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Association of G/T(rs222859) polymorphism in Exon 1 of YBX2 gene with azoospermia, among Iranian infertile males

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Summary

Animal model studies have shown that MSY2 gene has a potential role in spermatogenesis. Some mutations on this gene have been proposed to be associated with human male infertility. In this study, polymorphisms of exon 1 of YBX2 gene have been investigated. A total of 276 men were evaluated. They included 96 men with normal spermatogenesis, 60 men with nonobstructive azoospermia, 60 men with oligospermia and 60 men with asthenospermia. We extracted DNA from blood and testis tissues of samples, and analysed polymorphisms of exon 1 by sequencing method. Moreover, YBX2 gene expression was studied by real-time PCR on blood and testis tissue of samples. Sequencing results showed that among the studied polymorphisms, frequency of TT genotype in rs222859 polymorphism was significantly higher in azoospermic patients compared to control group ($P < 0.001$). Azoospermic men exhibited significant underexpression of YBX2 gene in blood and testis samples in comparison with controls, oligosperm and asthenosperm samples ($P < 0.001$), but there was no significant difference in gene expression of YBX2 gene in blood and testis tissues of azoospermic men, with and without mutation ($P > 0.05$). According to our results, the alterations of this gene might be involved in azoospermia among Iranian population.

Introduction

Male infertility comprises 50% of infertilities, but nearly 50% of infertile men have unexplained or idiopathic infertility where the causes cannot be identified (Huynh *et al.*, 2002; Zhang *et al.*, 2007). Genetic factors which involve in impaired spermatogenesis are the main causes of male infertility (Yang *et al.*, 2006). Accordingly, Yq microdeletion and chromosomal abnormality are two main causes of impaired spermatogenesis (Totonchi *et al.*, 2012). Moreover, mutation of autosomal genes is closely related to male infertility, but the underlying molecular mechanisms are still unknown (Fernando *et al.*, 2006; Yang *et al.*, 2006).

Protamines (PRMs) substitute somatic histones during spermiogenesis in a step-by-step manner and increase DNA packaging in spermatozoa (Ward & Coffey, 1991). PRM genes are only transcribed in round spermatids and are stored as silent mRNAs for later translation in elongating spermatids (Aoki *et al.*, 2005). It is clear that the regulation of PRM incorporation in chromatin is related to the temporal uncoupling of transcription and translation (Carrell *et al.*, 2007). In this regard, some messenger ribonucleoproteins (mRNPs) are introduced which present in round and elongating spermatids (Kleene, 1996). These mRNPs bind to PRM mRNA and inhibit translation; *Y box binding protein 2* (YBX2) is one of these mRNPs.

YBX2 is the human homologue of Xenopus DNA/RNA-binding and mouse MSY2 proteins and is on chromosome 17p13.1. (Kwon *et al.*, 1993; Gu *et al.*, 1998). Some studies in animal models show that this protein exists abundantly in testis tissue and is expressed in meiotic and post-meiotic germ cells (Kwon *et al.*, 1993; Gu *et al.*, 1998). YBX2 stabilises mRNA as a transcription factor of PRM genes, and its loss of expression is likely to contribute to the nuclear condensation defects that occur in Msy2-null late-stage spermatids (Horvath *et al.*, 2004; Meng *et al.*, 2005; Yang *et al.*, 2005a,b). In one study to show the functional role of MSY2 in germ cells, Yang

et al. (2005a,b) generated Msy2-null mice. They found that the mutant males had high number of apoptotic meiotic spermatocytes, lacked spermatozoa in the epididymis and were sterile; they underscored the main role of this protein in male fertility. It is shown that some mutations of YBX2 gene are associated with male infertility (Meng *et al.*, 2005; Deng *et al.*, 2008; Hammoud *et al.*, 2009). On the other hand, in our previous study, we showed that YBX2 gene had underexpression in testis tissue of azoospermic men with maturation arrest (Najafipour *et al.*, 2015). In this regard, we proposed for the first time the screening of exon 1 of YBX2 gene polymorphisms among the Iranian azoospermic, oligospermic and asthenospermic men. Moreover, the relation between the frequency of gene mutation and level of the YBX2 gene expression in the blood samples of all studied groups and testis tissues of azoospermic men was assessed.

Materials and methods

Subjects

This study was approved by the Ethical Committee of the Faculty of Medical Sciences of Qazvin Medical Science University (Qazvin, Iran) and patients gave their informed written consent. All samples were collected during 2012–2014 years from fertility and infertility centre of Shariati Hospital, Tehran, Iran. We obtained blood samples from four groups, 96 fertile men with normal spermatogenesis as control samples. Patients' samples were men with nonobstructive azoospermia, oligospermia and asthenospermia. There were 60 samples in each group. None of the fertile men had a clinical history of varicocele, cryptorchidism, and all of them had normal semen parameters. Infertile men were not previously diagnosed with other criteria of infertility (e.g. cystic fibrosis, Klinefelter syndrome, varicocele, chemotherapy, AZF genes micro deletions). To study polymorphisms and gene expression on the testicular biopsies, testis tissues were obtained from 60 men with nonobstructive azoospermia who were candidates for testicular sperm extraction (TESE) technique, and histologically, over 55% of samples had maturation arrest. In this regard, control samples were 12 patients with obstructive azoospermia after vasectomy; biopsies were carried out for diagnostic reasons during vasectomy reversal. These biopsies revealed normal spermatogenesis and served as controls.

Polymorphisms analysis

DNA was extracted from peripheral blood lymphocytes and testis tissues using Dyna Bio™ Blood/Tissue DNA Extraction Mini Kit (Takapouzist, Tehran, Iran). Primer sets were designed and optimised to amplify exon 1 (F: 5′-GGCTGTGGCCGGTACTGGAC-3′ R: 5′-AGGATCCG GAAAACCAAAGGC-3′). Sequencing of PCR products was carried out according to Sanger method using ABI 3730XL Capillary Sequencer. Sequencing results were compared with the sequence of normal YBX2 gene (NC_000017.11), obtained from NCBI website: http://www.ncbi.nlm.nih.gov; also, sequence traces were assembled using Chromas software (version 2.4, Technelysium company, South Brisbane, Australia).

Expression study on blood and tissue samples

Frozen testis tissues are homogenised using an Ultrasonic Processor UP100H (Hielscher, Germany). RNA was extracted with an RNeasy Mini Kit (Qiagen, Germany) from blood and tissue samples. The extracted RNA was then frozen at −80 °C. Quality and quantity of isolated total RNA was measured using NanoDrop 2000c (Thermo, USA). In this case, RNA samples with A260/A280 ratios of >2 were selected for quantitative analysis. First-strand complementary DNA (cDNA) synthesis was also performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Fermentas, Waltham, MA, USA).

GAPDH gene was used as an internal control for quantification of target gene expression. Two target genes, YBX2 and GAPDH (as internal control), were amplified with appropriate primers (Table 1). Real-time PCR assay was carried out in final reaction volumes of 20 μl with 10 μl of TaqMan master mix (Takara, Shiga, Japan), 0.2 μM of forward and reverse primers and 2 μl of cDNA.Thermal cycling was performed on ABI-7500 (Applied Biosystems, Foster, CA, USA) sequence detection system using the following cycling condition: 30 s at 95 °C as first denaturation step, followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s. The $2^{-\Delta CT}$ method of relative quantification was used to determine the fold change in expression. This was performed by normalising the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control (GAPDH) in treated and untreated samples ($\Delta CT = CT_{target} - CT_{GAPDH}$) (Schmittgen & Livak, 2008).

Statistical analysis

Statistical analysis was performed using SPSS statistical software (SPSS Inc., Chicago, Illinois, USA). The association between genotypes and infertility was assessed by computing the odds ratio (OR) and 95% confidence intervals (95% CI) from logistic regression analyses. The Hardy–Weinberg equilibrium was tested for polymorphisms. Additionally, one-way analysis of variance (ANOVA)

Table 1 Oligonucleotide primers used for real-time PCR assay

Target and internal control genes	Sequence	Amplicon size (bp)
YBX2	F:CCCTACCCAGTACCCTGCT	
	R:CCTTCCTTCAACCCTTGATAA	150
GAPDH	F: TCAAGAAGGTGGTGAAGCAG	
	R:CGCTGTTGAAGTCAGAGGAG	93

carried out to determine the significant differences in expression ratio between the studied groups. *P*-value of <0.05 was considered as statistically significant.

Results

Exon 1 of YBX2 gene has 10 single nucleotide polymorphisms (SNPs). We screened all of these SNPs in our samples, but only three polymorphisms had clear frequency difference among the studied groups. These polymorphisms were rs222859 G>T, rs8069533T>C and rs768015399 G>A. All of them created amino acid alterations: rs222859 G>T changes Gly into Val, rs8069533T>C substitutes Ser for a Pro, and rs768015399 G>A substitutes Val for a Met. Statistical analysis results showed that frequency of different genotypes of rs8069533T>C and rs768015399 G>A polymorphisms had no significant difference between patients and control groups (Table 2). Regarding rs222859 G>T polymorphism, frequency of TT genotype was significantly higher in azoospermic samples (22%) in comparison with normal ones (8%) ($P < 0.05$); In this regard, oligospermic and asthenospermic patients compared to normal group showed no significant difference ($P > 0.05$). Frequency of GT heterozygote genotype did not show any significant difference between normal and azoospermic patients ($P > 0.05$) (Table 2).

Since in rs222859 G>T polymorphism, frequency of TT genotype was significantly higher in blood samples of azoospermic men, we evaluated frequency of different genotypes of this polymorphism in testis tissues of candidates of TESE technique.

Our results showed that all of the 13 azoospermic men, who had TT genotype in their blood samples, had the same polymorphism in their testis tissues. In terms of GG genotype, 27 of 30 azoospermic patients had same genotype in their blood and testis samples, but three of them had TT genotype in their testis samples. Frequency of GT genotype was the same in blood and tissue samples (Table 3).

Evaluation of mRNA content of YBX2 gene in blood samples of the studied groups showed a significant underexpression in azoospermic patients when compared to the samples with normal spermatogenesis ($P < 0.001$), but there was no significant difference in expression of YBX2 gene among the normal, oligospermic and asthenospermic samples ($P > 0.05$). Comparison of expression ratio in blood samples of azoospermic men with and without mutation showed that there was no significant difference between these two groups ($P > 0.05$) (Fig. 1a).

In testicular tissues of azoospermic men, the fold change of YBX2 transcripts was significantly lower than testis tissues of men with normal spermatogenesis ($P < 0.001$). Evaluation of mRNA expression in testicular tissues of men with mutation and those without mutation showed no significant difference ($P > 0.05$) (Fig. 1b).

Discussion

There are more than 3000 genes which have specific roles in male fertility (Tekur *et al.*, 1999). In animal model studies, Yang *et al.* (2005a,b), for example, indicated that

Table 2 Distribution of genotypes of studied polymorphisms in case and control groups

YBX2 SNPs	Genotypes	Control $n = 96$	Azoospermic men $n = 60$	OR CI (95%)	Oligospermic men $n = 60$	OR CI (95%)	Asthenospermic men $n = 60$	OR CI (95%)
rs8069533	TT	62%	66%	–	68%	–	70%	–
	TC	34%	30%	1.09 (0.6–1.9)	27%	1.3 (0.7–2.5)	25%	1.5 (0.8–2.8)
	CC	4%	4%	0.49 (0.08–2.7)	5%	0.3 (0.07–2.04)	5%	0.3 (0.07–2.04)
rs222859	GG	58%	50%	–	53%	–	51%	–
	GT	34%	28%	1.68 (0.91–3.1)	45%	0.6 (0.3–1.1)	43%	0.6 (0.3–1.2)
	TT	8%	22%	0.23 (0.12–0.6)*	2%	0.2 (0.04–1.1)	6%	1.3 (0.4–4.08)
rs768015399	GG	51%	48%	–	48%	–	50%	–
	GA	44%	49%	0.8 (0.4–1.4)	43%	0.8 (0.5–1.5)	46%	0.9 (0.5–1.5)
	AA	5%	3%	1.7 (0.3–7.3)	9%	1 (0.6–1.7)	4%	1.2 (0.3–4.8)

*Significant difference between patients and control groups ($P < 0.05$).

Table 3 Comparison of rs222859 different genotypes frequency between blood and testis tissues of azoospermic men

Type of mutation	Azoospermic men which had TT genotype in their blood samples	Azoospermic men which had GG and GT genotypes in their blood samples
rs222859 (Exon 1)	GG – GT – TT 13 (100%)	GG 27 (57%) GT 17 (36%) TT 3 (7%)

Msy2-null mice had an abnormally high number of apoptotic meiotic spermatocytes, and the mutant males lacked spermatozoa in the epididymis and were sterile. Their results emphasised on the major role of this protein in male fertility.

This investigation is the first report of the association between some polymorphisms of a key spermatogenesis regulator (YBX2) gene and male infertility among Iranian population. We selected our samples based on semen analysis and divided them into four groups, control, azoospermic, oligospermic and asthenospermic. Studied polymorphisms were based on Deng *et al.* (2008) and Hammoud *et al.* (2009) investigations. They sequenced all exons and introns of this gene and reported some polymorphisms in exon and intron regions of the gene. Some of these polymorphisms were significantly higher in infertile men and were correlated with sperm production and/or sperm function.

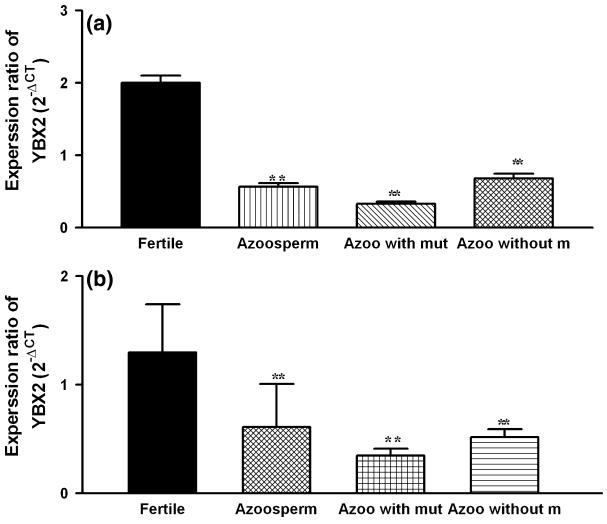

Fig. 1 Result of $2^{-\Delta ct}$ of YBX2 gene expression level relative to GAPDH (a) in blood samples of studied groups, the same letter shows significant underexpression of this gene in azoospermic patients ($P < 0.05$). (b) shows a significant underexpression of this gene in testis tissue of azoospermic patients compared to control group ($P < 0.05$). ** shows significant deference.

We studied exon 1 alterations because in other studies among the exonic regions, polymorphisms of exon 1 were higher than others. Exon 1 of this gene has a highly conserved cold-shock domain among the mammalian species and has a putative casein kinase 2 phosphorylation site (Sakthivel *et al.*, 2008). Our results showed that the frequency of only one polymorphism, TT genotype in rs222859 polymorphism, was significantly higher in azoospermic men when compared to control, oligospermic and asthenospermic men. Deng *et al.* (2008), unlike our study, noticed no significant difference in TT genotype of exon 1 between control and infertile men among the Chinese population. Deng *et al.* (2008) showed that frequency of C allele in rs8069533 T>C polymorphism was higher in control group compared to infertile men, and they concluded that this allele had a protective effect in fertility, but in our study, there was no significant difference between control groups and patients.

Polymorphisms of exon 1, which are reported in Hammoud *et al.* (2009) study, showed no significant difference between the studied groups in our study. Our results also showed that in rs222859 polymorphism, all of the patients who had mutant TT genotype in their blood samples had the same genotype in their testis tissues. GT genotype frequency was the same in blood and testis tissue, but 7% of patients which had normal GG genotype in their blood samples had TT or mutant genotype in their testis tissues.

Incoherence between blood and germ cells has been reported in different studies. In this regard, Dada *et al.* (2007) and Saktivel *et al.* (2008) have pointed out that frequency of Yq microdeletion was higher in sperm samples of patients in comparison with their blood samples. Khazamipour *et al.* (2009) showed MTHFR gene methylation in testis tissues of azoospermic patients, but they did not see this methylation in blood samples of their patients. These results reveal that screening of mutations in genes that are involved in spermatogenesis in blood samples only is not sufficient. It is better to screen sperm samples too.

In our previous investigation, we showed underexpression of this gene in testis tissues of azoospermic men with maturation arrest compared to control group (Najafipour *et al.*, 2015). In the same line to our previous study, expression evaluation in blood samples showed underexpression of this gene in azoospermic men, but in oligospermic and asthenospermic samples, we saw no under expression. In both blood and testis samples, there was no significant difference in expression between samples with and without mutation. In future studies, it is better to investigate the effect of this mutation on the 3D structure of protein. Underexpression of this gene in blood and testis samples of azoospermic men is probably

caused by other mutations in promoters and other exonic and intronic regions of this gene. We suggest screening of all the promoter, introns and exons of this gene among Iranian populations in future studies. In conclusion, the present data suggest that YBX2 gene might be associated with male infertility, and it is better to screen sperm samples mutations in addition to blood samples in future studies.

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