Original Article

Antiviral MicroRNA Expression Signatures are Altered in Subacute Sclerosing Panencephalitis

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Submitted: 30-Mar-2021
Revised: 21-Jun-2021
Accepted: 29-Jun-2021
Published: 20-Sep-2021

ABSTRACT

Background: Subacute sclerosing panencephalitis (SSPE) is a chronic, progressive disease caused by a persistent infection of the measles virus. Despite extensive efforts, the exact neurodegeneration mechanism in SSPE remains unknown. MicroRNAs (miRNAs) have emerged as an essential part of cellular antiviral defense mechanisms and can be modulated by antiviral cytokines Such as interferon-beta (IFN-β). Aims and Objectives: In this study, we aimed to elucidate the role of antiviral miRNAs in the pathogenesis of SSPE and analyze the interaction between host antiviral miRNAs and virus genes. **Materials and Methods:** Thirty-seven patients who were followed with SSPE and age-matched healthy children were included in the study. Peripheral blood mononuclear cell levels of miR-196b, miR-296, miR-431, and miR-448 were analyzed using quantitative polymerase chain reaction. Target predictions and pathway constructions of deregulated miRNAs were assessed. **Results:** Here, we showed that IFN-β-modulated miR-196b, miR-296, and miR-431 were significantly upregulated in patients with SSPE compared with healthy controls. Besides, sequence complementarity analysis showed that miR-296 and miR-196b predicted binding regions in measles virus genomic RNA. **Conclusion:** Our findings suggest that antiviral miRNAs are upregulated in patients with SSPE, which could be a part of the host antiviral defense mechanism.

KEYWORDS: *Antiviral microRNA, measles virus, microRNA, subacute sclerosing panencephalitis*

INTRODUCTION

Subacute sclerosing panencephalitis (SSPE) is a fatal disease of the central nervous system (CNS), which emerges secondary to a persistent measles virus infection.[1] Immunomodulatory and antiviral treatment options are possible for patients with SSPE in Stage 1; however, the disease is incurable in the later stages (Stage 2 and Stage 3). Several diagnostic methods are available for SSPE including electroencephalography (EEG) findings, anti-measles antibody titer in serum and cerebrospinal fluid (CSF), histologic examination of the brain tissue after biopsy, and polymerase chain reaction (PCR) to detect the measles virus genome in CSF.

An effective treatment regimen for SSPE has not yet been established; nevertheless, a combination of inosiplex and interferon (IFN)-alpha/beta has shown partial efficacy.[2] According to a nonrandomized study on patients with SSPE, 3 weeks of IFN-β treatment leads to a significant increase in survival and clinical response.[3]

In attempts to identify the role of cellular microRNAs (miRNAs) in defense against viral infection, Pedersen *et al.* showed that IFN-β modulated the expression of approximately 30 miRNAs.[4] Among these, hsa-miR-1, hsa-miR-30, hsa-miR-128, hsa-miR-196, hsa-miR-296, hsa-miR-351, hsa-miR-431, and hsa-miR-448 had nearly perfect complementarity in their seed sequences against the hepatitis C virus (HCV) RNA

Access this article online
Quick Response Code:
Website: www.nsnjournal.org
DOI: 10.4103/nsn.nsn_57_21

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How to cite this article: Tüfekçi KU, Allmer J, Çarman KB, Bayram E, Topçu Y, Hız S, *et al.* Antiviral microRNA expression signatures are altered in subacute sclerosing panencephalitis. Neurol Sci Neurophysiol 2021;38:166-72.

genome and were able to suppress HCV replication and infection.[4]

It is hypothesized that inflammation plays a major role in the pathogenesis of SSPE. Inflammatory cytokines, including interleukin (IL)-1 beta, IL-2, IL-6, and tumor necrosis factor-alpha, are found in brain lesions in SSPE.[5] Furthermore, several studies have linked alterations in miRNA expression levels to inflammatory disorders.[6] Of note, hsa-miR-146 and hsa-miR-155 are two important miRNAs that play crucial roles in inflammation. In our previous study, we showed that hsa-miR-146, hsa-miR-155, and hsa-miR-181a were significantly overexpressed in patients with SSPE compared with healthy controls.[7] Furthermore, miR-155 showed stage-specific expression in SSPE.[7] To our knowledge, this study is the first to investigate the roles of miRNAs in SSPE. Furthermore, in a recent study, the miR-548 family was reported to be elevated in patients with SSPE.[8] Still, the details of the miRNA-SSPE relationship, especially with respect to antiviral miRNAs, warrant further studies.

Here, we aimed to identify the potential role of antiviral miRNAs in the pathogenesis of SSPE. We analyzed the expression levels of hsa-miR-196b, hsa-miR-296, hsa-miR-431, and hsa-miR-448 and found that all miRNAs except miR-431 were overexpressed in patients with SSPE compared with controls. Moreover, our *in silico* analysis revealed that these miRNAs predicted binding sites in measles virus genomic RNA. Taken together, our findings suggest that altered antiviral miRNA expression can represent a part of the host antiviral defense mechanism against SSPE.

Subjects and Methods

Subjects

The study received institutional ethics approval from Dokuz Eylul University, School of Medicine (Protocol no: 48-SBKAEK). Structured written consent from the guardians of the children was obtained by researchers.

Thirty-seven patients who were followed with a diagnosis of SSPE in the pediatric neurology divisions of Dokuz Eylul University, School of Medicine (Izmir, Turkey), and Gaziantep Children's Hospital (Gaziantep, Turkey) were included in the study. The same patient and control cohorts were previously published.[7] SSPE was diagnosed by a pediatric neurologist according to the following diagnostic criteria: clinical features, increased measles virus antibody titer in the CSF, and a typical electroencephalography showing periodic slow waves early in the disease. Disease staging was performed as follows: Stage 1, mental and behavioral symptoms; Stage 2, stereotypical jerks; Stage 3, rigidity, extrapyramidal symptoms, and diminished responses to stimuli; and Stage 4, coma, vegetative state, autonomic failure, and akinetic mutism. Control subjects were selected from age-matched healthy children. The patient cohort, as well as the control subjects, were analyzed previously.[7]

Peripheral blood mononuclear cell isolation

Blood samples of patients with SSPE who were followed at Gaziantep Children's Hospital were transferred at 4°C to Dokuz Eylul University (Izmir). Ficoll separation was used to isolate peripheral blood mononuclear cells (PBMCs) from the patients with SSPE and controls. Briefly, 6 mL of ethylenediaminetetraacetic acid (EDTA) – Blood was diluted with phosphate-buffered saline (PBS), layered on 4 mL of Biocoll (Biochrom AG, Berlin, Germany) solution, and centrifuged at 830 ×*g* with no acceleration and deceleration. The PBMC layer was obtained and washed twice with PBS. After centrifugation, cell pellets were lysed in QIAzol reagent (Qiagen, Hilden, Germany) and stored at − 80°C until RNA isolation.

RNA isolation

miRNeasy mini RNA isolation kit (QIAGEN, Hilden, Germany) was used to isolate RNA from PBMCs. Quantification of RNA samples and quality checks were conducted on a NanoDrop 2000 spectrophotometer (Thermo Scientific). RNA samples were stored at – 80°C until further analysis.

Reverse-transcription and quantitative real-time polymerase chain reaction

An miScript II RT Kit (QIAGEN, Hilden, Germany) was used according to the manufacturer's instructions to reverse-transcribe RNA samples. An equal amount of RNA (200 ng) was used for reverse-transcription reactions. Before quantitative real-time PCR, cDNA samples were diluted in nuclease-free ultrapure water.

miScript SYBR Green PCR kit (QIAGEN, Hilden, Germany) and commercial hsa-miR-196b, hsa-miR-296, hsa-miR-431, and hsa-miR-448 primers (QIAGEN, Hilden, Germany) were used for quantitative real-time PCR. All qRT-PCRs were performed on a LightCycler 480 II (Roche, Penzberg, Germany) instrument. Melting curve analysis was performed to determine the specificity of the PCR products. The Human RNU6B gene was used as a housekeeping gene to normalize miRNA expression in the samples. Relative expression of each miRNA was calculated using the $2^{-\Delta\Delta Ct}$ method.[9]

Pathway construction

The Kyoto Encyclopedia of Genes and Genomes (KEGGs) provides manually curated pathways for human and other organisms.[10] Such pathways may show any interaction such as regulatory

or compositional. KEGG also provides compound pathways, and we downloaded the measles pathway, which contains human pathways and their interactios with the measles genes (http://www.genome.jp/kegg-bin/show_pathway?hsa05162). Unfortunately, the pathway displayed on the KEGG webpage is significantly different from the download that is provided on the same page. To overcome this issue and to add the miRNA-driven regulation to the compound human/measles pathway, we loaded the downloaded pathway into a network editor. This editor, Cytoscape, among other functionalities, allows formatting, layout, and additions to the pathway.[11-13] Thus, Cytoscape enabled us to establish the pathway similar to how it appeared on the KEGG website and to add the miRNAs analyzed in this study.

The overexpressed miRNAs were investigated in miRBase[14] and miRTarBase[15] and their source genes were determined. A total of four source genes lead to six miRNAs that had targets in the measles and or human pathways. Targets were determined using miRTarBase,[15] TarBase,[16,17] and computational target prediction (see below). Targets were either within the measles or associated human pathways or were targeting completely different processes (other targets). The miRNAs, their source genes, and their targets were manually added to the combined human and measles pathways using the Cytoscape editor.

Target prediction

The mature sequences for the miRNAs found in this study were reverse complemented and then compared with the measles genome using BLAST[18] for small sequences. The first binding of the sequences was forced to be among the first three nucleotides of the reverse complement because the seed sequences are the most important in target binding. The binding of 6–8 nucleotides in the seed sequence was found to be sufficient for target recognition and therefore at least this length of perfect matching was required. The targets were visualized on the National Center for Biotechnology Information (NCBI) measles genome browser using the NCBI facilities (Available at: http://tinyurl.com/po4ko6v).

Statistical analysis

SPSS v.20.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Data are presented as mean ± standard error. Student's *t*-test was used to compare parametric variables. The Kruskal–Wallis and Mann–Whitney U-tests were used to analyze nonparametric variables ($n < 30$). Pearson's correlation test was used to determine the correlation between miRNA expression levels and clinical variables. $P < 0.05$ was considered statistically significant.

Results

Clinical features of subacute sclerosing panencephalitis patients

Thirty-seven patients with SSPE (25 boys and 12 girls; mean age = 14.6 years) and 41 healthy controls (22 girls and 19 boys; mean age = 13.5 years) from the same patient and control cohort[7] were included in the study. Fifty-four percent of patients with SSPE and 36.6% of healthy control samples were obtained from Gaziantep Children's Hospital (Gaziantep, Turkey) ($P = 0.35$). The demographic and clinical features of the patients were previously published[7] [Table 1].

Antiviral microRNA expression levels are altered in subacute sclerosing panencephalitis

To determine the alterations in antiviral miRNAs expression levels, we used quantitative PCR to analyze hsa-miR-448, hsa-miR-196b, hsa-miR-296, and hsa-miR-431 expression levels. The mean hsa-miR-196b expression in patients with SSPE was 4.9-fold higher compared with the healthy controls ($P < 0.001$). The mean hsa-miR-431 expression in patients with SSPE was 5.6-fold higher compared with the healthy controls ($P < 0.001$). The mean miR-296 expression in patients with SSPE was 6.6-fold higher compared with the healthy controls ($P = 0.001$). The mean miR-448 expression in patients with SSPE was 1.5-fold higher compared with the healthy controls ($P = 0.32$). Overall, the expression levels of all miRNAs, except hsa-miR-448, were significantly higher in patients with SSPE compared with the healthy controls [Figure 1].

Given that the majority of the patients (26 of 37 patients) received IFN-β treatment, elevated miRNA expression levels may be a consequence of this treatment. When we grouped the patients concerning IFN-β treatment (treated vs. untreated), we found no significant difference in expression levels of any miRNA ($P > 0.05$).

Antiviral microRNAs have predicted binding sites in the measles genomic RNA

Our next question was whether the elevated miRNA species would target the measles genome. Previously, Pedersen *et al*. used a sequence complementarity analysis of several IFN-β-induced miRNAs against HCV genomic RNA and determined that eight IFN-β-induced miRNAs had nearly perfect complementarity in their seed sequences with the HCV RNA genome.[4] By employing a similar approach, we analyzed the complementarity between the seed sequences of miR-196b, miR-296, and miR-431 and the measles virus RNA genome. We identified that miR-296 and miR-196b targeted the RNA genome of the measles virus, as well as its

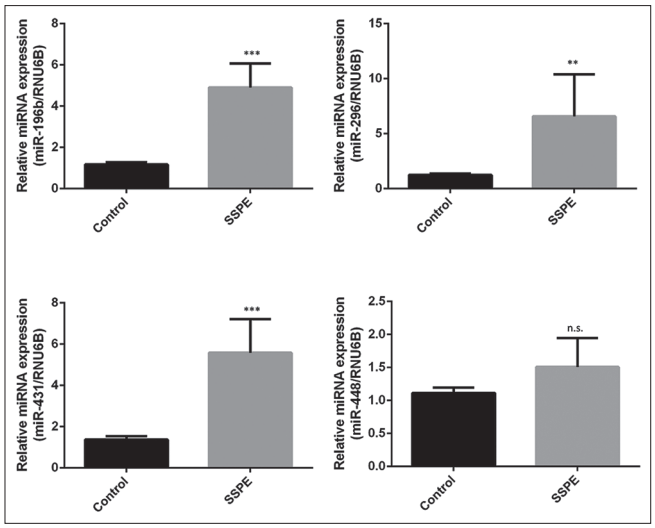

Figure 1: Antiviral microRNA expression levels are altered in subacute sclerosing panencephalitis. Peripheral blood mononuclear cells were isolated from patients with subacute sclerosing panencephalitis (n = 37) and healthy controls (n = 41). Total RNA was isolated from peripheral blood mononuclear cells, and hsa-miR-448, hsa-miR-196b, hsa-miR-296, and hsa-miR-431 expression levels were analyzed using quantitative polymerase chain reaction

variants [Table 2 and Figure 2]. Further analysis of these miRNAs showed that they also targeted different viruses including HCV, influenza virus, and poliovirus. This finding suggests that elevated miRNA expression profiles in patients with SSPE could represent a shared antiviral defense mechanism.

Pathway analysis

In the next step, we conducted pathway analysis to identify the potential measles genes that were targeted by the antiviral miRNAs. Our pathway analysis confirmed that 196b-5p, 296-5p, and 296-3p had targets within the KEGG measles pathway [Table 2 and Figure 3]. Furthermore, all miRNAs found in this study predicted targets in the measles genome [Table 3, Figure 3].

Discussion

During the past decade, miRNAs have emerged as important regulators of cellular antiviral defense mechanisms in different viral infections. However, we still do not know which miRNAs play a role in SSPE, or how. In the present study, we report that several antiviral miRNAs are overexpressed in patients with SSPE compared with healthy controls.

One of the pioneering studies on the antiviral role of miRNAs came from Lecellier *et al*., where the authors showed that cellular miRNA limited the replication of a mammalian virus, thus functioning as a cellular mediator of the antiviral response.[19] Later, Pedersen *et al*. reported that IFN-β modulated the expression of different cellular miRNAs, eight of which were predicted to target the HCV genomic RNA.[4] Subsequent studies indicated that miRNAs were major mediators of antiviral immunity, and analysis of virus-induced miRNA expression represented a rational way of studying virus-host interactions. In a recent study, Hou *et al*. demonstrated that hepatitis B virus (HBV) promoted miR-146a expression, which in turn suppressed STAT1 expression and led to interferon resistance.[20] Similarly, in our previous study, we found that miR-146a expression was significantly higher in patients with SSPE compared with healthy controls.[7] This finding could represent a shared mechanism of virus-induced alteration of miRNA expression. Still, the consequences of elevated miR-146a expression in SSPE are not completely identified.

We previously analyzed expression levels of several miRNAs involved in the immune system and inflammation in patients with SSPE and found that hsa-miR-146a, hsa-miR-181a, and hsa-miR-155 expression was significantly higher in patients with SSPE compared with healthy age-matched controls.[7] Together with the findings of the present report, these results suggest that miRNAs that are modulated by IFN-β might play a significant role in the pathogenesis of SSPE.

Interferon regulatory factors (IRFs) represent a group of transcription factors that play key roles in the innate immune system, especially during interferon production.[21] The measles virus nucleocapsid gene (N) activates IRF3,[22] but hsa-miR-296-5p counteracts that by repressing IKBKE, which also activates IRF3. Hsa-miR-196-5p shares GNB2 L1 as a target with hsa-miR-296-3p. MV-L is targeted by hsa-miR-448, hsa-miR-196 5p and 3p and by hsa-miR-196-3p, which may indicate that the measles polymerase (L) is an important target for human defense mechanisms. All miRNAs identified in our study targeted at least one measles gene, but usually multiple, which led to redundant repression of some virus genes such as L, H/F, and N. However, C, V, and M are also targeted but only by a single miRNA [Figure 3]. The distribution of the targets of these miRNAs is uneven and many target MV-L and MV-F more compared with other genes, which indicates that these two genes are important for defense against MV [Figure 3].

As summarized in Table 1, the majority of the patients with SSPE had an advanced stage disease and also received IFN-β. This information should be considered when interpreting the antiviral miRNA expression levels. It is likely that IFN-β treatment also contributed to the observed elevation in miRNA expression levels. Determining the actual increase in antiviral miRNA expression levels is possible through analysis of CSF

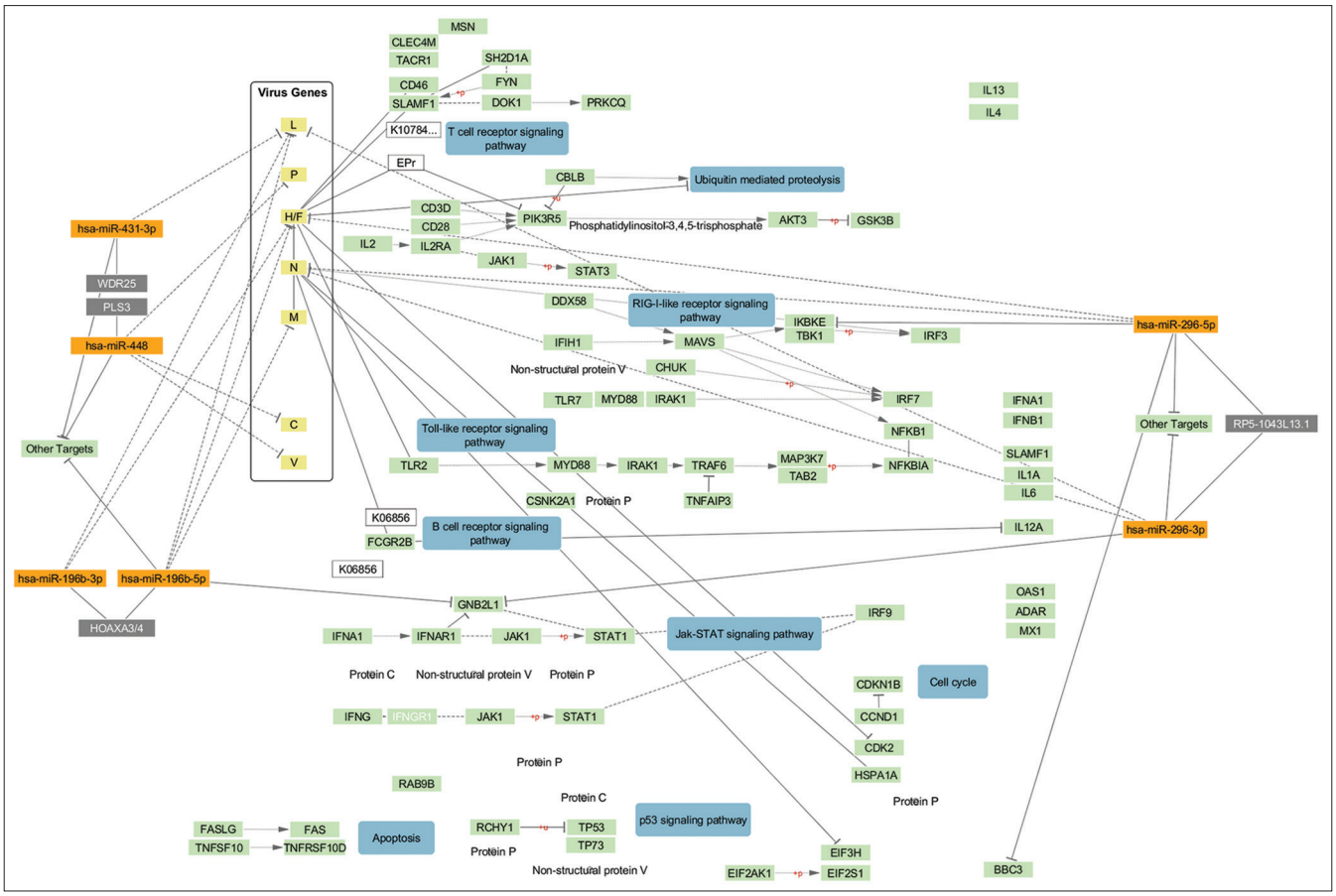

Figure 2: The transcripts of the measles virus and the predicted targets of the microRNAs. Due to space constraints, the microRNAs were abbreviated as follows: Hsa-miR-196b-3p (t13; dark red), hsa-miR-196b-5p (t15; orange), hsa-miR-296-3p (t23; olive), hsa-miR-296-5p (t25; green), hsa-mir-431 (t41, aquamarine), and hsa-mir-448-3p (t43, blue). Drop lines on the genomic view indicate the locations of the targets of these microRNAs. The horizontal bars art the collapsed features available in full on National Center for Biotechnology information website

Table 1: Classification of patients with subacute sclerosing panencephalitis with respect to disease stage

Stage	Patients (*n*)	IFN-β treatment		Mean age (years)	Vaccination status	
		Yes	No		Yes	No
1	3	1	2	11.5±2.3	1	1
2	6	3	2	15±2.61	5	1
3	28	22	7	14.6±2.3	25	3

IFN-β: Interferon-beta

Table 2: hsa-miR-296 and hsa-miR-196b predicted binding regions in the measles genome

miRNA	Predicted target genes (target sequences) in measles genome	Confirmed targets in human measles KEGG pathway
hsa-miR-196-5p	3 (4)	1
hsa-miR-196-3p	2 (2)	0
hsa-miR-296-5p	2 (4)	2
hsa-miR-296-3p	2 (5)	1
hsa-miR-431-3p	1 (1)	0
hsa-miR-448	3 (3)	0

miRNA: Microribonucleic acid, KEGG: Kyoto Encyclopedia of Genes and Genomes

samples and/or CNS cells; however, this approach relies on invasive sample collection procedures.

There are certain limitations of our present study. We were unable to compare miRNA expressions between patients convalescing from measles and those with SSPE; such information would allow us to have an accurate interpretation of the role of these four miRNAs in SSPE pathogenesis. We initially hypothesized that elevated miRNA expression levels were a response to measles infection; hence, it is possible to speculate that miRNAs would show differential expression between patients who recovered from measles infection and patients who progress to SSPE. Unfortunately, we do not have any follow-up information about the patients, and the only available information is the time of admission to the study. Another limitation of our study is that RNA profiles may vary in PBMC samples obtained from blood from different cities. However, in the pilot study, we conducted before our study, miRNA levels were compared by isolating PBMCs from fresh blood samples and from the same blood that was kept in EDTA tubes

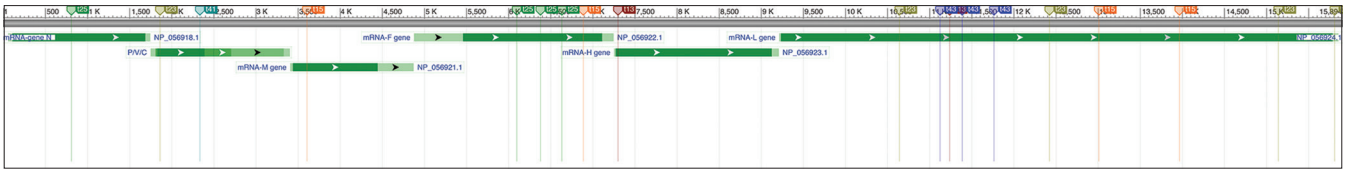

Figure 3: hsa-miR-448, hsa-miR-196b, hsa-miR-296, and hsa-miR-431 had targets in the measles Kyoto Encyclopedia of Genes and Genome pathway. The Kyoto Encyclopedia of Genes and Genome measles pathway (Accession: Hsa05162) was amended with the measles genes (khaki rectangles). The microRNAs found in this study and their targets were also added to the pathway (orange rectangles). Gray rectangles with diamond connection to the microRNAs indicate their source genes. Solid lines with perpendicular lines from microRNAs to genes indicate repression. If these lines are dotted, it indicates that the targets are computational predictions. Green rectangles (other targets) indicate the presence of further targets in the human genome

Table 3: *In silico* prediction of measles virus genes that are targeted by antiviral micro ribonucleic acid

miRNA	Match position		Score	Strand	Position in MV genes
	Start	End			
hsa-miR-196b-5p	3609	3615	14	+	Beginning of M
hsa-miR-196b-5p	6883	6889	14	+	In F
hsa-miR-196b-3p	11,233	11,240	15,9	+	In L
hsa-miR-196b-3p	11,233	11,240	15,9	+	In L
hsa-miR-196b-5p	13,009	13,020	15,9	+	In L
hsa-miR-196b-5p	13,967	13,976	19,6	+	In L
hsa-mir-296-5p	811	821	14	+	In N
hsa-mir-296-3p	1857	1863	14	+	After N
hsa-mir-296-5p	6093	6100	15,9	+	In F
hsa-mir-296-5p	6375	6382	15,9	+	In F
hsa-mir-296-5p	6631	6638	15,9	+	In F
hsa-mir-296-3p	10,644	10,650	14	+	In L
hsa-mir-296-3p	12,419	12,425	14	+	In L
hsa-mir-296-3p	15,138	15,144	14	+	In L
hsa-mir-296-3p	15,803	15,813	15,9	+	End of L
hsa-mir-431-3p	2337	2346	19,6	+	In PVC
hsa-mir-448-3p	11,126	11,137	17,7	+	In L
hsa-mir-448-3p	11,389	11,395	14	+	In L
hsa-mir-448-3p	11,761	11,767	14	+	In L

A selection of interesting target genes, as well as a complete list of targets for each antiviral miRNA are shown here. miRNA: Microribonucleic acid, PVC: Polyvinyl chloride

for 48 h at room temperature. There was no difference between the RNA concentrations, cycle threshold (Ct) values, and ΔCt values we obtained. Hence, our investigation showed that the stability and expression of miRNAs were not affected by external conditions during transportation of blood samples. We were also unable to analyze the potential correlation between the disease stage with measles virus titers because this parameter is not regularly used in routine clinical practice in Turkey. Finally, we were unable to validate *in silico* predictions due to the lack of a suitable *in vitro* SSPE model.

To identify the precise contribution of miRNAs to the pathogenesis of SSPE, future studies should focus on functional analyses of miRNA gain and/or loss in an *in vitro* model of SSPE. Given the lack of an existing model for this disease, this could represent a technical challenge, but the outcomes of such efforts will surely help us to determine the roles of miRNAs in SSPE.

Acknowledgment

The study received financial support from Dokuz Eylul University, Izmir, Turkey (Grant number: BAP 2012. KB.SAG.106).

Financial support and sponsorship

This study has been funded by Dokuz Eylul University (Project number: 2012.KB.SAG.106).

Conflicts of interest

There are no conflicts of interest.

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