Identifying mRNAs Bound by Human RBMY Protein in the Testis

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Abstract. Rbmy gene encodes a germ-cell specific nuclear RNA-binding protein and is involved in spermatogenesis. To further investigate the specific events of spermatogenesis in which Rbmy plays a role, the target mRNAs of human RBMY protein were isolated and identified. Through the isolating specific nucleic acids associated with proteins (SNAAP) technique, we isolated twenty potential target genes of human RBMY protein from the human testis in the present study. Some of these target genes play important roles during spermatogenesis and have alternative transcripts in the testis. In this study, we focused on the human- related (never in mitosis gene a) kinase 10 (Nek10) gene, which belongs to the Nek protein kinase subfamily. Nek10 has two transcripts, and the results of RT-PCR and Electrophoretic Mobility Shift Assays (EMSA) show that hRBMY protein can only bind to transcript variant 2 of Nek10 and that hRBMY may take part in alternative splicing of Nek10. Isolation and identification of target genes of hRBMY will be helpful to further investigate the biological function of RBMY in spermatogenesis.

Key words: Nek10, Rbmy gene, RNA-binding protein, SNAAP technique, Spermatogenesis

Spermatogenesis is a complex cell differentiation process that requires highly regulated expression of lots of genes [1–3]. Many genes are mainly or exclusively expressed in the testis, and in most situations, further research has shown that they are only expressed in the germline cells [4]. Microdeletions at a specific region called the AZF in the long arm of Y chromosome have been described in significant proportions of oligo and azoospermic patients, suggesting that some genes located at these regions play an essential role during spermatogenesis [5]. Of all the genes located at the AZF region, Rbmy was the first of these candidate genes to be identified [6]. There are many Rbm genes distributed throughout the Y chromosome, but only the genes in deletion interval AZFb give rise to detectable levels of protein [7]. According to the complete sequence of the human Y chromosome, it is now known that there are six Rbmy copies mapped in the AZFb region, but only one copy is actively transcribed [8]. Human and mouse Rbmy genes have a homologue on the human X chromosome (Rbmx) that maintains a widespread function, while Rbmy evolves a male-specific function in spermatogenesis [9]. There is another retrogene called hnRNPG-T that belongs to the same family as Rbmy and Rbmx, which is also specifically expressed in the testis. Previous study has shown that haploinsufficiency of hnRNPG-T results in abnormal sperm production in the mouse. Genetic defects resulting in reduced levels of hnRNPG-T may be a cause of male infertility in humans [10]. Although Rbmy is evolutionarily conserved in the human, mouse and marsupial [11–13], their expression patterns are not completely identical. The expression of mouse Rbmy is limited to spermatogonia and early spermatocytes, while human Rbmy gene is expressed in all of the transcriptionally active stages of germ cell development from spermatogonia through spermatocytes to round spermatids [14].

Perhaps the most distinctive character of the RBMY protein sequence is that there is an RNA recognition motif (RRM) in the N-terminal of RBMY protein that mediates an interaction with RNA, so Rbmy probably functions by binding RNA [6]. There is a repeated sequence motif that is enriched in the amino acids serine, arginine, glycine and tyrosine (SRGY) of human RBMY protein, and this motif corresponds to a protein interaction domain [6]. Previous study has shown that RBMY protein interacts with SAM68 and the closely related T-STAR protein (signal transduction and RNA binding), which are considered to be molecular transducers between cell signal and splicing regulation [15]. Furthermore, RBMY can also interact with Srp20 or Tra2β, which belongs to the family of SR-rich pre-mRNA splicing regulators [16, 17]. Alternative splicing is very prevalent in the testis. These suggest that Rbmy may affect alternative splicing during spermatogenesis.

To understand in depth the biological function of hRBMY during spermatogenesis, we intend to isolate the target mRNAs bound by hRBMY protein through the SNAAP technique. This assay adopts copurification of unknown mRNAs bound by an RNA-binding protein and subsequently isolates the mRNAs using differential display technology [18]. We herein report the testis-specific substrate mRNAs bound by hRBMY.

Materials and Methods

Preparation of total testis extracts

Informed consent was received from the participants, and the
electrodes of West China Hospital, Sichuan University, granted research approval prior to sample collection. Testicular tissue samples were obtained from adult males who died due to accidents (Body Donor Center, West China Hospital, Sichuan University). Testicular tissue was washed in phosphate-buffered saline (PBS) and placed into lysis buffer (100 mM NaCl, 10 mM MgCl2, 30 mM Tris–HCl, 1 mM dithiothreitol (DTT), protease inhibitor cocktail, 40 U/ml RNase OUT and 0.5% Triton X-100) [19]. The tissue was mashed with a razor blade, insoluble matter was separated by centrifugation for 10 min at 12,000 g and 4 C and the supernatant was collected.

**Generation of glutathione S-transferase fusion proteins**

The pGEX-hRBMY and pGEX-hNRBD plasmids, respectively, encoding the glutathione S-transferase (GST)-hRBMY and (GST)-hNRBD fusion proteins were constructed by placing the hRBMY and hNRBD coding regions (without RNA-binding domain, amino acids 83–380) into the pGEX-5X-3 vector. The coding region was reverse transcribed and amplified from the RNA of male testes with the following primers: the pGEX-hRBMY primers were, 5’ GGATCCCATGGTGAAGAATGCTTCCCTACGGTC 3’ (BamHI site in bold) and 5’ TGGGCGCCCTTCAATGCTTGCCTTAAATCTG 3’ (NotI site in bold), and the pGEX-hNRBD primers were, 5’ GGATCCCATGGTGAAGAATGCTTCCCTACGGTC 3’ (BamHI site in bold) and 5’ TGGGCGCCCTTCAATGCTTGCCTTAAATCTG 3’ (NotI site in bold). The GST-hRBMY and GST-hNRBD expression plasmids were confirmed by analysis of restriction digestion and DNA sequencing. GST, GST-hNRBD and GST-hRBMY were expressed in Escherichia coli BL21 cells, and extracts were prepared.

**SNAAP screen**

Jiao et al. have described the SNAAP technique [20]. Briefly, after GST, GST-hNRBD and GST-hRBMY proteins were bound to glutathione beads and unbound proteins were separated by washing, the washed beads containing the fusion proteins were incubated with 300 mg of total testis extracts precleared with 20 µl of glutathione sepharose beads (GE Healthcare, Piscataway, NJ, USA). After binding at 4 C for 1 h, the beads were washed by RNA-binding buffer RBB/0.25% Triton X-100, in succession through a 10-min wash in RBB/0.25% Triton X-100 containing 1 mg/ml heparin. Thereafter, the beads were washed four times in RBB/0.25% Triton X-100, and bound RNA was then collected by a standard procedure. Co-purifying RNAs were isolated by the differential display technique.

**RNA isolation and RT-PCR analysis**

In order to detect the alternative splices of Smad5, Nek10, Tex14, Nsf2, Tex11, C6orf182, Uspl and Stk31 in the male testis, total RNA was extracted from the adult male testis using Trizol reagent (Invitrogen, Carlsbad, CA, USA), cDNA was synthesized with a PrimeScript RT Reagent Kit (Takara, Japan), RT-PCR was carried out with specific primers. The RNAs acquired in the SNAAP assay bound by GST, GST-hNRBD and GST-hRBMY proteins were subjected to RT-PCR with specific gene primers. All primers were designed to span at least 1 intron to guard against genomic contamination.

**Electrophoretic mobility shift assays**

The electrophoretic mobility shift assay was performed on the basis of the King PH protocol [21]. RNA synthesis and labeling were carried out using an SP6/T7 Transcription and DIG RNA Labeling Kit (Roche, Germany). The binding buffer for the protein and RNA probe was buffer (50 mM Tris–HCl, pH 7.0, 150 mM NaCl, 67 mg/ml yeast tRNA, 0.25 mg/ml bovine serum albumin and 1.5 mg/ml heparin)+2% 2-mercaptoethanol. Potential protein–RNA complexes were formed at 37 C for 10 min. Subsequently, 2.5 µl of 6× loading buffer was appended, and the samples were loaded onto a 1% agarose gel in 1× TBE buffer. The gel was subjected to electrophoresis on ice until separation was attained. The RNA in the gel was electroblotted to a positively charged nylon membrane and subsequently cross-linked to the membrane. The membrane was washed with a detergent solution followed by a block solution. Detection of the RNAs on the membrane was probed with anti-digoxigenin-AP conjugate to bind digoxigenin-labeled RNA. The membrane was washed again, and a substrate (CDP-Star) was added to initiate the chemiluminescent reaction. The membrane was wrapped with a plastic wrap and then exposed to an X-ray film.

**Results**

**Isolation of target genes of hRBMY protein using the SNAAP technique**

Because the hRBMY protein contains an N-terminal RNA recognition motif (RRM), hRBMY protein likely functions by binding RNA, and therefore, we sought to isolate mRNAs bound by hRBMY protein. Using the SNAAP technique, we isolated 20 target genes of hRBMY protein (Table 1). Some target genes have alternative splicing transcripts. As far as the target genes of RNA-binding proteins are concerned, some RNA-binding proteins may interact with each other, so they may have identical target genes. In our study, we did not use other RNA-binding proteins as a control. Instead, the GST and GST-hNRBD proteins acted as control proteins and gave no enrichment in any of these target mRNAs. Among these target genes, our previous study showed that Spa17, Tcf5 and Tex15 were also the target genes of mouse RBMY protein [22]. These results suggest that mouse and human RBMY protein may have homologous target genes. Some target genes are exclusively expressed in the testis and play a role during spermatogenesis. Spa17, Tex14, Tex11, Tcf5, Tex15, Pgk2, Fih17, Taf7l, Nsf2 and Stk31 are reported to express specifically in the testis. Tex15 is required for DNA double-strand break repair and chromosomal synapsis during male meiosis [23]. Tex11 regulates the level of meiotic crossovers and thus influences homologous chromosome segregation during spermatogenesis [24]. Tcf5 is a basic helix-loop-helix transcription factor and interacts with the Calmegin gene promoter in spermatogenesis [25, 26]. TEX14 localizes to germ cell intercellular bridges and is vital for intercellular bridges in male mice [27].

Using a bioinformatic approach, the results showed that Smad5,
Nek10, Tex14, Tex11, Usp1 and Stk31 have two or three alternatively spliced mRNAs. Through RT-PCR using specific primers, we detected that Nek10, Tex14, Nxf2, Tex11 and Usp1 have two alternative spliceoforms and that Smad5, HN1 and Stk31 have three alternative spliceoforms in the human testis (Fig. 1).

Target genes of hRBMY were further confirmed by specific gene RT-PCR analysis

Although some mRNA substrates of hRBMY protein were iso-
lated through the SNAAP technique, the SNAAP technique has its
own shortcoming. So, we further demonstrated the result through
specific gene RT-PCR analysis, and the results showed that
Laptm4a and Thbs3 could not be bound by hRBMY protein.
Because some genes have alternative spliceoforms in the human
testis, the results show that partial transcripts of some target genes
cannot be bound by hRBMY protein. Human RBMY protein could
not bind to Nek10-1, Tex14-1, Tex11-2, HN1-1, HN1-2, Stk31-3
and Smad5-2, and hRbmy may affect alternative splicing of these

Table 1. A summary of mRNA substrates bound by mRBMY protein

<table>
<thead>
<tr>
<th>Name</th>
<th>Accession number</th>
<th>Tissue</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smad5 transcript variant 1 (Smad5-1)</td>
<td>NM_005903</td>
<td>Widespread</td>
<td>Involved in the regulation of spermatogonia differentiation</td>
</tr>
<tr>
<td>Smad5 transcript variant 2 (Smad5-2)</td>
<td>NM_001001419</td>
<td>Widespread</td>
<td>Involved in the regulation of spermatogonia differentiation</td>
</tr>
<tr>
<td>Smad5 transcript variant 3 (Smad5-3)</td>
<td>NM_001001420</td>
<td>Widespread</td>
<td>Involved in the regulation of spermatogonia differentiation</td>
</tr>
<tr>
<td>Nek10 transcript variant 1 (Nek10-1)</td>
<td>NM_001031741</td>
<td>Widespread; highly expressed in the testis</td>
<td>Protein serine/threonine and tyrosine kinase activity</td>
</tr>
<tr>
<td>Nek10 transcript variant 2 (Nek10-2)</td>
<td>NM_152534</td>
<td>Widespread; highly expressed in the testis</td>
<td>Protein serine/threonine and tyrosine kinase activity</td>
</tr>
<tr>
<td>Ehnt1</td>
<td>NM_024757</td>
<td>Widespread</td>
<td>Histone-lysine N-methyltransferase activity, plays a role in G0/G1 transition in cell cycle</td>
</tr>
<tr>
<td>Cinp</td>
<td>NM_032630</td>
<td>Widespread</td>
<td>Cyclin-dependent kinase 2-interacting activity, kinase activity</td>
</tr>
<tr>
<td>Thbs3</td>
<td>NM_007112</td>
<td>Widespread; highly expressed in the testis</td>
<td>Mediates cell-to-cell and cell-to-matrix interactions</td>
</tr>
<tr>
<td>Rpl39</td>
<td>NM_001000</td>
<td>Widespread</td>
<td>Belongs to the S39E family of ribosomal proteins</td>
</tr>
<tr>
<td>Tex11 transcript variant 1 (Tex11-1)</td>
<td>NM_001003811</td>
<td>Testis</td>
<td>Not known</td>
</tr>
<tr>
<td>Tex11 transcript variant 2 (Tex11-2)</td>
<td>NM_031276</td>
<td>Testis</td>
<td>Not known</td>
</tr>
<tr>
<td>Spa17</td>
<td>NM_017425</td>
<td>Testis</td>
<td>Sperm surface zona pellucida binding protein. Helps to bind spermatozoa to the zona pellucida with high affinity</td>
</tr>
<tr>
<td>Tcl15</td>
<td>NM_024885</td>
<td>Testis</td>
<td>Functions in a crucial role in spermatogenesis as a transcription factor by regulating cell proliferation or differentiation of cells through binding to a specific DNA sequence like other bHLH molecules.</td>
</tr>
<tr>
<td>Pgk2</td>
<td>NM_138733</td>
<td>Testis</td>
<td>Testis specific, phosphoglycerate kinase activity</td>
</tr>
<tr>
<td>Stk31 transcript variant 1 (Stk31-1)</td>
<td>NM_031414</td>
<td>Testis</td>
<td>Protein serine/threonine kinase activity</td>
</tr>
<tr>
<td>Stk31 transcript variant 2 (Stk31-2)</td>
<td>NM_032944</td>
<td>Testis</td>
<td>Protein serine/threonine kinase activity</td>
</tr>
<tr>
<td>Stk31 transcript variant 3 (Stk31-3)</td>
<td>NM_001122833</td>
<td>Testis</td>
<td>Protein serine/threonine kinase activity</td>
</tr>
<tr>
<td>Tex15</td>
<td>NM_031271</td>
<td>Testis</td>
<td>Is essential for DNA double-strand break repair and chromosomal synopsis during male meiosis</td>
</tr>
<tr>
<td>Usp1 transcript variant 1 (Usp1-1)</td>
<td>NM_003368</td>
<td>Widespread; highly expressed in the testis</td>
<td>Negative regulator of DNA damage repair</td>
</tr>
<tr>
<td>Usp1 transcript variant 2 (Usp1-2)</td>
<td>NM_01017415</td>
<td>Widespread; highly expressed in the testis</td>
<td>Negative regulator of DNA damage repair</td>
</tr>
<tr>
<td>Tex14 transcript variant 1 (Tex14-1)</td>
<td>NM_198393</td>
<td>Testis</td>
<td>Essential for intercellular bridges and fertility</td>
</tr>
<tr>
<td>Tex14 transcript variant 2 (Tex14-2)</td>
<td>NM_031272</td>
<td>Testis</td>
<td>Essential for intercellular bridges and fertility</td>
</tr>
<tr>
<td>Fth17</td>
<td>NM_031894</td>
<td>Testis</td>
<td>Ferritin, functioning in iron metabolism</td>
</tr>
<tr>
<td>Tkc11</td>
<td>NM_012253</td>
<td>Testis</td>
<td>Not known</td>
</tr>
<tr>
<td>Laptm4a</td>
<td>NM_014713</td>
<td>Widespread</td>
<td>Transporter protein; regulates cellular multidrug resistance</td>
</tr>
<tr>
<td>HN1 transcript variant 1 (HN1-1)</td>
<td>NM_016185</td>
<td>Widespread</td>
<td>Not known</td>
</tr>
<tr>
<td>HN1 transcript variant 2 (HN1-2)</td>
<td>NM_001002032</td>
<td>Widespread</td>
<td>Not known</td>
</tr>
<tr>
<td>HN1 transcript variant 3 (HN1-3)</td>
<td>NM_001002033</td>
<td>Widespread</td>
<td>Not known</td>
</tr>
<tr>
<td>Nxf2 transcript variant 1 (Nxf2-1)</td>
<td>NM_017809</td>
<td>Testis</td>
<td>Nucleocytoplasmic mRNA transporter</td>
</tr>
<tr>
<td>Nxf2 transcript variant 2 (Nxf2-2)</td>
<td>NM_022053</td>
<td>Testis</td>
<td>Nucleocytoplasmic mRNA transporter</td>
</tr>
</tbody>
</table>

Nek10, Tex14, Tex11, Usp1 and Stk31 have two or three alternatively spliced mRNAs. Through RT-PCR using specific primers, we detected that Nek10, Tex14, Nxf2, Tex11 and Usp1 have two alternative spliceoforms and that Smad5, HN1 and Stk31 have three alternative spliceoforms in the human testis (Fig. 1).

Target genes of hRBMY were further confirmed by specific gene RT-PCR analysis

Although some mRNA substrates of hRBMY protein were iso-
Human RBMY protein can bind to Nek10 transcript variant 2 mRNA

Because Nek10 has two transcripts, the results of the SNAAP technique and specific gene RT-PCR analysis showed that Nek10 transcript variant 2 mRNA may be the target mRNA of hRBMY protein. These results could not explain whether hRBMY protein can directly bind to Nek10 transcript variant 2 mRNA. The difference between the two transcripts is in the 1405-2025 nt of the Nek10 transcript variant 1 mRNA and in the 1-485 nt of Nek10 transcript variant 2 mRNA. In this study, we labeled the two mRNA fragments with DIG. The results of EMSA showed that hRBMY protein can directly bind to 1-485 nt of Nek10 transcript variant 2 mRNA and cannot bind to 1405-2025 nt of Nek10 transcript variant 1 mRNA (Fig. 3). In order to further confirm the results, competitive binding assays were performed (Fig. 4). The intensities of the retarded bands representing the protein–RNA complex reduced on the addition of increasing amounts of unlabeled RNA.

Identification of the binding region of the hRBMY on the Nek10 transcript variant 2 mRNA

The approaches employed above demonstrate that hRBMY protein can directly bind to 1-485 nt of Nek10 transcript variant 2 mRNA but do not provide detailed information about the binding region. The fragment was divided into four small segments, and the results of EMSA showed that the hRBMY–binding sequence is in the 1-118 nt Nek10 transcript variant 2 mRNA (Fig. 5).

Discussion

Alternative splicing enables gene to encode multiple protein isoforms that often play different biological roles [28–30]. Because germ cell expansion and differentiation need many cellular changes and regulatory steps, alternative splicing is especially important in the testis [28, 31]. A previous study has been conducted on 52 different tissues and more than 10,000 genes; the results showed that the highest rate of alternative splicing events in the testis is found between the human and mouse [32]. Protein-protein interactions and subnuclear location experiments suggest that Rbmy may take part in the alternative splicing in spermatogenesis, but its precise function is still not clear. Our results show that some target genes of hRbmy protein have two or three alternatively splicings, and this suggests that Rbmy may be involved in alternative splicing of these genes.

In this study, we identified 18 target genes through the SNAAP technique and further RT-PCR analysis with gene-specific primers. Among these target genes, there are six genes that exist in alternative spliceoforms (Fig. 2). Nek10, Tex14, Nxf2, Tex11 and Usp1 have two alternative spliceoforms, while Smad5, HN1 and Stk31 have three alternative spliceoforms.
ISOSATION AND IDENTIFICATION OF TARGET GENES OF HUMAN RbMY PROTEIN

We focused on Nek10, which belongs to the Nek protein kinase subfamily. The Nek protein kinase subfamily is named after the NIMA protein kinase of Aspergillus nidulans. NIMA has long been known to be involved in the control of mitotic entry and progression, inasmuch as niaA mutants arrest in G2 with high Cdc2 activity and generate aberrant spindle and nuclear envelope morphologies when allowed to enter mitosis by the additional mutation of an APC subunit (bimE7) [33]. So, Nek10 may take part in regulation of the cell cycle during spermatogenesis.

Nek10 has two transcripts, and our results show that hRbMY protein only can bind to Nek10 transcript variant 2 mRNA and that the binding region is in the 1-118 nt of 5'UTR of Nek10 transcript variant 2.

The major difference between the two transcripts is 5'UTR and 3'UTR. Previous study has shown that hRbMY is located in sites of active gene expression in the cell and affects splicing in trans by modifying the activity of SR and SR-like proteins or by preventing their access to their target mRNAs [34]. These findings suggest that hRbMY may affect the splicing of 5'UTR and 3'UTR of Nek10, but the mechanism needs further demonstration.

However, according to existing reports, six of the binding genes have two or three transcripts, and the other 12 genes have only one transcript. Besides alternative splicing, some studies have suggested that RbMty might be involved in storage, metabolic stability or transportation of specific mRNA from the nucleus during spermatogenesis [35]. hRbMty may transport their mRNAs or affect their expression. For example, Spa17 has only one transcript. But Spa17 is only expressed during some phases of spermatogenesis; it is expressed in spermatocytes and abundantly in spermatids, but...
not in spermatogonia [36]. Human Rbmy may be involved in this regulation process.

Previous studies have found that RNA stem-loops capped by a C^\text{UC/CA} pentaloop are high-affinity binding targets for hRBMY [37]. In our study, we isolated 31 potential target RNA sequences of hRBMY protein using the SNAAP technique. Among these sequences, Thbs3, RpL39, laptm4a and HNI transcript variant 3 do not contain a C^\text{UC/CA} sequence. Through further specific gene RT-PCR analysis, Laptm4a and Thbs3 could not be bound by hRBMY protein. Other target sequences contain the C^\text{UC/CA} sequence, but the sequence does not exist in the stem-loop motifs. This reflects the different experiment approaches taken and the multitude and complexity of in vivo interactions that perhaps result in different results. So far, there are little data available concerning the RNA-recognition properties of RBMY and related hnRNP proteins. Besides the fact that hRBMY is highly conserved between the mouse and human and contains an RRM with 74% similarity to mRBMY, it may have homologous RNA targets. This study shows that mRBMY and hRBMY have some homologous target genes. Further study is required to investigate whether these RNA targets are evolutionarily conserved in mammals and whether they interact with each other.

Acknowledgments

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References