

Macrozoospermia: screening for the homozygous c.144delC mutation in *AURKC* gene in infertile men and estimation of its heterozygosity frequency in the Tunisian population

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Abstract

Purpose Macrozoospermia is a rare condition of male infertility characterized by the presence of close to 100 % large-headed multiflagellar spermatozoa. The homozygous mutation (c.144delC) in aurora kinase C gene (*AURKC*) has been identified as the most frequent mutation causing macrozoospermia in North African patients. The aim of this study was to evaluate the prevalence of this condition in Tunisia and estimate the frequency of c.144delC mutation among infertile and control populations.

Methods Sequencing c.144delC mutation was carried out in 33 macrozoospermic patients among 6652 infertile men. Minisequencing of exon3 was performed in 250 unrelated control individuals to estimate the frequency of c.144delC heterozygosity.

Results More than 80 % of macrozoospermic patients were c.144delC homozygous. The prevalence of homozygous c.144delC was 0.4 % among infertile men (27/6652). The frequency of heterozygosity was 0.4 % among controls (1/250). Surprisingly, it is five times less common than established in the general population of North Africa (2 %) or in the Moroccan population (1.7 %).

Capsule The c.144delC mutation in *AURKC* gene was found to be recurrent in Tunisian infertile male suffering from macrozoospermia with a relative low frequency of c.144delC heterozygosity detected in the control population.

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Conclusions We show that this mutation is relatively less frequent in the Tunisian population than in other Maghreb populations. The occurrence of homozygous mutation among infertile men can be attributed to the high rate of consanguinity and its impact on the expression of this autosomal recessive male infertility disorder rather than a high frequency of heterozygous carriers among the general population. This highlights the importance of the molecular analysis of *AURKC* mutations for infertile men with high percentage of large-headed multiflagellar spermatozoa in order to limit unnecessary in vitro fertilization attempts for them.

Keywords Male infertility · Macrozoospermia · *AURKC* mutations · Tunisia

Introduction

Infertility concerns at least 70 million couples worldwide [1]. In about half of cases, infertility is due to the inability of the male partner to produce spermatozoa of sufficient number (oligozoospermia), adequate motility (asthenozoospermia), or normal morphology (teratozoospermia) or combinations of these defects. Most of these cases are believed to have a genetic component since many familial cases were described. The implementation of whole-genome approaches, such as linkage analysis involving a genome-wide microsatellite scan, SNP arrays, whole-genome, or exome analysis through next generation sequencing, has recently allowed identifying few causal genes directly implicated in and linked to different and rare forms of male infertility [2–5]. *AURKC* is one of the first identified genes associated to a particular form of monomorphic teratozoospermia referred to as “macrocephalic

multiflagellar spermatozoa syndrome” or “macrozoospermia” [2]. This rare condition (frequency <1 % [6]) is commonly associated with severe oligozoospermia (<2 millions/ml) and characterized in its typical profile by ejaculates consistently showing total teratozoospermia with close to 100 % large-headed and multiflagellar spermatozoa. However, it has been reported that macrozoospermia may sometimes appear in milder forms with the coexistence of various percentages of large-headed spermatozoa and morphologically normal spermatozoa [7]. Among these milder forms, different aspects were described depending on the morphology of these abnormal large-headed spermatozoa; regular, irregular, duplicated [8], or monoflagellar [9, 10]. So far, four *AURKC* mutations severely affecting the protein function have been identified and associated to the typical phenotype form [2, 11–13]. The homozygous frameshift mutation (c.144delC) has been identified as the most frequent mutation causing macrozoospermia in North African patients [12], and a carrier frequency of 1/50 was established in individuals from the Maghrebian general population [12]. A further study led to the identification of a second recurrent nonsense mutation p.Y248* in both European and North African infertile men [13].

AURKC protein is the third member of the Aurora subfamily of serine/threonine protein kinases and is preferentially expressed in the testis [14–16]. It was demonstrated 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. In addition, *AURKC* is involved in the spindle assembly checkpoint (SAC) which is essential in mitosis to limit the production of aneuploid cells with misaligned chromosomes or abnormal microtubule tension that would result in incorrect segregation and in meiosis to limit the conception of chromosomally abnormal gametes [13, 17]. Macrozoospermia typical phenotype caused by the absence of *AURKC* confirm therefore its role in the SAC since in the absence of *AURKC* meiotic cytokinesis is blocked, but spermiogenesis is not stopped leading to the production of large-headed multiflagellar tetraploid spermatozoa [12, 13, 17]. Consequently, a positive molecular diagnosis for infertile men presenting with typical macrozoospermia indicates a systematic contraindication for in vitro fertilization by intracytoplasmic sperm injection (ICSI). Moreover, the extent of chromosome segregation defects and division failure can therefore be variable, and abnormal aneuploid large-headed spermatozoa with different aspects (duplicated, monoflagellar...) associated to morphologically normal spermatozoa are produced and not fully incompatible with fertility [9, 18].

Here, we estimate the prevalence of macrozoospermia among infertile Tunisian men. As far as we know, this is the first report about screening results of *AURKC* gene in a large cohort of macrozoospermic Tunisian patients and determination of the heterozygosity frequency of the recurrent c.144delC mutation in a control Tunisian population.

Materials and methods

Patients and control population

We included, in this study, a total number of 33 patients referred to our laboratory over a period of 6 years (2009–2014) for routine semen analysis. All patients were described to have an absolute teratozoospermia (100 % abnormal morphology), and most of them (28 out of 33) presented the typical profile of macrozoospermia with close to 100 % macrocephalic multiflagellar spermatozoa. Hormone concentrations of FSH, LH, testosterone, and the seminal levels of biochemical markers of accessory sex glands were in the normal range.

To estimate the frequency of *AURKC* c.144delC mutation in a Tunisian control population, a total of 250 unrelated individuals were genotyped; (i) 80 were fertile men that fathered at least one child, (ii) 70 for whom we controlled their semen parameters and they were normospermic men, but we have no clear data if they have children or not, and (iii) 100 individuals (males and females) from different Tunisian regions about whom no fertility status data are available.

All patients, controls and anonymous donors, gave their written informed consent. The study was approved by the ethics committee of Farhat Hached University Hospital of Sousse (Tunisia).

Sperm analysis

The basic semen parameters were evaluated according to the World Health Organization guidelines [19]. All sperm analyses were performed at least twice within a period of 3 months. We determined the percentage of morphologically abnormal spermatozoa (irregular, large-headed, duplicated heads, multiflagellar spermatozoa) according to the David's classification [20] by analyzing 100 spermatozoa for each patient.

Molecular analysis

DNA extraction and DNA sequencing

Genomic DNA was extracted from peripheral blood leukocytes using the FlexiGene DNA Kit (Qiagen) according to the manufacturer's instructions. The seven *AURKC* exons and their intronic boundaries were amplified using primers described previously by Dieterich et al. [2]. Sequencing analyses were carried out using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI Prism Genetic Analyzer 310 (Applied Biosystems).

Multiplex minisequencing assay (SNaPshot)

Screening for the c.144delC deletion in the 250 control subjects was performed by Single Nucleotide Extension

technique using the SNaPshot Multiplex Kit (Applied Biosystems). Briefly, *AURKC* exon 3 was amplified and PCR products were purified with the QIAquick PCR Purification Kit (Qiagen). The minisequencing reaction was carried out in a total volume of 6 μ l containing 2.5 μ l of SNaPshot Multiplex Ready Reaction reagent (Applied Biosystems), 2.5 μ l of a specific primer (5 pmol/ μ l) complementary to the analyzed region plus a poly (T) tail, which was added to ensure spatial resolution of the extension product during capillary electrophoresis (5'-T₍₂₁₎ATGACTTTGAAATCGGGCGTCCC-3'), and 1 μ l of purified PCR product. The SNaPshot Multiplex Ready Reaction reagent set contains fluorescently labeled dideoxynucleotide triphosphates (ddNTPs), reaction buffer, and AmpliTaq DNA polymerase which extend the primer by one nucleotide, adding a single ddNTP to its 3' end. The minisequencing reaction consisted of 25 cycles at 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s. After extension, the SNaPshot products were treated with 1 unit of calf intestinal alkaline phosphatase (CIP) (Invitrogen) for 15 min at 37 °C, followed by 15 min at 65 °C and 7 min at 4 °C to remove excess fluorescent dye terminators. The purified products (1 μ l) were mixed with 17 μ l of formamide and 0.8 μ l of GeneScan-120 LIZ Size Standard (Applied Biosystems) and incubated at 95 °C for 2 min, followed by 2 min on ice. The reaction products were separated by capillary electrophoresis using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). To reveal the electrophoresis data, the peak signal was analyzed with GeneScan Analysis Software (Applied Biosystems); the dye color of the fragment was used to identify the nucleotide of interest. For the minisequencing technique, color was assigned to individual ddNTP as follows: green/A, black/C, blue/G, red/T. The minisequencing reaction produces one (homozygote) or two (heterozygote) peaks depending on the genotype at this locus (Fig. 3). Heterozygous cases were then confirmed by Sanger Sequencing.

Results

Characteristics of the patients

The majority of patients (93.9 %) consulted for primary infertility with a mean duration of 5.5 years. Their ages ranged from 25 to 56 (mean 37.16). Only two patients consulted for a secondary infertility with duration of 2 years, and they were aged 39 and 34 years old. All patients were unrelated apart three pairs of siblings. Twenty patients were issued from a consanguineous marriage (60.6 %) and including the sibling pairs, eight other patients had the notion of infertility in the family either brothers, cousins, or uncles who do not consult or consulting at another place. Analyzed macrozoospermic patients were from all the regions of Tunisia but about 75 %

of them were from the central region. All patients had normal somatic karyotype and none of them was positive for Y microdeletions.

Sperm morphology

The conventional semen parameters are summarized in Table 1. Semen analysis showed a “classical” macrozoospermia phenotype for the majority of patients (28/33) consisting of 100 % teratozoospermia with an average of 80 % large-headed, 86 % irregular head, 45 % multiflagellar spermatozoa, and 95 % acrosomal abnormalities (Fig. 1a, b). Fifteen out of the 28 patients had a low sperm count (<10 million/ml). Sperm concentration ranged from 0.62 to 48 million/ml, with a mean value of 12.5 million/ml. The average viability was 45 % and the sperm progressive mobility was 0 % for almost all patients, apart for P3 and P4 (5 % and 12 % respectively).

Despite a total teratozoospermia, the remaining five patients presented “milder” phenotypes of the syndrome. For patient P1, extreme oligozoospermia was observed (<0.1 million spermatozoa/ml) and sperm morphology was analyzed only for 50 spermatozoa instead of 100. For P2 and P3, spermocytogram showed an important presence of duplicated head (38 and 45 % respectively) with a lower frequency of large headed (8 and 26 % respectively) and multiflagellar (26 and 28 % respectively) (Fig. 1c). For P4 and P5, 45 and 41 % of large headed and 24 and 5 % multiflagellar were respectively noted (Table 1).

Over the last 6 years (2009 to 2014), among 6652 infertile men consulting from all regions of Tunisia, only 33 patients presented with macrozoospermia. Then, in our study, we estimated the frequency of this rare syndrome to be 0.5 % among infertile Tunisian patients.

Molecular analysis of the *AURKC* gene in patients

Direct sequencing of *AURKC* exon 3 revealed the presence of the c.144delC recurrent mutation at a homozygous state in 27 patients out of 33. The remaining six *AURKC* exons and their boundaries were then sequenced for the other six patients. In one patient the c.744C>G (p.Y248*) mutation in exon 6 was identified and no *AURKC* mutations were detected in the other five patients (Fig. 2). Overall, in our cohort of macrozoospermic patients, a molecular defect of *AURKC* was identified in 84.8 % (28/33) or in 83.3 % (25/30) considering only one proband in familial cases (30 genetically independent analyzed patients). Therefore, the frequency of the homozygous c.144delC deletion was 80 % (24/30) while p.Y248* accounted only for 3.3 % (1/30) and in 16.7 % (5/30) of cases no *AURKC* mutations were identified.

Table 1 Sperm parameters of analyzed patients ($n=33$) according to *AURKC* genotype

Sperm characteristics	<i>AURKC</i> mutation ($n=28$)		No <i>AURKC</i> mutation ($n=5$)				
	Average	Range	P1 ^a	P2	P3	P4	P5
Sperm volume (ml)	2.51	0.5–5.2	2.2	2.5	4	5	2
Sperm concentration ($\times 10^6$ /ml)	12.52	0.62–48	<0.1	6.2	11.2	8	0.7
Progressive Mobility (after 1 h) (%)	0	0	0	0	5	12	0
Viability (%)	45.06	10–88	(24/50)	47	67	37	44
Large-headed spz (%)	80.42	30–100	(38/50)	8	26	45	41
Irregular head (%)	86.48	53–100	(35/50)	57	54	79	88
Duplicated head (%)	0.68	0–6	(4/50)	45	38	0	0
Multiflagellar spz (%)	44.82	15–65	(14/50)	28	26	24	5
Abnormal acrosome (%)	94.85	57–100	(42/50)	100	97	95	100
Multiple Anomalies Index (%)	3.52	2.80–4.13	3.52	3.48	3.68	3.04	3.49

Spz spermatozoa

^a Due to the very low sperm concentration for this patient we analyzed only 50 spz instead of 100

AURKC c.144delC mutation analysis in the control population

A minisequencing assay (SNaPshot) was developed to genotype the c.144delC mutation on 250 unrelated individuals from the general population. Only one heterozygous female case was detected for this deletion from the third subgroup; (i) 0/80 fertile men, (ii) 0/70 normospermic men, and (iii) 1/100 individuals (males and females) from different Tunisian regions (Fig. 3). Thus, we estimate a frequency of *AURKC* c.144delC mutation heterozygous carriers to be 0.4 % (1/250) in a Tunisian control population.

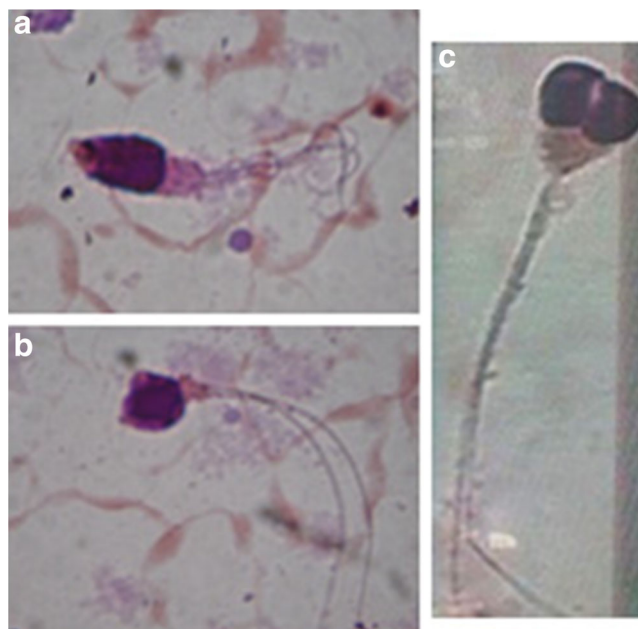
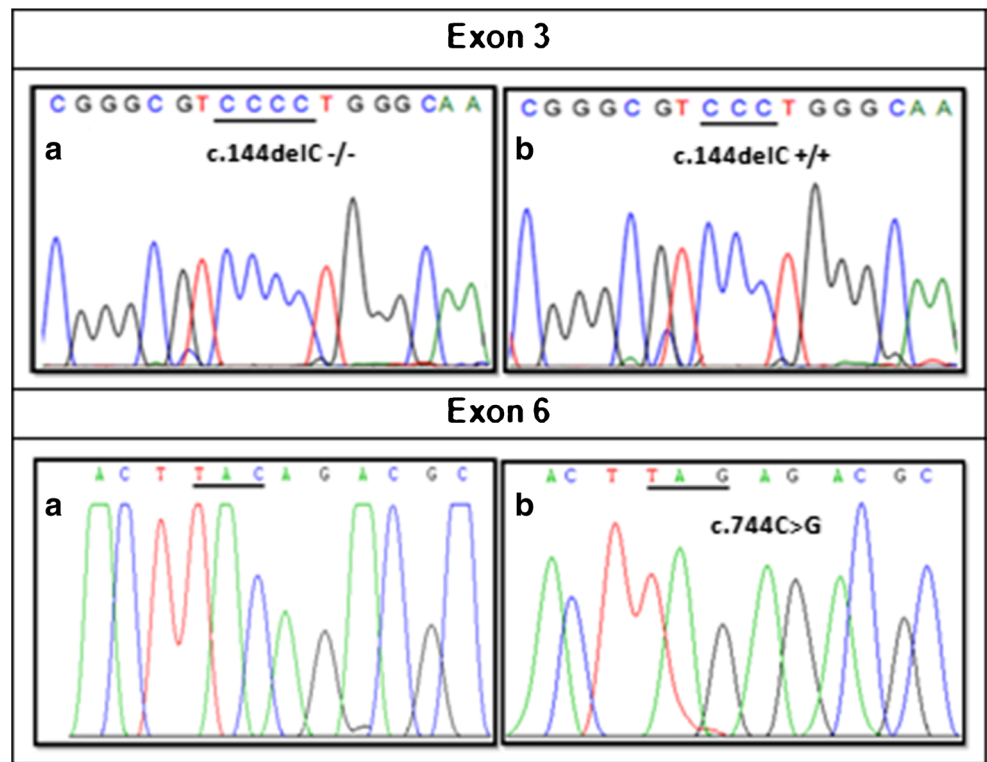


Fig. 1 Observation at light microscopy of large-headed multiflagellar spermatozoa (a and b) and spermatozoa with a duplicated head (c)

Discussion

Today's knowledge about the genetic causes of male infertility should be integrated in reproductive care when confronted with an infertile couple, especially in case of some particular infertility phenotypes. Macrocephalic multiflagellar spermatozoa syndrome is an autosomal recessive type of absolute teratozoospermia resulting in infertility or rather in sterility associated with a negative reproductive prognosis and a very poor outcome of infertility treatment. It was reported that among the infertile population, the frequency of this condition accounts for <1 %, and it was estimated by a French team to be 0.27 % [21, 22]. In our study we found a frequency of macrozoospermia among infertile patients to be 0.5 % which is double than that previously described. We assume that this high frequency is due to the high rate of consanguineous marriage in our country that increases the expression of this autosomal recessive male infertility form. Different mutations in *AURKC* gene were related to this sperm defect and two recurrent mutations (c.144delC and p.Y248*) were the most described. The encoded protein AURKC is a member of the cell cycle regulatory serine/threonine kinases (AURK A, B and C) which are essential during the mitotic cell division. AURKC is expressed specifically in the testis [14, 23] where it is involved in chromatin condensation and proper attachment of homologous chromosomes during the first meiotic division [24, 16]. The two recurrent truncating mutations, frameshift c.144delC mutation and p.Y248*, are expected to have a severe impact on the protein function. Transcript analysis showed that AURKC RNA is not present in mutated patients, suggesting that these two *AURKC* mutations were subjected to nonsense-mediated mRNA decay resulting in the complete absence of the protein [13, 11] leading to a defective meiosis with a

Fig. 2 Sequence chromatograms of part of *AURKC* exon 3 showing **a**) homozygous for the wild-type sequence (c.144delC -/-) and **b**) homozygous for the mutation (c.144delC +/+), and part of exon 6 showing **a**) homozygous for the wild-type sequence and **b**) homozygous for the mutation c.744C>G (p.Y248*)



blockage of spermatogenesis before the first meiotic divisions and the production of tetraploid large-headed multiflagellar spermatozoa [13, 11, 12].

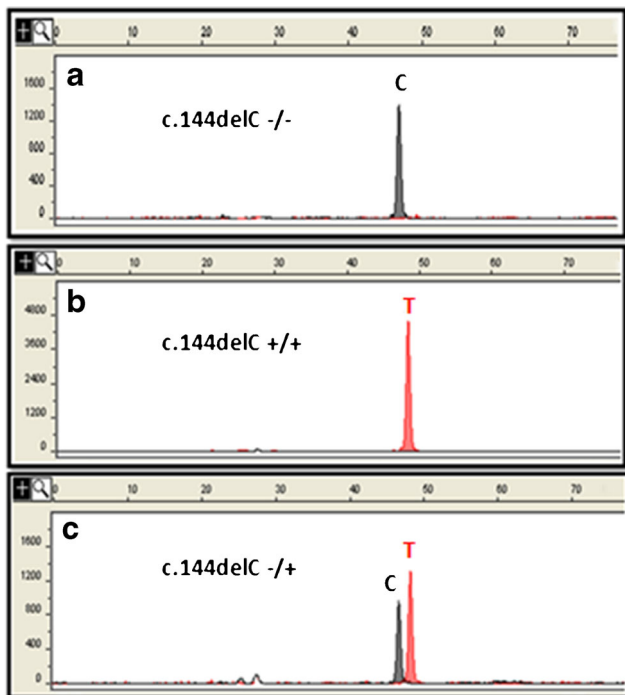


Fig. 3 Electropherogram of SNaPshot peaks showing the single extension base products **a**) homozygous wild type (c.144delC -/-), **b**) homozygous for the mutation (c.144delC +/-), and **c**) heterozygous for the mutation (c.144delC +/-)

Molecular analysis studies of *AURKC* gene demonstrated that most affected men were of North African origin. This was confirmed in Algerian and Moroccan populations [25, 26, 17]. We report here the first results of *AURKC* gene screening in a large cohort of Tunisian patients. It was found that, among 30 genetically independent patients, 80 % of them carried the most common mutation c.144delC and only 3.3 % carried the p.Y248* mutation. Our findings confirm again that the recurrent deletion c.144delC is the most frequent mutation causing macrozoospermia in Maghrebian patients and especially in Tunisian ones. Ben Khelifa et al., analyzed 83 probands from Maghrebian and European origin and identified the c.144delC deletion with a frequency of 85.5 % while the p.Y248* mutation accounted for 13 % [13]. El Kerch et al. found that among 18 Moroccan patients, 11 (61.1 %) carried the c.144delC mutation whereas no mutations were found for the remaining patients (38.9 %) [25]. By screening c.144delC mutation in 326 infertile Moroccan patients, Eloualid et al., found this mutation with an allelic frequency of 2.14 % (4 homozygous and 6 heterozygous out of 326) [26]. Ounis et al., analyzed 14 Algerian patients with macrozoospermia and found that c.144delC variant accounted for 71.4 % and p.Y248* accounting for 7.1 % [17].

Considering data from 24 (out of 27) homozygous patients for the common c.144delC mutation, 14 were issued from a consanguineous marriage (58.3 %). This confirms the very strong impact of consanguinity on increasing the rate of homozygous genotype and therefore on the expression of this autosomal recessive male infertility disorder. In Tunisia, as in

many Arab countries, there is a high preference for unions between relatives (first cousin marriages are the most represented). The rate of consanguinity is estimated to be more than 32 % and may reach 60 % in rural areas, which highly increases the risk of recessively inherited disorders [27]. Otherwise, 10 patients (41.7 %) were issued from non-consanguineous parents suggesting the relative frequency of this mutation in the general population.

To determine the c.144delC heterozygous frequency in a control Tunisian population, we applied a new method of genetic analysis called “minisequencing” or “SnapShot.” It is a relatively simple and affordable method that allows more efficient and quick results when it is compared with Sanger Sequencing. In total, 250 individuals were genotyped and unexpectedly, only 1 heterozygous case was found (0.4 %). It seems surprising that we found such a low frequency compared to that established in the general North African population found to be 2 % (8 heterozygous out of 385 analyzed individuals) [12] or to the frequency in the Moroccan population estimated to be 1.7 % (8 out of 459) [26]. The frequency of homozygous for the c.144delC mutation among infertile population in our study was estimated to be 0.4 % (27 homozygous out of 6652 infertile men) which is consistent with the frequency of heterozygous carriers in the control population that we found (0.4 %). Therefore, we can suggest that this mutation is less frequent in the Tunisian population than in the other Maghrebian population. We can assume that the higher prevalence of macrozoospermia observed in our study (0.5 %) among infertile population is, therefore probably, not due to a higher allelic frequency of c.144delC mutation in the general population but to the higher rate of consanguineous marriages which increase the rate of mutated homozygous c.144delC. Nevertheless, we can expect that many patients did not consult because of the taboo still frequently associated with male infertility in our society.

All patients with an *AURKC* mutation had a typical phenotype of macrozoospermia with 0 % normal spermatozoa and more than 80 % (on average) large-headed spermatozoa. No *AURKC* mutation was identified for five patients showing milder phenotype which is consistent with previous studies [2, 12, 25, 17]. In fact, semen analysis of these patients showed that one of them (P1) presented an extreme oligozoospermia; it is supposed then that, despite the total teratozoospermia he presents, he more likely suffered from extreme oligo-asthenozoospermia rather than macrozoospermia. For P2 and P3, both of them showed a particular milder form characterized by the presence of duplicated heads (respectively, 45 and 38 %), large-headed spermatozoa accounting only for 8 and 26 %, respectively. Duplicated heads can have different morphological aspects; either tight nuclei that may result from an absence of meiotic division and spermatozoa with this abnormality are considered as macrocephalic spermatozoa or two heads perfectly individualized that may result from

an abnormal spermiogenesis [28]. The absence of *AURKC* mutations in these two patients with this particular milder phenotype of macrozoospermia and in the three other patients indicates that it is clear that besides *AURKC*, other genes involved in cell cycle regulation and cell division could be responsible for the different milder forms of described macrozoospermia.

It was shown that large-headed spermatozoa from patients carrying c.144delC *AURKC* mutation are tetraploid [12]. This indicates that in case of typical form of macrozoospermia, unnecessary intracytoplasmic sperm injection (ICSI) cycles should be avoided. However, a few successful deliveries with birth of healthy babies after in vitro fertilization [28, 18, 17] or a pregnancy bound to miscarriage [9] were reported in patients suffering from milder forms of macrozoospermia. Two patients, in our cohort, presented with duplicated headed milder phenotype. One couple (patient P2) reported having suffered several spontaneous abortions and patient P3 fathered a healthy child after a spontaneous pregnancy proving that for this form of macrozoospermia fertilization and implantation could be possible, but the fetus could or not be viable.

In conclusion, our findings confirm the recurrent character of c.144delC *AURKC* mutation in macrozoospermic Tunisian patients. Contrary to what is expected, the frequency of c.144delC heterozygosity in our control population was found to be 1/250 (0.4 %). We noticed that this observed frequency is five times less than the frequency estimated in the general North African population (2 %) and approximately four times less than reported in the Moroccan population (1.7 %). Although we cannot demonstrate if this difference is statistically significant, we can suppose according to our finding that this mutation is less frequent in the Tunisian population. Among infertile Tunisian population, macrozoospermia represents 0.5 % which is more frequent than what was reported by others [21, 22]. This could be explained by the high rate of consanguinity (58.3 %) observed among analyzed patients which increase the rate of homozygous c.144delC mutation allowing the occurrence of this form of infertility. In 83.3 % of the cases, a molecular defect of *AURKC* was identified. This highlights the importance of *AURKC* molecular analysis for macrozoospermic patients for limiting unnecessary ICSI attempts. In fact, the chance of achieving pregnancy for a patient homozygous for an *AURKC* mutation is null, and in our country, adoption remains the best and only option for them. Nevertheless, in milder forms of macrozoospermia with an absence of *AURKC* mutations, fertility is not fully compromised. We recommend for these men fluorescence in situ hybridization (FISH) analysis of chromosomal aneuploidies and sperm DNA fragmentation in order to predict the chances of success in assisted reproductive technology. Furthermore, pre-implantation genetic diagnosis (PGD) as mentioned by others [29] could also be proposed after FISH analysis, but in our country, it is not yet allowed.

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Compliance with ethical standards All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare that they have no competing interests.

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