Comparison of Protamine 1 to Protamine 2 mRNA Ratio and YBX2 gene mRNA Content in Testicular Tissue of Fertile and Azoospermic Men

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Abstract

Background: Although aberrant protamine (PRM) ratios have been observed in infertile men, the mechanisms that implicit the uncoupling of PRM1 and PRM2 expression remain unclear. To uncover these mechanisms, in this observational study we have compared the PRM1/PRM2 mRNA ratio and mRNA contents of two regulatory factors of these genes.

Materials and Methods: In this experimental study, sampling was performed by a multi-step method from 50 non-obstructive azoospermic and 12 normal men. After RNA extraction and cDNA synthesis, real-time quantitative polymerase chain reaction (RT-QPCR) was used to analyze the PRM1, PRM2, Y box binding protein 2 (YBX2) and JmjC-containing histone demethylase 2a (JHDM2A) genes in testicular biopsies of the studied samples.

Results: The PRM1/PRM2 mRNA ratio differed significantly among studied groups, namely 0.21 ± 0.13 in azoospermic samples and -0.8 ± 0.22 in fertile samples. The amount of PRM2 mRNA, significantly reduced in azoospermic patients. Azoospermic men exhibited significant under expression of YBX2 gene compared to controls (P<0.001). mRNA content of this gene showed a positive correlation with PRM mRNA ratio (R=0.6, P=0.007). JHDM2A gene expression ratio did not show any significant difference between the studied groups (P=0.3). We also observed no correlation between JHDM2A mRNA content and the PRM mRNA ratio (R=0.2, P=0.3).

Conclusion: We found significant correlation between the aberrant PRM ratio (PRM2 under expression) and lower YBX2 mRNA content in testicular biopsies of azoospermic men compared to controls, which suggested that downregulation of the YBX2 gene might be involved in PRM2 under expression. These molecules could be useful biomarkers for predicting male infertility.

Keywords: Protamines, YBX2, JHDM2A, Azoospermia

Introduction

Protamines (PRMs) comprise the largest amount of nucleoproteins in mature human sperm. These proteins are transcribed in steps 1-4 of spermatids (1) while synthesis of the corresponding proteins starts, with temporal delay, in step 4 spermatids (2). During spermiogenesis, PRMs replace somatic histones in a step-by-step manner, and cause higher DNA packaging in sperm compared to somatic cells. On the other hand, the condensed and insoluble nature of the sperm chromatin protects the genetic integrity of the parental genome during its transport through the male and female reproductive tracts (3).

Various studies reported abnormal expressions of PRM genes in sperm of infertile men. In addition,
correlation of the altered PRM1/PRM2 ratio has been shown with low sperm counts, decreased sperm motility and morphology, decreased fertilization ability and increased sperm chromatin damage (4-7). Several factors have been postulated and studied as possible causes of PRM1/PRM2 deregulation (8-11). One of these candidate mechanisms is PRM gene polymorphisms that have also been reported for PRM1, 2 genes. However most of these studies suggest that none of the PRMs’ single nucleotide polymorphisms (SNPs) and transition protein genes is likely to be a common cause of PRM abnormalities. Other factors that have attracted attention in this regard are transcription and translation regulatory genes of PRM. Several genes and proteins involved in PRM1/PRM2 expression regulation have been identified and presented (12-16); until now, modification of these factors in infertile men with PRM deficiency attracted less attention. In this study we have proposed two PRM regulatory factors -Y box binding protein 2 (YBX2) gene and JmjC-containing histone demethylase 2a (JHDM2A) gene. These genes encode two important proteins involved in regulation of PRM1/PRM2 expressions (17, 18). YBX2 is the human homologue of Xenopus DNA/RNA-binding and mouse MSY2 proteins (16), animal model studies show that this protein exists abundantly in testis tissue and is expressed in meiotic and post-meiotic germ cells (17). YBX2 acts as an mRNA stabilizer and a transcription factor of PRM genes (18, 19). Consequently, YBX2 loss of expression is likely to contribute to the nuclear condensation defects that occur in Msy2-null late stage spermatids (20). JHDM2A specifically regulates the expression of genes that encode transition protein 1 (Tnp1) and PRM1; this is necessary for proper chromatin reorganization during spermatid maturation by directly promoting transcription of TNP1 and PRM1 genes (21). The current study analyzes the PRMs ratio in testicular tissue of azoospermic men. As YBX2 and JHDM2A are involved in expression regulation of these genes, we have additionally evaluated whether PRM deficiency is related to downregulation of these genes.

Materials and Methods

Testicular tissue

This experimental study was approved by the Ethical Committee of the Faculty of Medical Sciences of Qazvin Medical Science University (Qazvin, Iran). After patients gave their informed written consent, testicular biopsies were obtained from 50 infertile men with a mean age of 31.3 ± 3.7 years; these patients were candidates for assisted reproductive technique (ART) and exhibited impaired spermatogenesis. In 12 patients with obstructive azoospermia after vasectomy, biopsies were performed out for diagnostic reasons during vasectomy reversal. These biopsies revealed normal spermatogenesis which served as controls; the mean age of these individuals was 35 ± 2.9 years. In this study patients were excluded if they had the following criteria: Y chromosome microdeletion, cystic fibrosis, varicocele, Klinefelter syndrome, or exposure to chemotherapy and radiation. In non-obstructive azoospermia patients, one part of the testicular tissue specimen was used for testicular sperm extraction, while the other part was cut into two pieces. One piece was immediately prepared and frozen for the RNA extraction procedure and the other piece was fixed in Bouin’s fixative, then embedded in paraffin.

Histological evaluation

We stained 5 µm paraffin sections in hematoxylin and cosin, and then scored the sections according to the modified Johnsen scoring system for histological evaluation (22). In this system of classification, all tubular sections in each piece of the testicular biopsy are evaluated systematically, and each is given a score from 1 to 10. Complete spermatogenesis with numerous spermatozoa is evaluated as score 10; slightly impaired spermatogenesis, numerous late spermatids, disorganized epithelium as score 9, less than five spermatozoa per tubule and few late spermatids as score 8, no spermatozoa, no late spermatids, and numerous early spermatids as score 7, no spermatozoa, no late spermatids, and few early spermatids as score 6; no spermatozoa or spermatids, and many spermatocytes as score 5, no spermatozoa or spermatids, and few spermatocytes as score 4; spermatogonia only as score 3, no germinal cells and Sertoli cells only as score 2; no seminiferous epithelium as score 1 (11). To follow this classification method, we divided the samples into two groups based on the above scoring: normal spermatogenesis (scores 9-10) and impaired spermatogenesis (scores 1-8) (Table 1, Fig.1A-E). It should be mentioned that our samples with score 8 had severe hypospermatogenesis.
Table 1: Characterization of patient candidates for ICSI

|                  | Fertile men (Mean ± SD) | Azospermic men (Mean ± SD) | P value |
|------------------|--------------------------|----------------------------|---------|
| Age (Y)          | 35 ± 2.9                 | 31.3 ± 3.7                 |         |
| Histology (Score)| 10                       | 1-8                        |         |
| Serum FSH (mIU/ml) | 5.2 ± 2.3               | 14.26 ± 7.3                |         |
| ΔCt (PRM2-PRM1)  | -0.8 ± 0.22              | 0.21 ± 0.13                | <0.0001 |
| ΔCt (YBX2-GAPDH) | 3.3 ± 0.99               | 5.1 ± 1.2                  | <0.0001 |
| ΔCt (JHDM2A-GAPDH) | 6.1 ± 1.1               | 6.3 ± 0.89                 | 0.5     |

FSH; Follicle stimulating hormone and ICSI; Intracytoplasmic sperm injection.

Fig. 1: Results of hematoxylin and eosin staining of testis tissues. A. Hypospermatogenesis, B. Maturation arrest in round spermatid stage, C. Maturation arrest in spermatocyte stage, D. Maturation arrest in spermatogonial stage and E. Sertoli cell only. (magnification: ×1000).
Role of YBX2 Expression in Protamine Deficiency

RNA extraction and first strand cDNA synthesis

After homogenizing frozen testis tissues using an Ultrasonic Processor UP100H (Hielsher, Germany), RNA was extracted with an RNeasy Mini Kit (Qiagen, Germany). The extracted RNA was frozen at -80°C. We used a Nano Drop 2000c (Thermo, USA) to evaluate the quantity of isolated total RNAs. In this regard, RNA samples with A260/A280 ratios of >2 were selected for quantitative analysis. First strand complementary DNA (cDNA) synthesis was also performed using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Fermentas, Waltham, MA, USA).

Real time quantitative polymerase chain reaction (RT-QPCR)

We designed four target genes primers and probes (PRM1, 2, YBX2 and JHDM2A) using Gene Runner Software (Version 3.05, Table 2). A Taq Man RT-QPCR assay was carried out in final reaction volumes of 20 μl with 10 μl of Taq Man Master Mix (Takara, Shiga, Japan), 0.2 μM of forward and reverse primers, and 2 μl of cDNA. Thermal cycling was performed on the ABI-7500 (Applied Biosystems, Foster, CA, USA) sequence detection system by using the following cycling condition: 30 seconds at 95°C as the first denaturation step, followed by 40 cycles at 95°C for 5 seconds and 60°C for 34 seconds. Each assay was repeated at least twice. The log-ratio of the transcript content in the samples was determined by the ΔCt method of relative quantification. The log-ratio of PRM1 and PRM2 was also calculated by ΔCt = CtPRM2 – CtPRM1 for YBX2 and JHDM2A, these were ΔCt = CtYBX2 – CtGAPDH and ΔCt = CtJHDM2A – CtGAPDH. To study the correlation between YBX2 and JHDM2A mRNA content and PRM log concentration, we used the calculating pattern of Steger et al. (6). Since Ct of PRM1 did not change in the different groups, log-concentration of YBX2 and JHDM2A were normalized to PRM1 (ΔCt = CtYBX2 – CtPRM1) and ΔCt = CtJHDM2A – CtPRM1 (23).

Table 2: Primer and probe sequences of target and internal control genes

| Target and internal control genes | Sequence | Amplicon size (bp) |
|----------------------------------|----------|--------------------|
| PRM1                             | F: TCACTCACACGCCACAGGAT R: CTGGACACGATCTGTACCT P: AGGCCAGCCCACACCTGCAC | 124 |
| PRM2                             | F: GCAAGGAAAGGCACACAC R: GACACTCTGCACAGGAGG P: CGAGCAGCAGTGCTGCT | 98 |
| YBX2                             | F: CCCCTCCACACCACCTCTGCAT R: CCTTCCTCTCAACCTTGAAAT P: CAGGAGGCAAGCAAAGCGACGC | 150 |
| JHDM2A                           | F: GTTCACAAAGCATTGACTGG R: CTGTTGCTGTTGAACATCC P: TGCCAATCCTGACTGAC | 145 |
| GAPDH                            | F: TCAAGAAGGCTGTAAGGAC P: CCTCAAGGCACTGCTGCT | 93 |

YBX2; Y box binding protein 2, JHDM2A; JmjC-containing histone demethylase 2a, PRM; Protamin and GAPDH; Glyceraldehyde 3-phosphate dehydrogenase.
Statistical analysis

In order to determine the significant differences between the studied groups, statistical analysis that included mean, standard deviation (SD), correlation coefficients ($R^2$) and unpaired t test were performed with Prism (version 3) software. Additionally, linear correlations were tested using the Pearson coefficient of correlation. All tests were performed at a confidence level of 95%.

Results

The mean Ct of $PRMI$ in testis tissues were almost identical, $23.4 \pm 3.6$ (azoospermic) and $23.3 \pm 1.5$ (fertile). The mean Ct of $PRM2$ in azoospermic men was $23.6 \pm 1.8$ and in the fertile group, it was $22.5 \pm 0.41$. Hence the expression ratio of $PRM2$ was lower than the $PRM1$ expression ratio in azoospermic men. The logarithm of the $PRM1/PRM2$ mRNA ratio in azoospermic men was $0.21 \pm 0.13$ and for fertile men, it was $-0.8 \pm 0.22$. This difference of ratios between fertile and azoospermic men was statistically significant (P<0.0001, Table 2).

In testicular tissues of azoospermic men with impaired spermatogenesis, the fold change of $YBX2$ transcripts was $0.02 \pm 0.019$, and the log ratio of $YBX2$ expression between azoospermic ($5.1 \pm 1.2$) and fertile men ($3.3 \pm 0.99$) significantly differed (P<0.0001, Table 2). In terms of $JHDM2A$ gene expression, the differences of expression ratio were $6.1 \pm 1.1$ (fertile) and $6.3 \pm 0.89$ (azoospermic), which was not significant (P=0.5, Table 2).

The $YBX2$ mRNA content revealed a positive linear correlation (R=0.6, P=0.007) with the $PRM1/PRM2$ mRNA ratio or with $PRM2$ deficiency (Fig.2A). However, we observed no linear correlation between $JHDM2A$ mRNA content and the $PRM1/PRM2$ mRNA ratio (R=0.2, P=0.3, Fig.2B).

![Fig.2: A. Correlation between the protamine-1 ($PRM1$) to $PRM2$ log-ratio $\Delta$Ct ($PRM2$-$PRM1$) and normalized $YBX2$ log-concentration $\Delta$Ct ($YBX2$-$PRM1$, R=0.6, P=0.007) and B. Correlation between the $PRM1$ to $PRM2$ log-ratio $\Delta$Ct ($PRM2$-$PRM1$) and normalized $Jhdm2a$ log-concentration $\Delta$Ct ($JH-PRM1$, R=0.2, P=0.3).](https://www.SID.ir)
Discussion

In this research we observed a significant difference in PRM1/PRM2 ratio between azoospermic and fertile men testicular biopsies (0.21 ± 0.13, -0.8 ± 0.22). PRM2 changes were more than PRM1, which suggested different mRNA stabilities for the two molecules. A variety of studies reported a relationship between abnormal PRM1/PRM2 ratios and male infertility (4-6, 21, 23). On the basis of the studies which showed high PRM1/PRM2 ratios, it has been supposed that a reduction in PRM2 expressions was responsible for aberrant PRM1/PRM2 ratios in infertile males. Along this line, two studies reported complete selective absence of PRM2 in infertile men (4, 24, 25). Lewis et al. (26) observed that in sperm of infertile men, PRM2 downregulation occurred much more frequently than PRM1 deregulation, because PRM2 expression was more sensitive to the variation of regulatory controlling mechanisms than those for PRM1.

Our results confirmed the results from the above studies; we have observed significant downregulation of PRM2 in the studied azoospermic men. PRMs genes are only transcribed in round spermatids and stored as silent mRNAs for later translation in elongating spermatids, in which transcription is no longer active. Since it is well justified to consider altered PRMs mRNA levels as a potential origin of altered protein levels (1, 11, 27), we have evaluated PRM expression at the mRNA level. Generally speaking, the mechanisms that underlie the uncoupling of PRM1 and PRM2 expression remain unclear, but generally four pathways in regulation of PRM gene expression have received more attention: the PRM genes themselves, transcription regulation, translation regulation, and downstream protein processing.

The PRM1 and PRM2 genes exist in a single chromatin domain in human sperm, and their transcription is regulated by the same upstream regulatory elements, thus making transcriptional and translational regulation a possible cause for aberrant PRM1/PRM2 expression (28, 29). There are a number of regulatory proteins identified which are involved in repression or activation of PRM expression (29). In this regard, various animal models and in vitro studies have been performed, but scientists emphasize that future investigations should focus on aberrant expression, activation, and function of these regulatory factors in patients with deregulated PRM1/PRM2 ratios (30, 31). To this end, we have focused on the expression ratio of two factors of PRM gene expressions.

YBX2 is a transcription and translation regulatory factor, and a germ-cell-specific molecule essential for the production of functional spermatozoa. This gene is expressed in meiotic and post-meiotic germ cells, but its functional form is in round spermatids. Inactivation of YBX2 can lead to male infertility. YBX2, also known as Contrin, is the human homologue of Xenopus DNA/RNA-binding and mouse MSY2 proteins (32, 33). To clarify the functional role of MSY2 in germ cells, Yang et al. (19) have generated Msy2-null mice. They found that mutant males had an abnormally high number of apoptotic meiotic spermatocytes, lacked spermatozoa in the epididymis, and were sterile. Their results emphasized the major role of this protein in male fertility (33). Hammoud et al. (34) investigated YBX2 gene alterations in men with severe defects in spermatogenesis that included azoospermia, severe o oligozoospermia, and PRM deregulation samples. Their results showed 15 polymorphic sites, of which 7 polymorphisms were present at a statistically higher frequency in patients with infertility, particularly in men with abnormal PRM expression. On the same path they showed that some SNPs in the YBX2 gene occurred at a significantly higher incidence in men with PRM abnormalities than the control group. Our results, accordingly, showed significant downregulation of this gene in testicular tissues of azoospermic men compared to fertile men.

In terms of the molecular function of this protein, animal models and in vitro assay studies have shown that MSY2 acts as a transcription factor and an mRNA stabilizer which regulates expression of some testis specific genes at the transcription and translation levels, such as PRM1, 2. MSY2 marks specific mRNAs (those transcribed from Y-box promoters) in the nucleus for cytoplasmic storage, and thereby links mRNA transcription and storage/translational delay. In this process, MSY2 recognizes the CTG ATTTGC/TC/TAA sequence, a DNA motif in the promoter of numerous genes specifically expressed in male germ cells. After binding MSY2 to its consensus promoter
sequence, it binds to transcripts of this gene, and stabilizes and represses their translation in cytoplasmic RNA-protein complexes (19, 35-38). Since PRM1, 2 are regulated by the same upstream regulatory elements, we have expected significant under expression of YBX2 to cause simultaneous downregulation of PRM1, 2 in our azoospermic samples compared to fertile samples.

Unlike our expectation PRM2 downregulated in the studied samples. We observed a positive linear correlation between downregulation of PRM2 and YBX2 genes. Statistically, R=0.6 exhibited an intermediate or good correlation, but this correlation was not perfect or 100%. This probably indicated that factors other than YBX2 were involved in PRM2 downregulation. Consistent with our results, one study has shown that decreased PRM1 protein level is usually linked with post-translational deregulation, but decreased PRM2 is associated with low PRM2 mRNA (30). PRM2 transcripts are more susceptible to variations other than PRM1 transcripts, downregulation of YBX2 affects PRM2 transcripts more than PRM1 (26).

Another regulatory factor studied in this research was JHDM2A, also known as Jmjd1a or Kdm3a, was identified as an H3K9 demethylase (for monomethylation and dimethylation). JHDM2A was originally cloned as a testis-specific gene transcript. Results of immune histochemical analysis using anti- JHDM2A antibody showed an intense nuclear expression of this gene in round spermatids and a sub-nuclear distribution. Co-expression of JHDM2A gene with RNA polymerase II indicated and a sub-nuclear distribution. Co-expression of JHDM2A with RNA polymerase II indicated a positive linear correlation. Statistically, shows a weak or no correlation. Other studies have shown that this gene acts as a transcriptional activator of the PRM1 gene. In addition, our samples displayed downregulation of the PRM2 gene; therefore, expression of JHDM2A in our samples did not have an important role.

Conclusion
We found significantly aberrant PRM mRNA ratios and a lower YBX2 mRNA content in testicular spermatids of infertile men. In future studies the exact role of these molecules (YBX2 and JHDM2A) and other PRM expression regulatory factors must be determined in human spermatogenesis.

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