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Sperm cryopreservation in male infertility due to genetic disorders

Csilla Krausz* and Gianni Forti
*Andrology Unit, Department of Clinical Physiopathology, Viale Pieraccini, 6, Firenze, Italy; *Author for correspondence (email: c.krausz@dfc.unifi.it)*

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Abstract

Certain chromosomal and genetic anomalies, such as Klinefelter syndrome (47,XXY) and Y chromosome microdeletions, have been reported as potential causes of a progressive impairment of spermatogenesis. In these cases cryoconservation of ejaculated or testicular sperm represent a valuable tool for the preservation of fertility. However, dealing with genetic disorders, the transmission of genetic anomalies has to be taken into consideration. It is therefore important to be aware about the clinical importance and the related genetic risks of these anomalies. In this article we describe the clinical significance of these diseases.

Introduction

Cryopreservation of semen is considered the only currently available preventive therapy for the preservation of fertility in patients undergoing treatments with potential toxic effects on spermatogenesis.

However, with the advent and diffusion of new techniques of assisted reproduction, in particular 'Intracytoplasmic Sperm Injection' (ICSI) which requires only a few viable spermatozoa, the indications for cryopreservation have been extended also to other categories of patients. In fact, patients affected by severe oligozoospermia (sperm number <5millions/ml) or cryptozoospermia, especially if a progressive decrease of the number of spermatozoa over time is observed, are now candidates for preventive cryopreservation.

The progressive depletion of spermatozoa in the ejaculate may have testicular or post-testicular origin. The latest is generally related to a progressive occlusion of the seminal tract mainly due to inflammatory processes, whereas testicular causes are more heterogeneous. Certain chromosomal and genetic anomalies, such as Klinefelter syndrome (47,XXY) and Y chromosome microdeletions, have been reported as potential causes of a progressive impairment of spermatogenesis. In these cases cryopreservation represent a valuable tool of prevention, although the transmission of genetic anomalies has to be taken into consideration. It is therefore important to be aware about the clinical importance and the related genetic risks of these anomalies.

Klinefelter syndrome

Klinefelter syndrome (47,XXY) is the most common sex chromosome abnormality in humans with an incidence of 1:600 in live births and 1 in 300 in spontaneous abortion (Nielsen and Wolhert 1991). It is also the most frequent chromosomal anomaly in azoospermic men (14%). About 80% of patients bear a 47,XXY caryotype whereas the other 20% represented either by 47,XXY/46,XY mosaics or higher grade sex chromosomal aneuploidy or structurally abnormal X chromosome (Nieschlag et al. 2000). The syndrome is classically characterized by hypergonadotrophic azoospermia, small firm testes and symptoms of androgen deficiency.

Patients affected by this syndrome have an average of 15–20% of testicular sperm recovery rate. However, data in the literature are limited (Tournaye et al. 1996; Okada et al. 1999; Westlander et al. 2001; Kamischke et al. 2003; Westlander et al. 2003) and more studies are needed to better define predictive values for sperm recovery. Hormone levels (FSH, Inhibin B), testis volume do not seem to predict for the presence or absence of spermatozoa in the testis. Among the predictive factors, younger age is considered a positive predictor since patients at younger age have a higher incidence of sperm recovery and in some cases spermatozoa can be even found in the ejaculate (Kamischke et al. 2003). Sperm recovery was positive in 5/11 patients with <34 years whereas 0/7 with >34 years old (Westlander et al. 2003). The age of patients with ejaculated sperm was < 24years old (Kamischke et al. 2003) indicating the potential importance of an early diagnosis. Although men at their early 20s may not desire immediate conception, a preventive sperm cryopreservation of ejaculated spermatozoa should be a correct way to preserve their fertility. On the other hand, if patients are already azoospermic at a relatively young age, testicular sperm extraction may be a too invasive although potential useful treatment for preventive storage of extracted sperm. Due to its invasiveness and to the relative scarcity of data in the literature, decision for cryoTESE in young men with no immediate interest in procreation should be taken after careful counseling. If a patient reaches to the clinic for couple infertility and decides for a full diagnostic work-up including testis biopsy, cryoTESE (cryopreservation of testicular sperm) is mandatory before the partner is induced for multiple follicular growth. If spermatozoa is recovered from the wet preparation of testicular tissue subsequent ICSI can be performed. Concerns have been raised about the chromosomal normality of the embryos generated through this infertility treatment. To date, 34 healthy children have been born using ICSI without Preimplantation Genetic Diagnosis (PGD) and the conception of one 47,XXY fetus has been reported (for review see Staessen et al. 2003). However, a recent study based on ICSI combined with PGD on 113 embryos shows that there is a significant fall in the rate of normal embryos for couples with Klinefelter sdr. in respect to controls (54% versus 77.2%). Due to the significant increase of sex chromosomal and autosomal abnormalities in the embryos of Klinefelter patients, ICSI + PGD should be performed (Staessen et al. 2003).

Y Chromosome microdeletions and spermatogenic failure

While the X chromosome is shared by both sexes, the presence of the Y chromosome in somatic cells represents a unique peculiarity of males. Due to the abundance of tandemly repeated satellite DNA and the apparent paucity of gene content, the Y chromosome was considered for long time a 'genetic wasteland,' necessary only for sex determination. This has led to propose its future extinction in an evolutionary context (Marshall 2000; Aitken and Marshall 2002). This view has been recently challenged by the recent identification of both an unexpected number and variety of Y chromosome genes, many of which with an ubiquitous expression, and by the presence of a conversion-based system of gene copy 'correction,' acting to preserve Y genes from the gradual accumulation of deleterious mutations and thus ensuring their continuity in time (Repping et al. 2002; Skaletsky et al. 2003).

The human Y chromosome is classically divided into two functionally distinct regions: (i) the pseudoautosomal regions (PAR1 and PAR2), which are homologous with X chromosome sequences and are responsible for correct pairing between the two sex chromosomes during male meiosis; (ii) the male specific region Y (MSY), previously called the 'Non-recombining region Y' (NRY), in which, in certain parts, instead of classical recombination 'intrachromosomal gene conversion' (non-reciprocal transfer) takes place (Repping et al. 2002). This region comprises 95% of the length of the chromosome (Figure 1).

The role of the Y chromosome in reproductive functions is indisputable since it contains the master gene of testis determination (SRY) and a number of genes with specific expression pattern in spermatogenic cells (Figure 1). The extent of the MSY region is around 63 Mb, but only 23 Mb are transcriptionally active (euchromatic portion). A total of 156 transcriptions units have been identified with 78 protein coding units and 78 putative non-coding units (Skaletsky et al. 2003). The protein coding units are the product of 27 genes,

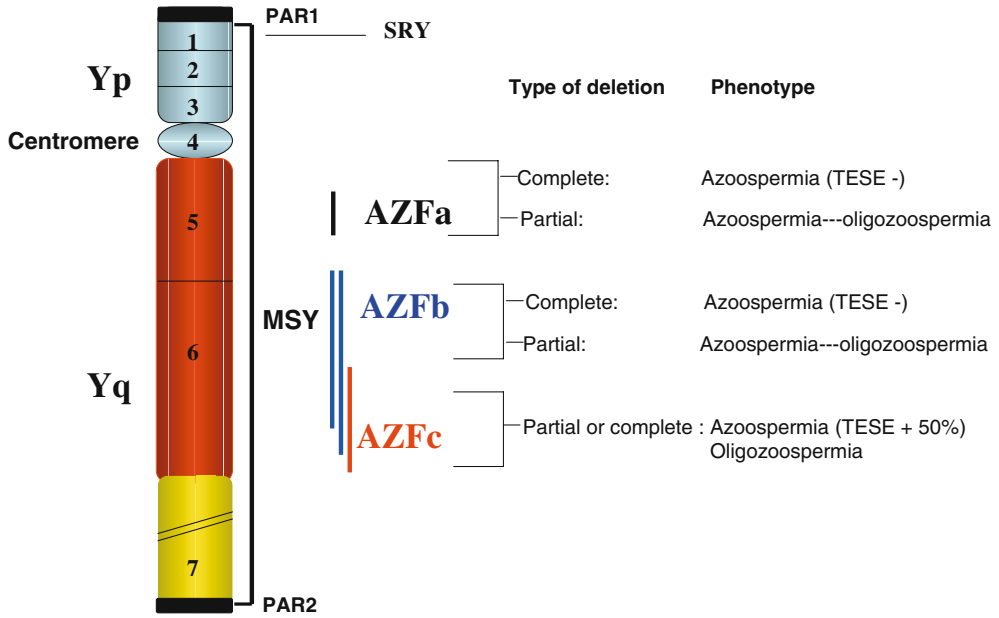

Figure 1. The 3 Azoospermia Factor (AZF) regions. Deletions of these regions result in spermatogenic failure. The type of deletions and the associated phenotype are indicated. AZFb deletions (two subtypes) overlap with the AZFc region. Yq: long arm of the Y chromosome, Yp: short arm of the Y chromosome, PAR: pseudoautosomal region, MSY: male specific Y with an extension of 63 Mb.

12 of which are expressed ubiquitously and 11 are exclusively, or predominantly, expressed in testes. Although the majority of the AZF candidate genes have been identified years ago, their exact function and role in spermatogenesis remains unclear. Genes involved in spermatogenesis are situated mainly in three regions of the long arm of the Y chromosome (Yq). Although it has been established in 1976 that deletions of the long arm of the Y chromosome are associated with spermatogenic failure (Tiepolo and Zuffardi 1976), it is only in the last few years have these regions been defined at a molecular level. Vogt et al. (1996) observed that Y chromosome microdeletions follow a certain deletion pattern, with three recurrently deleted non-overlapping subregions called Azoospermia Factor (AZF) in proximal, middle and distal Yq11, designated AZFa, AZFb and AZFc, respectively. Following further characterization of the AZF regions, the deletions previously thought to define AZFb were found to extend into the AZFc region (Repping et al. 2002). After resolving many initial contradictory issues such as specificity of Y deletions, variability in deletion frequency, markers to be tested, genotype–phenotype correlation, the clinical significance of AZF deletions have been well established.

Specificity and frequency

The etiopathogenetic role of Y deletions in male infertility has been questioned by reports describing Y microdeletions in 'proven fertile men,' however appropriate studies using normospermic men as controls and *not* 'fertile subjects with unknown sperm count,' have highlighted that AZF deletions are specific for spermatogenic failure (see Krausz and McElreavey 2001 and references herewith).

The incidence of Y deletions varies enormously between studies from 1–55% (van der Ven et al. 1997; Foresta et al. 1998). Many of the initial studies were suffering of technical problems, and papers presenting a mosaic type of pattern of deletions, especially with deletions of single STS without confirmation, are of dubious significance. A part from the lack of rigorous testing of negative results or inappropriate choice of markers, other factors such as differences in the composition of the study populations (clinical characteristics and ethnic background) have been evoked to explain this variability. We studied four different populations using a similar set of markers and similar clinical criteria for the definition of patients. The studies demonstrate that the main factor

influencing deletion frequency is the composition of the study population (highest frequency found in the two studies with the largest number of azoospermic men included) rather than the ethnic origin of a given population (Krausz et al, 1999a, b; Krausz et al. 2001; Krausz et al. 2003). The incidence of Y deletions is 10–15% in idiopathic azoospermic and 5–10% in idiopathic oligospermic men.

Indications for Y deletion analysis

Y deletions have been found almost exclusively in patients with <1 million spermatozoa/ml and are extremely rare with a sperm concentration of >5 millions of spermatozoa/ml (approximately 0.7%). Deletions have been found also (7%) as 'chance association' in the presence of other abnormal andrological findings such as varicocele, cryptorchidism, hypogonadotrophic hypogonadism etc. (Krausz et al. 1999b).

In two studies of the Danish population complete hormonal analysis was available in all patients and serum inhibin B concentration was uniformly below the normal range in patients with microdeletions due to their severe spermatogenic impairment (Krausz et al. 2001; Frydelund-Larsen et al. 2002). In a large study by Tomasi et al. (2003) inhibin B and FSH levels were undistinguishable in patients with idiopathic and microdeletion-associated oligo-azoospermia. These data do not support the hypothesis proposed by an other group (Foresta et al. 2002), that microdeleted patients have a less severe impairment of Sertoli cell function than patients with idiopathic oligo-azoospermia.

Therefore, indications for AZF microdeletion analysis are (1) a sperm concentration of <5 millions of spermatozoa/ml in the absence of other known causes of spermatogenic damage ('idiopathic cases'); (2) for 'non-idiopathic' cases the threshold of sperm number can be lowered to <1 million spermatozoa/ml.

Which markers have to be tested?

The most frequently deleted region is AZFc (approximately 60%) followed by deletions of the AZFb and AZFb + c or AZFa + b + c regions (35%) whereas deletions of the AZFa region are extremely rare (5%).

For diagnostic purposes it is important to use a relatively small number of well-chosen markers which cover all the three AZF regions. It is still debated if gene specific deletion screening which theoretically is more appropriate, gives any advantage in the clinical management of the patients. The absence of isolated gene specific deletions in >1600 severe male factor patients, is suggestive of no practical advantage (see for review Silber et al. 1998; Krausz et al. 2003 and references herewith). The minimal set of primers proposed by the European Academy of Andrology (Simoni et al. 1999) is able to detect almost 100% of clinically relevant deletions. Once a deletion is found, it is important to define, with a second set of primers, the extension of the deletion, especially in case of AZFa and AZFb deletions in order to distinguish between 'partial' and 'complete' deletions.

The experience of the European Academy of Andrology and European Molecular Genetics Quality Network external quality control scheme in Molecular diagnosis of microdeletions of the Y chromosome implicate further efforts for the standardization of the method.

Clinical significance of Yq deletions

Genotype–phenotype correlations

A precise genotype–phenotype correlation can be observed only for deletions removing the entire AZFa or AZFb regions ('complete' deletions). These deletions are associated with Sertoli Cell only syndrome (SCOS) and spermatogenic arrest, respectively (Krausz et al. 2000; Kamp et al. 2001).

'Variable and changing phenotype'

'Partial' deletions of these regions or 'complete' or 'partial' AZFc deletions are associated with a variable phenotype ranging from hypospermatogenesis (oligozoospermia) to SCOS (azoospermia) in different subjects. Possible explanations for such a variable phenotype in the presence of similar deletions in different individuals are: (i) influences of the genetic background and environmental factors; (ii) a progressive regression of the germinal epithelium over time, therefore patients with different age may have different grade of spermatogenic failure. There is an increasing evidence

in the literature showing a progressive decrease of sperm number over time both in the ejaculate and in the testis, in patients affected by AZFc deletions (Calogero et al. 2001).

Therefore the identification of Y deletions has a diagnostic, prognostic and preventive value as:

- The presence of Y microdeletions explains the aetiology of the infertility and, thereby enable unnecessary medical and surgical treatments (for example correction of varicocele, Cayan et al. 2001) to be avoided.
- In azoospermic men, the presence of a complete AZFa or AZFb deletion has a negative prognostic value for testicular sperm retrieval (Brandell et al. 1998; Krausz et al. 2000, Hoops et al. 2003; S. Silber personal comunication).
- In patients presenting oligozoospermia who are at risk for a progressive decrease of sperm concentration over time, cryopreservation of spermatozoa could avoid future more invasive techniques such as TESE/ICSI Krausz and McElreavey 1999). For patients affected by azoospermia due to 'partial' AZFa, b or 'partial' and 'complete' AZFc deletions the probability of sperm retrieval is around 50%. Although this percentage is higher than the one expected for Klinefelter patients, also in this case cryoTESE rather than TESE/ICSI is advised.

Genetic counselling

If *in vitro* fertilization is offered with fresh or frozen ejaculated or frozen testicular spermatozoa, patients should be counseled about the genetic risks they can expect.

Patients with Y microdeletions will obligatory transmit the deletions to their male offspring. Spermatozoa from patients with Yq microdeletions have been found to be fully fertile both following IVF and ICSI procedures and even by natural conception. However, it is not clear if the fertilization rate and embryo development are comparable to that observed in men without deletions (Mulhall et al. 1997; Rossato et al. 1998; Silber et al. 1998; van Golde et al. 2001).

The phenotype of son may vary substantially and the severity of spermatogenic failure cannot be predicted entirely due to different genetic background and the presence or absence of environmental factors with potential toxicity to reproductive function. However, due to the potential 'changing' phenotype, preventive cryoconservation of spermatozoa in the sons at a relatively young age should be advised to the parents.

Is there any other risk than infertility?

We have recently reported that a significant proportion of spermatozoa from men with Y microdeletion are nullisomic for sex chromosomes (Siffroi et al. 2000). This result indicates a potential risk for the offspring to develop 45,X0 Turner's syndrome and other phenotypic anomalies associated with sex chromosome mosaicism, including ambiguous genitalia. The screening for Y chromosome microdeletions in patients bearing a mosaic 46XY/45X0 karyotype with sexual ambiguity and/or Turner stigmata has shown a relatively high incidence of AZFc deletions (33%; Patsalis et al. 2002). These data suggest that some Yq microdeletions are associated with an overall Y chromosomal instability leading to the formation of 45,X0 cell lines.

Until now only 17 male and 18 female ICSI babies born from fathers affected by Yq microdeletions have been reported (Kent-First et al. 1996; Mulhall et al. 1997; Jiang et al. 1999; Kamischke et al. 1999; Kleiman et al. 1999; Cram et al. 2000; van Golde et al. 2001; Peterlin et al. 2002; Oates et al. 2002). It appears that the children are phenotypically normal, except for one son born with pulmonary atresia and a hypoplastic right ventricle (Page et al. 1999) and no ambiguous genitalia or Turner syndrome have been observed among them. Considering that embryos bearing a 45,XO karyoptye have a higher risk of spontaneous abortion, it would be important to know whether there is a higher incidence of spontaneous abortion among the partners of Y deleted men. Until this information is available, it should be mentioned to the patients with caution.

Considering that embryos bearing a 45,XO karyoptye have a higher risk of spontaneous abortion, it would be important to know whether among the partners of Y deleted men there is a higher incidence of spontaneous abortion. If this is the case, pre-implantation diagnosis could be offered to the couple.

Future directions

A part from the classical AZF deletions, a new type of Yq deletion has recently attracted the attention of geneticist and andrologist. A partial deletion in the AZFc region, termed 'gr/gr' has been described specifically in infertile men with varying degrees of spermatogenic failure (Repping et al. 2003). This deletion removes half the AZFc gene content including two copies of the major AZFc candidate gene called DAZ. The currently used method for the detection of gr/gr deletions is based on STS plus/minus type of analysis which alone does not provide information about the type of missing gene copies. This analysis may also detect false deletions due to rearrangements of the STS containing sequence and is also unable to rule out a duplication of the non-deleted part of the AZFc region. The only study in the literature in which gene dosage and gene copy analysis were performed in order to confirm the STS based assay and in which the control group was selected for normal sperm parameters shows that gr/gr deletion is a significant risk factor for spermatogenic disturbance (Giachini et al. 2005).

Animal models and gene expression data indicate that gene anomalies on the X and on autosomal genes may also be responsible of moderate/severe or even progressive impairment of spermatogenesis. Considering that the majority of the so called 'idiopathic' cases are probably due to yet unidentified genetic defects, a preventive cryoconservation can be offered (considering its relatively low cost and uninvasiveness) to patients presenting a progressive decrease of sperm number over time in the absence of other plausible explanation.

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