihydrotestosterone and testosterone. The AR mediates the action of androgens in male reproduction and mutations in the AR are associated with androgen insensitivity syndrome (AIS, MIM#300068). However, not all individuals with AIS carry a mutation in the AR gene; Lim et al. (2001) screened the NCOA4 gene for causative mutations without identifying any. However, a causative role of a deletion for GD cannot be excluded.

**Dup1q21.3**

A duplication affecting catenin, alpha 3 (CTNN3) gene was detected in patient 74 with partial XY-GD and some features of virilization. CTNN3 expression was originally discovered in testis, but is most abundantly expressed in heart. In tests, CTNN3 expression is confined to peritubular myoid cells and Sertoli cells. The heart- and testis-specific expression of CTNN3 is explained by a specific binding and activation of the transcription factor GATA-binding protein 4 (GATA4) to the CTNN3 promoter (Vanpoucke et al., 2004). In Sertoli cells, GATA4 transactivates a number of other testis-specific genes (Tremblay and Viger, 2001). One binding partner of CTNN3 is β-catenin in the testis. Activation of β-catenin in XY mice resulted in male-to-female sex reversal (Maatouk et al., 2008). Therefore, this duplication can be causative.

**Dup12q24.13-q24.21**

We detected in patient 57 a 240.7 kb duplication interval on the long arm of chromosome 12, which affects partly the RNA-binding motif protein 19 (RBM19) gene. Mice with a disruption of the Rbm19 gene due to a gene-trap insertion failed to develop beyond the morula stage, suggesting that nucleolar protein RBM19 is essential for preimplantation development (Zhang et al., 2008). An association between this gain and patients phenotype cannot be excluded.

**Dup20p13**

We found a duplication affecting the proteasome inhibitor subunit 1 (PSMF1) gene in patient 6, a male with a syndromic form of 46,XY partial dysgenesis. An up-regulation of PSMF1, an inhibitor of 20S proteasome, was found after administration of exogenous testosterone (Cui et al., 2008). The authors suggested that an increase of PSMF1 may participate in the abnormal spermatogenesis by impairing the accumulation of ubiquitin-proteasome pathway, which is important for normal spermatogenesis. A correlation between XY-GD and this duplication is questionable.

**DupXp11.22**

In patient 17, who is affected by a complete form of 46,XY-GD, we determined a 507.8 kb duplication including four genes. Unfortunately, no DNA was available from her affected sister. The partly duplicated AKAP4 gene codes for the highly conserved A-kinase anchor protein 4, which is transcribed late in spermatogenesis and plays an essential role in fibrous sheath assembly during spermatogenesis and in the regulation of flagellar function in spermatozoa. Male mice lacking Akap4 showed no decrease in sperm number but were infertile due to a loss of sperm motility (Miki et al., 2002). Furthermore, in an infertile patient with sperm fibrous sheath dysplasia, an intragenic gene deletion involving AKAP3 and AKAP4 genes has been found (Baccetti et al., 2005). Since AKAP4 is involved in late spermatogenesis, it is unlikely that a partial duplication provokes complete XY-GD. Also duplicated is cyclin B3 (CCNB3) gene, which encodes a mammalian meiosis-specific cyclin and is expressed in leptotene and zygotene spermatocytes during the first round of meiosis in adult tests. A prolonged expression of CCNB3 after zygote leads to a disruption in spermatogenesis in male mice (Refik-Rogers et al., 2006).

In summary, we identified the definite cause for gonadal dysgenesis in 25% of syndromic cases and in 5.6% of non-syndromic cases. Therefore, array CGH is a very useful diagnostic tool in the management of XY-GD. Additionally, array CGH can also be used for the identification of potential candidate genes. In this study we could delineate 11 regions. However, for none of these 11 regions, did we detect recurrent or overlapping aberrations. Even if no obvious candidate gene for gonadal dysgenesis could be identified, it is striking that many genes involved in spermatogenesis are compromised by these rearrangements.
Authors’ roles

S.L.: design of the study, acquisition, analysis and interpretation of the data, main author; O.H.: acquisition and interpretation of the data, critical review; G.S.: acquisition and interpretation of the data, critical review; M.H.: acquisition and analysis of the data; G.W.: acquisition of the data, critical review; S.M.: acquisition of the data, critical review; A.K.: acquisition of the data, critical review; P.W.: conception and design of the study, interpretation of the data, critical review.

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