SCIENTIFIC REVIEWS AND LECTURES

M.R. Sapronova, K.D. Yakovleva, A.A.Usoltseva, Yu.S. Panina, S.N. Zobova, D.V. Dmitrenko

BIOMARKERS OF EPILEPSY: MICRO-RNA (PART II)

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This article considers the use of microRNAs as a possible biomarker of epilepsy.

The presented studies have shown that microRNAs can be involved in the process of epileptogenesis by regulating the inflammatory response, apoptosis of neurons, and transcription factors involved in cell differentiation. Biological fluids (blood and CSF) of patients with epilepsy showed differences in the number of circulating microRNAs, which may allow further use of microRNAs as a diagnostic biomarker. Recent discoveries providethe sufficient source of new microRNA targets, but there are still significant problems of studying their role in pathogenesis and the possibility of their application in clinical practice.

Keywords: epilepsy, epileptic seizures, biomarker, microRNA, epileptogenesis.

Introduction. Epilepsy is a chronic neurological disease characterized by recurrent seizures resulted from pathological synchronous excitation of brain neurons [26]. The diagnosis of epilepsy is usually based on clinical manifistations of the disease, electroencephalogram (EEG) data, and magnetic resonance imaging (MRI) of the brain. In some cases, clarifying a seizure type is not an easy task and it makes the choice of the therapy even more challenging.

There is not enough evidence proving that antiepileptic drugs affect the process of epileptogenesis. No biomarkers of epileptogenesis to prevent the development of the disease and assess the effectiveness of therapy are revealed, it allowing to predict the response to antiepileptic drugs. There is a great need to search for new biomarkers of epileptic seizure, status epilepticus, as well as of epileptogenesis [25]. Moreover, molecular biomarkers are especially attractive, since their determination is possible using minimally invasive methods with the study of biofluids, e.g. blood [9].

The usage of gene expression profiling, which dates back to early 2000s, has made the understanding of the scale of changes in the brain of patients with epilepsy [1, 49]. Nowadays, a number of molecules have been studied, including proteins and ribonucleic acids (RNA), but most of them not characterized with specificity and sensitivity [47]. The research results published about 10 years ago showed changes in the levels of microRNA in blood after an epileptic seizure in rats [13].

MicroRNA: definition and mechanisms of functioning. The existence of microRNAs was first reported in 1993 by Victor Ambros and Gary Ruvkun [34, 58]. Currently, miRNAs are recognized as the main regulators of the translation stability of messenger RNA (mRNA) [20].

MicroRNAs are molecules from a class of short noncoding RNAs that regulate the level of gene expression by affecting the mRNA stability. Specific microRNAs bind to complementary sequences of the non-coding part of mRNA (3'-untranslated region, 3'-UTR) . It was shown that the synthesis of about 60% of all human proteins is regulated by microRNA molecules [13].

MicroRNA sinthesis occurs in the nucleus by transcription of the encoding gene. When entering in the cytoplasm of the cell and reaching its functional state, the miRNA molecule is able to bind to the matrix molecule (mRNA), inhibiting the subsequent translation of the protein of this mRNA, which is a matrix for protein's synthesis in the ribosome with the transport RNAs' involvement [4, 5, 19, 56].

MicroRNA's role in the pathogenesis of epilepsy. Over the past 5 years, several targeted and genome-wide studies of the microRNA level of expression in epilepsy have been carried out. According to the obtained data, changes were found in more than 100 different miRNAs in animal modelx and in patients with epilepsy, which proves the correlation between miRNA expression and epilepsy [24, 39, 55]. The very first study of miRNAs in human epilepsy was published in 2010, when an increase in hippocampal miRNA-146a levels associated with the control of inflammatory responses was reported [29].

Recently, a potential clinical use of miRNAs has been proposed using agomyres or inhibitory miRNA sequences as potential therapeutic molecules in epilepsy [2, 14, 21]. Altered microRNA profiles in biological fluids can be useful biomarkers of epileptogenesis [40].

The control of microRNA expression in epilepsy. The mechanism of changes in microRNA expression in patients with epilepsy remains uncertaion. Both direct and indirect disregulation theouries have been discussed [18].

Epigenetic mechanisms may be important regulators of microRNA expression in epilepsy. Epigenetic processes comprise DNA methylation and histone protein modification. Increased DNA methylation generally promotes chromatin compaction and decreases gene transcription in these regions. For example, after an epileptic seizure induced by kainic acid in rats, the acetylation of histones of the microRNA-124 gene locus decreases. This epigenetic mechanism of expression's suppression may explain the decrease in the level of miR-124 in the hippocampus [17]. Genomic analysis

SAPRONOVA Margarita Rafailevna – Ph.D., associate Professor, Department of medical genetics and clinical neurophysiology, V.F. Voyno-Yasenetsky Krasnoyarsk State Medical University, Krasnoyarsk. sapronova.mr@yandex.ru, **YAROVLEVA Kristina Dmitrievna** – postgraduate student, laboratory assistant, Department of medical genetics and clinical neurophysiology, V.F. Voyno-Yasenetsky Krasnoyarsk State Medical University, Krasnoyarsk Kris_995@mail.ru; **USOLTSEVA Anna Alexandrovna** – the resident, laboratory assistant, Department of medical genetics and clinical neurophysiology, V.F. Voyno-Yasenetsky Krasnoyarsk State Medical University, Krasnoyarsk a.usoltseva@list.ru, **PANINA Julia Sergeevna** – research scientist, Department of medical genetics and clinical neurophysiology of Institute of postgraduate education, V.F. Voyno-Yasenetsky Krasnoyarsk State Medical University, Krasnoyarsk. mrs.yuliapanina@mail.ru, **ZOBOVA Svetlana Nikolaevna** – Ph. D, research scientist, Department of medical genetics and clinical neurophysiology of Institute of postgraduate education V.F. Voyno-Yasenetsky Krasnoyarsk State Medical University, Krasnoyarsk; Krasnoyarsk Scientific Center of the Siberian Branch of the Russian Academy of Sciences, the Separate Division «Research Institute of Medical Problems of the North», Krasnoyarsk. snzobova80@gmail.com, **DMITRIENKO Diana Viktorovna** – MD, Ph. D., head of the Department of medical genetics and clinical neurophysiology, V.F. Voyno-Yasenetsky Krasnoyarsk State Medical University, Krasnoyarsk. mart2802@yandex.ru

of DNA methylation in hippocampal tissue in patients with temporal lobe epilepsy revealed differences in the methylation state of several miRNA genes. Inverse correlations were found between methylation status and miRNA expression in hippocampal samples [37].

Mutations in microRNA genes can also affect the risk of epilepsy development. A number of studies have reported the association of candidate genes carriage and the disease's manifestation [7, 42]. Single-nucleotide variants of genes encoding microRNAs can influence the function of microRNAs in one of the following ways: altering the primary transcription of miRNAs, processing primary miRNAs (pri-microRNA) and precursor miRNAs (pre-miRNA), and by indirect modulation of microRNA – mRNA interactions [8, 10]. Changes in the global profiles of microRNA expression in various animal models of epilepsy have been shown [27]. Specific miRNAs in brain tissue have been associated with seizure-induced neuronal death or neuroprotection [28]. But still, no convincing evidence has been found that genetic forms of epilepsy are caused by mutations or single nucleotide variants' carriage of genes encoding microRNAs (Fig. 1).

Figure 1 - This schematic representation illustrates the potential epigenetic mechanisms, including DNA methylation, histone alterations, RNA-based transcriptional control, and bro domain reading activity which can alter the cellular gene expression profile and thus promote inhibition of epileptogenesis progression.). Figure has been adaptated from the article of Younus I. et al. 2017 [35].

The EpimiRNA Consortium studies the role of changes in genes encoding microRNAs in epilepsy development. In the project, a group of patients with epilepsy is compared with healthy individuals from the control group. The sequencing strategy focuses on those genomic regions in which changes are most likely to cause epilepsy. The changes affecting miRNAs are analyzed together with the entire subset of their predicted mRNA targets [59]. These microRNAs are expressed in the hippocampus of the human brain [33] and are likely to take part in the development of epilepsy. Thus, the search and selection of possible microRNA targets is carried out, the role of which has been confirmed in functional studies [60]. It is expected that the EpimiRNA sequencing strategy will highlight the regions of the genome encoding microRNAs, variations in which contribute to the development of epilepsy [15].

On the other hand, experimental and

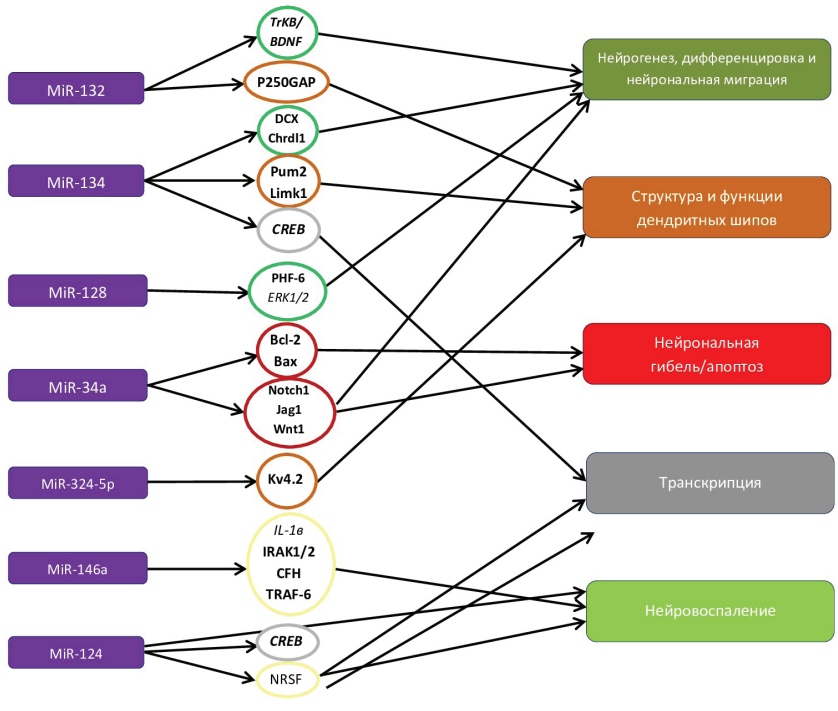

Fig. 1. Targeted approaches to the discovery of epigenetic inhibitors in epilepsy

human epilepsy are associated with network-wide changes in the levels of transcripts encoding proteins and aberrant protein production, which leads to significant suppression of transcription. Thus, seizures are likely to increase the levels of microRNA, which will subsequently lead to a decrease in the amount of mRNA of the genes encoding the protein. It was shown that epileptic seizures increase the levels of 28 miRNAs in the CA3 subfield of the mouse hippocampus [45]. For some epilepsy-related miRNAs, such as miRNA-134, transcriptional mechanisms are well-known. The Mef2 protein is activated by neuronal activity and stimulates the expression of miRNA-134 in neurons [31].

However, the question is not as simole as it seems, it has been proven that one microRNA molecule can have dozens of targets that regulate several genes of the same pathogenetic pathway or separate genes belonging to different pathogenetic pathways, which allows it to act at different levels of the pathological process [60].

The targets of microRNA in epileptogenesis. The studies have shown that microRNAs can be involved in the process of epileptogenesis by regulating the inflammatory response, apoptosis of neurons, and transcription factors involved in cell differentiation [38, 46]. The role of various miRNAs in epileptogenesis is shown on Figures 2 and 3.

Figure 2 - Simplified model depicting targets of candidate microRNAs and the associated epileptogenic pathways. Targets that have not been shown to be directly regulated by the respective microRNA are printed in italics). Figure has been adaptated from the article of Tiwari D. et al. 2018 [34].

Early functional studies linked the influence of microRNAs on the development of epileptic seizures with neuroinflammation and changes in the microstructure of neurons. For example, dysregulation of miRNA-134 changed the number and volume of dendritic spines of excitatory neurons, presumably through the target kinase of the LIM-domain [16, 43, 48], whereas miRNA-146a, miRNA-221, and miRNA-222 can control immune responses through interleukin-1β and cell adhesion molecules [6, 23, 52].

Moreover, the altered microRNA levels are likely to affect a wide range of molecular and cellular pathways in epilepsy, including cell differentiation, migration, and proliferation. For example, the identification of the axon guidance occurs under the microRNA contro, which underlines that if miRNAs associated with epilepsy were identidied, the mechanisms underlying epilepsy would be discovered [9, 43]. Although the effect of expression's deregulation of axon guidance signals on epileptogenesis is not well understood, these molecular signals contribute to various neurological disorders due to their ability to control axon growth, neuronal migration, and synapse development and functioning [36, 41]. Axon-guiding molecules may be important for the inte-

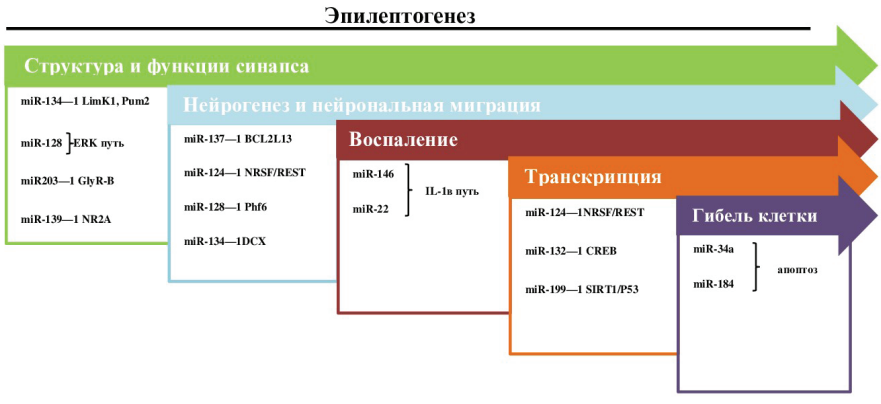

Fig. 2. The simplified model depicting candidate micro-RNA targets and associated epileptogenic pathways

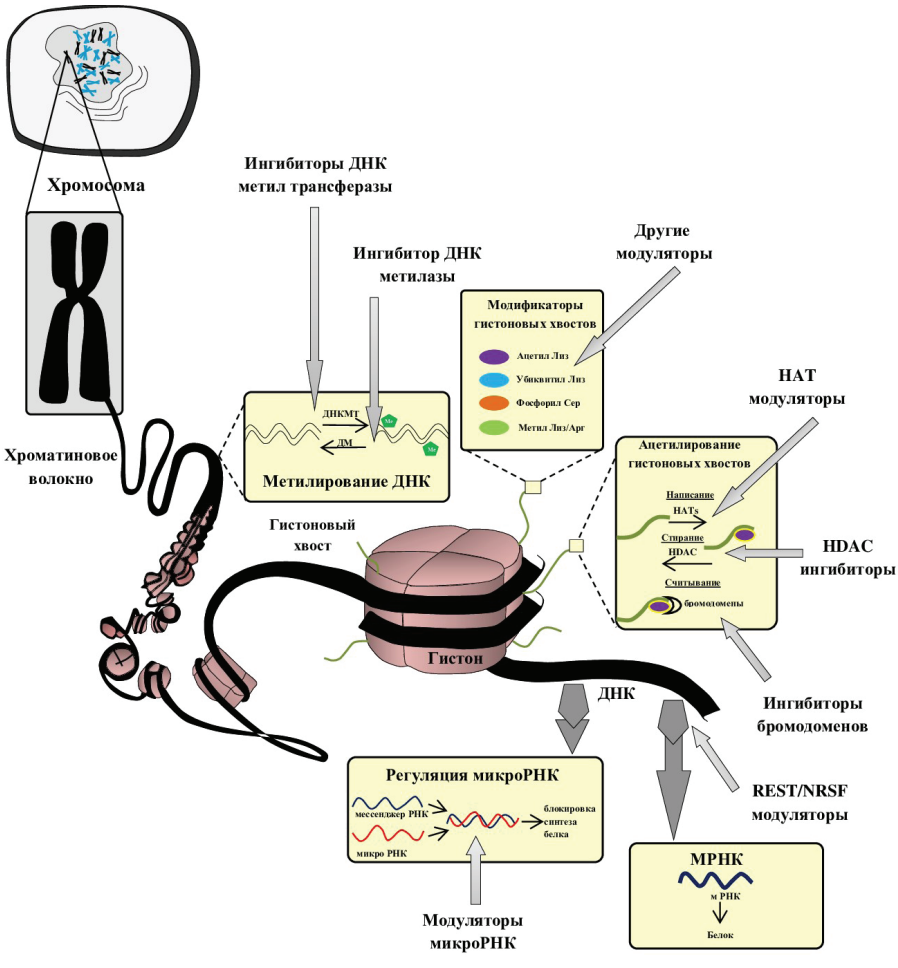

Fig. 2. Micro-RNA and their targets in epileptogenesis

gration of newly differentiated neurons, for example, in the dentate gyrus of the hippocampus. These changes are traced in models of epilepsy and in patients with mediobasal temporal lobe epilepsy [3, 4, 54] (Fig. 3).

Figure 3 – microRNAs and their targets in epileptogenesis. This figure summarise examples of microRNAs and their mRNA targets and pathways associated with key processes in epileptogenesis). Figure has been adaptated from the article of Brennan G.P. et al. 2018 [14].

In an animal sample of epilepsy and in patients, it was shown that microRNAs are differentially expressed in the brain, and functional studies have shown their role in modulating the susceptibility and severity of epileptic seizures [14, 30, 53]. The main microRNA-regulated pathogenetic pathways of epilepsy are: control of the cell cycle; ion channel modification; tissue remodeling and neural plasticity; regulation of transcription and gene expression; neuroinflammation; apoptosis; the emergence of stem cells (stemness). The immune system, cell cycle, apoptosis as well as neurotrophin signaling pathway were identified as main and most inriched pathways [15].

Neuroinflammation. The importance of the adaptive and innate immune system is increasingly recognized in the pathogenesis and constant maintenance of the epileptic state. MicroRNAs have become potent regulators of inflammation, targeting components of both innate and adaptive immune responses. Thus, it was shown that miRNA-132 has an anti-inflammatory effect by acting on acetylcholinesterase and multiple miRNAs involved in the regulation of the transcription factor NF-kB and other pathways [33].

The first inflammation-related miRNA that was identified in epilepsy was miRNA-146a, which regulates the expression of toll-like receptors (TLRs) and cytokine signaling pathways [33]. Toll-like receptors (TLRs) have been identified as direct targets for microRNAs in the brain. It has been shown that TLR4 is activated after experimentally induced seizures in mice. TLR4 levels are partially controlled by microRNAs, including let-7i. Apparently, the ligand for TLR4 is the HMGB1 protein, which is thought to be released from damaged neurons upon stimulation of seizures. Thus, RNA-sensitive TLRs are expressed in the brain both in glia and in neurons. MicroRNA let-7b expressed in the brain can activate TLR-7 to promote neuronal death. Evidence that this is a relevant mechanism *in vivo* has been presented in an intrathecal model of neurodegeneration [15].

It was also shown that interleukin 1β regulates the expression of microRNA - 146a in cultures of human astrocytes. There is an assumption that the immune system is not only regulated by microRNA, but can regulate microRNA itself [23]. It was revealed that in the normal brain microRNA-146a is expressed by neurons, not glia. After the status epilepticus, hippocampal levels of microRNA-146a were increased both in rats of different ages and in the resected hippocampus in adults and children with drug-resistant temporal lobe epilepsy. An increased level of miRNA-146a was present in neurons and astrocytes, but not in microglia, which indicates the specificity of the cell types producing this microRNA. However, these studies involved patients with hippocampal sclerosis, so it is un-

clear whether microRNA-146a increases in epilepsy without this pathology. The mechanism of an increase in the level of microRNA can be mediated through IL-1, whereas TNFα does not stimulate the expression of microRNA-146a at all [56

On the other hand, microRNA-15b-5p has been shown to be specifically activated in the cerebrospinal fluid of patients with Alzheimer's disease [11]. In epilepsy, it was found that this miRNA is suppressed, which suggests a possible loss of control over the ASM enzyme and leads to the conversion of sphingomyelin into a proinflammatory and proapoptotic ceramide [57]. Studies have also shown that microRNA-106b is involved in AD-associated inflammation in epilepsy [22], and miRNA-451 is involved in maintaining the inflammatory status in several brain pathologies. Additionally, some works describe the activation of microRNA-451 in temporal lobe epilepsy [23] and its connection with the inflammation in the brain [44].

Cell cycle. Another target for microRNA in epileptogenesis is neurogenesis, control of the cell cycle and cell proliferation, called "cell cycle". This pathway includes all those genes that are involved in the proliferation and differentiation of neuronal cells, which are controlled by microRNA-15a-5p, microRNA-34a, microRNA-106b-5p, and microRNA-146. Thus, in epilepsy, the observed suppression of microRNA-15a-5p can lead to a decrease in its control activity on targets, including the ubiquitin ligase FBXW7, which destabilizes Cyclin E and leads to the blockage of the cell cycle in the S-phase. This may partially reflect the inhibition of neurogenesis, which, with a parallel increase in neuronal apoptosis, leads to the loss of neurons observed in patients with epilepsy [57].

It has been demonstrated recently that the microRNA-34/449 family is a key regulator of mitotic spindle orientation during cerebral cortex development. In addition to this, members of the microRNA-34 family are the most activated microRNAs in differentiated neurons. They play a role in controlling the cell cycle and blocking apoptosis, suggesting that the observed suppression of miR-34a in epilepsy may lead to the termination of the cell cycle, activation of apoptosis, and consequently to the neurons' loss. What is more, in a rat sample of epilepsy induced by electrical stimulation, it was shown that microRNA -106b-5p is activated at an early stage of epilepsy development, which indicates a potential role of this microRNA in the induction of neuronal cell cycle termination and neuronal apoptosis [44].

Apoptosis. Apoptosis includes apoptosis-associated genes and pathways involved in pro- or anti-apoptotic signaling, which are confirmed targets for microRNA-15a-5p (downregulated), microRNA-106b-5p, microRNA-146 and microRNA-451 [31].

MiRNA-15a-5p has been as a modulator of ischemia in the human brain: the level of the miR-15a / 16 cluster usually increases after cerebral ischemia. It has been observed that antagomir treatment or genetic loss of this microRNA cluster is capable of inducing the activation of anti-apoptotic proteins (such as Bcl2 and Bcl-w) and suppressing pro-inflammatory molecules. It is possible that the observed suppression of miRNA-15a-5p in epilepsy mainly works due to the effect of miRNA-15a-5p decrease on the modulation of neuroinflammatory cytokines [59].

MicroRNA-106b-5p regulates caspase 6 (CASP6) and MAPK-binding protein 1 (MAPKBP1) (inflammation and apoptosis of neurons). Status epilepticus induces the expression and activation of CASP6 in the rat hippocampus, which leads to neuronal apoptosis in various models of epilepsy [50].

Upregulation of microRNA-146 has been found in several models of epilepsy and probably also plays a role in the regulation of neuronal apoptosis [57] as it reduces proliferation and promotes apoptosis in other pathologies. For instance, activation of microRNA-451a in the cerebrospinal fluid was associated with several pathologies of the central nervous system. Regarding the role of microRNA-451, it is known that this microRNA is able to control the AMPK-mTOR pathway. The increase in microRNA-451 observed in patients with epilepsy may modulate autophagy and neuronal loss that is observed in the brain after cerebral ischemia [58].

Synaptic structures & functions. MicroRNA-134 is constitutively expressed in the adult brain in neurons and is found in the body of the neuron as well as in their dendrites [15]. It was found that overexpression of miR-134 in neurons *in vitro* reduces the volume of the neuronal dendritic process, whereas the inhibition of miR-134 leads to a slight increase in its volume [45]. The mechanism of these changes was determined by local directed translation of miRNA-134 of the Lim domain containing kinase 1 (Limk1) inside the dendrites. Limk1 phosphorylates and inhibits actin depolymerization factor (ADF / cofilin), thereby promoting the formation of F-actin, which is critical for enlargement of the dendrite (and induction of long-term depression). By inhibiting Limk1, microRNA-134 promotes process collapse by increasing G-actin in neuronal processes. It is noteworthy that the dendritic phenotype of miRNA-134 overexpression is similar to that in Limk1 mice. The relationship between the volume of the dendritic process and excitatory synaptic strength may implicate hyperexcitability pathology such as epilepsy. Overexpression of microRNA-134 *in vivo* using viral vectors led to a small but still significant decrease in the complexity of basal dendrites in pyramidal neurons of MicroRNA-134 is constitutively expressed in the adult brain in neurons and is found in the body of the neuron, as well as in dendrites. It was found that overexpression of miR-134 in neurons in vitro reduces the volume of the neuronal dendritic process, while inhibition of miR-134 leads to a slight increase in its volume. The mechanism of these changes was determined by local directed translation of miRNA-134 of the Lim domain containing kinase 1 (Limk1) inside the dendrites. Limk1 phosphorylates and inhibits actin depolymerization factor (ADF / cofilin), thereby promoting the formation of F-actin, which is critical for enlargement of the appendix (and induction of long-term depression). By inhibiting Limk1, microRNA-134 promotes process collapse by increasing G-actin in neuronal processes. It is noteworthy that the dendritic phenotype of miRNA-134 overexpression is similar to that in Limk1 mice. Given the relationship between the volume of the dendritic process and excitatory synaptic strength, this has obvious implications for hyperexcitability pathology such as epilepsy. Overexpression of microRNA-134 in vivo using viral vectors led to a small but significant decrease in the complexity of basal dendrites in pyramidal neurons of layer V of the cerebral cortex. Since then, other miRNA-134 targets have been identified, including the Pum2 RNA-binding protein, CREB, and DCX. Thus, miR-134 is a potentially important regulator of brain development and synaptic plasticity V layer of the cerebral cortex. Since then, other miRNA-134 targets have been identified, including the Pum2 RNA-binding protein, CREB, and DCX. Thus, miR-134 is a potentially important regulator of brain development and synaptic plasticity [15].

The initial studies have shown that neuronal depolarization causes a significant increase in neuronal miR-134 levels, but it is not known whether increased neuronal activity *in vivo* affects miR-134 expression. Expression profiling studies have identified miR-134 among activated miRNAs in mice and rat models of status

epilepticus. Detailed studies showed that miR-134 induction occurred in areas of the hippocampus that were damaged by seizures, as well as in less damaged populations of neurons. The increase in miR-134 was accompanied by a decrease in the protein level in both Limk1 and CREB, which suggests that they may be targets *in vivo*. Seizures also increased miR-134 levels, implying functional absorption. MiR-134 levels were also increased in the hippocampus of epileptic mice and in the surgically obtained temporal neocortex from patients with drug-resistant epilepsy. Thus, miR-134 activation appears to be a general response to pathological brain activity. However, inhibition of miR-134 can affect other targets when it is used *in vivo*. Although the functional significance of dendrite alteration is unknown, temporary contractions of dendritic spines are belived to unleash NMDA-dependent signaling and create protection against excitotoxic damage. Consistent with this hypothesis, the mice in which miR-134 was silent were highly seizure-resistant in the kainic acid model of status epilepticus, experiencing less than 50% of normal seizures and significantly reducing hippocampal damage. Antagomirs of miR-134 also prevented the toxicity of kainic acid *in vitro*. These results showed for the first time that targeting one microRNA (provided that antagomir only affects this microRNA) can reduce pathological brain activity *in vivo* [15].

Conclusion. These studies have significantly expanded the number of microRNAs with a potential role in epileptogenesis and improved our understanding of their practical use. The crucial test for miRs clinical translation will be an evidence of whether treatment can affect or reverse epilepsy in epileptogenic tissue. The results of the presented studies are an abundant source of new targets for microRNAs, but significant problems are about to solve before their role in the pathogenesis, diagnosis, and treatment of epilepsy can be used in clinical practice. There is currently insufficient evidence that miRNAs have multiple targets in patients with epilepsy. Moreover, antiepileptic drugs may affect microRNA levels in the brain, which requires more research.

Although the data are promising, detailed microRNA validation will be essential for the clinical use of these biomarkers.

All listed authors have no competing interests to declare.

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