MLPA and sequence analysis of DPY19L2 reveals point mutations causing globozoospermia

Charles Coutton1,2,3,4, Raoudha Zouari5, Farid Abada1,2,4, Mariem Ben Khelifa1,2,4, Ghaya Merdassi6, Chema Triki7, Denise Escalier8, Laetitia Hesters9, Valérie Mitchell10, Rachel Levy11, Nathalie Sermondade11, Florence Boitrelle12, François Vialard12, Véronique Satre1,2,3, Sylviane Hennebicq1,2,3, Pierre-Simon Jouk2,3, Christophe Arnoult1,2, Joël Lunardi2,4,13, and Pierre F. Ray1,2,4,*

1Laboratoire AGIM, CNRS FRE3405, Equipe ‘Génétique, Infertilité et Thérapeutiques’, La Tronche F-38700, France 2Université Joseph Fourier, Grenoble F-38000, France 3Département de Génétique et Procréation, CHU de Grenoble, Grenoble cedex 9 F-38043, France 4UM de Biochimie et Génétique Moléculaire, Département de Biochimie Pharmacologie et Toxicologie, Poche de Biologie, CHU de Grenoble, Grenoble, F-38000, France 5Clinique de la Reproduction les Jasmins, 23, Av. Louis BRAILLE, 1002 Tunis, Tunisie 6Unité de Procréation Médicalement Assistée, Hôpital Aziza Othmana, Tunis, Tunisia 7CMRDP, 5, rue Ibn Hazem, 1002 Tunis Behdèrè, Tunisie 8INSERM U654, Hôpital Armand Trousseau, Paris F-75571, France 9APHP, Departments of Biology and Genetics of Reproduction, Antoine Béclère Hospital, Clamart, France 10Institut de biologie de la reproduction – Spermiologie – CECOS, EA 40308 CHRJ- 59035 Lille Cedex, France, and EA 4308 spermatogenesis and quality of the male gamete 11Histologie Embryologie Cytogénétique CECOS, CHU Jean Verdier, 93143 Bondy, France 12Department of Reproductive Biology, Centre Hospitalier Poissy Saint Germain, Poissy, France 13Grenoble Institut des Neurosciences, INSERM U.836, F-38000 Grenoble, France

*Correspondence address. UM de Biochimie et Génétique Moléculaire, Institut de Biologie et Pathologie, CHU de Grenoble, 38 043 Grenoble cedex 9, France. Tel: +33-476-765-573; Fax: +33-476-765-837; E-mail: pray@chu-grenoble.fr

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STUDY QUESTION: Do DPY19L2 heterozygous deletions and point mutations account for some cases of globozoospermia?

SUMMARY ANSWER: Two DPY19L2 heterozygous deletions and three point mutations were identified, thus further confirming that genetic alterations of the DPY19L2 gene are the main cause of globozoospermia and indicating that DPY19L2 molecular diagnostics should not be stopped in the absence of a homozygous gene deletion.

WHAT IS KNOWN ALREADY: Globozoospermia is a rare phenotype of primary male infertility characterized by the production of a majority of round-headed spermatozoa without acrosome. We demonstrated previously that most cases in man were caused by a recurrent homozygous deletion of the totality of the DPY19L2 gene, preventing sperm head elongation and acrosome formation. In mammals, DPY19L2 has three paralogs of yet unknown function and one highly homologous pseudogene showing >95% sequence identity with DPY19L2. Specific amplification and sequencing of DPY19L2 have so far been hampered by the presence of this pseudogene which has greatly complicated specific amplification and sequencing.

STUDY DESIGN, SIZE, DURATION: In this cohort study, 34 patients presenting with globozoospermia were recruited during routine infertility treatment in infertility centers in France and Tunisia between January 2008 and December 2011. The molecular variants identified in patients were screened in 200 individuals from the general population to exclude frequent non-pathological polymorphisms.

PARTICIPANTS/MATERIALS, SETTING, METHODS: We developed a Multiplex Ligation-dependent Probe Amplification test to detect the presence of heterozygous deletions and identified the conditions to specifically amplify and sequence the 22 exons and intronic boundaries of the DPY19L2 gene. The pathogenicity of the identified mutations and their action on the protein were evaluated in silico.

MAIN RESULTS AND THE ROLE OF CHANCE: There were 23 patients who were homozygous for the DPY19L2 deletion (67.6%). Only eight of the eleven non-homozygously deleted patients could be sequenced due to poor DNA quality of three patients. Two patients were compound heterozygous carrying one DPY19L2 deleted allele associated respectively with a nonsense (p.Q342*) and a missense mutation (p.R290H). One patient was homozygous for p.M358K, another missense mutation affecting a highly conserved amino acid. Due to
the localization of this mutation and the physicochemical properties of the substituted amino acids, we believe that this variant is likely to disrupt one of the protein transmembrane domains and destabilize the protein. Overall, 84% of the fully analysed patients (n = 31) had a molecular alteration of DPY19L2. There was no clear phenotypic difference between the homozygous deleted individual, patients carrying a point mutation and undiagnosed patients.

**LIMITATIONS, REASONS FOR CAUTION:** Globally poor fertilization rates are observed after intracytoplasmic sperm injection of round spermatozoae. Further work is needed to assess whether DPY19L2 mutated patients present a better or worse prognosis than the non-diagnosed patients. Evaluation of the potential benefit of treatment with a calcium ionophore, described to improve fertilization, should be evaluated in these two groups.

**WIDER IMPLICATIONS OF THE FINDINGS:** In previous work, deletions of DPY19L2 had only been identified in North African patients. Here we have identified DPY19L2 deletions and point mutations in European patients, indicating that globozoospermia caused by a molecular defect of DPY19L2 can be expected in individuals from any ethnic background.

**STUDY FUNDING/COMPETING INTEREST(S):** None of the authors have any competing interest. This work is part of the project ‘Identification and Characterization of Genes Involved in Infertility (ICG2I)’ funded by the program GENOPAT 2009 from the French Research Agency (ANR).

**Key words:** DPY19L2 / globozoospermia / point mutations / MLPA / sequence analysis

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**Introduction**

Approximately 15% of all couples are confronted with infertility (Evers, 2002; Gurunath et al., 2011). A male factor is believed to be present in nearly half the cases, often manifested by a qualitative and/or quantitative defect of sperm parameters. Globozoospermia (MIM #613958), characterized by the presence in the ejaculate of a majority of round-headed spermatzoa devoid of acrosome, is a rare (incidence <0.1%) but severe disorder in male infertility (Dam et al., 2007a). In a previous work, we demonstrated that a recurrent homozygous deletion of the DPY19L2 gene was found in a large majority of globozoospermia patients. This 200 kb deletion encompasses the totality of DPY19L2 coding sequence without infringing on other surrounding genes (Harbuz et al., 2011). The mechanism leading to the deletion of DPY19L2 is based on the non-allelic homologous recombination (NAHR) between two highly homologous sequences, or low-copy repeats (LCR), surrounding the breakpoint regions. DPY19L2 deletion which has been described to account for 75% (Harbuz et al., 2011) and 19% (Kosinski et al., 2011) of studied patients is now recognized as the mutation responsible for most cases of globozoospermia. As a NAHR caused by the presence of a highly homologous LCR, this mutational event is expected to be recurrent. Analysis of public databases compiling data from comparative genomic hybridization experiments in the general population showed the presence of heterozygous deletions centred around DPY19L2 at a frequency of about 1/220, implying a theoretical disease frequency close to 1/200 000, which is concordant with the rarity of the phenotype. DPY19L2 is a transmembrane protein (Harbuz et al., 2011) of yet unknown function. Previous work has indicated that DPY-19, a DPY19L2 ortholog in Caenorhabditis elegans, is involved in the establishment of cell polarity in the worm (Honigberg and Kenyon, 2000), a function coherent with the failure to achieve sperm head elongation that is observed in our patients.

Other genetic causes are, however, involved in globozoospermia. A mutation in SPATA16, encoding a protein present in the Golgi vesicles of spermatids which serve to fill the acrosome, was described to be responsible for a familial case of globozoospermia (Dam et al., 2007b). No other SPATA16 mutations could, however, be identified in a large cohort of globozoospermic patients (Dam et al., 2007b). Recently, using a candidate genes strategy, heterozygous mutations in ZPBPI were described in patients presenting with abnormal sperm head morphology, but their involvement in the disease has not been formally demonstrated (Yatsenko et al., 2012). Similarly, a homozygous missense PICK1 mutation was identified in a Chinese family (Liu et al., 2010), however, with only one familial case it is also difficult to make formal conclusions. Several knockout mice have also been described to present a globozoospermia-like phenotype when the following genes are mutated: Csnk2a2 (Xu et al., 1999), Hrb (Kang-Decker et al., 2001), Gopc (Yao et al., 2002), Pick1 (Liu et al., 2010), Hsp90b1 (Audouard and Christians, 2011), Vps54 (Paiardi et al., 2011) and Zpbp1 (Lin et al., 2007). Only a few mutations have been identified in the human orthologs of theses genes, but with no formal proof of causality. Overall, these data suggest that despite a common phenotype, globozoospermia is somewhat phenotypically heterogenous and genetically heterogenous but with a predominance of cases involving DPY19L2. The full involvement of DPY19L2 has, however, not been investigated as DPY19L2 non-deleted patients have not been sequenced to identify DPY19L2 point mutations.

In order to fully assess the involvement of DPY19L2 in globozoospermia, we analyzed a large cohort of globozoospermic patients. When a positive diagnosis was not obtained (absence of a homozygous deletion), a sequence analysis of DPY19L2 22 exons was carried out to search for potentially deleterious nucleotide variants. We identified three novel point mutations in DPY19L2 confirming the prevalence of DPY19L2 mutational events in globozoospermia (Fig. 1). Ultimately, these new findings might help improve our understanding of the structure-function of the protein and its role in acrosome formation and sperm head elongation.

**Materials and Methods**

**Patient and control individuals**

We included in this study the 20 patients described in Harbuz et al. (2011). We had reported that 15 out of 20 patients with globozoospermia had a
homozygous deletion of the DPY19L2 region. Multiplex Ligation-dependent Probe Amplification (MLPA) was carried out here on the five non-deleted patients. Unfortunately, we ran out of DNA for three patients and could not obtain any additional biological material. Sequence analysis could therefore only be carried for the two remaining patients.

An additional 14 unrelated and unpublished globozoospermic patients were included. These patients were analysed by MLPA and genomic sequencing of all exons and intron–exon junctions was carried out on all who did not carry a homozygous deletion.

Overall, 24 patients consulted for infertility in Tunisia and were of Tunisian origin, and of the 10 who consulted in France, 6 were of North African origin.

A total of 200 anonymous individuals were screened by high-resolution melting (HRM). All had agreed to donate their DNA for research purposes. The fertility of these individuals was not documented. All were French citizens. Half (100) were of North African origin (Algeria, Morocco and Tunisia) and half were of European origin. All patients and anonymous donors gave their written informed consent, and all national laws and regulations were respected.

**Sperm analysis**

Sperm analysis was carried out in the source laboratories during the course of the routine biological examination of the patient, according to World Health Organization (WHO) guidelines (WHO, 1999). Small protocol variations might be observed between the different laboratories. The different parameters were compared between the different groups (Table I) using a two-tailed t-test.

**Molecular analyses**

**DNA extraction**

DNA was extracted from blood or saliva. Blood DNA extraction was carried out from 5 to 10 ml of frozen EDTA blood using the quick guanidium chloride extraction procedure (Jeanpierre, 1987). Saliva was collected with an Oragene DNA Self-Collection Kit (DNAgenotech, Canada) and DNA extraction was performed using the manufacturer’s recommendations.

**Mutation detection**

The 22 DPY19L2 exons and intronic boundaries were amplified as indicated in Table II. Sequencing analyses were carried out using the BigDye Terminator v3.1 sequencing kit and an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using protocols described in Ben Khelifa et al. (2011).

The nomenclature of the identified variants was established according to Human Genome Variation Society (HGVS) as indicated in: www.hgvs.org/rec.html.

Sequence numbering referred to NP_776173.3 for the protein sequence and to DPY19L2-001 ENST00000324472 for the cDNA sequence.

**HRM analysis**

HRM analysis was performed with the LightCycler 480 (Roche), using the LightCycler 480 HRM master kit. Results were analyzed with the Gene scanning software (Roche) as described in Harbuz et al. (2010).

**MLPA analysis**

The design of the MLPA probes, MLPA reaction and data analysis were performed according to the recommendation of the MRC-Holland synthetic protocol (www.mlpa.com).

For this study, three synthetic MLPA probes specific for exons 1, 17 and 22 of DPY19L2 were designed (Table III). Because of the high homology between DPY19L2 and the other DPY19L paralogs and pseudogenes, MLPA probes were designed in order to match specific DPY19L single nucleotide mismatches at the ligation site (Schouten et al., 2002). In addition, three MLPA control probes specific to the OCRL1 gene were included to serve as control probes for copy number quantification. Information about sequences and ligation sites of these control probes can be obtained in Coutton et al. (2010). The comparative height of the control probes...
### Table I  Semen parameters measured according to the patient’s genotype.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Homozygous deleted (n = 23)</th>
<th>Point mutation carriers (n = 3)</th>
<th>No identified mutation (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm volume (ml)</td>
<td>3.5 (1.7–7.5)</td>
<td>5.6 (3.7–7.5)</td>
<td>3.2 (0.9–6.3)</td>
</tr>
<tr>
<td>Nb spz (× 10⁶ per ml)</td>
<td>60 (0.6–108)</td>
<td>37.1 (7.5–53)</td>
<td>20 (14–25)</td>
</tr>
<tr>
<td>Round cells (× 10⁶ cells)</td>
<td>3.1 (0–16)</td>
<td>0.1 (0–0.2)</td>
<td>0.8 (0.4–1.4)</td>
</tr>
<tr>
<td>Motility A + B, 1 h</td>
<td>27 (1–40)</td>
<td>27 (20–34)</td>
<td>35 (20–50)</td>
</tr>
<tr>
<td>Vitality</td>
<td>60.2 (48–81)</td>
<td>59 (42–76)</td>
<td>62 (47–76)</td>
</tr>
<tr>
<td>Normal spermatozoa</td>
<td>0</td>
<td>0</td>
<td>2 (0–6)</td>
</tr>
<tr>
<td>Globozoospermes</td>
<td>90.2 (29–100)</td>
<td>71 (53–89)</td>
<td>63 (12–100)</td>
</tr>
<tr>
<td>Rolled flagella</td>
<td>24 (8–38)</td>
<td>26 (26–27)</td>
<td>20 (0–41)</td>
</tr>
<tr>
<td>Intermediate piece angulation</td>
<td>12.4 (1–26)</td>
<td>13 (8–19)</td>
<td>19 (0–34)</td>
</tr>
<tr>
<td>Flagella of irregular calibre</td>
<td>14.2 (0–42)</td>
<td>15 (0–31)</td>
<td>4.3 (1–16)</td>
</tr>
<tr>
<td>Shortened flagella</td>
<td>2.8 (0–16)</td>
<td>0.7 (0–1)</td>
<td>1 (0–2)</td>
</tr>
<tr>
<td>Absence of flagella</td>
<td>1 (0–4)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Multiple flagella</td>
<td>0.2 (0–2)</td>
<td>0.7 (0–1)</td>
<td>1 (0–2)</td>
</tr>
<tr>
<td>Multiple heads</td>
<td>0.4 (0–4)</td>
<td>0.7 (0–1)</td>
<td>3.8 (0–10)</td>
</tr>
<tr>
<td>Multiple Anomalies Index</td>
<td>2.7 (2.1–3)</td>
<td>2.9 (2.3–3.4)</td>
<td>2.8 (1.9–3.3)</td>
</tr>
</tbody>
</table>

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

### Table II  DNA sequences of the 22 DPY19L2 primers pairs and respective melting temperatures (Tm).

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer sequences (S' → 3')</th>
<th>Reverse</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GGCCCAACTTTTCTACTCGGAC</td>
<td>ATTTCACAGTGCATGACG</td>
<td>65</td>
</tr>
<tr>
<td>2</td>
<td>GCTTGTTCATATGAG</td>
<td>AAAGCAGCTATTAAGAC</td>
<td>52</td>
</tr>
<tr>
<td>3</td>
<td>GACACAGCTGACGGGCAC</td>
<td>ATTTCTGCTGGTGCACAGAT</td>
<td>56</td>
</tr>
<tr>
<td>4</td>
<td>TGGCCATTATATCCAATACAG</td>
<td>GGGAAATGTGATGAAGTTT</td>
<td>58</td>
</tr>
<tr>
<td>5</td>
<td>ATAGTCAAGATTGCGATCTAG</td>
<td>TAATATCAAAACACGCA</td>
<td>56</td>
</tr>
<tr>
<td>6</td>
<td>ATGACTTTTGAGATAGAA</td>
<td>AACTATATAATCAGCTAATA</td>
<td>47</td>
</tr>
<tr>
<td>7</td>
<td>TAAAGCAAGAGATTTTGTAG</td>
<td>GTAAGGCTGAGATGACAGA</td>
<td>52</td>
</tr>
<tr>
<td>8</td>
<td>GCCCTTGTTTTATATAATCG</td>
<td>GGTAGTTATGCTGCTTATT</td>
<td>57</td>
</tr>
<tr>
<td>9</td>
<td>GCTACATTTTCTACAT</td>
<td>AGTTTCATTTATGATATTTT</td>
<td>53</td>
</tr>
<tr>
<td>10</td>
<td>CCAAGAGGAGGTACCGTATAA</td>
<td>GCCATCCATCTTTTATATTCA</td>
<td>59</td>
</tr>
<tr>
<td>11</td>
<td>AACCTCCTCAATGACTTATG</td>
<td>TGGGCAAAGACTATT</td>
<td>57</td>
</tr>
<tr>
<td>12</td>
<td>GAAGGTTTAATGAGCCTAGA</td>
<td>ATTAGCCTGACGAAATGGT</td>
<td>58</td>
</tr>
<tr>
<td>13</td>
<td>AGAACTTTTCATTTTAAA</td>
<td>TCTCTTTCTGCTATT</td>
<td>42</td>
</tr>
<tr>
<td>14</td>
<td>CTTAGAGGAGTGTCTAAATAT</td>
<td>TCCAAAGTGCCTAGATTAT</td>
<td>54</td>
</tr>
<tr>
<td>15</td>
<td>CCGGTGTACCTACAATGTTAA</td>
<td>AATGTTAAAATTTGAGTAAACC</td>
<td>55</td>
</tr>
<tr>
<td>16</td>
<td>TTTAATACTTGGATGGTGCA</td>
<td>GCCATCTATAGTGGCTGCAG</td>
<td>60</td>
</tr>
<tr>
<td>17</td>
<td>GCTCAGGCGCTGAGGACTAAG</td>
<td>GCCACATCGGAACAC</td>
<td>60</td>
</tr>
<tr>
<td>18</td>
<td>AATTAGTCCAGCAAGGCACCA</td>
<td>TAGACATCTGATAAAATTTAGC</td>
<td>54</td>
</tr>
<tr>
<td>19</td>
<td>GGTTTTAATGGTGCATTAC</td>
<td>AATTTATGTTGGACCTTACT</td>
<td>55</td>
</tr>
<tr>
<td>20</td>
<td>CAGAGGGCAACAGGTACGTTAT</td>
<td>ACCCTTGAACGTGTAAGTATTA</td>
<td>56</td>
</tr>
<tr>
<td>21</td>
<td>AGGGTTAAATACCTCTTATGTC</td>
<td>TATATTCTGAAAACCTGTGAA</td>
<td>46</td>
</tr>
<tr>
<td>22</td>
<td>GTGTCAATTTAAGGCTTGTG</td>
<td>ATTTGTCTCAGACAGAATCAT</td>
<td>57</td>
</tr>
</tbody>
</table>
DMP19L2 point mutations in globozoospermia

In silico analyses of sequence variants and prediction of protein conformation

The pathogenicity of the identified false sense variants was evaluated using the MutPred (http://mutpred.mutdb.org/) and MuStab (http://bioinfo.ggc.org/mustab/) webserver as recommended by Thusberg et al. (2011). The potential effect of these variants on RNA splicing was assessed with http://www.umd.be/HSF/ (Yeo and Burge, 2004; Desmet et al., 2009).

Protein alignments [DMP19L2 paralogs and orthologs (Fig. 3b)] were realized with CLC Sequence viewer 6 (http://mac.softpedia.com/get/Math-Scientific/CLC-Free-Workbench.shtml).

Prediction of transmembrane helices in proteins was performed using TMHMM server v.2 based on a hidden Markov model (http://www.cbs.dtu.dk/services/TMHMM/).

Results

MLPA analysis

Of the 34 analysed patients, 23 (67.6%) had a homozygous DMP19L2 deletion, 2 were heterozygous (5.9%) and 9 were non-deleted (26.4%; Fig. 1). For illustration of the MLPA technique, see Fig. 2.

DMP19L2 sequencing and HRM analysis

Due to lack of DNA, full sequence analysis could not be performed on three patients. A total of six deletion-negative and two heterozygous patients were analysed by DNA sequencing (Fig. 1). All 22 DMP19L2 exons and intron boundaries were sequenced for these eight patients. No mutations were identified in five patients. Gene sequencing revealed a total of three point mutations in three individuals. A point mutation was identified on the remaining allele of the two
heterozygously deleted patients who were both of European origin. The first patient carried a heterozygous missense mutation in exon 8 altering the DPY19L2 869th nucleotide: c.869G>A and modifying the 290th amino acid: p.R290H. The second patient carried a heterozygous nonsense mutation in exon 9: c.1024C>T; p.Q342* (Fig. 3). A third patient, of North African origin, carried a homozygous missense mutation in exon 10: c.1073T>A; p.M358K. We did not have access to DNA from this patient’s parents and could not verify that both were heterozygous for this variant to exclude the presence of a small deletion (not detected by MLPA) centred on exon 10. As the parents were first cousins, we consider that it is much more likely that the variant is indeed homozygous.

To exclude the possibility that the identified variants may be common in the studied populations, we performed an HRM of DPY19L2 exon 8 and 9 in 100 individuals of French origin and of exons 10 for 100 individuals of North African origin. The HRM technique represents an efficient way of detecting variants in amplified fragments. Three artificial heterozygous control DNA were tested by HRM for each mutation and showed a characteristic green profile (Fig. 3c). The homozygous or hemizygous patients were also passed in triplicate and are shown in red. Each profile (homozygous, heterozygous and non-mutated) clearly showed a distinct profile (Fig. 3c). None of the control DNA samples, shown in blue, presented any abnormal profile for any of the exons tested. Moreover, M358K and Q342* were absent from the most recent database: dbSNP build (build 135, October 2011; http://www.ncbi.nlm.nih.gov/snp/) which regroups sequence data from several thousand individuals. One R290H allele was identified out of 4471 alleles. This low allelic frequency (0.022%) is however not surprising for a recessive trait.

**Figure 3** DPY19L2 novel point mutations. (a) Electropherogram of DPY19L2 exon 8, 10 and 9 showing the mutated sequence and sequence obtained from a control individual. Three patients carried a hemizygous missense mutation (p.R290H) in DPY19L2 exon 8, a hemizygous nonsense mutation (p.Q342*) in DPY19L2 exon 9 or a homozygous missense mutation (p.M358K) in DPY19L2 exon 10. (b) The arginin in position 290, the methionine in position 358 and the glutamine in position 342 are all conserved within species and DPY19 paralogs. Amino acid sequence alignment of exon 7, 8, 9 and 10 of the human DPY19L2 with paralogs and interspecies DPY19 sequences was realiszed with CLC Sequence viewer 6. (c) HRM profiles of DPY19L2 exon 8, 10 and 9 from the three patient carrying exon mutations respectively in red (replicated) and from control individuals in blue. The green lines represent artificial heterozygous DNAs. All mutations found in these patients were not present in 100 control subjects (blue lines).

**Prediction of the effects of the variants on the mRNA and the protein**

The nonsense mutation, p.Q342*, changed a glutamine codon (CAA) into a stop codon (TAA) at amino acid position 342 in exon 9 (Fig. 3a). This premature stop codon is expected to produce a truncated
protein missing 416 amino acids out of the 758 residues of DPY19L2. The other two mutations described here are missense mutations which cause non-conservative amino acid substitutions. Both concern highly conserved amino acids which are located in evolutionary conserved domains of the protein (Fig. 3b).

The first missense mutation (p.R290H) is an arginine to histidine substitution in exon 8 (Fig. 4a and b). The utilization of the MutPred algorithms (Li et al., 2009) indicates that p.R290H is deleterious with a probability of 0.785. Furthermore, analysis with MuStab webserver (Teng et al., 2010) predicts that this substitution would decrease the protein stability with a confidence of 83.6%.

The second missense mutation (p.M358K) concerns the substitution of a conserved methionine by a lysine in exon 10. For this mutation, MutPred indicates a deleterious effect with a probability of 0.766. This software also indicates a very probable loss of stability (P = 0.0076), the gain of a catalytic residue at M358 (P = 0.0116) and a gain of ubiquitination at M358 (P = 0.0311). MuStab prediction is also in favour of a decreased stability with a prediction confidence of 89.6%.

False sense or silent exonic variants can also alter RNA splicing by modifying key regulatory signals. We therefore analysed the effect our two variants could have on splicing using HSF Matrice and MaxEnt software. Neither of the two variants introduced an acceptor or a donor site nor were they predicted to have any other obvious effect on splicing.

**Prediction of protein conformation**

To be able to predict the potential effect of the identified variants we wanted to obtain a 2D model of DPY19L2. All prediction software indicate that DPY19L2 is a multipass membrane protein. We used a TransMembrane prediction program using Hidden Markov Models (TMHMM), a membrane protein topology prediction method which discriminates between soluble and membrane domains with a
specificity and sensitivity better than 99% (Khsay et al., 2005). TMHMM indicates that the human and mices DPY19L2 proteins have nine transmembrane domains. In the human, the fourth domain is just above the threshold whereas the same sequence in mice is below and is not considered as a transmembrane domain. The opposite happens for mouse exon 7. As the protein function is preserved in both species [DPY19L2 knock-out mice perfectly mirror the human phenotype (unpublished data)], we believe that it is highly unlikely that the protein structure differs between the two species. We therefore think that it is likely that both proteins have 8 transmembrane domains as shown in Fig. 4d. We, however, cannot rule out the possibility that both proteins may have 10 transmembrane domains.

Comparison of sperm parameters
The values measured during routine spermogram and spermocytogram were compared between homozygously deleted patients, those carrying point mutations and those for whom no DPY19L2 molecular alteration had been identified (Table I). No significant phenotypic difference was observed between these three groups. Patients came from different centres, and despite a common observance of WHO guidelines (WHO, 1999), important scoring variations were observed even in genotypically identical individuals. Additionally to the head morphological defect, we observed frequent midpiece and flagellar defects.

Discussion
In total, 23 and 2 patients out of 34 were diagnosed respectively with a homozygous and a heterozygous DPY19L2 deletion. The detection of heterozygous individuals was realized by MLPA and allows a quantitative assessment of DPY19L2 allelic status with more sensitivity and specificity than with other amplification-based techniques. MLPA is less sensitive to DNA fragmentation than other long PCR or other gene dosage techniques (Kozlowski et al., 2007). According to our experience, this can be particularly useful for the analysis of DNA extracted from saliva which can sometime be of substandard quality (personal experience). A long PCR amplification allowing amplification across the deleted region has been described previously (Kosciński et al., 2011). Such an approach, combined with exon-specific amplification allows the detection of heterozygous deletions. A negative result will, however, be obtained if the breakpoints fall outside the region covered by the deletion-specific primers. MLPA does not present this shortcoming and allows the detection of any deletion of DPY19L2 whole coding the sequence, irrespectively of the localization of the breakpoints. For the diagnosis of globozoospermia, we therefore recommend initiating the molecular diagnosis by DPY19L2 MLPA to identify all homo- and heterozygously deleted individuals.

Following MLPA analysis, DPY19L2 sequence analysis was carried out when enough DNA was available from the non-homozygously deleted patients. We identified the first point mutations in DPY19L2 in globozoospermia patients thereby confirming the predominance of DPY19L2 molecular abnormalities in the globozoospermia phenotype. These results also further confirm that it is the absence of a functional DPY19L2 protein that is responsible for the globozoospermia phenotype and not an indirect effect of the deletion. The first mutation was a non-sense (stop) mutation identified in a compound heterozygous individual carrying a heterozygous deletion. We wanted to assess whether a truncated protein was produced or if the aberrant mRNA was degraded by non-sense mediated mRNA decay. Since DPY19L2 presents a testis restricted expression, we realized an RT–PCR on fresh sperm cells from fertile control individuals but did not obtain any amplification (data not shown). As there was no clinical rationale for conducting a testis biopsy, we could not obtain any mRNA from this mutated patient and could not assess whether the transcript was degraded or not. We believe that the presence of a shortened protein lacking more than half of its sequence would be at least detrimental to the cell as the total absence of that protein. Irrespective of the molecular physiopathology, we therefore consider that this mutation provokes a complete loss of function. We then wanted to understand the action of the two amino acid substitutions on the protein function. Both variants involve amino acids that are highly conserved throughout evolution and concern amino acids with very different physicochemical properties. We used several software analyses to evaluate the effect these variants have at the protein level and on mRNA splicing. Both variants were predicted to have a highly deleterious effect on the protein but no obvious effect on mRNA splicing. We therefore wanted to better evaluate the localization of these variants on the protein domains. Comparing the prediction of the localization of the transmembrane domains between man and mouse, we postulate that DPY19L2 has 8 transmembrane domains (Fig. 4d). Based on this prediction, we see that p.R290H is located on an extra-membrane domain. Physicochemical changes resulting from this substitution could modify an interaction site between DPY19L2 and an essential protein partner. The second missense mutation (p.M358K) is predicted to be located in the fifth transmembrane domain. It substitutes a non-polar (M methionine) with a polar amino acid (K lysine). We can therefore predict that this amino acid variation is likely to disrupt a transmembrane domain and potentially the whole anchorage of DPY19L2 in the membrane. These three novel mutations are all located in the central part of the protein (exon 8, 9 and 10), suggesting that this region is likely to have a particularly important function (Fig. 4). Transfection and expression of wild-type and mutant protein could be carried out ex vivo to better understand the action of these mutations. These mutations could be used to identify DPY19L2 partners by subtractive pull down with wild-type and mutant peptides. This approach would be particularly suited to p.R290H, which is predicted to be located on an extra-membrane loop.

The patients presented here consulted for infertility at fertility centres in France and Tunisia. Of the 31 fully analysed patients, 22 (68%) consulted at procreation centres in Tunis and of 10 who consulted in France, 6 were of North African descent. The four patients of European origin carried DPY19L2 alterations: two were the compound heterozygotes mentioned previously and two were homozygously deleted. Occurrence of genomic recombinations by NAHR such as the DPY19L2 deletion is expected to occur at a similar frequency irrespective of the genetic background of the individuals. This is indeed confirmed by the observation that this CNV was observed in different populations (Shaikh et al., 2009). The higher incidence of globozoospermia observed in North African men is therefore not likely due to
an increased allelic frequency but is more likely to be the manifestation of the high rate of intra-familial marriages specific to this population which strongly favours the emergence of recessive traits.

The values measured during routine spermogram and spermocytogram were compared between homozgyously deleted patients, patients carrying point mutations and patients for whom no DPY19L2 molecular alteration had been identified (Table I). No significant phenotypic difference was observed between these three groups. Additionally to the head morphological defect that is the hallmark of globozoospermia, we observed frequent midpiece and flagellar defects that have seldom been highlighted in the description of globozoospermia. This kind of defect is logical as acrosome formation, sperm head elongation and flagellar elongation are concomitant during spermiogenesis and depend on highly interconnected processes (Kierszenbaum and Tres, 2004). We currently do not have any clear genotype, phenotype (IVF success rate) correlation as we obtained poor fertilization rates, but could obtain a few pregnancies after ICSI in both groups. Recent work suggests that the low fertilization rate observed with globozoospermatozoa is at least partially caused by a decrease or a defect in PLCγ2, a protein involved in the induction of calcium oscillations triggering oocyte activation (Yoon et al., 2008; Heytens et al., 2009). PLCγ2 mutations have been demonstrated to cause infertility by preventing oocyte activation without inducing a globozoospermia phenotype (Kashir et al., 2012). Several centres have tried to overcome this problem using calcium ionophores which facilitate the transport of Ca2+ across the plasma membrane and artificially activate the oocyte. The utilization of such a chemical is not consensual as it does not mimic the physiological activation of the oocyte, but its use does seem to improve the overall pregnancy rate (Dam et al., 2007a). Prospective studies evaluating the success rate of ICSI with or without calcium ionophore on mutated and non-mutated patients should be performed to evaluate the prognosis of these patients with different protocols.

A total of 34 globozoospermic patients are presented in this study. Overall 23 (67.6%) were homozygous for the DPY19L2 deletion. MLPA analysis indicated that two (5.9%) were heterozygous for the genomic deletion. A point mutation was indentified in the non-deleted allele of these two patients. Nine individuals (26.5%) did not carry a genomic deletion. An increased allelic frequency but is more likely to be the manifestation of the high rate of intra-familial marriages specific to this population which strongly favours the emergence of recessive traits.

The values measured during routine spermogram and spermocytogram were compared between homozgyously deleted patients, patients carrying point mutations and patients for whom no DPY19L2 molecular alteration had been identified (Table I). No significant phenotypic difference was observed between these three groups. Additionally to the head morphological defect that is the hallmark of globozoospermia, we observed frequent midpiece and flagellar defects that have seldom been highlighted in the description of globozoospermia. This kind of defect is logical as acrosome formation, sperm head elongation and flagellar elongation are concomitant during spermiogenesis and depend on highly interconnected processes (Kierszenbaum and Tres, 2004). We currently do not have any clear genotype, phenotype (IVF success rate) correlation as we obtained poor fertilization rates, but could obtain a few pregnancies after ICSI in both groups. Recent work suggests that the low fertilization rate observed with globozoospermatozoa is at least partially caused by a decrease or a defect in PLCγ2, a protein involved in the induction of calcium oscillations triggering oocyte activation (Yoon et al., 2008; Heytens et al., 2009). PLCγ2 mutations have been demonstrated to cause infertility by preventing oocyte activation without inducing a globozoospermia phenotype (Kashir et al., 2012). Several centres have tried to overcome this problem using calcium ionophores which facilitate the transport of Ca2+ across the plasma membrane and artificially activate the oocyte. The utilization of such a chemical is not consensual as it does not mimic the physiological activation of the oocyte, but its use does seem to improve the overall pregnancy rate (Dam et al., 2007a). Prospective studies evaluating the success rate of ICSI with or without calcium ionophore on mutated and non-mutated patients should be performed to evaluate the prognosis of these patients with different protocols.

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Authors’ roles

C.C., F.A. and M.B.K. undertook all the molecular work. R.Z., G.M., C.T., D.E., L.H., V.M., R.L., N.S., F.B., F.V., V.S. and S.H. handled the recruitment of patients, sample collection, sperm analyses and supervised the clinical aspects of the work. C.C., C.A., J.L., P.-S.J. and P.F.R. contributed to data analysis. P.F.R. designed the overall study, supervised all molecular laboratory work, had full 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 the report.

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Conflict of interest

None declared.

References


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