Spermatogenic failure. A genetic Odyssey
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CHAPTER 6

Heterogeneous nuclear ribonucleoprotein G-T (HNRNP G-T) mutations in men with impaired spermatogenesis


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Abstract

The genetic cause of male subfertility due to impaired spermatogenesis is unknown in the majority of cases, but the general assumption is that it is a complex disorder. The aim of this study was to determine whether mutations occur in the HNRNP G-T gene in men with idiopathic impaired spermatogenesis. The heterogeneous nuclear ribonucleoprotein G-T (HNRNP G-T) gene is located in chromosomal region 11p15 that has been shown to be associated with impaired spermatogenesis. It 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.

We identified eight single nucleotide variants in our patient group of 153 subfertile men by sequencing the HNRNP G-T gene. Two of the mutations, R100H and G388del, did not occur in a control group of 143 normozoospermic men. The R100H mutation causes loss of a conserved arginine, thereby affecting a putative site of methylation possibly required for RNA-binding. Interestingly, this mutation was inherited from the mother. The G388del mutation causes loss of one non-conserved glycine located in a glycine stretch at the end of the protein that is not a known functional motif or domain.

Our data show that HNRNP G-T mutations are not a frequent cause of impaired spermatogenesis. Nevertheless, the R100H mutation detected suggests that in some men mutations in the HNRNP G-T gene can cause impaired spermatogenesis.
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Introduction

Subfertility, defined as the inability to conceive after one year of unprotected intercourse, affects 10-15% of couples. In ~50% this subfertility is due to impaired semen parameters (Hull et al., 1985; de Kretser, 1997; Evers, 2002). Although frequently suggested, evidence of a genetic aetiology of impaired spermatogenesis is scarce (Turek and Pera, 2002). The only accepted genetic causes of impaired spermatogenesis in men are numerical and structural chromosomal abnormalities and Y chromosome deletions (Maduro and Lamb, 2002). Taken together, these genetic abnormalities explain only ~15% of the cases of impaired spermatogenesis (Hargreave, 2000; Foresta et al., 2001). Thus, in the majority of cases, the cause of reduced sperm quality remains unclear.

Recently, we described an association of chromosomal region 11p15 with impaired spermatogenesis. This association was found by SNP analysis of two 11p15 genes, ZNF214 and ZNF 215. In addition, some mutations in those two genes were identified in patients with impaired spermatogenesis (Gianotti et al., 2003). An additional candidate gene for impaired spermatogenesis in this region is heterogeneous nuclear ribonucleoprotein G-T (HNRNP G-T), which is located 68 kb proximal to ZNF214 (Elliott et al., 2000).

HNRNP G-T is a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) gene family. It is a single-copy and highly conserved gene. Although it is retroposon-derived and thus does not contain any introns, HNRNP G-T is transcribed into a functionally active protein (Elliott et al., 2000). HnRNP G-T is a germ cell-specific nuclear protein that is expressed predominantly in pachytene spermatocytes and, to a much lesser extent, in round spermatids (Elliott et al., 2000; Maymon et al., 2002). HNRNP G-T is homologous to RBMY on the Y chromosome and to HNRNP G (RBMX) on the X chromosome (Chai et al., 1998; Elliott et al., 2000; Venables et al., 2000). RBMY is located in the P5/P1 region, which is deleted in ~2% of men with azoospermia (Repping et al., 2002). These three family members all encode RNA-binding proteins (Weighardt et al., 1996; Chai et al., 1998; Venables et al., 2000). Although an essential role of RNA-binding proteins in spermatogenesis is generally accepted, their individual and specific functions have yet to be clarified (Venables and Eperon, 1999). However, HNRNP G-T is thought to play a role in cell-specific pre-mRNA splicing in germ cells, thereby producing testis-specific isoforms (McGuffin et al., 1998; Venables et al., 1999, 2000; Nasim et al., 2003).

In the present study we sought to determine whether mutations in the HNRNP G-T gene occur in men with idiopathic severe oligozoospermia orazoospermia.
Materials and Methods

Patients
Male partners of subfertile couples who were referred to the Center for Reproductive Medicine of the Academic Medical Center were consecutively included in this study from January 1998 until December 2002. Written informed consent was obtained from all participants. This study was approved by the Institutional Review Board of the Academic Medical Center.

Patients were men with idiopathic azoospermia or severe oligozoospermia, defined as a total sperm count (TC) of <20x10⁶ in two consecutive semen samples. Men with normozoospermia, defined as a total sperm count of >40 x 10⁶ with a progressive motility and normal morphology of ≥40% in two consecutive semen samples, were included as controls.

Semen analyses were performed according to WHO guidelines (World Health Organization, 1999). Patients with a history of orchitis, alcohol abuse, surgery of the vasa deferentia, bilateral orchidectomy, chemo- or radiotherapy, obstructive azoospermia (confirmed by testicular biopsy), bilateral cryptorchidism and numerical chromosomal abnormalities or deletions of the Y chromosome were excluded.

Of all patients, data concerning testicular volume and levels of FSH and testosterone were retrieved from the medical records. DNA was extracted from peripheral blood leucocytes according to standard procedures. Whenever possible, parental DNA was used to check for the inheritance pattern of any identified mutation.

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**Mutation analysis**

We amplified the entire genomic sequence of the *HNRNP G-T* gene in five overlapping fragments. Primers were designed using the MELT program (Jones et al., 1999) (Table I). PCR was carried out in a total volume of 25 microl and contained 25 microg DNA, 0.2 mmol/l dNTPs and 0.5 IU *superTaq* polymerase. Specific conditions for each fragment are listed in Table I. For amplification of all fragments we used a Touchdown PCR program with a temperature range of 69 – 62 °C with a 1°C decrement per cycle and one cycle increment per temperature step and a final amplification for 20 cycles at 94°C for 30 s., 62°C for 30 s. and 72°C for 30 s with a final extension at 72°C for 5 min.

Mutation screening was performed by direct sequencing of both sense and antisense strands, using the same primers as those used for PCR, on an automated ABI Prism 3100 Genetic Analyser (Applied Biosystems). Overlapping sequences of the five regions were aligned to produce a contiguous sequence which was then compared with the available sequence of *HNRNP G-T* from GenBank (NT_028310.10).

For each variant detected, we subsequently designed an restriction fragment length polymorphism assay to evaluate the frequencies of these variants in the control group. Statistical differences in genotype frequencies and distribution between the patient and control groups were evaluated using a $\chi^2$ test. A $p$-value < 0.05 was considered significant.
<table>
<thead>
<tr>
<th>Fragment no.</th>
<th>Primer sequence</th>
<th>Product size (bp)</th>
<th>PCR conditions</th>
</tr>
</thead>
</table>
| 1            | F: GCCGCTGACCAGTAGGAGCCG  
R: CGAACCGCCGTTTGGGTGCGCTGG | 383            | 1 mol/l betaine, 5 microl 5x betaine buffer*, 2 mmol/l MgCl₂, 8 pmol each primer |
| 2            | F: CAGACGCAAAGCCCGCCCGCACAG  
R: CATTCCACCACCCCGCTGCTGGAGC | 343            | 1 mol/l betaine, 5 microl 5x betaine buffer*, 1.5 mmol/l MgCl₂, 8 pmol each primer |
| 3            | F: CAGGGTCGCGCACCACCCCAAGA  
R: TAGTCACCGGTCGACCTCCGTAG | 346            | 1 mol/l betaine, 5 microl 5x betaine buffer*, 1.5 mmol/l MgCl₂, 8 pmol each primer |
| 4            | F: ACGGCCACCTCAGTTCCGGGCAC  
R: GTCTGCTGGCTGCCCACCGGCTGC | 315            | 1 mol/l betaine, 5 microl 5x betaine buffer*, 2 mmol/l MgCl₂, 16 pmol each primer |
| 5            | F: CGACAGTTACAGCAGCAGTTATG  
R: GGAGAGCTTAAACAGGAATTTC | 351            | 1x Roche buffer b, 10% DMSO, 3 mmol/l MgCl₂, 12 pmol each primer |

*a betaine buffer: 0.25 mol/l Tris (pH 9.2), 70 mmol/l NH₄SO₄, 10% dimethylsulphoxide (DMSO) and 1.5% Tween

b Roche buffer: cat no. 1699105

F = forward; R = reverse
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Results

Patients
The clinical features of the patients included in this study are listed in Table II.

Table II. Patient’s clinical data

<table>
<thead>
<tr>
<th>Patients</th>
<th>No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azoospermia</td>
<td>29/153 (19)</td>
</tr>
<tr>
<td>Severe oligozoospermia</td>
<td>124/153 (81)</td>
</tr>
<tr>
<td>Reduced testis volume (&lt; 15 ml)</td>
<td>78/134 (58)</td>
</tr>
<tr>
<td>FSH (&gt; 7.5 IU/L)</td>
<td>71/135 (53)</td>
</tr>
<tr>
<td>Reduced testosterone (&lt; 11 nmol/L)</td>
<td>28/131 (21)</td>
</tr>
<tr>
<td>Unilateral cryptorchidism</td>
<td>23/153 (15)</td>
</tr>
</tbody>
</table>

Figure 2. Alignment of the human and mouse hnRNP G-T proteins

All variants found are shown in bold superscript above the amino acid concerned. The RNA binding domain is indicated by a dark gray box. The RXR cluster is indicated by a light gray box. A star indicates identical amino acids, a double dot similar amino acids and a single dot non-conserved amino acids.
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Mutation screening

Eight sequence variants were identified (Table III). Two variants, A4A (dbSNP: 4758158) and A66V, occurred with high frequency in both patients and controls, in the heterozygote state as well as in the homozygote state. The frequencies of both variants did not differ significantly between the patient group and the control group ($p=0.12$ and $p=0.68$ respectively). Two other variants, G9G and R371R, were silent mutations. In addition, two missense mutations, A134T and G262V, were identified in both patients and controls. However, we discovered two unique missense mutations in two patients. The first mutation resulted in a G→A nucleotide change at position 298 and was present in the heterozygote state. This nucleotide change caused a substitution of a conserved amino acid, altering arginine at codon 100 into histidine (R100H). This amino acid is closely located to the RNA-binding motif and within an RXR cluster known to be involved in protein methylation (Figure 1). The second mutation resulted in an in-frame-deletion of three nucleotides, causing a deletion of a glycine at codon 388 (G388del) and was also in the heterozygote state. This amino acid is located in a non-conserved region at the end of the HNRNP C-T gene (Figure 1).

None of the 143 normozoospermic control men analysed carried either of these mutations. The carrier of the R100H mutation was an azoospermic man. His medical history showed unilateral cryptorchidism, corrected at the age of 5 years through hormone injections. The patient had a reduced testicular volume (11 ml). Hormone screening showed an elevated FSH level (42.0 IU/L) and a normal testosterone level (17.9 nmol/L). Analysis of the pattern of inheritance of the mutation in the carrier's family showed that the patient had inherited this mutation from his mother (Figure 2).

The carrier of the G388del suffered from severe oligoasthenoteratospermia (TC 0.24x10⁶). He had a reduced testicular volume (6 ml) and an elevated FSH level (19.7 IU/L). His testosterone level was normal (15.1 nmol/l). Blood samples from his parents were not available for analysis of the inheritance pattern.
Table III. Variants found in patients and controls.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Amino acid</th>
<th>Patients (n=153)</th>
<th></th>
<th>Controls (n=143)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+/+</td>
<td>+/-</td>
<td>-/-</td>
<td>+/+</td>
</tr>
<tr>
<td>12</td>
<td>G→A</td>
<td>A4A</td>
<td>79 (52%)</td>
<td>53 (34%)</td>
<td>21 (14%)</td>
</tr>
<tr>
<td>87</td>
<td>C→A</td>
<td>G9G</td>
<td>152</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>177</td>
<td>C→T</td>
<td>A66V</td>
<td>87 (57%)</td>
<td>58 (38%)</td>
<td>8 (5%)</td>
</tr>
<tr>
<td>298</td>
<td>G→A</td>
<td>R100H</td>
<td>152</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>399</td>
<td>G→A</td>
<td>A134T</td>
<td>152</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>785</td>
<td>G→T</td>
<td>G262V</td>
<td>151</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>1110</td>
<td>A→C</td>
<td>R371R</td>
<td>152</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1160-1162</td>
<td>delGAA</td>
<td>G388del</td>
<td>152</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

+/- = homozygote wild type; +/- = heterozygote; -/- = homozygote mutant; ND = not determined.
**Figure 2. Variants found in patients and controls**

Shown are the PCR products of fragment 2 of the HNRNP G-T gene of the father (lane 1), the mother (lane 2) and the affected son (lane 3) respectively. PCR products were incubated with the restriction enzyme BbvI and run on an agarose gel. The R100H mutation created an additional restriction site in the affected patient and his mother, thus producing an extra band on the gel.

**Discussion**

In this study we identified eight single nucleotide variants in 153 men with azoospermia or severe oligozoospermia by sequencing of the HNRNP G-T gene. Two of these variants, A4A and A66V, are common polymorphisms, as they occurred with high frequency in patients as well as in controls. Two silent mutations, G9G and R371R, were irrelevant, as they did not cause an amino acid substitution nor affect a possible splice site, as HNRNP G-T is an intronless gene. Two missense mutations, A134T and G262V, are probably rare polymorphisms without functional consequences, as they do not occur in a functional motif or domain of the hnRNP G-T protein and were present in both patients and controls. However, we found two unique mutations, G388del and R100H, in two patients with impaired spermatogenesis, which were not present in our control group. The G388del mutation causes loss of one non-conserved glycine located in a glycine stretch at the end of the protein that is not a known functional motif or domain. Therefore this mutation appears to be of low significance. Interestingly, however, the R100H mutation causes a single amino acid substitution of a conserved arginine located within an RXR cluster, just a few amino acids downstream of the RNA binding motif of the gene.

We suspect that the R100H mutation might cause impaired spermatogenesis by disrupting the hnRNP G-T protein function for the following two reasons. First, the mutation involves a conserved amino acid, changing an arginine into a histidine. An arginine residue at this position is consistently found in both human and mouse hnRNP G-T, as well as in RBMY and hnRNP G. In addition, the overall region is strictly conserved in the mouse orthologue.
as well as in the human homologues. Thus, because there is such a high degree of evolutionary conservation, indicating that hnRNPG-T is under strict selection, even a subtle alteration might have phenotypical consequences.

Second, the amino acid change probably affects protein methylation. Previous studies have shown that most hnRNPs undergo post-translational modifications, with methylation at arginine residues being the most relevant of such adjustments (Liu and Dreyfuss, 1995; Kim et al., 1997; Kzyshkowska et al., 2001; Pawlak et al., 2002). A specific biological role for arginine methylation in hnRNPs has not yet been defined, but it is thought that it might affect the RNA-binding activity of hnRNPs with other proteins (Shen et al., 1998; Smith et al., 1999; Pawlak et al., 2002; Wada et al., 2002). RGG motifs, containing a variable number of closely spaced Arg-Gly-Gly repeats, are the preferential sites of methylation in hnRNPs (Liu and Dreyfuss, 1995). An additional site of methylation is in the RXR (Arg-X-Arg) cluster (Smith et al., 1999). Both hnRNPG and hnRNPG-T contain such a cluster. Since the R100H mutation involves an arginine residue that is located within an RXR cluster, methylation at this site might be altered with subsequent consequences for the function of the hnRNPG-T protein.

The observed maternal inheritance of the R100H mutation in the carrier’s family supports our line of reasoning that this mutation is pathological. Indeed, a number of the variants that we identified in the ZNF214 and ZNF215 genes, located in the same 11p15 region, were also maternally inherited (Gianotte et al., 2003). In general, inheritance of impaired spermatogenesis through the mother might explain how male subfertility can segregate through generations (Lilford et al., 1994; Gianotten et al., 2004).

The R100H mutation is in the heterozygote state. In general, autosomal heterozygote mutations are not considered to be sufficient to result in a pathological condition. Several arguments can be offered to explain how this heterozygote mutation can lead to the impaired spermatogenesis phenotype. First, the R100H mutation might be an autosomal dominant gene defect inherited from the mother. Second, HNRNP G-T might be an imprinted gene, meaning that just one allele is expressed based on the parental origin. Several imprinted genes are located in chromosomal region 11p15, including ZNF215, for which the maternal allele is preferentially expressed in several tissues (Alders et al., 2000). Finally, the HNRNP G-T gene might show haploinsufficiency. In this situation, the unaffected allele would not be able to compensate for the dysfunctional one.

In summary, mutations in the HNRNP G-T gene do not seem to be a frequent cause of impaired spermatogenesis in our patient population. Nevertheless, the R100H mutation detected suggests that in some men mutations in the HNRNP G-T gene can account for the impaired spermatogenesis phenotype.
Acknowledgements
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References


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Chapte rr  6


