Chapter 8

Summary

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Summary

Over the last decades the Y chromosome has been studied extensively to identify genetic abnormalities that affect spermatogenesis and as a result, male fertility. In 1976, large deletions of the long arm of the Y chromosome (Yq) were first identified, in 1996 followed by the report of three non-overlapping regions that could be deleted in men with spermatogenic failure and that were termed AZFa, AZFb and AZFc (Tiepolo and Zuffardi, 1976; Vogt et al., 1996). With the use of sequence tagged site (STS) PCR and Fluorescence in situ Hybridization (FISH), additional deletions of the Y chromosome were identified between 2002 and 2004, namely the b1/b3, b2/b3, gr/gr, P5/proximal-P1 and the P5/distal-P1 deletion (Fernandes et al., 2002; Repping et al., 2002; Repping et al., 2003; Fernandes et al., 2004; Repping et al., 2004). Of these five deletions, the latter two result in spermatogenic failure, whereas the gr/gr deletion is a risk factor for spermatogenic failure and the b1/b3 and b2/b3 deletion do not affect spermatogenesis (Repping et al., 2002; Fernandes et al., 2004; Repping et al., 2004; Visser et al., 2009). Nearly all deletions are the result of homologous recombination between amplicons (Kamp et al., 2000; Sun et al., 2000; Blanco et al., 2000; Fernandes et al., 2002; Repping et al., 2002; Silber and Repping, 2002; Repping et al., 2003; Fernandes et al., 2004; Simoni et al., 2004; Repping et al., 2004; Ferlin et al., 2005; Lepretre et al., 2005; Lynch et al., 2005; Hucklenbroich et al., 2005; Repping et al., 2006; Ferlin et al., 2007).

In this thesis, we investigated the effect of intragenic variation of the DAZ genes, (partial) duplications of the AZFc region, gene copy number variations of the AZFc region, deletions within the AZFc region caused by non-homologous recombination, pericentric inversions of the Y chromosome, and pseudo iso-Y chromosomes on spermatogenesis.

In this thesis, we aimed at identifying additional Y-chromosome aberrations that are causative in male infertility. To do so, we used rigorously defined inclusion criteria, designed specific STSs for plus-minus PCR, designed a novel qPCR method and did not use sequence nucleotide variant (SNV) analysis to avoid incorrect interpretations due to the repetitive nature of the Y chromosome. The importance and significance of these criteria and molecular techniques are described below.

Inclusion criteria: patient and control definition

In association studies, rigorously defined inclusion criteria are essential so that patients and controls are identical with the exception of the phenotype of interest. Therefore, we used patient and control groups that were categorized solely by their semen quality. Unfortunately, many published studies in the field of spermatogenic failure fail to do so (Visser and Repping, 2010). Patient groups in these case-control studies do not always consist of men with impaired semen quality, but of men that are part of an infertile couple regardless of their semen quality. In addition, control groups often consist of proven fathers, men that had previously undergone a vasectomy, or population samples, all of unknown semen quality. The problem here is that a proven fertility status of a man does not per se equal normal semen quality, nor can subfertility automatically be associated with low(er) semen quality: the fertility status of a couple is based on both male and female factors. Currently, patients and controls are categorized on the basis of the WHO criteria for normozoospermia (World Health
Organization, 1999). Using these criteria, men with sperm concentrations <20x10^6/ml are classified as “patients” and men with concentrations >20 x10^6/ml, in the presence of normal motility and morphology, are classified as “controls”. This dichotomy does not take into account the wide range of semen quality among men, which is best visualized by their total sperm count that can range from zero to over one billion spermatozoa in the entire ejaculate.

In addition, it is of great importance to include both patients and controls from the same geographical region so that the genetic background of both groups is in principal the same. This holds true especially for studies involving the Y chromosome, as it well known that the distribution of Y chromosome haplotypes varies tremendously across the world (Jobling and Tyler-Smith, 2003).

We therefore used an alternative method for the patient-control design based on the WHO criteria (World Health Organization, 1999), namely a cohort-based approach where we considered spermatogenesis to be a quantitative trait like—for instance—cholesterol levels, blood pressure and osteoporosis. This cohort-based approach ruled out any form of population stratification as semen parameters of men with Y-chromosomal variants are compared to men without these variants from the same cohort/population.

Sequence tagged sites

Most studies use STSs for a rapid screen for Y-chromosomal deletions but many of these STSs were designed prior to the publication of the MSY sequence (Skaletsky et al., 2003). This can lead to incorrect interpretations due to the repetitive complexity of the ampliconic sequences where most of these markers are located.

A classic example is the illusive AZFd region. This ‘region between AZFb and AZFc’ or ‘proximal part of AZFc’ was first described in 1999 and later screened for by others (Kent-First et al., 1999; Muslumanoglu et al., 2005). With current knowledge on the sequence of the MSY, it is clear that the so called AZFd specific and single-locus STSs sY145 and sY153 are in fact multi-locus STSs that even have copies at the distal part of the AZFc region (Noordam et al., 2006). Another example of such a misinterpretation is the 1.5 Mb overlap of the P5/proximal-P1 region with the AZFc region that went undetected until 2002 (Vogt et al., 1996; Repping et al., 2002).

We therefore designed specific STS using the MSY sequence as a guide to determine breakpoints in men carrying chromosomes with deletions caused by non-homologous recombination (chapter 6) or pseudo iso-Y chromosomes (chapter 7).
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qPCR

To screen for (partial) deletions and duplications of the AZFc region, we designed a novel qPCR method. This method allowed us to determine the actual copy numbers of specific amplicons. As a result, we could rapidly and with high accuracy, locate breakpoint regions or determine which regions of the AZFc region were duplicated. Via breakpoint analysis or FISH conformation, the reliability of this novel qPCR method was demonstrated (chapters 4, 5 and 6).

Sequence nucleotide variants

One of the techniques that has been used in the past for the detection and characterization of (partial) AZFc deletions is the detection of sequence nucleotide variants (SNV) (Fernandes et al., 2002; Machev et al., 2004; Fernandes et al., 2004). Most SNVs of the AZFc region are based on variations within the DAZ genes that were initially described based on a few different DAZ copies of a few men (Saxena et al., 2000). With the absence of some SNVs, some researchers claim the absence of specific DAZ genes and thus the existence of a specific partial AZFc deletion such as the gr/gr or the b2/b3 deletion. Similarly, SNVs of other parts of the AZFc region, including the green and yellow amplicons, are also used to search for partial deletions. The SNVs of the AZFc region can however be polymorphic due to Y-Y gene conversion, and therefore the absence of an SNV does not stand for the absence of a specific DAZ gene or amplicon but merely for the absence of that specific SNV (Rozen et al., 2003; Lepretre et al., 2005). Therefore, we did not use SNVs for identification of (partial) deletions in the AZFc region.
In summary, this thesis describes the following findings

**Chapter 2** reviews the anatomy of the human Y chromosome prior to the start of this thesis.

Most knowledge of the chromosomal makeup of the human Y chromosome and its effect on spermatogenesis has been obtained via deletion analyses and analyses of associations between Y chromosomal makeups and semen parameters. Through this research we know that AZFa, P5/proximal-P1 and P5/distal-P1 deletions result in azoospermia and the AZFc deletion in azoospermia or oligozoospermia, whereas the gr/gr deletion is a risk factor for spermatogenic failure. The b1/b3 and b2/b3 deletions do not appear to affect spermatogenesis. We also discuss SNV and STS analyses including their pitfalls when interpreting results of these analyses and finally, we propose future research topics.

**Chapter 3** reports DAZ gene variation and semen quality.

In this study, we screened for intragenic variations of DAZ in 47 different Y haplotypes and in men with partial AZFc deletions. For this study we used DNA samples reported in earlier studies (Repping et al., 2003; Repping et al., 2004; Repping et al., 2006) and samples of men that attended the Center for Reproductive Medicine of the Academic Medical Center as part of an infertile couple and in whom it was established that they carried a gr/gr or a b2/b3 deletion. We found that the DAZ Y repeat is the most variable structural element of DAZ varying between zero and fourteen copies. In addition, we found significant structural variation in the number of the DAZ EX repeats and limited variation in the number of RNA recognition motives (RRMs). In patients with partial deletions of AZFc none of these identified variations were associated with a spermatogenic phenotype.

**Chapter 4** investigates (partial) duplications of the AZFc region.

In a cohort of 845 men with various spermatogenic phenotypes we screened for (partial) duplications of the AZFc region. We excluded men with known clinical causes of spermatogenic failure, i.e. retrograde ejaculation, obstructive azoospermia, hyperprolactinemia, hypogonadotrophic hypogonadism, previous chemo- or radiotherapy, bilateral cryptorchidism, surgery of the vas deferens, orchitis, and bilateral orchidectomy. Men were also excluded if molecular diagnostics identified an AZFa, P5/proximal-P1, P5/distal-P1, AZFc, b1/3, b2/b3, gr/gr deletion or numerical or structural chromosome abnormalities. We identified 34 men that carried (partial) duplications of the AZFc region. In contrast to other studies that were performed on (partial) duplications of the AZFc region, we were able to distinguish duplications that lead to six or eight DAZ genes, to four, five, six or eight BPY2 genes and to three or four CDY1 genes. Increased DAZ gene copy number was significantly associated with decreased semen quality. More precisely, men with eight DAZ genes had a significantly reduced total motile sperm count as compared to men with four DAZ genes (2x10⁶ vs 50x10⁶, p=0.017). Taking together with previously described reduced semen quality in men with (partial) AZFc deletions, these data suggest that variation in AZFc gene copy number is limited due to reduced reproductive fitness of men with too few or too many DAZ genes.
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Chapter 5 studied gene copy number reduction in the AZFc region and semen quality. In a cohort of 840 men with various spermatogenic phenotypes we screened for (partial) deletions using STS PCR. We excluded men with known causes of spermatogenic failure according to the same clinical criteria as listed in chapter 4. Men were also excluded if molecular diagnostics identified an AZFa, P5/proximal-P1, P5/distant-P1, AZFc deletion, numerical or structural chromosome abnormalities or (partial) AZFc duplications in the absence of an STS deletion that were identified in chapter 4. We identified 31 men that carried Y chromosomes with (partial) deletions of the AZFc region. We then determined the copy number of DAZ, BPY2 and CDY/CSPG4LY/GOLGA2LY using qPCR. Of these 31 men, six men carried Y chromosomes that underwent additional duplications of the (initial) partial deleted AZFc region. A reduction in copy numbers of DAZ or CDY1/CSPG4LY/GOLGA2LY displayed a significantly reduced TMC. In contrast, not every gene copy number reduction of BPY2 was associated with a reduced TMC. We therefore hypothesize that either DAZ or CDY1/CSPG4LY/GOLGA2LY affects spermatogenesis in a dose sensitive relationship. This would imply that duplications of partial deleted AZFc regions that restore AZFc gene copy number should restore spermatogenesis to normal levels. We examined the semen qualities of men that carried such deleted/duplicated chromosomes and found their semen quality to be indeed equal to that of men carrying the reference gene copy numbers. This finding strengthened our hypothesis that gene copy number of the AZFc region affects semen quality and hence reproductive fitness.

Chapter 6 focuses on deletions in the AZFc region caused by non-homologous recombination. For this study, we set out to identify deletions in the AZFc region caused by non-homologous recombination between palindromes. We screened 1,237 men that originated from a previous study (Repping et al., 2003) or from a consecutive cohort of men that attended the Center for Reproductive Medicine of the Academic Medical Center as part of an infertile couple. We identified eight unrelated men with such deletions. These deletions all removed the proximal part of AZFc encompassing palindrome P3 and we categorized these deletions into four distinct classes which we termed P3a, P3b, P3c and P3d. The P3a and P3b deletion were found in single instances, while the P3c and P3d deletion were both found in three unrelated men. The P3c and P3d deleted chromosomes both occurred specifically in a single Y-chromosomal haplotype branch (branch 11* for P3c and branch E1a* for P3d). Unexpectedly, the three men with a P3c deletion had a significantly higher total sperm count than men without a deletion, even though they lacked seven testis-specific genes from the AZFc region. This result contradicts with all previous findings that demonstrated that a reduction in AZFc genes results in reduced semen quality. However, P3c stands out from other (partial) AZFc deletions by a deletion of a single copy of PRY. Since PRY is thought to regulate apoptosis of spermatids and spermatozoa, the deletion of one PRY gene copy in men with a P3c deletion could theoretically result in a decreased rate of apoptosis and therefore to an increase in the number of spermatids and thus, spermatozoa.

Taken together with previously identified deletions resulting from homologous recombination between amplicons, our findings demonstrate that the palindromic nature of the human Y
chromosome renders it highly susceptible for deletions, some of which affect spermatogenesis and hence reproductive fitness.

Chapter 7 describes intrachromosomal homologous recombination between inverted amplicons on opposing Y chromosome arms. Here, we report four novel cases of intrachromosomal homologous recombination events involving inverted amplicons of the Y chromosome of which one copy is located on the short arm (Yp) and the other on the long arm (Yq). The men studied originated from sample collections of the Academic Medical Center, Onze Lieve Vrouwe Gasthuis, Maastricht University Medical Center, and the Whitehead Institute. They were selected based on a karyotype analysis that was performed as part of a fertility workup or parental screening as a follow-up of abnormalities detected during prenatal screening and that indicated the presence of a pericentric inversion or a pseudo isoYp chromosome. In two men, the intrachromatid crossing over between inverted amplicons on opposing arms generated pericentric inversions. Both men had normozoospermia. In two other men, the crossing over resulted in pseudoisochromosome formation, loss of long arm heterochromatin, loss of the 2nd pseudoautosomal region (PAR2) and, in one of these two men, loss of the AZFc region. The latter two men had oligozoospermia and azoospermia, respectively. These findings highlight the intrinsic tendency of the human Y chromosome towards recombination, as intrachromosomal homologous recombination occurs for all amplicons on the Y chromosome, even if these amplicon copies are located on opposing arms.

Future implications

Our results provide evidence that DAZ gene copy number has a significant effect on semen quality and thus reproductive fitness. This effect of DAZ on spermatogenesis was noticed previously in DAZL-knockout mice and in studies where human DAZ rescued spermatogenesis in the mouse DAZL-knockout and the degree of rescue was determined by the number of human DAZ copies that integrated into the mouse genome (Ruggiu et al., 1997; Sosl et al., 1999). This suggests that DAZ by itself could restore spermatogenesis in AZFc deleted men with severely impaired spermatogenesis. This will be difficult to assay in humans as currently there are no culture systems that allow complete in vitro human spermatogenesis. Recently, our group established an in vitro culture system that allows the propagation of human spermatogonial stem cells (SSCs) (Sadri-Ardekani et al., 2009). As DAZ is known to be expressed in human spermatogonia, this culture system will allow the study of the function of DAZ in human spermatogonia either by establishing SSC culture from men with AZFc deletions or by specifically blocking DAZ expression in SSC culture using shRNA (Menke et al., 1997). In addition, if indeed DAZ has a function in spermatogonia, the potential rescue capability of specific DAZ genes could be studied in SSC cultures of AZFc deleted men. Ultimately, if these experiments have positive results, spermatogenic failure caused by an AZFc deletion could be rescued by introducing specific DAZ transcripts in isolated spermatogonia followed by
autotransplantation of these spermatogonia to restore or increase spermatogenesis thereby omitting the need for TESE and ICSI procedures in men with AZFc deletions.

Future studies on male infertility should also focus on the X chromosome as, similar to the Y chromosome, this chromosome is only present in single copy in males and studies in mice have suggested that it contains multiple testis specific genes (Wang et al., 2001). Similar to the Y chromosome, the human X chromosome contains repetitive regions, but it contains significantly more genes than the Y chromosome, namely 2,000 (Ross et al., 2005). Of these genes, roughly 10% are associated with cancer/testis (CT). These CT genes of the X chromosome account for roughly 90% of the total CT genes of the human genome. Interestingly, these CT genes are expressed in the testis of healthy men and are hypothesized to influence the reproductive fitness of men (Chomez et al., 2001; Ross et al., 2005). They may therefore be potential genes that can influence spermatogenesis by gene copy number as well.

**Conclusion**

This thesis describes the presence and phenotypic effect of variations that can occur within the AZFc region of the Y chromosome.

We elucidated the intragenic variation of DAZ, identified duplications of the AZFc region, deletions in the AZFc region that occurred via non-homologous recombination, large scale inversions on the Y chromosome, pseudo iso-Yp chromosomes, and established their associated spermatogenic phenotypes.

The results described in this thesis have one important implication for clinical practice. In addition to screening men with spermatogenic failure for deletions of the AZFc region, we now advise to also screen for duplications of AZFc that lead to eight DAZ genes as these clearly result in spermatogenic failure. The novel qPCR method described in this thesis should allow rapid screening for such duplications.

We found that it is not the intragenic variation of DAZ, but its copy number that affects spermatogenesis. This is best visualized by our finding that men carrying Y chromosomes with gr/gr deletions followed by a duplication did not have different semen qualities as men with Y chromosomes that had no deletion of (part of) AZFc. The fact that DAZ gene copy number and not the intragenic variation of DAZ affects semen quality could be due to its position within AZFc. In contrast to all other AZFc genes, DAZ genes are located in such positions that their copy number is affected by every homologous recombination that can occur within the AZFc region. Thus, it is the DAZ gene copy number that controls reproductive fitness and thereby safeguards the stability of the AZFc region on the rapidly evolving human Y chromosome. In light of the absence of meiotic recombination on the Y chromosome since its divergence from the X chromosome 166 million years ago and the subsequent massive gene loss on the Y chromosome, the human Y chromosome has been portrayed as a decaying chromosome (Stern, 1957; Warren et al., 2008; Veyrunes et al., 2008; Visser et al., 2009). Indeed, as also shown in this thesis, modern day Y chromosomes show significant structural variability suggesting rapid and continuous structural rearrangements. Critical
regions of the Y chromosome, such as the AZFc region, have however remained relatively stable over millions of years.

Clinically our aim was to identify additional Y-chromosome aberrations that cause male infertility. Indeed, we have discovered that duplications of AZFc that result into a Y chromosome with eight DAZ genes are a risk factor for spermatogenic failure. Furthermore, we have found that gr/gr deletions with a subsequent duplication do not affect semen quality. As such we advise that AZFc duplications should be included in the counseling of subfertile couples as a potential cause of male infertility and that gr/gr deletion screening must include DAZ gene copy number analysis to detect subsequent duplications. It is of note that although Y-chromosome deletion screening has been performed as a routine practice for many years, there is still an ongoing debate on the usefulness of this screening. This is due to the low frequency at which these aberrations are normally found –less than 10% of men with azoo- or severe oligozoospermia have a Y-chromosome aberration–, to the absence of a clear cut clinical consequence –almost all couples of which the male partner has been
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diagnosed with a Y-chromosome aberration proceed with ICSI–, and to the costs of screening. The findings in the current thesis do not defer from this.

Our current results together with previous described reduced semen quality in men with (partial) AZFc deletions suggest that this stability is due to a direct relationship with sperm production and hence reproductive fitness: The Y chromosome is here to stay!
References


