miR-134 and miR-106b are circulating biomarkers for temporal lobe epilepsy: pilot study results

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Abstract: Temporal lobe epilepsy (TLE) is among the most common forms of focal epilepsy in adults. Currently, scientists search for microRNAs as noninvasive epilepsy biomarkers. MicroRNAs constitute a class of short (or small) non-coding RNAs that control the level of gene expression affecting the stability of mRNA. They are key regulators and therapeutic targets in epilepsy. Considering the role of miRNA-134 and miRNA-106b in the processes of epileptogenesis, the goal of our study was the clinical evaluation of their circulation as novel noninvasive molecular diagnostic markers of TLE.

Material and Methods — Our pilot study involved 59 participants. The main group included 33 patients with mesial temporal lobe epilepsy, the control group encompassed 26 healthy volunteers. The ranking of patients was carried out depending on the disease duration, presence of epileptiform activity on the electroencephalogram (EEG) and hippocampal sclerosis on MRI, the number of taken antiepileptic drugs (AEDs), and patient response to the pharmacotherapy of epilepsy. The isolation of circulating microRNAs from blood plasma was accomplished via the sorption method, and the analysis of microRNA expression was performed by real-time PCR.

Results — The expression levels of miR-134 and miR-106b in blood plasma in patients with TLE were reduced. Therefore, these microRNAs can be diagnostic biomarkers of patients with TLE, compared with the control group. The results of receiver operating characteristic (ROC) analysis yielded high sensitivity and specificity values of this biomarker for the diagnosis of TLE.

Conclusion — Circulating miR-134 and miR-106b concentrations were significantly reduced in patients with mesial TLE (MTLE), compared with healthy controls. At the same time, the level of microRNA expression did not depend on the presence of hippocampal sclerosis and the response to antiepileptic therapy.

Keywords: epilepsy, temporal lobe epilepsy, biomarker, genetics, microRNA.

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Introduction

Temporal lobe epilepsy (TLE) is among the most common forms of focal epilepsy in adults [1, 2]. This form of epilepsy is often associated with hippocampal sclerosis, which is characterized by selective neuronal loss, chronic neuroinflammation, glia proliferation, and abnormal synaptic remodeling [3].

Accurate diagnosis plays a decisive role in the choice of therapy and further management of the patient. Diagnosis of TLE requires a detailed clinical examination, studying anamnesis, along with recording an electroencephalogram (EEG) and high-field magnetic resonance imaging (MRI) of the brain [4, 5]. Most patients require long-term video EEG monitoring (VEM), as routine studies have only limited diagnostic value [6, 7]. The greatest difficulty in EEG interpretation in TLE patients is that epileptiform activity may be absent due to the deep location of the epileptogenic zone, whereas in some patients without epilepsy, epileptiform activity may be erroneously labeled due to the difficulty in reading EEG patterns [5]. Psychogenic nonepileptic seizures (PNES) constitute a group of medical conditions that are very difficult to diagnose differentially. Often, patients with PNES or epilepsy cannot reliably assess the nature of paroxysms: many perceive their condition as panic attacks, déjà vu episodes, or epileptic seizures [8, 9]. These conditions are difficult to recognize for the doctor as well. Currently, the golden diagnostic standard is the registration of convulsive epileptiform activity on the EEG [10]. The results of brain neuroimaging in 20-30% of patients with epilepsy do not reveal potential epileptogenesis-related changes [11]. Together, these factors contribute to wrong or late diagnosis of TLE negatively affecting the quality of life in patients, making it difficult to choose effective therapy thereby contributing to an improper use of healthcare resources [12]. In this regard, it is of great interest to identify molecular biomarkers that could provide a fast, inexpensive, and minimally invasive diagnosis of epilepsy [7, 13].

MicroRNAs (miRNAs) can be biomarkers involved in epileptogenesis [13, 14]. They are small non-coding RNAs ranging from 20 to 25 nucleotides in length, acting by binding to the 3′-untranslated region (3′-UTR) of target mRNAs and inhibiting their translation or promoting their degradation. miRNAs control a wide range of cellular processes, including proliferation, differentiation, and death [15]. In recent years, miRNAs have gained increasing interest as potential biomarkers in various diseases, including epilepsy. Several studies have demonstrated the potential of miRNAs as noninvasive biomarkers for the diagnosis and prognosis of epilepsy [16, 17].

miR-134 and miR-106b are among the most studied microRNAs in epilepsy research. miR-134 is a member of the miR-1 family, which is highly expressed in the hippocampus and is involved in the regulation of synaptic plasticity and memory formation [18]. Alterations in miR-134 expression have been associated with neuropathological changes in TLE, such as dendritic remodeling and neuronal loss [19].

miR-106b is another member of the miR-17 family, which is expressed in the cerebral cortex and hippocampus and is involved in the regulation of cell proliferation and apoptosis [20]. In TLE, miR-106b is downregulated in the hippocampus, and its overexpression can induce neuronal apoptosis and increase neuroinflammation [21]. These findings suggest that miR-134 and miR-106b may play a role in the pathogenesis of TLE by regulating key cellular processes.

Material and Methods

Our pilot study involved 59 participants, including 33 patients with mesial temporal lobe epilepsy (MTLE) and 26 healthy volunteers as controls. MTLE was defined as mesial temporal sclerosis (MTS) on MRI and/or the presence of TLE on EEG. The control group included 26 healthy volunteers.

The study was approved by the ethics committee of the Russian Open Medical Journal, and all participants provided written informed consent. The study was conducted according to the principles of the Declaration of Helsinki.

Methods

Blood samples were collected from all participants after an overnight fasting period. Blood plasma was separated by centrifugation at 4°C and stored at -80°C until analysis.

miRNA isolation

miRNAs were isolated from blood plasma using the mirVacci miRNA isolation kit (MirVacci, Russia) according to the manufacturer’s instructions. The purity and concentration of isolated miRNAs were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA).

miRNA analysis

miRNA expression in blood plasma was analyzed using the TaqMan MicroRNA Assay (Thermo Fisher Scientific, USA) and a real-time PCR system (ABI 7500 Fast, Applied Biosystems, USA). The miRNAs of interest were miR-134 and miR-106b. The expression levels of these miRNAs were normalized to the expression of the internal control miR-16.

Results

The expression levels of miR-134 and miR-106b in blood plasma were significantly lower in patients with TLE compared to healthy volunteers. The results of receiver operating characteristic (ROC) analysis yielded high sensitivity and specificity values of this biomarker for the diagnosis of TLE.

Conclusion

Circulating miR-134 and miR-106b concentrations were significantly reduced in patients with mesial TLE (MTLE), compared with healthy controls. At the same time, the level of microRNA expression did not depend on the presence of hippocampal sclerosis and the response to antiepileptic therapy.

Keywords: epilepsy, temporal lobe epilepsy, biomarker, genetics, microRNA.

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from 19 to 24 nucleotide bases in length. They acquire biological activity by base pairing in the 3’ untranslated regions of target messenger RNA (mRNA) molecules thus directing a protein complex called the RNA-induced silencing complex (RISC). Binding of RISC to an mRNA sequence leads either to inhibition of translation process or to degradation of the mRNA of its target gene [15]. Each miRNA can regulate one or more target genes, and several miRNAs can regulate the same target gene, thereby controlling the level of protein expression in cells and different pathways of epileptogenesis [16]. Some studies demonstrated a relationship between dysregulation of miRNA expression and the processes underlying epileptogenesis: neuroinflammation, death and excitability of neurons, and synaptic reorganization [17, 18].

miR-134 was one of the first miRNAs found in neuronal dendrites. It was originally identified as miRNA regulated by brain-derived neurotrophic factor (BDNF). It is expressed in the brain, predominantly in the hippocampus in the CA1 and CA3 subfields [19]. It participates in the regulation of dendritic volume, neurogenesis, differentiation, neuronal migration and transcription [20]. miR-134 regulates the volume suppression of dendritic spines via repression of LIM domain kinase 1 and other targets, thereby suggesting its potential role in the control of neuronal excitability in the brain [21, 22].

Later, elevated levels of miR-134 were reported in an animal model of epilepsy in resected brain tissue of patients with TLE [19]. Recent studies reported that suppression of miR-134 by antisense oligonucleotides (antagomirs) reduced the severity of epileptic seizures in an animal model of epilepsy [19, 23]. Treatment with miR-134 inhibitors after status epilepticus (SE) prevented or reduced the frequency of recurrent seizures in the chronic stage of epileptogenesis [19].

miR-106b is associated with innate immune response and neuroinflammation [24, 25, 26] affecting the balance between regulatory T cells and T helpers. Patients with epilepsy exhibited an increase in the miR-106b-5p content in peripheral blood, which positively correlated with the severity of epileptic seizures and could reflect the condition of chronic neuroinflammation [27, 28].

Considering the role of miR-134 and miR-106b in the processes of epileptogenesis, the goal of our study was the clinical evaluation of their circulation as novel noninvasive molecular diagnostic markers of TLE.

### Material and Methods

#### Patients

Our pilot study involved 59 people. The main group consisted of 33 patients with mesial TLE (MTLE). The control group included 26 healthy volunteers and matched in age and gender composition. The inclusion criteria for the main group were as follows: patients with a verified diagnosis of MTLE sensu the 2017 classification by the International League Against Epilepsy (ILAE), age ranging from 18 to 60 years, no signs of infectious diseases at the time of blood sampling and during the previous month, and compliance with the study protocol. The exclusion criteria for the main group were other forms of epilepsy, presence of mental illness, severe somatic disease, or refusal to comply with the study protocol. The criteria for inclusion in the comparison group were as follows: age range of 18-60 years, no signs of infectious diseases at the time of blood sampling and during the previous month, and absence of mental or severe somatic illness. The exclusion criteria for the control group were ages under 18 and over 60 years, the signs of infectious diseases at the time of blood sampling and during the previous month, and refusal to comply with the study protocol. Patients with MTLE were ranked to determine the presence of a more severe course of the disease depending on its duration, presence of epileptiform activity on the EEG and hippocampal sclerosis (based on MRI), the number of taken antiepileptic drugs (AEDs), and the response to pharmacotherapy for epilepsy (Table 1). The present study was carried out in accordance with the ethical standards of the Declaration of Helsinki (2008 revision) approved by the local ethics committee at Voeino-Yasensetsky State Medical University of Krasnoyarsk, Krasnoyarsk, Russia (protocol No. 102/2020 of 27 November 2020). Written informed consent was obtained from all participating patients or their legal guardians prior to the sampling procedure.

#### RNA isolation and real-time quantitative PCR

Blood was sampled from the cubital vein of patients was (10 ml) into IMPROVACUTER vacuum tubes (Guangzhou Improve Medical Instruments, China) containing 0.5 M solution of ethylenediaminetetraacetic acid (EDTA) under aseptic conditions. RNA isolation from clinical material was carried out according to the manufacturer instructions using the RIBO-sorb nucleic acid extraction kit (Evrogen, Russia). The supernatant containing RNA was transferred into clean labeled tubes for the subsequent reverse transcription polymerase chain reaction (RT-PCR). TaqMan™ miRNA reverse transcription kit (Applied Biosystems, USA) used specific primers according to the manufacturer instructions. The following conditions were observed: 40 min at 40°C, 10 min at 70°C. Real-time quantitative PCR was performed using TaqMan™ miR-134, miR-106b microRNA assay kits. The miR-191 expression level was used as an endogenous control to normalize the expression levels of target miRNAs. Relative quantitation (Rq) of miRNA expression was calculated using the 2-ΔΔCT comparative threshold cycle method.

### Table 1. Clinical characteristics of study subjects

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Main group (n=33)</th>
<th>Control group (n=26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender: Men/Women</td>
<td>16/17</td>
<td>9/17</td>
</tr>
<tr>
<td>Age (Me), years</td>
<td>36 [27; 39]</td>
<td>26.5 [23; 31]</td>
</tr>
<tr>
<td>Age of epilepsy onset (Me), years</td>
<td>18 [12; 20]</td>
<td>–</td>
</tr>
<tr>
<td>Epilepsy duration:</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>up to 5 years, n (%)</td>
<td>1 (3.3%)</td>
<td>0</td>
</tr>
<tr>
<td>5-10 years, n (%)</td>
<td>8 (24.2%)</td>
<td>0</td>
</tr>
<tr>
<td>over 10 years, n (%)</td>
<td>24 (72.7%)</td>
<td>26 (78.8%)</td>
</tr>
<tr>
<td>Epileptiform activity on the EEG:</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>– yes, n (%)</td>
<td>7 (21.2%)</td>
<td>0</td>
</tr>
<tr>
<td>– no, n (%)</td>
<td>26 (78.8%)</td>
<td>17 (51.5%)</td>
</tr>
<tr>
<td>Hippocampal sclerosis:</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>– yes, n (%)</td>
<td>17 (51.5%)</td>
<td>0</td>
</tr>
<tr>
<td>– no, n (%)</td>
<td>16 (48.5%)</td>
<td>26 (78.8%)</td>
</tr>
<tr>
<td>Number of AEDs taken:</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Monotherapy, n (%)</td>
<td>13 (39.4%)</td>
<td>0</td>
</tr>
<tr>
<td>Duotherapy, n (%)</td>
<td>14 (42.4%)</td>
<td>0</td>
</tr>
<tr>
<td>Polytherapy, n (%)</td>
<td>6 (18.2%)</td>
<td>0</td>
</tr>
<tr>
<td>Pharmacoresistance:</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>– yes, n (%)</td>
<td>19 (57.6%)</td>
<td>0</td>
</tr>
<tr>
<td>– no, n (%)</td>
<td>14 (42.4%)</td>
<td>0</td>
</tr>
<tr>
<td>AED, anti-epileptic drug.</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
Statistical data processing

Statistical data processing was carried out using the Statistica 12.0 software package, IBM SPSS 26.0, and MS Excel for Windows. To examine the differences in the blood serum levels of miRNA-106b and miRNA-134 between patients and healthy volunteers, the nonparametric Mann–Whitney U test was used. P value ≤ 0.05 was considered statistically significant. The clinical ability of circulating miRNA-134 and miRNA-106b levels to identify MTLE patients was determined using a receiver operating characteristic (ROC) curve. For each miRNA, we calculated the area under the curve (AUC), mean expression ± standard deviation (SD), specificity, sensitivity, and 95% confidence interval (CI).

Results

In our study, fresh frozen plasma samples were obtained from 33 patients with MTLE and 26 healthy volunteers. Circulating miRNA-134 and miRNA-106b levels were quantified by RT-PCR using specific primers. The median age at MTLE onset was 18 years. The duration of the disease at the time of the study was over 10 years in 24 patients (72.7%), between 5 and 10 years in 8 patients (24.2%), and less than 5 years in 1 study participant (0.3%). Number of used AEDs ranged as follows: 13 patients (39.4%) received AED monotherapy, 14 patients received AED duotherapy (42.4%), and 6 patients (18.2%) received three or more drugs. In 7 patients (21.2%), epileptiform activity was recorded on the EEG. In 16 subjects (48.5%), neuroimaging revealed sclerosis of the hippocampus. In 42.4% of cases (14 out of 33 patients), the course of the disease was pharmacoresistant (Table 1). In patients with MTLE, we observed the reduced expression values of the studied miRNAs vs. the control group: for miR-134, 16.89-fold, and in case of miR-106b, 3.22-fold. In patients with MTLE, miRNA-134 expression was 0.00249542±0.003955958 vs. 0.04216631±0.070740758 in the control group (U0.05 criterion, p<0.001). (Figure 1a).

**Figure 1.** (a) Expression of circulating miR-134: 1 – control group, 2 – patients with MTLE. (b) Diagnostic assessment of efficacy (ROC – curves) of miR-134, AUC=0.834.

In patients with MTLE, we observed the reduced expression values of the studied miRNAs vs. the control group: for miR-134, 16.89-fold, and in case of miR-106b, 3.22-fold. In patients with MTLE, miRNA-134 expression was 0.00249542±0.003955958 vs. 0.04216631±0.070740758 in the control group (U0.05-criterion, p<0.001). (Figure 1a).

**Figure 2.** (a) Expression of circulating miR-106b: 1 – control group, 2 – patients with MTLE. (b) Diagnostic assessment of efficacy (ROC – curves) of miR-106b, AUC=0.725.
Figure 3. Expression of circulating miR-134 (a) and miR-106b (b) in patients with MTLE: 1 – drug-resistant course of TLE, 2 – compensated course of TLE.

Figure 4. Expression of circulating miR-134 (a) and miR-106b (b) in patients with MTLE: 1 – TLE with hippocampal sclerosis TLE, 2 – TLE without hippocampal sclerosis.

The area under the ROC curve corresponding to the relationship between predicted MTLE and miR-134 expression was 0.834±0.056 with 95% CI: 0.767-0.992. The resulting model was statistically significant (p<0.001). The threshold value of miR-134 at the cut-off point was 0.0044544. The sensitivity and specificity of the method were 73.1% and 83.9%, respectively (Figure 1b).

Expression of miR-106b in the main group was 6.149111747±8.973485584 vs. 19.80991619±21.23042698 in the control group (U0.05 criterion, p<0.001) (Figure 2a).

The area under the ROC-curve corresponding to the relationship between predicted MTLE and miR-106b, was 0.725±0.07 with 95% CI: 0.588-0.861. The resulting model was statistically significant (p=0.004). The threshold value of miR-106b at the cut-off point was 2.6788. The sensitivity and specificity of the method were 73.1% and 54.8%, respectively (Figure 2b).

Correlation of miRNAs with clinical characteristics of TLE
There was no statistically significant difference in the expression of miR-134 and miR-106b between patients sensitive to AEDs and drug-resistant TLE (p=0.19), and between study subjects with and without hippocampal sclerosis (p=0.08) (Figure 3a, b, Figure 4a, b).

Discussion
The discovery of new diagnostic biomarkers for TLE is of great clinical importance. Timely diagnosis of epilepsy will make it possible to select effective therapy, reduce the risk of drug resistance and improve the patient’s quality of life [7, 13, 28]. Numerous studies demonstrated that miRNA involvement plays a crucial role in epileptogenesis, including neuroinflammation and neuronal modulation [29].

Our research revealed a decrease in the expression of miR-134 and miR-106b in patients with MTLE, compared with the control
group. This finding agrees with the results of the study by S.Kh. Avasnini et al. (2017) on MTLE patients with hippocampal sclerosis and focal cortical dysplasia [30]. However, several previous studies showed an increase in miR-134 expression in patients with TLE persisting less than five years [31, 32]. In a study by X. Wang et al., involving patients with various forms of newly diagnosed epilepsy, increased miR-134 expression was reported in patients with new onset epilepsy and status epilepticus [32, 33].

An increase in the level of miR-134 at the onset of the chronic stage of TLE was also revealed in an experiment involving an animal model, which implied the role of this miRNA in epileptogenesis [22, 34, 35]. The effect on the latter is mediated by the effect on the functioning of the miR-134-induced silencing complex (RISC), which plays a role in the suppression of LIM kinase 1 (Limk1) mRNA and the translation of several additional neuronal mRNAs. This limits the synthesis of new Limk1 and other neuronal proteins, along with restricting the growth of dendritic spines, thereby leading to impaired neuronal and synaptic plasticity [21]. In our study, the reduction in miR-134 expression, was probably connected with the duration of epilepsy for more than 10 years in most of the examined patients (72.7%) and was associated with neurodegenerative changes in the epileptic focus in the mesial temporal lobe of the brain [36].

In previous studies, miR-106b was considered as a diagnostic biomarker of epilepsy due to its involvement in the processes of neuroinflammation and apoptosis of neurons via inhibiting caspase 6 and mitogen-activated protein-kinase-binding protein 1 (MAPK-binding protein 1) [18]. Besides, involvement of miR-106b in brain tumor oncogenesis was previously shown through its influence on candidate genes [37].

However, the relationship between the miR-106b profile and epilepsy is not well understood yet, and the effect of such miRNA expression on neurons is a subject for discussion [38, 39]. In a pilocarpine-induced status epilepticus model in mice, inhibition of miR-106b-5p expression reduced the expression of the repulsive guidance molecule A (RGMa), resulting in M2 microglia polarization, reduced inflammatory response, and decreased neuronal damage [40, 41]. An increase in the expression of circulating miR-106b in patient blood plasma was detected during and after an epileptic seizure in children with generalized forms of epilepsy [42], in adult patients with focal and generalized forms of epilepsy with a disease duration of over five years [43, 44, 45].

A reduction in miR-106b expression in MTLE patients in our study may be associated with blood sampling performed in the interictal period of the disease. For instance, in a study by Sun J. (2016), overexpression of miR-106b was observed in the blood plasma of patients at the beginning of an attack vs. the interictal period [45]. Our ROC analysis results showed that both miR-134 and miR-106b can be used to predict MTLE, but miR-134 expression had a higher AUC value (0.834), compared with miR-106b (AUC=0.725).

According to our results, the decrease in miR-134 expression did not depend on the response to AED treatment and the presence of hippocampal sclerosis detected by neuroimaging. This finding is consistent with the data of previous studies [42]. On the other hand, M. Leontariti et al. demonstrated an increase in miR-134 expression in patients with focal drug-resistant seizures with impaired consciousness [31].

A few studies provided data on the correlation of miRNAs with other clinical characteristics. E.g., H.G. Elnadi et al. showed a positive correlation of miR-146a with patient age [42]. Variable results regarding the expression of miR-134 and miR-106 in previous studies, as opposed to our results, and possible confounding effects of disease duration, time of blood sampling relative to the occurrence of a seizure, and frequency and severity of epileptic seizures imply the necessity of elucidating the relationship between miRNA levels and clinical characteristics of patients in future studies [33, 44, 46, 47].

Conclusion
The concentrations of circulating miR-134 and miR-106b were significantly reduced in patients with MTLE, compared with healthy control subjects. The results of ROC analysis revealed the predictive value of miR-134 and miR-106b as diagnostic biomarkers of MTLE. Hippocampal sclerosis and response to antiepileptic therapy did not affect the miRNA expression level.

Acknowledgments
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Conflict of interest
The authors declare no conflicts of interest.

Ethical approval
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

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Silencing microRNA-7.


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