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Homozygous mutation of *AURKC* yields large-headed polyploid spermatozoa and causes male infertility

Klaus Dieterich[1,2], Ricardo Soto Rifo[3,4], Anne Karen Faure[1,2,5], Sylviane Hennebicq[1,2,5], Baha Ben Amar[6], Mohamed Zahi[7], Julia Perrin[1,2], Delphine Martinez[1,2], Bernard Sèle[1,2,5], Pierre-Simon Jouk[1,2], Théophile Ohlmann[3,4], Sophie Rousseaux[1,2,5], Joel Lunardi[1,2] & Pierre F Ray[1,2]

The World Health Organization conservatively estimates that 80 million people suffer from infertility worldwide. Male factors are believed to be responsible for 20–50% of all infertility cases, but microdeletions of the Y chromosome are the only genetic defects altering human spermatogenesis that have been reported repeatedly[1]. We focused our work on infertile men with a normal somatic karyotype but typical spermatozoa mainly characterized by large heads, a variable number of tails and an increased chromosomal content (OMIM 243060)[2–4]. We performed a genome-wide microsatellite scan on ten infertile men presenting this characteristic phenotype. In all of these men, we identified a common region of homozygosity harboring the aurora kinase C gene (*AURKC*) with a single nucleotide deletion in the *AURKC* coding sequence. In addition, we show that this founder mutation results in premature termination of translation, yielding a truncated protein that lacks the kinase domain. We conclude that the absence of AURKC causes male infertility owing to the production of large-headed multiflagellar polyploid spermatozoa.

Semen from men with large-headed multiflagellar polyploid spermatozoa consistently showed close to 100% morphologically abnormal spermatozoa with low motility, oversized irregular heads, abnormal midpiece and acrosome and up to six flagella (**Fig. 1** and **Table 1**). Increased DNA content has been recurrently documented using Feulgen-stained preparations[5], spermatocyte C-banding[6] and FISH analysis[7–9]. More specifically, most gametes were polyploid with several flagella (**Fig. 1**), implying an incomplete partition of chromosomes and chromatids during meiosis I and II respectively, associated with a failure of cytokinesis followed by normal spermiogenesis events[8]. Most studies so far have explored candidate genes in populations of infertile men and have generated negative or ambiguous results probably due to genetic heterogeneity of the infertility phenotype and the relatively limited size of the tested populations. Here, we applied a classical identical-by-descent genetic procedure, which had not been used so far for the identification of genes causing infertility, to a few well selected individuals having a clear large-headed spermatozoa phenotype.

We carried out a genome-wide microsatellite scan of ten infertile men with a large-headed sperm phenotype. Four were unrelated French citizens of North African descent (G1–G4), all born from first-degree cousins, leading us to suspect a recessive mode of inheritance. The small genealogical distance between the four index individuals and a postulated morbid ancestor chromosome present in their respective great-grandmother or great-grandfather (three meioses) was compatible with large homozygous regions detectable with the low-coverage 10cM kit used. The other six men all came from the Rabat region in Morocco, suggesting the possibility of a founder effect. In that case, owing to probable greater genealogical distances, we expected smaller regions of homozygosity. Initial results led to the identification of six regions with at least two adjacent homozygous markers present in six of ten affected individuals. Five out of six

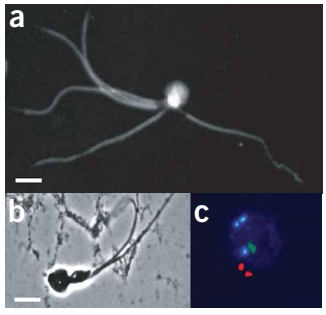

Figure 1 Large-headed spermatozoa. (**a**) DAPI staining, (**b**) phase contrast microscopy and (**c**) after FISH detection of chromosomes 18 (blue), X (green) and Y (red). Scale bar, 10 μm.

[1]Centre Hospitalier Universitaire (CHU) de Grenoble, Département de Génétique et Procréation, Unite Fonctionelle (UF) de biochimie génétique et moléculaire, F-38700 La Tronche, France. [2]Faculté de Médecine-Pharmacie, Domaine de la Merci, Université Joseph Fourier, F-38700, Grenoble, France. [3]Inserm, U758, Lyon, F-69364 France. [4]Ecole Normale Supérieure de Lyon, Unité de Virologie Humaine, IFR 128, Lyon, F-69364 France. [5]Inserm U309, Institut Albert Bonniot, Grenoble, F-38700, France. [6]Clinique Alboustane, Centre de FIV, 7 rue Descartes, Rabat, Morocco. [7]Centre privé de Fécondation in vitro, 13 rue A. Doukkali, les Orangers, Rabat, Morocco. Correspondence should be addressed to P.F.R. (pray@chu-grenoble.fr).

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Table 1 Sperm parameters of 14 subjects with *AURKC* homozygous mutation

Sperm characteristics	Average	Range
Large-headed spz (%)	56	34–100
Multiflagellar spz (%)	28.5	16–50
Abnormal acrosome (%)	76	31–100
Normal morphology (%)	2	0–5
Sperm volume (ml)	3.8	1.6–8
Number spz x 10^6 per ml	3.2	0.2–7.76
Motility (after 1 h) (%)	13	7–26
Motility (after 4 h) (%)	7.3	5–13

Spz, spermatozoa.

regions were excluded by analysis of additional interstitial microsatellite markers (data not shown), leaving 19qter as the last candidate region. At that stage we included four additional affected individuals from Rabat in the study. A detailed microsatellite analysis of the region established homozygosity in these four men, confirmed the status of the original six homozygous individuals and allowed the identification of a smaller region of homozygosity in three index cases that had passed unnoticed with the first low-coverage screen. In addition, 13 of 14 affected men were homozygous and haploidentical for marker D19S214, indicating the possible proximity of a single common causal genetic event (**Supplementary Fig. 1** online). We noticed that the gene encoding aurora kinase C (AURKC), known to be highly expressed in the testis[10,11] and putatively involved in cytokinesis, mitosis and meiosis[12,13], was located less than 40,000 bp away from D19S214. We sequenced the coding sequence and detected a homozygous cytosine (C) deletion in exon 3 (c.144delC) in all 14 affected individuals (**Fig. 2a**), including subject R3, who is heterozygous for D19S214 (**Supplementary Fig. 1**). Additional biological material was obtained from subject R6's family members. We confirmed that both parents were heterozygous for c.144delC; brother II:2 was also heterozygous and had fathered two children, and II:3 and II:4 were homozygous unaffected (**Fig. 2b**). We did not detect any mutant alleles among 100 chromosomes from European men, and one was identified in 100 chromosomes from North African men of proven fecundity. We observed a common haplotype in 20 of 28 of the mutant chromosomes studied between *AURKC* and D19S218 (460 kb). The genetic distance indicated in the Genethon map between D19S214 and D19S218 is 1.3 cM, or 1 out of 77 meiotic events. If we approximate that *AURKC* colocalizes with D19S214 and use an average generational time of 20 years, we conclude that the most recent common ancestor of 10 of our 14 subjects lived about 1,500 years ago.

At the protein level the alteration L49W fsx22 introduces a frameshift, inducing incorrect translation from amino acid 49 until premature protein arrest upon reaching the first stop codon at position 71 (**Fig. 3a**). Since the conserved catalytic domain of the protein begins at position 42, seven residues before the first modified amino acid, the mutant protein is unlikely to retain any kinase activity. Although the premature insertion of a stop codon usually leads to the production of a truncated protein, reinitiation of translation can take place in eukaryotes[14]. To verify that the described mutation actually leads to the premature arrest of protein synthesis we performed *in vitro* and *ex vivo* experiments using the wild-type and mutated sequence of *AURKC* (**Fig. 3a**). The *in vitro* assay consisted in translating the two mRNAs in the rabbit reticulocyte model. This resulted in the efficient synthesis of the wild-type protein at all the RNA concentrations tested (**Fig. 3b**). In contrast, no full-length protein product could be visualized on the autoradiography when the mutated *AURKC* transcript was used, indicating that translation reinitiation did not take place *in vitro*. The peptide resulting from the translation of the first 71 codons of the *AURKC* gene, containing only the first seven amino acids of the kinase catalytic domain, was detected. We then carried out *ex vivo* studies in which the capped and polyadenylated mRNAs encoding the wild-type and mutant AURKC were coupled to the *Renilla* luciferase enzyme and were directly transfected into HeLa cells. Seven hours after transfection, we measured luciferase activity in these cells (**Fig. 3c**). Again, virtually no activity could be detected from the mutated transcript, indicating that reinitiation of translation did not occur within human cells. Taken together, these experiments show that ribosomes are unable to resume translation of *AURKC* after the stop codon introduced following the cytosine deletion. As a result, translation of the mutated gene produces a short 71–amino acid peptide that lacks nine-tenths of its kinase catalytic domain.

We performed fluorescent *in situ* hybridization (FISH) of chromosomes 18, X and Y on spermatozoa from five AURKC-deficient individuals. Only 8% of the analyzed spermatozoa were haploid; in others the number of signals varied from one to four and zero to two for chromosome 18 and each of the sex chromosomes, respectively. Within these values, all combinations of chromosome abnormalities were detected (**Fig. 4**). Although a high proportion of hyperhaploid spermatozoa were found, there was no sign of hypohaploid

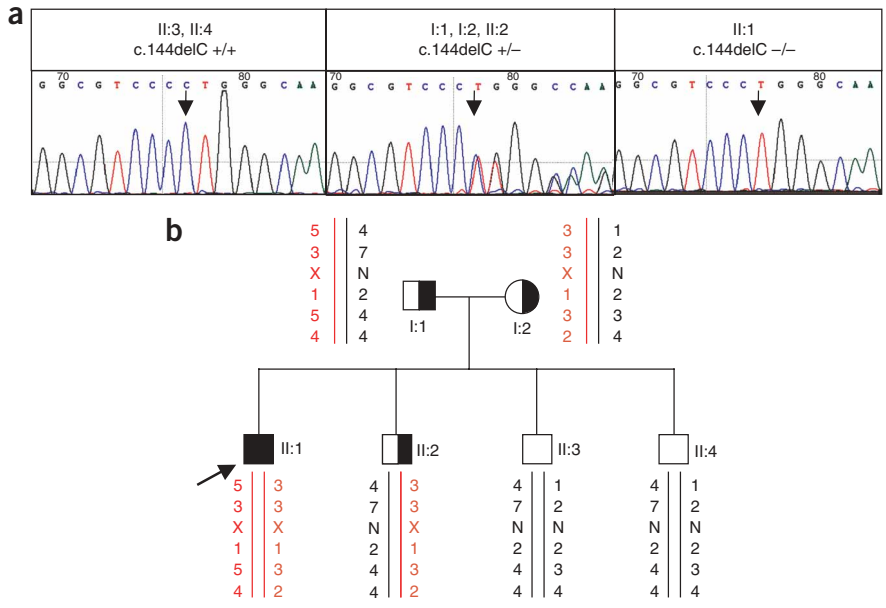

Figure 2 Segregation of the *AURKC* deletion in family R6. (**a**) Raw sequence results: proband II:1 shows a homozygous C deletion. Both parents (I:1 and I:2) and brother II:2 are heterozygous and brothers II:3 and II:4 are homozygous for the wild-type allele. (**b**) Family tree with haplotypes.

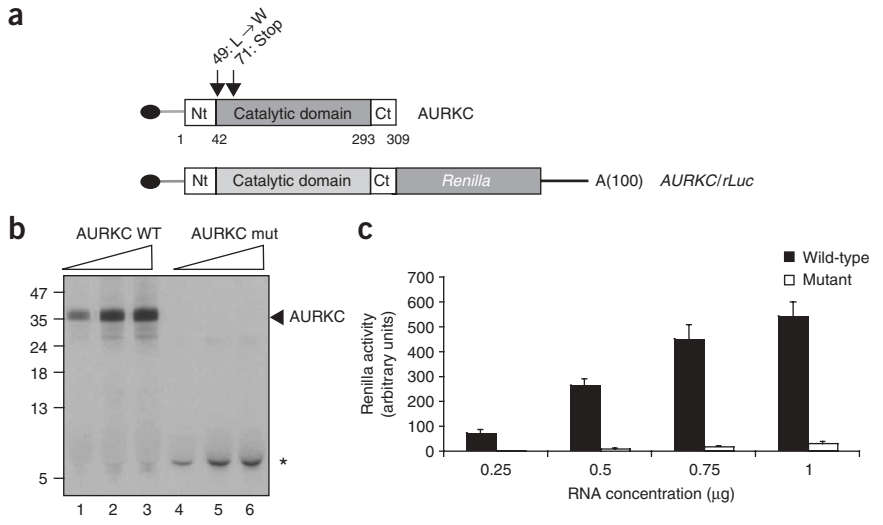

Figure 3 L49W fsx22 leads to premature termination of translation. (**a**) Schematic representation of the constructs used in this study. The position of the C deletion and the resulting stop codon are indicated by the arrows. (**b**) Different concentrations of capped wild-type (lane 1, 10 ng; 2, 30 ng; 3, 50 ng) and mutated (lane 4, 10 ng; 5, 30 ng; 6, 50 ng) *AURKC* transcripts were translated in the rabbit reticulocyte lysate for 30 min. Samples were then resolved on a 15% SDS-PAGE and submitted to autoradiography. The position of the molecular mass markers and the wild-type AURKC are indicated (kDa). The star indicates the position of amino acids 1–71 comprising the peptide resulting from premature termination of translation of the *AURKC* gene. This result is representative of four independent experiments. (**c**) Different amounts of capped and polyadenylated transcripts containing the wild-type or mutated version of *AURKC* in frame with *Renilla* luciferase were directly transfected in the cytoplasm of HeLa cells. Luciferase activity was measured 7 h after transfection, and the results obtained from four independent experiments are expressed as means ± s.e.m.

spermatozoa, suggesting that nuclear partition did not take place or was extremely disturbed. Recent work indicates that in mouse spermatogenesis, Aurkc is involved in chromosome segregation and cytokinesis[13]. Non- or mis-segregated homologous chromosomes or chromatids are expected to remain close to each other within the sperm nucleus; it is therefore likely that many of the FISH signals for such chromosomes will be superimposed and appear as a single fluorescent spot. As each sex chromosome is marked with a different fluorophore, superimposed X and Y signals are distinguishable and the presence of both sex chromosomes should be a good indicator of the failure of the first meiotic division. Ninety-two percent of the analyzed spermatozoa (65–99.8%) showed at least one signal for both the X and Y, indicating that a very large majority of the spermatozoa have a defective meiosis I division. It is probable that the second division would also be impaired in these cells, which would thus remain tetraploid. The overall FISH data depict a seemingly random chromosomal content. These observations can probably be explained by partial chromosomal and chromatid segregation resulting sometimes in the distinction of sister chromosomes and chromatids, sometimes in signal overlaps. The fact that two or more chromosome 18 signals were detected in 55% of the analyzed spermatozoa (44–64%), at a much lower frequency than that observed with the differently labeled sex chromosomes (92%), is an indirect cue indicating that signal overlap is likely to be a very frequent occurrence in these experiments. It is thus probable that most spermatozoa are tetraploid, indicating total cytokinesis failure, which would explain the large size of the gametes. Taking into consideration the intracellular localization of the mouse protein during spermatogenesis, we may speculate that AURKC deficiency impairs chromosomal segregation and that spermatozoa with blocked meioses then experience cytokinesis failure.

Aurora kinases A, B and C are cell cycle regulatory serine/threonine kinases believed to be essential to the successful execution of mitotic cell division by ensuring the formation of a bipolar spindle and accurate chromosome segregation[15]. AURKC, the last to be discovered and the least understood of the three, shares a high amino-acid sequence identity with AURKB but is present mainly in the testis[10,11], its expression seemingly restricted to meiosis[13]. Abnormal cell division was observed *in vitro* upon depletion of AURKB[16,17] as well as upon overexpression of AURKB and AURKC mutant proteins. In each case large multinucleated cells accumulated, reminiscent of the large-headed spermatozoa observed here. It was suggested that the overexpression of mutant AURKC had a dominant negative effect by denying AURKB its physiological localization on the spindle[18,19]. AURKC could rescue the AURKB-silenced multinucleation phenotype, suggesting that its function can overlap with and complement AURKB during mitosis[20]. In this study we observed that despite their infertility phenotype, c.144delC homozygous men did not show any obvious physiological or anatomical defects, demonstrating that AURKC expression is not essential in human somatic tissues. We also observed that heterozygous men did not have a reduced fecundity: all affected men were (more than likely) born from heterozygous fathers and had numerous siblings (three to nine). These facts are in disfavor of a dominant negative effect acting through AURKB such as the one observed in cells overexpressing a mutant AURKC. The possibility cannot be completely excluded, however, that the small N-terminal portion of the mutant protein visualized in the *in vitro* translation study (**Fig. 3b**) could interfere with some restoration of function by AURKB. *Aurkc*

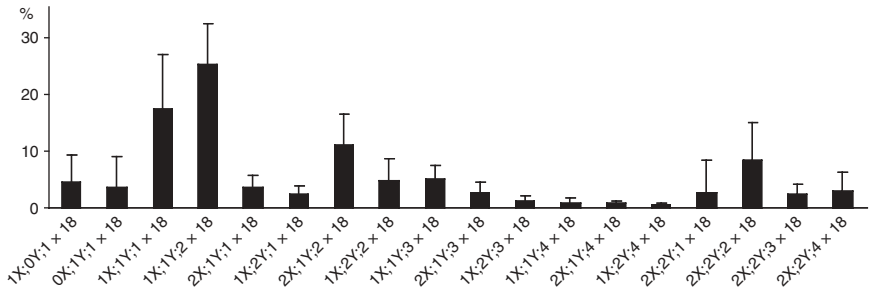

Figure 4 Proportion of the different chromosomal constitutions detected by FISH for chromosomes X,Y and 18 on a total of 3,689 spermatozoa from subjects G1, G2, G3, R1 and R3. The number of chromosomes detected is indicated by 'x'. Error bars indicate the s.d. of the mean number of spermatozoa for each chromosomal constitution for each individual.

null mice have recently been produced[21]. As observed in humans, homozygous mutant animals have no apparent somatic defect and both male and female heterozygous mice show normal fertility. Reduction of fecundity in homozygous females was not reported, whereas 40% of homozygous males failed to produce pups, the remaining having litters that were 31% smaller than their wild-type littermates. Sperm count and testis weight in *Aurkc*$^{-/-}$ were normal, but 20% of the spermatozoa were abnormal, with condensation and acrosome defects associated with a large head size. Although less severe, the phenotype observed in mouse Aurkc mutants is consistent with what was observed here in humans, the differences probably being due to interspecies variations.

AURKC has also been described to be expressed in ovaries[22] and in oocytes[23]. Its key role in male meiosis has been confirmed but its role in oogenesis remains to be investigated. We also intend to carry out a larger epidemiological study of both fertile and infertile North Africans to estimate the prevalence of *AURKC* c.144delC and evaluate the impact it may have the reproductive fitness of this population.

METHODS

Clinical information. All subjects showed typical multiflagellar large-headed spermatozoa (**Table 1**). All sperm analyses were performed at least twice in accordance with the World Health Organization recommendations[24]. All subjects had normal somatic karyotypes.

Subjects R1–R10 came from the Rabat region. R4b and R5b were brothers; all others were unrelated with no known consanguinity. Subjects G1–G4 lived in the Grenoble region (France) but were all of North African descent. The families of G1–G3 came from Algeria, and the family of G4 came from Tunisia. All four were born from first-degree cousins, but the four families were not related and lived in different area of the Rhone Alpes region.

No testicular biopsy was carried out from any of the 14 subjects. Because of the nature of the specimen and cultural beliefs, additional semen samples for research purpose could not be obtained from any of them. A total of 100 unrelated anonymous fertile men were genotyped by sequencing of *AURKC* exon 3. Half were of European origin and half of North African origin. The study was approved by the local ethics committee of Grenoble University Hospital, and informed consent was obtained from all participants.

Molecular analyses. DNA was extracted from 5–10 ml of frozen EDTA blood using a guanidium chloride extraction procedure. A genome-wide scan was performed for the 22 autosomes with the 10cM Linkage Mapping v2.5 (Applied Biosystems). The sequences of the PCR and sequencing primers used in this study are indicated in **Supplementary Table 1** online. Sequencing reactions were carried out with BigDye Terminator v3.1 (Applied Biosystems). All sequences and microsatellite electrophoresis were carried out on ABI 3100 (Applied Biosystems).

DNA constructs and translation assays. AURKC wild-type and mutant clones were derived from the original *AIE1* clone[25] (see Acknowledgments), renamed at a later stage *AURKC* isoform 1. Directed mutagenesis of the c.144delC mutation was carried out from the AURKC isoform 1 wild-type in PET32a vector by a service provider (Genecust). All constructs were resequenced in their entirety to verify that the mutation had been reproduced accurately and that no other changes had been introduced. Both wild-type and mutant sequences were amplified by PCR and cloned downstream the globin 5′ UTR and in frame with the coding region of the *Renilla* luciferase reporter gene. Capped wild-type *AURKC*, mutated *AURKC*, wild-type *AURKC/rLuc* and mutated *AURKC/rLuc* RNAs were synthesized by *in vitro* transcription using T7 RNA polymerase as previously described[26]. For *in vitro* translation, the Flexi Rabbit Reticulocyte Lysate System (Promega) was programmed with 10, 30 or 50 ng of capped wild-type and mutated *AURKC* RNAs. The reaction was incubated at 30 °C for 30 min and stopped with SDS loading buffer. Translation products were analyzed by 15% SDS-PAGE and autoradiography. For *ex vivo* translation assays, hybrid capped and polyadenylated *AURKC/rLuc* mRNAs were transfected directly into HeLa cells using the Mirus reagent (Mirus Bio Corporation). Transfected cells were incubated at 37 °C and 5% CO_2 for 7 h and *Renilla* luciferase activity was measured in a Veritas luminometer (Turner Biosystems) using the Dual Luciferase Reporter Assay (Promega).

FISH analysis. Semen preparation and FISH analysis were carried out as previously described[8]. The three fluorescent DNA probes (Abbott Diagnostics) corresponded to centromeric sequences. Chromosome X and Y probes were labeled green and red, respectively (CEP spectrum green and orange, Vysis), and chromosome 18 was labeled in blue (CEP aqua, Vysis).

Accession codes. All DNA and amino acid numeration in this manuscript refers to *AURKC* isoform 1, the longest isoform (references NM_00101587 and NP_001015878, respectively). *AURKC* isoforms 2 and 3 have a modified 5′ terminal end, leading to differential splicing of exon 1 and 2. All three variants are identical from exon 3 onwards; the described mutation (c144delC) therefore affects all three isoforms in the same manner. Translation experiments were carried out on isoform 1.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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