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Next Generation Sequencing in diagnosis of mitochondrial epilepsy

Abstract. Epilepsy is a common manifestation of mitochondrial disorders, in addition mitochondrial oxidative stress may play an important role in epileptogenesis since it affects the excitability of neurons. Mitochondrial diseases are often difficult to diagnose unless the symptoms are clearly identified as a part of a specific mitochondrial mutation. The use of next-generation sequencing would lead to the rapid identification of genes associated with epilepsy syndromes. In this study, we evaluate the applicability of the NGS method for diagnosis of mitochondrial epilepsy by clinicians and laboratories in Kazakhstan. We performed complete mitochondrial genome sequencing on the Illumina MiSeq platform for 6 patients with epilepsy. Using the MITOMAP and HmtDB databases, we identified three pathogenic variants (MT-ND1 m.3697G> A, m.5628T> C and m.7547T> C) leading to the development of epilepsy, additionally we found 6 variable sites (m.5586C> T, m.10095C> T, 514_515delCA, 16180_16181delAA, C514.CACACA, A955. ACCCC), the clinic of which was not previously mentioned in the literature. Our preliminary study suggests that mitochondrial genes potentially play a role in the pathogenesis of epilepsy and mutations in these genes cause various forms of epilepsy. It is necessary to elucidate the main mechanisms and participation of variants of mtDNA haplogroups in the development of epilepsy to apply the NGS method in diagnosis of mitochondrial epilepsy.

Key words: Epilepsy, Next Generation Sequencing, mitochondrial DNA, mutation.

Introduction

Epilepsy is a chronic neurological disease with a high degree of genetic heterogeneity, which is accompanied by recurrent unprovoked seizures and has different clinical manifestations. The overall incidence of epilepsy is 61.4 per 100,000 people [1]. Long course, low cure rates, and high rates of disability from illness have a profound impact on patients and society. In recent years, numerous studies have been conducted on the pathogenesis of epilepsy, but despite this, the exact mechanism of the disease has not been fully understood. Presumably 20-30% of epilepsy cases are caused by acquired conditions, such as skull trauma, previous stroke, brain tumors, previous infectious diseases of the brain [2, 3], but the remaining 70-80% of cases are caused by one or more genetic factors [4].

Mitochondrial dysfunction and oxidative stress are considered as factors that play a role in the pathogenesis of a number of neurological diseases, including epilepsy [5]. Many studies show the relationship between mitochondrial dysfunction and epilepsy, but most of them does not study mitochondrial genome (mtDNA) of patients suffering mitochondrial diseases which would be extremely important for determining the role of mitochondrial genome in the development of disease and for the treatment of patients as mitochondrial epilepsy is often difficult to treat and resistant to antiepileptic drugs. Diagnosis of mitochondrial disease is often challenging unless the symptoms are clearly identified as part of a specific mitochondrial mutation [6].

Screening for mutations of the underlying genetic defects in epilepsy is often complicated by the many presumably responsible genes. Next Generation Sequencing (NGS) is a valuable and reliable diagnostic tool for massively parallel sequencing of as many genes as possible, and it is a fast and cost-effective diagnostic method to analyze genetic cause of epilepsy [7].

The use of NGS technologies in research and diagnostic laboratories has led to the rapid identifica-

tion of genes associated with epilepsy syndromes. In this study, we will evaluate the suitability of the NGS method for diagnosis of mitochondrial epilepsy by clinicians and laboratories in Kazakhstan.

Materials and methods

Patients. The study protocol was approved by the local ethics committee of the Kazakh-Russian Medical University (protocol No. 51 from 05.09.2017). The studies included three patients with epileptic encephalopathy and three patients with myoclonic epilepsy. All patients were treated at SVS clinic (Almaty, Kazakhstan). When collecting biological material, patients filled out a questionnaire and signed an informed consent form.

DNA isolation and sequencing. Total DNA was extracted by GeneJet Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) according to the protocol recommended by the manufacturer. We used MiSeq Sequencing Platform (Illumina, USA) and Nextera XT Library Prep Kit to sequence the entire human mitochondrial DNA (mtDNA) genome. During sample preparation, the mtDNA genome was amplified in two PCRs to generate two long fragments spanning the entire human mitochondrial genome (16,569 bp). The amplicons were quantified and pooled before library preparation.

Primers
MTL-F1 5'- AAA GCA CAT ACC AAG GCC AC -3'
MTL-R1 5'- TTG GCT CTC CTT GCA AAG TT -3'
MTL-F2 5'- TAT CCG CCA TCC CAT ACA TT -3'
MTL-R2 5'- AAT GTT GAG CCG TAG ATG CC -3'

Data analysis. Raw FASTQ files were processed and mapped to the mitochondrial reference rCRS using Eager [8]. Genotyping was performed using GATK HaplotypeCaller and Genotype GVCF [9]. Geneious software version 2019.2.1 was used to visually assess the sequencing depth and the quality of genotype calling [10]. The genome for alignment is the Revised Cambridge Reference Sequence (rCRS), a mtDNA genome used by the Forensic Genomics community. Confirmed differences from the CRS were compared to previously reported mutations or polymorphisms listed in the MITOMAP database (http: //www.mitomap.org) and HmtDB (http://www.hmtdb.uniba.it).

Results and discussion

Mitochondrial diseases encompass a genetically and clinically heterogeneous group of diseases caused by defects in mitochondrial oxidative phosphorylation. Epilepsy is a common manifestation of mitochondrial disorders; in addition, mitochondrial oxidative stress may play an important role in epileptogenesis, as it affects the excitability of neurons [11].

mtDNA mutation analysis. We sequenced using NGS the mitochondrial genome of 6 patients with epileptic encephalopathy and myoclonic epilepsy. Bioinformatic analysis of NGS data showed the presence of 115 mutations, of which 80 mutations are localized in genes encoding proteins; the D-loop was also a sensitive region in which we found 35 variable sites. Using the MITOMAP and HmtDB databases, we found 21 missenses, 41 synonymous variants, 11 mutations in the 12S rRNA and 16S rRNA genes, 5 mutations in the tRNA genes (MT-TA, MT-TC, MT-TD, MT-TH, MT- TR) and two mutations in the regulatory region of the MT-NC3 gene. The distribution of variable sites in each gene is shown in Figure 1.

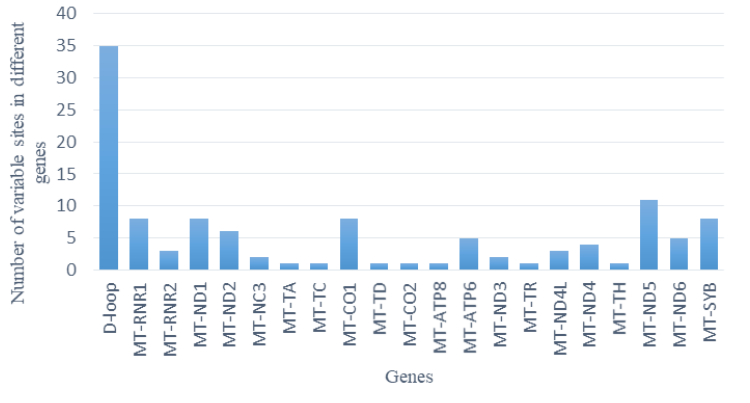

Figure 1 – The distribution of variable sites in each gene

Variable sites in the MT-ND5 gene were more frequent (11 mutations), 8 mutations were found in the MT-RNR1, MT-CO1, MT-ND1 and MT-CYB genes, 6 mutations were in the MT-ND2 gene, 5 variable sites were identified in the MT-ND6 and MT-ATP6 genes, 4 variable sites were in the MT-ND4 gene, the MT-RNR2 and MT-ND4L genes each had 3 variable sites, the MT-ND3 and MT-NC3 genes had 2 mutations. The least variable sites were in the genes of the transport RNA, 1 each in the genes MT-TA, MT-TC, MT-TD, MT-TH, and MT-TR. According to the MITOMAP and HmtDB databases, 26 mutations were associated with the development of epilepsy, 17 of them (Table 1) of them occurred in positions associated with haplogroups, and 9 mutations (Table 2) were directly involved in the development of epilepsy.

Table 1 – Variable sites of mtDNA associated with epilepsy and haplogroups

POS	Locus	Mutation type	AA Change		Population frequency	
				dbSNP ID	MITOMAP	1000G
152	MT-HV2	T→C		rs117135796	26.319%	NA
183	MT-HV2	A→G		rs113913230	0.6%	0.0078
961	12S rRNA	T→C		rs3888511	0.9%	0.0066
1736	16S rRNA	A→G		rs193303006	2.7%	0.0477
5628	MT-TA	T→C		rs1556423015	0.19%	0.0062
6671	MT- CO1	T→C	Syn: His256 His	rs1978028	0.7%	0.0033
6962	MT-CO1	G→A	Syn: Leu353Leu	rs1970771	2.4%	0.0271
7547	MT-TD	T→C		rs879076142	0.107%	NA
7738	MT-CO2	T→C	Syn: Thr51Thr	rs878875354	0.2%	NA
8794	MT-ATP6	C→T	Non-syn: His90Tyr	rs2298007	2.8%	0.045
8887	MT-ATP6	A→G	Non-syn: Ile121Val	rs1556423565	0.2%	0.0029
10310	MT-ND3	G→A	Syn: Leu84Leu	rs41467651	3.6%	0.0382
10410	MT-TR	T→C		rs200478835	0.5%	0.0054
10609	MT-ND4L	T→C	Non-syn: Met47Thr	rs200487531	2.4%	0.0255
13368	MT-ND5	G→A	Syn: Gly344Gly	rs3899498	5.0%	0.023
14569	MT-ND6	G→A	Syn: Ser35Ser	rs386420019	2.5%	0.0233
14668	MT-ND6	C→T	Syn: Met2Met	rs28357678	4.1%	0.0468

Mainly single nucleotide substitutions were found in genes encoding proteins, most of them are synonymous (7 variants: His256 His, Leu353Leu, Thr51Thr, Leu84Leu, Gly344Gly, Ser35Ser, Met-2Met). The frequency of these mutations according to the MITOMAP database was less than 5% in the population. The frequency of the three missense mutations found in the protein encoding genes (His90Tyr, Ile121Val, Met47Thr) was less than 2.8%. Non-synonymous single nucleotide substitutions may result in dysfunction of the protein, which may cause epileptic seizures. Thus, single nucleotide substitutions found in the MT-ATP6 gene can cause the development of epileptic syndromes NARP and Leigh [12].

Thus, we identified 4 synonymous variants (Met-100Met, Ala375Ala, Ile123Ile, Leu36Leu); three non-synonymous (Gly131Ser, Thr112Ala, Hr194 Ala), and two variable regions were found in rRNA genes (m.955A> C, m.2706A> G).

The pathogenic mutation of MT-ND1 gene (Non-syn: Gly131Ser) was previously described by Kirby et al. in patients with MELAS syndrome [13]. Our study confirms the clinical significance of this mutation, as it was found in a patient with epileptic encephalopathy and was possibly the cause of epileptic seizures.

The protein encoded by the MT-ND1 gene is one of the main components that form the hydrophobic core of complex I of the mitochondrial OXPHOS complexes [14]. Defects in the respiratory chain disrupt its functioning, reduce the gradient of the mitochondrial proton potential and interfere with the synthesis of ATP in mitochondria, even if ATP synthetase is not affected [15].

Table 2 – Variable sites of mtDNA associated with epilepsy

POS	Locus	Mutation type	AA Change		Population frequency	
				dbSNP ID	MITOMAP	1000G
955	12S rRNA	A→C		rs1556422497	0.006%	0.0004
2706	16S rRNA	A→G		rs2854128	78.9%	NA
3697	MT-ND1	G→A	Non-syn: Gly131Ser	rs199476122	NA	0.0004
4769	MT-ND2	A→G	Syn: Met100Met	rs3021086	97.6%	NA
7028	MT-CO1	C→T	Syn: Ala375Ala	rs2015062	80.8%	NA
8860	MT-ATP6	A→G	Non-syn: Thr112Ala	rs2001031	98.5%	NA
12705	MT-ND5	C→T	Syn: Ile123Ile	rs193302956	41.8%	NA
14854	MT-CYB	C→T	Syn: Leu36Leu	rs1057516071	0,012%	NA
15326	MT-CYB	A→G	Non-syn: Hr194Ala	rs2853508	98.65%	NA

Two mutations found in the transport RNA genes (m.5628T> C and m.7547T> C) are predicted to be pathogenic (disease Score: 0.75 and 0.5) according to the HmtVar Pathogenicity Prediction database. Over the past 20 years, many studies have found links between inherited population variants of mtDNA and neurological diseases. Since the first association of Leber hereditary optic neuropathy (LHON) with mitochondrial haplogroup J in the late 90s was revealed [16-19], many other mitochondrial disorders along with classic neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), disseminated sclerosis (MS) and amyotrophic lateral sclerosis (ALS) were also associated with mitochondrial disfunction [20].

In the case of MELAS, a study of 142 unrelated french families with the m.3243A> G mutation found statistically significant under-representation of the mutation in patients with haplogroup J [21]. Analysis of the same mutation in a smaller sample of the Spanish population found no association [22].

The increased risk of diseases with a mitochondrial contribution to pathogenesis is most likely determined by the balance between beneficial and damaging mtDNA mutations in haplogroups. In the case of some haplogroups, the balance tends more towards the harmful side. Deleterious mtDNA mutations are supposed to be population-specific, that is one variant may be associated with disease in one population but not in another [23].

Using the next-generation sequencing we revealed 6 variable sites in patients with epilepsy, the clinic of which was not previously mentioned in the literature (Table 3).

Table 3 – Variants of unknown significance

POS	Locus	Mutation typy	AA Change	dbSNP ID	Population frequency	
					MITOMAP	1000G
514	D-loop	GCA→ GCACACA			0.061%	
514-515	D-loop	GCA→ G			23.838%	
955	12S rRNA	A→ACCCC			NA	
5586	MT-NC3	C→T		rs879067503	0,104%	
10095	MT-ND3	C → A	Non-syn: Leu13Met		0.002%	
16179	D-loop	CAA → C		rs371240719	0,00%	0.0084

Genetic variants with unknown significance were mainly found in regulatory regions and were represented by deletions (514_515delCA and 16180_16181delAA), insertions (C514.CACACA and A955.ACCCC) and single nucleotide substitutions (m.5586C> T and m.10095C> A).

Deletion of 514_515delCA was detected in 3 patients with epileptic encephalopathy and in 1 with myoclonic epilepsy. The pathogenicity of these variable sites has been tested in the MITOMAP and HmtDB databases and has not been confirmed. A non-synonymous mutation in the MT-ND3 gene resulted in the substitution of the amino acid leucine for methionine at position 13. The pathogenicity of this mutation was confirmed in the HmtDB database (disease Score: 0.71) and its population frequency is 0.002%, but despite this, the clinic of this mutation is not described. Previously, mutations in the MT-ND3 gene have been described for patients with Lee syndrome and MELAS [24]. Xiao-Li Fu et al. described the T10158C mutation in the MT-ND3 gene for patients with mitochondrial encephalopathy [25]. In our case, the MT-ND3 gene mutation m.10095C> A was found in a patient with epileptic encephalopathy.

Conclusion

Advances in DNA sequencing and interpretation of genetic variation are rapidly changing our understanding of the etiology of epilepsy, which affects clinical diagnostic and treatment protocols. In developed countries, genetic testing for epilepsy is rapidly entering clinical practice, helping to accurately diagnose the type of epilepsy, establish hereditary epileptic syndromes, predict the course of the disease, choose specific treatment, and identify the risk of developing epilepsy for other family members. Unfortunately, in Kazakhstan, genetic methods for diagnosing epilepsy have not yet been introduced into medical practice. Many patients from Kazakhstan and Central Asia apply to laboratories in other countries for molecular genetic diagnosis of epilepsy.

Our preliminary study suggests that mitochondrial genes potentially play a role in the pathogenesis of epilepsy and identify subgroups of patients with different clinical phenotypes. To apply the NGS method in the diagnosis of mitochondrial epilepsy by clinicians and laboratories, it is necessary to determine the main mutations associated with the development of the disease and elucidate the role of haplogroup variants associated with the development of epilepsy. For this purpose, we plan to increase the sample and sequence of mtDNA in patients with epilepsy in the Kazakhstani population.

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