Aberrations of the X chromosome as cause of male infertility

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Abstract

Male infertility is most commonly caused by spermatogenetic failure, clinically noted as oligo- or azoospermia. Today, in approximately 20% of azoospermic patients, a causal genetic defect can be identified. The most frequent genetic causes of azoospermia (or severe oligozoospermia) are Klinefelter syndrome (47,XXY), structural chromosomal abnormalities and Y-chromosomal microdeletions. Consistently with Ohno’s law, the human X chromosome is the most stable of all the chromosomes, but contrary to Ohno’s law, the X chromosome is loaded with regions of acquired, rapidly evolving genes, which are of special interest because they are predominantly expressed in the testis. Therefore, it is not surprising that the X chromosome, considered as the female counterpart of the male associated Y chromosome, may actually play an essential role in male infertility and sperm production. This is supported by the recent description of a significantly increased CNV burden on both sex chromosomes in infertile men and point mutations in X-chromosomal genes responsible for male infertility. Thus, the X chromosome seems to be frequently affected in infertile male patients. Four principal X-chromosomal aberrations have been identified so far: 1) aneuploidy of the X chromosome as found in Klinefelter syndrome (47,XXY or mosaicism for additional X chromosomes). 2) Translocations involving the X chromosome, e.g. nonsyndromic 46,XX testicular disorders of sex development (XX-male syndrome) or X-autosome translocations. 3) Copy Number Variations (CNVs) affecting the X chromosome. 4) Point mutations disrupting X-chromosomal genes. All of these are reviewed herein and assessed concerning their importance for the clinical routine diagnostic workup of the infertile male as well as their potential to shape research on spermatogenetic failure in the next years.
Introduction

Male infertility is most commonly caused by spermatogenetic failure, clinically noted as oligo- or azoospermia. Concerning the underlying pathophysiology, a substantial genetic contribution can be assumed from family studies as well as from animal - mostly mouse knockout - models (1, 2, 3, 4). The most frequent genetic causes of severe oligo- or azoospermia are Klinefelter syndrome (47,XXY), structural chromosomal abnormalities (e.g. translocations) and microdeletions of the AZF (“AZoospermia Factor”) regions on the long arm of the Y chromosome (5). Beyond these well-established causes of male infertility, more and more genes are reported to be associated with spermatogenetic failure in males. NR5A1 (6, 7, 8, 9) and DMRT1 (10, 11) are examples of autosomal genes in which heterozygous mutations are causative. However, sex-chromosomal genes are prime candidates because of their hemizygous state in males and TEX11 (12, 13) and RHOX (14, 15) are recent examples which, if mutated, cause male infertility.

The Y chromosome triggers embryonic male development. Specifically, the SRY gene (Sex Determining Region Y), located on the short arm of the Y chromosome, is responsible for male sex-determination (16, 17). Concerning infertility in otherwise healthy men, three partially overlapping but discrete regions were identified on the long arm of the Y chromosome that are essential for normal spermatogenesis. The deletion of any of these regions, designated as AZF microdeletions, regularly leads to infertility due to severe oligo- or azoospermia (18). Ampliconic regions in the male specific region of the Y chromosome (MSY) explain the mechanism of the different AZF deletions by non-homologous recombination (19). Besides, the Y chromosome of higher mammalian lacks most genes originally located on ancestral Y chromosomes and other genes were moved to autosomes (20, 21). These inserted genes are of special interest because the autosomal gene copies show increased expression in the testis, suggesting an essential role in spermatogenesis, and these genes are possible candidates causing male infertility (20).

In contrast to this evolutionary dramatical loss of genes from the Y chromosome (22), genes on the other sex chromosome, the X chromosome, were predicted to vary little in mammals (23). In fact, the X chromosome is evolutionary stable, but it has been evolving towards a kind of male specialization. It seems that independently acquired
X-linked genes show an important role in male infertility and sperm production (24, 25, 26).

**The human X chromosome**

The human sex chromosomes (X and Y) originate from an ancestral homologous chromosome pair, which during mammalian evolution lost homology due to progressive degradation of the Y chromosome (20, 27, 28, 29). In contrast to females who inherit an X chromosome from each parent, males own a single, maternal X chromosome. In females, gene dosage resulting from the two X chromosomes is compensated through inactivation of almost the entire genetic content of the second X chromosome (30, 31, 32, 33, 34). Every supernumerary X chromosome, as in 47,XXY or 47,XXX, will be inactivated in the same manner. However, a rather large fraction of about 10% of X-chromosomal genes escape X-inactivation (35). The ‘pseudoautosomal’ regions (PAR1 and PAR2) are short regions of homology between both distal ends of the X and Y chromosomes as they behave like an autosome and recombine during meiosis (36, 37, 38, 39, 40, 41). Genes inside the PARs share copies on both sex chromosomes, whereas the vast majority of genes outside the PARs are present in only a single copy in the male genome.

Altogether, 1098 genes were annotated to the X chromosome, of which 99 encode proteins expressed in testis and in various tumor types (25). In 1967, it was predicted by Ohno that the X chromosome was evolutionary very stable, and he assumed that X-linked genes would vary little among mammals (23). To test this hypothesis, the so-called ‘Ohno’s law’, Mueller and colleagues very recently first improved and then compared the sequences of the human and mouse X chromosomes (24). In accord with Ohno’s law, they found that most X-linked single-copy genes are shared by humans and mice. However, and in contrast to Ohno’s law, approximately 10% of human and 16% of mouse X-chromosomal genes did not have orthologs in the other species. In addition, the majority of these unique genes were found to reside in ampliconic regions, making them rather difficult to sequence. Re-analysis of RNA sequencing data indicated that nearly all of these independently acquired genes are expressed in males, but not females, and predominantly in the testes. The authors concluded that, in fact, the X is a ‘male chromosome’ (24) highly enriched for genes relevant for spermatogenesis.
Sex chromosome aneuploidy

More than 60 years ago, in 1956, the first human chromosomes were made visible by Tjio and Levan (42) and three years later the first clinical disorders were identified that are caused by chromosomal aberrations including Klinefelter syndrome (43).

In contrast to the 22 autosomes, which are present in pairs, the X and Y chromosomes are of special interest concerning male infertility because they are only present once in normal males. Thus, any gene located on the sex chromosomes is only present in a single copy (hemizygous) with the exception of genes located in PAR1 and PAR2, respectively.

Four major sex chromosome aneuploidies are relatively common. Of these, 47,XXY Klinefelter syndrome (1 in 500 newborn boys) and 45,X Turner syndrome (1 in 2,500-3,000 newborn girls) are regularly associated with infertility. In contrast, 47,XYY (1 in 1,000 newborn boys) and 47,XXX Triple X syndrome (1 in 1,000 newborn girls) both apparently have little effect on fertility in most patients. However, the supernumerary sex chromosomes are also considered to impair meiosis leading to varying degrees of infertility, but the cause-effect relationship is difficult to assess in individual patients (44, 45, 46, 47).

In contrast to autosomal trisomies, additional copies of the X chromosome are associated with mild phenotypic abnormalities possibly because of dosage compensation by inactivation of the other X chromosome (45, 48).

Another difference to autosomal trisomies concerns the parental origin of the aneuploidies. While autosomal trisomies in the vast majority (>90%) arise during oogenesis, male gametogenesis plays a major role in the generation of sex chromosomal aneuploidies in the offspring (49, 50). For example, the supernumerary X in Klinefelter syndrome is roughly equally often of maternal and paternal origin (51, 52).

Meiosis and XY pairing

In mammals, meiotic cell division is necessary to produce male and female germ cells. Haploid DNA content in germ cells is achieved by one round of DNA
replication, followed by two successive rounds of cell division (meiosis). To successfully segregate chromosomes to daughter cells in the first meiotic cell division, homologous chromosomes must find each other and stably pair. Autosomal chromosomes are homologous along their entire length and are hence able to pair from end to end. In contrast, the X and Y chromosomes are not homologous save for the short segments at the end of each chromosome arm, the PARs (39). Both chromosomes differ dramatically in size and gene content and X–Y chromosome homology search and pairing can, therefore, only be mediated by the PARs, conceivably making X–Y segregation particularly difficult (22, 28, 53).

**Klinefelter syndrome**

A supernumerary X chromosome resulting in the karyotype 47,XXY as the genetic cause for the Klinefelter Syndrome (KS) was first described in 1959 (43). KS is one of the most frequent cytogenetic anomalies found in infertile men. The prevalence increases from approximately 3% in unselected to up to 15% in azoospermic patients (54, 55, 56). The most common karyotype found in 80-90% of KS men is 47,XXY and the others comprise sex-chromosomal mosaicism (e.g. 47,XXY/46,XY), additional sex chromosomes (48,XXXXY; 48,XXYY; 49,XXXXY), or structurally abnormal X chromosomes (4, 57, 58). The supernumerary X chromosome is derived either from meiotic non-disjunction during gametogenesis of the parents or from post-zygotic mitotic cell divisions during early embryogenesis (48).

As hallmark features, KS men regularly have small testes (<6 ml bi-testicular volume), azoospermia, and high gonadotropin levels (LH and FSH). In rare cases, some spermatozoa may be found in the ejaculate of KS men and very few natural conceptions by KS men have been described. Depending on the testosterone levels, KS men often exhibit varying symptoms of hypogonadism such as undervirilization, gynecomastia, or erectile dysfunction (58, 59, 60).

The phenotype of patients with polysomy of the sex chromosomes (48,XXXXY; 48,XXYY; 49,XXXXY; 49,XXYY; 49,XXXXY; 49,XXXXY) has to be seen quite separate from patients with a 47,XXY karyotype (48, 58, 61). These types of sex chromosome aneuploidy are very rare and only few case reports have been
published so far. Although, it has been shown that also the additional X chromosomes are inactivated (62, 63), as mentioned above some loci escape inactivation and may function in a disomic (in case of XXY, XXYY or XXYYY), trisomic (XXXY or XXXYY) or tetrasomic (XXXXY) state. This is likely the predominant reason for the more severe phenotypic abnormalities associated with these karyotypes (64, 65, 66).

Nonsyndromic 46,XX testicular Disorders of Sex Development (XX-male syndrome/DSD)

Nonsyndromic 46,XX testicular disorders of sex development (46,XX testicular DSD; formerly known as XX-males) are characterized by the combination of male external genitalia, testicular differentiation of the gonads and a 46,XX karyotype identified by conventional cytogenetic analysis. Most 46,XX testicular DSD-males arise from translocations of parts of the short arm of the Y chromosome to one of the X chromosomes (67, 68). Translocations of a DNA-segment that contains the testis-determining gene SRY from the Y to the X chromosome takes place during paternal meiosis (69). The presence of the SRY gene is sufficient to cause the initially indifferent gonad to develop into a testis. Individuals with 46,XX testicular DSD typically have normal external genital development, but micropenis, hypospadia or cryptorchidism may be seen. In addition, males with 46,XX karyotype regularly have decreased testosterone levels with high levels of LH and FSH (70). As reported above, 46,XX testicular DSD-males are infertile because of azoospermia and Sertoli-Cell-Only syndrome or complete degeneration of the seminiferous tubules upon histological examination (70, 71). The X-Y rearrangements cannot be detected using conventional cytogenetic analysis and, thus, molecular cytogenetic analysis using a specific probe for the SRY locus or array-Comparative Genomic Hybridization (CGH) should be carried out in all cases of 46,XX testicular DSD.

Very few cases have been reported on Y-autosomal translocations including the SRY gene leading to 46,XX testicular DSD (72, 73).

SRY-positive X-Y rearrangements are generally not inherited because they result from de novo translocations and the affected males are infertile. When SRY is
translocated to another chromosome or when fertility is preserved, sex-limited autosomal dominant inheritance is observed (69, 74).

Males with an 46,XX karyotype having no SRY gene are rare with about 10% of all 46,XX testicular DSD and the testicular development may be activated due to other genetic aberrations (70). Copy Number Variations (CNVs) affecting SOX9 or SOX3 as well as RSPO1 mutations have been implicated in this respect (75, 76, 77).

**X-autosome translocations in males**

Meiosis in males with X-autosome translocations is typically affected due to failure in XY pairing (see above) and practically all males with X-autosome translocations are infertile due to spermatogenic arrest (54, 78) for which disruption in the formation of the sex vesicle is the obvious cause (53, 79). The proper sex vesicle formation is necessary for normal spermatogenesis, and any interference will compromise the process of sperm development (53). In cases of X-autosome translocations the formation of quadrivalents was proposed, in which the PARs of the Y chromosome associate with the homologous parts of the X chromosome on the derivative X chromosome and the derivative autosome (80). In this context, also Y-autosomal translocations, with an autosomal breakpoint other than an acrocentric short-arm, may result in disruption of the sex vesicle leading to infertility (81). However, for both scenarios, if spermatozoa can be recovered, albeit in very small numbers, in vitro fertilization (IVF) using intracytoplasmic sperm injection (ICSI) may be attempted, but associated with a potentially high risk for generation of unbalanced chromosomal aberrations in the offspring (82). Prenatal genetic diagnostics (PGD) can be offered to the couple after genetic counselling to screen for balanced or unaffected embryos.

**X-chromosomal deletions**

Since genome-wide technologies (array-Comparative Genomic Hybridization [array-CGH] or Single Nucleotide Polymorphism [SNP]-arrays) have been available to identify submicroscopic deletions and duplication, these are analyzed on a large scale in many diseases and are termed Copy Number Variants (CNVs). It immediately became clear that CNVs add to the normal variation in our genome and that the majority of CNVs has probably no or little relevance for disease (83). At the
same time, however, many novel microdeletion syndromes are being described with increasing pace, of which many are associated with intellectual disability and malformations (84).

The Y-chromosomal AZF deletions are an example for CNVs with a clear cause-effect relationship for male infertility and have been part of the clinical routine diagnostic for many years. In contrast, genome-wide CNV analyses in infertile men are thus far only performed in research settings (10, 85, 86, 87), of which some have focused specifically on the X-chromosome (88, 89, 90). When comparing infertile (oligo- or azoospermic) with fertile (or normozoospermic) men, the consistent finding of the available studies is a significantly higher overall “burden” of microdeletions, especially on the sex chromosomes (10, 85, 88, 89). In addition, one study has reported an increase of single nucleotide variations (SNVs) in infertile men compared with controls (10) and it has been speculated that infertility may be associated with an increased overall genome instability. To date, no CNVs with a definite cause-effect relationship comparable to the Y-chromosomal AZF deletions have been identified and replicated in an independent study. Thus, CNV analyses can currently not be advised for clinical diagnostics. However, analyses of CNVs can be used to identify novel candidate genes for male infertility. A recent example is \textit{TEX11}, in which CNVs as well as SNVs cause non-obstructive azoospermia (see below) (12).

\textbf{X-linked candidate genes}

Numerous mouse models that have linked hundreds of genes with azoospermia and infertility provide insight into the molecular mechanisms responsible for this condition in mice (91). However, over the last few years, some of these genes have been reported to be mutated in men but most of these studies have so far not been replicated and their role in human gonadal development and function currently remains unclear. A recurring problem is the interpretation of detected ‘mutations’, which may comprise rare but still non-pathogenic variants or variants that are associated with specific ethnic groups. This underlines the need for 1) strict \textit{in silico} assessment of the pathogenicity, which should follow established guidelines (e.g. American College of Medical Genetics and Genomics, ACMG (92)), and 2) functional studies to ascertain the pathogenicity of mutations.
Nevertheless, genes on the sex chromosomes are prime factors for sexual differentiation and gonadal function. Many X-linked genes are expressed in the testis and are thought to be involved in gametogenesis.

**Androgen Receptor (AR)**

Mutations in the X-linked AR gene cause three different diseases I) androgen insensitivity syndrome (AIS) (93), II) spinal and bulbar muscular atrophy (SBMA or Kennedy disease) (94), and III) prostate cancer (95, 96, 97).

For the wide spectrum of AIS phenotypes, AR mutations may lead to complete androgen insensitivity (CAIS) with a female phenotype in karyotypic males, partial forms (PAIS) in patients with ambiguous genitalia, or mild forms (MAIS) in men with hypospadias, gynecomastia and spermatogenic impairment (98, 99, 100). However, patients with MAIS may have normal male genitalia and infertility as the only symptom. Therefore, AR mutational analysis should be prompted in cases of a high androgen sensitivity index (ASI) but mutations in the AR gene seem to be a rare cause of isolated male infertility (101, 102, 103).

Two polymorphisms, the CAG and GGN polymorphisms, located in exon 1 of the AR gene, code for polyglutamine and polyglycine stretches, respectively. Longer lengths of the CAG-repeat are associated with decreased transcriptional activity of the AR protein *in vitro*, suggesting that longer polyglutamine tracts may be related to male infertility (104, 105, 106). Thus, the CAG repeat length has been extensively studied for their role in male infertility. However, overall statistically significant, but very small and thus clinically irrelevant differences have been determined between infertile men and controls (105, 107, 108, 109). Variations of the GGN polymorphism are frequently found in the general male population and are not associated with male infertility (109, 110, 111, 112, 113).

**TEX11**

The X-linked TEX11 (testis-expressed 11) gene encodes a protein critical for male germ cell meiotic DNA recombination (114). Tex11-knockout male mice exhibit azoospermia with meiotic arrest at the pachytene stage due to an inability to repair
double-strand DNA breaks (114). Recently, Yatsenko et al. identified TEX11 as a new genetic marker for spermatogenic arrest in men with idiopathic infertility (12). Using array-CGH, a loss of approximately 99 kb on chromosome Xq13.2, involving three exons of TEX11, was found in two azoospermic patients. Subsequent mutational screening identified five additional TEX11 mutations in overall 2.4% of azoospermic patients, which were absent in normozoospermic controls. Importantly, five of those TEX11 mutations were detected in 33 patients (15%) diagnosed with azoospermia and meiotic arrest, resembling the Tex11-deficient mouse meiotic arrest phenotype. Immunohistochemical analysis showed specific cytoplasmic TEX11 expression in late spermatocytes, as well as in round and elongated spermatids, in normal human testes. In contrast, testes from azoospermic patients with TEX11 mutations showed meiotic arrest and lacked any TEX11 expression (12). This finding relied on the combination of genetics and phenotyping by testicular histology. Hemizygous mutations in TEX11 were confirmed as an important cause for meiotic arrest already in another study (13). Exome sequencing was also recently used to identify a homozygous mutation in the closely related, but autosomal gene TEX15 as a cause for meiotic arrest (115).

**RHOX**
The human Reproductive Homeobox (RHOX) genes are clustered on the X chromosome and comprise three genes: RHOXF1, RHOXF2 and RHOXF2B, which are selectively expressed in human oocytes and male germ cells (116, 117, 118). Several mutations in RHOXF1 and RHOXF2/2B found in patients with severe oligozoospermia stress the importance of these genes in male infertility (14, 15). It has been shown that RHOXF2/2B mutations significantly impair the ability to regulate downstream genes such as transcription factors and chaperons from the HSP70 family (15).

**ANOS1 (KAL1)**
ANOS1 (formerly known as KAL1), located on the short arm of the X chromosome (Xp22.31), encodes the extracellular matrix protein anosmin 1 that plays an important role in the migration of gonadotropin-releasing hormone (GnRH)-producing neurons
to olfactory axons of the hypothalamus (119, 120). Gene deletions and point mutations were identified in patients with Hypogonadotropic Hypogonadism (HH) with or without anosmia (121, 122, 123, 124, 125).

**USP26**

The *USP26* gene is located on Xq26 and belongs to a large family of deubiquitinating enzymes (DUBs) (126), which are responsible for processing inactive ubiquitin precursors, removing ubiquitin from cellular adducts, and rescuing macromolecules from degradation (127, 128, 129). The USP26 protein assembles with the androgen receptor (AR) and modulates its ubiquitination. Therefore, USP26 influences AR transcriptional activity, which is, as described above, fundamental for the proper maintenance of spermatogenesis (130). In recent years, numerous nucleotide variations in *USP26* gene have been reported both in fertile and infertile men, but the conflicting results of these studies render the association of variations in *USP26* with male infertility unclear (108, 131, 132, 133, 134).

**TAF7L**

In mice, *Taf7l* is highly expressed in germ cells and *Taf7l* knockout mice exhibit structurally abnormal sperm, a reduced sperm count, motility, and fertility (135, 136). Thus, it has been speculated that the human *TAF7L* gene may be essential for maintenance of spermatogenesis (137). However, sequence analysis of infertile patients and controls revealed no clear association to male infertility (108, 138).

**Summary**

The supposedly “female” human X chromosome plays a predominant role in spermatogenesis and several X-chromosomal aberrations are known to cause male infertility. The genetic spectrum ranges from an additional X chromosome found in Klinefelter patients to point mutations in several recently published genes. Furthermore, patients with spermatogenic failure carry a higher CNV burden on the sex chromosomes compared with fertile males.
Two different types of genes are located on the X chromosome: highly conserved genes according to Ohno’s Law combine with acquired adaptive genes in ampliconic gene families. These acquired X-linked genes are predominantly expressed in the testes and mutations may impact male reproduction as it was recently found in *TEX11* or *RHOXF1* and *RHOXF2/2B*. These genes emphasize the importance of the X chromosome for male gonadal development.

In summary, the diverse X-chromosomal defects found in patients with spermatogenic failure point out that the X chromosome is, besides the well-known X-linked developmental genes, also a “male” chromosome, containing a multitude of genes orchestrating different stages of male gonadal and especially germ cell development.

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