Spermatogenic failure. A genetic Odyssey
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CHAPTER 8

Summary, conclusions,
and implications for future research

Judith Gianotten
Summary

Azoospermia and severe oligozoospermia due to testicular failure are significant causes of male subfertility. In up to 40% of subfertile men the aetiology is unknown and therefore these men can not be treated. To date, their only option to achieve a pregnancy is in vitro fertilization (IVF) combined with intracytoplasmic sperm injection (ICSI). The therapeutic efficacy of ICSI is generally accepted and the incidence of major birth defects seems not to be increased after ICSI. However, there are some negative side effects accompanying this treatment.

Assisted reproductive techniques (ART) place a heavy burden on the normal fertile female partners of the subfertile men. These women have to undergo controlled ovarian hyperstimulation and, in case of IVF and ICSI, follicle aspiration, which both are potentially risky procedures. In addition, there is a high rate of multiple pregnancies with more complications for mother and child, compared to singleton pregnancies. More general negative aspects include that IVF and ICSI are very expensive. Above all, genetic factors are transmitted to the next generation by ICSI. If unknown genetic factors play a role in impaired spermatogenesis, these genetic abnormalities might also be transmitted to the next generation by ICSI with a possible negative impact on fertility. Therefore, further research into specific genetic causes of male subfertility due to impaired spermatogenesis is warranted.

Several genetic causes have been identified in men with subfertility due to testicular failure. Numerical chromosomal abnormalities of which the Klinefelter syndrome is most common and structural chromosomal abnormalities have been found in patients with azoo- or oligozoospermia. Also, five classes of Y chromosome deletions, AZFa (Azoospermia Factor a), P5/proximal-P1, P5/distal-P1, P4/distal-P1 and b2/b4 (AZFc) deletions, cause spermatogenic failure. In addition, several families with multiple subfertile male family members have been described in which a genetic defect segregates through the family and familial clustering have been found in a case-control study. This indicates that unknown genetic defects might be involved in idiopathic impaired spermatogenesis. Therefore, the aim of this thesis was to identify unknown genetic causes involved in idiopathic impaired spermatogenesis.
Chapter 1 gives an outline of this thesis.

Chapter 2 focuses on the first aim of this thesis:

To study whether male factor subfertility due to impaired spermatogenesis has a familial component and, in case there is, to study the mode of inheritance.

Several families with multiple subfertile male family members in which a genetic defect segregates in the family have been described in previous studies. This indicates that impaired spermatogenesis can be a heritable condition. In addition, familial clustering of male subfertility has been observed in a case control study, which could be explained by an autosomal recessive mode of inheritance in the majority of cases. Conclusions were based on a significantly increased number of subfertile brothers of men with reduced sperm counts as compared to fertile controls. These findings were supported by a study describing that men with an ICSI indication have fewer siblings than fertile controls. The fertility status of a couple, however, is not exclusively based on semen parameters, as several additional female factors contribute to the subfertile phenotype. In both studies, the control group consisted of fertile men for whom no semen parameters were available. Reporting bias might have influenced the data found in the case control study, as fertile controls are probably less informed about fertility problems among their relatives, than men with reduced semen parameters. Therefore, we compared family data from men with severe impaired spermatogenesis with data of men with normal semen parameters.

We determined the prevalence of subfertility among brothers and sisters of 160 patients with severe idiopathic impaired spermatogenesis attending the Center for Reproductive Medicine of the Academic Medical Center. These data were compared with the prevalence of subfertility among brothers and sisters of 285 men with normal semen parameters, attending the same fertility clinic. In addition, we determined whether the subfertility of the brothers was due to reduced semen parameters or caused by other factors. Furthermore, we performed a segregation analysis to detect the best fitting mode of inheritance.

We found an equal distribution of family size and number of siblings between the cases and the controls. No differences were found in the average number of children conceived by the siblings between both groups. In the patient group, however, 16.3% of the brothers who had tried to father a child were subfertile, compared to 5.8% in the control group, which is statistically different (OR 3.18 (95% CI 1.59-6.37)). Subfertility was defined as the inability to conceive a pregnancy within one year of unprotected intercourse. The subfertility among the brothers in the patient group was more often due to reduced semen parameters than in the control group. There were no significant differences in subfertility among the sisters in the patient group compared to the sisters of the control group (OR 2.03 (95% CI 0.90-4.60)). Segregation analysis of subfertility among brothers did not fit with frequent autosomal...
dominant or recessive segregation.
We conclude that male subfertility due to impaired spermatogenesis can cluster in families. Therefore, it is very likely that heritable genetic factors are involved in the aetiology of impaired spermatogenesis. However, as simple Mendelian segregation patterns did only fit in a limited number of cases, it is not to be expected that impaired spermatogenesis is caused by a common genetic defect. Male subfertility due to impaired spermatogenesis is therefore most likely a complex disease in which several different factors play a role.

Chapter 3 deals with the second aim of this thesis:
To establish the possible genetic basis of impaired spermatogenesis in a family with five subfertile brothers with severely reduced semen parameters.

We studied a unique family with five brothers who are subfertile because of non-obstructive azoospermia or severe oligozoospermia. We constructed a pedigree of this family and screened all five brothers as well as their parents and two other paternally related family members for known genetic causes of impaired spermatogenesis. As a common genetic cause for the joint impaired spermatogenesis seemed very likely, we performed an extensive analysis of the Y chromosomal DAZ genes, X chromosomal linkage analysis, and mutation analysis of the mitochondrial DNA.
A reduced copy number of the DAZ genes was found in all individuals tested. The five brothers did not share an identical X chromosomal locus, and no mutations in the mtDNA of the index patient were found. The deletion of two of the DAZ genes, found in all five patients, was also present in the father of these brothers, who had normal sperm counts. Moreover, two other paternally related family members, a father and a son, also had a reduced copy number of the DAZ genes. The father had undergone a vasectomy, but the son had completely normal semen parameters as well.
Until now, a reduced copy number of the DAZ genes has not been described in individuals with normozoospermia. Apparently, other unknown factors contribute to the subfertile phenotype in this family and partial DAZ deletions can be transmitted to the next generation spontaneously. This illustrates that the phenotype associated with a reduced copy number of the DAZ genes can be extremely variable.

Chapter 4 handles the third aim of this thesis:
To evaluate whether genetic epidemiology provides proper tools to identify genes involved in idiopathic impaired spermatogenesis.

We now know that there is a substantial familial component in male subfertility, and therefore shared autosomal loci segregating through families can be assumed. Genetic
mapping studies are in general a good approach to detect disease-causing genes that are segregating through a population; they can provide a shortcut to unravelling the biochemistry of a disease. Classical linkage studies, however, require large families with multiple affected family members and a specification of the genetic model of the disease. Until now, large families with multiple subfertile men have not been identified and general genetic models involved in impaired spermatogenesis in such families are not exactly known. Moreover, in pedigrees, men with subfertility due to impaired spermatogenesis can not easily be identified and non-paternity will influence the analysis of the family data negatively. Shared segment methods are model free linkage methods and do not require large families. However, such model free linkage methods as well as classical linkage studies only identify shared loci responsible for the phenotype. In association studies a homogeneous population derived from one ancestor is the ideal study population. In heterogeneous populations large sample sizes are needed to obtain sufficient statistical power to detect genetic risk factors.

We discuss the possible genetic models involved in male subfertility due to impaired spermatogenesis and conclude that it is very likely that many different genes might be involved in impaired spermatogenesis and, in addition to heritable genetic aberrations, de novo mutations as well as environmental factors might play a role. Therefore, it is at this moment not realistic to expect that association or linkage studies are powerful enough to detect disease-causing genes involved in impaired spermatogenesis.

Based on genetically manipulated animal models and specific expression in testis, a large number of genes has been put forward as candidates. At this moment direct screening of candidate genes for mutations is the only realistic method to identify genes involved in impaired spermatogenesis. However, this is a very time-consuming method, needs many well-defined patients and will lead to many negative results.

Chapter 5 focuses on the fourth aim of this thesis:

To test whether chromosomal region 11p15 is associated with impaired spermatogenesis and to screen the ZNF214 and ZNF215 genes, which are located on this region, for mutations in men with spermatogenic failure.

Chromosomal region 11p15 plays a role in the aetiology of the Beckwith Wiedemann Syndrome (BWS). This is a congenital overgrowth disorder defined by a diversity of symptoms that can occur in various combinations. Cryptorchidism and reduced fecundity are among the clinical findings described in male BWS patients. Furthermore, a balanced translocation with a breakpoint at chromosome 11p15 has been described in a patient with oligozoospermia. The Zinc Finger genes (ZNF) 214 and 215 are localized on this chromosomal region and are predominantly expressed in testis. Therefore, we hypothesized
that chromosomal region 11p15 and in particular the ZNF214 and ZNF215 genes might play a role in impaired spermatogenesis.

We conducted an association study based on allele and estimated haplotype frequencies of nine common single nucleotide polymorphisms (SNPs) in the ZNF214 and ZNF215 genes. In total, 62 men with idiopathic azoo- or oligozoospermia, 64 men with azoo- or oligozoospermia and cryptorchidism in their medical history and 72 men with normozoospermia were included in this study. In patients with idiopathic impaired spermatogenesis significantly different allele frequencies were found for one SNP in the ZNF214 gene compared with the control group ($p=0.032$). In patients with reduced semen parameters and cryptorchidism in their medical history a significantly increased frequency was found for another SNP in the ZNF214 gene compared with the control group ($p=0.019$). All other variants showed no differences in allele frequencies between the two patient groups and the controls.

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In addition, 27 haplotypes were estimated based on the allele frequencies of the nine SNPs used in this study. These estimated haplotype frequencies differed significantly between the patients with idiopathic impaired spermatogenesis and the controls ($p=0.021$). The differences in estimated haplotype frequencies between the patients with cryptorchidism and the controls were not statistically significant.

We concluded that chromosomal region 11p15 is associated with impaired spermatogenesis in patients with and without cryptorchidism. Therefore, we screened all patients in both patient groups for mutations in the ZNF214 and ZNF215 genes by SSCP analysis. Aberrant patterns were confirmed by DNA sequencing. In addition to the known SNPs, five heterozygous sequence variants were identified in the patients that were not present in the controls. Three of these variants were located in ZNF214. The first variant, a heterozygous 671 G→A transition which results in a C224Y conversion, was found in a patient with cryptorchidism and was inherited from the mother. The second variant, a heterozygous 816-817 TA A insertion that results in an asparagines insertion at position 271, was found in a patient with idiopathic impaired spermatogenesis. The third variant, a heterozygous 1221 C→T transition which results in a H408Y conversion, was found in a patient with idiopathic impaired spermatogenesis. This variant was inherited from the mother and was not present in the patient's brother who had normal semen parameters. Two variants were found in the ZNF215 gene. One variant, a heterozygous 1198 A→G transition which results in an I400V conversion, was found in a patient with idiopathic impaired spermatogenesis and was inherited from the mother. The fifth variant, a heterozygous 1487 G→T transition resulting in a S496I conversion, was found in a patient with idiopathic impaired spermatogenesis as well as in a patient with cryptorchidism in his medical history.

Unfortunately, at this moment the specific biological function of the proteins encoded by the ZNF214 and ZNF215 genes is not known, although it has been suggested that they might act...
as transcription factors. Therefore it is not yet possible to define the functional consequences of these mutations and firmly establish a causal relationship between the presence of the variants and the phenotype.

Chapter 6 deals with the fifth aim of this thesis:
To screen the candidate gene HNRNP G-T for mutations in men with idiopathic impaired spermatogenesis.

The Heterogeneous Nuclear Ribonucleoprotein G-T (HNRNP G-T) gene is located in close neighbourhood of the ZNF214 and ZNF215 genes, on chromosomal region 11p15, which we showed to be associated with impaired spermatogenesis. The HNRNP G-T gene is a member of the HnRNP gene family and is predominantly expressed in pachytene spermatocytes and round spermatids, where it is thought to affect splicing and signal transduction.

To detect mutations, we sequenced the entire HNRNP G-T gene in 153 men with idiopathic impaired spermatogenesis. Variants detected in the patient group were screened by RFLP analysis in the control group, which consisted of 143 men with normozoospermia. We identified eight single nucleotide variants in our patient group. Two of these mutations were not present in the control group. The first variant, a heterozygous 298 G→A transition which results in a R100H substitution and loss of a conserved arginine, thereby affecting a putative site of methylation possibly required for RNA binding. Therefore, we suspect that this mutation might be causal in the patient's phenotype, which was azoospermia, an elevated FSH level and reduced testicular volume. The mutation was inherited from the patient's mother. The second variant detected was a heterozygous in-frame-deletion of three nucleotides resulting in a deletion of a glycine at codon 388 (G388del). This amino acid change is located at the end of the protein that is not a known functional motif. Therefore, this mutation appears to be of low significance. Blood samples from the patient's parents were not available for analysis of the inheritance pattern.

We conclude that the R100H mutation found in the HNRNP G-T gene in an azoospermic man might be responsible for the patient's phenotype. Nevertheless, our data show that mutations in the HNRNP G-T gene are not a frequent cause of impaired spermatogenesis.

Chapter 7 addresses the last aim of this thesis:
To screen the candidate gene PCI for mutations in men with idiopathic azoospermia or teratozoospermia.

Protein C inhibitor (PCI) might be involved in male reproductive function. PCI is detected on the head of human spermatozoa and acts as an inhibitor of activated protein C, but also inhibits various other proteases such as acrosin. It is suggested that PCI may function as an
inhibitor of acrosin during storage of spermatozoa in the epididymis. This inhibitory function could possibly prevent the proteolytic activity of acrosin, released from the acrosomes of degenerating spermatozoa, upon other cells. In mice it has already been shown that the presence of PCI is an absolute requirement for reproduction. Male homozygous PCI knockout mice appeared to be healthy but infertile. This was caused by abnormal spermatogenesis due to destruction of the Sertoli cell barrier, probably due to unopposed proteolytic activity. The resulting epididymal spermatozoa in these mice were malformed.

We screened 27 men with idiopathic azoospermia, 34 men with severe teratozoospermia and 34 men with normozoospermia for mutations in the PCI gene by direct sequencing. Variants found in the patient group which were not present in the initial 34 controls, were tested in an additional control group of 80 men with normozoospermia by RFLP analysis. In addition, PCI antigen levels were measured in the seminal plasma of the patients in which a potential mutation was found.

We found three new variants that were exclusively present in men with idiopathic azoospermia. The first variant, a heterozygous -806 G→A transition in the promoter region, potentially disrupts a binding site, which can strongly stimulate transcription. However, the PCI antigen level in the seminal plasma was not reduced and therefore this mutation is not likely causing the patient’s phenotype. The second variant is a heterozygous 735-6 C→T transition in intron 2. Analysis of the consensus nucleotide frequency pattern at the 3’ splice sites shows that at this position C or T occurs with almost the same frequency. In addition, the PCI antigen level in the seminal plasma of this patient was within the normal range and therefore it is not likely that this variant causes azoospermia. The third potential mutation, a heterozygous 985 G→T transition, results in a W271C conversion. The PCI antigen level in the seminal plasma of this patient was within the normal range. Furthermore, this variant was inherited from the patient’s mother but was also present in the patient’s brother who had normal semen parameters. Therefore it is not likely that this variant is responsible for the patient’s phenotype.

In addition to these point mutations the sequence of a large part of the gene was present in homozygous form in 4 patients and 1 control. We excluded the presence of a partial deletion of one allele in these men by quantifying the number of PCI alleles by Southern blot analysis. We conclude that mutations in the human PCI gene are not a common cause of reduced semen parameters in men.
Conclusions

- Male subfertility due to impaired spermatogenesis can cluster in families. It is most likely a complex disease in which several different factors play a role.

- A reduced copy number of DAZ genes can be transmitted to the next generation spontaneously. The phenotype associated with a reduced copy number of DAZ genes can be extremely variable.

- Genetic epidemiological studies provide no promising tools to detect genes involved in impaired spermatogenesis. At this moment direct screening of candidate genes for mutations is the only realistic method to identify such genes.

- Chromosomal region 11p15 is associated with impaired spermatogenesis in patients with and without cryptorchidism.

- Mutations in the candidate genes ZNF214, ZNF215, HNRNP G-T and PCI are found in patients with impaired spermatogenesis in low frequencies. Most mutations are inherited from the patient’s mother. The functional consequences of these mutations on the phenotype are not yet clear.
Implications for future research

It is now clear that the search for genes involved in impaired spermatogenesis is extremely complicated. There are no large families with multiple affected family members, the mode of inheritance is still unknown, and there is a large variability in the phenotype. Therefore mapping studies are no promising means to detect yet unknown candidate genes. However, the incidence of impaired spermatogenesis among ICSI children, when they have reached adulthood, will increase our understanding about the genetic model in the near future. For this, a proper follow up of the ICSI children is necessary. In this thesis we conclude that direct screening of candidate genes is a worthwhile approach to detect some of the genes we are searching for, although it is time consuming, needs many well-defined patients, and will reveal many negative results. As the list of candidate genes is exhaustive, the number of genes to be screened has to be narrowed down. Genome wide expression profiling represents a possible approach to identify promising candidate genes. Comparison of the expression pattern of thousands of genes in men with impaired spermatogenesis with the expression pattern of those genes in men with normal spermatogenesis will indicate the involvement of particular genes in the phenotype. For this approach, however, testicular tissue from healthy men and from men with testicular failure is needed and, given the complexity and different cell types present in the testicular tissue, cell fractionation is required prior to micro-array analysis.

A further problem in screening candidate genes is that the functional consequences of identified mutations can not easily be studied, as the biological function of the genes is usually unknown. Therefore more insight in the biochemistry of normal spermatogenesis is needed to study functional effects of genetic aberrations found in our patients. To gain more insight in basic mechanisms regulating fertility, mouse models might help to define mechanisms of reproductive function. Using this approach, primary defects in candidate genes and consequences on the phenotype can be investigated. Alternatively, an accessible human in vitro model could possibly lead to a major breakthrough in our understanding of molecular mechanisms of human spermatogenesis. For this to happen, a reliable method for isolation of spermatogonial stem cells and the ability to culture spermatogonial stem cells in vitro, is urgently needed to study the effect of mutations on human spermatogenesis.

References


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