# Molecular Genetic Diagnosis and Analysis of Male Infertility

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## INTRODUCTION

Current estimates indicate that approximately 15% of couples are infertile; a male factor has been identified in 50% of infertile cases (Bhasin et al., 1994). Although male infertility is common, little is known about genetic aetiologies that causes spermatogenic defects. Recently, advances in molecular biology have allowed researches to identify and characterize underlying genetic causes of male infertiltiy. While the genetics of many conditions remain unknown, it is becoming clear that a significant proportion of men with infertility, particularly those with azoospermia or severe oligozooseprmia, have a genetic aetiology for reproductive failure.

Development in reproductive technologies make it possible and practical for many subfertile men, including those with severe male factor infertility, to father children. While these exciting advances in reproductive medicine have given new hope for men who previously had no possibility of impregnation, they have also raised new concerns about passing genetic abnormalities to the offspring of these men. With increasing evidence of a genetic cause of many infertile conditions, it is thus incumbent on clinicians to initiate appropriate genetic investigations and counseling for these couples before embarking on procedures that might help them conceive.

Here, an up-to-date summary of the genetic aspects of male infertility is presented. Recent informations obtained from transgenic studies are also given to suggest likely directions that research into the fundamental biology will take.

## OVERVIEW OF SPERMATOGENESIS

All humans nucleated dipoloid cells contain 22 pairs of autosomes and 1 pair of sex chromosomes, for a total of 23 pairs, which provides the normal 46,XX (female) or 46,XY (male) chromosomal configuration. These 46 chromosomes consist of approximately 6 to 7 billion base pairs of DNA. It is estimated that the human genome contains 50,000 to 100,000 genes that encode proteins. An alteration in even a single base pair of a gene can result in an absence or abnormal protein, with potentially serious phenotypic consequences.

Spermatogenesis occurs in successive mitotic, meiotic and postmeiotic phases. Upon compltion of meiosis, 4 haploid cells, bearing one sex chromosome, are produced. In men all 4 cells persist as functional spermatozoa, while in women only 1 ovum persists, with remaining products becoming

nonfunctional polar bodies. Depending on which sex chromosome the sperm is carrying, the resultant zygote may be 46,XX or 46,XY.

Genes central to the process of spermatogenesis cannot be identified easily by family stidies or mapped by linkage approaches beacause of the limitations imposed on family size by the nature of the defect. The Y chromosome presents a unique situation in this context. Not only is it thought to be a favored location for genes involved in male physiology for evolutionary reasons based on the concept of genetic hichhiking, but it is also dispensable for normal development of the female. In effect, this mens that individuals with deletions of the Y chromosome can be viable.

## CYTOGENETIC CAUSES OF MALE INFERTILITY

### *Chromosomal abnormalities*

There are several checkpoints during male meiosis to monitor the quality of the process. If pairing is not occurred between homologous chromosomes (an asynaptic chromosome), there is an arrest at first meiotic metaphase and the arrested cells are eliminated by apoptosis. This apoptotic process is p53-independent. Although double stranded DNA breaks are initiated to form chiasmata between homologous chromosomes, these breaks can not be repaired by the formation of chiasmata in asynaptic chromosomes (no homologous chromosomes or part of chromomosome present). The possible trigger for the synapsis check point might be the unrepaired double stranded DNA breaks at the end of pachytene in the asynaptic chromosome(s) as shown in mice models (Odirisio et al., 1998). These data might explain why some chromosomal abnormalities are associated with a deficient spermatogenesis.

### *Sex chromosmes*

*Kleinefelter syndrome (47,XXY)*: This occurs in one in 500 of male births, and accounts for approximately 14% of azoospermic males (De Braekeleer & Dao, 1991; Nordenson et al., 1984). Although a vast majority of Kleinefelter males have a 47,XXY karyotype resulted from X chromosome meiotic non-disjunction (60% maternal, 40% paternal in origin), mosacism XX-XY accounts for 10%. In rare cases, a 48,XXXY karyotype can also be found. The extra X chromsome does not allow the survival of germ cells in the testis, resulting in azoospermia due to germ cell aplasia. However, aproximately half of mosaic cases may actually have some degree of spermatogenesis with resultant parternity.

*47,XYY*: Males with 47,XYY karyotype are potentially fertile. Sperm count ranges from azoospermic to normal, due to maturation arrest and germ cell absence. They are quite tall but phenotype is otherwise normal. The incidence varies between one and four in 1000.

*45,XO/46,XY mosaic or mixed gonadal dysgenesis*: Phenotypes of individuals vary from female to male with sometimes a testis on one side and a streak gonad on the other. Different degrees of gondal dysgenesis as well as development of intersexual gonads are observed. Most individuals are sterile. One third display features of Turner syndrome with short statue, webbed neck etc. The internal gonads are at risk for malignancy and should be removed (De Braekeleer & Dao, 1991; Nor-

denson et al., 1984).

*Y chromosomal translocations*: Numerous reports in the literature detail different chromosmal translocations which are associated with impaired spermatogenesis. Translocations of terminal portions of the short arms of sex chromosmes (Xp & Yp) result in an X chromosome with translocated SRY. This results in a 46,XX male, occurring in one out of 20,000. In these men, a 46,XX genotype is associated with male gonadal differentiation. Absence of azoospermia factor (AZF) gene(s) which is located on the long arm of Y (Yq), will lead to sterility in these men (Vogt et al., 1995).

Y chromosomal DNA can be translocated onto autosomes. Autosomal translocations of the Y chromosome are observed in balanced or non-balalnced karyotypes. Azoospermic males with balanced autosomal-Yq translocations can show an arrest of spermatogenesis before, during meiosis or during the formation of spermatids. In these males breakpoints are usually in the Yq11 region where the spermatogenesis genes are localized. Translocations with the breakpoint in the inert Yq12 region do not result in severe spermatogenesis problems. However, improper pairing and segregation of the chromosomes during mitosis and meiosis, may result in a mildly reduced spermatogenesis. Unbalalced trnaslocations with partial loss of the long arm can be associated with early disruption of germ cell development if the genes in the AZF region are deleted (Vogt et al., 1995).

*Microscopic Y chromosomal deletions*: De novo deletion is among the most common causes of severe spermatogenic defects. Either recognizable structural aberrations of the Y chromosome or mutations involving the SRY/AZF genes themselves may result in a variety of clinical states ranging from bilateral testicular absence due to absence of functional SRY, to sterility because of faulty or absence of AZF region genes in the presence of normal SRY. Besides dysfunction of genes in Yq11, diminished spermatogenesis may also be caused by disruption of the Yq11 chromosome structure. Interference with the X-Y pairing process contributes to the male infertility. Spermatogenesis is often blocked before meiosis. Different karyotypes with deletions in Yq11, including at least part of the AZF locus have been described in sterile man. There is no obvious correlation between the size of the Y deletion and the severity of the spermatogenic defect (Reijo et al., 1995). Gross structural aberrations of the Y cromosome resulting in AZF deletions causing azoospermia can be observed such as monocentric Yq chromosomes (46,XYq$^-$), due to terminal deletion of distal Yq11, dicentric iso Yp$^-$ chromosomes, which carry two pseudoautosomal regionsin Yp, and ring Y chromosomes. These men are often phenotypically normal but sterile (Haaf et al., 1990; Micic et al., 1990; Iwamoto et al., 1995; Beverstock et al., 1989; Chandley et al., 1989).

### *Autosomal chromosmes*

Blanaced reciprocal autosomal translocations and Robertsonian translocations are also associated with impaired fertility due to spermatogenesis defects (Johannisson et al., 1993). Robertsonian translocations between the acrocentric chromosomes 13, 14, 15, 21 and 22 are frequently observed in series of male infertiltiy and the t(13,14) translocation is the most frequent one. Although the reason and the mechanism for this are not clear, current hypotheisis is that there may be a problem with pairing homologous chromosomes during the first meiotic division.

# MOLECULAR GENETIC CAUSES OF MALE INFERTILITY

## Genes on the Y chromosome

### *Microdeletions in the AZF region*

*AZF subregions and correlation between microdeletions and phenotypes*: Molecular analysis of interstitial microdeletions using sequence-tagged-sites (STSs) of the Y chromosome revealed an additional class of mutations in the Y chromosme. These deletions removes submicroscopic portions of Yq, encompassing larger or smaller portions of the previously defined AZF region (Tiepolo & Zuffardi, 1976). Recent results aimed at mapping this locus in azoospermic men with much smaller Y chromosmome microdeletions have complicated the picture and suggest that rather than a single locus, three non-overlapping subregions in intervals 5 and 6 are important for male fertiltity. These subregions are now called AZFa, AZFb and AZFc (Vogt et al., 1996).

Association of specific defects in spermatogenesis with specific Y-linked microdeletions is difficult. Men with larger deletions tend to have azoospermia or sever oligozoopsermia. However, specific non-polymorphic small deletions can have similarly drastic effects on fertility. It has been suggested previously that men with microdeletions in AZFa, AZFb and AZFc subregions present with SCO-type I, spermatogenic arrest at a meiotic phase, and SCO type II, respectively (Vogt et al., 1996; Brandell et al., 1998). Other studies have suggested that germ cells may occasionally be present and mature sperm may occur in patients with microdeletions in AZFa. It has also been shown that patients with SCO have microdeletions in AZFb and AZFc, while those with spermatogenic arrests have microdeletions restricted to the AZFc (Kim et al., 1999). Therefore, the correlations described previously are refuted, though certain patients with large deletions involving multiple AZF subregions or AZFa alone tend to present with more severely compromised spermatogenesis than those with microdeletions restricted to AZFb, AZFc. Recently, a fourth subregion, localized between AZFb and AZFc, have been suggesed and microdeletions restricted to this subregion may lead to mild oligozoospermia (Kent-First et al., 1999).

At least three spermatogenesis-regulating genes or gene families have been mapped to deletion intervals 5 and 6 of the Y chromosme AZF region, namely RNA binding motif (RBM, Ma et al., 1993), deleted in azoospermia (DAZ, Reijo et al., 1995; Saxena et al., 1996) and possibly Drosophila fat facets regulated Y (DFFRY, Brown et al., 1998).

*RBM family members as candidate(s) for AZFb gene*: Notably, RBM encodes a RNA-binding protein with a single RNA recogntion motif. Its expression is restricted to the male germline in man (Elliott et al., 1997) and mouse (Elliott et al., 1996). RBM is closely related to the hnRNP family of proteins (Soulard et al., 1993; Leconiat et al., 1992), and both are members of the hnRNP family of proteins, which are associated with nuclear polyadenylated RNAs and are involved in pre-mRNA packaging, transport to the cytoplasm, and splicing (Weighardt et al., 1996). However, an important difference is that hnRNP is ubiquitously expressed, suggesting that its function is required in all cell types. Rather than encoded by a single gene, there is a family of 20~50 RBM genes and pseudo-

genes spread over the AZFb region (Prosser et al., 1996). The multicopy nature of the RBM gene family in human (and mouse) has complicated attempts to prove an essential role for the protein in spermatogenesis. An important question has been whether all the RBM family memebrs will dispartate Y chromosomal locations are functionally redundant. Recently, it has been demonstrated that active copies are localized to AZFb subregion (Elliott et al., 1996). However, it remains possible that deletion of genes other than RBM that lies within this subregion may partly or wholly responsible for causing the observed spermatogenic arrest.

*DAZ gene family as candidate(s) for AZFc gene*: More recently, two other AZF candidates have been isolated from the AZFc subregion. Although distinct from RBM, DAZ/SPGY encode proteins with a similar modular structure, with a single RNA recognition motif and an internally repeated sequence (Reijo et al., 1995; Affara et al., 1996). The best estimate of the number of genes in the family is 6～10. This region of the Y chromosome is deleted in 4～13% of patients presenting at infertility clinics with either azoospermia or severe oligozoospermia (Najmababadi et al., 1996; Nakahori et al., 1996; Reijo et al., 1996; Querishi et al., 1996). This frequency of de novo deletion is the highest yet reported for any region of the human genome and presumably reflects structural features of the DNA or chromatin in this region of the Y chromosome. However, the existence of oligozoospermic individuals with DAZ/SPGY deletions implies that these genes are not absolutely required for the completion of spermatogenesis, a conclusion strengthened by the observation that at least one man deleted for DAZ/SPGY fathered a son who inherited the microdeletion (Vogt et al., 1996). If this son becomes azoospermic, other factor(s) may affect the penertrance of this deletion since earlier observations have found diverse testicular phenotypes in men with AZFc deletions.

*The AZFa deletion interval*: This is the deletion interval with the most severe spermatogenic failure (SCO). However, no convincing candidate gene has been identified to date. A copy of DFFRY (also known as USP9Y) maps to interval 5C (Brown et al., 1998), but unlike the mouse homologue, it is expressed in a wide range of tissues. Furthermore, one patient deleted for DFFRY was found to be oligospermic; this appears to exclude DFFRY as the gene underlying the SCO phenotype, although it may contribute. More recent study involving reanalysis of same patients, however, have demonstrated that deletion in a splice-donor site and a gene deletion are closely associated with spermatogenic failure (Sun et al., 1999). Other genes have been localized to this interval (Lahn & Page, 1997), including UTY, a homologue of the mouse Uty, that maps to the $Sxr^b$ deletion interval. Like DFFRY, these new genes have X chromosome homologues and are expressed ubiquitously, but this does not preclude them from having an impact on spermatogenesis.

## Autosomal genes with functions in the testis

Recent methods for mainpulating the mouse genome in transgenic animals are starting to yield large numbers of other genes with involvement in spermatogenesis. One approach to identifying and subsequently analyzing mouse genes with specific and essential roles in spermatogenesis has been to use recombination in cultured cells to disrupt genes known to be specifically expressed or overexpressed in spermatogenesis and to generate knock-out mice from these cells.

*Genes for ubiquitin-conjugating enzymes*: Several genes have been identified as key players in normal spermatogenesis. Evidence for the important role of ubiquitinylation in spermatogenesis has come from homozygous male mice disrupted for mHR6B, the autosomal homologue of the yeast Rad6 gene encoding ubiquitin-conjugating enzyme (Roest et al., 1996). From mouse to human, the sequence conservation is remarkable; there is 100% interspecies amino acid sequence identity. Ubiquitinylation modifies the properties of the target protein, resulting in a change of the biological activity or stability of that protein. There are also indications that ubiquitin pathway palys a very active role during spermatognesis (Baarends et al., 1998). A possible substrate protein would be histones. However, postmeiotic codensation of chromosomes in round spermatids is defective in knock-out mice. Therefore, it has also been proposed that its X chomosomal homologue, mHR6A, may involed in the replacement of histones with transition proteins before genome repackaging with protamines (Koken et al., 1996).

*Genes for signalling molecules*: Mice with homozygous disruptions of two genes encoding signalling molecules, BMP8B and Dhh, are infertile as a result of the absence or arrested development of germ cells (Zhao et al., 1996; Bitgood et al., 1996). These mice are otherwise viable, showing that cell-to-cell interactions are critical for normal spermatogenic development. BMP8B, a member of the TGFβ family, is expressed and secreted at low levels by spermatogona at puberty, and then at much higher levels in round spermaitds in the adult testis. The gene is thought to be involved in autocrine interactions between germ cells and/or short-range paracrine interactions between germ cells and Sertoli cells. In contrast Dhh is secreted by Sertoli cells from early embryogenesis to adulthood and is thought to mediate an interaction between Sertoli and Leydig cells.

*Genes essential for meiosis*: Targeted disruption of the heat shock protein Hsp70-2, which is specially expressed in meiotic spermatocytes, results in a failure of meiosis and male infertility, possibly through an effect on synaptonemal complex formation (Dix et al., 1996). A second heat shok protein, Hsc70tp, is specially expressed in round spermatids (Mastumoto et al., 1993; Maekawa et al., 1989).

One of the most intriguing sets of genes involved in meiosis are the mismatch repair genes Pms1 and Mlh1. These are hotly investigated because of the association between their biochemistry and malignancy, but when Pms2 (Baker et al., 1996) and Mlh1 (Baker et al., 1996; Edelman et al., 1996) are homozygously deleted, they have effects on chromosome pairing in meiosis. In the case of Mlh1, this results in male and female infertility, with the males producing no sperm and females producing reduced number of oocytes. The gene products localizes to discrete foci on paired axes of the synaptonemal complexes at junctions btween paired and unpaired regions.

Other proteins with functions in meiosis are ATM and ATR (Keegan et al., 1996; Xu et al., 1996). Both gene products are closely required for cell cycle checkpoint pathways. Deletions of ATM in mice results in infertility and chromosomeal fragmentation during meiosis as well as other features of ataxia-telangiectasia (Xu et al., 1996). It has been shown both gene products localize at complementary sites on pairing forks in mitotic prophase, ATR protein on the unsynapsed axes and ATM protein on the synapsed side (Keegan et al., 1996).

## CONCLUSIONS

Although spermatogenesis requires a good number of gene products, a smaller number have effects exclusively in spermatogenesis. Observations in humans have shown us some of these genes, and experiments in mice have revealed others. Currently, analysis of human Y chromosome has been a focus of male infertility studies. However, it is still early to draw a clear picture of the absolute nature of the link between Y chromosome deletions and spermatogenic defects. Further studies are needed to determine the exact frequency of various Y microdeletions in men.

General screening for mutations in autosomal genes implicated in infertility alone seems to be unwarranted at this point of time. This is because no single gene is likely to contribute a large fraction of cases. In those cases with known functions other than fertility, as seen in cystic fibrosis, the frequency of mutations justifies screening.

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