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O. Kh. Khamdiyeva[1], Zh. B. Tileules[1], G. S. Baratzhanova[1], A. V. Perfilyeva[1], Z. A. Menlimuratova[2], Y. E. Akchurina[2], L. B. Djansugurova[1]

[1]Laboratory of Experimental Mutagenesis, Institute of General Genetics and Cytology, Almaty, Kazakhstan,
[2]SVS clinic by V. M. Savinov, Almaty, Kazakhstan.
E-mail: azadahamdieva@gmail.com, janerke.tileules@mail.ru

ANALYSIS OF CANDIDATE POLYMORPHISMS AT EPILEPSY PATIENTS WITHOUT MECHANICAL DISTURBANCES

Abstract. The article presents the results of a molecular-genetics research on patients with diagnosed epilepsy without mechanical disturbance. The aim of this research was the analysis of candidate gene polymorphisms in development of different forms of epilepsy excepting mechanical reasons. 78 patients of V.M. Savinov SVS clinic with different forms of epilepsy were selected for the molecular-genetic analysis. Genotyping on candidate polimorphisms of gene coding the methyl-CpG-binding protein 2 (*MECP2,* 3 polymorphisms), the genes of sodium (*SCN1A,* 4 polymorphisms) and potassium (*KCNT1,* 2 polymorphisms) channels was performed by a site-specific PCR-RFLP method. Molecular genetic analysis revealed the presence of normal functioning alleles for 3 investigated candidate polymorphisms (p.Thr158Met, p.Thr197Met, p.Arg306Ter) of 3^{d} exon of *MECP2* gene at all epilepsy patients. However, 1 case (patient suffering from Dravet syndrome) of *de novo* mutation was defined for sodium channel gene (*SCN1A* p.Ala1783Thr) and 3 cases (2 patients suffering from temporal epilepsy and 1 patient with residual encephalopathy) of new mutations in gene responsible for potassium channel (*KCNT1* p.Ala934Thr). To determine the inherited *SCN1A* and *KCNT1* mutations, the molecular-genetics analysis was conducted for close relatives of patients. As a result, we conclude that, candidate polymorphisms of SCN1A p.Ala1783Thr and *KCNT1* p.Ala934Thr, disrupting the ion channels normal functioning, can be involved in development of non-mechanical forms of epilepsy.

Keywords: epilepsy, gene polymorphism, mutation, *MECP2, SCN1A, KCNT1*.

Introduction. Epilepsy is one of the most common and heterogeneous neurological diseases with chronic appearance characterizing by recurrent, unprovoked seizures.

Non-mechanical forms of epilepsy are diagnosed if patient had two unprovoked seizures that were not caused by a known and reversible disease, such as seizures after a brain concussion on the fever background, alcohol withdrawal, or an excessively low level of sugar in the blood.

According to the Code of the Republic of Kazakhstan "About people health and health care system" (Article 7, item 89), dated by September 18, 2009, epilepsy refers to socially significant diseases. This disease is one of the most common serious neurological disorders that affects about 1% of people worldwide (50 millions) [1]. In Kazakhstan, more than 45,000 people suffer from epilepsy, 40% of them are children, adolescents and young people, 38% of patients become disabled, and their life quality reduces by 85% on average [2].

The greatest number of children suffering from epilepsy is registered at the age of 4 to 7 years (31.75%), which is probably due to better diagnosis and clinical manifestations in this age group. The next age range for epilepsy frequency is the age from 1 year to 3 years - 27.48%. The frequency of epilepsy in age from 8 to 14 years is 20.78%. And the lowest frequency of epilepsy patient (19.97%) is registered for children before 1 year [2]. In recent decades, in view of untimely diagnosis and wrong treatment, infant mortality from epilepsy remains at a high level. For an example, mortality rate from sudden unexpected

death in epilepsy (SUDEP) reaches 8.2-10 per 1000 individuals. The main perspectives in reducing such high rates of morbidity and mortality associate with the improvement of diagnostic methods that have scientifically based effectiveness.

Both hereditary and environmentally acquired factors are involved in epilepsy pathogenesis. The molecular mechanisms underlying the various epileptic seizures have been intensively studied for more than two decades. The genetic impact plays a big role in the etiology of epilepsy idiopathic forms. Approximately 20-30% of epilepsy cases by acquired conditions, such as stroke, tumor, or head trauma. However, recent data indicate that remaining 70-80% of cases development due to genetic background [3].

Most of the epilepsy hereditary forms with established gene mutations are caused by the damage of ion channels that ensure the neuronal membrane polarization. Such epilepsy forms are referred to the channelopathy group. First of all, they include the genes of sodium, potassium, calcium and chloride channels (SCN1A, SCN2A, CACNA1A, KCNJ10, KCNQ2) [4-9].

Mutations in the sodium channel genes - SCN1A and SCN2A, were described for 70% of children suffering from Dravet syndrome, most of the mutations had spontaneous nature [4, 6]. SCN1A mutations can cause the development of severe myoclonic epilepsy of infancy (SMEI), which related to symptomatic forms [10]. The dominant mutations in the KCNT1,sodium potassium channel gene intensely expressed in the brain, cause autosomal dominant night frontal lobe epilepsy (ADNFLE) and malignant migrating partial seizures of infancy (MMPSI) [7]. Mutations in this gene increase the membrane permeability that leads to unregulated excitation of neurons in the brain.

The genes responsible for DNA methylation are also involved in the pathogenesis of epilepsy and the development of mental retardation. Using a systematic approach to gene screening, Zogby and coauthors [11] identified mutations in the gene for methyl-CpG-binding protein 2 (MECP2), which were responcible for development of some cases of Rett's syndrome. MeCP2 is a chromosome-binding protein that selectively binds 5-methylcytosine residues in symmetrically located CpG-dinucleotides [12].

The list of candidate genes for epilepsy is not restricted by mentioned variants. The spectrum of epilepsy genes acquires specificity, largely due to the results of large-scale genome-wide studies (GWASs) [13]. Leading manufacturers develop genetic panels and biochips for epilepsy diagnosis based on massive parallel sequencing (NGS) and full exome sequencing. But, despite the obvious progress in epilepsy genetics a lot remains to be understood.

The aim of this research was the analysis of candidate gene polymorphisms of *MECP2* (c.473C>T - p.Thr158Met; c.590C>T - p.Thr197Met; c.916C>T - p.Arg306Ter)*, SCN1A* (c.5492T>C - p.Phe1831Ser; c.5020G>C - p.Gly1674Arg; c.5347G>A - p.Ala1783Thr; c.4969C>G - p.Pro1657Ala)*, KCNT1* (c.2782C>T - p.Arg928Cys and c.2800G>A - p.Ala934Thr) genes in development of different forms of epilepsy excepting mechanical reasons.

The identification the association of key genes mutations or polymorphisms with epilepsy symptoms will help to develop the successful early diagnosis and therapy tools.

Materials and methods.

Study objects. For molecular genetic analysis, we collected the EDTA-treated peripheral blood samples presenting 78 patients of SVS clinic named after V.M. Saminov, who were epilepsy diagnosed in accordance with the criteria of the ILAE Commission for Classification and Terminology (1989). The excepting criterium was mechanical reason of epilepsy development. Before collection of blood samples we asked people the voluntary consent to participate in genetic research. A detailed questioning was done after obtaining the signed voluntary informed consents. The study was approved by the Ethics Committee of the Kazakh-Russian Medical University.

Isolation of genomic DNA. DNA was isolated from frozen (-20°C) peripheral blood samples containing EDTA as an anticoagulant. Isolation was carried out using the GeneJet Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) in accordance with protocol recommended by the manufacturer. Quantitative and qualitative evaluation of DNA preparations was carried out by spectrophotometric and electrophoretic analysis. After isolation, the DNA samples were stored at -20°C.

Site specific PCR for critical regions of MECP2, SCN1A, and KCNT1 genes. PCR was carried out with specific primers, the design of which was selected using the online program - PrimerQuest Tool, the PCR conditions were optimized for each primer (table 1).

Table 1 – Sequence of primers for site specific PCR

Gene, location	Primers, 5'→ 3'	PCR conditions
SCN1A, 26 exon	F-CCCGACTGTGACCCTAATAAAG	94°C - 4 min. 94°C - 40 sec. 55°C - 30 sec. 72°C - 40 sec. 72 °C - 8 min. 35 cycles
	R-GTTTGGTTGTGGCAGATTGAG	
	F -GTTTCTTGCCGAGCTGATAGA	
	R -CGATCCCAACTTCCCTCTTAAC	
	F-ACCGGATCCACTGTCTTGATA,	
	R-CGTCTGTAAGCACGCTGAATAA.	
KCNT1, 24 exon	F-CACCCTGAGACCTCCTACAA	95°C - 3 min. 95°C - 30 sec. 58°C - 30 sec. 72°C - 30 sec. 72 °C - 10 min. 35 cycles
	R-CCCTTTCTCCCACTCTTTCTG	
MECP2, 3 exon	F-ATGGGAGTTGATTGCGTACTT	95°C - 3 min. 95°C - 30 sec. 58°C - 30 sec. 72°C - 30 sec. 72 °C - 10 min. 35 cycles
	R-CAGTCCTTTCCCGCTCTTC	

The PCR was carried out in 0.2 ml microtube on the Thermocycler Eppendorf ™ Mastercycler ™ Nexus Thermal Cycler with a set of programs that determine the temperature of the PCR. To evaluate the amount and specificity of the resulting PCR products, the amplified DNA fragments length were checked by electrophoresis in 1.5% agarose gel. The correspondence of the molecular weights of the amplicons of each gene was assessed using the DNA Ladder GeneRuler 100 bp marker (ThermoFisher Scientific, USA). A samples which not contained DNAs were used as a negative control of PCR.

RFLP – analysis of candidate mutations/polymorphisms. For each polymorphic site, a restriction enzyme was selected using the online software WatCut. Table 2 indicates the restrictases, the restriction products for each selected candidate polymorphism.

Table 2 – RFLP identification of candidate polymorphisms

Gene, location	Mutation/Polymorphism	Restriction endonuclease	DNA fragments length and corresponding genotype
SCN1A, 26 exon	c.5492T>C (p.Phe1831Ser)	PstI	TT - 321bp; CC -282 и 39 bp; TC - 321, 282 и 39 bp
	c.5020G>C (p.Gly1674Arg)	HaeIII	GG - 90, 83 и 75 bp; CC - 173 и 75 bp; GC - 173, 90, 83 и 75 bp
	c.4969C>T (p.Pro1657Ala)	BamHI.	CC - 140 и 108 bp;TT - 248 bp;CT - 248, 140 и 108 bp.
	c.5347G>A (p.Ala1783Thr)	Acc II	GG - 188 и 133bp; AA - 321 bp; GA - 321, 188 и 133bp
KCNT1, 24 exon	c.2782C>T (p.Arg928Cys)	HpyF10VI	CC - 116 и 117 bp; 233 bp; 233, 117 и 116 bp
	c.2800G>A (p.Ala934Thr)	Acc II	GG - 128 и 105 bp; AA - 233 bp; GA - 233, 128 и 105 bp
MECP2, 3 exon	c.473C>T (p.Thr158Met)	TaaI	CC - 424, 75, 70 и 36 bp; TT - 493, 75 и 37 bp CT - 493, 424, 75, 70, 37 и 36 bp
	c.590 C>T (p.Thr197Met)	Hin1II	CC - 334 и 207 bp;TT - 207, 187 и 141 bp;CT - 334, 207, 187 и 141 bp.
	c.916 C>T (p.Arg306Ter)	HhaI.	CC - 513 и 91 bp;TT - 604 bp;CT - 604, 513 и 91 bp.

The PCR products were subjected to restriction by endonuclease (all used restrictases were taken from ThermoFisher Scientific, USA) digestion at an incubation temperature of 37°C for 5 hours. Then the RFLP-products were analyzed in an 8% polyacrylamide gel (PAGE) with staining by ethidium bromide. Evaluation of the fragments obtained was carried out using a DNA Ladder GeneRuler 100 bp marker (Thermo Fisher Scientific, USA) and a gel-documenting system QuantumSTS (VilberLourmat France).

Results and their discussion. The demographic and clinical data of 78 epilepsy patients, who voluntary agreed to participate in the study, are summarized in Table 3. Clinical examination of studied cohort with different forms of epilepsy revealed that the neurological status of all 78 patients was without meningeal signs and cerebral symptoms. A decrease of psycho-emotional memory, attention and emotional lability were registered at 1 patient.

Table 3 – Clinical characteristics of patients

Total number of patients	78
Age	
Average age	34±11,15
Year of birth	1954-2015 yy
Sex	
Males	44
Females	34
EEG data	
Pathological variant of EEG	72
Flat-EEG	6
Seizures type	
Generalized seizures	45
Partial seizures	33

According to the clinical diagnosis, 11 individuals were suffering from temporal epilepsy, 10 individuals had epilepsy with tonic clonic seizures, 4 individuals - juvenile and child absent epilepsy, 6 individuals - primarily generalized epilepsy, 5 individuals - secondary generalized epilepsy, 6 - residual encephalopathy, 6 - juvenile myoclonic epilepsy, 4 persons had autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE), 10 persons - idiopathic epilepsy, 3 - frontal epilepsy, 1 patient was diagnosed by Vest syndrome, and 12 persons had symptomatic epilepsy.

The DNA samples were extracted, characterized and genotyped using PCR-RFLP analysis for screening the fallowing candidate polymorphisms: *MECP2* (c.473C>T - p.Thr158Met; c.590C>T - p.Thr197Met; c.916C>T - p.Arg306Ter), *SCN1A* (c.5492T>C - p.Phe1831Ser; c.5020G>C - p.Gly1674Arg; c.5347G>A - p.Ala1783Thr; c.4969C>G - p.Pro1657Ala), *KCNT1* (c.2782C>T - p.Arg928Cys and c.2800G>A - p.Ala934Thr).

The molecular genetic analysis of 3 exon of *MECP2* gene coding the methyl-CpG-binding protein 2 did not revealed the mutant alleles in critical sites (c.473C>T, c.590C>T, c.916C>T) in all studied DNA samples. The figure 1 demonstrates the normal alleles by 3 investigated sites of 3 exon *MECP2*.

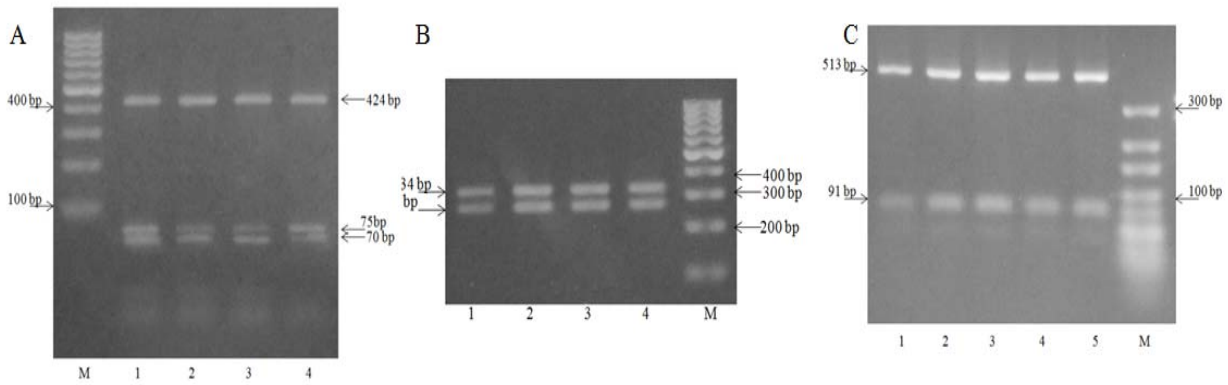

Figure 1 – PCR-RFPL analysis for mutation of *MECP2*gene:
M - 100 bp DNA ladder. A - RFPL analysis for mutation p.Thr158Met,
B - RFPL analysis for mutation p.Thr197Met, C - RFPL analysis for mutation p.Arg306Ter

As known from literature data, the mutations of the X chromosome gene-*MECP2*, which encodes the methyl-CpG-binding protein 2, can result in development of Rett syndrome, which clinically characterized by epilepsy and mental retardation [26]. Mutations in the *MECP2* gene exclusively affects the females because for the males they associate with lethal effect. But recently, there has been published evidence of detection of *MECP2* mutations at males, including the epilepsy patients who suddenly died from unknown reasons [26].

Genotyping of 4 candidate *SCN1A* gene polymorphisms of 26 exon (c.5492T>C - p.Phe1831Ser; c.5020G>C - p.Gly1674Arg; c.5347G>A - p.Ala1783Thr; c.4969C>G - p.Pro1657Ala) revealed only 1 case of mutation (c.5347G> A) in heterozygouse state at patient (2.5 years old) with the Dravet syndrome (figure 2). The analyses of close relatives (father, mother, 3 month-aged sister) of this child did not revealed the mutant alleles. We conclude that the detected variant represent *de novo* mutation of sodium channel gene *SCN1A*.

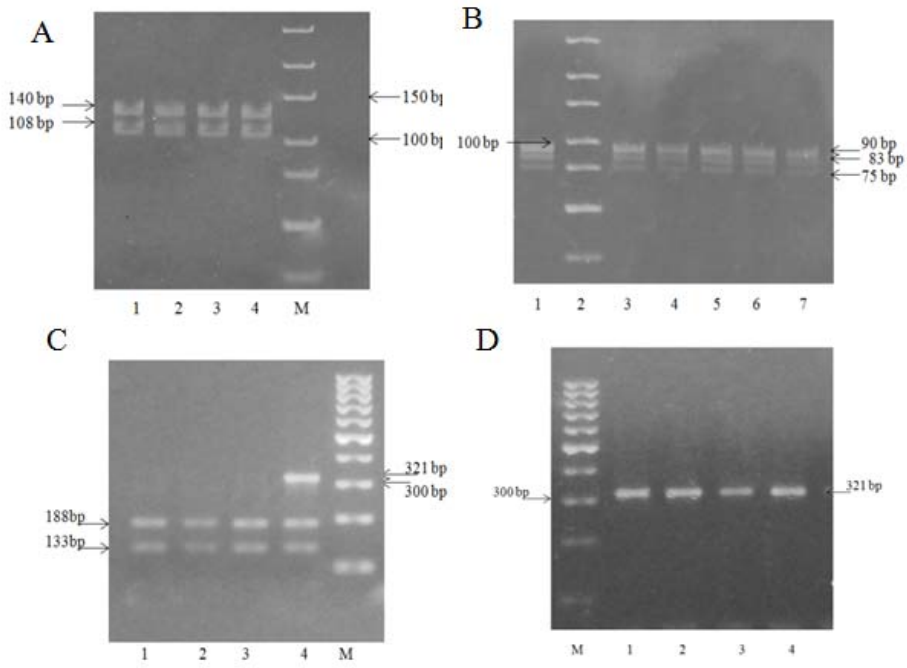

Figure 2 – PCR-RFPL analysis for mutation of *SCN1A* gene:
M - DNA ladder. A - RFPL analysis for mutation p.Pro1657Ala, B - RFPL analysis for mutation p.Gly1674Arg,
C - RFPL analysis for mutation p.Ala1783Thr (Lanes 1–3 normal samples, lane 4 – sample with a heterozygous mutation),
D - RFPL analysis for mutation p.Phe1831Ser

The clinical data indicate that convulsions at this patient first time detected at the age of 3 months and were repeated 2 times per month with different semiotics. The febrile convulsions were not detected. *De novo* mutation of the gene *SCN1A* (p.Ala1783Thr), which led to a disruption of the sodium channel, is evidence of the Dravet syndrome. The Dravet syndrome is a cryptogenic epileptic syndrome that has features of both focal and generalized seizures and in which convulsions usually do not respond to treatment and are associated with mental disability.

The treatment of the patient by Valproate led to only slight improvement. The replacement of therapy by Topiramate led to decreasing the frequency of seizures, but not significantly. The treatment by Oksarbazepine was unsuccessful because of worsening of the patient's condition. Based on this experience, the patient was again appointed to Topiramate in combination with Valproate and Dexamethasone. But despite this, seizures arose daily with myoclonus of the eyes and shoulders [13-15].

We also conducted the molecular genetic analysis of 2 critical for epilepsy candidate sites (c.2782C>T - p.Arg928Cys and c.2800G>A - p.Ala934Thr) of potassium channel *KCNT1* gene, exon 24. The result of PCR-RFLP analysis of *KCNT1* gene is presented on figure 3. The 3 cases of mutant variants (c.2800G>A) were detected regarding the polymorphism of 934 codon. All mutations were in heterozy-

gous state. 2 (1972 and 1988 yy. of birth) of 3 patients, who carrying the mutation, were suffered from temporal epilepsy. They had partial and generalized attacks of psychomotor automatism. The seizures frequency was 1-2 times per month. The another patient (born in 1987), carrying the mutation of 934 codon of *KCNT1* gene in heterozygous state, was diagnosed by residual encephalopathy. He had primary generalized convulsions. The frequency of seizures was 1 time per 1-2 months.

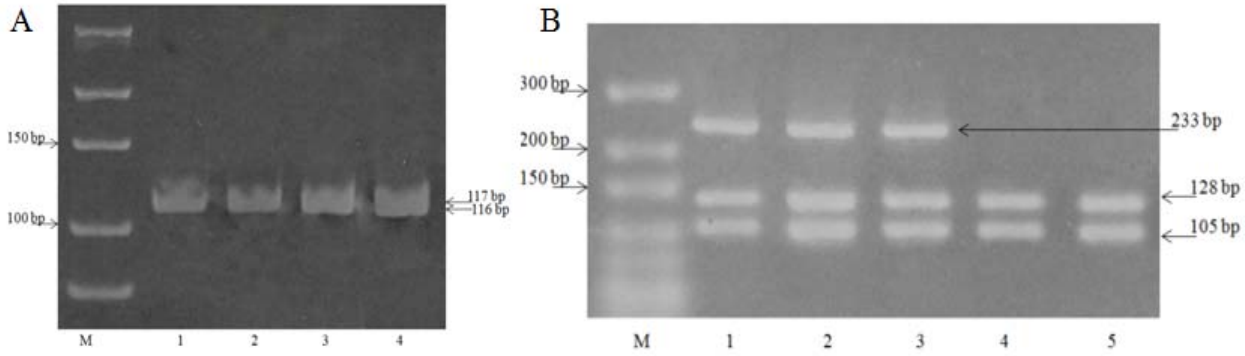

Figure 3 – PCR-RFPL analysis for mutation of *KCNT1* gene:
M - 25 bp DNA ladder. A - RFPL analysis for mutation p.Arg928Cys (Lanes 1-4 – normal samples),
B - RFPL analysis for mutation Ala934Thr (Lanes 1-3 – sample with a heterozygous mutation, lanes 4 and 5 normal samples)

Molecular genetic analysis of close relatives of these 3 patients (mothers, farthers, sisters, brothers) did not revealed the mutant variants of 934 codon of *KCNT1* gene. That confirmed the *de novo* occurrence of *KCNT1* p.Thr934 allele in all 3 families.

Mutations in the potassium channel gene of *KCNT1* were detected at various epileptic syndromes: ADNFLE [16], epilepsy of infancy with migratory focal seizures (EIMFS), previously known as malignant migratory partial seizures of infancy (MMPSI), or recently, as malignant migratory fetal seizures of infancy (MMFSI) [17], early onset epileptic encephalopathy (EOEE) [18], and Okhtahara Syndrome (OS) [19]. Patients with mutation in the *KCNT1* gene were characterized by high level of severe psychic inferiors and mental retardation.

Literature data [20] shows that the indicated mutation of 934 codon of the *KCNT1* potassium channel gene should be specific for malignantly migrating partial infantile seizures (MMPSI). But we have identified the *de novo* mutation p. Thr934 *KCNT1* in patients suffering from temporal epilepsy (TLE). And we did not detected this mutation at 4 studied ADNFLE patients.

Thus, we conclude that mutations in the *KCNT1* potassium channel gene can cause not only an autosomal dominant nocturnal epithelial frontal lobe (ADNFLE), but other forms of epilepsy.

So, the primary analysis of the range of candidate polymorphisms of key epilepsy genes allows to conclude that candidate polymorphisms of *SCN1A* p.Ala1783Thr and *KCNT1* p.Ala934Thr, disrupting the ion channels normal functioning, can be involved in development of non-mechanical forms of epilepsy. Mutations of *MECP2* are rare, and, possibly, to detect them we need to increase the case number and examined the lethal cases.

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О. Х. Хамдиева[1], Ж. Б. Тілеулес[1], Г. С. Баратжанова[1], А. В. Перфильева[1], З. А. Менлімұратова[2], Я. Е. Акчурина[2], Л. Б. Джансугурова[1]

[1]Экспериментальды мутагенез лабораториясы,
Жалпы Генетика және Цитология институты, Алматы, Қазақстан,
[2]В. М. Савинов атындағы SVS клиникасы, Алматы, Қазақстан

МЕХАНИКАЛЫҚ СИПАТТАҒЫ БҰЗЫЛЫССЫЗ ЭПИЛЕПСИЯМЕН АУЫРАТЫН НАУҚАСТАРДА КАНДИДАТ ПОЛИМОРФИЗМДЕРДІ ТАЛДАУ

Аннотация. Мақалада механикалық сипаттағы емес эпилепсия диагнозы қойылған науқастарды молекулярлы-генетикалық зерттеу нәтижелері келтірілген. Жұмыстың мақсаты механикалық бұзылыстарды қоспағанда эпилепсияның әртүрлі формаларының дамуына кандидат полиморфизмдердің әсерін талдау. Молекулярлы-генетикалық талдау жүргізу үшін В.М. Савинов атындағы SVS клиникасында емдеуде болған эпилепсияның әртүрлі формаларымен ауыратын 78 науқас таңдалынып алынды. Генотиптеу метил-CpG-байланыстырушы белок 2 (*MECP2,* 3 полиморфизм) гені, натрий (*SCN1A,* 4 полиморфизм*)* және калий (*KCNT1,* 2 полиморфизм) каналдары гендерінің кандидат полиморфизмдері бойынша сайт-спецификалық ПТР-ПДРФ әдістерінің көмегімен жүргізілді. Молекулярлы-генетикалық талдау нәтижесі барлық науқастардың *MECP2* генінің 3-ші экзонының 3 зерттелген кандидат полиморфизмі (p.Thr158Met, p.Thr197Met, p.Arg306Ter) бойынша қалыпты функциональды аллелді көрсетті. Алайда 1 науқаста (Драве синдромымен ауыратын) натрий каналы гені бойынша *de novo* мутация (*SCN1A* p.Ala1783Thr) және 3 науқаста (2 науқас самайлық

эпилепсиямен және 1 науқас резидуальды энцефалопатиямен ауыратын) калий каналы генінің жаңа мутациялары (*KCNT1* p.Ala934Thr) анықталды. *SCN1A* және *KCNT1* гендерінің тұқым қуалаушы мутацияларын анықтау үшін науқастардың жақын туыстарына молекулярлы-генетикалық талдау жүргізілді. Нәтижесінде *SCN1A* p.Ala1783Thr және *KCNT1* p.Ala934Thr кандидат полиморфизмдері ионды каналдардың қалыпты жұмысын бұзып, механикалық емес сипаттағы эпилепсияның дамуымен байланысты болуы мүмкін деген қорытынды жасалынды.

Түйін сөздер: эпилепсия, гендер полиморфизмі, мутациялар, *MECP2, SCN1A, KCNT1*.

О. Х. Хамдиева[1], Ж. Б. Тілеулес[1], Г. С. Баратжанова[1], А. В. Перфильева[1], З. А. Менлимуратова[2], Я. Е. Акчурина [2], Л. Б. Джансугурова[1]

[1]Лаборатория экспериментального Мутагенеза,
Институт Общей генетики и Цитологии, Алматы, Казахстан,
[2]SVS Клиника им. В. М. Савинова, Алматы, Казахстан

АНАЛИЗ КАНДИДАТНЫХ ПОЛИМОРФИЗМОВ У БОЛЬНЫХ ЭПИЛЕПСИЕЙ БЕЗ НАРУШЕНИЙ МЕХАНИЧЕСКОГО ХАРАКТЕРА

Аннотация. В статье приведены результаты молекулярно-генетического исследования пациентов с диагностированной эпилепсией не механического характера. Целью данной работы был анализ участия кандидатных полиморфизмов в развитии различных форм эпилепсии, за исключением механических повреждений. Для проведения молекулярно-генетического анализа были выбраны 78 пациентов с разными формами эпилепсии, которые находились на лечении в SVS клинике им. В. М. Савинова. Генотипирование проводили методами сайт-специфической ПЦР-ПДРФ по кандидатным полиморфизмам гена метил-CpG-связывающего белка 2 (*MECP2,* 3 полиморфизма), генам натриевого (*SCN1A,* 4 полиморфизма*)* и калиевого (*KCNT1,* 2 полиморфизма) каналов. Молекулярно-генетический анализ показал наличие нормальных функциональных аллелей по 3-м изученным кандидатным полиморфизмам (p.Thr158Met, p.Thr197Met, p.Arg306Ter) 3 экзона гена *MECP2* у всех пациентов с эпилепсией. Однако, 1 случай (пациент с синдромом Драве) *de novo* мутации был установлен в гене натриевого канала (*SCN1A* p.Ala1783Thr) и 3 случая (2 пациента с височной эпилепсией и 1 пациент с резидуальной энцефалопатией) новых мутаций гена калиевого канала (*KCNT1* p.Ala934Thr). Для установления наследуемых мутаций генов *SCN1A* и *KCNT1* проводили молекулярно-генетический анализ родственников пациентов ближайшей степени родства. В результате установлено, что кандидатные полиморфизмы *SCN1A* p.Ala1783Thr и *KCNT1* p.Ala934Thr, нарушающие нормальную работу ионных каналов, могут быть связаны с развитием эпилепсии немеханического характера.

Ключевые слова: эпилепсия, полиморфизм генов, мутации, *MECP2, SCN1A, KCNT1*.

Information about authors:
Озада Хамдиева – https://orcid.org/0000-0002-3990-4512
Тилеулес Жанерке – https://orcid.org/0000-0001-8099-6503
Джансугурова Лейла – https://orcid.org/0000-0002-6745-9903
Баратжанова Гульминям – https://orcid.org/0000-0002-3542-9881

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