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ORIGINAL RESEARCH

Association of mutations in the zona pellucida binding protein 1 (*ZPBP1*) gene with abnormal sperm head morphology in infertile men

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ABSTRACT: Nearly 7% of men are afflicted by male infertility worldwide, and genetic factors are suspected to play a significant role in the majority of these patients. Although sperm morphology is an important parameter measured in the semen analysis, only a few genetic causes of teratozoospermia are currently known. The objective of this study was to define the association between alterations in the genes encoding the Golgi-associated PDZ- and coiled-coil motif containing protein (GOPC), the protein interacting with C kinase 1 (PICK1) and the acrosomal protein zona pellucida binding protein 1 (ZPBP1/sp38) with abnormal sperm head morphology in infertile men. Previous reports demonstrated that mice lacking *Gopc*, *Pick1* and *Zpbp1* are infertile due to abnormal head morphology. Herein, using our validated RNA-based method, we studied spermatozoal cDNA encoding the human *GOPC*, *PICK1* and *ZPBP1* genes in 381 teratozoospermic and 240 controls patients via direct sequencing. Among these genes, we identified missense and splicing mutations in the sperm cDNA encoding *ZPBP1* in 3.9% (15/381) of men with abnormal sperm head morphology. These mutations were not observed in 240 matched controls and the dbSNP database ($\chi^2 = 9.3$, $P = 0.002$). In contrast, statistically significant and functionally relevant mutations were not discovered in the *GOPC* and *PICK1* genes. In our study *ZPBP1* mutations are associated with abnormal sperm head morphology, defined according to strict criteria, resembling the mouse *Zpbp1* null phenotype. We hypothesize that missense mutations exert a dominant-negative effect due to altered ZPBP1 protein folding and protein:protein interactions in the acrosome.

Key words: male infertility / teratozoospermia / abnormal head morphology / *ZPBP1* cDNA mutations / sp38

Introduction

Male infertility is a multifactorial condition encompassing a variety of disorders (Matzuk and Lamb, 2002, 2008). A plethora of genetic and environmental factors can cause spermatogenic deficiencies leading to a block in spermatogenesis, defects in spermiogenesis and/or defective function of mature spermatozoa. Ultimately, structural and/or functional sperm defects may result in male infertility. In the majority of infertile men, the etiology of impaired spermatogenesis remains unknown (Guzick *et al.*, 2001). Clinical laboratory diagnosis of infertile men employs a routine semen analysis to assess the semen parameters of the ejaculate such as sperm count, motility and morphology (WHO, 2010). Sperm morphology (SM) provides important information regarding the process of spermiogenesis (the differentiation steps in spermatogenesis culminating in the production of the spermatozoa) that may ultimately impact sperm function. In general, while sperm with abnormal morphology frequently function abnormally, the converse is not true; namely, sperm with normal morphology may function abnormally. Furthermore, in the clinical andrology laboratory the molecular cause of an identified sperm defect is almost never defined. Nevertheless, defects in the sperm cell structure or function clearly underlie some types of male infertility (Barratt *et al.*, 2011).

Teratozoospermia (abnormal SM) is characterized by morphological defects in spermatozoa and is defined as an ejaculate with <4% normal spermatozoa (WHO, 2010) as defined according to strict

morphologic criteria first described by Kruger *et al.* (1987, 1988). Assessment of SM has become increasingly strict since the 1950s. Routine classification criteria and evaluation procedures were developed by Eliasson in 1971 (Eliasson and Treichl, 1971) and these criteria have changed with the successive editions of the publication on semen evaluation by the World Health Organization (WHO, 1992, 2010). According to the strict criteria defined by Kruger *et al.* (1988), a normal spermatozoon has specific dimensions with an oval head configuration featuring a smooth shape and no defects in midpiece and tail. SM defects are generally classified as head, neck, midpiece and/or tail defects. Examples of teratozoospermia include globozoospermia (round-headed sperm lacking an acrosome), macrocephaly (large-headed sperm), two-headed or two-tailed sperm, bent midpiece or the presence of a cytoplasmic droplet (Dam *et al.*, 2007a; WHO, 2010).

Genetic studies of infertile men designed to detect familial segregation of subfertility markers using linkage analyses are hindered by the absence of large affected pedigrees and a high degree of clinical and genetic heterogeneity (Hackstein et al., 2000; Matzuk and Lamb, 2008). Accordingly, much of our understanding of the genes required for male fertility comes from studies of mouse models. Targeted gene disruption strategies in mice have revealed hundreds of genes causing abnormal spermatogenesis and/or fertility defects; however, translation of these findings to define a specific etiologies of infertility is not always successful (Matzuk and Lamb, 2008; Yatsenko *et al.*, 2010). A genetic cause of teratozoospermia is suggested by the discovery of this phenotype in several mouse models (Matzuk and Lamb, 2008). Mutations in the aurora kinase C, protamine 1 and *SPATA16* genes are examples of gene mutations associated with teratozoospermia in humans (Iguchi *et al.*, 2006; Dieterich *et al.*, 2007, 2009; Dam *et al.*, 2007b).

Golgi-associated PDZ- and coiled-coil motif containing protein (GOPC) is involved in Golgi vesicle trafficking and is essential for acrosome formation in developing spermatids localizing to the proacrosomal vesicles (Yao *et al.*, 2002; Ito *et al.*, 2004). Mice homozygous for a null mutation in *Gopc* are infertile as a result of acrosome formation defects and abnormalities with mitochondrial arrangement. *Gopc* null mouse sperm are characterized by rounded head morphology and occasional tail defects similar to that seen in teratozoospermic men (Yao *et al.*, 2002). Protein interacting with C kinase 1 (PICK1) is associated with the Golgi and membrane proteins and is necessary for normal acrosome formation (Xu and Xia, 2006; Xiao *et al.*, 2009). PICK1 encodes a protein with PDZ and BAR domains and interacts with over 40 proteins (Xu and Xia, 2006). PICK1 is expressed in various tissues, demonstrating the highest protein expression in the brain and testis (Xia *et al.*, 1999). *Pick1*$-/-$ mice are infertile (Xiao *et al.*, 2009), and their reproductive phenotype is similar to *Gopc*$-/-$ mice (Yao *et al.*, 2002).

Zona pellucida binding protein 1 (ZPBP1/sp38), which localizes to the acrosomal membrane and likely interacts with multiple acrosomal matrix proteins, was named for its function—binding to the oocyte zona pellucida (ZP) protein following the acrosome reaction. Using an *in silico* search for germ cell-specific genes in mice, we discovered *Zpbp1* and its novel paralog, *Zpbp2* (*sp17*) (Katoh, 2003; Lin *et al.*, 2007). Indeed, the mouse ortholog ZPBP1 (sp38), is required for sperm acrosome formation, compaction and sperm:oocyte binding (Mori *et al.*, 1993, 1995; Lin *et al.*, 2007). Mice homozygous for a null mutation in *Zpbp1* have acrosome fragmentation, disrupted Sertoli-spermatid junctions and a defective sperm head morphology with characteristics reminiscent of teratozoospermia in infertile men with a type of head defect (Lin *et al.*, 2007).

Using a non-invasive approach that exploits mRNA from germ cells, and is capable of efficiently detecting splicing and missense mutations in a high-throughput fashion (Yatsenko *et al.*, 2006), we tested the hypothesis that *GOPC*, *PICK1* and *ZPBP1* gene defects are associated with abnormal SM. We screened 381 teratozoospermic patients and 240 controls for mutations in the *GOPC*, *PICK1* and *ZPBP1* genes. Novel mutations were found in some infertile men in *ZPBP1*, but not in *GOPC* and *PICK1*. These mutations were associated with defects in sperm head morphology in infertile men.

Materials and Methods

Patient recruitment and classification

The study was approved and oversight provided by the Institutional Review Board at Baylor College of Medicine (IRB # H-12083 and H-19753). Male patients in this study were evaluated for infertility by Larry I. Lipshultz, M.D. in the Division of Male Reproductive Medicine and Surgery in the Scott Department of Urology at Baylor College of Medicine. Residual excess semen samples to be discarded were collected for research after the samples were de-identified. The patients were anonymous to the investigators. Semen samples were classified as teratozoospermic based upon the semen analysis performed according to WHO guidelines (WHO, 1999). Using Kruger's strict criteria, abnormal SM is defined as semen in which $\leq$4.4% of the sperm show normal head, tail and/or midpiece/neck morphology. For this study, this lower limit is the statistically determined value obtained for men with proved fertility in the clinical andrology laboratory, but this lower limit is also consistent with the new fifth edition of the WHO guidelines for semen analysis (WHO, 2010).

When multiple semen analyses were performed, the semen parameters (i.e. count, motility, etc.) represent the single result closest to the mean value. The control population in this study ($n = 240$) is comprised of infertile men with a normal SM of $\geq$4.4% ($n = 90$) and infertile normozoospermic men with a sperm count $>60 \times 10^6$/ml some of whom were tested for SM ($n = 150$; Supplementary data, Table S1). These normozoospermic men with randomly tested strict morphology ($\geq$4.4%) served as the morphology controls (Supplementary data, Table S1). In addition, we analyzed results in dbSNP database from the 1000 genomes project (1000 Genomes Project Consortium, 2010). Procedures for semen analysis (e.g. cell count, motility and strict morphological analysis of spermatozoa) are described in the WHO guidelines (WHO, 1999). All patients underwent a complete history and physical, including evaluation of possible endocrine causes of male infertility. Teratozoospermic men were not tested for chromosomal aberrations and Y chromosome aberrations in accordance with the AUA/ASRM guidelines for the evaluation of the infertile male (AUA, 2010). Men with known causes of infertility, such as endocrine defects, cryptorchidism, chromosome abnormalities and Y chromosome deletions, were excluded from the study. Because patients' samples were de-identified, information on IVF/ICSI outcome could not be obtained.

RNA and DNA extraction, RT–PCR, sequencing and sequence analysis

Semen RNA and DNA extraction was performed as previously described (Yatsenko *et al.*, 2006). Briefly, freshly ejaculated semen

was liquefied for 30 min at 37°C. After all clinical testing was completed and the samples de-identified, the semen was thoroughly mixed with equal volume of Sperm Washing Medium (Irvine Scientific, Santa Ana, CA, USA), and the suspension was centrifuged for 10 min at 652*g* at room temperature. The sperm were resuspended and lysed with 1 ml of TriZOL reagent (Invitrogen, Carlsbad, CA, USA). Total RNA and DNA were extracted and resuspended in DEPC-treated water and sterile 10 mM Tris 1 mM EDTA solution, pH 7.0 (Ambion, Austin, TX, USA), respectively.

For first strand cDNA synthesis, we used M-MLV Reverse Transcriptase (Invitrogen) with random primers and total spermatozoal RNA (Yatsenko *et al.*, 2006). PCR for *GOPC* and *PICK1* were performed with KAPA HiFi HotStart DNA Polymerase and 40 ng of cDNA according to the manufacturer's protocol (Kapa Biosystems, Woburn, MA, USA). PCR for *ZPBP1* was performed with JumpStart RedTaq DNA polymerase and 40 ng of cDNA or DNA according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO, USA), we used 37 cycles and Ta 60°C. Three PCR primers were designed for amplification of three cDNA fragments of *GOPC* (NM_020399.3) and three cDNA fragments of *PICK1* (NM_012407.3) each, and two PCR primers were designed for amplification of two cDNA fragments of *ZPBP1* (NM_001159878; Supplementary data, Table S2). Primer sequences for amplifying *ZPBP1* exons and PCR conditions are shown in Supplementary data, Table S3. The PCR products were sequenced with BigDye V3.1 sequencing reagent and run on an ABI Prism Sequencer 3130XL (Applied Biosystems, Foster City, CA, USA). Sequence analyses were performed with Sequencher 4.2 (Gene Codes, Ann Arbor, MI, USA). Multiple protein alignments were made via ClustalW (http://www.ebi.ac.uk/clustalw). Novel single nucleotide polymorphisms (SNPs) were crosschecked against dbSNP database (build 37.1).

To test for an association between teratozoospermia in patients and the presence of SNPs in *ZPBP1*, the frequencies of the alleles were compared between the group with abnormal SM and the control group (normozoospermia and normal SM) separately and combined, using a likelihood ratio χ^2 test. All tests were two-sided with an alpha level of 0.05 considered to indicate statistical significance, and unadjusted *P* values were reported. All statistical analyses were performed using JMP Start Statistics software (SAS Institute Inc., Cary, NC, USA).

Results

To test the hypothesis that *GOPC*, *PICK1* and *ZPBP1* gene defects are associated with abnormal SM, we screened for *GOPC*, *PICK1* and *ZPBP1* gene mutations in 381 teratozoospermic patients ($\leq$4.4% of sperm with normal morphology) and 240 controls. We used our non-invasive and cost-effective approach that exploits mRNA isolated from ejaculated spermatozoa. This method allows detection of both splicing and missense mutations in a high-throughput fashion (Yatsenko *et al.*, 2006).

We amplified the *GOPC* and *PICK1* open reading frames and flanking regions, covering 1389 and 1248 bp, respectively, using three overlapping PCR fragments. Sequence analysis identified one novel *GOPC* heterozygous alteration (p.L123V) and one novel *PICK1* heterozygous alteration (p.Q26E) in 381 patients (Table I). Statistical estimation for the frequencies of each novel SNP [0.3% (1/381)] evaluated, indicated a lack of statistical significance for the identified changes. Further computational analysis indicated that the amino acid alterations resulting in these changes will have no effect on the structure and function of the GOPC and PICK1 proteins. We also identified two frequent non-coding SNPs: in *GOPC*, a cDNA change c.695G>A (p.K147K, rs41302045, 3/381, 0.78%) and in *PICK1*, a cDNA change c.1435C>T (p.Y349Y, novel SNP, 3/381, 0.78%) (Table I). The frequency of these non-synonymous alterations was not tested in a control population.

We efficiently amplified the *ZPBP1* 1177 bp open reading frame (NM_001159878.1) via two overlapping cDNA fragments (Fig. 1). Sequence analysis identified 16 *ZPBP1* alterations in 3.9% (15/381) of the patients (Table II, Fig. 1). Analysis of the gene in the dbSNP database did not identify these *ZPBP1* changes as polymorphic, and these alterations were not present in the 240 control men, providing strong statistical evidence for these mutations to be deleterious ($\chi^2 = 9.3$, $P = 0.002$). These teratozoospermic patients are characterized by abnormal head morphology (for these men, $\geq$90% sperm analyzed were abnormal) (Table II), resembling the abnormal head morphology phenotype of *Zpbp1* mouse knockout model. Six of the 15 patients with novel mutations were oligoteratozoospermic.

Table I GOPC and PICK1 SNPs identified by analysis of 381 teratozoospermic patients.

Patient	Vol. (ml)	Count $\times 10^6$/ml	Mot %	FP	SM % normal	Head % defects	Neck % defects	Tail % defects	cDNA change	Protein	Zygocity	dbSNP Acc. #
GOPC												
1	2.5	4.5	40	2.5	1	98	1	0	621C>G	L123V	hetero	novel
2	1	34	55	2.5	3	90	6	1	695G>A	K147K	hetero	rs41302045
3	4.6	11	60	2.5	0	100	0	0	695G>A	K147K	hetero	rs41302045
4	3.5	6.5	50	2.5	0.5	89.5	10	0	695G>A	K147K	hetero	rs41302045
PICK1												
5	2.5	47.5	75	3	1	95.5	2.5	1	466C>G	Q26E	hetero	novel
6	4	50	45	2.5	0	100	0	0	573C>T	D61D	hetero	rs117707976
7	3	55	75	3	0	88	12	0	789C>T	T133T	hetero	novel
8	5.5	14	70	2.5	3	86	9.5	1.5	1437C>T	Y349Y	hetero	novel
9	2	25.5	60	2.5	3	91	6	0	1437C>T	Y349Y	hetero	novel
10	2	21	60	2.5	1	92	7	0	1437C>T	Y349Y	hetero	novel

Count, sperm count; Mot %, percent motile; FP, forward progression; SM, strict morphology (percent of spermatozoa with normal morphology defined by Kruger's strict criteria); hetero, heterozygous; homo, homozygous.

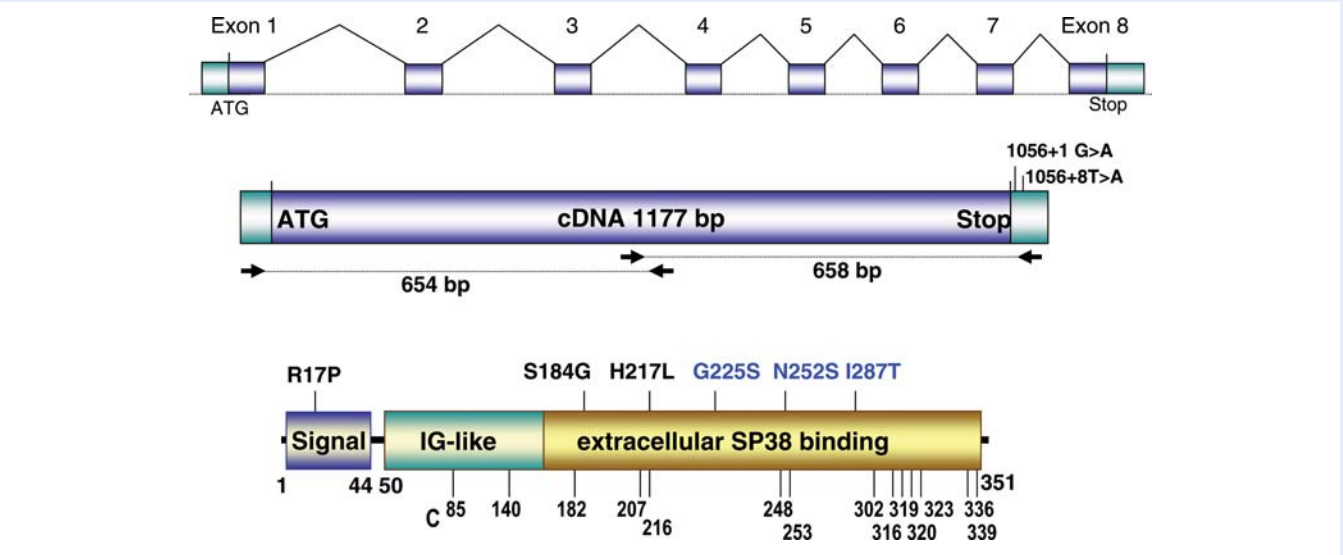

Figure 1 *ZPBP1* gene and cDNA and point mutations identified in teratozoospermic patients. *ZPBP1* gene, cDNA amplification scheme and mutations in the 3′UTR are shown in the upper two diagrams. The position of the missense mutations in the ZPBP1 protein is shown in the lower diagram. The predicted protein structure is based on Interproscan, Prosite, Pfam and Blocks algorithms. ZPBP1 has a signal peptide (aa 1–44) and an extracellular region consisting of two predicted overlapping domains, an immunoglobulin-like (IG; aa 50–156) domain and an sp38 domain (aa 86–351). Homozygous mutations are depicted in blue, and heterozygous mutations are depicted in black. The positions of the cysteines (C) are shown at the bottom.

Table II ***ZPBP1*** **mutations identified by analysis 381 teratozoospermic men.**

Patient	Vol. (ml)	Count $\times 10^6$/ml	Mot %	FP	SM % normal	Head % defects	Neck % defects	Tail % defects	cDNA change	Protein	Zygocity
1	1.5	225	45	3	2	64	7	27	**c.50G>C**	R17P	hetero
2	4	55	35	2.5	3	95	2	0	**c.50G>C**	R17P	hetero
3	4	44	75	3	4	90	4	2	**c.550A>G**	S184G	hetero
4	1	28	55	3	1	96	2	1	c.650 A>T	H217L	hetero
5	4	13	35	2	1	90	3	6	c.673G>A	G225S	homo
5	4	13	35	2	1	90	3	6	c.860T>C	I287T	homo
6	3.5	5.5	35	2.5	0	98	1	1	c.755A>G	N252S	homo
7	3	52.5	75	3	1.5	92.5	6	0	**c.1056+1G>A**	3′UTR	hetero
8	5.5	31	70	2.5	2	94.5	2.5	1	**c.1056+8T>A**	3′UTR	hetero
9	3.5	43	60	3	4	89	3	4	spl ex5–7 del	del158 aa	~50%
10	5	19	55	2.5	1	95.5	3	0.5	spl ex6–7 del	del 85 aa	~90%
11	3	13	65	2.5	2	98	0	0	spl. ex7 del	FS	~90%
12	2	7	55	2.5	3.5	95.5	1	0	spl. ex7 del	FS	~50%
13	7	14	40	2.5	3.5	83.5	12	1	spl. ex7 del	FS	~50%
14	1	15	15	2	0.5	97	1	1.5	spl.ex7a, ins144 (*Alu*)	ins 48aa	~50%
15	2	27.5	60	2.5	0	93	7	0	spl.ex7a, ins144 (Alu)	ins 48aa	~50%

Mutations shown in bold were analyzed and confirmed by analysis of genomic DNA from sperm. Semen analysis (WHO, 1999) results for teratozoospermic patients with identified *ZPBP1* genetic defects are shown. This analysis includes sperm count, motility and strict morphology with three subcategories (percentile of head, neck and tail defects). Count, sperm count; FP, forward progression; SM, strict morphology (percent of spermatozoa with normal morphology defined by Kruger's strict criteria); nt, nucleotides; hetero, heterozygous; homo, homozygous.

Missense mutations were present in six patients. Seven patients had gene defects predicted to cause splicing defects in the *ZPBP1* mRNA, and two infertile men had mutations in the 3′UTR (Table II and Figs 1 and 2). The missense changes were distributed throughout the *ZPBP1* coding region. Two patients showed a predicted p.R17P change in the ZPBP1 signal peptide; however, prediction programs suggest that the

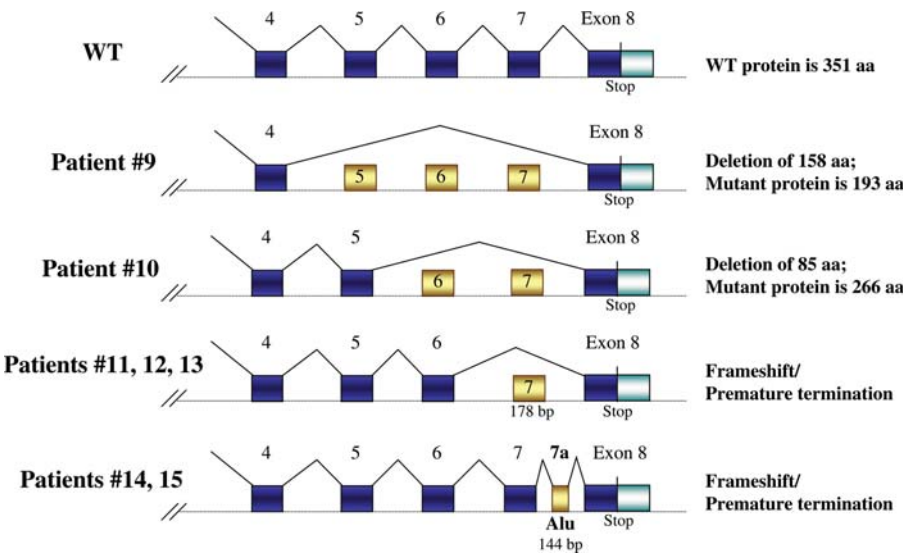

Figure 2 Schematic diagrams for *ZPBP1* splicing mutations identified in teratozoospermic patients. The upper diagram depicts the normal splicing pattern; the middle schematics show abnormal splicing defects of *ZPBP1* exons 5–7 (patient 9), exons 6–7 (patient 10) and exon 7 (patients 11, 12, and 13) resulting in in-frame deletions or frameshifts as shown. The bottom schematic shows inclusion of exon 7A that includes partial retention of an *Alu* repeat.

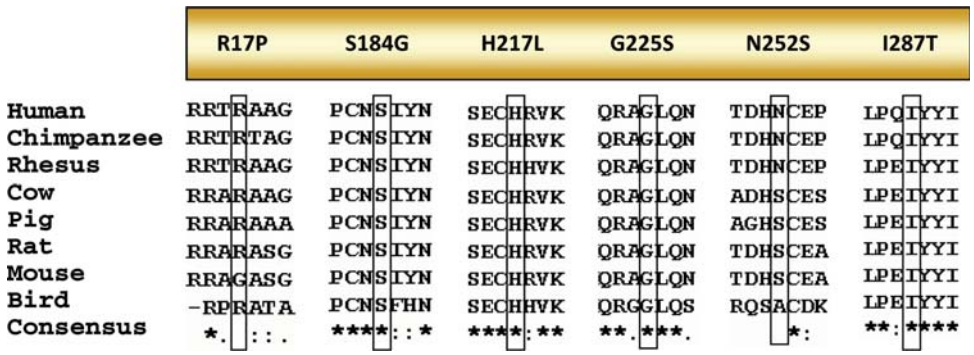

Figure 3 Evolutionary conservation of amino acids affected by missense mutations. Alignments of species' ZPBP1s reveal evolutionary conservation of amino acids affected by missense mutations among mammalian and bird orthologs. The mutant alleles are boxed.

ZPBP1 signal peptide would continue to be cleaved after amino acid 44. In addition, the arginine at position 17 is not 100% conserved between several species (Fig. 3). Likewise, at position 252, an asparagine is present in primates, whereas a serine is present in other mammals and birds suggesting that the p.N252S change does not impart a major effect on the protein structure despite its 'homozygous' presence in patient #5.

The other four missense mutations identified (p.S184G, p.H217L, p.G225S and p.I287T) alter amino acids that are 100% conserved in mammals and birds. In the mature (processed) portion of the ZPBP1 sequence, there are 15 cysteines (Fig. 1) and 3 putative *N*-linked glycosylation sites (N-X-S/T) at asparagine located at positions 114, 187 and 340. Using two independent prediction algorithms, SIFT 2.0 and PolyPhen, the missense amino acid alterations in the mature region are predicted to cause significant effects on the ZPBP1 protein structure by at least one algorithm.

The p.G225S and p.I287T mutations (present as a double mutation in one patient) are observed as 'homozygous' mutations indicating that these are true homozygous defects in the genome or that they are present in one *ZPBP1* allele and the second *ZPBP1* allele is not expressed or absent. In contrast, the p.H217L and p.S184G mutations are heterozygous mutations, each in one patient. These mutations are present in the cysteine-rich C-terminus of the ZPBP1 protein with the p.H217L mutation adjacent to the fifth cysteine in the ZPBP1 sequence (position 216) and the p.S184G change in close proximity to cysteine 182; these mutations are predicted to disrupt intramolecular disulfide bond formation of the ZPBP1 protein, an effect that is expected to disrupt protein function (Fig. 1). These patients exhibit

high levels of sperm head defects with minimal or no neck and tail defects. Accordingly, these missense mutations may act in a dominant-negative manner and/or as null mutations in the four men who express these alleles.

We also identified seven teratozoospermic patients with splicing defects (≥50% abnormal product relative to the normal RT–PCR product) that are predicted to lead to large disruptions of the protein (Table II, Fig. 4). Skipping *ZPBP1* exons 5, 6 and 7 is predicted to result in in-frame deletions of 158 amino acids (17 kDa), skipping of exons 6 and 7 is predicted to cause an 85 amino acid deletion (9 kDa) and omission of exon 7 is predicted to cause a frameshift with truncation of the protein (Fig. 2). An insertion of abnormal exon 7a, containing 144 bp segment of an *Alu* repeat, is predicted to introduce a premature stop codon (Fig. 2). Since these splicing defects delete regions of the cysteine-rich region of ZPBP1, we anticipate that these mutations would not permit ZPBP1 disulfide bond formation and protein folding and would impair ZPBP1 function. With the exception of patient 13, the other 7 patients with splicing defects demonstrate ≥89% sperm head defects and ≤8% neck and tail defects (Table II).

Two teratozoospermic patients have single heterozygous nucleotide alterations in the *ZPBP1* 3′UTR of unknown significance (Fig. 1).

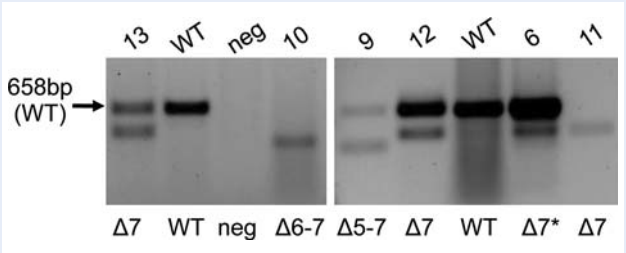

Figure 4 Detection of splicing defects in the *ZPBP1* gene in teratozoospermic patients. Gel electrophoresis of RT–PCR products are shown. WT denotes wild-type sample and neg denotes negative control. Δ5–7, Δ6–7 and Δ7 denote splicing defects in the patients as shown that result in omission of exons 5–7, 6–7 and 7, respectively. * denotes patient with a mild abnormal splicing product.

Lastly, we identified several rare *ZPBP1* SNPs in patients and controls (Table III). We also found a low-abundance splicing alterations in some patients and controls at comparable frequencies [i.e. low expression of the exon 7 deletion (25–30% of the wild-type RT–PCR product) was detected in ∼14% of teratozoospermic patients and ∼16% of controls (Table III)].

Discussion

Male infertility affects ∼4 million men in the USA (Abma *et al.*, 1997; Anderson *et al.*, 2009). Today, investigators suspect that perhaps 50% or more of these infertile men have a genetic cause for their infertility (Matzuk and Lamb, 2008). While some infertile men have cytogenetic defects (Retief *et al.*, 1984; Bourrouillou *et al.*, 1985), a majority of these genetic factors that underlie male infertility remain undiagnosed. Another issue to consider is that while infertile men can be normozoospermic, closer laboratory inspection can reveal the presence of subtle sperm defects (i.e. ultrastructural morphology defects causing immotile cilia, globozoospermia causing failure of the acrosome reactions, etc.). These defects and other as yet unidentified genetic defects are treated with ICSI together with *in vitro* fertilization. ICSI is associated with an increase in congenital birth defects, perinatal complications, growth delay, general health problems and disturbed normal genomic imprinting (Hansen *et al.*, 2002; Olson *et al.*, 2005; Knoester *et al.*, 2008; Wen *et al.*, 2010; Yan *et al.*, 2011). This fact, along with the limitations of current methods for diagnosis of male infertility, suggests that affected males may be transmitting unrecognized male infertility-causing mutations to their offspring. Accordingly, the development of improved diagnostic tools is paramount for couples seeking treatment for their infertility (WHO, 1987; Lipshultz and Lamb, 2007).

Here, we tested the hypothesis that mutations in *GOPC*, *PICK1* and *ZPBP1* are associated with abnormal SM and specifically head defects. We did not identify any homozygous mutations in *PICK1* or *GOPC* in 381 teratozoospermic patients. A previous study of three globozoospermic infertile men, identified a single homozygous mutation in one patient in *PICK1* (p.G393R) and no mutations in *GOPC*. Both parents were heterozygous for the *PICK1* mutation and were fertile (Liu *et al.*, 2010). This previous study and our current study indicate that

Table III Rare ZPBP1 SNPs and polymorphic splicing variants identified in teratozoospermic patients and controls.

#	DNA, nt	Protein, aa	Zygosity	Patients freq	Controls freq	dbSNP
1	74G>C	R25P	hetero	1/381 (0.3%)	0	rs61696422
2	312G>A	G104G	hetero	1/381 (0.3%)	0	
3	375T>C	L125L	hetero	1/381 (0.3%)	1/240 (0.4%)	rs76027725
4	498G>A	E166E	hetero	1/381 (0.3%)	1/240 (0.4%)	
5	645T>C	L215L	homo	1/381 (0.3%)	0	
6	732A>G	G244G	homo	2/381 (0.5%)	0	
7	735C>T	P245P	hetero	0	1/240 (0.4%)	
8	1047C>A	T349K	hetero	0	1/240 (0.4%)	
9	ex7 del	SpI	low level	52/381 (14%)	39/240 (16%)	
10	ex6–7 del	SpI	low level	8/381 (2%)	6/240 (2.5%)	
11	ex5–7 del	SpI	low level	4/381 (1%)	2/240 (0.8%)	

mutations in *PICK1* and *GOPC* are rare in the infertile population. Alternatively, *PICK1* and *GOPC* may be critical for human spermatozoon maturation; aberrations in these genes may not be tolerated. Our studies and those of others suggest that high-throughput technologies (e.g. next generation sequencing) should be applied for such heterogeneous conditions such as male infertility.

Previously, we showed that disruption of *Zpbp1* led to male sterility secondary to abnormal sperm head morphology and no progressive motility (Lin *et al.*, 2007). Ultrastructural studies demonstrated that absence of ZPBP1 prevents proper acrosome formation, resulting in acrosome fragmentation as well as disruption of the Sertoli-spermatid junctions (Lin *et al.*, 2007). Recent studies suggest that ZPBP1 and other yet unknown acrosomal proteins play important roles in the intricate fertilization process including the acrosome reaction, ZP recognition, ZP binding and ZP penetration (Tardif *et al.*, 2010; Jin *et al.*, 2011). In this study, we identified homozygous and heterozygous missense mRNA alterations and high-level splicing mutations of *ZPBP1* mutations in 15 out of 381 (3.9%) teratozoospermic men tested ($P \leq 0.002$). *ZPBP1* alterations in teratozoospermic patients may contribute to the dysmorphic shape of the sperm head. Although the null ZPBP1 mouse model demonstrates head morphology and forward progression defects, we observed that teratozoospermic patients have abnormal sperm head morphology, implying a different effect of presumably 'hypomorphic' mutations on protein function.

We noted that a patient with the splicing defect causing the absence of exon 7 (#13) has milder morphology defects (84% sperm head defects). This observation could be explained by a residual (~50%) level of normal functional ZPBP1 that partially compensates for the abnormal protein. In contrast to the teratozoospermic patients, *ZPBP1* point mutations were not present in control men or the dbSNP database; we identified several rare *ZPBP1* SNPs and polymorphic weakly expressed splicing mutations in controls and believe that they have mild or negligent/neutral effect on ZPBP protein function and SM phenotype. In addition, weakly expressed splicing mutations in 14–16% of patients and controls suggest that abnormal morphology is a quantitative trait.

The low frequency of *ZPBP1* mutations in the teratozoospermic population (~4%) is not unexpected given the predicted complexity and heterogeneous nature of this defect. Importantly, ~90% of germ cells with mutations in the human *ZPBP1* gene have a dysmorphic appearance of the sperm head. The abnormal head morphology in these cases may be due to absence of functional ZPBP1 proteins or due to dominant-negative effects of the mutant ZPBP1 proteins with other acrosomal proteins that are required for formation of the sperm head. The mutations identified may also have a dominant-negative effect. The mechanism by which disruption of ZPBP1 leads to teratozoospermia in men is under further investigation. Importantly, other proteins involved in spermiogenesis may be responsible for synergistic effects as well.

In summary, analysis of full-length mRNAs isolated from transcriptionally inert human spermatozoa revealed multiple, novel germline splicing and missense mutations in the *ZPBP1* gene but no novel mutations in *PICK1* or *GOPC*. The *ZPBP1* variants were associated with sperm head morphology defects in 3.9% of teratozoospermic men. These findings are reminiscent of the phenotype observed in our *Zpbp1* knockout mouse model, which displays male infertility due to abnormal sperm head morphology (Lin *et al.*, 2007). Therefore, we hypothesize that ZPBP1 is important for spermiogenesis and sperm:egg binding in mammals. Both mouse and human studies suggest that mutations in *ZPBP1* are associated with teratozoospermia, providing a previously unrecognized underlying etiology for a subgroup of infertile men with sperm head morphology defects. Genetic diagnosis of men with defective human spermiogenesis might provide new insights into the cause of abnormal sperm head morphology in men with teratozoospermia.

Accession numbers

GenBank accession number for the human *GOPC* gene is NM_020399.3, for the *PICK1* gene is NM_012407.3, and for the *ZPBP1* gene is NM_001159878.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

Authors' roles

A.N.Y., D.S.O., A.R., D.J.L. and M.M.M. designed the study and prepared the manuscript, to which other authors added their comments. Laboratory work was undertaken by A.N.Y., D.S.O., A.R., P.A.A., R.C. and L.J.M.; R.C. and L.J.M. collected and processed samples.

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