A new AURKC mutation causing macrozoospermia: implications for human spermatogenesis and clinical diagnosis

Mariem Ben Khelifa1,2,3, Raoudha Zouari4, Radu Harbuz1,2, Lazhar Halouani4, Christophe Arnoult1, Joël Lunardi2,5, and Pierre F. Ray1,2,*

1Laboratoire AGIM, FRE 3405 CNRS – UJF, Equipe Génetique Infertilité et Thérapeutique (GIT), campus santé de Grenoble, Grenoble, France 2UM de Biochimie et Génétique Moléculaire, CHU Grenoble, 38700 Grenoble, France 3Molecular Investigation of Genetic Orphan Diseases Research Unit UR04/SP03, Pasteur Institute, Tunis, Tunisia 4CPSR les Jasmins, 23, Av. Louis BRAILLE, 1002 Tunis, Tunisia 5INSERM U836, Grenoble Institut des Neurosciences, Equipe Muscle et Pathologies, Grenoble F-38000, 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

Submitted on June 8, 2011; resubmitted on June 8, 2011; accepted on June 28, 2011

ABSTRACT: The presence of close to 100% large-headed multi-tailed spermatozoa in the ejaculate has been described as a rare phenotype of male infertility with a very poor prognosis. We demonstrated previously that most cases were caused by a homozygous mutation (c.144delC) in the Aurora Kinase C gene (AURKC) leading to the absence or the production of a non-functional protein. AURKC deficiency in these patients blocked meiosis and resulted in the production of tetraploid spermatozoa unsuitable for fertilization. We describe here the study of two brothers presenting with large-headed spermatozoa. Molecular analysis of the AURKC gene was carried out in two brothers presenting with a typical large-headed spermatozoa phenotype. Both affected brothers were heterozygous for the c.144delC mutation. After complete sequencing of the gene a new heterozygous variant, c.436-2A>G, was identified in both patients. This mutation is located in the acceptor consensus splice site of exon 5. AURKC transcripts were extracted from one of the patient’s leukocytes and reverse transcription polymerase chain reaction could be realized showing the presence of a truncated transcript indicating that c.436-2A>G leads to the skipping of exon 5. These results indicate that AURKC molecular analysis of patients with large-headed spermatozoa should not be stopped in the absence of a homozygous recurrent mutation on exon 3 but complete sequence analysis should be performed. This diagnosis is important as the identification of AURKC mutations in patients indicates that all spermatozoa will be chromosomally abnormal and that ICSI should not be attempted.

Key words: gene mutations / genetic diagnosis / spermatogenesis / spermatozoa / meiosis

Introduction

Infertility concerns a minimum of 70 million couples worldwide. An important proportion of cases are believed to have a genetic component, yet few causal genes have been identified so far. Patients with large-headed multiflagellar spermatozoa or ‘macrozoospermia’ present with a majority of large-headed, multiflagellar polyploid spermatozoa in the ejaculate. This syndrome was first described in 1977 (Nistal et al., 1977) and cases have been described regularly ever since (Escalier, 1983; In’t Veld et al., 1997; Pieters et al., 1998; Benzacken et al., 2001; Devillard et al., 2002; Mateu et al., 2006), these studies highlighting a highly abnormal chromosomal content in these patient’s spermatozoa. In 2007, we demonstrated that a homozygous mutation (c.144delC) in the Aurora Kinase C (AURKC) gene was found in a large majority of macrozooecephalic patients (Dieterich et al., 2007). A carrier frequency of 1/50 was established from individuals from the Maghrebian general population, comparable to that of Y-microdeletions, thus far the only known recurrent genetic event altering spermatogenesis. We then could demonstrate that large-headed spermatozoa from AURKC deficient patients were tetraploid indicating that without a functional AURKC protein, meiosis could not be completed (Dieterich et al., 2009).

A few other genes have been linked with other infertility phenotypes and in particular globozoospermia, a phenotype characterized...
Materials and Methods

Patients and control subjects

Both patients (II.1, II.2) are brothers (Fig. 1) from Tunisian descent and were treated for infertility at the CPSR les Jasmins in Tunis. They were diagnosed with macrozoospermia following routine sperm analysis. Both had close to 100% large-headed spermatozoa with a few non-megalophead spermatozoa. In total 11 ICSI had been unsuccessful attempted. Molecular analysis revealed the presence of an unknown AURKC mutation. Transcript analysis confirmed the pathogenicity of the newly identified mutation, showing that the encoded protein would lack one of its seven exons. Furthermore, this strengthens the prognostic value of AURKC genotyping for patients with large-headed spermatozoa, reinforcing the fact that ICSI should not be attempted for AURKC mutated patients.

Molecular analyses

Genomic DNA was extracted from peripheral blood leukocytes using a guanidium chloride extraction procedure. Patients and family member’s DNA was extracted from saliva using Oragene DNA Self-Collection Kit (DNAgenotech, Canada) according to the manufacturer’s recommendations.

The seven AURKC exons and intronic boundaries were amplified as described previously (Dieterich et al., 2007). All 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).

RNA was extracted from nucleated cells isolated from the whole blood using Ficoll\hexF\x80400 by Sigma-Aldrich Corporation (St. Louis, MO, USA) following the manufacturer’s protocol. RNA extraction was carried out on isolated white blood cells using Macherey Nagel NucleoSpin pin\hexF\x80microRNA II columns (Macherey Nagel, Hoerdt, France) according to the manufacturer’s protocol. Reverse transcription (RT) was carried out with 5 μl of extracted RNA (~500 ng). Hybridization of the oligo (dT) was realized by incubating for 5 min at 65°C the following mixture: 5 μl of RNA, 3 μl of poly T oligo primers (dT)12–18 (10 mM, Pharmacia), 3 μl of the four dNTPs (0.5 mM, Roche Diagnostics) and 2.2 μl of H2O followed by ice quenching. RT was then carried out during 30 min at 55°C after the addition of 4 μl of 5× buffer, 0.5 μl RNase inhibitor and 0.5 μl of Transcriptor Reverse Transcriptase (Roche Diagnostics). Five microliters of the obtained cDNA mix was used for the subsequent polymerase chain reaction (PCR).

Primers were designed to amplify exons 4–6 from cDNA to characterize the consequences of the c.436-2A>G mutation. The 5’ primer was located on exon 4 (CAATATCCTGCGCCTGTATAACT) and the 3’ primer on exon 6 (TCATTTCGTGCGCGCAAGT). Two microliters of the reverse transcribed RNA was amplified with these primers (40 cycles) at an elongation temperature of 58°C.

High resolution melting (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).

Results

Both patients (II.1, II.2) were treated for infertility at the Clinique des Jasmin in Tunis between 1999 and 2005. They were diagnosed with (Algeria, Morocco or Tunisia). All patients, family members and anonymous donors gave their written informed consent, all national laws and regulations were respected.
macrozoospermia following routine sperm analysis. Both had close to 100% large-headed spermatozoa with a sperm count, 1 M/ml (Table I). However, after centrifugation and careful examination, a few smaller, ‘normal-looking’ spermatozoa which could fit into an injection pipette were identified in all semen samples analyzed from each patient. Six and five ICSI cycles were attempted for Patients 1 and 2, respectively (Table II), before the characterization of the role of AURKC in macrozoospermia (Dieterich et al., 2007) and the identification of the two AURKC mutations in the patients. There was no major difference observed in the ICSI results obtained from both brothers (Table II), we therefore comment on the figures obtained from data averaged from the 11 ICSI cycles. Transfers were realized on D2 unless D2 was a Sunday in which case transfer was carried the next day on D3. There was one attempt of blastocyst transfer on D5 but none of the six embryos developed to the blastocyst stage, so there were no embryos transferred (Table II attempt P2.5). The average number of cumulus–oocyte complexes (COCs) retrieved and of M2 oocytes obtained was good, with an average of 14.4 and 8, respectively. The overall fertilization rate (59%) was lower than what was achieved with testicular sperm for azoospermic patients [around 70% (De Croo et al., 2000)]. After fertilization most zygotes cleaved and reached the four cell-stage (96%) with 65% of grade 1 or 2 embryos (Table II). One to four embryos were transferred, and in cycle P2.2 (Table II) an additional transfer of thawed embryos was carried out. There were no pregnancies initiated from any of these cycles.

Saliva samples were collected from both patients II:1 and II:2 and family members for AURKC analysis. Sequencing of the AURKC exon 3 containing the c.144delC mutation revealed a single copy of the recurrent deletion in both brothers (Fig. 2). The other exons were sequenced and the c.436-2A>G variant was identified in both brothers (Fig. 2). This substitution takes place two nucleotides prior to exon 5 and alters an AG consensus acceptor splice signal likely to be crucial for adequate splicing. Both splice-prediction programs http://www.fruitfly.org/seqtools/splice.html and http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi indicated that the substitution would obliterate the acceptor site and that the splicing machinery would skip exon 5. To verify this hypothesis we performed RT–PCR on cDNA obtained from controls and patient II:1. Amplification of a sequence ranging from cDNA exon 4–6 yielded a normal band of 329 bp in controls whereas a single smaller band of 179 bp was obtained from II:1 cDNA (Fig. 3A). Sequence analysis of the amplified products confirmed that the mRNA from patient II:1 was indeed devoid of exon 5 (Fig. 3B) as shown in Fig. 3C.

Furthermore, to exclude the possibility that c.436-2A>G may be a common variant in the population studied we analyzed AURKC exon 5 from 100 individuals of North African descent. The analysis was carried out by HRM which highlights the presence of variants in amplified fragments. Three c.436-2A>G heterozygous individuals (II:1,2,4) were tested by HRM and showed a characteristic red profile clearly different from that of all the tested control individuals giving a flat profile in blue (Fig. 4). This indicated that none of the 100 North African controls tested had any nucleotide variant in exon 5 or in its bordering intronic sequences.

**Discussion**

We have demonstrated previously that all patients with a typical macrozoospermia phenotype carried an AURKC mutation. In a series

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**Table II** Details of the 11 ICSI realized (COCs).

<table>
<thead>
<tr>
<th>Patients</th>
<th>Exon 3</th>
<th>Exon 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>I:2, II:3</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>I:1, II:1,2,4</td>
<td>c.144delC</td>
<td></td>
</tr>
<tr>
<td>I:1, II:3</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>I:1, II:1,2,4</td>
<td>c.436-2A&gt;G</td>
<td></td>
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**Figure 2** Electropherogram of AURKC exon 3 and 5. Electropherogram showing the presence of heterozygous mutations c.144delC and c.436-2A>G in individuals I:1, II:1,2,4 and in I:1, II:1,2,4, respectively.
Figure 3  Transcript analysis from control subjects from the general population (C1–C3) and patient II:1 [c.436-2A>G, c.144delC]. (A) Electrophoresis showing the RT–PCR amplification of AURKC exon 4–6. Controls C1–C3 yield a normal fragment of 329 bp and patient II:1 a shortened fragment of 180 bp devoid of exon 5. (B) Electropherogram showing the exons boundaries of the bands is show in (A). Sequence analysis indicates that exons 4–6 are present in control C1 whereas exon 5 is removed from II:1 transcript. (C) Illustrates the exon 5 skipping as observed in patient II:1.

Figure 4  HRM analysis of AURKC exon 5 on subjects from the North African general population (blue) and c.436-2A>G heterozygotes (II:1,2,4) (red).
of 32 typical macrozocephalic patients 31 were homozygous for the c.144delC mutation and 1 was a compound heterozygote carrying the recurrent mutation and a missense mutation in exon 6, p.Cys229Tyr (Dieterich et al., 2009). Here we characterized two brothers carrying the recurrent mutation and the new c.436-2A>G variant. This mutation is located on a consensus acceptor splice site predicted to be necessary for adequate splicing of AURKC mRNA. We demonstrate that a normal mRNA could be consistently amplified from control subjects whereas only a truncated transcript devoid of exon 5 could be amplified by RT–PCR from patient II:1. This confirms the results predicted by the splice site prediction softwares tested, which predicted the abrogation of the acceptor site leading the prolongation of intron 5 until the following acceptor site preceding exon 6 (Fig. 3C). Sequence analysis of exon 5 truncated product indicates that there is no disruption of the reading frame caused by this splicing mutation. The mutant protein will lack the 50 amino acid coded by exon 5 but there will be no premature stop codon introduced in the coding frame. Amino acid 146–195 will be missing from the protein. They are localized in the middle of the catalytic domain and their absence is therefore very likely to severely hamper the functionality of the protein.

We could also observe that there was no band of normal size corresponding to the c.144del allele. This indicates that the c.144del mutation induced non-sense mRNA decay, at least in leukocytes. Nonsense-mediated mRNA decay is a cellular surveillance mechanism that results in the degradation of transcripts containing premature translation termination codons. It also influences the expression of certain wild-type transcripts [for review, see (Maquat, 2004)]. This mechanism is important to limit the presence of truncated protein which could have a dominant-negative effect and are likely to be much more damageable to the cell and the organism than a half amount of gene product expected in heterozygotes. In our previous work, we had demonstrated that the surrexpression of a c.144delC mutant protein in Hela cells lead to the production of a 71aa truncated protein stopped by the first stop codon read after the frame-shift deletion. In the Hela cells the mRNA concentration was, however, ~50 times greater in cells transfected with the normal AURKC sequence than with the c.144delC mutated clone, indicating the presence of a strong mRNA decay of the c.144delC allele in Hela cells (Dieterich et al., 2007). Here, this mRNA decay is confirmed in vivo on blood leukocytes. This suggests that c.144delC homozygous patients (the vast majority of macrozocephalic patients yet described) do not have any AURKC protein. As stated previously the new mutation described here does not create any nonsense signal/stop codon in the mRNA sequence and logically we did not observe any nonsense mRNA decay of the mutant allele.

Aurora kinases (AURK A, B and C) are cell cycle regulatory serine/threonine kinases essential to the successful execution of mitotic cell division by ensuring the formation of a bipolar spindle and accurate chromosome segregation (Bischoff et al., 2002). AURKC, shares a high amino acid sequence identity with AURKB but its expression is mainly testis-specific (Bernard et al., 1998; Tang et al., 2001) where it is involved in chromatin condensation and proper attachment of homologous chromosomes during the first meiotic division (Tang et al., 2006). Abnormal cell division was observed in vitro upon dephosphorylation of AURKB as well as upon overexpression of AURKB and AURKC mutant proteins (Tatsuka et al., 1998; Honda et al., 2003).

In each case large multinucleated cells accumulated, reminiscent of the large-headed spermatozoa observed in macrozoospermia. AURKC could rescue the AURKB-silenced multinucleation phenotype, suggesting that its function can overlap with and complement AURKB during mitosis (Sasai et al., 2004). Aurora C knockout mice are viable and males have normal testis weights, but reduced litter size, with some males being sterile. Homozygous male have a higher rate of morphologically abnormal spermatozoa (21%) compared with normal controls (5%; Kimmins et al., 2007). Abnormalities include heterogeneous chromatin condensation, loose acrosomes and blunted heads. Original work suggested that there might be several AURKC copies in the mouse genome (Hu et al., 2000) and this could explain the mild phenotype observed in Aurkc−/− mice (Yang et al., 2010; Avo Santos et al., 2011). Careful search of the updated mouse genome, however, does not support this information. We therefore believe that the milder phenotype observed in mouse compared with human is likely due to a greater overlap of AURB and C functions in mice spermatogenesis compared with human, allowing for AURB to compensate the absence of AURKC only in mice. AURKC is also described to be present in both human and mice oocytes. The fecundity of Aurkc−/− female mice was not discussed (Kimmins et al., 2007), presumably because it was not affected, which is what was observed in human (Dieterich et al., 2009). Recent work, however, indicates that microinjection of a kinase-deficient Aurora-C (Aur-CKD) mRNA into mouse oocytes led to the production of cytoplasmic failure in meiosis I, resulting in producing large polyploid oocytes, a pattern similar to Aurora-C deficiency human spermatozoa (Yang et al., 2010). The authors conclude that Aurkc but not Aurkb plays an essential role in mouse oogenesis. One can, however, wonder whether the injected deficient mRNA and the protein it subsequently produces would not interfere with both Aurkc and b, potentially by saturating the physiological localization of both native proteins. The observed phenotype would therefore be equivalent of oocyte-specific Aurkb and c double mutant, thus explaining the effect on oogenesis. Whether the action AURKC is necessary for oogenesis therefore remains to be clarified. Interestingly AURKC has also been described to be highly expressed in early human preimplantation embryos (Avo Santos et al., 2011). The authors logically suggest that AURKC is likely involved in chromosome segregation in the first few embryonic divisions and speculate that it could be linked with the high aneuploidy rate observed in preimplantation embryos (Avo Santos et al., 2011). Overall these data confirms the implication of AURKC in gametogenesis and early human reproduction. The question that now needs to be addressed is what is the specificity of AURKC compared with AURKB in male meiosis?

The brothers studied here have a typical macrozocephalic phenotype but with a low sperm concentration (<1 M/ml) whereas the average sperm concentration measured in a series of 32 c.144delC homozygous men was 7.2 M/ml. This could indicate that the presence of the abnormal protein—with a truncation of exon 5—has an effect on cell concentration. On the other hand a few normal-looking spermatozoa could be observed and selected for each ICSI attempts. Such spermatozoa were seldom observed in c.144delC homozygous patients indicating that the truncated protein might preserve a small functionality permitting a few spermatozoa to pass meiosis. Careful selection by Motile Sperm Organelle Morphology Examination (MSOME) has previously been applied to select the more normal-looking spermatozoa
in AURKC c.144delC deleted patients (Chelli et al., 2010). In that study only six normal-looking spermatozoa were selected and FISH analysis was carried out on these spermatozoa. All six were aneuploid confirming that ICSI should not be attempted for AURKC mutated patients even after a very thorough morphological selection (Chelli et al., 2010). Here, in the course of 11 ICSI attempts a total of 88 normal-looking spermatozoa could be used for ICSI. Sperm morphology was assessed by standard light microscopy. As almost all spermatozoa were abnormal, the main criterion for selection and injection was whether the spermatozoa could fit into the ICSI pipette. Some spermatozoa with a slight defect in head or flagella morphology were therefore sometimes selected. Fertilization could be achieved by 47 of these gametes but no pregnancy was obtained after eight embryo transfer. We can speculate that these embryos were most likely carrying some gross chromosomal abnormalities which prevented sustained embryo development. Our results therefore reinforce our view that ICSI should not be attempted for AURKC mutated men.

We identified here a third mutation in the AURKC gene causing macrozoospermia opening the door for yet more allelic variants of this gene. We demonstrate again that there can be no hope of autologous fertilization for AURKC deficient men who can only be directed towards gamete donation or adoption. Other groups propose a rapid screening of exon 3 only to detect the presence of the recurrent c.144delC mutation in patients with macrozoospermia (El Kerch et al., 2011). Our findings indicate that the molecular analysis should not be stopped after a negative screening of exon 3 and that it is important to sequence the whole of AURKC coding sequence for men with macrozoospermia to assure that no rarer genetic variants will be missed and to avoid unnecessary ICSI cycles for these patients and their spouses. The prognosis for non-mutated men—normally with milder forms of the pathology—is more open. To better assess the reproductive potential of these patients a FISH analysis should be performed on spermatozoa. Depending on the results, ICSI, potentially accompanied by preimplantation genetic diagnosis could be proposed as mentioned and realized previously (Kahraman et al., 2004).

Authors’ roles
M.B.K., R.H.: undertook all the molecular work. R.Z.: supervised all the sperm analyses and embryo work. L.H., R.Z.: oversaw sample collection and supervised all clinical aspects of the work. P.F.R., C.A., J.L., R.Z.: contributed to data analysis. P.F.R. supervised all molecular laboratory work and interpreted the results. P.F.R. designed the overall study, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to the report.

Acknowledgements
We thank our patients and family members for their participation. We thank Nabila Ben Khoud for her precious help in sample collection.

Funding
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). This work was also funded in part by program CIBLE 2009 from the Rhône-Alpes Région.

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