

New Sequence Variations in Spermatogenesis Candidate Genes

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ABSTRACT

Objective: The aim of this paper was to estimate the frequency and types of mutations in key candidate genes involved in spermatogenesis, and their potential role as a cause of azoospermia /cryptozoospermia.

Patients and Methods: The sequencing of the coding region of genes DBY, RBMY, DAZ, CDY and BPY2, excluding the promoter region, was performed in a series of 25 patients with azoospermia or severe oligozoospermia without AZF microdeletions. The exon 3 from the DAZL gene (DAL3) was also sequenced. The sequences obtained were analyzed by ProSeq, DnaSP v5 and compared with the database using Blastn and tblastx.

Results: 16 of the 25 patients showed some type of variants, such as transversions, transitions, deletions and/or insertions in the DAZ, DAZL, CDY and RBMY genes. The mutated sequences had between 97 and 99% homology with the specific protein of every gene, except the DAZL (73%) and DAZ (94%) proteins.

Conclusions: The variants found have not been described previously, suggesting they could be mutations that might affect protein function.

Keywords: Male infertility, Spermatogenesis candidate genes, New sequence variants

INTRODUCTION

It is well recognized that the regions called AZFa, AZFb and AZFc of the long arm of chromosome Y are associated with azoospermia or severe oligozoospermia, because in that region there are several candidate genes for normal spermatogenesis (Simoni *et al.* 2004; Raicu *et al.*, 2003; Sadeghi-Nejad & Farrokhi, 2007)

The long arm of the Y chromosome contains genes involved in male infertility (Stuppia *et al.*, 2000). Some of them are DAZ, CDY, BPY2, RBMY and DBY. DBY (Dead box polypeptide Y) is located in the AZFa region (Ditton *et al.*, 2004) and the RBMY (RNA-binding motif) is in AZFb (Skrisovska *et al.*, 2007; Dreumont *et al.*, 2010). On the other hand, DAZ (Deleted in Azoospermia), CDY1 (chromodomain Y1) and BPY2 (basic protein2) are located in AZFc (Sadeghi-Nejad & Farrokhi, 2007). These genes are involved in spermatogenesis. Many of these genes have not been characterized yet, and their study is not used for infertility diagnosis.

A recent study has shown that CDY2 is overexpressed in patients with Sertoli cell syndrome, suggesting that this gene is located within a segment of the Y chromosome - which is important for sperm maturation, and it is overexpressed in patients with spermatogenic maturation arrest (Stahl *et al.*, 2012). Furthermore, it was discovered that the BPY2 gene is deleted in some infertile patients, playing a probable role in male gametogenesis (Stuppia *et al.*, 2000).

The DAZ gene has an autosomal homologue, DAZL (DAZ-like), on chromosome 3p24, which is highly homologous to the DAZ gene, with 83% similarity to the coding region of the cDNA. Both genes encode RNA binding proteins. It is believed that the DAZ gene appeared some 40 million years ago during primate evolution by transposition, reamplification, and loss of the ancestral autosomal gene DAZL. It has been suggested that more than 60% of undiagnosed male infertility could be due to recessive autosomal mutations. The DAZL gene appears to be an interesting candidate for autosomal recessive infertility. To date, DAZL mutations have not been reported yet; however, a single nucleotide polymorphism (SNP) in exon 3 of the DAZL gene has been identified in patients with azoospermia or severe oligozoospermia (Poongothai *et al.*, 2009).

Our goal was to estimate the frequency and types of variants in candidate genes involved in spermatogenesis, in a series of 25 infertile men with azoospermia or severe oligozoospermia without Yq deletions or AZF microdeletions.

MATERIALS AND METHODS

Fragments of six genes (BPY2, DBY, DAZ, RBMY, CDY and DAZL) involved in spermatogenesis were sequenced from 25 patients with abnormal semen. All of them had azoospermia or severe oligozoospermia, these patients had normal karyotype and did not have AZF microdeletions in the Yq11region. The sequence involved in the coding regions of the aforementioned genes determines azoospermia or severe oligozoospermia when they are deleted (Sadeghi-Nejad *et al.*, 2007) (Table 1).

The infertile men were referred to the Association of Human Genetics from Mar del Plata by different Reproductive Medicine Centers. All patients were informed that the sequencing of such candidate genes is not a routine genetic testing in the medical practice of male infertility and they expressed their consent in written.

The DNA extraction from peripheral blood leucocytes was performed according to the protocol described by Rada & Taboada, 1998. Table 2 depicts the different primers used in the PCR reaction according to the suggestions from Krausz *et al.*, 1999 and Umeno *et al.*, 2006.

The final PCR reaction volume was 25ml constituted by 50ng of genomic DNA, 10mM dNTPs of primer 10X, 25 mM MgCl₂, and 10X Buffer. 1U of Taq polymerase was used per tube. Cycling conditions were: 1- 94° 10', 2- 94° 30'', 3- 56° 1', 4- 72° 1', 5- 72° 10'. Steps 2, 3 and 4 comprised 30 cycles. The amplified fragments were purified using the Biopur® kit and subsequently sequenced by MacroGen™. The sequences obtained were analyzed by ProSeq (Filatov, 2002), DnaSP v5 (Rozas *et al.*, 2010) and compared with the database through Blastn and tblastx (in blast.ncbi.nlm.nih.gov).

Table 1 . Details of the genes involved in spermatogenesis. Details of the genes involved in spermatogenesis and the length of the gene fragments analyzed. Quotations of the chromosomal variants described (HGVS and NCBI).

GEN	ID	NM	LENGTH (pb)	FRAGMENT DESIGNED (pb)	PROTEIN	CHROMOSOME VARIANTS DESCRIBED
BPY2	9083	_004678	1212	102	Basic Y2 testis-specific protein	Redon 2006; Perry 2008; Locke 2006
BPY2B	442867	_001002760	1206	102	Y2 testis-specific basic protein - isoform B	Redon 2006; Perry 2008; Locke 2006
BPY2C	442868	_001002761	1206	102	Basic testis-specific protein Y2 - C isoform	Redon 2006; Perry 2008; Locke 2006
RBM1A1	5940	_005058	1900	174	Proteins because of A1 RNA binding member	Redon 2006; Perry 2008; Locke 2006; Sharp 2005; Kim 2009
DAZL	1618	_001351.3	3054	130	1 and 2 isoforms DAZL3	Conrad 2010; Park 2010; Xu 2011; Teague 2010; Pang 2010
CDY1	9085	_004680.2	2373	72	Chromodomain protein specific Y1 testicular - isoforms b	Redon 2006; McCarroll 2008; Locke 2006; Campbell 2011
DBY	8653	_004660	4488	148	DDX3Y - ATP-dependent RNA helicase - isoform histone demethylase UTY 4	not found
DAZ2	57054	_001005785.2	3781	264	Protein 2 deleted in azoospermia - isoforms 2 and 3	Redon 2006; Perry 2008; Sharp 2005; Locke 2006; Matsuzaki 2009; Mc Carroll 2008
DAZ3	57055	_020364.3	3515	264	3 protein deleted in azoospermia	Redon 2006; Perry 2008; Sharp 2005; Locke 2006; Matsuzaki 2009; Mc Carroll 2008

Table 2 . Sets of primers for sequencing and size of the product. Sequences of primers used for amplification of the spermatogenesis candidate genes and the size of their respective products.

Locus	Primers	Sequence	PCR product size
DBY	Forward	5'CTTACACTTGCAGACTTTGC3'	192bp
	Reverse	5'TTTAGTGGTGGACAATCATC3'	
RBM1A	Forward	5'CGCATATAGTAATACACGAG3'	239bp
	Reverse	5'TGCTTTAATATCTGCTCGAG3'	
DAZ	Forward	5'CTGGTGACTGAATTACAATG3'	317bp
	Reverse	5'GTTCATGATGTATGTTAAGG3'	
CDY	Forward	5'TCATAACAATCCAATTGTAAGG3'	132bp
	Reverse	5'TTCTATCCCTCGGGCTGAGCTC	
BPY2	Forward	5'CAGCGTATCATAGAAAATGT3'	142bp
	Reverse	5'AGTACTTTATTTGCAGGTTCTG3'	
DAZL3	Forward	5'GAATGCTGAATTTTACTCTTGAAG3'	181pb
	Reverse	5'CTCTATACGTGGCTAGAGTTC3'	

Table 3 . Findings from the variants with the sequencing of the genes DBY, RBMY, DAZ, DAZL, CDY and BPY2. Details of variants found in the sequencing of the genes involved in spermatogenesis. In cases of deletion and/or insertion, the specific amino acid where the reading frame shift is generated. (NC_00024.9). All variants found are not reported in the international literature.

GENE	PATIENT	CHROMOSOME POSITION (pb)	FUNCTION	db SNP ALLELES	PROTEIN RESIDUE
DAZ2	5	25338454	Reading frame shift INSERT	A	Tyr [Y] Ser [S]
DAZ3	5	26952837	Reading frame shift INSERT	A	Tyr [Y] Ser [S]
RBMY	3	23710770	Non-synonymous reference	T A	Phe [F] Ile [I]
	15	23710816	Non-synonymous reference	C G	Phe [F] Ile [I]
	15	23710848	Non-synonymous reference	C G	Leu [L] Val [V]
CDY	1	27770162	Non-synonymous reference	C G	Thr [T] Arg [R]
	1	27770151	Reading frame shift INSERT	C	Gly [G] - Ser [S] Gly [G] - ALA [A]
	1	27770157	Reading frame shift INSERT	T	Ser [S] Gln [Q]
	1	27770156	Reading frame shift DELETION	A-G	Ser [S] Gln [Q]
	3 - 7 - 17 - 21 - 23 - 24	27770161	Non-synonymous reference	G A	Gly [G] Arg [R]
	21	27770160	synonymous reference	A C	Pro [P] Pro [P]
	11 - 20 - 22	27770157	Reading frame shift DELETION	G	His [H] Gln [Q]
DAZL	10	16635718	Non-synonymous reference	T G	Ile [I] Ser [S]
	12 - 18	16635641	synonymous reference	A G	[Ter[*]] [Ter[*]]
	12	16635747	Non-synonymous reference	C G	Pro [P] Ala [A]
	18	16635636	Non-synonymous reference	A G	Asn [N] Asp [D]
	18	16635673	Non-synonymous reference	A G	Lys [K] Arg [R]
	18	16635678	Non-synonymous reference	A G	Lys [K] Glu [E]
	18	16635757	Non-synonymous reference	A G	Lys [K] Arg [R]
	18	16635644	Non-synonymous reference	T G	Asn [N] Lys [K]
	18	16635656	Non-synonymous reference	T G	Tyr [Y] [Ter[*]]
	18	16635662	Non-synonymous reference	T G	Cys [C] Trp [W]
	18	16635666	Non-synonymous reference	T A	Cys [C] Ser [S]
	18	16635692	Non-synonymous reference	C A	Cys [C] [Ter[*]]
	18	16635719	Non-synonymous reference	G T	Arg [R] Ser [S]
	18	16635671	synonymous reference	G A	Glu [E] Glu [E]
	18	16635695	synonymous reference	C G	Ser [S] Ser [S]
	25	16635681	Non-synonymous reference	A G	Asn [N] Asp [D]
25	16635695	synonymous reference	A G	Ser [S] Ser [S]	

Table 4. Types of variants found in the sequences of the gene fragments studied. Results of the sequencing of the genes studied and their relation with the type of semen abnormality.

INDIVIDUAL	SEMEN	CDY ID 9085	RBMV ID 5940	DAZ ID 57055 y/o ID57054	DAZL ID 1618	BPY2 ID 9083	DBY DAZ ID 8653
1	Azoospermia	One non-synonymous mutation Reading frame shift deletion insertion	-	-	-	-	-
2	Azoospermia	-	-	-	-	-	-
3	Azoospermia	One non-synonymous mutation	One non-synonymous mutation	-	-	-	-
4	Oligozoospermia	-	-	-	-	-	-
5	Oligozoospermia	-	-	Reading frame shift by insertion	-	-	-
6	Oligozoospermia	-	-	-	-	-	-
7	Oligozoospermia	One non-synonymous mutation	-	-	-	-	-
8	Azoospermia	-	-	-	-	-	-
9	Oligozoospermia	-	-	-	-	-	-
10	Azoospermia	-	-	-	One non-synonymous mutation	-	-
11	Azoospermia	Reading frame shift by deletion	-	-	-	-	-
12	Azoospermia	-	-	-	One synonymous mutation One non-synonymous mutation	-	-
13	Oligozoospermia	-	-	-	-	-	-
14	Oligozoospermia	-	-	-	-	-	-
15	Azoospermia	-	-	Two non-synonymous mutations	-	-	-
16	Azoospermia	-	-	-	-	-	-
17	Azoospermia	One non-synonymous mutation	-	-	-	-	-
18	Oligozoospermia	-	-	-	Ten non-synonymous mutations Three synonymous	-	-
19	Azoospermia	-	-	-	-	-	-
20	Azoospermia	Reading frame shift by deletion	-	-	-	-	-
21	Oligozoospermia	One non-synonymous mutation	-	-	-	-	-
22	Oligozoospermia	Reading frame shift by deletion	-	-	-	-	-
23	Azoospermia	One non-synonymous mutation	-	-	-	-	-
24	Oligozoospermia	One non-synonymous mutation	-	-	-	-	-
25	Azoospermia	-	-	-	One non-synonymous mutation One synonymous mutation	-	-

RESULTS

Different variants have been observed in the DAZ, DAZL, CDY and RBMY genes, corresponding to transversions, transitions, deletions and/or insertions in the gene fragment studied. Table 3 summarizes the molecular findings.

No variations in BPY2 and DBY were observed in the sequences analyzed.

From the sequencing of the 6 key candidate genes from the 25 patients, 16 had some type of variant. All patients had a single gene mutated, except one patient with azoospermia who showed mutations in two genes (CDY and RBMY) (Table 4). The mutations were found in CDY, RBMY, DAZ and DAZL. There were no variations in the BPY2 and DBY in the analyzed sequences.

CONCLUSIONS

In this series we found 16 patients with some type of mutation. The DBY gene presents as a single copy and is only detected in male germ line cells, mainly spermatogonia (Ditton *et al.*, 2004). The DBY gene fragment of 148pb appears to be the most conserved in man; however several SNPs were found, but outside of our fragment sequenced. Therefore, it would be interesting to evaluate other parts of the DBY gene sequence.

BPY2 is a family of multicopy genes, specifically expressed in spermatogonia, spermatocytes and round spermatids (Lahn & Page 2000, Tse *et al.*, 2003). The sequencing of the 102 pb fragment did not allow to find mutations.

The DAZ gene corresponds to another family of multicopy genes, expressed in spermatogonia and implicated in the development of haploid gametes (Menke *et al.*, 1997; Kee *et al.*, 2009). This gene associated with azoospermia showed a mutation in a patient with oligozoospermia with a shift in the reading frame of the fragment studied (264pb). Although it is not possible to ensure that this change generates a negative effect on fertility, it alters the protein and its function. There are only two SNPs described in the DAZ2 gene, with little information about the possible effects on spermatogenesis.

The exon 3 of the DAZL gene (DAZL3) showed transversions and transitions in the sequences of four patients, three with azoospermia and one with oligozoospermia (Table 3). The sequence obtained (130pb) from the patient with oligozoospermia had 73% of homology compared with the protein in the database, which could indicate impaired function and altered protein structure due to the presence of various synonymous and non-synonymous mutations (Table 4), but we cannot be sure whether the cause of the disorder is due to a particular mutation or to the sum of them.

The CDY gene, which is expressed in mature spermataids (Lahn *et al.*, 2002), had mutations in ten patients (10/25: 40%). All types of variants, transitions, transversions, insertions and/or deletions were observed (Table 3). Six patients (three with azoospermia and three with oligozoospermia) had the same non-synonymous mutation, with an adenine switched for guanine at position 27770161 of the Y chromosome, replacing the amino acid arginine for glycine at the CDY protein. Furthermore, three patients (two with azoospermia and one with oligozoospermia) showed a reading frame shift by deletion of a guanine in position 27770157. It is possible that these mutations affect the protein function, but proteomic studies should be carried out beforehand, to infer if these variants cause adverse effects on male fertility. Since this region presented the greatest variation in the sequences analyzed, it is likely that the terminal region of the protein encoded by this gene is more prone to mutate.

The RBMY gene belongs to a family of multicopy gene

and it is expressed only in testis (Skrisovska *et al.*, 2007; Dreumont *et al.*, 2010). Two patients with azoospermia had non-synonymous mutations (Table 4). Four SNPs have been described in this gene, but the variants found in our patients were not coincident with the ones previously reported (Table 3).

From all patients analyzed, only one had non-synonymous mutations in more than one gene (RBMY and CDY) (Table 4), which could be the cause of azoospermia. SNPs for the RBMY, DAZ2, DAZL and DBY genes have been described; however, the mutations found in the patients analyzed (Table 3) did not match the SNPs described. Existing data on sequenced genes involved in spermatogenesis are still scarce and contradictory. We need further studies that reveal the relation between new sequences with spermatid quality and the functionality of the proteins they encode.

Summarizing, the type of mutations found in the sequencing of the fragment studied in this series do not match those described in the literature. Therefore, at present they only represent new sequences that need to be confirmed with further studies before confirming whether they are normal or pathogenic variants. Regarding the fragments of the genes studied in infertile patients, DBY and BPY2 were the most conserved while the CDY gene presented the highest number of sequence variants.

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CONFLICT OF INTERESTS

No conflict of interest have been declared.

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