Identification of a new recurrent Aurora kinase C mutation in both European and African men with macrozoospermia

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STUDY QUESTION: Can we identify new sequence variants in the aurora kinase C gene (AURKC) of patients with macrozoospermia and establish a genotype—phenotype correlation?

SUMMARY ANSWER: We identified a new non-sense mutation, p.Y248*, that represents 13% of all mutant alleles. There was no difference in the phenotype of individuals carrying this new mutation versus the initially described and main mutation c.144delC.
WHAT IS KNOWN ALREADY: The absence of a functional AURKC gene causes primary infertility in men by blocking the first meiotic division and leading to the production of tetraploid large-headed spermatozoa. We previously demonstrated that most affected men were of North African origin and carried a homozygous truncating mutation (c.144delC).

STUDY DESIGN, SIZE, DURATION: This is a retrospective study carried out on patients consulting for infertility and described as having >5% large-headed spermatozoa. A total of 87 patients are presented here, 43 patients were published previously and 44 are new patients recruited between January 2008 and December 2011.

PARTICIPANTS/MATERIALS, SETTING, METHODS: All patients consulted for primary infertility in fertility clinics in France (n = 44), Tunisia (n = 30), Morocco (n = 9) or Algeria (n = 4). Sperm analysis was carried out in the recruiting fertility clinics and all molecular analyses were performed at Grenoble teaching hospital. DNA was extracted from blood or saliva and the seven AURKC exons were sequenced. RT–PCR was carried out on transcripts extracted from leukocytes from one patient homozygous for p.Y248*. Microsatellite analysis was performed on all p.Y248* patients to evaluate the age of this new mutation.

MAIN RESULTS AND THE ROLE OF CHANCE: We identified a new non-sense mutation, p.Y248*, in 10 unrelated individuals of European (n = 4) and North African origin (n = 6). We show that this new variant represents 13% of all mutant alleles and that the initially described c.144delC variant accounts for almost all of the remaining mutated alleles (85.5%). No mutated transcripts could be detected by RT–PCR suggesting a specific degradation of the mutant transcripts by non-sense mediated mRNA decay. A rare variant located in the 3′ untranslated region was found to strictly co-segregate with p.Y248*, demonstrating a founding effect. Microsatellite analysis confirmed this linkage and allowed us to estimate a mutational age of between 925 and 1325 years, predating the c.144delC variant predicted by the same method to have arisen 250–650 years ago. Patients with no identified AURKC mutation (n = 15) have significantly improved parameters in terms of vitality and concentration of normal spermatozoa, and a decreased rate of spermatozoa with a large head and multiple flagella (P < 0.001).

LIMITATIONS, REASONS FOR CAUTION: Despite adherence to the World Health Organization guidelines, large variations in most characteristic sperm parameters were observed, even for patients with the same homozygous mutation. We believe that is mainly related to inter-laboratory variability in sperm parameter scoring. This prevented us from establishing clear-cut values to indicate a need for molecular analysis of patients with macrozoospermia.

WIDER IMPLICATIONS OF THE FINDINGS: This study confirms yet again the importance of AURKC mutations in the aetiology of macrozoospermia. Although a large majority of patients are of North African origin, we have now identified European patients carrying a new non-sense mutation indicating that a diagnosis of absence of a functional AURKC gene should not be ruled out for non-Magrebian individuals. Indirect evidence indicates that AURKC might be playing a role in the meiotic spindle assembly checkpoint (SAC) during meiosis. We postulate that heterozygous men might have a more relaxed SAC leading to a more abundant sperm production and a reproductive advantage. This could be the reason for the rapid accumulation of the two AURKC mutations we observe in North African individuals.

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 programme GENOPAT 2009 from the French Research Agency (ANR).

Key words: male infertility / spermatogenesis / gene mutations / genetic diagnosis / aurora kinase C

Introduction

Men presenting with a primary infertility characterized by the presence of the ejaculate of 100% morphologically abnormal spermatozoa with a majority of large-headed, multi-tailed gametes have been reported in the scientific literature for over three decades (Nistal et al., 1977; German et al., 1981; Escalier, 1983; Escalier, 2002). This sperm abnormality is listed in Online Mendelian Inheritance in Man (#243060) and referred to as ‘male infertility with large headed multiflagellar spermatozoa’, ‘macronuclear spermatozoa’ or ‘macrozoospermia’. Studies of Feulgen-stained preparations (German et al., 1981), spermatocyte C-banding (Pieters et al., 1998) and fluorescence in situ hybridization (FISH) analysis (In’t Veld et al., 1997; Pieters et al., 1998; Viville et al., 2000; Benzacken et al., 2001; Devillard et al., 2002; Guthauser et al., 2006; Mateu et al., 2006; Perrin et al., 2008; Chelli et al., 2010) soon demonstrated that these spermatozoa had a large excess of genetic material, suggestive of a meiotic dysfunction. The description of affected siblings and the high incidence of cases from consanguineous couples were suggestive of a genetic cause with an autosomal recessive inheritance. We therefore carried out a whole genome scan on a small series of North African patients which allowed us to identify a shared region of homozygosity in 19q13 which was present in a large majority of the tested patients. The Aurora Kinase C gene (AURKC), reported to be expressed preferentially in the testis and involved in chromosomal segregation and cytokinesis, was localised in the centre of this candidate region and thus appeared as a good candidate. The same homozygous mutation, c.144delC, was identified in all 14 patients analysed, suggesting a role for AURKC in genesis of the macrozoospermia phenotype (Dieterich et al., 2007). We subsequently analysed 18 additional patients with a pure phenotype: 17 were c.144delC homozygous and one was a compound heterozygote with the recurrent mutation and p.C229Y, a new missense mutation (Dieterich et al., 2009). A heterozygous splicing mutation was also identified in two affected brothers who also carried the c.144delC mutation (Ben Khelifa, et al., 2011). Previous
FISH studies had highlighted the presence of a large chromosomal excess in these abnormal spermatozoa with a heterogeneous population of haploid, diploid and tetraploid gametes. Analysis of the DNA content of four patients by flow cytometry after propidium iodine staining demonstrated that all analysed spermatozoa were in fact tetraploid, indicating that meiosis was blocked before the completion of the first division. These results, discordant with data from the previous FISH studies, suggested that FISH analyses on such chromosomally abnormal gametes led to a large underestimate of the number of chromosomes/chromatids present in the analysed spermatozoa, probably owing to the frequent overlapping of signals (Dieterich et al., 2009).

Aurora kinases A and B (AURKA, B) are ubiquitous cell cycle regulatory serine/threonine kinases which are essential to the successful formation of a bipolar spindle prior to chromosome segregation (Bischoff et al., 2002). AURKC shares a high amino-acid sequence identity with AURKB but it is expressed predominantly in male germ cells (Bernard et al., 1998; Tang et al., 2001; Tang et al., 2006). It has been demonstrated that AURKC and AURKB share the same substrates as both phosphorylate in vitro the centromeric histone Centromere Protein-A (CENP-A) and Borealin (Slattery et al., 2008). An abnormal cell division was observed in vitro upon the depletion of AURKB or overexpression of AURKB and AURKC mutant proteins (Tatsukuri 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). Interestingly, AURKC has also been described as being highly expressed in early human preimplantation embryos (Avo Santos et al., 2011). It has been suggested that AURKC is likely involved in chromosome segregation in the first few embryonic divisions and speculated that it could be related to the high aneuploidy rate observed in preimplantation embryos (Avo Santos et al., 2011). The importance of AURKC during preimplantation development has been confirmed in mice as it was demonstrated that Aurkc endogenous expression alone in Aurkb-deficient mice could sustain preimplantation development up to the late blastocyst stage (Fernandez-Miranda et al., 2011). Furthermore, the authors demonstrated that the timing of the developmental arrest of the Aurkb knock-out mice coincides with the switching off of Aurkc suggesting again that Aurkc could replace Aurkb during these first few meiotic divisions (Fernandez-Miranda et al., 2011). The opposite is, however, not true as Aurkb is expressed in male germ cells (Tang et al., 2006) but the absence of Aurkb leads to macrozoospermia by provoking meiosis I arrest (Dieterich et al., 2007; Dieterich et al., 2009). Interestingly, the specificity of AURKC in male and female meiosis seems to be reversed in man and mouse. Homozygous male knockout mice produce abnormal spermatozoa but are fertile and produce litters of reduced size, whereas all homozygous mutated men are sterile (Kimmins et al., 2007). Conversely, women with homozygous AURKC mutations are fertile (Dieterich et al., 2009), whereas the repression of Aurkc in mouse oocytes was shown to cause cytokinesis failure in meiosis I, resulting in the production of large polypliod oocytes, a pattern similar to AURKC-deficient human spermatozoa (Yang et al., 2010).

Here we present the genetic analysis of a large cohort of macrozoospermic patients. We identified a second ancestral mutation presenting with a geographic range of Europe to North Africa. We assessed the effect of this mutation at the mRNA level. In the light of AURKC function in spermatogenesis, we discuss the possible mechanisms that might have contributed to the spreading and accumulation of these two deleterious mutations over time.

Materials and Methods

Patient information

Forty-four patients were recruited between January 2008 and December 2011. Patients were included when they were described to have >5% 'large-headed' spermatozoa. To obtain meaningful epidemiological data we also included data from two of our previous publications: 41 patients (out of 62 patients described) with >5% large-headed spermatozoa who were presented in Dieterich et al. (2009) and the two patients who were described in Ben Khelifa et al. (2011).

All 87 patients consulted for primary infertility in fertility clinics in France (n = 44), Tunisia (n = 30), Morocco (9) or Algeria (4). A large majority of the French patients originated from North Africa (30/44), while the other 14 French patients had no known ascendants from North Africa. The patients were unrelated apart from four sets of two brothers recruited in France (n = 1 set), Algeria (n = 1) and Tunisia (n = 2). A total of 83 index cases (166 alleles) are therefore presented in Fig. 1. None of the patients had chromosomal abnormalities detected by karyotype analysis.

Control DNAs were extracted from blood of anonymous French donors originating from North Africa (n = 100) and Europe (n = 100). All patients, family members and anonymous donors gave their written informed consent, and all national laws and regulations were respected. Ethical approval was obtained from Grenoble CHU review board.

Sperm analysis

Sperm was carried out in the source laboratories during the course of the routine biological examination of the patient, according to World Health Organization (WHO, 1999) guidelines. Small variations in protocol might occur between the different laboratories.

Molecular analysis

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 guanidinium chloride extraction procedure (Jeanpierre, 1987). Saliva was collected with a Oragene DNA Self-Collection Kit (DNAgenotech, Canada) and DNA extraction was performed following the manufacturer’s recommendations.

RNA extraction

Nucleated cells were isolated from whole blood using ficoll® 400 (Sigma-Aldrich Corp., St. Louis, MO, USA) following the manufacturer’s protocol. RNA extraction was carried out on the isolated white blood cells using Macherey Nagel NucleoSpin® RNA II columns (Macherey Nagel, Hoenrdt, France) using the manufacturer’s protocol.

Mutation detection

The seven AURKC exons and intron/exon boundaries were amplified by PCR and sequenced, 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 Biosoysms, Foster City, CA, USA).
**RT–PCR**
Reverse transcription was carried out with 5 µl of extracted RNA (≏500 ng). Hybridization of the oligo dT was performed by incubating for 5 min at 65°C and quenching on ice with the following mix: 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. Reverse Transcription then was carried out for 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). Two microlitres of the obtained cDNA mix was used for the subsequent PCR. AURKC 5′ primer was located on exon 4 (5′ CAATATCCTGCGCCTGTATAACT 3′) and the 3′ primer on exon 6 (5′ TCATTCTGGCGGCAAGT 3′). Two microlitres of the reversed transcribed RNA was amplified with these primers (40 cycles) at an elongation temperature of 58°C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as control. Thirty-five cycles of PCR amplification were carried out at an elongation temperature of 60°C with the following GAPDH primers (5′ to 3′): GAGTCAACGGATTTGGTCGT and TTGATTTTGGAGGGATCTCG.

**Statistical analysis**
The different sperm parameters (Table I) were compared in different groups using a two-tailed t-test. A value of P < 0.05 was considered significant. Analyses were carried out with the GraphPad software.

**Results**
Here we present new sequence data from 44 patients with macrozoospermia and analyse these together with our previous data from an additional 43 patients.

**AURKC sequencing and HRM analysis**
In a previous study (Dieterich et al., 2009) we reported that 31/41 patients presenting with >5% large-headed spermatozoa were c.144delC homozygous, one was a compound heterozygote carrying c.144delC with a false-sense variant: p.C229Y and 9 (classified as atypical) were non-deleted. No variants had been detected in...
19 patients with <5% large-headed spermatozoa (Dieterich et al., 2009), as they do not fulfil the criteria for this study (i.e. >5% large-headed spermatozoa) these 19 patients are not included here. In a subsequent work we described two brothers who were compound heterozygotes carrying c.144delC with a splicing variant: c.436–2A>G (Ben Khelifa et al., 2011). In these two studies we therefore had recruited and analysed a total of 43 patients with macrozoospermia, of whom 34 carried two AURKC mutated alleles.

A total of 44 patients were recruited between January 2008 and December 2011 who we had not described previously. The AURKC exon 3 was sequenced in all 44 patients. The remaining 6 exons and intron boundaries were sequenced for all who did not present a c.144delC homozygous mutation. No mutations were identified in 6 patients and the remaining 38 carried two mutations. A new nonsense mutation: p.Y248*; c.744C>G (NM_001015878) was identified in 11 patients (Fig. 2). Overall 27 men were c.144delC homozygous, 9 were p.Y248* homozygous (including 2 brothers) and 2 were compound heterozygotes for these two mutations. An additional variant located in AURKC 3′UTR (c.930+38G>A) was homozygous in all p.Y248* homozygous patients and heterozygous in the two compound heterozygotes (Fig. 2).

When all patients are pooled, we have a total of 72 patients with a mutation out of 87 (83%) or 68 out of 83 probands (82%). The c.144delC variant represents 85.5% of the mutated alleles (taking into account only one proband in familial cases), the p.Y248* represents 13% of the alleles and two familial mutations account for the remaining 1.5% (Fig. 1). Probands were recruited equally in France (43) and in North Africa (40). All patients recruited in North Africa originated from North Africa and the majority of the French patients

<table>
<thead>
<tr>
<th>Table I</th>
<th>Semen parameters of patients according to aurora kinase C (AURKC) genotype.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>AURKC mutation (n = 72)</td>
</tr>
<tr>
<td>Sperm volume (ml)</td>
<td>2.5 (0.1–5.5)</td>
</tr>
<tr>
<td>Sperm concentration (10⁶ per ml)</td>
<td>13.1 (0.01–98.7)</td>
</tr>
<tr>
<td>Round cells (10⁶ per ml)</td>
<td>4.9 (0–27.95)</td>
</tr>
<tr>
<td>Motility A + B, 1 h</td>
<td>19.5 (0–70)</td>
</tr>
<tr>
<td>Sperm vitality</td>
<td>24.0 (0–66)</td>
</tr>
<tr>
<td>Normal spermatozoa</td>
<td>0.1 (0–1)</td>
</tr>
<tr>
<td>Large-headed spermatozoa</td>
<td>79.6 (34–100)</td>
</tr>
<tr>
<td>Multiflagellar spermatozoa</td>
<td>37.9 (7–100)</td>
</tr>
<tr>
<td>Multiple anomalies index</td>
<td>3.0 (2.95–4.1)</td>
</tr>
</tbody>
</table>

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

*Significant P < 0.001.

Figure 2 Electropherogram and HRM analysis of AURKC exons 6 and 7 with the 3′UTR. (A) Electropherogram showing part of AURKC exon 6 for a wild-type subject (Control) and a p.Y248* homozygous patient (P hmz). (B) Electropherogram showing part of AURKC 3′ UTR from the same individuals showing the presence (or absence) of the c.930+38G>A variant. (C) and (D) HRM analysis of AURKC exon 6 and 7 (with 3′UTR) respectively, of control subjects (blue), heterozygous (green) and homozygous (red) patients.
were also of North African origin (67%). The frequency of positive diagnoses was much higher in probands of North African origin (93%) compared with probands of European origin (29%) (Fig. 1).

The p.Y248* was identified in six homozygous probands of North African origin and in four probands of European origin including two homozygotes and two compound heterozygotes who also carried the c.144delC variant. These individuals had no known relative of North African descent. These two compound heterozygotes were the only two patients of European origin identified as carrying the c.144delC variant.

To exclude the possibility that the identified p.Y248* and its associated c.930+38G>A variant may be common in the studied populations, we performed an HRM of AURKC exon 6 and 7 in 100 individuals of French origin and 100 individuals of North African origin. Homozygous and heterozygous patients were passed in triplicate and are shown in red and green, respectively, in Fig. 2. Each profile (homozygous, heterozygous and non-mutated) was clearly distinct (Fig. 2C and D). There was no abnormal exon 6 profile from any of the 200 tested DNAs, confirming that the p.Y248* mutation is not frequent in either European or North African individuals. HRM of exon 7 allowed us to identify three c.930+38G>A heterozygotes among the European and five among the North African control subjects indicating a frequency of heterozygosity of ≏4% or an allelic frequency of 2%. UCSC genome bioinformatics presents a pool of 1097 individuals with a heterozygosity rate of 41%, concordant with our findings. These results indicate that c.930+38G>A is a rare variant and exclude a chance association of c.930+38G>A with p.Y248*.

Transcript analysis
As a truncating mutation, p.Y248* is expected to have a severe impact on the protein function. A residual activity could however result from a shortened peptide. We therefore carried out RT–PCR on transcripts extracted from leukocytes from one p.Y248* homozygous patient. Control amplification with a house keeping gene (GAPDH) was positive from the patient and control subjects, whereas AURKC amplification could only be obtained from the controls (Fig. 3). This indicates that AURKC RNA is not present in p.Y248* patients, suggestive of mRNA decay associated with this mutation.

Sperm analysis
The sperm parameters of the patients carrying c.144delC were compared with those carrying p.Y248* (including the two heterozygous individuals). There was no difference between the two groups. Data from both groups were therefore pooled and compared with data from the patients without a mutation (Table 1).

All patients with a mutation had a large majority of macrozoosperms (average of 80%) and 37% of the spermatozoa were reported as multiflagellar. There was no difference in sperm volume, spermatozoa, round cells concentration, mobility or multiple anomaly index. There was, however, a significant increase in the proportion of large-headed and multiflagellar spermatozoa in patients with a mutation compared with those with no mutation, and there was a decrease in the frequency of typical spermatozoa and sperm vitality in the individuals with a mutation.

Dating of the mutations
To compute the age of p.Y248*, we used the number of ancestral haplotypes at the different microsatellite markers that are linked to the locus of interest (Fig. 4). There are 9, 14, 10 and 5 (among 18) ancestral haplotypes between the locus of interest and D19S210, D19S214, D19S218 and D19S890, respectively. The probability to observe a non-ancestral haplotype is given by \(1 - c\), where \(g\) is the allele age in generations and \(c\) is the crossover rate (Slatkin and Rannala, 2000). The crossover rate in the region of chromosome 19 between D19S210 and D19S890 is 3.1 cM/MB as provided by the genetic map of Marshfield (Broman et al., 1998). The number of ancestral haplotypes is a binomial random variable with \(n = 18\) and \(P = (1 - c)^g\). Multiplying the four binomial distributions corresponding to the four microsatellite loci, we calculate a pseudo-likelihood function which is maximal at \(g = 37\) generations.

Labuda et al. (1996) have shown that the dating arising from such genetic clock methods is biased downwards because this method does not account for the demographic expansion of the population. To account for an exponential expansion of rate \(r\) per generation, the mathematical expression: 
\[
(1/r) \ln(c'/c' - 1)
\]
should be added to the estimates provided by genetic clock methods. Assuming that an upper bound for the expansion rate is given by the expansion \(r = 0.4\), as was calculated for the Ashkenazy Jewish populations (Risch et al., 1995), we found that the age of the mutation is in the range of 37–53 generations. Taking 25 years for the generation time, the mutation would have then arisen some 925–1325 years ago.

We then estimated the age of the c.144delC mutation using the same method. For that we used the haplotypes of 14 individuals published previously (Dieterich et al., 2007). There are 27, 20 and...
15 (among 28) ancestral haplotypes between the locus of interest and D19S214, D19S218 and D19S890, respectively. After using the adjustment of Labuda et al. (1996), this results in a mutation occurring 10–26 generations (250–650 years) ago.

**Discussion**

We presented data from a total of 87 patients (44 new patients and 43 reported previously) presenting with macrozoospermia and have identified a new mutation, p.Y248*, in 10 unrelated individuals of both Maghrebian and European origin. We observed that this variant accounts for 13% of the mutant alleles and that 85% of the remaining alleles bear the initially described c.144delC mutation. We show that p.Y248* is also a founding mutation that largely predates the c.144delC variant. As a nonsense mutation, we anticipated that p.Y248* would have a severe effect on the protein. We wanted to assess whether p.Y248* transcripts could lead to the production of a truncated protein which could potentially retain some functionality, or if the mutated transcript was subjected to nonsense-mediated mRNA decay (NMD) resulting in the absence of the protein (Maquat, 2004). Although AURKC is preferentially expressed in the testis, it is also weakly expressed in several tissues including the lung, ovary or skeletal muscle (Yan et al., 2005) and we had also noticed that AURKC transcripts were present in blood leukocytes.

We carried out RT–PCR on leukocytes from one of the p.Y248* homozygous patients. Contrary to what was observed in a fertile control, we could not detect any AURKC transcript in our patient, demonstrating that the p.Y248* mutation was subjected to NMD, at least in leukocytes (Fig. 3). We had previously observed that the recurrent c.144delC mutated transcript, which contains several premature stop codons, was also subject to NMD (Ben Khelifa et al., 2011). From what we observed in blood we can conclude that both recurrent...
mutations are very likely to have the same effect and both lead to a total absence of the protein.

The values measured during the routine spermogram and spermocytogram were compared between patients with c.144delC, Y248* and patients without a mutation (Table I). Not surprisingly, as we demonstrated that both c.144delC and p.Y248* resulted in the absence of the protein, there was no phenotypic difference between these two groups of patients. Patients with no identified AURKC mutation (n = 15) have significantly improved parameters in terms of vitality and concentration of normal spermatozoa, and a decrease in spermatozoa with a large head and multiple flagella (Table I). Patients came from different centres and despite a common observance to the WHO guidelines (WHO, 1999) important variations in scoring were observed, even in genotypically identical individuals. In our experience, patients with a mutation never present any typical spermatozoa and have a large majority of large-headed spermatozoa. We believe that inter-laboratory variability in scoring of the most relevant morphological features, such as the concentration of large-headed and multi-flagellar spermatozoa, is responsible for some of the overlap observed between patients with and without mutations. It is perhaps the presence of >1% normal spermatozoa that is the most discriminating parameter as this was never reported in individuals with a mutation. The absence of normal spermatozoa was observed in a few individuals with no mutation but this was only observed in patients with very severe oligozoospermia who more likely suffered from severe oligoteratozoospermia rather than pure macrozoospermia. As a routine diagnosis strategy in men, we propose the sequencing of AURKC exons 3 and 6 for all patients presenting with a high proportion of large-headed spermatozoa. In the absence of a mutation, the sequencing of the remaining exons could be proposed only to individuals with a sperm concentration >1 million and described as having <1% normal spermatozoa.

We have estimated here the age of the two AURKC recurrent mutations. We calculated that p.Y248* arose 925–1325 years ago and therefore largely predates c.144delC, dated as having occurred 250–650 years ago. The fact that p.Y248* is more ancient than c.144delC is concordant with the wider geographical coverage of p.Y248* that is found in individuals of both European and North African origin. We had calculated previously that the c.144delC variant was more ancient than reported here (Dieterich et al., 2007). We had used a simplified method and considered that the smallest conserved region spanned from the mutation to D19S214, not taking into account the conserved sequences centromeric to AURKC. We believe that the method used here is likely to give a much better estimate of the age of the mutations.

We had previously measured that 1 in 50 individuals originating from North Africa carried a copy of c.144delC (Dieterich et al., 2009). It seems surprising that a pathological variant having a negative effect on reproduction reached such a high frequency in a relatively short period. It is also surprising to identify a second ancestral founding allele with a wide geographic coverage.

Research carried out on aurora B and C, as summarised in the introduction, demonstrated that AURKC has an essential role in meiotic interkinesis (the equivalent of cytokinesis in mitosis) and also that AURKC participates in the control of microtubule–kinetochore attachment, verifying the bi-orientation of the tensions preceding chromosome segregation, thus ensuring the production of euploid gametes. During meiosis, germ cells with misaligned chromosomes or abnormal microtubule tension likely to result in missegregation, are blocked in prometaphase. This control, called spindle assembly checkpoint (SAC), is essential in mitosis to limit the production of aneuploid cells, which are likely to transform into malignant cells, and in meiosis to limit the conception of chromosomally abnormal gametes and embryos. There is now an increasing body of evidence indicating that the action of AURKC in microtubule–kinetochore attachment is an integral part of the SAC, Lane et al. (2010) studied the effect of ZM447439, a general inhibitor of aurora kinases on oocyte meiosis and they showed that while a continuous exposure to ZM447439 logically led to a meiosis I block, a short exposure at the prometaphase stage accelerated polar body extrusion. They also demonstrated that this treatment allowed an overriding of the SAC induced by nocodazole treatment, therefore indicating that the accelerated meiosis was linked to a speedier, and thus relaxed, SAC provoked by the inactivation of the aurora kinases. They observed a 15% increase in aneuploid oocytes in the ZM447439-treated group therefore supporting a role for an alteration in the checkpoint mechanism.

The involvement of AURKC in the SAC might explain some intriguing features concerning large-headed spermatozoa. First, to our knowledge, macrozoospermia is the only sperm phenotype associated with meiotic arrest. All other genetic alterations leading to meiotic arrest result in azoospermia (following appropriate activation of the SAC). We can take, for example, chromosome Y microdeletions or the effect of Dmc1 deficiency in mice (Bannister et al., 2007). The macrozoospermia phenotype caused by the absence of AURKC therefore constitutes an in vivo confirmation of the predominant role of AURKC in the SAC, as in the absence of AURKC interkinesis is blocked but spermiogenesis is pursued leading to the production of tetraploid flagellated spermatozoa. Second, we are surprised to find two deleterious mutations which have reached a relatively high frequency (c.144delC heterozygous frequency is 1/50 in the North African population (Dieterich et al., 2009)) over a relatively short period of time. We can wonder if the reduced amount of AURKC protein in heterozygous men could have an effect similar to that of ZM447439 in the oocyte: the SAC could be slightly relaxed leading to sperm and thus much more abundant spermatogenesis. We therefore would expect heterozygotes to have an increased sperm count, probably accompanied by a small increase in aneuploidy. This could entail a small reproductive advantage that could explain the rapid propagation of AURKC mutations. We intend to verify this theory but we are having difficulties in recruiting heterozygous donors.

Most genetic diseases have manifestations that are dependent on the effect of the mutation(s) on the protein. Cystic fibrosis is a classic example; more than 1500 mutations have been identified so far leading to symptoms ranging from typical manifestation with severe lung and pancreatic impairment to male infertility only, owing to atresia of the vas deferens. In macrozoospermia we have detected only four mutations: the two recurrent mutations described here, a missense mutation identified in only one patient and a splicing variant resulting in loss of an exon identified in two brothers. All patients with a mutation present with the most severe form of the disease: 0% normal spermatozoa with close to 100% large-headed spermatozoa and we have not identified any mutations in patients presenting with a mosaic of normal and large-headed spermatozoa. There can be three explanations for these two observations (1) ‘milder’
mutations are infrequent and we have not yet sampled the right patients. (2) AURKC protein sequence is extremely sensitive to change and all/most mutations give a severe phenotype but this does not explain why so few variants have been identified so far and 3) ‘milder’ mutations result in a distinct phenotype, thus explaining why we identified so few variants and why we did not identify any AURKC mutations in mosaic macrozoospermia. We favour this last hypothesis as we can imagine that less severe mutations (which would not result in protein loss by mRNA decay) might have an effect on microtubule–kinetochore attachment and/or on interkinetin kinase without blocking the SAC activity of the protein. If that were the case these less severe mutations would lead to azoosperminia, a phenotype considered, paradoxically, to be more severe than macrozoospermia. We therefore would not be surprised to identify ‘milder’ AURKC variants in non-obstructive azoospermia patients. We plan to sequence the exome of a cohort of azoospermia patients, and will be particularly attentive to the presence of AURCK variants that could reinforce this hypothesis.

This study confirms yet again the importance of AURKC mutations in the aetiology of macrozoospermia. Although a large majority of patients are of North African origin, we have now identified European patients carrying mainly a new non-sense mutation indicating that AURKC diagnosis should not be ruled out for non-Magrebian individuals. Indirect evidence concerning AURKC function in meiosis raises the possibility that the rapid accumulation of two AURKC mutations could be the consequence of a reproductive advantage for heterozygous individuals. We also postulate that other AURKC molecular alterations might induce a different and unexpected phenotype, namely azoospermia.

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Authors’ roles

M.B.K., C.C., F.A and R.H. carried out and interpreted all the molecular work. M.B., P.S.J., C.A. and J.L. and PR realised the analysis and interpretation of data; R.Z., A.G., P.M.P., V.M., J.R., C.T., G.M., F.V., I.K., S.V., L.K., J.P.S., N.R., B.D., F.L., L.H., C.P., B.B. and S.H. supervised all the patient’s information and contributed to the acquisition of data. All authors revised the manuscript and approved its final version. P.F.R. designed the overall study, supervised all molecular laboratory work, wrote the main draft of the manuscript, had full access to all of the data and takes responsibility for the integrity of the data and its accuracy.

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Conflict of interest

None declared.

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