Expression of MicroRNA 146a, 155, 181 and 223 in febrile seizure

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ABSTRACT

Background. We studied microRNAs (miRNAs) -146a, -155, -181 and -223 expressions and proinflammatory cytokine levels in children with Febrile seizure (FS) and compared to febrile controls.

Methods. This prospective multicenter study examined representative populations in eight different cities in Turkey between June 30, 2018 and July 1, 2019. Blood samples were taken from all children at presentation. The real time (RT) polymerase chain reaction (PCR) were used to measure the expressions of microRNAs and tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), and interleukin 6 (IL-6) levels were studied by enzyme-linked immuno-sorbent assay.

Results. The study was conducted with 60 children; 30 children with FS and 30 children in the febrile control group. The seizure was classified as simple FS in 73.3 % and half of the children were experiencing their first FS episode.

Although the expression levels of miRNAs-146a, -181a and -155 were higher in febrile seizure patients, only miRNAs 146a level was significantly higher in FS patients. Serum TNF-α, IL-1β, IL-6 levels were higher in the FS group than the controls. The results of statistical analysis showed that there were correlations within miRNA expressions in children with FS. No differences were found considering miRNA expression between FS type, number of FS experienced.

Conclusions. miRNAs-146a, -181a, -155 and -223 may be involved in FS pathogenesis. Altered miRNA expression levels might be an adaptive response to inflammation. New therapeutic approaches might be developed based on miRNA expressions in children with FS.

Key words: febrile seizure, microRNA, proinflammatory cytokine.

The pathophysiology of febrile seizure (FS) is not fully understood, and research results have revealed a complex interaction of inflammation, genetic tendency and cytokines. Tumor necrosis factor alpha (TNF- α), interleukin 1 beta (IL-1β) and interleukin 6 (IL-6) are the main cytokines involved in the pathophysiology of FS.[1,2]

MicroRNAs (miRNAs) are small non-coding RNAs, and their main target is messenger RNAs (mRNAs). More than two thousand miRNAs have been identified thus far, and more than half of these are expressed in the human brain.[3] Alteration in the expression levels of specific miRNAs has been suggested as a possible cause in the pathophysiology of different diseases, such as cancer, Parkinson disease and epilepsy.[3,4]

miRNAs have emerged as potent regulators of inflammation.[4,5] For example, miRNA-146a is the first inflammation-associated miRNA.

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It has been shown to regulate the expression of toll-like receptors and cytokine pathways. Both miRNA-146a and IL-1β were upregulated in astrocytes in epilepsy models. The purpose of miRNA-146a expression in astrocytes may be to modulate the inflammatory response stimulated by pro-inflammatory cytokine IL-1β.[5,6] miRNA-146a is significantly upregulated in tissues obtained from patients with mesial temporal lobe epilepsy (MTLE), more than half of whom had a history of FS in childhood.[5-7] In addition, increased expression of miRNA-155 has been observed in the hippocampal tissue of children with MTLE, and this increase was correlated with an increase in TNF-α in nervous tissue.[8]

Respiratory viral infections are the most common cause of fever in children with FS.[9] The miRNA system modulates viral replication and pathogenesis.[10] Studies have shown that many miRNAs have high expression, whereas thirty-five miRNAs have low expression following adenovirus infection, which commonly affects children with FS. Downregulation of miRNA-146a and miRNA-155 in patients with influenza A virus has also been reported.[11] The role of miRNAs in the pathophysiology of FS has rarely been investigated.[12,13] Thus, in the present study, we aimed to investigate miRNAs 146a, 155, 181 and 223 expressions in children with FS and compare these to levels in febrile controls.

Material and Methods

This prospective multicenter study was conducted in eight different cities between June 30, 2018 and July 1, 2019. The study was approved by the Local Ethical Committee of Eskisehir Osmangazi University (IRB Number 2020/55, Date: 02.03.2020) and supported by a university research grant. Written informed consents were obtained from the parents of all the participating children.

At each site, all children with seizures were evaluated for a one-year period, and children with FS were enrolled in the study. FS in each child was classified.[14] The childrens' previous medical history and demographical features were recorded. Detailed physical examinations were performed, including neurological examinations. The control group included age-matched children who were diagnosed with febrile disease but who did not have seizures and had no known history of previous FS. The febrile diseases include respiratory tract infections, gastrointestinal infections and other viral infections.

Blood samples (3 cc) were obtained within one hour after the seizure in a serum separation tube (SST). The serum samples were immediately separated by centrifugation and stored at -80 °C in a freezer.

Cytokine analysis: All those stored were analyzed using commercially available assay kits according to the manufacturer's instructions. Serum IL-6 values were determined using ELISA technique (Bioassay Technology Laboratory, Shanghai, China). The minimum detectable IL-6 concentration was 0.092 pg/ml. Serum TNF-α values were also measured using an ELISA technique (Bioassay Technology Laboratory, Shanghai, China). The minimum detectable TNF-α concentration was 1.52 pg/ml. Serum IL-1β values were determined using ELISA technique (Bioassay Technology Laboratory, Shanghai, China). The minimum detectable IL-1β concentration was 0.02 pg/mL. The intraassay coefficient of variations for all three cytokines were below 8%, and inter-assay coefficient of variations were below 10%.

Total RNA Extraction: Total RNA extraction was done by using a TRIzol™ Reagent kit according to the manufacturer's protocol for serum/plasma samples. In short, 200 μl human serum was added into 1000 μl TRIzol™ reagent and after vortexing incubated at room temperature for 2 min. Following the phase separation by adding chloroform with centrifugation, upper aqueous phase (containing RNA) was mixed with 500 μl of 2-Propanol. The supernatant was discarded after centrifugation. The pellet was resuspended with 75% ethanol, centrifuged

and air dry. Thirty µl of nuclease-free elution buffer was carefully added to resuspend the pellet. Then the amount of nucleic acid in the total RNA samples by fluorescence spectrophotometry (Colibri Microvolume Spectrometer, Titertek-Berthold, Germany) was measured. RNA concentrations ranged from 100 to 400 ng/µl, and the total RNA purity was verified using of A260/A280 ratios (range 1.81–1.97). The integrity of the serum RNA could not be assessed by gel electrophoresis because the levels were very low amounts in the serum and plasma. DNase I enzyme (Arcticzymes, Norway) was used to remove DNA from serum RNA by fixing 100 ng/µl.

Poly(A) Polymerisation and Reverse Transcription: In this step, poly(A) tails were added to the 3' end of miRNAs with Poly(A) Polymerase, Yeast (ABM) kit. The reaction mixture consisted of 5 µl of 5X buffer, 11.25 µl of nuclease-free water, 1 µl Poly(A) Polymerase (1 U/µl), 1.25 µl of ATP (10mM), 2.5 µl of MnCl2 (25 mM), 5 µl (500 ng) of RNA for a 25 µl reaction and incubate at 37 °C for 20 min and 65 °C for 20 min in in Veriti™ 384-Well Thermal Cycler (Life Technologies, Carlsbad, CA, USA). These modified miRNAs were then reverse transcribed using a stem-loop poly(A) tailed RT-PCR. The reaction mixture consisted of 2 µl of M-MuLV reaction buffer (2x), 10.5 µl of nuclease-free water, 1 µl of M-MuLV RT enzyme (200U/µl), 1.25 µl of dNTP (10mM), 1 µl of miRNA specific RT primer (10mM), 0.25 µl of RNase Inhibitor (40U/µl) and 5 µl of poly(A) tailed RNA for a 20 µl reaction. Reverse transcription was performed for OneScript® Plus cDNA Synthesis (ABM) in in Veriti™ 384-Well Thermal Cycler (Life Technologies, Carlsbad, California, United States) at 42 °C for 60 min, heat inactivate the reverse transcriptase at 85 °C for 10 min.Then the product was immediately cooled to 4 °C and stored at -20 °C.

Detection of miRNAs by qPCR: The RT-qPCR reactions were performed and monitored using a real-time ABI Prism 7500 FAST qPCR System from Applied BioSystems (Life Technologies, Carlsbad, California, United States). Real time

PCR was performed in duplicate for each miRNA and included non-template control. Human small non-coding RNAs miR181a, miR155, mir146a and miR223 designs (Diagen, Ankara, Turkey) were used for study and endogenous control small ncRNA SNORD47 was chosen as the internal control for normalization. The PCR reaction consisted of, 10 µl of Diagen 2X PCR Master Mix solution (2x), 3.5 µl of nuclease-free water, 3.5 µl of miRNA specific Primer Mix and 3 µl of poly(A) tailed miRNA for 20 µl reaction. Amplification was performed and monitored using a real-time instrument ABI Prism 7500 FAST qPCR System from Applied BioSystems (Life Technologies, Carlsbad, California, United States) at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 sec, 55 °C for 30 sec, 72 °C for 5 sec. Real time PCR raw fluorescence data were analyzed using 7500 Fast Software v2.0.6 with automatic baseline and threshold setting for quantification cycle (Ct) determination. PCR amplification efficiencies test were calculated to verify the primer specificity and sensitivity by use of the formula E = (10-1/slope - 1) 9 100 and to control the PCR yield was demonstrated by the synthetic miRNA sequence of each miRNA. The qPCR Ct values of the samples included in the target and control groups were calculated as fold changes in the gene expression analysis. Fold Change value is qPCR gene expression ratio of each sample. In this study, fold change values were calculated of the miRNAs miR181a, miR155, mir146a and miR223 with the comparative $2(-\Delta\Delta Ct)$ relative expression method to reference gene SNORD47. The gene expression fold change values for each gene in each sample were transformed to log2.The fact that the fold change data give extremely low or high expression gene expression coefficients affects the data distribution in statistical analyses. The purpose of logarithmic transformation is to make gene expression level more specific and to show normal distributions.[15,16] In this way, the expression load is equalized in cases of over-expression and low expression of genes, and the effect of extreme values is also eliminated.

Statistical analysis: The expression levels of miRNAs and serum cytokine levels were

presented as median, minimum and maximum values. The non-parametric tests were performed because of small sample size and the data that were not normally distributed were analyzed with Shapiro-Wilk's test. The Mann-Whitney U test was used to compare miRNA and cytokine levels between the two groups. To further potential any interrelationship, Spearman rank correlation test was performed. A p value <0.05 was deemed statistically significant. The analyses were performed with SPSS for Windows 15.0 (Chicago, IL, United States)

Results

The study was conducted with 60 children: 30 children with FS and 30 children in the febrile control group. Half of the children (15 out of 30; 50%) were experiencing their first FS episode. In 73.3% of the children (22/30), the seizures were classified as simple FS. A total of 56.6% (17/30)

of the children had a family history of FS. The children's demographic and clinical features are summarized in Table I. Serum IL-6, TNF-α, and IL-1β were higher in febrile seizure group than in the controls (p<0.01, p<0.01 and p<0.05, respectively) (Table I).

miRNA expression analysis revealed an alteration in children with FS compared to the controls. Serum median miRNA-181, miRNA-155, miRNA-146a, and miRNA-223 levels were higher in febrile seizure group than in the control group, however, the expression levels of miRNAs 146a were significantly increased in FS patients (p<0.05) (Table II). No differences were found in miRNA expression between FS type and the number of seizures experienced (p>0.05). There were correlations between levels of TNF-α, IL-1β and IL-6 (p<0.001). However, no relation was found between miRNA expression and TNF-α, IL-1β and IL-6 (Table III).

Table I. Demographic, clinical findings and serum TNF-α, IL-1β, and IL-6 levels of the study group.

	Febrile seizures n (%)	Control group n (%)	p-value
Age (months) (mean ± SD; min-max)	25.17±13.23 (10–60)	40.16±18.23 (6–60)	p>0.05
Gender (boys/girls)	15/15	20/10	p>0.05
Source of fever			
Upper respiratory tract infections	23 (79.3)	25 (83.3)	p>0.05
Other infections	7 (10.7)	5 (16.7)	p>0.05
TNF-α (pg/ml)	515.58	212.58	p<0.01
Median (min-max)	(31.96–1127.71)	(39.27- 423.42)	
IL-1β (pg/ml)	3923.54	1747.13	p<0.05
Median (min-max)	(280.83–8220.80)	(221.48–7817.39)	
IL-6 (pg/ml)	326.79	109.59	p<0.01
Median (min-max)	(13.64–728.53)	(21.42–610.72)	

Table II. MicroRNA 146a, 155, 181 and 223 expression.

MicroRNA	Ct Value	Febrile Seizure Group Fold Change Median $2^{-\Delta\Delta Ct}$ (min–max)	Control Group Fold Change Median $2^{-\Delta\Delta Ct}$ (min–max)	p-value
MicroRNA-181	20.4 – 28.1	-0.4240 (-1.50–1.57)	-0.1490 (-1.77–2.17)	p>0.05
MicroRNA-155	26.1 – 32.2	-0.6200 (-1.92–0.88)	-0.0150 (-2.69–1.57)	p>0.05
MicroRNA-146a	15.2 - 27.4	-1.0600 (-2.39–1.60)	-0.0980(-1.66–2.68)	p<0.05
MicroRNA-223	17.7 - 23.7	-0.4010 (-1.69–2.15)	-0.0820 (-2.04–2.39)	p>0.05

Table III. The correlations between MicroRNAs; MicroRNAs and cytokines.

	MicroRNA-181	MicroRNA-155	MicroRNA-146a	MicroRNA-223	TNF-α	IL-1β	IL-6
MicroRNA-181	-	0.663	0.365	0.206	-0.92	0.037	-0.77
		<0.001	0.047	0.274	0.629	0.845	0.685
MicroRNA-155			0.469	0.377	0.218	0.288	0.199
			0.009	0.040	0.247	0.123	0.291
MicroRNA-146a				0.299	0.66	0.130	0.36
				0.109	0.728	0.495	0.850
MicroRNA-223					0.095	0.157	-0.039
					0.617	0.406	0.837
TNF-α						0.940	0.951
						<0.001	<0.001
IL-1β							0.871
							<0.001
IL-6							-

The level of miRNA values in predicting patient and control were analyzed using ROC (Receiver Operating Characteristics) curve analysis. When a significant cut-off model was observed, it was presented as p value, area under curve (AUC) with 95% confidence interval and cut-off value which has maximum value calculated by dividing sensitivity/specificity. According to these; the cut-off value for miR155 was 0.834 (AUC:0.69, 95%CI: 0.55-0.82, p=0.013). The cut-off value for miR146 was 1.443 (AUC:0.67, 95%CI: 0.53-0.81, p=0.028).

Discussion

This prospective multicenter study found that the expression levels of miRNA 146a were significantly increased in FS patients. Although previous reports support the hypothesis that miRNAs may contribute to the pathogenesis of epilepsy, studies on the role of microRNAs in the pathophysiology of FS are limited.[5,6,12,13,17-22]

Several pro-inflammatory cytokines increase during FS, and, as a consequence, their related miRNAs are also affected.[4,6] Omran et al.[7] revealed a negative correlation between IL-1β and miRNA146a expression. This significant increase in miRNA146a expression and its association with the low level of IL-1β might suggest that the purpose of miRNA146a expression is to modulate the inflammation stimulated by IL-1β. In contrast, the results of the present study showed a positive correlation between miRNA146a and IL-1β level. The genetic polymorphisms might play a role in this correlation. A study conducted by Issac et al.[12] revealed that rs2910164 polymorphism in the pre-microRNA-146a gene might be accompanied by an upregulation of proinflammatory cytokines.

The present study found increased miRNA-223 expression in FS patients than controls, without statistical significance. Wang et al.[13] used the hot water-bath box method with the FS model and examined the role of miRNA-223 in pathogenesis of FS. They found that, compared to normal controls, the expression of miRNA-223 in hippocampal tissues of rats in the FS group was significantly decreased, suggesting low expression levels of miRNA-223. The seizure latency was markedly prolonged while the seizure duration was significantly shortened for rats injected with miRNA-223. The results demonstrate that upregulating the expression of miRNA-223 can improve seizures in FS rats.

Lumbar puncture and cerebrospinal fluid (CSF) analysis are not recommended routinely

to evaluate FS patients so was not conducted in the present study. In a recent study, Kim et al.[23] searched the miRNA profile of CSF in FS patients. They reported 95 miRNAs were significantly higher in patients than in controls and the top 5 highly expressed miRNAs were miRNA-4486, miRNA-6850-5p, miRNA-642-3p, miRNA-7107-5p and miRNA-4281. None of these miRNAs were searched in the present study.

FS is associated with a higher risk of epilepsy in general and especially in temporal lobe epilepsy (TLE).[24] Ren et al.[25] found miRNA-181a to be increased in the temporal lobe tissues of children with intractable epilepsy. In addition, miRNA-146a and miRNA-155 were also significantly upregulated in tissues obtained from patients with MTLE.[5-8] Another study found that increased miRNA-155 expression was correlated with increased TNF-α in nervous tissue.[6] However, our results revealed no relationship between miRNAs and proinflammatory cytokines (TNF-α, IL-1β, IL-6).

It has been speculated that modulation of miRNA expression might be a therapeutic model for seizures. In a pilocarpine-induced TLE mouse model, intranasal delivery of miRNA146a antagonist reduced the percentage of animals with seizure onset to 6.7% and also resulted in an increase in seizure latency.[26] This may have been the result of a decrease in inflammatory modulators and cytokines, such as nuclear factor kB (NF-kB), TNF-α, IL-1β and IL-6. An intraventricular injection of miRNA-181a-mimic produced neuronal death in rats, whereas antagomirs against miRNA-181a reduced neuronal death after status epilepticus.[12,27]

Viral respiratory infections are commonly associated with FS. Our previous study showed that the most frequently detected viruses were adenovirus, influenza A and influenza B.[16] Human miRNAs have been suggested to have their evolutionary origin as an innate immune defense mechanism against viral infection. The miRNA system modulates viral replication and pathogenesis in several ways: a) respiratory cell miRNAs can affect viral replication; b) some viruses also encode miRNAs, and viral-encoded miRNAs target cellular genes involved in cell proliferation and anti-viral response; and c) viral-encoded miRNAs may regulate viral gene expression.[11,28]

A study conducted by Qi et al.[29] revealed that the expression levels of many miRNAs increase while the levels of more than thirty miRNAs decrease after adenovirus infection, which is one of the common viruses that affect FS patients. A recent study showed a unique expression pattern of microRNAs in influenza infection and decreased expression of miRNA-155, miRNA-146b, miRNA-29, miR-150, miRNA-299-5p and miRNA-335.[30] Huang et al.[31] showed an accumulation of miRNA-146a, miRNA-7, miRNA-132, miRNA-187, miRNA-200c and miRNA-1275 in lung tissue infected with the influenza A virus. In the present study, we did not search for respiratory viruses; therefore, we cannot analyze the correlation between viruses and miRNA.

The present study has some limitations. miRNA expression was not searched in healthy children. Since most of miRNAs analyzed in the present study were inflammation-related, febrile children were preferred as controls.

In conclusion, miRNAs -146a, -181a, -155 and -223, especially miRNA-146a might be involved in FS pathogenesis. Altered miRNA expression levels might be an adaptive response to inflammation. New therapeutic approaches based on miRNA expression could provide new perspectives for FS treatment.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: KBÇ; data collection: YK, GGM, AE, PP, ÇY; analysis and interpretation of results: KBÇ, DA, EÇD; draft manuscript preparation: KBÇ, EÇD. All authors reviewed the results and approved the final version of the manuscript.

Ethical approval

The study was approved by the Local Ethical Committee of Eskisehir Osmangazi University (IRB Number 2020/55, Date: 02.03.2020) and supported by a university research grant.

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Conflict of interest

No potential conflict of interest was reported by the authors.

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