**DPY19L2 gene mutations are a major cause of globozoospermia: identification of three novel point mutations**

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**ABSTRACT:** Globozoospermia, characterized by round-headed spermatozoa without acrosomes, is a rare and severe teratozoospermia causing primary male infertility. Homozygous DPY19L2 deletions have been identified as the main cause of globozoospermia, blocking sperm head elongation and acrosome formation. Several previous studies showed a very different prevalence of DPY19L2 gene deletions among globozoospermic patients in cohorts with different sample sizes and in different ethnic background. And all the patients previously analyzed were mainly of European, North African and Middle Eastern origins. So far, only 11 different point mutations of the DPY19L2 gene have been reported. To investigate the prevalence of DPY19L2 gene mutations in Chinese patients with globozoospermia and whether we can identify new sequence variants in this study, we recruited a total of 16 globozoospermic patients. Excluding one of two brothers, molecular analysis for deletions and mutations in the DPY19L2 gene was performed on 15 genetically independent individuals. Four of the 15 genetically independent patients with globozoospermia were homozygous for the DPY19L2 deletion, 5 were homozygous for a point mutation including a nucleotide deletion c.1532delA (two patients), a multi-mutation consisting of a nucleotide deletion c.1679delT and a two-nucleotide deletion c.1681_1682delAC (c.[1679delT; 1681_1682delAC]) (one patient), a recurrent missense mutation R290H (one patient) and a missense mutation L330P (one patient). One additional patient had a heterozygous deletion in one allele but with no mutation identified in another allele. Overall, 60% of the patients (9/15) have a sequence variant of DPY19L2 in both alleles. This study confirms that the DPY19L2 mutations are the major cause of globozoospermia. Three novel point mutations and a recurrent missense mutation were found in this study, further broadening the spectrum of DPY19L2 mutations.

**Key words:** DPY19L2 / genetics of infertility / globozoospermia / point mutations

**Introduction**

Globozoospermia (MIM 102530) is a rare (incidence <0.1%) but severe form of teratozoospermia causing primary male infertility, characterized by the production of a majority of the round-headed spermatozoa lacking an acrosome (Dam et al., 2007a; Harbuz et al., 2011). The characterized teratozoospermia was mostly described in family cases, pointing to a genetic contribution to this disorder (Dam et al., 2007a). Three brothers of an Ashkenazi Jewish family affected with total globozoospermia were found to carry a homozygous mutation of SPATA16 (MIM 609856), encoding a protein localized in the Golgi vesicles and proacrosomal vesicles which are transported to the acrosome in spermatids during spermiogenesis (Dam et al., 2007b). However, no other SPATA16 mutations were identified in 29 other affected men with globozoospermia, indicating that SPATA16 was not the main locus associated with globozoospermia (Dam et al., 2007b). Recently, two studies identified that most cases in men with complete globozoospermia were caused by a recurrent homozygous deletion of the totality of the DPY19L2 gene (MIM 613893) (Harbuz et al., 2011; Koscinski et al., 2011), encoding a transmembrane protein necessary for sperm head elongation and acrosome formation (Pierre et al., 2012). This DPY19L2 deletion was again identified in unrelated patients and recognized as a major cause of globozoospermia (Coutton et al., 2012; Elimati et al., 2012). The mechanism underlying this recurrent deletion is most probably a non-allelic homologous recombination (NAHR) between the flanking low-copy repeats (LCRs) (Koscinski et al., 2011). So far, there have been nine breakpoints (BPs) for the DPY19L2 NAHR-driven deletion characterized in 27 patients from 9 different geographic regions, indicating that the deletions result from recurrent events linked to the specific genomic architectural feature of...
this locus rather than from a founder effect, even a recent one (Koscinski et al., 2011; Elinati et al., 2012).

It seems that globozoospermia shows some phenotypic and genetic heterogeneity (Dam et al., 2007a). However, no clear correlation between phenotype and genotype has been established. Therefore, the preference of gene analyzed in the molecular diagnosis for globozoospermia may depend on the prevalence of the gene mutations in the affected individuals. Although four previous studies demonstrated the DPY19L2 deletion as the major cause of globozoospermia, a large difference was observed in the prevalence of this deletion (range 44–84%) among globozoospermic patients in cohorts with different sample sizes and in different ethnic background (Harbuz et al., 2011; Koscinski et al., 2011; Coutton et al., 2012; Elinati et al., 2012). And all the previously analyzed patients were mainly of European, North African and Middle Eastern origins. To fully assess the contribution of DPY19L2 in globozoospermia, DPY19L2 point mutations were also sequenced in the DPY19L2 non-deleted patients in two studies (Coutton et al., 2012; Elinati et al., 2012). So far, only 11 different point mutations of the DPY19L2 gene have been identified, and by fine genotyping of DPY19L2 in globozoospermic patients, the differences of phenotypes can be compared among those patients with homozygous deletion, those with point mutations and those with no identified DPY19L2 mutations. However, to date, no significant phenotypic difference has been observed (Coutton et al., 2012).

In the present study, we aimed to fully investigate the prevalence of DPY19L2 mutations in a large cohort of globozoospermic patients of Chinese origin and to screen for new DPY19L2 mutations in a large cohort of globozoospermic patients of Citic-Xiangya, P.R. China. All subjects were natives of China. The patients were unrelated apart from two brothers. Thereafter, we included 15 genetically independent patients in this study. We confirmed that DPY19L2 gene mutations are the main cause of globozoospermia in this group of Chinese patients. We identified three novel mutations and discussed the possible effects on the structure and function of the DPY19L2 protein. These new findings would broaden the mutation spectrum of DPY19L2.

Materials and Methods

Patients and control individuals
Apart from 3 patients described in our previous study (Liu et al., 2010), 16 additional patients including 1 affected individual from a consanguineous family, 2 brothers and 13 sporadic affected individuals were recruited between January 2007 and September 2012. Patients were included when they were described to have 100% round-headed spermatozoa.

All 16 patients consulted for primary infertility in Reproductive and Genetic Hospital of Citic-Xiangya, P.R. China. All subjects were natives of China. The patients were unrelated apart from two brothers. Therefore, we included 15 genetically independent patients in this study. None of the patients had an abnormal somatic karyotype.

A total of 100 unrelated, anonymous, native male donors were screened by sequencing analysis of DPY19L2. All patients, family members and anonymous male donors gave their informed consent, and all national laws and regulations are respected. This study was approved by the ethical committee of Reproductive and Genetic Hospital of Citic-Xiangya, P.R. China.

Sperm analysis
Sperm analysis was carried out in the same laboratory of Reproductive and Genetic Hospital of Citc-Xiangya, P.R. China, during the course of the routine biological examination of the patient, according to World Health Organization guidelines (WHO, 1999). The different semen parameters were compared between the different groups (Table I) using a two-tailed t-test.

Molecular analyses
DNA extraction
DNA was extracted from 5–10 ml of frozen EDTA blood using quick guanidium chloride extraction procedure (Jeanpierre, 1987). DNA extraction was performed using the manufacturer’s recommendations.

Deletion screening
Based on the fact that six of nine BPs (BP 1–6) (Elinati et al., 2012) are covered by the PCR sequences of ‘a’ and ‘e’ (Koscinski et al., 2011) (Fig. 1a), DPY19L2 deletions were detected by a long PCR method using the primers ‘LCR1a forward’ and ‘DPY19L2-BP reverse’, combined with a PCR amplification of DPY19L2 exon 10 (Koscinski et al., 2011). For those patients with a double-negative result, PCRs of ‘A’–‘G’ (they are ‘a’ to ‘g’, respectively, described by Harbuz et al. (2011)) were then performed. In addition, a duplex PCR using pairs of DPY19L2 exon 10 primers and internal control (In Ct) primers (specific to the exon 6 of SEDL described in Gedeon et al. (1999)) was also carried out. Because of high conservation of the duplicated regions, special care was taken to choose specific oligonucleotides with a unique sequence specifying a single location. All amplicons were sequenced in order to control for the specificity of the PCR.

Based on the sequencing results of long PCR amplicons, localization of the BPs was performed using the BLAT (GRCh37/hg19) of UCSC genome browser (http://genome.ucsc.edu/).

Mutation screening
PCR products covering all the 22 exons of DPY19L2 and their exon—intron boundaries were amplified using the primers described in Coutton et al. (2012). Sequencing analyses were carried out using the BigDye Terminator v3.1 sequencing kit and ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

The identified sequence variants were described following the guidelines of Human Genome Variation Society (www.hgvs.org/rec.html).

Sequence numbering was referred to the sequence of DPY19L2 (uc001srp.1) provided by UCSC genome browser (GRCh37/hg19) (http://genome.ucsc.edu/) for the protein sequence and cDNA sequence.

In-silico analysis of sequence variants and prediction of protein conformation
The pathogenicity of the identified missense variants on the DPY19L2 protein was assessed using the SIFT (http://sift.jcvi.org/www/SIFT_score_submit2.html) (Ng and Henikoff, 2001), Polyphen v2 (http://coot.embl.de/PolyPhen/) (Ramensky et al., 2002) and PAHTHER (http://www.pantherdb.org/tools/csnpScoreForm.jsp) (Thomas et al., 2003) web servers.

The potential effect of the identified missense variants on RNA splicing was evaluated with NNsplice (http://fruitfly.org/seq_tools/splice.html) (Reese et al., 1997) and HSF (http://www.umd.be/HSF/) (Desmet et al., 2009).

Multiple sequence alignment of the human DPY19L2 protein with its paralogs and orthologs was realized with MultAlin (http://multalin.toulouse.inra.fr/multalin/) (Corpet, 1988).

Prediction of the novel missense variants on the properties of the involved extramembrane loops of the DPY19L2 protein was performed using TMHMM server v.2 (http://www.cbs.dtu.dk/services/TMHMM/).
Table I Semen parameters of Chinese globozoospermic patients according to DPY19L2 genotype.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Homozygous deleted (n = 4)</th>
<th>Point mutation carriers (n = 5)</th>
<th>No. identified mutation (n = 5)</th>
<th>Reference values (WHO, 1999)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm volume (ml)</td>
<td>3.4 (1.5–5.5)</td>
<td>4.5 (3.0–6.0)</td>
<td>3.0 (1.1–5.5)</td>
<td>≥2.0</td>
</tr>
<tr>
<td>Sperm (×10⁶ per ml)</td>
<td>55 (0.8–90)</td>
<td>40 (8.0–50)</td>
<td>20 (14–25)</td>
<td>≥20</td>
</tr>
<tr>
<td>Round cells (×10⁶ cells)</td>
<td>3.1 (0–15)</td>
<td>0.1 (0–0.2)</td>
<td>0.8 (0.4–1.4)</td>
<td>≤5</td>
</tr>
<tr>
<td>Motility A + B, 1 h</td>
<td>24 (1–40)</td>
<td>22 (20–30)</td>
<td>35 (25–50)</td>
<td>≥50</td>
</tr>
<tr>
<td>Vitality</td>
<td>65 (45–80)</td>
<td>60 (40–75)</td>
<td>65 (45–75)</td>
<td>≥50</td>
</tr>
<tr>
<td>Normal spermatozoa</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>≥15%</td>
</tr>
<tr>
<td>Globozoosperma</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>–</td>
</tr>
<tr>
<td>Rolled flagella</td>
<td>20 (9–35)</td>
<td>25 (20–30)</td>
<td>24 (0–35)</td>
<td>–</td>
</tr>
<tr>
<td>Multiple flagella</td>
<td>0.1 (0–1)</td>
<td>0.2 (0–1)</td>
<td>0.2 (0–1)</td>
<td>–</td>
</tr>
<tr>
<td>Multiple anomalies index</td>
<td>2.7 (2–3)</td>
<td>2.9 (2.2–3.5)</td>
<td>2.8 (1.9–3.5)</td>
<td>1.0–3.0</td>
</tr>
</tbody>
</table>

Values are expressed as the mean with the lower and higher values between brackets. Values are expressed in percent, unless specified otherwise.

Figure 1 Deletion analysis of DPY19L2 in 15 globozoospermic patients (Globo 1 to Globo 15) of Chinese origin. (a) Localization of nine BPs and PCR sequences used to determine the BPs at the two LCR loci flanking the DPY19L2 gene. BPs are colored in blue, PCR sequences of ‘a’ to ‘e’ are colored in green, and ‘A’, ‘B’, ‘F’ and ‘G’ in orange (see the section Materials and methods). (b) PCR results of DPY19L2 exon 10 and BPs. Amplification of exon 10 (upper panel) was used to indicate the presence or absence of DPY19L2. The PCR of BPs (lower panel) was performed using the primers of ‘LCR1a forward’ and ‘DPY19L2 reverse’ (see the section Materials and methods). A fertile man (positive control) was used to test for PCR specificity. No template control (NTC or negative control) was used to test for contaminating DNA. (c) PCR results for ‘A’ to ‘G’ and duplex PCR for DPY19L2 exon 10 (upper band) and internal control (In Ct) (lower band). ‘C’, ‘D’ and ‘E’ represent DPY19L2 exons 22, 11 and 1, respectively. A fertile man (control) was used to test for PCR specificity. (d) Table presenting the BP zone and its corresponding zygosity determined by PCR and sequencing for 15 globozoospermic patients. UBP indicates undetermined BP, ‘Homo-’ indicates homozygosity, ‘hetero-’ indicates heterozygosity and ‘-’ indicates undetermined zygosity.
Results

Deletion analysis

Four of the 15 genetically independent patients were homozygous for the DPY19L2 deletion. First two patients (Globo 1 and 2) had an amplification of the long PCR of 1700 bp encompassing the deletion of BPB and no amplification of exon 10 (Fig. 1b). Sequencing of the two long-PCR products revealed the same previously reported BPB, BP2 (Koscinski et al., 2011; Elinati et al., 2012). Another two patients (Globo 3 and 4) did not have any amplification of exon 10 or the 1700-bp BP fragment (Fig. 1b), suggesting that these patients may have a homozygous deletion but the BPBs may lie outside the region covered by the primers used in this study. Negative PCR results of ‘A’–‘G’ verified the deletion and suggested an absence of BP 7 and BP 8 and 9 (Fig. 1c). We speculated that the BPBs for these two patients may lie outside the LCR1 and LCR2. The positive result of the internal control in duplex PCRs excluded a poor DNA quality or a technical problem (Fig. 1c). One patient (Globo 5) heterozygous for the deletion had an amplification of both exon 10 and the 1700-bp BP fragment (Fig. 1a). Sequencing analysis of the 1700-bp BP fragment also showed a previously reported BPB, BP 5 (Elinati et al., 2012).

Overall, of 15 genetically independent patients, 4 (26.7%) were homozygous for the DPY19L2 deletion, 1 (6.7%) was heterozygous for this deletion and 10 (66.7%) were non-deleted (Fig. 1d).

Mutation analysis

In a second step, we sequenced all the exons of DPY19L2 and their exon–intron boundaries in a total of 10 non-deleted patients and one heterozygous patient. No mutation was identified in this heterozygous patient. Among 10 non-deleted patients, 5 were found homozygous for a mutation. The first patient, descending from a consanguineous family (Fig. 2a), was homozygous for a novel nucleotide deletion at position 1532 in exon 15: c.1532delA, causing a frameshift mutation and introducing a premature stop codon (p.K511RfsX8) (Fig. 2b). His mother and unaffected father and brother were homozygous for this deletion (Fig. 2a and b). The second patient, who was unrelated to the first patient and had no history of consanguinity, were also found homozygous for the same frameshift mutation: c.1532delA. His parents were heterozygous for this mutation. The third patient carried a novel homozygous multi-mutation in exon 18 consisting of a nucleotide deletion c.1679delT and a two-nucleotide deletion c.1681_1682delAC (c.[1679delT; 1681_1682delAC]), creating a newly combined premature stop codon (p.LS60X) (Fig. 2b). His normal brother was not found to carry this mutation (Fig. 2a). His parents are not available and thus we could not test the presence of a small deletion encompassing exon 18 (could not detected by long PCR in this study) to exclude the possible compound heterozygosity of this deletion and the point mutation in this patient. The fourth patient, one of two affected brothers descending a non-consanguineous family (Fig. 2a), was found homozygous for a recurrent missense mutation in exon 8: c.869G>A; p.R290H (Fig. 2b) (Coutton et al., 2012; Elinati et al., 2012). His affected brother was also homozygous for this mutation while his mother and unaffected father were heterozygous (Fig. 2a and b). And the fifth patient carried a novel homozygous missense mutation in exon 9: c.989T>C; p.L330P (Fig. 2b). Both above missense mutations concern highly conserved amino acids of the DPY19L2 protein (Fig. 2c). His parents were heterozygous for this mutation.

To exclude the possibility that the identified variants may be common in studied population, we performed a sequencing analysis of DPY19L2 exons 8, 9, 15 and 18 in 100 individuals of Chinese origin. No abnormal variants in any of the exons tested were identified in any of the control DNA samples. Moreover, the c.1532delA, c.[1679delT; 1681_1682delAC] and p.L330P were not found in the dbSNP (build 135) [all SNPs (135) track] in the UCSC genome browser (GRCh37/hg19) (http://genome.ucsc.edu/). This dbSNP (build 135) shows one R290H allele was identified out of 4472 alleles. This low allelic frequency (0.022%) indicated that R290H is a rare variant and would become a recurrent mutation in patients with globozoospermia, concordant with our findings.

Predictions of the effects of the variants on the mRNA and the protein

The homozygous frameshift mutation, resulting from a single nucleotide deletion c.1532delA, would introduce a premature termination codon (PTC) in the mRNA of DPY19L2. This PTC is predicted to produce a truncated protein, lacking 248 amino acids out of 758 residues of DPY19L2 and ending with an aberrant peptide of 9 amino acids in the COOH terminus. The homozygous multi-mutation c.[1679delT; 1681_1682delAC], creating a new combined PTC at the 560th amino acid: p.LS60X, would cause disease with a similar pathogenic mechanism and is expected to produce a truncated protein missing 199 amino acids in the COOH terminus of DPY19L2.

The missense mutation R290H was a known deleterious mutation indicated by multiple prediction tools: MutPred, MutStab (Coutton et al., 2012), SIFT and Polyphen (Elinati et al., 2012). Here we also showed that R290H was predicted to be pathogenic by an additional program: PAHTHER with a probability of 0.8162. The other missense mutation L330P concerns the substitution of a conserved leucine by a proline in exon 9 (Fig. 2c) that was predicted to be deleterious by SIFT (with a score of 0.00), Polyphen (with a score of 1.00) and PAHTHER (with a probability of 0.9418).

To exclude the possibility that sequence variants can disrupt RNA splicing through modifying key regulatory elements, we also evaluated the effects of the novel frameshift mutation p.K511RfsX8 and missense mutation p.L330P on DPY19L2 mRNA splicing using NNSplice, HSF Matrice and MaxEnt software. It was predicted that the missense mutation would neither introduce an acceptor site or a donor site nor have any other remarkable effect on mRNA splicing.

Predictions of the effect of the variants on the protein conformation

The DPY19L2 protein was predicted to have eight transmembrane helices using TMHMM in a previous study, although it cannot exclude the possibility that this protein may have 10 transmembrane helices (Coutton et al., 2012). In either case, the missense mutation L330P was predicted to be located on one of the extra- membrane loops of the DPY19L2 protein and significantly decrease the hydrophobic properties of the involved extramembrane loop (Fig. 3).
Comparison of sperm parameters according to genetic status

The sperm parameters of the patients carrying a homozygous deletion of DPY19L2 were compared with those carrying point mutations and those with no mutation of DPY19L2 identified (Table I). The patient with a heterozygous deletion of DPY19L2 with no mutation identified in another allele was not included in any of these three groups. There was no significant phenotypic difference between these three groups. And even in those two brothers with the same homozygous point mutation, small variations can be observed in the sperm parameters (detailed data not shown). In addition to the head morphological defect, the rolled flagella were frequently observed. Other semen parameters such as the number of spermatozoa were frequently observed in a normal range for most patients.

Discussion

We presented the analysis of a large cohort of 16 patients (2 brothers and other 14 unrelated individuals) presenting with 100% round-headed spermatozoa, which allowed us to define the frequency distribution of the DPY19L2 mutation types in patients of Chinese origin and to enlarge the mutation spectrum. In total, out of 15 genetically independent patients, 4 patients (26.7%) were diagnosed with a homozygous DPY19L2 deletion and 5 patients (33.3%) with a homozygous point mutation (Fig. 4a). Therefore, through molecular analysis of DPY19L2, 60% of globozoospermic patients can have a clear genetic diagnosis, which strongly suggests that DPY19L2 molecular abnormalities are the predominant cause of globozoospermia in Chinese patients and should be preferentially analyzed during the genetic diagnosis. Two previous studies showed that the homozygous DPY19L2 deletion accounted for 67.6% (23/34) (Coutton et al., 2012) and 44.4% (24/54) (Elinati et al., 2012) of the total DPY19L2 genotypes. This difference may be due to a bias in patient recruitment or a limited number of patients analyzed in our study. However, it could be also explained that a different frequency distribution of DPY19L2 mutation types may occur in patients of pure Chinese descent with such an autosomal recessive disorder, modified by social-cultural factors such as consanguinity. Although the frequency distribution of...
Figure 3  Prediction of the missense mutation L330P on the properties of the involved extramembrane loop of the DPY19L2 protein using TMHMM server. Plot of the posterior probabilities for wide-type (upper panel) and mutant (lower panel) sequences of the DPY19L2 proteins, based on a hidden Markov model approach. The hydrophobicity as one of the most important parameters incorporated into this model can be revealed by the plot. The dashed box highlights the significant decrease of the hydrophobicity for the involved extramembrane loop as a result of an alternation from the wild-type amino acid (Leucine, L) in position 330 to the mutant amino acid (proline, P).

Figure 4  Pie chart showing the overall frequency of the DPY19L2 mutation types among 15 globozoospermic patients (a) and schematic representation of the DPY19L2 gene (b). (a) Among these 15 patients, 26.7% (4 patients; n = 4) have a homozygous deletion of DPY19L2, 6.7% (n = 1) have a heterozygous deletion, 33.3% (n = 5) have a homozygous point mutation and 33.3% (n = 5) have no deletion or mutation identified (non-mutated). (b) Exons are indicated as a black box, untranslated region as a clear box, introns as a line, the localization of the identified point mutations is shown by a line and the span of the identified small deletions encompassing two or three exons is indicated by a line with two arrows at the end. The numbers under the boxes depict the exons. Missense mutations are indicated in black, nonsense and frameshift mutations in red, splicing mutations in blue and small deletions in green. The point mutations identified in this study were highlighted in a clear box.
DPY19L2 mutation types would largely influence our diagnostic strategy, other factors should also be considered such as the costs and the complexity of the diagnostic procedure. A long PCR method combined with exon-specific amplification (Kosinski et al., 2011; Elinati et al., 2012) allows us to detect the homozygous and heterozygous deletion of the whole DPY19L2 gene and defines the BPs with a relative high sensitivity and specificity. However, it cannot identify the heterogeneous large deletions with BPs falling outside of the long PCR primers or the heterozygous small deletions encompassing one or more exons. Other quantitative PCR methods such as multiplex ligation-dependent probe amplification (MLPA) are expected to overcome this shortcoming (Coutton et al., 2012); however, it requires special care to choose highly specific probes with a unique sequence matching specific DPY19L2 nucleotides to overcome the disturbance from the pseudogenes of DPY19L2 due to a very high identity shared by DPY19L2 and its pseudogenes (Carson et al., 2006). Any heterozygous deletions indicated by MLPA should be further confirmed by a long-range PCR defining the BPs. A recent study developed an MLPA method and identified two patients with heterozygous deletions of the whole DPY19L2 gene without defining the BPs to confirm the results (Coutton et al., 2012). In addition to current methods for detecting heterozygous deletions of DPY19L2 (Coutton et al., 2012; Elinati et al., 2012), a more sophisticated MLPA method combined with a long-range PCR still needs to be developed to identify heterogeneous small deletions encompassing one or two exons, using highly DPY19L2-specific primers.

Through sequencing the long PCR products, two recurrent BPs located in the LCRs, BP 2 and BP 5, were identified in three patients of Chinese origin, which further confirmed the fact that the whole DPY19L2 gene deletions mostly result from recurrent events of NAHR between the flanking LCRs, rather than from a founder effect (Kosinski et al., 2011; Elinati et al., 2012).

Two previous studies (Coutton et al., 2012; Elinati et al., 2012) totally identified 11 different point mutations, including 4 missense mutations (p.R290H, p.R298C, p.M358K and p.T493R), 3 non-sense mutations (p.Q342X, p.Q345X and p.K680X), 1 frameshift mutation (c.1183delT; p.S395LfsX77), 1 splice-site mutation (c.1218+1G>A) and 2 exonic deletions (Ex5_6del and Ex5_7del) (Fig. 4b). Here, we identified three additional novel point mutations of DPY19L2 and a recurrent missense mutation, which would further expand the mutation spectrum of DPY19L2 (Fig. 4b). The first mutation was a homozygous single nucleotide deletion c.1532delA identified in two unrelated patients, causing a frameshift mutation and introducing a PTC (p.K511RfsX8). And the second mutation was a homozygous missense mutation c.1679delT; 1681_1682delAC, creating a newly combined PTC (p.L560X). These two mutations were predicted to cause the corresponding aberrant mRNAs rapidly degraded through the pathway of non-sense mediated mRNA decay (NMD), resulting in the absence of the protein (Mendell et al., 2004). Even if the aberrant mRNAs were not all degraded, the produced truncated proteins lacking a large fragment in the COOH-terminus would be non-functional with impaired conformation of the whole transmembrane protein, which may otherwise have a detrimental effect to the cell and thus be trapped in the endoplasmic reticulum of the cell and subsequently be quickly degraded (Kaufman et al., 2002). Therefore, through either pathway of degradation, we consider that these two mutations cause a complete loss of function. Since the DPY19L2 proteins are expressed predominantly in the testes (Harbuz et al., 2011), a testicular biopsy could be done to help to evaluate whether a predicted truncated protein is produced and then subjected to degradation in the cell and whether the mutated transcripts with PTC are degraded by NMD. However, we are not allowed to perform this procedure without a clinical rationale. Furthermore, we cannot obtain any mRNA or protein from the fresh sperm cells of these mutated patients either, due to the fact that the DPY19L2 proteins are present in human testis but absent from ejaculated human sperm (Harbuz et al., 2011). We then evaluated the possible effects of the novel missense mutation L330P on the function of the protein and mRNA splicing, using different multiple prediction software tools. This novel mutation was consistently predicted to have a deleterious effect on the protein but no remarkable effect on mRNA splicing. We then wanted to further evaluate the possible effect of the mutation on the structure of the protein. In mice, Dpy19l2 was demonstrated to be a transmembrane protein located in the inner nuclear membrane of the nucleus facing the acrosome, with both NH2-terminus and COOH-terminus located in the nucleoplasm (Pierre et al., 2012). Since the described phenotype of Dyp19l2 knockout mice concurs perfectly with the observed phenotype of human DPY19L2 deletion (Harbuz et al., 2011; Pierre et al., 2012), we have no doubt that the transmembrane structure of the proteins were highly conserved in these two species. Therefore, using different prediction programs and comparing the possible transmembrane structures of DPY19L2 in several species, it was estimated that the protein could only have 8 or 10 putative transmembrane helices (Coutton et al., 2012; Pierre et al., 2012), and thus both NH2-terminus and COOH-terminus could be located on the same side of the membrane. We notice that the missense mutation L330P is located on an extramembrane loop in either number of transmembrane helices but would be on the different side of the membrane according to the transmembrane helix number. This mutation predicted to significantly decrease the hydrophobic properties of the involved loop located in the central part of the protein would abolish or impair possible interaction between DPY19L2 and its essential partners. As for the recurrent missense mutation R290H identified in this study, it was also predicted to be located on an extramembrane loop but on the different side of the membrane from the L330P. Therefore, these two missense mutations could be used to identify Dpy19l2 partners on both sides of the nuclear membrane through subtractive pull down with wild-type and mutant peptides, facilitating a better understanding of the protein participating in the anchoring of the acrosome to the nucleus and bridging the nuclear envelope to both the nuclear dense lamina and the acroplaxome during the process of acrosome formation in the spermadids (Pierre et al., 2012). There is one patient with a heterozygous deletion of DPY19L2 in one allele but with no mutation identified in the remaining allele. Other mutations located in the intronic or promoter region of DPY19L2 could not be excluded, otherwise, DPY19L2 may not be the cause of globozoospermia in this patient. In addition, we still have five patients with no mutations identified in both alleles of DPY19L2, suggesting that other candidate genes remain to be analyzed. In a previous study, we identified a homozygous missense mutation of PICK1 (MIM 605926) in a patient with total globozoospermia of a Chinese family by screening mutations in candidate genes for globozoospermia (Liu et al., 2010). However, no other PICK1 mutations
proved to be associated with a complete form of globozoospermia in humans. Other genes including Cnk2a2 (Xu et al., 1999), Pirrello et al., (2005), Hbb (Kar-Dekker et al., 2001), Gprc (Yao et al., 2002), Hsp90bi (Audouard and Christians, 2011), Vps54 (Paired et al., 2011) and Zp3pl (Lin et al., 2007), are only associated with a globozoospermia-like phenotype in knockout mice but not yet fully proved to be involved in human globozoospermia. However, they are all candidate genes for human globozoospermia and could be analyzed if no mutations of the above three genes, DPY19L2, SPATA16 and PCK1, were identified in patients with globozoospermia.

All nine patients with DPY19L2 molecular defects declared no other medical impairment apart from their primary fertility, which concurs perfectly with the fact that DPY19L2 is predominantly expressed in the testis. And so far, no females with DPY19L2 molecular abnormalities in both alleles have been reported and thus no data concerning the corresponding potential medical impairment are available. However, it can still be anticipated that no significant clinical phenotype would present in these females, based on the sperm-specific function of DPY19L2 (Harbuz et al., 2011). On the other hand, it could be hard to exclude the possibility that other DPY19L family members having a transmembrane structure highly similar to DPY19L2 but presenting a slightly different expression pattern in tissues (Carson et al., 2006) could at least partially compensate for the loss of DPY19L2 function in other tissues apart from the testis, resulting in no obvious clinical manifestations in other systems.

Sperm analyses were routinely performed in the male patients with primary infertility. In this study, all the sperm analyses were performed in the same laboratory and thus no inter-laboratory variability in sperm parameter scoring could be observed. We compared the semen parameters between patients with homozygous deletions, patients carrying point mutations and patients with no molecular defects of DPY19L2 identified, in order to evaluate whether there is a potential correlation between the phenotype and the genotype. There was no significant phenotypic difference observed between these three groups and on the other hand, small variations in the sperm parameters observed even in those two brothers with the same genotype, suggesting that no clear correlation between genotype and phenotype could be obtained through semen analyses, consistent with the results in a previous study (Coutton et al., 2012). In addition to the head morphological defect, the coiled tails were frequently observed that have been frequently reported in the description of globozoospermia (Dam et al., 2007a). Although the coiled tail was highlighted in Gopc-deficient mice with round-headed spermatozoa (Suzuki-Toyota et al., 2004), it has not yet been proved in human globozoospermia. Other semen parameters such as the number of spermatozoa were observed normal in most patients. Although oligospermia was described in three affected brothers with globozoospermia carrying SPATA16 mutations (Dam et al., 2007b), it still requires studies of more patients with SPATA16 defects to evaluate whether a normal spermatozoa number would predict a lower possibility of SPATA16 defects in globozoospermic patients. After all, semen analyses have a limited power for morphological description of the sperm. A much finer phenotyping could be performed by immunofluorescence staining visualized using confocal microscopy, by transmission electronic microscopy and by scanning microscopy. Through such finer phenotyping, it was revealed that the acrosome could be either absent or atrophied and misplaced in globozoacephalic spermatozoa (Harbuz et al., 2011), and it would be valuable to investigate whether these two described statuses of the acrosome are correlated with any genetic status concerning DPY19L2. A recent study showed that 1% of the total round-headed spermatozoa in a patient presented a small bud of acrosome revealed by an inverted microscopy with \( \times 10,000 \) magnification and confirmed by transmission electron microscopy and anti-CD46 staining analysis (Sermondade et al., 2011). The patient couple had a healthy childbirth after intracytoplasmic morphologically selected sperm injection without assisted oocyte activation (AOA) (Sermondade et al., 2011). Although no DPY19L2 deletions were detected in this patient, it was not excluded that DPY19L2 point mutations may be possibly involved in this pathology. Therefore, we suggest that it would be valuable to establish a clear genotype and phenotype (absence of acrosome, atrophied and misplaced or a small bud of acrosome in a very low percentage) correlation from studies on large cohorts of globozoospermic patients using finer phenotyping methods besides semen analyses and a thorough genetic diagnosis strategy. Due to an absence of acrosome, the round-headed spermatozoa were unable to penetrate the zona pellucida of an oocyte without the help of ICSI. However, overall frequencies of fertilization and pregnancy after ICSI are low, although some successful pregnancies have been reported (Liu et al., 1995; Dam et al., 2007a). Recent work reported that fertilization rates could be improved after ICSI with AOA, via a treatment with calcium ionophore, in patients with globozoospermia (Taylor et al., 2010), suggesting the globozoospermia could be associated with the decrease or defect of phospholipase C \( \mathrm{PLC}_\zeta \), a protein involved in the induction of calcium oscillations triggering oocyte activation (Yoon et al., 2008; Heytens et al., 2009). A recent study of a large cohort of globozoospermic patients has shown that the fertilization rates after ICSI with AOA are restored to normal when compared with conventional ICSI in globozoospermic patients regardless of the presence of a mutation in DPY19L2 (Kuentz et al., 2013). Although the fertilization rates after conventional ICSI in patients with a DPY19L2 mutation are observed to be slightly better (but still very low) when compared with that in patients with no DPY19L2 mutations, it is proposed that the first-line therapeutic approach for complete globozoospermia should imply AOA regardless of the DPY19L2 status (Kuentz et al., 2013). Therefore, it could be expected that a thorough molecular diagnosis for globozoospermic patients would not provide a strong indication for any assisted reproductive treatment but it does facilitate adequate genetic counseling.

In previous work, molecular defects of DPY19L2 have only been identified in European, North African and Middle Eastern patients. Here, we have identified deletions and point mutations of DPY19L2 in a majority of Chinese patients with globozoospermia, confirming again that DPY19L2 genetic abnormalities are the main causes responsible for globozoospermia, also in Chinese patients. We identified three novel mutations including the first multi-mutation c. [1679delT; 1681_1682delAC] and a recurrent missense mutation R290H. The frequency rate of R290H in our cohort is 6.7% (1/15) while two previous studies showed a frequency rate of 2.9% (Coutton et al., 2012) and 1.9% (1/54) (Einat et al., 2012), suggesting this mutation could be a relative hotspot in DPY19L2 mutational
spectrum. For a thorough genetic diagnosis strategy for globozoospermia, we preferred to perform a long PCR method combined with a PCR amplification of DPY19L2 exon 10 first, which is relatively low in cost and complexity. DNA sequencing is then required for individuals with a heterozygous deletion and those with no deletions identified. Then, we can perform an MLPA approach to identify potential heterozygous exonic deletions on individuals with no mutations identified. A positive MLPA result is recommended to be verified by a following long-PCR method to define the BPs of the identified deletions. A thorough genetic diagnosis for globozoospermia remains challenging and costly, but given that the molecular diagnosis would facilitate an adequate genetic counseling, it would be recommended as an optional procedure in clinical practice for globozoospermic patients.

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Authors’ roles

F.Z. undertook all the molecular work. F.G. and G. Lin handled the recruitment of patients, sample collection, sperm analyses and supervised the clinical aspects of work. F.Z. and G. Lin contributed to data analysis. F.Z. and G. Lu designed the overall study and wrote the first draft of the manuscript. G. Lu supervised all molecular laboratory work, had all access to all of the data in the study and takes responsibility for the integrity of the data and its accuracy. All authors contributed to discussion of the results and final manuscript preparation.

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Conflict of interest

None of the authors have any competing interest.

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