Mitochondrial DNA polymerase gamma gene polymorphism is not associated with male infertility

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Abstract

Purpose CAG repeat length of human mitochondrial DNA Polymerase gamma (POLG) gene is polymorphic with a major allele at 10 repeats and considered as the common allele whereas the mutant alleles (not 10/not 10 CAG repeats) were found to be associated with oligospermia / oligoasthenospermia in male infertility. To explore whether CAG trinucleotide repeat expansion in exon 1 of POLG gene is associated with spermatogenic failure.

Methods One hundred twenty four infertile men (sperm count <20 million/ml) and 60 normozoospermic (sperm count >20 million/ml) control Indian men of Tamil Nadu, were enrolled. DNA was extracted from 10 ml of peripheral blood and from semen using standard procedures. CAG repeat expansion was analyzed by polymerase chain reaction. Amplified products were quantified by 2 % agarose gel electrophoresis and subjected to genescan analysis to ascertain the size of POLG-CAG alleles.

Results This analysis interestingly revealed that the common allele 10 (10-CAG repeats) was widespread in infertile and normozoospermic control men with a frequency of 79 % and 71.7 % respectively. No statistical significance was found in POLG genotypic frequency distribution between infertile men and normospermic men.

Conclusion The present study confirmed no association between the POLG gene polymorphism and male infertility. Thus, if associated with infertility, the POLG gene polymorphism should be only considered as a minor possible contributing factor in infertile male patients with no impact on obtaining a pregnancy.

Keywords Infertility · POLG · Noormospermia · Tamil Nadu

Introduction

Infertility is a reproductive health problem, either in male or female that inhibits the ability to conceive and deliver a child. It affects every fifth couple in the fertile age. Infertility is one of the major medical issue associated with social stigma. This is exaggerated due to occupational hazards. According to current research surveys, more than 50 % of the cause is attributed to male and is the result of acquired and/or congenital abnormalities, in which ~60–75 % is found to be idiopathic (unexplained). A large proportion of male infertility cases are due to systemic defects like reproductive organ impairment and defective sperm production with imbalance in levels of gonadal steroids most of which can be treated. Genetics contributes to infertility by influencing a variety of physiological processes including hormonal homeostasis, spermatogenesis, and sperm quality [1]. Therefore understanding of the genetic basis of reproductive failure is essential to appropriately counsel an infertile couple. Until now, the role of Y chromosome micro deletions, X-linked and autosomal genes in male infertility has been extensively studied. However, in many infertile cases, they are not sufficient to explore the factors that lead to infertility. Mitochondrial DNA polymerase gamma (POLG) gene is recognized as one of the strong tool to reveal the mystery behind the idiopathic infertility.

Sperm mitochondria play an important role in its functionality; therefore, genetic alterations of mitochondrial DNA (mtDNA) might affect fertilization. It has a single DNA polymerase (Pol γ) [2], mapped on 15q25 responsible for replication and repair of mtDNA. A CAG repeat motif (CAG)₁₀CAACAGCAG coding for a stretch of 13 glutamines is located in the first coding exon of this gene. CAG

Capsule Evaluation of influence of mtDNA POLG gene polymorphism in spermatogenic failure.

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repeat length is polymorphic with a major allele with 10 repeats [3, 4]. Ten copies of CAG repeat were found to be uniformly high (0.88) in different ethnic groups [5] and considered as the common allele whereas the mutant alleles (not 10/ not 10 CAG repeats) were found to be associated with oligospermia / oligoasthenospermia in male infertility [6]. Expansion of CAG repeat in some genes is associated with various human pathologies. Implication of POLG CAG repeats in infertility is suggested by copious studies [6–10], but are debated [11–15]. As it is not clear whether the variable CAG genotype is one of the contributing factor for male infertility, an attempt was made to screen the CAG repeat motif of POLG gene exclusively in infertile men from two different districts of Tamil Nadu, South India.

Methods

Patient selection

A total of 124 infertile men from Erode and Nilgiri Districts of Tamil Nadu, South India, of which 63 were oligozoospermic ($1-20 \times 10^6$ spermatozoa/ml), 45 were asthenozoospermic (>60 % of non-motile sperms), and 16 were oligoasthenozoospermic ($<20 \times 10^6$ spermatozoa/mL; <50 % motile spermatozoa; <30 % abnormal shape and size of spermatozoa) men and 60 normal males (sperm count >20 million/ml) of proven fertility served as controls for this study. Further, these 124 infertile samples included 64 blood and 60 semen samples whereas normal controls included 45 blood, and 15 semen samples. All the procedures followed were in accordance with the ethical standards of Centre for Cellular and Molecular Biology, Hyderabad.

Ten milliliter of blood sample was collected from the individuals with EDTA as anticoagulant. Semen samples were obtained by masturbation on two different occasions, separated by a 3-week interval, following a 3-day period of sexual abstinence. Semen samples were allowed to liquefy for 30 min at 37°C . Appropriate written informed consent was obtained from all participants in this study. Only patients with an apparently normal 46,XY karyotype and who did not exhibit obstruction in the sperm delivery, endocrinological defect, pelvic injury, and Y chromosome abnormalities [16] were included in this study.

DNA extraction and polymerase chain reaction for POLG-CAG repeats

DNA was extracted from 10 ml of peripheral blood [17] and from semen [18] using standard procedures. A pair of primers were used to amplify the region containing the CAG repeats, of which forward primer (5' CCAGGGCCGGTTCCAGC TC3') was designed, while the sequence of the reverse primer

(mip31-5'GTGCTATCCTCGGAGGGCGGGCAGC3') was obtained from elsewhere [7]. While synthesizing, forward primer was tagged with 5' FAM fluorescence dye in order to analyze the PCR products in the automated DNA analyzer (ABI 3730).

DNA samples were analyzed for CAG repeat expansion by Polymerase Chain Reaction (PCR) in a 0.2-mL thin-wall tube under the following conditions: 5.0 ng of DNA, 1.2 mM MgCl_2 , 200 mM dNTPs, 10.0 pMol of each specific primer, and 1- 2U AmpliTaq Gold in a 10 μl reaction volume. Amplification was carried out in a MJ Research Thermal Cycler (Waltham, MA, USA) using the following cycling conditions: After an initial denaturation step at 94°C for 10 min, cycle parameters were 94°C for 71 min, 60°C for 45 s, and 72°C for 2 min for 30 cycles with a final extension of 72°C for 7 min. Amplified products were quantified by 2 % agarose gel electrophoresis. The PCR products were then visualized under UV light in transilluminator. On obtaining a single band devoid of any primer-dimer bands the PCR products were then subjected to Genescan analysis.

Genotyping

1.0 μl of the PCR product was mixed with 0.3 μl of GS LIZ500 size standard and 8.7 μl of Hi-Di formamide and analysed on 3730 DNA analyzer (Applied Biosystems). The raw data were further analysed using GeneMapper software (v3.7) to ascertain the size of POLG-CAG alleles (Fig. 2a). PCR and genotyping were repeated for all the samples to confirm the number of repeats.

Statistical analysis

We performed the χ^2 test (Sys, version 10.0, from SPSS Inc, Chicago, IL, USA), odds ratio and obtained p value (<http://faculty.vassar.edu/lowry/VassarStats.html>) to analyze the significance of the POLG polymorphism in male infertility.

Result and discussion

Polymorphisms in CAG repeat number of the coding region (exon 1) of the catalytic subunit of the mitochondrial DNA polymerase gamma (POL G) in 124 infertile men and 60 normozoospermic proven fertile control were analyzed to explore the possible associations between the POLG polymorphism and semen quality and fecundity. This analysis interestingly revealed that the common allele 10 (10-CAG repeats) was widespread in infertile and normozoospermic control men with a frequency of 79 % and 71.7 % respectively (Fig. 1a and b). District wise analysis of POLG-CAG repeats in infertile men had shown almost similar incidence (Fig. 2).

Fig. 1 **a** Gel picture showing CAG repeats of *POLG* gene. **b** Gene scan electrophorograms showing CAG repeats of *POLG* gene

Fig. 2 Distribution of genotypic frequency of the CAG repeats of *POLG* gene in infertile men of Erode and Nilgiri districts of Tamil Nadu

Table 1 Number and frequency of each genotype in oligozoospermic, asthenozoospermic, oligoasthenozoospermic, infertile men and normospermic men. Odds ratio, chi-square, and *p* value were also given

Alleles	Oligozoospermic men (n)	Oligozoospermic men (%)	Asthenozoospermic men (n)	Asthenozoospermic men (%)	Oligoasthenozoospermic men (n)	Oligoasthenozoospermic men (%)	Infertile men (n)	Infertile men (%)
9/10	1	1.6	—	—	—	—	1	0.8
10/10	51	80.9	36	80	11	68.8	98	79
10/11	7	11.1	8	17.8	5	31.3	20	16.2
10/12	2	3.2	—	—	—	—	2	1.6
11/11	2	3.2	1	2.2	—	—	3	2.4
Total	63		45		16		124	

Normozoospermic men (n)	Normozoospermic men (%)	odds ratio	Chi square ratio(Yates)	p value	Chi square ratio(Pearson)	p value
—	—	—	—	—	—	—
43	71.7	1.4902	0.85	0.356552 ^a	1.22	0.269361 ^a
13	21.7	0.6953	0.51	0.475139 ^a	0.84	0.359397 ^a
—	—	—	—	—	—	—
4	6.7	0.3471	—	—	—	—
60						

Bold values indicate total number of samples of each category

P<0.05 - significant

^a Not significant

The same tendency was observed when the allelic frequency of the common allele (10) in oligozoospermic men (80.9 %), asthenozoospermic men (80 %) and oligoasthenozoospermic men (68.8 %) of Tamil Nadu State in South India was analyzed. Allele 11 was the next common allele observed in all the three categories of the infertile samples (16.1 %), viz., oligozoospermic men (11.1 %), asthenozoospermic men (17.8 %), oligoasthenozoospermic men (31.2 %) and 21.7 % in normozoospermic men (Table 1).

Four alleles (9–12 repeats) and five genotypes (Fig. 3) of which two (10/10, 11/11) were homozygous and the remaining three were heterozygous (9/10, 10/11, 10/12)

Fig. 3 Bar diagram indicating the genotype frequency distribution between infertile and control men of Erode and Nilgiri districts of Tamil Nadu

were noticed in infertile men. Normozoospermic control men showed a narrow range of alleles from the repeat length 10 to 11 (two alleles) and three genotypes (Fig. 3) of which two (10/10, 11/11) were homozygous and the remaining one was heterozygous (10/11). Another important observation made in this entire study was the absence of allele with more than 12 CAG repeats in infertile group.

Yates and Pearson χ^2 test, odds ratio and *P* value (<http://faculty.vassar.edu/lowry/VassarStats.html>) were performed to find out the significance of the POLG polymorphism in male infertility. A *P*-value<0.05 was considered as significant. No significance was found in statistical analysis of POLG genotypic frequency distribution between infertile men and normospermic men (Table 1).

Average sperm count of 31.8 million /ml of semen out of which 18 % actively motile, 24 % sluggishly motile, 58 % non motile were found in 60 infertile men carrying POLG polymorphism. Sperm morphology showed 98 % normal, 2 % pin headed, 5 % amorphous, 1 % giant headed and 1 % double headed sperms. Clinical profile revealed that 23 out of 60 infertile men had consanguineous marriage; 2.5 % of infertile men suffered from mumps and measles; 19.4 % suffered from small pox at their infantile period; Occupational, environmental and recreational factor analysis revealed that 21.4 % of infertile men were addicted to smoking and alcohol intake; 19.4 % suffered from stress; 18.4 % have disturbances in sleep cycles; 20.4 % have prolonged usage of hot bath; 15.5 % were exposed to heat at their working place.

The search for new genetic cause represents one of the major tasks in present andrology, since majority of the

idiopathic cases of male infertility are of genetic origin. The sole molecular genetic test that is routinely proposed in severe spermatogenic disturbances is screening for Yq microdeletions. Recently, genetic polymorphisms involved in spermatogenesis is being considered. The most debated and controversial issue concerns the polymorphic CAG repeat-length variations of POLG gene. Mutated alleles in POLG would produce suboptimal mtDNA polymerase leading to accumulation of mutations in the mtDNA with consequence of impaired energy metabolism of the spermatogenic cells and finally bring about a disturbance of sperm production and/or differentiation [19].

In this study, normozoospermic proven fertile control men showed the presence of homozygous mutant genotype (non 10/non 10) in almost the same frequency as seen in infertile men. Jensen et al. 2004 [10], reported a significantly higher frequency of non 10/non 10 genotype in normozoospermic infertile men affected by unexplained sub fertility and normal spermograms. In concert with the previous report [6, 8, 15], this study revealed that homozygotes with 10 repeats in both alleles (10/10) were the most common and constituted 79 % of all infertile subjects which was almost equivalent to the normospermic control frequency (71.7 %). Rovio et al. 1999 [5], have shown the frequent (88 %) occurrence of genotype 10/10 in different ethnic group. As found by Krausz et al. 2004; Akinin-Seifer et al. 2005 [11, 12], this study also suggested that the CAG repeat of the POLG gene does not play a significant role in men with severe male factor infertility.

The frequency of the common allele was not significantly different ($P>0.05$) in infertile and control men nor was any evidence found for an association between a specific unusual allele (Table 1). Only 3 ‘homozygous mutants’ out of 124 infertile patients were found, which was in disagreement with the data from Rovio et al. 2001 [6], where nine ‘homozygous mutants’ were identified out of 99 infertile men. The same group had also found an association between the absences of common genotype (10/10) and male infertility in moderate oligozoospermic men which was debated by this study. The genetic cause behind abnormal semen morphology and various clinical parameters should be elucidated in these 60 infertile men carrying POLG polymorphism.

Conclusion

In conclusion, the present study confirmed no association between the POLG gene polymorphism and male infertility. Thus, if associated with infertility or with one of the rare infertility syndromes, the incidence of which is too low to make it statistically significant when compared with general population it may be considered as minor contributing

genetic risk factor for male infertility with no impact on obtaining a pregnancy.

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