Idiopathic impaired spermatogenesis: genetic epidemiology is unlikely to provide a short-cut to better understanding

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The aetiology of impaired spermatogenesis is unknown in the majority of subfertile men. From several studies of concordance for involuntary childlessness among men, we can conclude that there is a substantial familial component in male subfertility and that shared loci segregating through families can be assumed. We now know that deletions on the Y chromosome, which do not penetrate fully, account for some of these cases. There are good reasons to suspect that other cases result from mutations in genes located elsewhere in the genome. In this article, we discuss different approaches to unravelling the molecular basis of impaired spermatogenesis originating from genetic abnormalities in chromosomes other than the Y chromosome. Genetic mapping studies are in general a good approach to detect disease-causing genes that are segregating through a population; they can provide a short-cut to unravelling the biochemistry of a disease. In this paper, we explain our reasons for arguing that linkage and association studies are no promising means to identify the genes causing impaired spermatogenesis. We conclude that direct screening of candidate genes for mutations will be necessary to detect genes involved in impaired spermatogenesis. However, this approach requires studies of the biochemical pathways of normal and abnormal spermatogenesis. Since we have a poor understanding of these pathways, more research is needed into the biochemistry of spermatogenesis.

Key words: gene mutations/male infertility/spermatogenesis/Y chromosome/genetic epidemiology

Introduction

Subfertility, defined as 1 year of unprotected intercourse without conception, affects 10–15% of couples (Hull \textit{et al.}, 1985; De Kretser 1997; Snick \textit{et al.}, 1997; Evers, 2002). Obviously, these figures depend on the definition of subfertility and the study population (Irvine, 1998). According to the World Health Organization (1987) in 47% of subfertile couples semen parameters are decreased. The prevalence of subfertility based exclusively on testicular failure is not known precisely.

Evidently, subfertility is only diagnosed in couples that are trying to conceive a pregnancy. A combination of various female and male factors influences the fertility of a couple. Male subfertility can be categorized as due to pre-testicular, testicular and post-testicular factors (De Kretser, 1997). Endocrine disorders and sexual dysfunction are among the pre-testicular factors and epididymal or vasal obstruction is among the post-testicular factors. Testicular dysfunction results in reduced semen parameters due to impaired spermatogenesis. In this paper, we are interested in this group of otherwise healthy subfertile men with impaired spermatogenesis.

Several genetic abnormalities are involved in impaired spermatogenesis. Structural and numerical chromosomal abnormalities are found in ~4% of patients with azoo- or oligozoospermia (Tuerlings \textit{et al.}, 1998). Structural chromosomal aberrations cause meiotic abnormalities, resulting in spermatogenic failure (Chandley, 1979; Quack \textit{et al.}, 1988).

Five classes of Y chromosome deletions, AZFa (Azoospermia Factor a), P5/proximal P1, P5/distal P1, gr/gr, and b2/b4 (AZFc) deletions, cause spermatogenic failure (Reijo \textit{et al.}, 1995; Vogt \textit{et al.}, 1996; Repping \textit{et al.}, 2002, 2003). Deletions of the AZFc region are the most frequent and are found in 6–12% of azoospermic or severely oligozoospermic men (Kremer \textit{et al.}, 1997; Kuroda-Kawaguchi \textit{et al.}, 2001).

In addition, mutations or an expanded CAG repeat length in the androgen receptor (AR) gene have been described in subfertile men (Yong \textit{et al.}, 1998; Dowsing \textit{et al.}, 1979; Komori \textit{et al.}, 2004).
1999; Mifsud et al., 2001; Wallerand et al., 2001). In a recent review on aberrations in the androgen receptor, it is suggested that the expression of defects in the AR might differ in different populations and therefore the genetic background of a patient might play a significant role in the expression of the AR defects (Yong et al., 2003). However, strong arguments for a causal role are still lacking.

**Idiopathic impaired spermatogenesis**

In the majority of subfertile men, the aetiology of the impaired spermatogenesis is unknown (World Health Organization, 1987; Bhasin et al., 1994; De Kretser, 1997). For clinical practice, this is not of great concern since treatment is not influenced by the cause of subfertility given the limited range of effective therapeutic options. Intrauterine insemination (IUI) is best first-line cause of subfertility given the limited range of effective therapy between ICSI and imprinting disorders is based on case reports and uncontrolled cohorts. Therefore the exact impact of the risk is not yet clear and prospective controlled studies are needed to clarify whether imprinting disorders play a role in ICSI more often than in the general population (Gosden et al., 2003; Devroey and Van Steirteghem, 2004).

Although the fertility status of ICSI children is not known, as the oldest children are yet to enter puberty (Van Steirteghem et al., 2002), it is to be expected that if idiopathic impaired spermatogenesis has a genetic component, unknown genetic abnormalities are also transmitted via ICSI. For Y chromosome deletions it is already known that these are transmitted to sons by ICSI, and therefore these boys are likely to be infertile as adults (Kamischke et al., 1999; Page et al., 1999). In the same line of reasoning, yet unknown autosomal genetic defects underlying impaired spermatogenesis might be transmitted to ICSI children too, with a possible negative impact on fertility.

For this reason it is important to evaluate whether idiopathic impaired spermatogenesis does have a genetic component and whether it is a heritable condition. Furthermore, it is important to detect genes involved in testicular failure in order to implement diagnostic tools and offer appropriate counselling to patients who need ICSI. In this paper, we describe what is known about the genetic background of idiopathic impaired spermatogenesis and discuss different approaches to identify the genes that may be involved.

**Genetic basis of idiopathic impaired spermatogenesis**

Genetic studies in several different animal models such as the yeast *S. cerevisiae*, the worm *C. elegans*, the fly *Drosophila* and the mouse have provided evidence for the existence of hundreds of X chromosomal and autosomal genes that can mutate to male sterile alleles (Hackstein et al., 2000). From these studies it can be estimated that >4000 genes may be involved in human spermatogenesis (Venables and Cooke, 2000).

The classical method, in humans, to identify whether a condition has a genetic basis is twin studies. In the case of male subfertility, only one underpowered pilot twin study has been performed so far. Despite its small size, this study showed a clear familial component to normal human spermatogenesis (Handelsman, 1997).

In addition to this study, however, several case reports with multiple affected family members have been published (Chaganti and German, 1979; Leonard et al., 1979; Shabtai et al., 1980; Cantu et al., 1981; Rivera et al., 1984; Meschede et al., 1994; Chang et al., 1999; Saut et al., 2000; Rolf et al., 2002; Tuerlings et al., 2002; Gianotten et al., 2003a). In two families an autosomal recessive mode of inheritance was suggested but no cause for the shared infertility could be identified (Chaganti and German, 1979; Cantu et al., 1981). In one family an autosomal dominant trait of male infertility with sex-limited expression was suggested by segregation analysis (Tuerlings et al., 2002). In four families, structural chromosomal abnormalities were found (Leonard et al., 1979; Shabtai et al., 1980; Rivera et al., 1984; Meschede et al., 1994), while in three families a Y chromosome deletion was transmitted from the father to his infertile sons (Chang et al., 1999; Saut et al., 2000; Rolf et al., 2002; Gianotten et al., 2003a).

These reports indicate that the affected family members share a genetic trait that accounts for their male subfertility. In addition, familial clustering of male subfertility has been observed in a case–control study (Lilford et al., 1994). In this study, conclusions were based on a significantly increased number of subfertile brothers of men with reduced sperm counts as
compared to fertile controls. Recently, we partly confirmed these data in a case-control study comparing familial occurrence of male subfertility between families of patients with azoospermia or severe oligozoospermia and families of patients with normozoospermia (Gianotten et al., 2004). This study indicated that male subfertility due to impaired spermatogenesis has a familial component, but in only about half of the subfertile population.

Considering all these data, we can conclude that there is a familial component in male subfertility. This suggests a shared genetic or a shared environmental aetiology. As it is very likely that idiopathic impaired spermatogenesis has a genetic background, shared genetic loci segregating through these families can be assumed.

**Genetic mapping in impaired spermatogenesis**

The strategy to identify disease-causing genes depends on how much is known about the pathogenesis of the disease and on what resources are available (Strachan and Read, 1999). As the pathogenesis of impaired spermatogenesis and the biochemical functions of the genes involved are not known currently, functional cloning strategies cannot be used to identify the genes involved in human testicular failure. Positional cloning strategies, on the other hand, are independent of the gene product and might therefore be a good approach for identifying the genes in impaired spermatogenesis. Positional cloning is based on genetic mapping. The aim of genetic mapping studies is to discover how often two loci are separated by meiotic recombination. This can be done by linkage analysis and by association analysis (Strachan and Read, 1999).

**Linkage studies in impaired spermatogenesis**

A marker is linked with a disease if there is a non-random co-segregation between the marker and the phenotype. Linkage analysis can be performed on the whole genome (multipoint analysis) as well as on specific candidate loci. Classical linkage analysis is performed on data of families with multiple affected family members. Linkage analysis using shared segment methods is used in nuclear families. The analysis of linkage studies can be very difficult for several reasons, which we will discuss in relation to the male subfertility phenotype.

First, a classical linkage study requires large families with multiple affected family members in which co-segregation of numerous genome-wide polymorphic markers can be studied. However, families with the disease under study, especially when subfertility is studied, are seldom large enough for results from one family alone to reach statistical significance. Therefore, it is necessary to combine data from several families assuming that the genetic disease locus involved is the same in those families. This may be particularly problematical if indeed there are up to 4000 genes involved, as the chance that different families have the same defect may be quite small.

Second, to detect genes which are causing impaired spermatogenesis, we are only interested in the subfertile males with reduced semen parameters and not in men of subfertile couples with normal semen parameters. In pedigrees, however, the exact cause of subfertility is not always known and therefore in family studies, men with subfertility due to impaired spermatogenesis cannot easily be identified. In addition, men who conceived children spontaneously will be indicated as fertile, even though they may have reduced semen parameters.

Third, in families with subfertile males, the degree of unknown non-paternity might be higher than in the general population. This will influence the analysis of the family data negatively.

The fourth problem is that conventional linkage studies require a specification of the genetic model of the disease. Unfortunately, we can only speculate about the genetic model involved in impaired spermatogenesis, as there is no clear pattern of segregation. Determining the genetic model behind impaired spermatogenesis is difficult, as the phenotype influences the mode of inheritance by itself. An autosomal recessive mode of inheritance was the best-fitting model in the majority of cases in both case-control studies (Lilford et al., 1994; Gianotten et al., 2004). Indeed, inheritance of autosomal recessive single gene defects might be one explanation of how male factor subfertility can be transmitted to the next generation, as only homozygotes are affected and recessive males would not be subfertile, but pass the defect to their children. However, autosomal dominant defects of maternal alleles or X-chromosomal aberrations might also run in families and can possibly be transmitted through the maternal line. In addition, defects in mitochondrial DNA might also be involved since mitochondrial DNA is passed to offspring only by mothers. Finally, the results of the case–control and twin studies are also compatible with cases arising from mutations of variable penetrance on the Y chromosome.

To resolve the problems of shortage of large families and the unknown genetic model, shared segment methods can be used instead of classical linkage analysis. Shared segment methods are model free linkage studies that can be used within nuclear families. When studying impaired spermatogenesis, a nuclear family includes at least two affected brothers with their parents. Many alleles of these brothers are then compared, looking for shared segments inherited from the same parent. These shared segments are candidate loci for impaired spermatogenesis. To narrow down the number of shared segments, these comparisons are done in many different nuclear families; such analyses are much less powerful than conventional linkage studies but do not require any assumption about the underlying genetic model and unaffected people can be ignored in the analysis.

The most important difficulty, however, is that linkage analysis in nuclear families as well as conventional linkage studies identifies shared loci responsible for the phenotype. As human sperm cells and seminal plasma contain hundreds of different proteins, it has been suggested that many genes can potentially be involved in testicular failure (Lilford et al., 1994; Hackstein et al., 2000). Spermatogenesis is a complex process and problems at several different stages of spermatogenesis can result in male infertility. Therefore, probably many subgroups of patients with a different pathophysiological background and thus with different genetic aberrations contribute to the male subfertile phenotype (Cooke and Saunders, 2002). In this respect, it is likely that many different genes and their interaction predispose for reduced semen parameters. These different disease loci will not be identified by linkage analysis.
In summary, because of all the reasons mentioned above it is at this moment not realistic to believe that linkage studies are powerful enough to detect disease-causing genes involved in impaired spermatogenesis.

**Association studies in impaired spermatogenesis**

Linkage is a relationship between loci, but association is a relationship between alleles. Allelic association means that across the whole population, people who have a certain allele at one locus have a statistically greater than random chance of having a particular allele at a second locus (Strachan and Read, 1999). Association studies are generally performed on candidate genes but also polymorphic markers distributed over the whole genome can be analysed. This method offers the opportunity to study unrelated affected subjects but results have to be compared with control subjects with the same genetic background. The ideal population for association studies is genetically homogeneous, because all case–control studies are prone to selection bias (Strachan and Read, 1999). Moreover, the patients have to be descended from a small number of original founders.

In general, the statistical power to detect a real association is limited by the heterogeneity of a population due to environmental and other genetic factors. Therefore, in heterogeneous populations such as subfertile patients, large sample sizes are needed to obtain sufficient statistical power to detect genetic risk factors. More homogeneous populations such as genetically isolated populations might be an alternative, because environmental variation might be lower and the genetic background is expected to be less complex due to founder effects (Heutink and Oostra, 2002). However, this approach would miss different alleles, which might be important in other or more diverse populations.

As the patients under study in an association analysis are not directly related to each other, the chance that the disease phenotype is caused by a shared locus is even more unlikely than in linkage studies. Furthermore, in association studies, a candidate locus has to be defined and except the Y chromosome, we do not know of any specific regions with a high probability of being involved in impaired spermatogenesis.

In summary, association studies in testicular dysfunction have only a low probability of detecting a disease locus involved in the male subfertile phenotype.

**Alternative genetic models**

Although familial clustering of male subfertility due to impaired spermatogenesis has been convincingly demonstrated, subfertility appears not to be segregating in the majority of the cases. Therefore, we might be dealing with de novo gene defects in a substantial number of cases and environmental factors can also play a role. Moreover, as it is likely that many genes are involved in impaired spermatogenesis, we have to consider more complex genetic models that theoretically could play a role in the subfertile phenotype.

In genetically complex disorders, which are diseases in which a multifactorial model plays a role, various susceptibility genes, each with only moderate impact, influence the phenotype and exogenic non-genetic factors have substantial effects on phenotype expression. The aetiology of these disorders is therefore polygenic and there might be a reduced penetrance of the disease causing genes on the phenotype. In the multifactorial major gene model, only a few major susceptibility genes affect the risk for reduced spermatogenesis, but their expression depends on smaller effects of minor susceptibility genes and exogenous factors.

In the genetic heterogeneity model, different genes influence phenotype expression in different patients. In this model, the phenotype is not a distinct disorder but includes several different disorders of distinct aetiology. For impaired spermatogenesis, all models mentioned could play a role, each in a subgroup of patients, the so-called mixed model.

In addition to deletions of the Y chromosome, de novo mutations, the various genetic models, the phenomenon of genetic imprinting could also be important in impaired spermatogenesis. Imprinting is a mechanism in which the allele from one parent is expressed and the allele from the other parent is silenced (Hall, 1997). Because of this kind of differential expression, the effects of certain genes depend on whether they are inherited through the mother or through the father. Genetic imprinting has been observed in a number of disorders dealing with growth, behaviour and abnormal cell growth (Hall, 1997).

Imprinted genes show an unusual mode of inheritance, since mutant genes have an effect on the phenotype only if they come from the parent from which they are not silenced. Moreover, in several recent studies, it has been shown that allele-specific expression is also relatively common among non-imprinted autosomal genes (Knight, 2004). If differential expression plays a role in spermatogenesis, this will make the understanding of the molecular background of male subfertility even more complicated.

Finally, abnormalities in the mitochondrial genome and in nuclear genes that function in mitochondria, might cause infertility, although the relative importance of errors in the mitochondria for causing infertility and reduced sperm quality is unknown (Lestienne et al., 1997; Ruiz-Pesini et al., 2000; Holyoake et al., 2001; Rovio et al., 2001; St John et al., 2001; Spiropoulos et al., 2002; Jensen et al., 2004).

In summary, although impaired spermatogenesis has a familial component, the mode of inheritance is not clear. In addition to simple Mendelian segregation patterns and Y chromosome deletions, de novo mutations, mitochondrial defects, more complex genetic models, reduced penetrance and allele-specific expression might be involved.

**Environmental factors**

Although a genetic component is likely to be involved in impaired spermatogenesis, environmental factors will influence the phenotype too. In fact, there are many reports suggesting an adverse trend in male reproductive function over the past decades that cannot be caused by changes in genetics. Semen parameters have possibly declined in recent decades and the incidence of testicular cancer in Caucasian men has increased. At the same time the incidence of cryptorchidism and hypospadias seems to have increased in certain regions. In addition, testicular germ cell cancer is associated with a higher incidence in maldescent of the testis and with reduced semen quality before
the cancer is diagnosed. In this respect, it was proposed that testicular cancer, hypospadias, cryptorchidism and reduced semen parameters are all symptoms of the testicular dysgenisis syndrome (TDS), with a common origin in fetal life. As the rise in the incidence of the various symptoms of TDS occurred rapidly over a few generations, the aetiological effect of adverse environmental factors, probably acting upon a susceptible genetic background, must be considered (Asklund et al., 2004). An important factor involved in reduced semen quality, for example, might be the interplay between the sensitivity to estrogens and the exposure in utero (Spearow et al., 1999, 2001).

Screening of candidate genes for mutations

As we are probably looking for many different genes, and male subfertility might be a complex disease, we need a more direct approach to detect those genes than genetic mapping studies. Such an approach is screening of candidate genes by looking for sequence variations in cases which are not present in controls. A candidate gene is a gene that is considered, for different reasons, as a possible locus for the disease phenotype. If mutations in the gene under study can be identified in affected patients and not in the control samples, the gene is likely to be a locus for the disease (Strachan and Read, 1999).

In spermatogenic failure, the list of suggested candidate genes is exhaustive in accordance with the complex pathophysiology of spermatogenesis (Hackstein et al., 2000; Venables and Cook, 2000; Cooke and Saunders, 2002; Matzuk and Lamb, 2002). In humans, however, only very few candidate genes have been investigated until now.

Most research has been done on the Y chromosome. The first report indicating that the Y chromosome is involved in human spermatogenesis showed that some men with azoospermia had a deletion of the entire long arm of the Y chromosome (Tietopol and Zuffardi, 1976). Thereafter, it took several years before smaller deletions of the long arm of the Y chromosome were detected in azoospermic men (Ma et al., 1992). Since then, five classes of Y chromosome deletions have been described which cause spermatogenic failure: AZFa (Azoospermia Factor a), P5/proximal P1, P5/distant P1, gr/gr, and B2/B4 (AZFc) deletions (Reijo et al., 1995; Vogt et al., 1996; Repping et al., 2002, 2003). Recently, the sequence of the male-specific region of the human Y chromosome has been completed (Skaletsky et al., 2003). This region harbours ≥78 genes of which 60 are expressed exclusively or predominantly in the testis (Skaletsky et al., 2003). Until now, only one patient with a de novo point mutation in the USP9Y (Ubiquitin Specific Protease 9 Y) gene has been described resulting in azoospermia (Sun et al., 1999).

Furthermore, several autosomal genes have been screened in DNA of subfertile men; the HOXA10 (Homeobox) gene (Kolon et al., 1999), the INSIL3 (Leydig insulin-like hormone) gene (Krausz et al., 2000; Tomboc et al., 2000; Lim et al., 2001; Marin et al., 2001), the DAZL (Deleted in Azoospermia Like) gene (van Golde et al., 2001; Teng et al., 2002), the ZNF214 and ZNF215 (Zinc Finger) genes (Gianotten et al., 2003b), the SYCP3 (synaptonemal complex protein) gene (Miyamoto et al., 2003) and the HNRNP G-T (heterogeneous nuclear ribonucleoprotein G-T) gene (Westerveld et al., 2004) respectively. In addition the POLG (mitochondrial DNA polymerase) gene has been screened in sperm DNA of infertile men (Rovio et al., 2001; Jensen et al., 2004). Although several polymorphisms were identified in some of these genes, functional disruption of the genes could not be proven. The only exception is the SYCP3 gene, in which a deletion that results in a premature stop codon was identified in two of the 19 azoospermic patients with maturation arrest. This mutation is the only described autosomal gene defect that is proven causative in men with testicular failure.

Screening of candidate genes might be very appealing if the function of the candidate gene is known. To separate the biologically relevant mutations from harmless single nucleotide polymorphisms, the biological effect of each of the variants found in the mutation screen has to be tested (Heutink and Oostra, 2002). Unfortunately, in impaired spermatogenesis, the biological function of the genes is often unknown and therefore a causal role can only be assumed if a mutation is identified. Another problem in screening candidate genes is that mutations can be located outside the coding region of the gene in elements that have a regulatory function of the gene (Heutink and Oostra, 2002). Such mutations will not be detected in screening the genes. In summary, at this moment, mutation screening on candidate genes is the only realistic method to identify genes that might be involved in impaired spermatogenesis. However, candidate screening is time-consuming, needs many well-defined patients and will reveal many negative results. This method would be more appealing if there is more knowledge about the biological function of candidate genes.

Conclusions

Although there is evidence for a genetic basis for impaired spermatogenesis and in a subgroup of the population the phenotype segregates through their families, identifying the genes involved in impaired spermatogenesis is very difficult. It is very likely that male subfertility is a complex disease in which many susceptibility genes and environmental factors play a role. If impaired spermatogenesis is such a complex disease, in which different genes involved in different populations play a role, identification of the disease genes would be almost impossible.

In this paper we have discussed the reasons for arguing that linkage and association studies are not promising means to identify the genes causing testicular failure. At this moment, direct screening of candidate genes for mutations is the only realistic method to identify genes involved. However, this is a very time-consuming method with a low probability of detecting causal genes. To increase this probability, the number of good candidate genes has to be narrowed down. For example, this might be realized by expression studies. Moreover, more research is needed into the biochemistry of spermatogenesis in order to study the functional effect of genetic aberrations found by mutation screening in our patients.

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