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 polymorphisms 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 3rd 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 chanelopathy 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 responsible 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 bioships 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 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

<table>
<thead>
<tr>
<th>Gene, location</th>
<th>Primers, 5′→3′</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCN1A, 26 exon</td>
<td>F-CCCAGCTGTGACCCCTAATAAG&lt;br&gt;R-GTGTGGTGTTGGGACGATCGAG&lt;br&gt;F-GTTTCTTTCGCGACTGATAGA&lt;br&gt;R-CGATTCCAACCTCTTCCTTAAAC&lt;br&gt;F-ACCCGATTCCACTGTCTGATA,&lt;br&gt;R-CGTCGTGTAAGCAGCGCTGAAAT.</td>
<td>94°C - 4 min. 94°C - 40 sec. 55°C - 30 sec. 72°C - 40 sec. 72°C - 8 min. 35 cycles</td>
</tr>
<tr>
<td>KCNT1, 24 exon</td>
<td>F-CCACCTGAGACCTCCCTACAA&lt;br&gt;R-CCCTTCTCCCTCCTTCTTG</td>
<td>95°C - 3 min. 95°C - 30 sec. 58°C - 30 sec. 72°C - 30 sec. 72°C - 10 min. 35 cycles</td>
</tr>
<tr>
<td>MECP2, 3 exon</td>
<td>F-ATGGGAGTGTGATTGCGTACCT&lt;br&gt;R-CAGTCCTTCCGCGCTCTTC</td>
<td>95°C - 3 min. 95°C - 30 sec. 58°C - 30 sec. 72°C - 30 sec. 72°C - 10 min. 35 cycles</td>
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</table>

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

<table>
<thead>
<tr>
<th>Gene, location</th>
<th>Mutation/Polymorphism</th>
<th>Restriction endonuclease</th>
<th>DNA fragments length and corresponding genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCN1A, 26 exon</td>
<td>c.5492T&gt;C (p.Phe1831Ser)</td>
<td>PstI</td>
<td>TT - 321bp, CC - 282 and 39 bp, TC - 321, 282 and 39 bp</td>
</tr>
<tr>
<td></td>
<td>c.5020G&gt;C (p.Gly1674Arg)</td>
<td>HaeIII</td>
<td>GG - 90, 83 and 75 bp, CC - 173 and 75 bp, GC - 173, 90, 83 and 75 bp</td>
</tr>
<tr>
<td></td>
<td>c.4969C&gt;T (p.Pro1657Ala)</td>
<td>BamHI</td>
<td>CC - 140 and 108 bp, TT - 248 bp, CT - 248, 140 and 108 bp</td>
</tr>
<tr>
<td></td>
<td>c.5347G&gt;A (p.Ala1783Thr)</td>
<td>Acc II</td>
<td>GG - 188 and 133bp, AA - 321 bp, GA - 321, 188 and 133bp</td>
</tr>
<tr>
<td>KCNT1, 24 exon</td>
<td>c.2782C&gt;T (p.Arg928Cys)</td>
<td>HpyFII0VI</td>
<td>CC - 116 and 117 bp, 233 bp, 233, 117 and 116 bp</td>
</tr>
<tr>
<td></td>
<td>c.2800G&gt;A (p.Ala934Thr)</td>
<td>Acc II</td>
<td>GG - 128 and 105 bp, AA - 233 bp, GA - 233, 128 and 105 bp</td>
</tr>
<tr>
<td>MECP2, 3 exon</td>
<td>c.473C&gt;T (p.Thr158Met)</td>
<td>Taal</td>
<td>CC - 424, 70 and 36 bp, TT - 493, 75 and 37 bp, CT - 493, 424, 75, 70, 37 and 36 bp</td>
</tr>
<tr>
<td></td>
<td>c.590C&gt;T (p.Thr197Met)</td>
<td>HinfI</td>
<td>CC - 334 and 207 bp, TT - 207, 187 and 141 bp, CT - 334, 207, 187 and 141 bp</td>
</tr>
<tr>
<td></td>
<td>c.916C&gt;T (p.Arg306Ter)</td>
<td>HhaI</td>
<td>CC - 513 and 91 bp, TT - 604 bp, CT - 604, 513 and 91 bp</td>
</tr>
</tbody>
</table>

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

<p>| | |</p>
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Total number of patients</td>
<td>78</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>Average age</td>
<td>34±11.15</td>
</tr>
<tr>
<td>Year of birth</td>
<td>1954-2015 yy</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>44</td>
</tr>
<tr>
<td>Females</td>
<td>34</td>
</tr>
<tr>
<td>EEG data</td>
<td></td>
</tr>
<tr>
<td>Pathological variant of EEG</td>
<td>72</td>
</tr>
<tr>
<td>Flat-EEG</td>
<td>6</td>
</tr>
<tr>
<td>Seizures type</td>
<td></td>
</tr>
<tr>
<td>Generalized seizures</td>
<td>45</td>
</tr>
<tr>
<td>Partial seizures</td>
<td>33</td>
</tr>
</tbody>
</table>

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 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-RFLP analysis for mutation of MECP2 gene](image)


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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 heterozygous 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-RFLP analysis for mutation of SCN1A gene:

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 KCN7I gene, exon 24. The result of PCR-RFLP analysis of KCN7I 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 yv. 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-RFLP analysis for mutation of KCNT1 gene: M - 25 bp DNA ladder. A - RFLP analysis for mutation p.Arg928Cys (Lanes 1-4 – normal samples), B - RFLP analysis for mutation Ala934Thr (Lanes 1-3 – sample with a heterozygous mutation, lanes 4 and 5 normal samples)](image)

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 inferiories 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 identifed 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 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.

Acknowledgment. We would like to thank the personnel of SVS clinic by V.M. Savinov and all patients who were participated in this study.

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МЕХАНИКАЛЫҚ СИПАТАТТАУ БҰЗЫЛЫССЫЗ ЭПИЛЕПСИЯНИҢ АУЫРЫТЫН НАУҚАСТАРДА КАНДИДАТТАП ПОЛИМОРФИЗМДЕРІ ТАЛДАУ

Аннотация. Макалада механикалық сипаттагы емес эпилепсия диагнозы қойылған науқасдарда молекулярла-генетикалық зерттеу нетікелері келтірілген. Құрылыстарының мақсаты механикалық бұзылымдардың коспапанған эпилепсияның әр түрлі формалықтарының дайынын қандайдықты пайдасын анықтау. Молекулярла-генетикалық талдада жұруғу үшін В.М. Савиных атындағы SYС клиникасында емділігі бар жұруғы ортақтықтау 78 науқас таңдағалық алдынды. Генотиптерде метил-СрG-байланыстыруы белок 2 (MECPR2, 3 полиморфизм) гені, натрий (SCN1A, 4 полиморфизм) және қалыпты (KCNT1, 2 полиморфизм) каналдарды қандайдықтын ортақтықтары бойынша сайт-спецификалық ПГДРФ едістерінің комегімен жұруғын қабылдайды. Молекулярла-генетикалық талдада нетікесі барлық науқасдардың MECPR2 генінің 3-ші экзондары 3 зерттейге кандайдық пайдасы (рТhr158 мет, рТhr197 мет, рАс306 тер) бойынша қылыми функционалдық алелді қорсетеді. Алдыңда 1 науқаста (Драpeat синдромының ауырлығы) натрий каналы гені бойынша de novo мутация (SCN1A p.Ala178Thr) және 3 науқаста (2 науқас самалайлық
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 СВС клинике им. В. М. Савинова. Генотипирование проводили методами сайт-специфической ПЦР-PDRF по кандидатным полиморфизмам гена метил-CpG-связывающего белка 2 (MECP2, 3 полиморфизма), гена натриевого (SCN1A, 4 полиморфизма) и калиевого (SCN1, 2 полиморфизма) каналов. Молекулярно-генетический анализ показал наличие нормальных функциональных аллелей по 3-m изученным кандидатным полиморфизмам (p.Thr158Met, p.Thr197Met, p.Asp306Ter) 3 эзона гена MECP2 у всех пациентов с эпилепсией. Однако, 1 случай (пациент с синдромом Драве) de novo мутации был установлен в гене натриевого канала (SCN1A, Ala1783Thr) и 3 случая (2 пациента с височной эпилепсией и 1 пациент с резидуальной эпилепнопатией) новых мутаций гена калиевого канала (SCN1T, Ala934Thr). Для установления наследуемых мутаций генов SCN1A и KCNT1 проводили молекулярно-генетический анализ родственников пациентов ближайшей степени родства. В результате установлено, что кандидатные полиморфизмов SCN1A, Ala1783Thr и KCNT1, p.Ala934Thr, нарушающие нормальную работу ионных каналов, могут быть связаны с развитием эпилепсии немеханического характера.

Ключевые слова: эпилепсия, полиморфизм генов, мутация, MECP2, SCN1A, KCNT1.

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