Polymorphism of the \textit{GRTH/DDX25} gene in normal and infertile Japanese men: a missense mutation associated with loss of GRTH phosphorylation

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The gonadotropin-regulated testicular RNA helicase (GRTH/Ddx25) is present in Leydig and germ cells of rodents, and is essential for fertility in mice. This study evaluated the incidence of \textit{GRTH/DDX25} gene mutations in a group of infertile patients with non-obstructive azoospermia (NOA), 85\% with a preponderance of Sertoli cells in the seminiferous tubule and 15\% with spermatogenic arrest, and compared them to a group of fertile subjects. Exonic sequences in the \textit{GRTH} gene were screened using denaturing high-performance liquid chromatography of the genomic DNA from 143 NOA and 100 fertile Japanese men. A unique heterozygous missense mutation Arg\textsuperscript{242}His in exon 8 was identified in 5.8\% of Sertoli cell-only patients and in 1\% of normal subjects. Although the mutant protein was efficiently expressed in COS-1 cells, only the 56 kDa nuclear/cyttoplasmic non-phosphorylated species was present, whereas the 61 kDa cytosolic phosphorylated species was absent. In addition, a silent mutation was identified in exon 11 in NOA subjects. The Arg\textsuperscript{242}His missense mutation of the \textit{GRTH/DDX25} gene associated with expression of a protein with reduced basicity, and the absence of the phospho-GRTH species, could be of relevance to some of the functional aspects of the protein that impact on germ cell development and/or function.

\textit{Keywords: GRTH/DDX25} mutation; missense mutation; single nucleotide polymorphism; azoospermia; infertility

\textbf{Introduction}

Although Y chromosomal microdeletions, including the gene families of AZF (Azoospermia factor) regions, are the most updated causes of non-obstructive male infertility, these account for only 8–15\% of the infertile population (Ma \textit{et al}.\textsuperscript{,} 1993; Simoni, 2001; Thangaraj \textit{et al}.\textsuperscript{,} 2003; Vogt, 2005; Krausz and Degl’Innocenti, 2006). The cause of the abnormality in most of the infertile patients with non-obstructive azoospermia (NOA) is still poorly understood. In addition to the Y chromosome, it has been speculated that the autosomal genes have a role in male fertility. For example, the mouse functional homologue of human autosomal DAZL (Deleted in azoospermic like), known as Dazl and located in chromosome 17, has been shown to be essential for the differentiation of germ cells, by gene knockout studies (Ruggiu \textit{et al}.\textsuperscript{,} 1997). The activity of Dazl was regained when a human DAZ gene was injected into the Dazl null mice, indicating a functional connection between the Y chromosome DAZ and the autosomal Dazl gene (Slee \textit{et al}.\textsuperscript{,} 1999). The SYCP3 gene, which is located on chromosome 12 and encodes a DNA-binding protein (Miyamoto \textit{et al}.\textsuperscript{,} 2003), has been reported to have an essential meiotic function in human spermatogenesis.

Our laboratory has cloned and characterized a novel gonadotropin regulated testicular RNA helicase (GRTH/DDX25) gene located in human chromosome 11, i.e. essential for spermatid development and the completion of spermatogenesis in mice (Tang \textit{et al}.\textsuperscript{,} 1999; Sheng \textit{et al}.\textsuperscript{,} 2003; Tsai-Morris \textit{et al}.\textsuperscript{,} 2004a,b; Dufau and Tsai Morris, 2007). GRTH/Ddx25 is present in Leydig cells, meiotic spermatocytes and spermatids of pubertal and mature rodent testis. It displays ATPase activity and ATP dependent bi-directional RNA helicase activity (Tang \textit{et al}.\textsuperscript{,} 1999). This gene product functions as a component of mRNP particle to regulate the translation of specific genes required for the progression of spermatogenesis (Tsai-Morris \textit{et al}.\textsuperscript{,} 2004a,b; Sheng \textit{et al}.\textsuperscript{,} 2006). GRTH/Ddx25 gene transcription is up-regulated by human chorionic gonadotropin (hCG) via cAMP induced androgen production in testicular Leydig cells. A cell specific and hormonally regulated expression of GRTH results from alternative utilization of translation initiation codons in the rat testis (Sheng \textit{et al}.\textsuperscript{,} 2003). A 56 kDa non-phosphorylated species and a 61 kDa phosphorylated GRTH species are present in spermatocytes and round spermatids of adult mice and rats. The 61/56 kDa species are associated with the cytoplasmic compartment, whereas the 56 kDa species is present in the nucleus of germ cells where it exerts specialized aspects of GRTH multiple functions (Sheng \textit{et al}.\textsuperscript{,} 2006). Male mice with the disruption of \textit{GRTH/DDX25} gene (GRTH\textsuperscript{−/−}) display a basal normal gonadotropin and androgen profiles; however, the mice are sterile with azoospermia caused by a complete arrest of spermiogenesis at step 8 of spermatids (Tsai-Morris \textit{et al}.,
2004a,b). Since GRTH/DDX25 is crucial for fertility in male mice, we initiated studies to determine the relevance of this gene to human spermatogenesis. In this study, two polymorphic forms of GRTH were identified by genetic screening of GRTH/DDX25 in 100 fertile and 143 infertile Japanese men with NOA, using denaturing high-performance liquid chromatography. The missense mutation found in this study impacted on the post-transcriptional modification of the expressed GRTH protein.

**Materials and Methods**

**Genomic DNA source and subjects background**

Genomic DNA samples of 100 fertile male subjects (age: 30 ± 4.4 years old) with normal sperm parameters and at least one offspring (Table I), and 143 NOA patients (age: 35 ± 5.6 years old) (Table II) diagnosed by testicular biopsy, were obtained from Kanazawa University, Kanazawa, Japan. No NOA patients had AZF deletion or other significant chromosomal abnormalities (Fukushima et al., 2006). All the patients underwent physical examination and endocrine profiles were assessed. FSH, LH, prolactin and testosterone were evaluated using chemiluminescent immunoassays (Elmlinger et al., 2002). Seminal samples were collected after 3–10 days of abstinence and analyzed for volume, sperm concentration and motility, and at least two consecutive semen analyses were obtained. Semen analyses were performed according to the World Health Organization recommendations (WHO, 1999). Genomic DNA and corresponding biopsy slides, hormonal values and patient characteristics were labeled via the non-identifier route. DNA samples were prepared from patients’ peripheral blood lymphocytes following the manufacturer’s protocol (Qiagen, Hilden, Germany). The study was approved by the Office of Human Subject Research, National Institutes of Health, Bethesda, Maryland, and the Institutional Review Board, Ethical Committee of Kanazawa University, Kanazawa, Japan.

**PCR amplification**

We designed primer pairs homologous to human GRTH DNA sequences encompassing individual coding exons of the human GRTH/DDX25 gene (12 exons) (Tsai-Morris et al., 2004a,b) from genomic DNA. DNA fragments in this study were generated between 120 and 270 bp targeting for the optimal range of dHPLC analysis (see below). PCR were carried out using high fidelity platinum Taq DNA polymerase (invitrogen) with a thermal program of GeneAmp-PCR9700 (Applied Biosystem). An initial denaturing step of 3 min at 95°C was followed by 32 cycles of amplification (30 sec at 95°C, 30 sec at 68°C and 30 sec at 68°C) and a final extension at 68°C for 5 min. A single band of PCR fragment was obtained under the established primer design and PCR conditions (Table III).

**Denaturing high-performance liquid chromatography analysis and DNA sequencing**

We optimized the screening protocol for detecting the potential mutations on all individual exons by denaturing high-performance liquid chromatography (dHPLC) (Transgenomic Wave Navigator dHPLC system, Transgenomic) (Table III). PCR products were subjected to dHPLC analysis to evaluate the profiles of the NOA patients compared to that of wild-type DNA. The identified variants, verified by PCR and dHPLC analysis (n = 3), were subcloned into a PCR2.1Topo vector (Invitrogen) for sequencing analysis in both orientations.

**Animal and tissue source**

Adult male mice (C57BL/6, Charles River Laboratories, Inc., Wilmington, MA, USA) were housed in pathogen-free, temperature- and light-controlled conditions (20 C, alternating 14 h light, 10 h dark cycles). All animal studies were approved by the NICHHD Animal Care and Use Committee (protocols 06-006). Frozen human testicular tissue (37 years old normal male) was obtained from the Southern Division of Cooperative Human Tissue Network (University of Alabama, Birmingham, Alabama).

**Mutagenesis**

The point mutation of Arg at aa. 242 to His (R242H) was prepared following the protocol described in the QuikChange Site-Directed mutagenesis kit (Stratagene). PCR was performed using mutagenesis-grade Pfu Turbo DNA polymerase with the oligonucleotide primers containing the required point mutation R242 to H (human: forward primer: TTGACTAAGATTGTGTTGTCCTG; reverse primer: CAGGACAAACACGATGTCATTGCTAAG). The PCR product was treated with DpnI. The DpnI was used to digest the methylated parental DNA template and select for the newly synthesized DNA containing mutations. The nicked vector DNA incorporating the desired mutation was then transformed into XL1-Blue cells. The mutated plasmid was isolated, sequenced and checked for expression.

**Transient transfection analysis of GRTH cDNA in COS-1 cells**

The full-length human GRTH cDNA (1 μg) (pGRTH-SPORT) was isolated from superscript human cDNA library in pCMV-SPORT (Life Technologies) (Tang et al., 1999) using the full-length rat GRTH cDNA as probe (Sheng et al., 2004a,b). Since GRTH/DDX25 is crucial for fertility in male mice, we initiated studies to determine the relevance of this gene to human spermatogenesis. In this study, two polymorphic forms of GRTH were identified by genetic screening of GRTH/DDX25 in 100 fertile and 143 infertile Japanese men with NOA, using denaturing high-performance liquid chromatography. The missense mutation found in this study impacted on the post-transcriptional modification of the expressed GRTH protein.

**Table I. Normal male sperm parameters**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Volume (ml)</th>
<th>Concentration (million/ml)</th>
<th>A (%)</th>
<th>B (%)</th>
<th>A + B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>30 ± 4.4</td>
<td>3.6 ± 1.6</td>
<td>104.0 ± 75.8</td>
<td>30.4</td>
<td>17.3</td>
<td>47.8 ± 12.4</td>
</tr>
<tr>
<td>N8</td>
<td>26</td>
<td>6</td>
<td>22.1</td>
<td>22.1</td>
<td>33.5</td>
<td>55.6</td>
</tr>
<tr>
<td>WHO criteria</td>
<td>≥2</td>
<td>≥20</td>
<td></td>
<td></td>
<td></td>
<td>≥50</td>
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</tbody>
</table>

**Table II. Background information of NOA patients**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (years)</th>
<th>Semen volume (ml)</th>
<th>Lt size (ml)</th>
<th>Rt size (ml)</th>
<th>FSH (mIU/ml)</th>
<th>LH (mIU/ml)</th>
<th>PRL (ng/ml)</th>
<th>Testo. (ng/ml)</th>
<th>Chromosomea</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOA</td>
<td>35 ± 5.6</td>
<td>3.2 ± 1.8</td>
<td>8.3 ± 4.2</td>
<td>8.8 ± 3.9</td>
<td>27.5 ± 11.8</td>
<td>7.9 ± 4.9</td>
<td>11.3 ± 9.8</td>
<td>4.5 ± 1.6</td>
<td>46, XY</td>
</tr>
<tr>
<td>I25</td>
<td>31</td>
<td>1.0</td>
<td>5</td>
<td>6</td>
<td>26.9</td>
<td>9.0</td>
<td>5.0</td>
<td>3.3</td>
<td>46, XY</td>
</tr>
<tr>
<td>I29</td>
<td>29</td>
<td>2.5</td>
<td>8</td>
<td>10</td>
<td>52.9</td>
<td>22.1</td>
<td>6.0</td>
<td>4.2</td>
<td>46, XY</td>
</tr>
<tr>
<td>I37</td>
<td>35</td>
<td>2.0</td>
<td>6</td>
<td>6</td>
<td>24.4</td>
<td>3.0</td>
<td>4.2</td>
<td>4.2</td>
<td>46, XY</td>
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<tr>
<td>I66</td>
<td>30</td>
<td>5.8</td>
<td>8</td>
<td>10</td>
<td>52.9</td>
<td>22.1</td>
<td>6.0</td>
<td>4.2</td>
<td>46, XY</td>
</tr>
<tr>
<td>I68</td>
<td>40</td>
<td>1.8</td>
<td>6</td>
<td>5</td>
<td>30.0</td>
<td>10.0</td>
<td>3.8</td>
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<tr>
<td>I74</td>
<td>33</td>
<td>4.5</td>
<td>10</td>
<td>10</td>
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<td>3.9</td>
<td>8.0</td>
<td>5.0</td>
<td>46, XY</td>
</tr>
<tr>
<td>I113</td>
<td>38</td>
<td>5.8</td>
<td>12</td>
<td>12</td>
<td>21.4</td>
<td>6.0</td>
<td>10.8</td>
<td>4.6</td>
<td>46, XY</td>
</tr>
<tr>
<td>Normal</td>
<td>≥2</td>
<td>12–15</td>
<td>12–15</td>
<td>2.0–8.3</td>
<td>0.8–5.7</td>
<td>3.6–12.8</td>
<td>2.2–10.4</td>
<td>46, XY</td>
<td></td>
</tr>
</tbody>
</table>

n = 143 NOA patients. Mean ± SD; 125, 29, 37, 66, 68, 74, 113: NOA patients with Sertoli cells only and missense heterozygous mutation in exon 8. Lt, left; Rt, right; T, testis; Testo., testosterone.

*No AZF microdeletion or other significant chromosomal abnormality.*
...were detected by the super-signal chemiluminescence system (Pierce). Purified by protein A-Sepharose (Tang ...16% SDS–PAGE gel were transferred to nitrocellulose membrane and the cells were incubated for an additional 24 h prior to harvesting for western blot analysis.

**Western blot analysis**

Protein extracts were prepared from COS-1 cells, mouse tissue (brain, testis) and human testis. Cells, frozen human testicular tissue or freshly prepared mouse tissues, were homogenized in tissue protein extraction reagent (1× complete protease inhibitor cocktail (Roche, Mannheim, Germany) and centrifuged at 10,000g for 5 min to pellet tissue debris. Supernatant was collected for either direct use as total tissue protein extract or for further purification of cytoplasmic and nuclear fractions. The cytoplasm and nuclear fractions of mouse testis and COS-1 cells were prepared using a nuclear and cytoplasmic extraction reagents kit (#78833, Pierce) in the presence of a mixture of protease inhibitors (Roche Applied Sciences) and phosphatase inhibitor mixture (Pierce Biotechnology). Protein (20 μg) separated by 8–16% SDS–PAGE gel were transferred to nitrocellulose membrane and assessed using the specific GRTH antibody (aa. 465–477) peptide of rat GRTH purified by protein A-Sepharose (Tang et al., 1999). Goat anti-rabbit IgG horseradish peroxidase (1:10 000) was used as the secondary antibody. Immunosignals were detected by the super-signal chemiluminescence method (Pierce).

**Statistical analysis**

The differences in genotypic frequencies of single nucleotide polymorphism (SNP) between the fertile and NOA patients were evaluated by χ² test from Georgetown Linguistics (http://www.georgetown.edu/faculty/ballc/webtools/web_chi.html).

**Results**

Semen analyses of the 100 control male subjects indicated that all parameters were within the normal range (Table I). The endocrine profile, semen volume and testicular size of the NOA patients of this study and of the individual patients with a missense mutation in exon 8 (see below) are shown in Table II. Testicular size was lower in NOA patients than in normal male subjects. Testicular histology revealed seminiferous tubules containing Sertoli cells with an absence of germ cells in 85% of the patients of the infertile NOA group, whereas 15% presented maturation arrest. Serum FSH levels were significantly elevated in all NOA patients (27.5 ± 11.8 mIU/ml in NOA compared with 2.0–8.3 mIU/ml in normal males), whereas serum LH levels (7.9 ± 4.9 mIU/ml in NOA compared to 0.8–5.7 mIU/ml in normal males) were above the normal range in 81 NOA subjects. Within this population, 31 patients had LH levels above 9 mIU/ml, and this included three patients with the missense mutation G725A (I25, I66 and I68). Prolactin levels (11.3 ± 9.8 ng/ml in NOA compared to 3.6–12.8 ng/ml in normal males) were elevated in 39 patients. All seven patients with missense mutation showed normal prolactin levels. Testosterone levels in all patients (4.5 ± 1.6 ng/ml in NOA compared to 2.2–10.4 ng/ml in normal males) were within the normal range.

dHPLC analysis revealed abnormal elution profiles in exon 8 and 11 of the GRTH/DDX25 gene of NOA patients from a Japanese population (Fig. 1). Other exons (exons 1–7, exons 9–10 and exon 12) showed patterns that were identical to those of the normal subjects. Seven abnormal elution profiles of exon 8 (125, 129, 137, I66, I68, I74 ans I113) were detected in 143 infertile patients and one abnormal profile (N8) was observed in 100 normal males (Fig. 1A and B). Profiles of normal males (N4–7, 9) and infertile males (I126, I64, I73 and I112) with identical patterns to normal male were used as reference (Fig. 1A–C). Abnormal profiles were detected in exon 11 of two infertile male subjects (I103 and I126), while profiles in infertile male I112 and normal males N21 and N1686 (DNA purchased from Clonetech) were used as references (Fig. 1D).

Sequence analysis showed that exon 8 variants displayed a consistent missense mutation at nt 725 from G to A resulting in an amino acid change from Arg to His at 242 aa position (Fig. 2, Table IV, GenBank Acc# AF155140). Approximately 50% of the clones (20 clones from each variant) showed the consistent nucleotide change at nt 725, whereas 50% displayed the wild-type sequence. Also, this mutation at nt 725 was detected in one of the 100 normal male subjects (N8). Both exon 11 variants (I103 and I112) showed a silent mutation at nucleotide position 1245 from C to G without change in amino acid sequence (Fig. 2 and Table IV, GenBank Acc# AF155140). For both mutations, there was no statistically significant difference in the genotype frequencies between the normal and infertile males based on the χ² test [exon 8, G725A, χ² = 3.59, (P ≥ 0.05); exon 11, C1245G, (χ² = 2.0, P ≤ 0.2)]. However, the χ² value (3.59) is only slightly below the value of 3.84 which is significant at P < 0.05.

Western blot analysis revealed molecular weights of endogenous GRTH protein species whereas only the 56 kDa species was present in the mouse testis (Fig. 3A). The 56 kDa phosphorylated species (Sheng et al., 2006) were observed in the human and mouse testis (Fig. 3A) and in COS-cells transfected with full-length wild type and mutant human cDNA construct (Fig. 3B, left). The cytoplasmic fraction of COS-1 cells transfected with the wild-type human construct contained both 56/61 kDa protein species whereas only the 56 kDa species was present in the nuclear compartment (Fig. 3A and B). The 61 kDa protein species was enhanced by cAMP treatment of cells expressing wild-type GRTH (Fig. 3B, left). In contrast, the 61 kDa phosphorylated
species was absent in COS-1 cells transfected with R to H mutant cDNA construct (Fig. 3B, right) and only the non-phosphorylated form was present in the cytoplasm. The nuclear fraction showed the characteristic non-phosphorylated form (Fig. 3B, right).

Discussion

Male infertility frequently results from genetic defects, many of which are unidentified and require further investigation. Genetic analysis should provide further information and identification of genes that are involved in impaired spermatogenesis. In this study, we have used dHPLC (reviewed in Xiao and Oefner, 2001), a fast and sensitive system, to detect DNA sequence variations including SNPs to search for mutation(s) of the GRTH/DDX25 gene that could be associated with male infertility. We could not detect any homozygous mutations and/or deletion in any of GRTH/DDX25 exons. However, we found heterozygous mutation in exons 8 (missense) and 11 (sense) from dHPLC analysis in patients from Japan with NOA. One missense mutation in exon 8 resulted in a single amino acid change from Arg to His at aa. 242.

Although the present study revealed no statistical significance in the frequency of the missense mutation of exon 8 between normal and NOA patients, we cannot exclude the possibility that this mutation may play a role in the infertility of NOA patient. When compared to only 8–15% of Y chromosome abnormalities that are known to cause human male infertility, the 5.8% mutation rate in a single GRTH gene associated with the patient population in this study could be clinically significant. A larger sample size would be

Table IV. Summary of GRTH/DDX25 gene mutations

<table>
<thead>
<tr>
<th>Location</th>
<th>Type</th>
<th>Base pair change</th>
<th>Amino acid change</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 8</td>
<td>Missense</td>
<td>724CGT→CAT</td>
<td>242R→H</td>
<td>7/121*</td>
</tr>
<tr>
<td>Exon 11</td>
<td>Silent</td>
<td>1243CTC→CTG</td>
<td>415L→L</td>
<td>2/100</td>
</tr>
</tbody>
</table>

*aTotal NOA patients = 143; Missense mutation occurred in NOA with Sertoli cell only group (121 patients); P ≥ 0.05.
ideal to further assess the GRTH gene mutation in association with the disease.

Since development of germ cells appears to be normal in GRTH/DDX25 heterozygous mice containing one normal allele (Tsai-Morris et al., 2004a,b), the functional significance of the heterozygous mutation found in this study requires further analysis. It is possible that the mutant protein resulting from the defected allele may play a dominant negative interference role with the wild-type protein, and consequently disrupt spermatogenesis. Such a dominant negative effect has been proposed in the azoospermia patients heterozygous for a mutation in SYCP3 gene (Miyamoto et al., 2003). A future study examining the function of the human wild type and mutated GRTH protein in GRTH null mice model will be essential to determine whether the mutated protein at aa. 242 from Arg to His has a dominant negative effect on the recovery of spermatogenesis by wild-type GRTH protein.

All patients with the G725A mutation revealed a similar endocrine profile (Table II) (elevated serum FSH and normal prolactin and testosterone levels). Only one of these seven patients presented a high serum LH level (22.1 mIU/ml compared to the normal range of 0.8–5.7 mIU/ml). Histological examination revealed only Sertoli cells in the seminiferous tubules of all patients with missense mutation of GRTH (5.8% of the NOA infertile patients with Sertoli cell only). However, it is not unusual that a few spermatozoa could be present in patients with Sertoli cells-only phenotype. The failure to observe those germ cells is limited by the section of the testis under histological examination. Some Sertoli cell-only phenotype cases display focal spermatogenesis and the observed pathology is usually much more heterogeneous in azoospermic men. It is possible that those patients with Sertoli cells-only phenotype and NOA could still have offspring via assisted reproductive technology which includes testicular sperm extraction followed by intracytoplasmic sperm injection (ICSI) of a single sperm into the cytoplasm of the mature oocyte (Iammarrone et al., 2003).

Nevertheless, our study has provided evidence for a GRTH/DDX25 gene mutation at G725A that may be involved in male infertility of some patients with idiopathic NOA. It remains to be determined whether the presence of this or other mutations in NOA patients with meiotic and post-meiotic abnormalities is related to our previously identified germ cells arrest in the null mice (Tsai-Morris et al., 2004a,b).

There have been 97 SNPs reported for the GRTH/DDX25 gene using the NCBI SNP program (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=Display&dopt=gene_snp&from_uid=29118). Only three SNPs are located within exons, including exon 4 (rs4329703), exon 5 (rs561704) and exon 10 (rs683155). Missense mutation is associated with exon 4 (TCTCCC to GCC, Pro89Ala) and exon 5 (A244TG to AGG, Met115Arg). In exon 10, a single nucleotide mutation did not change the amino acid sequence of the GRTH protein (TGT1194 to TGC, Cys398Cys). A recent report on a Chinese population of infertile patients with idiopathic azoospermia and severe oligozoosperma identified four SNPs of the GRTH gene that were suggested to be associated with impairment of spermatogenesis (Zhoucun et al., 2006). It is noted that two of these mutations were within introns and two others, located in exons 10 and 11, were silent with no change in the amino acid sequence of the protein. However, it was proposed that the polymorphism of c.852C of exon 10, located in one of the binding motifs of splicing factor 2 (SF2/ASF) (Sanford et al., 2005), could affect pre-RNA splicing of the GRTH gene and ultimately its expression.

In the present study, we have shown that mutation of R242H to H of the GRTH gene abolished the phosphorylation of the expressed protein and only the unphosphorylated form (56 kDa) was observed when the mutated gene was expressed in COS-1 cells. The functional significance of this mutation in exon-8 could be implied by absence of the 61 kDa cytoplasmic phosphorylated species in transfected cells. This species is present in germ cells of rodents only at cytoplasmic sites where it is widely distributed, and associates with polyribosomes and may regulate the translation of specific germ cell transcripts during the progress of spermatogenesis [i.e. histone 4, and high-mobility group protein (HMG2)] (Sheng et al., 2006). We demonstrated the effect of the mutation in COS-1 cells expressing the human construct utilizing total cells and most importantly in isolated cytoplasm where the GRTH phosphorylated species is normally present. Expression of the wild-type human constructs demonstrated an increase in phosphorylated species (61 kDa) by cAMP from very low basal levels and this was achieved with optimal resolution to clearly discern the phosphorylated (61 kDa) and non-phosphorylated (56 kDa) species. In contrast, the phosphorylated band was not induced by cAMP activation of cells expressing the mutant protein. The 56 kDa non-phosphorylated species was the only form expressed in the cytoplasm and nucleus of cells transfected with the mutant construct. It is also the only form found in the nucleus of rodent testis (Sheng et al., 2006) and COS-1 cells transfected with the wild-type human construct (this study). The 56 kDa functions as a component of mRNP and participates in the nuclear transport of specific messages of relevance to spermatogenesis (Sheng et al., 2006). Phosphorylation of GRTH, in addition to facilitating the initiation of translation of its target genes, may induce conformational changes to recruit protein(s) to gain access of mRNAs to relevant cytoplasmic sites of spermatocytes and round spermatids (i.e. chromatoid bodies) for storage and/or degradation (Dufau and Tsai-Morris, 2007). Thus the lack of the 61 kDa may have important functional consequences in germ cell development. Five putative pKA phosphorylation sites (aa. 39, 212, 239, 355 and 408) were found in the GRTH based on a phosphorylation motif (R/KXXT) scanning analysis. One phosphorylation site (aa. 239) is located closely adjacent to the mutation at

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Figure 3: Expression of GRTH protein

(A) Western analysis of human testis extracts was performed to assess endogenous human testicular GRTH protein expression. Total mouse tissue (T), total tissue extract) and cytoplasmic (C) and nuclear (N) extracts were used as positive controls for the human GRTH protein (T) and total mouse brain extracts (T) were used as the negative control. (B) GRTH protein expression in COS-1 cells transfected with full-length wild type (WT) or with missense mutation R242H to H of the GRTH-SPORT-CMV (pGRTH-Sport) construct, in the presence (+) or absence (−) of cAMP. Nuclear HDAC1 and mitochondrial Smac proteins were used as the purity index of cell fractionation for nucleus and cytoplasm, respectively.

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GRTH/DDX25 mutations in non-obstructive azoospermia
aa. 242. Mutation of R to H might result in the conformational change of the structure and consequently abolish the phosphorylation status through this adjacent putative phosphorylation site. Furthermore, based on the crystal structure of the N-terminal RecA-like domain of the DEAD-box helicase Dugesia japonica vasa-like gene B protein (Kurimoto et al., 2005), the relevant basic amino acid R242 in our study (which is a K in the DEAD-box helicase of several species of the Vasa orthologues and PL10 subfamily) resides in a conserved hydrophobic pocket on the non-ATPase side. Since this region has been proposed to form a RNA-binding domain site of DEAD-box helicases, and/or to engage in protein–protein interactions, the decrease in basicity resulting from the R242H mutation may impact on the biochemical function(s) of GRTH/DDX25 (Tang et al., 1999; Sheng et al., 2006).

Modulation of RNA structure by members of the DEAD-box family of RNA helicases is a crucial step in many fundamental biological processes (reviewed in Schmid and Linder, 1992; Luking et al., 1998; de la Cruz et al., 1999; Linder and Stutz, 2001; Silverman et al., 2003; Rocak and Linder, 2004; Abdelhaleem, 2005). Only a few of these genes have regulatory roles in male germ cell development, including the mouse homolog of Drosophila Vasa gene (Mvh) (Tanaka et al., 2000; Noce et al., 2001) and GRTH/DDX25 (Tsai-Morris et al., 2004a,b). Here, we provide the first evidence for the presence of a missense mutation of GRTH/DDX25 gene. Its association with loss of the phosphorylated cytoplasmic species of the expressed mutant protein indicates the relevance of the Arg242 residue for generation of these species in the human.

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References


