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Expression Analysis of RNA-Binding Motif Gene on Y Chromosome (RBMY) Protein Isoforms in Testis Tissue and a Testicular Germ Cell Cancer-Derived Cell Line (NT2)

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ABSTRACT

Background: RNA-binding motif gene on Y chromosome (RBMY), a germ cell-specific nuclear protein, is known as a key factor in spermatogenesis and disorders associated with this protein have been recognized to be related to male infertility. Although it was suggested that this protein could have different functions during germ cell development, no studies have been conducted to uncover the mechanism of this potential function yet. Here, we analyzed the expression pattern of RBMY protein isoforms in testis compared to NT2, a testicular germ cell cancer-derived cell line, to test probability of differential expression of RBMY protein isoforms at different spermatogenesis stages. **Methods:** Full length and a segment of RBMY gene were cloned and expressed in *E. coli*. Anti-human RBMY antibody was produced in rabbit using the recombinant proteins as antigen. Western-blot and immunofluorescence were conducted for detection and comparison of RBMY protein isoforms. **Results:** Selected segment of RBMY protein resulted in producing a mono-specific antibody. As results shows, only the longest isoform of RBMY was expressed at protein level in NT2 cell line, while three isoforms of this protein were detected in the whole testis lysate. **Conclusion:** The results imply that different alternative splicing may happen in testis cells and probably difference of RBMY function during spermatogenesis is due to the differential expression of RBMY protein isoforms. These results and further experiments on RBMY isoforms can help to obtain a better understanding of the function of this protein, which may increase our knowledge about spermatogenesis and causes of male infertility. *Iran. Biomed. J. 17(2): 54-61, 2013*

Keywords: Protein isoforms, Spermatogenesis, Male infertility

INTRODUCTION

RNA-binding motif (RBMY), previously known as RNA recognition motif (YRRM) [1], encodes a germ cell-specific nuclear protein, which is an important factor for spermatogenesis [2-4]. In addition, it is a kind of heterogeneous nuclear ribonucleoproteins, which are responsible for regulation of alternative splicing [5, 6], and it has a conserved sequence in male mammals [1, 7, 8]. Another function that has recently been proposed is its role in spermatozoa motility [9]. Deletion of azoospermia factor b region and also the RBMY microdeletion has been associated with germ cell arrest in meiotic division I, thus resulting in azoospermia or severe oligospermia in patients [1, 10-12]. RBMY was evolved from a ubiquitously transcribed X-Y identical gene [13]. Nevertheless, regulation of different sets of pre-mRNA observed in RNA-binding motif protein, X-linked (RBMX) compared to RBMY may speak about the contribution of RBMY to molecular sex differences [5]. The recognition and splicing events of pre-mRNA are done by interactions with other proteins, including SFRS3/SRP20 [14], KHDRBS1/SAM68, KHDRBS3/ T-STAR [15], TRA2B/SFRS10 [16-18] and splicing factor 9G8 [16].

There are at least 30 copies of RBMY gene on Y chromosome, some of which are pseudo genes [19, 20] and are distributed in both short and long arms [1, 2, 19]. However, only 6 loci are transcribed and 2 of them

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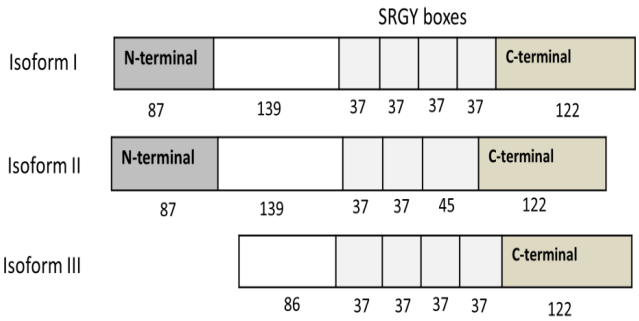

Fig. 1. Schematic representation of the RBMY isoforms structures. N-terminal domain (87 aa) is known as RNA recognition motif, which does not exist in isoform 3. In addition, the blank region is truncated in isoform3. Next domain comprises four repeated boxes (serine-arginine-glycine-tyrosine); in isoform 2, the third box is truncated. The C-terminal domain is similar in all isoforms.

are translated. As Figure 1 shows, three isoforms of RBMY out of its 12 exons have been detected that results in 496-, 459- and 356-amino acid proteins [19, 21].

While RBMX was shown to be expressed ubiquitously [5, 22], RBMY is expressed exclusively in testis [18]. However, the expression of this protein in hepatoblastoma and hepatocellular carcinoma has also been reported [23]. It was shown that RBMY is activated in hepatocellular carcinoma and has oncogenic effects [23, 24]. Knocked down RBMY HepG2 cells also showed a decrease in transformation and anti-apoptotic efficiency, implying that one of its potential role could be the regulation of androgen receptor activity in liver cancer and contribution to male predominance in this type of cancer [24].

The difference of RBMY structure compared to its homolog, RBMX, has been indicated to lead to special characteristics and novel functions of this protein, and it recognizes different sets of RNA during splicing process [5, 21]. The structural difference is mainly due to the serine-arginine-glycine-tyrosine RNA binding motif. This domain exists as four tandem peptide repeats in RBMY but only one in RBMX. These differences result in more complexity of this protein [1, 19, 22]. The differences of RBMY isoforms are related to this motif as well as the N-terminal domain (Fig. 1). Considering the fact that most of the protein interactions in RBMY are mediated via this domain [18], accordingly, unique functions of RBMY isoforms are expected.

Elliot and colleagues [21, 25] analyzed the RMBY protein isoforms by using Western-blot technique in whole testis lysate. Furthermore, they demonstrated the RBMY expression in different cells in this tissue. According to their results, RBMY protein is expressed between two stages, spermatogonial and round spermatid, in testis. However, they did not study the isoforms in different cells of testis tissue.

There are a number of studies investigating the function of RBMY and its domains in model cell lines [5, 18, 24] and transgenic mice [24]. To the best of our knowledge, however, there is no research analyzing the RBMY isoforms in cell specific expression context. This issue is important since there are different cell types in testis on one hand, and also different functions of RBMY during spermatogenesis have been proposed on the other hand [9, 21]. Therefore, expression pattern of RBMY isoforms may differ among them and the isoforms may have different functions in germ cell developmental stages.

In this study, we analyzed the expression of RBMY protein isoforms in both testis and NT2, which is a germ cell cancer-derived cell line, to examine whether the alternative splicing of RBMY and, as a result, its protein isoforms are different during spermatogenesis.

MATERIALS AND METHODS

Sample preparation. Testis tissue obtained from testis biopsy was frozen in liquid nitrogen and crashed with a mortar and pestle. The obtained powder was used for RNA and protein extraction. For preparation of cell lines (breast cancer, LNCap and DU145), the cell lines were grown in RPMI 1640 media containing 10% FBS in 125 ml tissue culture flasks. NT2 cell line was grown in a feeder free media containing 10% FBS. Cells were harvested when the desired confluency was observed.

Expression vector construction. Standard cloning techniques were used to construct the recombinant plasmid [26]. First, the full-length gene (RBMY) and also a segment ($RBMY_{pep}$) corresponding to the amino acids 220-320 were PCR amplified from cDNA of a normal testis tissue (Table 1). Following this, PCR products were digested with restriction enzymes and subsequently cloned into the same restriction sites of multiple cloning sites from pET-28a. Target gene expression was under direct control of T7 promoter and terminator (Fig. 2). Finally, for achieving a high level of expression, the expression constructs (RBMY and $RBMY_{pep}$) were transformed to *E. coli* Bl21 (DE3).

Recombinant protein expression. After sequence confirmation, validated vectors were transformed to *E. coli* BL21 (DE3) strain. The recombinant *E. coli* cells were cultured in 10 ml of Luria Bertani medium containing 80 µg/ml kanamycin at 37°C and shaked at 180 rpm overnight. The next day, the Luria Bertani medium containing 50 µg/ml kanamycin was maintained at 37°C until cells reached the log phase

Table 1. Primer sequence used for cloning entire RBMY and a segment of this protein

Gene name		Sequence	Restriction enzyme
Entire RBMY	sense	*ATTAGGATCC*ATGGTAGAAGCAGATCATC	*Bam*HI
	anti-sense	*ATTAGAATTC*CTTTTATATCTGCTCGAGTC	*Eco*RI
Segment RBMY	sense	*ATTAGGATCC* ATGGGAAATCATCCAAGTTG	*Bam*HI
	anti-sense	*ATTAAAGCTT*TCAATAACTATATCCTCTAGAGG	*Hind*III

Restriction sequences have been shown in underline.

(OD_{600} ~0.6). The expression of recombinant proteins was induced by 1 mM isopropyl-β-thiogalacto-pyranoside for 5 h. Recombinant proteins were extracted by sonication and lysed by a urea buffer (pH 8.8; 8 M urea and 0.1 M sodium phosphate) and purified by 6×His-Ni-NTA chromatography. Obtained recombinant proteins were desalted and concentrated using Amicon columns (Millipore, USA).

Recombinant protein confirmation and database search. The purified recombinant proteins were run on SDS- PAGE, stained with Coomassie brilliant blue, and then the corresponding protein bands were cut and analyzed using a Bruker Ultraflex III MALDI TOF/TOF mass spectrometer as described previously [27]. In order to perform the spectral processing and generate the peak lists for the MS and MS/MS spectra, Bruker Flex analysis software was used. Then, MS and MS/MS data were combined and subjected to database searching using a copy of Mascot version 2.1 (Matrix Science Ltd.UK), which was run locally through the Bruker BioTools interface (version 3.1). The procedure was run using the following criteria: trypsin was used as enzyme, peptide tolerance was set to 100 ppm, MS/MS tolerance was set to 0.5 dalton and instrument was MALDITOF/TOF. Carbamidomethyl was considered as a fixed modification for all alkylated samples. The database search was run against IPI_human (86845 sequences; 35122444 residues) on 4 May 2011. Protein score of greater than 62 was the threshold for significant (P<0.05) identification. RBMY protein was identified with a score of 120.

Raising antibodies against RBMY. Desalted recombinant proteins (400 µg, entire RBMY and $RBMY_{pep}$) were emulsified with Freund's complete adjuvant and injected to young female New Zealand white rabbits (Albino). The rabbits were boosted after one month. Two other boosters were given at three-week intervals, and bleeding was done two weeks after last booster. Antisera were used for antibody titration and immunodetections.

Immunoblotting. Total cellular protein was obtained by lysing cells with Trizol (Invitrogen 15596-026) lysis reagent and quantified for total protein by Bradford's assay with a standard curve generated using a human serum albumin/gamma-globulin standard (Fluka P8119**)**. Normal testis tissue, NT2, DU145 cell line, testis tissue with sertoli cell-only syndrome (SCO) were used for RBMY expression analysis. Breast cancer cell line was used as negative control. Total protein extract (40 µg) from each sample was separated on 12% SDS-polyacrylamide gels for 120 min at 100 V and transferred to PVDF membrane (Bio-Rad, USA) by a wet transfer system (Bio-Rad, USA) at 20 V overnight. Transferred blots were blocked with 5% BSA and 0.1% Tween-20 (Sigma-Aldrich). Afterward, blots were incubated with primary antibodies from entire and segment RBMY (antisera, 1:10000 in blocking solution). Blots were then washed three times, 20 min each, with TBST and incubated with secondary antibody, HRP-conjugated donkey anti-rabbit antibody (Abcam ab16284). After three washes, 15 min each, with TBST blots were incubated with chemilumin-escent peroxidase substrate (Sigma-Aldrich, PQ0201, Germany) in dark room and exposed to X-ray films (GE, 28906835). For a negative control, blots were incubated with none-immunized serum as primary antibody.

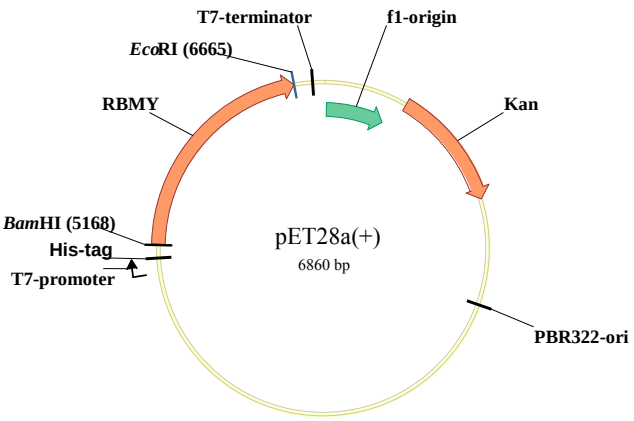

Fig. 2. The schematic view of RBMY construct on pet-28a vector. The restriction site sequences, *Bam*HI and *Eco*RI for entire RBMY and *Bam*HI and *Hind*III for the segment of RBMY, were added to the forward and reverse primers, respectively. CDNA and vector were digested with corresponding enzymes and ligated by T4 DNA ligase.

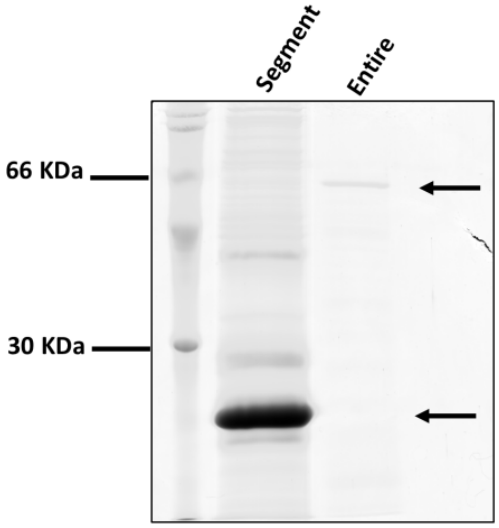

Fig. 3. Recombinant protein expression of entire and segment RBMY using Pet-28a vector transformed to BL21. 6×His-Ni-NTA chromatography purified proteins were loaded on 12% SDS-PAGE and stained with Coomassie brilliant blue. Arrows show the corresponding protein bands.

Immunofluorescence. Testis tissue was fixed in 4% paraformaldehyde, dehydrated in ascending concentrations of ethanol, cleared in xylene and finally embedded in paraffin. Then, embedded samples were cut into 5 µm thick sections and stained by hematoxylin and eosin stain method prior to immunodetection as a quality control checkpoint for tissue samples. Fresh slides were subjected to antigen retrieval (50:50, 0.5% trypsin and 1% $CaCl_2$) at 37°C for 20 min and washed two times, 5 min each, with PBST (phosphate buffer saline and 0.05 Tween-20). Then, the samples were permeabilized with Triton-X100 (0.2%) at 37°C for 15 min. After washing with PBST two times, 5 min each, the samples were incubated with blocking solution (10% goat serum; 5% BSA, 0.01% Tween-20 in PBS) at 37°C for one hour and with primary antibody diluted in 5% BSA (1:300) at 37°C for one hour. After three times washing with PBST, secondary antibody (FITC-conjugated goat anti-rabbit IgG [1:500 in 5% BSA]) was added to the slides. After one-hour incubation at 37°C, followed by three times washing with PBST, 4',6-Diamidino-2-Phenylindole, dihydrochloride was added to the slides for 30 seconds. Finally, slides were washed adequately and imaged by fluorescence microscopy (IX71, invert, USA).

Statistical analysis. Vector NTI software was employed for vector construction and design. Primers were designed by Gene Runner software. Image J software was used to analyze the Western-blot images. Microsoft excel was used for performing student's *t*-test and standard error estimation.

Ethical considerations. Tissue sample was obtained from a patient with obstructive azoospermia who underwent biopsy at Royan institute, Tehran, Iran. Karyotyping was carried out using a blood sample. The present study was approved by the Institutional Review Board of the Royan Institute and the participant provided a written informed consent, permitting the use of his tissue sample in this study.

RESULTS

Recombinant protein production. We cloned both the entire RBMY and also a segment of this protein ($RBMY_{pep}$). In spite of the same conditions, the shorter sequence, $RBMY_{pep}$, showed significantly more expression and yield (Fig. 3).

Antibody confirmation. Antisera obtained from rabbits were initially tested with recombinant proteins via Western-blot technique. Results showed that the titers of antibodies are considerably high in the both antisera. For further confirmation of antibodies, a panel comprising RBMY protein and also those irrelevant recombinant proteins which shared the vector tag (the initial sequence of Pet-28a vector consisting His tag, thrombin and T7 tag) were prepared and subjected to Western-blot analysis. It would be expected that the antisera react with all proteins because of the same tag, but less or no reactivity was expected with irrelevant proteins when the antisera and recombinant proteins were used in correct concentrations. In line with our expectation, there was no reactivity with other proteins when using very low concentrations (10 and 1 ng) of recombinant proteins (Fig. 4). According to our experiments, a dilution of 1:10,000 was selected for Western-blot analysis. Negative control blots incubated with none-immunized serum showed no protein bands (data not shown).

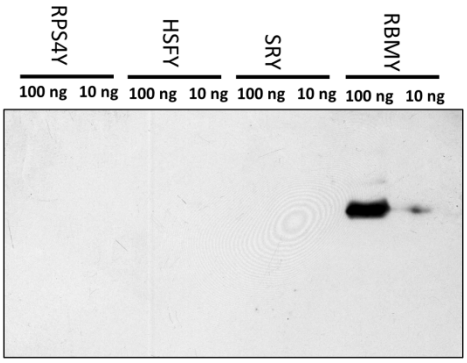

Fig. 4. RBMY antibody confirmation by using Western-blot analysis of recombinant RBMY. Two concentrations (100 and 10 ng) of each recombinant protein were loaded on SDS-PAGE and after separation were transferred to PVDF membrane. Blots were exposed to X-ray films.

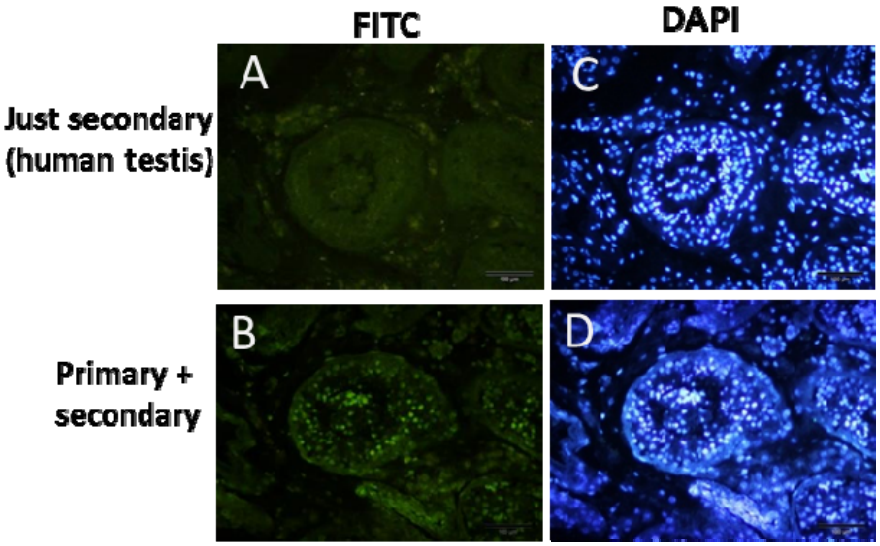

Fig. 5. Immunofluorescence analysis of human RBMY protein in human testis by using home-made antibody against a segment of RBMY protein. Testis tissue sections were probed with **(A)** only secondary FITC-conjugated antibody, and **(B)** primary antibody raised against a segment of RBMY protein followed by secondary FITC-conjugated antibody. The sections were stained with 4',6-Diamidino-2-Phenylindole, dihydrochloride, which stains nuclei blue (**C** and **D**). The analysis showed a nuclear localization of RBMY protein when both primary and secondary antibodies were used.

Identification of RBMY in testis tissue. For biochemical analysis of RBMY, positive control tissue (testis) and negative (breast cancer cell line) were used. Since the anti-$RBMY_{pep}$ antibody was more specific, we used this antiserum for RBMY detection. Western-blot analysis showed three protein bands at 53, 50 and 43 KDa, whereas no bands were detected in breast cancer cell line. Immunofluorescent analysis of testis tissue slides showed nuclear localization of this protein (Fig. 5). Expressing only in nucleus was another evidence for mono-specificity of our home-made antibody. Another slide was incubated just with secondary antibodies to make sure that there is no cross-reactivity of secondary antibody.

Differential expression of RBMY isoforms. In order to make a comparative expression analysis of RBMY, we compared the expression pattern of the RBMY isoforms in both testis and NT2. We also added prostate cancer cell line, DU145, SCO and breast cancer cell line. While there were three isoforms of RBMY in testis tissue, only the longest isoform was identified in NT2, which was used as a model of germ cell in testis (Fig. 6). There were no protein bands in breast cancer cell line, confirming the specificity of produced antibody and indicating no cross reaction with its homologs, RBMX and RBML. A slight level of expression of longest RBMY isoform was seen in DU145 cell line and SCO tissue.

DISCUSSION

In this study, expression of RBMY protein isoforms was emphasized. This point of view was not considered in previous studies on this protein. For this purpose, we started our work by cloning the gene and expressing recombinant RBMY protein as well as a segment of this protein ($RBMY_{pep}$). According to the results, $RBMY_{pep}$ showed a remarkably more expression compared to the entire RBMY gene. Generally, rare codons (mainly arginine, leucine, isoleucine and proline) are limiting factors for mammalian recombinant protein over-expression in *E. coli* [28, 29]. To evaluate if the sequence of entire RBMY contains more rare codons than $RBMY_{pep}$, the sequences were analyzed using online software developed by NIH MBI Laboratory for Structural Genomics and Proteomics (http://nihserver.mbi.ucla.edu /RACC/). This analysis confirmed that the entire RBMY has considerably more rare codons compared to $RBMY_{pep}$ (Fig. 7).

In addition, we produced a mono-specific antibody for this protein, which works well in both denatured (Western-blot) and native conditions (such as immunofluorescence). This antibody can be used for further experiments on RBMY protein and assures precise detection. In our experiment, all three isoforms of RBMY, just the same as previously reported isoforms [21], were detected. In addition, RBMY expression was detected only in nucleus, which was consistent with the previous studies [21, 25].

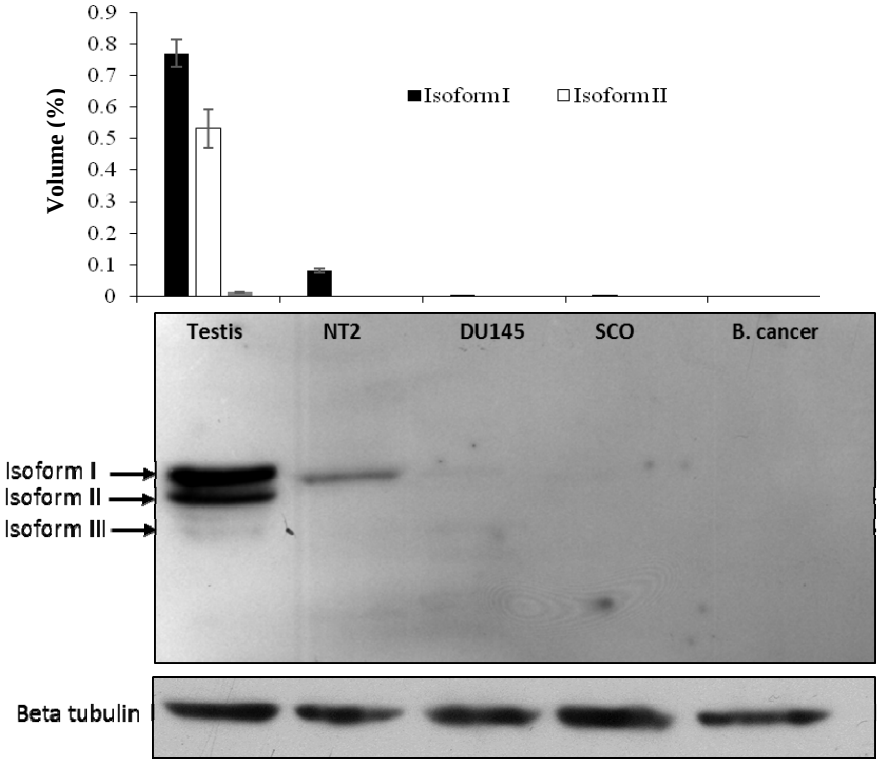

Fig. 6. Western blot of proteins extracted from human testis tissue (testis), NT2 and DU145 cell lines, testis tissue with sertoli cell only syndrome (SCO) and breast cancer cell line (B. cancer). Upper blot shows the expression pattern of RBMY protein isoforms in subjected samples. Three isoforms are expressed in testis, while only the longest isoform is expressed in NT2 cell line. Lower blot shows the beta tubulin as a housekeeping protein for loading control.

Comparative expression analysis of RBMY protein isoforms implied that alternative splicing of RBMY does not either happen in NT2 cell line or the isoforms are not translated, resulting in the presence of only one isoform of this protein. This result could somehow answer the question of whether RBMY undergoes any differential splicing during germ cell development stages. Elliot *et al.* [21] suggested that RBMY could have different functions from spermatogonial to round spermatid stages. Also, they indicated that the function of RBMY is regulated during spermatogenesis because of dynamic modulations in RBMY spatial location among different cell types. Their observations showed a transient association of RBMY with nuclear speckles enriched in splicing factors. These phenomena, however, was limited to the first two stages found in spermatogonia and spermatocytes, but there was no evidence of co-localization in round spermatids. Accordingly, the difference in function in different cell types could be due to the RBMY isoforms, probably being differentially expressed in aforementioned cells.

Although several studies showed a high association between RBMY deletion and microdeletion with spermatogenesis failure, there are some experiments reporting no deletion or microdeletion of RBMY in infertile men [30]. Certainly, there are a lot of factors involved in spermatogenesis. Nonetheless, the role of RBMY has been known to be critical in this procedure [10, 12, 16]. We hypothesize that the RBMY isoforms could play important roles in different stages of germ cell development. In addition, analysis of the isoforms to determine which isoform is expressed at each

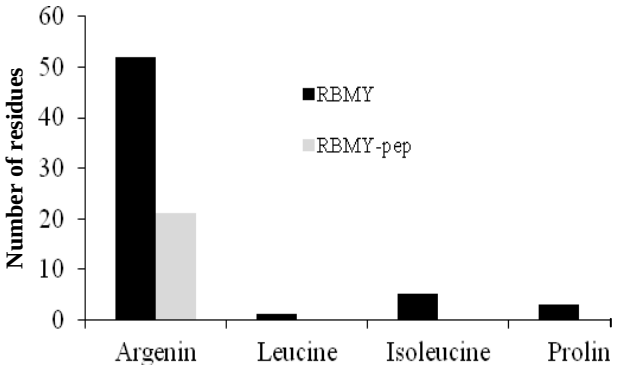

Fig. 7. The graph illustrates the number of rare codons in RBMY and RBMYpep. RBMY contains 61 rare codons among which 52 codons belong to arginine, the rarest codon for *E. coli*. RBMYpep sequence showed no rare codons for leucine, isoleucine and proline. However, it has 21 codons for arginine.

stage could shed lights on the isoforms function. Alternative splicing in testis happens regularly for some important transcripts such as CREM protein. Alternative splicing of this protein results in isoforms, of which some act as activator and some act as transcription repressor [21, 31]. In this process, before puberty, repressor isoforms are synthesized in germ cells, and then at puberty activator isoforms are induced. The induction is made under the impact of sexual hormones such as follicle-stimulating hormone, which finally results in stimulating some structural genes in round spermatids [31]. This process might exist for RBMY as well.

According to our results, it is probable that RBMY is differently spliced during spermatogenesis in different cell types. As a result, recognizing the cell-specific splicing of RBMY during germ cell development will shed light on better understanding of the function of this protein. For this purpose, separating and sorting normal testis cells at different developmental stages is critical. Another approach would be the analyzing the testis samples from patients bearing different types of spermatogenesis failures or arrests. In addition, we used a testicular germ cell cancer-derived cell line (NT2), and it should be noted that this cancer cell line does not necessarily reflect the normal cell performance. Therefore, further experiments are needed to confirm this study.

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