Molecular Biology Reports
https://doi.org/10.1007/s11033-023-08849-9

ORIGINAL ARTICLE

Single nucleotide polymorphism rs527236194 of the cytochrome B gene (*MT-CYB*) is associated with alterations in sperm parameters

Yulia Yu. Gromenko[1,2] · Kamil S. Galimov[3] · Irina R. Gilyazova[2] · Elmira F. Galimova[2] · Kirill V. Bulygin[3,4] · Sergey N. Ryagin[5] · Shamil N. Galimov[2] · Peter F. Litvitskiy[3] · Gennadii A. Piavchenko[3] · Valentin N. Pavlov[2]

Received: 8 March 2023 / Accepted: 26 September 2023

Abstract

Background The mitochondrial genome is substantially susceptible to mutations and has high polymorphism due to structural features, location, and lack of recombinant variability, as its inheritance is strictly maternal. All of these events can be accompanied by the accumulation of mitochondrial single nucleotide polymorphisms (mtSNPs) in the sperm. The aim of this research was to analyze the influence of mutations in the *MT-CYB* gene on sperm quality.

Methods and results We conducted a case–control study to identify mutations in the mitochondrial cytochrome B (*MT-CYB*) gene in men with asthenoteratozoospermia (89 cases) and oligoasthenoteratozoospermia (65 cases). The comparison group consisted of 164 fertile men. Somatic cell lysis followed by mtDNA extraction was conducted to analyze three mtDNA polymorphisms, rs28357373 (T15629C (Leu295=), rs527236194 (T15784C (p.Pro346=), rs2853506 (A15218G, p.Thr158Ala). Detection and genotyping of polymorphic loci in the *MT-CYB* gene was performed using the TaqMan allelic discrimination assay. To verify mutations in the *MT-CYB* gene, automated Sanger DNA sequencing was used. We found that rs527236194 was associated with asthenoteratozoospermia. rs28357373 in the *MT-CYB* gene did not show any polymorphism in the analyzed groups, which indicates a rare frequency of the TT genotype in our region. Rs28357373 and rs2853506 are not associated with male sperm abnormalities in the Volga-Ural region.

Conclusion The association of the rs527236194 polymorphic variant with sperm parameter alterations suggests its role in the pathophysiology of male infertility and requires further investigation in larger samples.

Keywords Idiopathic infertility · Asthenoteratozoospermia · Oligoasthenoteratozoospermia · Mitochondrial DNA · Cytochrome B · Mutations

✉ Gennadii A. Piavchenko
gennadii.piavchenko@staff.sechenov.ru

Yulia Yu. Gromenko
info@medufa.ru

Irina R. Gilyazova
gilyasova_irina@mail.ru

Elmira F. Galimova
efgalimova@mail.ru

Kirill V. Bulygin
kirill-bh-red@yandex.ru

Sergey N. Ryagin
ryagin_sn@mail.ru

Shamil N. Galimov
sngalim@mail.ru

Peter F. Litvitskiy
litvitskiy_p_f@staff.sechenov.ru

Valentin N. Pavlov
rectorat@mail.ru

[1] Medical Center "Family", Ufa 450054, Russia

[2] Bashkir State Medical University, Ufa 450008, Russia

[3] Department of Human Anatomy and Histology, I.M. Sechenov First Moscow State Medical University (Sechenov University, 8/2 Trubetskaya str, Moscow 119991, Russian Federation

[4] M.V. Lomonosov Moscow State University, Moscow 119991, Russia

[5] Moscow University for Industry and Finance «Synergy», Moscow 125190, Russia

Published online: 03 November 2023

Introduction

Infertility is the failure to achieve a pregnancy after 12 months of unprotected sexual intercourse. Up to 15% of couples worldwide are infertile, and approximately half of the cases are caused by male factors, including defects in sperm morphology and motility [1, 2]. Sperm motility is one of the most important factors in male fertility. Movement to the location of fertilization and normal fertilization are enabled by high-energy compounds that are synthesized mainly by sperm mitochondria [3, 4]. In gametes, mitochondria are positioned at the periphery of the flagellum, as the microtubule apparatus that comprises the spermatozoa flagellum is the most energetically costly. Mitochondria are organelles that have their own genome that comprises different extrachromosomal circular double-stranded DNA molecules and codes for 13 proteins. Mitochondrial DNA (mtDNA) is inherited maternally and has a unique mechanism of subcellular transcription and replication [5].

An important feature of the mitochondrial genome is its high rate of accumulation of mutations and high polymorphism. This feature of mtDNA is explained by a lack of protective histones and repair mechanisms, which increases the number of replication errors, and, considering the proximity of mtDNA to the complexes of the respiratory chain, by the impact of reactive oxygen species (ROS) [6, 7]. Many mitochondrial single nucleotide polymorphisms (mtSNPs) have been conserved in various populations throughout human evolution [8]. Because of the sole maternal inheritance of mtDNA and the fact that there is no recombination in the mitochondrial genome, mitochondrial SNPs accumulate and are passed on maternally [9].

Earlier research data show that mtDNA mutations are associated with cellular energy deficiency, probably reflecting chronic health problems, although not the lifestyle-associated ones [10]. Such a deficiency can lead to various pathologies, including the development of asthenozoospermia, oligozoospermia, and teratozoospermia. The functional activity of mitochondria is closely linked to acrosin activation, acrosome reaction capability, and chromatin integrity. To date, there is a large body of research dedicated to the role of mtDNA in the development of male infertility; however, data on the role of mutations in the cytochrome B gene (*MT-CYB*) in male infertility are scarce [11–14]. Mutations in this gene are associated with various dysfunctions, particularly in complex III [15], which can lead to impaired ATP production.

Thus, the aim of this study was to screen three mtDNA polymorphisms, rs28357373 (T15629C (Leu295=), rs527236194 (T15784C (p. Pro346=), rs2853506 (A15218G, p. Thr158Ala) in the *MT-CYB* gene in cases with asthenoteratozoospermia and oligoasthenoteratozoospermia.

Materials and methods

Participants

The study included men aged 24–46 years. All patients were examined by urologists and andrologists. The collected medical data included age, clinical findings, presence of varicocele, genetic pathology, and history of chronic disease. Semen specimens were collected in accordance with WHO criteria (2010). Fertile men (25–46 years, mean $29{,}8 \pm 0{,}5$) who had fathered at least one healthy child were recruited for the study. In the fertile group ($N = 164$), participants had normal semen analysis parameters (sperm count over 15×10^6/ml, total motility $\geq 40\%$, progressive motility $\geq 32\%$, normal morphology $\geq 4\%$). The group of cases included 154 males with asthenoteratozoospermia ($N = 89$) and oligoasthenoteratospermia ($N = 65$). The criteria for abnormal parameters were as follows: sperm count under 15×10^6/ml or under 39×10^6 in total sample volume, progressive motility (sum of grade $A + B) < 32\%$, and $< 4\%$ of normal sperm morphology (Kruger criteria). We did not exclude cases with chromosome aberrations, AZF deletions in the Y chromosome or genomic structural variants. Biomaterials were collected in the period of 2018–2022.

The study was carried out in accordance with the principles of the Declaration of Helsinki and approved by the local ethics committee (Protocol No. 8, Dec 14, 2022). All participants signed informed consent forms.

DNA analysis

Prior to DNA extraction, we performed somatic cell lysis to remove any potentially contaminating somatic cells. Following somatic cell lysis, we subjected the samples to DNA extraction using a QIAamp DNA Micro Kit (QIAGEN, Hilden, Germany). The concentration and purity of isolated DNA were assessed by measuring the optical density using a NanoDrop ND-1000 (Thermo Scientific, Waltham, USA) spectrophotometer. Genotyping of polymorphic loci in mitochondrial *MT-CYB* was performed using the TaqMan allelic discrimination assay. Allelic discrimination analysis was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, USA). The results of each allelic discrimination were analyzed using CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, USA) software.

To verify mutations in mitochondrial *MT-CYB*, we used an automated Sanger DNA sequencer (Life Technologies, Carlsbad, USA) at the UFRC RAS Institute of Biochemistry and Genetics. Sequencing was carried out using fluorescently labeled ddNTP sets in accordance with the manufacturer's recommendations (Big Dye Terminators v.3.1 RR kit, Life Technologies). Next, the DNA sequences of the participants

were compared with the reference sequence of the human mitochondrial genome.

Statistical analysis

Statistical analysis of the acquired data was performed using Origin Pro software (OriginLab, Northampton, USA). Pairwise comparisons of mutation rates in cases and controls were performed using $\chi 2$ (P) for 2×2 contingency tables, with Yates correction for continuity. The strength of the association was estimated using the odds ratio (OR) measure.

Results

We analyzed three mutations in *MT-CYB* in males with sperm abnormalities (oligoasthenoteratospermia and asthenoteratozoospermia) and in healthy men with normal semen parameters. The results are presented in Table 1.

Rs28357373, causing replacement of thymine by cytosine at position 15,629 of mtDNA was found neither in the group of males with sperm abnormalities nor in the control group of men. Based on the acquired data, it can be concluded that this mutation is rare in the region where the study was carried out and does not significantly contribute to the development of sperm abnormalities. Rs2853506, leading to replacement of adenine by guanine at position 15,218 of mtDNA, which causes a missense mutation p. Thr158Ala, was found at a low frequency both in cases (3% in males with oligoasthenoteratospermia and 3.3% in males with asthenoteratozoospermia) and in the fertile group (2.6%). Statistical analysis did not detect significant differences in the frequency of mutations between the case and control groups ($p > 0.05$).

Comparison of the frequencies of the polymorphic variant rs527236194, which causes replacement of the [CCT] triplet with [CCC] at the 15,784 position of mtDNA and causes synonymous substitution revealed a statistically significant difference between cases with asthenoteratozoospermia and the fertile participant group ($p = 0.04$).

Discussion

Due to the peculiarities of mtDNA structure, semi-autonomy, lack of recombination and DNA repair mechanisms, mtDNA is tens of times more frequently subjected to mutations compared to nuclear DNA, and mutations in mtDNA can lead to disease development, primarily due to changes in oxidative phosphorylation (OXPHOS) process mechanisms and coding for protein subunits in the respiratory chain. Oxidative damage and mutations can occur in sperm mtDNA, which can affect human sperm parameters and the development of male infertility. mtDNA is responsible for the production of ATP in spermatozoa through the OXPHOS process, and this process is also the production of reactive oxygen species that can damage DNA. During spermatogenesis, due to defects in mitochondrial DNA, there is an increased likelihood of free radical formation, which in turn can lead to impaired semen quality and sperm function. In addition, abnormalities in OXPHOS can lead to impaired ATP synthesis, and as a consequence, mutations in mtDNA can lead to impaired gene expression [16].

We attempted to analyze the influence of mutations in the MT-CYB gene on sperm quality. The study showed no associations of rs28357373 and rs2853506 with sperm abnormalities in the case-control study, but rs527236194 was associated with a decrease of sperm quality. Despite the fact that the rs527236194 variant does not alter protein structure, it may have a regulatory role, and according to some researchers, codon displacement can be a mechanism that controls gene expression levels [14].

It is necessary to note that the mtDNA polymorphic variant rs527236194 (T15784C (p.Pro346=) is a defining nucleotide substitution for a number of haplogroups, such

Table 1 Frequency of mtDNA variants in fertile and infertile men

SNP	Changes in mtDNA sequence (changes in protein)	Geno-types	Patients with oligoasthenoteratospermia		Patients with asthenoteratozoospermia		Fertile men		P-value (OR, 95%CI)
			N	%	N	%	N	%	
rs28357373	T15629C (Leu295=)	CC	0	0	0	0	0	0	$p > 0.05$
		CT	0	0	0	0	0	0	
		TT	65	100	89	100	164	100	
rs527236194	T15784C (p.Pro346=)	CC	6	9	12	13.5	10	6.1	$p = 0.04$* (OR = 2.4; 95%CI = 0.99–5.8)
		CT	0	0	0	0	0	0	
		TT	59	91	77	86.5	154	93.9	
rs2853506	A15218G (p.Thr158Ala)	GG	2	3	3	3.3	4	2.4	$p > 0.05$
		AG	0	0	0	0	0	0	
		AA	63	97	86	96.7	160	97.6	

* - comparison of fertile men and infertile men with asthenoteratozoospermia

as L1c2a, Z, N1b2, W3a, V13, H65, T2c1d2, F3b1, B2b3a, and U2e1b1 [8], which is why it is possible to identify this variant in ethnic groups that have been previously studied as part of population genomics research. Haplogroup Z is one of the most widespread mtDNA throughout Siberia, with the highest frequencies being over 10% in populations of Yukaghir (27.3%), Altai (15.4%), Even (15.2%), Udmurt (15.2%), Khakas (13%), Itelmen (12.8%), Nogai (12%), and Koryak (11.6%) people [17, 18]. In the Volga-Ural region, the mtDNA variant T15784C is found in the Z, V13, and N1b2 haplogroups and comprises no less than 8.6% of the Chuvash population, 7.1% of the Kazan Tatars population, 6.4% of the Mari population, 5.5% of the Komi population, 3% of the Besermyan population, and 2% of the Perm Bashkir population. These haplogroups and the mtDNA T15784C variant have not been previously encountered in the populations of Burzyan Bashkirs, Arkhangelsk Bashkirs, and Mordvins [19].

Changes in mitochondrial DNA can cause a number of disorders via various mechanisms, including the disruption of OXPHOS. These mutations can lead to serious alterations, ranging from mild impairments with insignificant clinical manifestations to life-threatening dysfunction of mitochondrial physiology [20]. Human sperm mtDNA molecules are particularly susceptible to oxidative stress and mutations, which have been demonstrated to play an important role in male infertility [21, 22]. One of the well-known major functions of mtDNA is the initiation of ATP production in spermatozoa through the process of OXPHOS by coding for protein subunits of the respiratory chain complexes. The process of OXPHOS is also the source of ROS generation and the DNA damage induced by them. During spermatogenesis, mitochondrial DNA damage can increase the probability of free radical production, which impairs the development and function of spermatozoa. Moreover, sperm motility is dependent upon ATP levels, and sperm mtDNA variations lead to the formation of defective proteins [23].

The present study analyzed three polymorphic variants in *MT-CYB*, and the results showed an association of the rs527236194 variant with the risk of one of the abnormalities in semen parameters. Previously, Talebi et al. [24] identified several complex alterations – deletions – in the mtDNA of abnormal sperm. These results allowed us to establish the association between 4977 b.p. deletion and pathospermia, and moreover, the sperm-quality stratified analysis showed a significant association between this anomaly and an increased risk of asthenozoospermia, oligoasthenoteratozoospermia, and asthenoteratozoospermia. In contrast, Zhang et al. indicated that some substitutions, such as mtDNA C3398T, are associated with a lower risk of developing asthenozoospermia [25].

Earlier studies demonstrated that the mtDNA mutation A3243G and large-scale deletions of mtDNA are linked to asthenozoospermia [26]. Moreover, in infertile men, various mtDNA mutations can accumulate throughout life, and these mutations reach high rates in selected sperm progenitor cells, which leads to impaired sperm motility and infertility.

Mughal et al. performed a PCR analysis of an 8.7 k.b.p. segment and identified multiple deletions [27], which were found at a much higher rate in patients with infertility than in fertile men. Comparison of various subtypes of infertility demonstrated that the highest rate of deletions was observed in oligoasthenoteratozoospermia. Statistical analysis of the case and control groups showed a significant association of the 8.7 k.b.p. deletion with infertility ($p = 0.031$), particularly in oligoasthenoteratozoospermia ($p = 0.019$). Two other mtDNA deletions – 4977 and 7599 b.p. were also associated with pathospermia and can be genetic risk factors for male infertility [24].

Mitochondrial markers for male infertility can be classified into three types of genetic defects: increased mtDNA copies in sperm, large-scale deletions of mtDNA (several thousand b.p. long), and synonymous and nonsynonymous mtSNPs (e.g., the rs527236194 variant that we identified in asthenoteratozoospermia). The first two types of defects are relatively well studied and have been linked to the decreased reproductive capacity of gametes and the probability of achieving a successful pregnancy in couples in the general population [28, 29]. At the same time, there are studies that offer alternative points of view. There is no consensus yet [30, 31].

The strength of the study is the population coverage of the Volga-Ural region. A limitation is related to the small cohorts enrolled in the investigation, and there were few fully categorized populations or concomitant interventions.

Conclusion

There is abundant evidence indicating the role of mtDNA in the development of pathospermia. The complex nature of male infertility and conflicting results of research studies warrant large prospective studies to validate the role of these mutations in the development of sperm abnormalities. Considering that the frequencies of genotypes/alleles of polymorphic variants and mutations differ in ethnic groups, our research is the attempt to verify the association of the polymorphic variant rs527236194 with the risk of sperm abnormalities in the Volga-Ural region. We conclude that single nucleotide polymorphism rs527236194 of the cytochrome B gene (*MT-CYB*) is associated with alterations in sperm parameters.

Funding This study was partially funded by the Bashkir State Medical University Strategic Academic Leadership Program (PRIORITY-2030).

Data Availability The allelic discrimination data may be provided upon request to the corresponding author.

Declarations

Ethical approval The study was approved by the Local Ethics Committee of Bashkir State Medical University (Protocol No. 8, Dec 14, 2022). The study was carried out in accordance with the principles of the Declaration of Helsinki.

Conflict of interest The authors declare no competing interests.

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