Original Article

MicroRNAs as Immune Regulators of Inflammation in Children with Epilepsy

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Epilepsy is a chronic clinical syndrome of brain function which is caused by abnormal discharge of neurons. MicroRNAs (miRNAs) are small non-coding RNAs which act post-transcriptionally to regulate negatively protein levels. They affect neuroinflammatory signaling, glial and neuronal structure and function, neurogenesis, cell death, and other processes linked to epileptogenesis. The aim of this study was to explore the possible role of miR-125a and miR-181a as regulators of inflammation in epilepsy through investigating their involvement in the pathogenesis of epilepsy, and their correlation with the levels of inflammatory cytokines. Thirthy pediatric patients with epilepsy and 20 healthy controls matched for age and sex were involved in the study. MiR-181a and miR-125a expression were evaluated in plasma of all subjects using qRT-PCR. In addition, plasma levels of inflammatory cytokines (IFN-γ and TNF-α) were determined using ELISA. Our findings indicated significantly lower expression levels of miR-125a (P=0.001) and miR-181a (P=0.001) in epileptic patients in comparison with controls. In addition, the production of IFN-γ and TNF-α was non-significantly higher in patients with epilepsy in comparison with the control group. Furthermore, there were no correlations between miR-125a and miR-181a with the inflammatory cytokines (IFN-γ and TNF-α) in epileptic patients. MiR-125a and miR-181a could be involved in the pathogenesis of epilepsy and could serve as diagnostic biomarkers for pediatric patients with epilepsy.

Key words: Epilepsy, MiR-181a, MiR-125a, inflammation, TNF-α, IFN-γ

Epilepsy is a chronic neurological disorder characterized by frequent aberrant electrical activity in brain, which is estimated to affect about 65 million individuals' worldwide (1). This complex disorder is associated with comprehensive changes in brain function at the molecular, cellular, and circular levels. It can occur due to genetic defects or it can be acquired through epileptogenic insults, such as "traumatic brain injury, brain infections, stroke, or status epilepticus" (2). In

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recent years, some clinical and experimental studies have found that encephalitis is an essential manifestation of pathological brain tissue of high risk in pharmaco-resistant epilepsy from various etiologies (3).

MicroRNAs (miRNAs) are small non-coding RNAs that control gene expression post-transcriptionally, and either encourage the degradation of their target mRNAs or promote translational suppression (4). They mainly regulate the innate immune response through modifying astrocyte-mediated inflammation, and are involved in regulating the function of T lymphocytes in the immune response. Unsurprisingly, dysregulation of miRNAs was observed in various diseases expanded from cancer and immune disorders to brain diseases (5). MiR181 and miR-125 families are highly preserved miRNAs in humans; miR181 family has four members miR181a, miR181b, miR181c, and miR181d (6). Abnormal expression of miR-181 family is associated with many nervous system disorders. Also, it plays a major role in immune cells development and function, including differentiation and activities of B and T lymphocytes (7). Previous studies demonstrated that miR-181a can prevent negative regulatory factors in the pathway of T-cell receptor signals (8), and through targeting IFN-γ, it influences on the naïve $CD4^+$ T cells differentiation, prevents cell proliferation, and promotes programmed cell death, and thus, affects the function of T cells (9). On the other hand, miRNA-125 family consists of miR-125a and miR-125b (10). MiR-125a has shown to inhibit innate macrophage responses through suppressing macrophage differentiation (11). A large number of studies showed high levels of specific inflammatory mediators, and noticed upregulation of their correlated receptors in the chronic epileptic brain, which indicates that some of the proinflammatory pathways are mostly activated in foci of seizure (12).

Nudelman *et al.* first indicated relations bet--ween seizures and altered miRNAs expression (13). It has been demonstrated that modulation and inflammation of the neuron morphology may be two of the greatest significant controlling roles of miRNAs in epilepsy formation (14). In addition, miRNAs are known as chief regulators for the production of protein during and after seizures; so, miRNAs might regulate neuronal excitability and also remodeling responses (15). Therefore, miRNAs may be related to epilepsy pathogenesis and therapies (16). In nearly 30% of all epileptic individuals, seizures cannot be controlled with the currently available medications (17). Thus, studies in epilepsy that revealed altered expression and function of miRNAs, which are supposed to regulate many downstream targets and cellular processes simultaneously, have aroused great interest in miRNAs defects as pathological mechanisms and potential therapeutic targets in epilepsy (5). Consequently, the aim of this study was to determine the association of circulating miR-125a and miR-181a expression with the pathogenesis of epilepsy and with the production of IFN-γ and TNF-α in pediatric patients with epilepsy.

Materials and methods

Study subjects

This study follows the guidelines of the ethics committee of National Research Centre, Giza, Egypt, and written informed consents were collected from the parent/guardian of all children involved in our study before their enrollment. The study involved 50 subjects; their age ranged from 5 to 15 years. They included 2 groups: group 1 involved 30 patients with epilepsy; group 2 involved 20 healthy subjects with the same age range, as a control group.

Patients were recruited from Pediatrics Neurology clinic, Al-Zahraa University Hospital, Cairo, Egypt during January 2019 to June 2019. Inclusion criteria: presence of idiopathic epilepsy

Table 1. Demographic and clinical characteristics of subjects.

Characteristic		Epileptic patients	Normal healthy controls
No of cases		30	20
Gender, no. male/female		20/10	11/9
Age range (years)		5-15	5-15
Disease duration (years), mean±S.E.		6.8±0.95	-
IQ (% of patients)	Low average	5	
	Average	45	
	High average	25	
	Excellent	15	
	Very excellent	10	
Medications (tegretol, depakine, tiratam)		23/30	0/20

IQ: Intelligence quotient

by clinical examination and electroencephalogram (EEG). Patients with other chronic diseases or psychiatric disorders, history of autoimmune diseases, history of infection 2 weeks before sample collection were excluded from the study. Patients in this study were subjected to full medical history with special emphasis on character of seizures (type, age of onset, and duration of the seizures), and full neurological examination. Intelligence quotient (IQ) of children was assessed by Stanford Binet test. Clinical characteristics and treatments of epileptic patients are summarized in Table 1.

RNA extraction and quantitative real-time PCR

MiRNAs were isolated and extracted from plasma of all subjects of the study using miRNeasy Mini kit (Qiagen, Germany), and by following the manufacturer's instructions. For miRNA-specific reverse transcription, using TaqMan® MicroRNA Reverse Transcription Kit and specific primers (Applied Biosystems, USA) and by following the manufacturer's instructions, miRNAs were reverse-transcribed to cDNA. Reverse transcription was done under the following conditions: 30 min at 16 °C, 30 min at 42 °C, followed by 5 min at 85 °C, and the cDNA was stored at −80 °C until use.

A real-time quantitative PCR (qRT-PCR) was done using TaqMan® MicroRNA Assay kit (miR-125a, assay ID: 002198; miR-181a, assay ID:

000480; Applied Biosystems, USA) and TaqMan® Universal Master Mix (Applied Biosystems, USA) to measure the expression levels (in triplicate) of mature miR-181a and miR-125a using 7500 fast real-time PCR system by following the manufacturer's instructions. The endogenous control used was *RNU48* to normalize the expression levels of target miRNAs. We calculated the relative quantification (Rq) of miRNA expression using the $2^{-\Delta\Delta CT}$ threshold cycle method. ΔCt was obtained by subtracting the Ct values for *RUN48* from the Ct values for the gene of interest. qRT-PCR was done under the following conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 50 cycles at 95 °C for 15 s and at 60 °C for 1 min (18).

Enzyme-linked immunosorbent assay (ELISA)

Plasma IFN-γ and TNF-α levels of all study subjects were determined in duplicate using Human IFN-γ and TNF-α ELISA kit (Elabscience, Elabscience Biotechnology Co., Ltd) by following the manufacturer's protocol.

Statistical analysis

By using SPSS version 19.0 software (SPSS Inc., Chicago, Illinois, USA), data were statistically analyzed. Non-parametric Mann-Whitney U Test was used for comparing the expression levels among groups, and Spearman′s rank correlation to test the association of clinical data and

inflammatory cytokines of patients with miRNA expression levels. Data were presented as median. A p value <0.05 was considered statistically significant. Receiver operating characteristic (ROC) curve was made for each miRNA to assess the efficacy of miRNAs as biomarkers for epileptic patients against controls. Area under curve (AUC) values, sensitivity, specificity, and 95 % confidence interval to each miRNA was calculated.

Results

Dysregulation of plasma miR-125a and miR-181a expression pattern in patients with epilepsy

Our findings indicated highly significant down-regulation of miR-125a (4.2-fold) in epileptic patients in comparison with controls (Figure 1).

In addition, the results of this study demonstrated that miR-181a showed significantly lower (3.9 fold) expression levels in epileptic patients in comparison with healthy controls (Figure 1).

Association of plasma miR-125a and miR-181a expression with demographic and clinical features

Association analysis showed that miR-125a expression levels have a significant positive association with the age and weight of epileptic patients but have no association with other
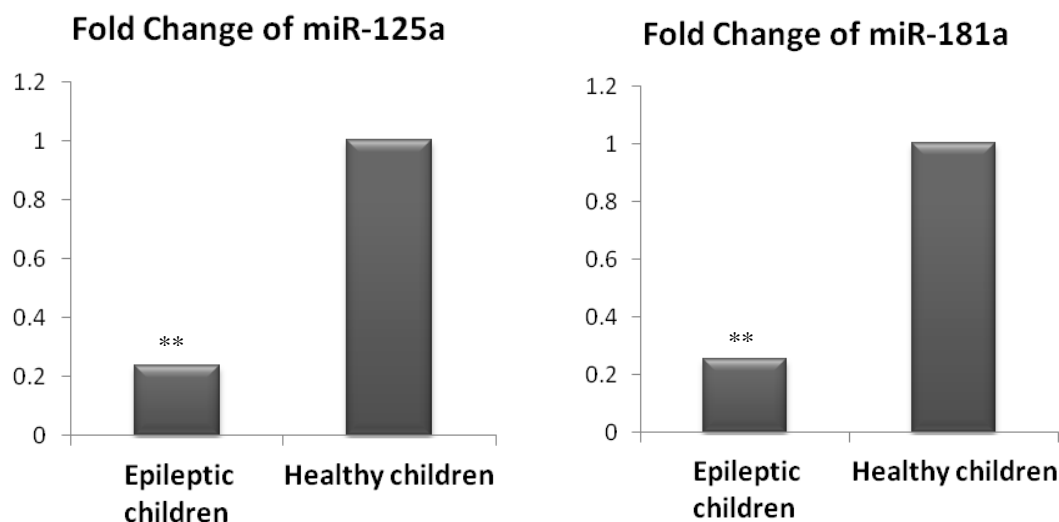

Fig. 1. Fold change of miR-125a and miR-181a in epileptic children relative to healthy controls. Bars show the median of fold change. (**: significant at P < 0.01 versus controls, by non-parametric Mann-Whitney U test).

Table 2. Association of plasma miR-125a and miR-181a expression levels with clinical data of epileptic patients.

	MiR-125a		MiR-181a	
	R (Spearman correlation)	P value	R (Spearman correlation)	P value
Age	0.667	0.050*	0.233	0.546
Gender	0.000	1	-0.087	0.825
Weight	0.723	0.028*	0.100	0.797
Seizure duration	0.050	0.899	0.211	0.586
IQ	-0.343	0.367	-0.052	0.894

*: Correlation is significant at 0.05 level (2-tailed).

demographic or clinical data (gender, seizure duration, and IQ). Furthermore, no association was found between miR-181a expression levels and any demographic or clinical data of studied patients (Table 2).

Correlation of miR-125a and miR-181a with inflammatory cytokines in patients with epilepsy

The findings of the study clarified that the production of IFN-γ and TNF-α was non-significantly higher in patients with epilepsy in comparison with the control group (Figure 2).

In addition, our data indicated that there were no correlations between miR-125a and miR-181a with the inflammatory cytokines (IFN-γ and TNF
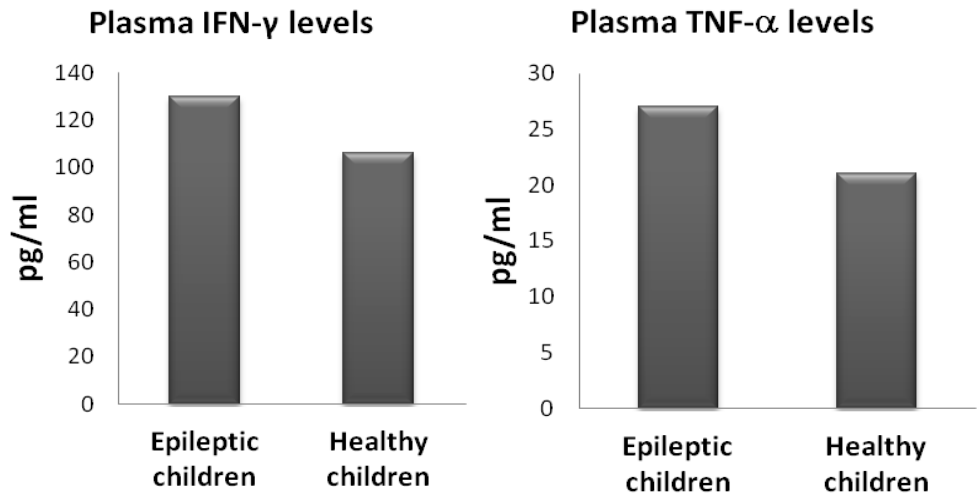

Fig. 2. Plasma levels of IFN-γ and TNF-α in epileptic and healthy children. Bars show the results as the median. (Non-significant versus controls, P = 0.435 for IFN-γ, and *p* = 0.172 for TNF-α, by non-parametric Mann-Whitney U test).
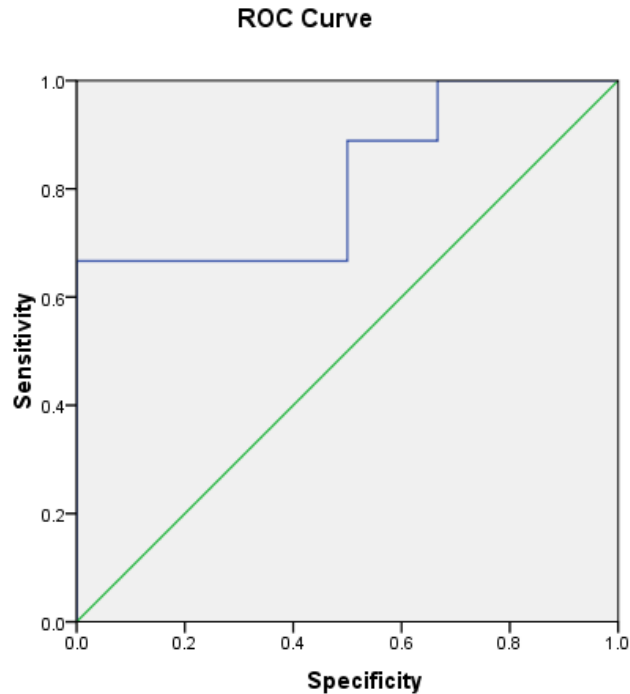

Fig. 3. Roc curve of miR-125a for epileptic patients versus normal controls. AUC = 0.815 (95 % CI 0.593-1.036); sensitivity = 70%; specificity = 83%. (Statistically significant versus normal controls, P = 0.045, under the non-parametric assumption).

Table 3. Correlations of plasma miR-125a and miR-181a expression levels with plasma inflammatory cytokines of epileptic patients.

	MiR-125a expression		MiR-181a expression	
	R	P value	R	P value
IFN-γ	-0.317	0.406	0.050	0.898
TNF-α	0.250	0.516	0.183	0.637

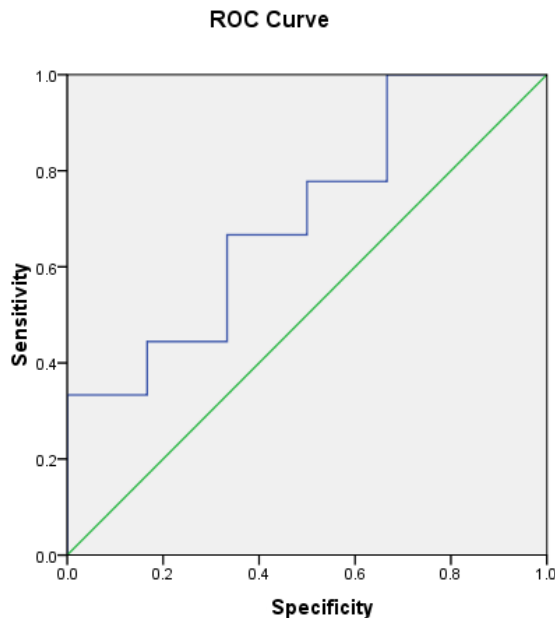P value was calculated using Spearman correlation analysis.

Fig. 4. Roc curve of miR-181a for epileptic patients versus normal controls. AUC = 0.704 (95 % CI 0.429–0.978); sensitivity = 66.7%; specificity = 67% (Non-significant versus normal controls, P = 0.195, under the non-parametric assumption).

-α) in epileptic patients (Table 3).

ROC curve of miR-125a and miR-181a

ROC curve analysis showed that miR-125a has an AUC value of 0.815 (95 % CI 0.593-1.036), a sensitivity of 70%, and a specificity of 83% (P = 0.045) (Figure 3). In addition, the ROC curve of miR-181a has an AUC value of 0.704 (95 % CI 0.429–0.978), a sensitivity of 66.7%, and a specificity of 67% (P = 0.195) (Figure 4).

Discussion

Epilepsy is considered as a chronic clinical syndrome of brain function, which is caused by abnormal discharge of neurons. Regarding the clinical manifestations of epilepsy, about 30-40% of patients are often associated with attention dispersion, memory impairment,and other cognitive dysfunctions (19). Unfortunately, one third of the patients are usually resistant to currently existing antiepileptic drugs (AEDs) which act mainly on the brain to reduce the severity and frequency of seizures (17). The potential role of miRNAs in the therapies and pathogenesis of epilepsy has been provided by many recent studies. Most of those studies usually used animal models of epilepsy (20). By using these models, more recent work has determined that the loss of miRNA biogenesis components by the mature brain will result in progressive tissue dysmorphogenesis, neurodege-neration, and seizures (21).

The majority of expressed miRNAs were upregulated in status epilepticus (SE) mice, while in tolerant mice only 18% of the expressed miRNAs were upregulated and 82% were down regulated (22).

The results of our study showed highly signi--ficant lower expression levels of miR-125a and miR-181a in epileptic patients in comparison with

controls. In addition, the production of IFN-γ and TNF-α was non-significantly higher in patients with epilepsy in comparison with the control group. Furthermore, there were no correlations between miR-125a and miR-181a with the inflammatory cytokines (IFN-γ and TNF-α) in epileptic patients. These findings agree with what was previously found by Dan *et al.* (2015) who verified that miR-181a was up-regulated and acted as negative regulators in lipopolysaccharide mediated inflammation via targeting *TNF-α* mRNA (23). Relatively, Jing *et al.* (2017) studied the effects of high mobility group box-1 protein (HMGB1) on the maturation and activation of splenic dendritic cells from mice, and proposed that miR-181a-5p may bind the 3′UTR of *TNF-α* mRNA to negatively regulate its expression (24). Furthermore, Sang *et al.* (2015) reported that, miR-181a affects the function of T lymphocytes by down-regulating *IFN-γ* in a dose-dependent manner (9). On the other side, Saki *et al.* (2015) stated that, miR-125 may reduce the effector function and activation of T-cells, which is shown by decreased levels of intracellular IFN-γ and IL-13 (25).

In a previous study, Liu *et al.*, (2015) found that miR-181a expression was significantly up-regulated in memory impairment group of the pentylenetetrazol (PTZ)-induced epileptic rats, but it remains unknown whether miR-181a can influence the cognitive function of PTZ-induced epileptic rats. This is in contrast with our results which demonstrated that the level of miR-181a expression was significantly lower in epileptic patients. In patients with epilepsy, the miR-181a expression level was 3.9-fold lower than the control group (26). In another study, Ashhab *et al.* (2013) observed a significant upregulation in the chronic stage, downregulation in the acute stage, and no change in the latent stage of miR-181a in comparison with the control group in a rat model (27). This phenomenon was similarly found in children. Inflammation related to miR-181a can

cause this condition (28). It is essential to investigate the role of miR-181a in different stages of epilepsy in the future.

It was observed that miR-125a, was significantly deregulated in rat peripheral blood and in hippocampal tissues at 24 h following SE, which suggests a role for this miRNA in epilepsy diagnosis. At 24 h after epileptic seizures, Liu *et al.* (2010*)* identified 147 miRNAs in blood and 91 miRNAs in brain which showed abnormal relative quantification > 1.5 in three different statuses of brain damage including intercerebral ischemic injury, hemorrhagic injury, and kainate seizures in rats. Among them, seven down-regulated miRNAs were found in the blood (one of them is miR-125a-5p.33) and other miRNAs were demonstrated to have defective expression in blood and brain tissue of all three conditions (29). This is in agreement with our findings that indicated a significant lower expression level of miR-125a in epilepsy patients. Moreover, association analysis showed that miR-125a expression levels have a significant positive association with the age and weight of epilepsy patients.

In another study, *Liu et al.* (2019) detected miR-125a-5p down-regulation in the hippocampus of PTZ-induced epilepsy rats. They observed that overexpression of miR-125a-5p reduced seizures and diminished inflammation in the hippocampus of PTZ-induced rats by suppressing its target gene, calmodulin-dependent protein kinase IV (*CAMK4*) (30).

The comparative analysis of miRNAs in the hippocampus from epilepsy model showed that miR-125a-5p increased. This study also presented the results of miRNAs profiling in peripheral blood samples of the same epileptic model. MiR-125a was confirmed to be increased. The deregulated miRNAs were predictable to control the mitogen-activated protein kinase (MAPK)-signaling pathway, tight junction pathway, TGF-β-signaling pathway, long-term potentiation pathway, axon

guidance and glycan structure-biosynthesis (31), which is in contrast with our results that demonstrated low expression of miR-125a. These differences in results may be due to different disease stages or different sample size.

MiRNAs have an important role in inflammatory pathways that have been discovered to be involved in epilepsy (32). Pharmacological modulation of proinflammatory signaling that inhibited seizures and changes in the risk of developing seizures in transgenic mice, proved the concept that brain inflammation might have a role in the etiopathogenesis of seizures (33).

TNF-α and IFN-γ have been involved in seizure generation and were shown to be up-regulated during a seizure (34, 35). This is in agreement with our findings which clarified that the production of IFN-γ and TNF-α was non-significantly higher in patients with epilepsy in comparison with the control group. Wang *et al.* (2015) noticed that serum levels of IFN-γ were correlated with seizure severity (36). According to our data, there were no correlations between miR-125a and miR-181a with the inflammatory cytokines IFN-γ in epileptic patients. On the other hand, other miRNAs may have correlations with increased IFN-γ levels in epilepsy as miR-146a (32) and miR-155 (37).

TNF-α is an inflammatory marker, and its level is also increased after seizures (38, 39). As our results indicated that there were no correlations between miR-125a and miR-181a with TNF-α in epileptic patients, other miRNAs such as miR 155 might have a regulatory role on TNF-α in nervous tissue (40).

It has been proven that miR-181a-5p suppresses IFN-γ in human CD4$^+$ T cells. I Infection of the activated human CD4$^+$ T lymphocytes with a lentivirus encoding pre-miR-181a, significantly decreased the protein level of that cytokine in both CD4$^+$ T cells and culture media (41, 9).

In the present study, miR-125a showed a sensitivity of 70% and a specificity of 83% ($P < 0.05$) while miR-181a showed a sensitivity of 66.7% and a specificity of 67% ($P < 0.05$).

Any discrepancy between our results and others may be due to assessing their levels at different stages of epilepsy, or to different sample sizes. Thus, miR-125a and miR-181a may be considered as promisingly sensitive, easily detectable, and specific biomarkers that may improve the diagnosis and treatment outcome of epilepsy.

In conclusion, miRNAs and brain inflammation might endorse epileptogenesis and epileptic seizures, and could be considered as diagnostic biomarkers for epilepsy. MiR-125a and miR-181a might serve as regulators of inflammation in epilepsy pediatric patients. Thus, further studies are needed to clarify whether prohibition of inflammatory signals activation and follow-up of miRNAs are useful as potential targets for pharmacological intervention, especially for patients with epilepsy who are resistant to antiepileptic drugs.

Acknowledgment

Conflict of interest

There is no conflict of interest for any of the authors.

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