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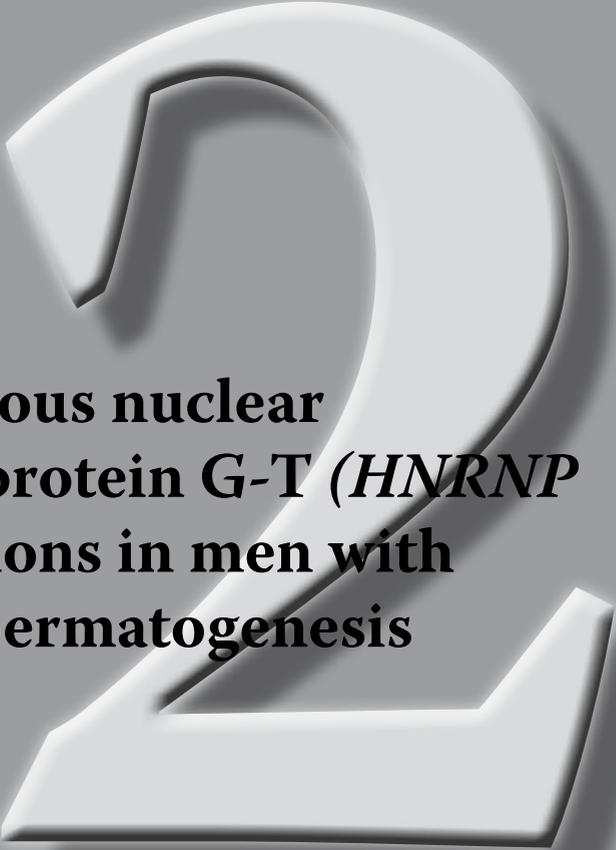
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**Heterogeneous nuclear  
ribonucleoprotein G-T (*HNRNP*  
*G-T*) mutations in men with  
impaired spermatogenesis**

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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 normospermic 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 point out 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.

## Introduction

Subfertility, defined as the inability to conceive after one year of unprotected intercourse, affects 10-15% of couples. In approximately 50% this subfertility is due to impaired semen parameters (1-3). Although frequently suggested, evidence of a genetic aetiology of impaired spermatogenesis is scarce (4). The only accepted genetic causes of impaired spermatogenesis in men are numerical and structural chromosomal abnormalities and Y chromosome deletions (5). Taken together, these genetic abnormalities explain only ~15% of the cases of impaired spermatogenesis (6, 7). 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 (8). 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* (9).

*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 (9). 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 (9, 10). *HNRNP G-T* is homologous to *RBMY* on the Y chromosome and to *HNRNP G (RBMX)* on the X chromosome (9, 11, 12). *RBMY* is located in the P5/P1 region, which is deleted in approximately 2% of men with azoospermia (13). These three family members all encode RNA-binding proteins (11, 12, 14). Although an essential role of RNA-binding proteins in spermatogenesis is generally accepted, their individual and specific functions have as of yet not been clarified (15). However, *HNRNP G-T* is thought to play a role in cell-specific pre-mRNA splicing in germ cells, thereby producing testis-specific isoforms (16-18).

In the present study we sought to determine whether mutations in the *HNRNP G-T* gene occur in men with idiopathic severe oligozoospermia or azoospermia.

## 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 less than  $20 \times 10^6$  in two consecutive semen samples. Men with

normozoospermia, defined as a total sperm count of more than  $40 \times 10^6$  with a progressive motility and normal morphology of at least 40% in two consecutive semen samples, were included as controls. Semen analyses were performed according to WHO guidelines (19). 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 record. 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.

### 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 (20) (Table I). PCR was carried out in a total volume of 25  $\mu$ l and contained 25 ng DNA, 0.2 mM dNTPs and 0.5 U *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 1 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 that was then compared with the available sequence of *HNRNP G-T* from GenBank (NT\_028310.10).

**Table I.** PCR primers and conditions

Fragment no.	Primer sequence	Product size	PCR conditions
1	F: GCCGCCTGACCAGTAGGAGCCG R: CGAACGCCGTTTGGTGGCCTGG	383 bp	1 M betaine, 5 $\mu$ l 5x betaine buffer <sup>a</sup> , 2 mM MgCl <sub>2</sub> , 8 pmol each primer
2	F: CAGACGCCAAGGCCGCCAGAG R: CATTCCACCGCCGCTGCTGCGAGC	343 bp	1 M betaine, 5 $\mu$ l 5x betaine buffer <sup>a</sup> , 1.5 mM MgCl <sub>2</sub> , 8 pmol each primer
3	F: CAGGGTCGGCCACCCCAAGA R: GTAGTCACGGTCGCGACCTCCGTAG	346 bp	1 M betaine, 5 $\mu$ l 5x betaine buffer <sup>a</sup> , 1.5 mM MgCl <sub>2</sub> , 8 pmol each primer
4	F: ACGGCCACTCCAGTGTCCGGGAC R: GATCTGGTCTGCCACCCGGTGTC	315 bp	1 M betaine, 5 $\mu$ l 5x betaine buffer <sup>a</sup> , 2 mM MgCl <sub>2</sub> , 16 pmol each primer
5	F: CGACAGTTACAGCAGCAGTTATG R: GGAGACGATCTTAACAGGAAATTC	351 bp	1x Roche buffer <sup>b</sup> , 10% DMSO, 3 mM MgCl <sub>2</sub> , 12 pmol each primer

<sup>a</sup>Betaine buffer: 0.25 M Tris (pH 9.2), 70 mM NH<sub>4</sub>SO<sub>4</sub>, 10% DMSO and 1.5% Tween

<sup>b</sup>Roche buffer: cat no 1699105

F = forward; R = reverse

For each variant detected, we subsequently designed an RFLP-assay to evaluate the frequencies of these variants in the control group.

Statistical analysis of differences in genotype frequencies and distribution between the patient and control group were estimated using a  $\chi^2$  test. A p-value < 0.05 was considered statistically significant.

## Results

### Patients

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

### 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.

**Table II.** Patient's clinical data

Patients	Number
Azoospermia	29/153 (19)
Severe oligozoospermia	124/153 (81)
Reduced testis volume (< 15 ml)	78/134 (58)
FSH (> 7.5 U/L)	71/135 (53)
Reduced testosterone (< 11 nmol/L)	28/131 (21)
Unilateral cryptorchidism	23/153 (15)

Values in parentheses are percentages

**Table III.** Variants found in patients and controls

Nucleotide	Amino acid	Patients (n=153)			P	Controls (n=143)		
		+/+	+/-	-/-		+/+	+/-	-/-
12 G → A	A4A	79 (52)	53 (34)	21 (14)	0.12	65 (45)	64 (45)	14 (10)
87 C → A	G9G	152	1	0		ND	ND	ND
177 C → T	A66V	87 (57)	58 (38)	8 (5)	0.68	78 (55)	54 (38)	11 (8)
298 G → A	R100H	152	1	0		143	0	0
399 G → A	A134T	152	1	0		140	3	0
785 G → T	G262V	151	2	0		142	1	0
1110 A → C	R371R	152	1	0		ND	ND	ND
1160_1162 delGAA	G388del	152	1	0		143	0	0

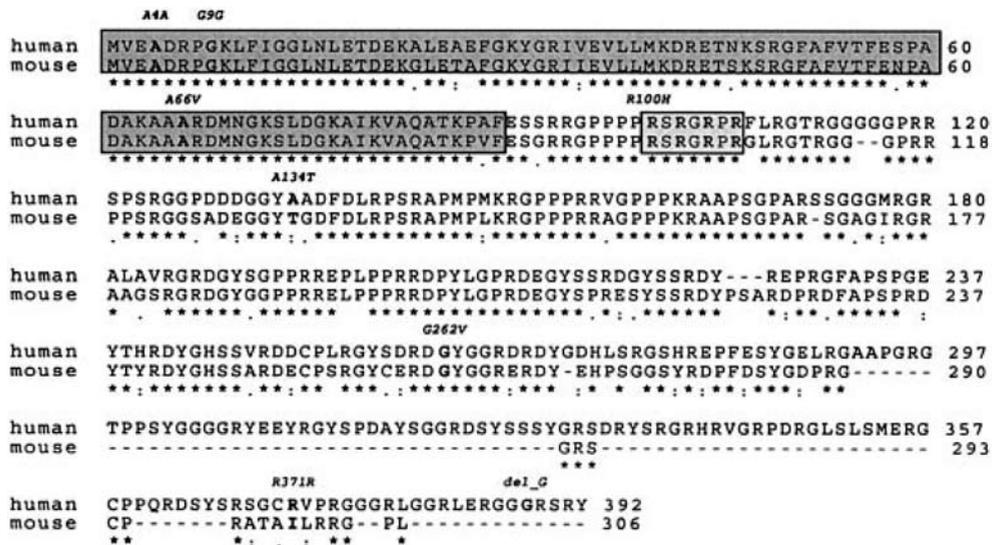
Values in parentheses are percentages.

+/+ = homozygote wild type; +/- = heterozygote; -/- = homozygote mutant; ND = not determined

However, we discovered two unique missense mutations in two patients. The first mutation resulted in a G-to-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 3 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 G-T* gene (Figure 1). None of the 143 normospermic 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 five 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).

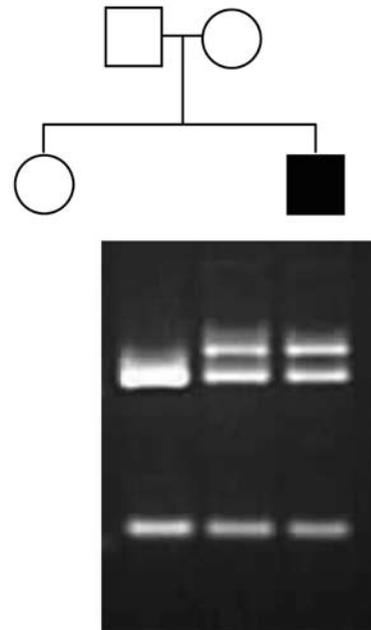
The carrier of the G388del suffered from severe oligoasthenoteratospermia (TC 0.24x10<sup>6</sup>). He had a reduced testicular volume (6 ml) and an elevated FSH level (19.7 IU/L). His testosterone level was normal (15.1 nmol/L). Blood samples from his parents were not available for analysis of the inheritance pattern.



**Figure 1.** Alignment of the human and mouse hnRNP G-T proteins.

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

**Figure 2.** Inheritance pattern of the R100H mutation. 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 runned 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, for *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 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 hnRNP G-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 (21-24). A specific biological role for arginine methylation in hnRNPs has not been defined yet, but it is thought that it might affect the RNA-binding activity of hnRNPs with other proteins (24-27). RGG motifs, containing a variable number of closely spaced Arg-Gly-Gly repeats, are the preferential sites of methylation in hnRNPs (23). An additional site of methylation is in the RXR (Arg-X-Arg) cluster (26). Both hnRNP G and hnRNP G-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 hnRNP G-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 maternally inherited as well (8). In general, inheritance of impaired spermatogenesis through the mother might explain how male subfertility can segregate through generations (28, 29).

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 (30). 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.

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