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ORIGINAL ARTICLE

Serum MicroRNA Expression Profiling in Mice Infected with Rabies Virus

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Abstract
Objectives: Serum or plasma microRNAs (miRNAs) are potential biomarkers for the diagnosis for cancer and prenatal diseases. This study was conducted to investigate whether rabies virus causes a change in serum miRNA expression.
Methods: ICR mice were intramuscularly inoculated with rabies virus and were sacrificed weekly to collect serum and brain tissue for 4 weeks postinoculation. Mice were assigned to four groups based on the results of indirect immunofluorescent assays, enzyme-linked immunosorbent assay, and nested reverse transcription-polymerase chain reaction and the expression profiles of serum miRNAs were compared using a commercial mouse miRNA expression profiling assay.
Results: The expression levels of miRNAs changed significantly with the different stages of the disease. The expression level of 94 serum miRNAs in infected mice changed at least twofold. Seven microRNAs of them were significantly upregulated or downregulated in all infected mice regardless of disease status. The number of miRNAs with an expression level change decreased with the progression of the disease. In a hierarchical cluster analysis, infected mice clustered into a group separate from uninfected control mice.
Conclusions: Based on the relationship of miRNAs to gene expression regulation, miRNAs may be candidates for the study of viral pathogenesis and could have potential as biomarkers.

1. Introduction

Rabies virus is a negative-stranded RNA virus of the family *Rhabdoviridae* and the genus *Lyssavirus*, and the cause of rabies, a fatal neurological disease in humans and animals [1]. Although rabies can be deadly, the disease is preventable by preexposure immunization and by the timely and appropriate application of postexposure prophylaxis [2]. Reverse transcription-polymerase chain reaction (RT-PCR), immunohistochemical staining, and

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direct fluorescent assays have been used to detect rabies virus from skin biopsy, cornea, saliva, urine, and cerebrospinal fluid for intra vitam diagnosis and from brain tissue for postmortem diagnosis [3]. Rapid fluorescent focusing inhibition test, enzyme-linked immunosorbent assay (ELISA), and indirect immunofluorescent assay (IFA) have been widely used for the serologic diagnosis of rabies. Rabies virus-specific antibodies have been detected in the late phase of infection and at the terminal stage of the disease using these procedures [4].

MicroRNAs (miRNAs) are highly conserved, noncoding, small RNAs of approximately 22 nucleotides that regulate gene expression posttranscriptionally by facilitating translational repression or mRNA degradation [5,6]. miRNAs play important roles in the control of many biologic processes such as development, metabolism, cellular differentiation, proliferation, cell-cycle control, and cell death [7,8]. miRNA-specific expression profiles have been reported for several pathogenic conditions, such as viral infections, cancer, and cardiovascular disease [7,9]. Thus, disease-associated miRNA may be a viable target for the development of diagnostic markers.

The fatal outcome of rabies may be due to a neuronal dysfunction of infected neurons [10,11]. In this study, it was hypothesized that miRNAs may be involved in alternation or change of neuronal functions and that infection with rabies virus may be initially evident as altered miRNA expression. To begin to examine this hypothesis, the current pilot study investigated whether rabies virus infection could lead to a change in the expression of serum miRNAs.

2. Materials and Methods

2.1. Rabies viruses

The rabies virus KGH strain was isolated from a patient bitten by a rabid raccoon dog (*Nyctereutes procyonoides*) in Korea in 2001. The supernatant of homogenized skin tissue from the nape of the patient's neck was inoculated intracerebrally into suckling mice. The brain tissue of mice was collected when clinical signs of the disease developed (6−7 days after inoculation) and stored at −70°C until use. The 50% lethal dose (LD_{50}) of KGH strain was determined in 5-week-old female ICR mice using the stored brain tissue. The supernatant of the brain tissue was serially diluted 10-fold with phosphate-buffered saline (pH 7.4). Five mice for each dilution were inoculated intramuscularly in the femoral region of the mice. Clinical signs were observed daily for 12 weeks after inoculation (wpi).

2.2. Experimental design

The protocol of the animal experiments was approved by the Animal Care and Use Committee of the National Institute of Toxicological Research on behalf of Korea Centers for Disease Control and Prevention (#NIH-08-14). Female, 5−6-week-old ICR mice (Samtako, Osan, Korea) were reared in Iso cages in a Biosafety Level 3 facility. They had access to food and water *ad libitum*. Control and experimental mice were inoculated intramuscularly in a femoral region with phosphate-buffered saline (pH 7.4) and with $10^{2.9}$ LD_{50} of the KGH strain, respectively. Brain tissue and blood of mice were collected weekly from 0 to 4 wpi for serologic and molecular assays. When moribund, mice were sacrificed to sample brain tissue and blood regardless of experimental schedule. Serum obtained was divided into aliquots and stored at −70°C for serologic assays and miRNA extraction. Nested RT-PCR was used to detect the virus from brain tissue and serum. All surviving mice were sacrificed on 12 wpi for sampling.

2.3. Serologic assays

Rabies antibodies were analyzed using IFA and ELISA. To prepare slides for IFA, baby hamster kidney cells were infected with the ERA strain of rabies virus for 1 hour and incubated for 45−48 hours at 37°C. After incubation, the cells were treated with trypsin-EDTA solution and resuspended in Eagle minimum essential medium (1 x 10^4 cells/mL). The cells were dispensed into wells of an eight-well slide glass (Lab-Tek Chamber Slide, Nalgen Nunc International, Rochester, NY, USA) and incubated 5−6 hours to allow cells to attach to the slide. Adherent cells were fixed with cold acetone for 10 minutes and kept at −20°C until further use. For IFA, 20 μL of serum diluted 1:16 was loaded in each well of another slide and incubated for 30 minutes at 37°C. Goat anti-mouse immunoglobulins G and M antibodies conjugated with fluorescein isothiocyanate (FITC; Jackson Immunoresearch Laboratories, West Grove, PA, USA) were treated in the same manner. FITC signal was observed with a fluorescent microscope (Carl Zeiss, Oberkochen, Germany). PLATELIA RABIES II kit (Bio-Rad, Marnes-la-Coquette, France) was used for ELISA. Mouse serum was diluted 1:100 with the sample diluent included in a kit following the manufacturer's protocol.

2.4. RNA extraction

Viral RNA was extracted from 100 μL of 10% tissue homogenate and 200 μL serum using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) and Trizol reagent (Invitrogen, Carlsbad, CA, USA), respectively. Extracted RNAs were resuspended in RNase-free distilled water. RNAs extracted from the brain tissue were used to determine virus infection. Before miRNA expression profiling, the concentration and quality of serum RNAs were measured using a Nanodrop ND-100 spectrophotometer (NanoDrop, Wilmington, DE, USA) and a model 2100 bioanalyzer (Agilent, Santa Clara, CA, USA).

2.5. Nested one-step RT-PCR

A nested one-step RT-PCR was designed to amplify the nucleoprotein gene of rabies virus. Forward and reverse primers were composed of forward primer RVND-F1 (5′-ctacaatggatgccgac-3′) and reverse primer RVND-R1 (5′-cgaatatgtcttgtttagaaactcg-3′) for one-step RT-PCR, and forward primer RVND-F2 (5′-gacatgtccggaagactgg-3′) and reverse primer RVND-R2 (5′-gtattgcctctctagcggtg-3′) for nested polymerase chain reaction (PCR). For one-step RT-PCR, 5 μL of extracted RNA were mixed with a RT-PCR mixture consisting of 5 uL 10 × PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% Triton X-100), 5 μL 25 mM $MgCl_2$, 2 μL 25 mM deoxynucelotide triphosphates, 1 μL of each primer (10 mM), 1 μL Taq DNA polymerase (5 U/μL), 0.5 μL RNaseOut RNase inhibitor (40 U/μL), and 0.5 μL SuperScript III reverse transcriptase (200 U/μL) in total volume of 50 μL. The conditions of the one-step RT-PCR were one cycle of 50°C for 60 minutes and 95°C for 5 minutes; 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 80 seconds; and one cycle of 72°C for 10 minutes. Nested PCR was performed with 1 μL of RT-PCR product and the same RT-PCR mixture composition without RNase inhibitors and reverse transcriptase. The conditions of the nested PCR was one cycle of 95°C for 5 minutes; 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and one cycle of 72°C for 80 seconds; and 72°C for 10 minutes. Amplified nested RT-PCR products were electrophoresed in 1.2% agarose gel containing 0.25% bromophenol blue and 0.25% xylene cyanol FF.

2.6. Analysis of miRNAs expression profiling

Mice for miRNA expression profiling analysis were assigned to four groups based on the results of IFA, ELISA, and nested RT-PCR (Table 1). The expression levels of serum miRNAs were measured using a mouse miRNA expression profiling assay (Illumina, Inc., San Diego, CA, USA). The assay panel contains 380 assays for mouse miRNAs described in the miRBase database v9.1. According to the manufacturer's instructions, miRNAs were polyadenylated and converted to complementary DNA after hybridization with miRNA-specific oligos and PCR-amplified. BeadStudio v3.1.3 (Illumina) was used to extract the array data. The signal value was log transformed and normalized by the quantile method. Hierarchical clustering was used to determine similarity using complete linkage and euclidean distance. miRNAs with at least a twofold difference in expression level in inoculated mice compared to uninoculated control mice were selected to determine the expression profile.

3. Results

3.1. Serologic assays, virus detection, and clinical signs

Each mouse was given an identification number, which was combined with group and individual numbers. The first digit and letter indicated the week of sampling collection after inoculation (e.g., 1 W denoted 1 week after inoculation) and the last digit denoted the individual number. Signs such as depression, ruffled fur, and tremors were present in mice 2W4, 2W5, 3W1, and 3W2. Mice 1W4 and 2W4 were positive for rabies antibodies by IFA but not by ELISA. Rabies antibodies were detected in mice 2W5, 3W1, and 3W2 by both IFA and ELISA (Table 1). Mouse 1W8 was serologically negative for the rabies virus. Rabies virus was detected in brain tissue of mice 1W4, 2W4, 2W5, 2W8, 3W1, and 3W2 by nested one-step RT-PCR (Table 1). However, rabies virus was not detected in sera of any mice, including uninoculated control mice. All uninoculated control mice (0W4, 0W8, and 0W10) were negative for rabies antibody and for virus.

3.2. Expression levels of serum miRNAs

Inoculated mice were divided into two groups based on the results of serologic and molecular assays to compare the expression profiling of serum miRNAs with three uninoculated control mice (Table 1). Mouse 1W8 was selected for analysis of miRNA expression profile because it was serologically and virologically negative

Table 1. Mouse groups classified by the results of serologic and molecular assays for comparing serum microRNA expression profile[a]

Group	Identification number of mouse	Results of assays		
		IFA	ELISA	Nested RT-PCR[a]
Control	0W4, 0W8, and 0W10	Negative	Negative	Negative
1A	1W8	Negative	Negative	Negative
1B	1W3, 1W6, 2W6, and 2W7	Positive	Negative	Negative
1C	1W4, 2W4, 2W4, 2W5, 2W8, 3W1, and 3W2	Positive	Positive or negative	Positive
2A	1W3, 1W6, 2W6, and 2W7	Positive	Negative	Negative
2B	1W4, 2W4, and 2W8	Positive	Negative	Positive
2C	2W5, 3W1, and 3W2	Positive	Positive	Positive

[a]*Rabies virus was only detected from brain tissues of some inoculated mice, not from serum by nested RT-PCR.*
ELISA = enzyme-linked immunosorbent assay; IFA = indirect immunofluorescent assay.

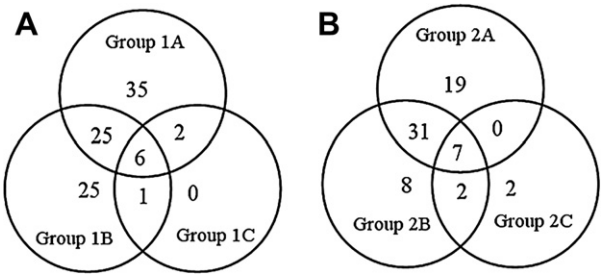

Figure 1. Expression profiling of serum miRNAs in mice inoculated with rabies virus KGH strain. miRNAs with at least a twofold change of expression level are denoted in the Venn diagram. Digits in the Venn diagram indicate the change in the miRNA expression level of each group and between groups. (A) Groups assigned based on results of IFA and nested RT-PCR. (B) Groups assigned based on the results of IFA, ELISA, and nested RT-PCR.

for the rabies virus. After preprocessing and normalization of data, 352 of the 380 miRNAs were selected for analysis of miRNA expression profiling. miRNAs with upregulated or downregulated expression are summarized (Figure 1). miRNA expression profiles were categorized into two major groups (Groups 1 and 2) based on IFA, ELISA, and nested RT-PCR results. Each major group was also subdivided into three subgroups (A, B, and C). First, Group 1 was classified by IFA and nested RT-PCR results as follows: 1W8 for Group 1A; 1W3, 1W6, 2W6, and 2W7 for Group 1B; and 1W4, 2W4, 2W5, 2W8, 3W1, and 3W2 for Group 1C. The expression levels of 94 serum miRNAs changed at least twofold in mice inoculated with rabies virus, and all but two miRNAs were downregulated or upregulated regardless of the groups. Two miRNAs, mmu-miR-701 and mmu-miR-142-5 p, were downregulated in group 1A but were upregulated in group 1B. Expression levels of six miRNAs (mmu-miR-98, mmu-miR-100, mmu-miR-187, mmu-miR-322, mmu-miR-466, and mmu-miR-707) changed significantly in all inoculated mice, and the expression levels of three miRNAs (mmu-miR-98, mmu-miR-466 and mmu-miR-706) were downregulated. Expression levels changed in 57 and 9 miRNAs in Groups 1B and 1C, respectively. Mouse 1W8 displayed altered expression of 68 miRNAs. Of the 68 miRNAs, 25 and 2 miRNAs also changed expression levels in Groups 1B and 1C, respectively.

Group 2 was divided based on the results of IFA, ELISA, and RT-PCR as follows: 1W3, 1W6, 2W6, and 2W7 for Group 2A; 1W4, 2W4, and 2W8 for Group 2B; 2W5, 3W1, and 3W2 for Group 2C (Figure 2). Mouse 1W8 was excluded from Group 2. As shown in Figure 2B, 69 miRNAs in serum of all groups displayed altered expression levels compared with uninoculated control mice. Among these, the expression of 19, 8 and 2 miRNAs were upregulated or downregulated only in Groups 2A, 2B, and 2C, respectively. The average expression levels of mmu-miR-130b and mmu-miR-142-50 increased 3.71- and 3.19-fold in Group 2A ($p < 0.04$ and $p < 0.02$) and mmu-let-7c, mmu-let-7f, mmu-let-7e, mmu-miR-346, and mmu-miR-546 were significantly down-regulated in Group 2A ($p < 0.00$), suggesting that there are some miRNAs expressed in the early stage of rabies infection. Two miRNAs (mmu-miR-155 and mmu-miR-465-5 p) and one miRNA (mmu-miR-103) were significantly downregulated in Groups 2B and 2C, respectively. The expression of mmu-miR-466 and mmu-miR-706 were significantly decreased in all inoculated mice.

3.3. Cluster analysis

The similarity of miRNA expression profiles among was analyzed using hierarchical clustering (Figure 3). Mice were divided into two major clusters based on expression pattern similarity. Uninoculated control mice grouped with mouse 1W8, which was serologically and clinically normal, and mice 3W1 and 3W2, which showed signs of the terminal stage of the disease. All other inoculated mice clustered together. Mice 1W3, 1W6, and 2W7, which were negative for antibody but positive for virus, clustered closely with each other. Mice 1W4 and 2W5, which were positive for both

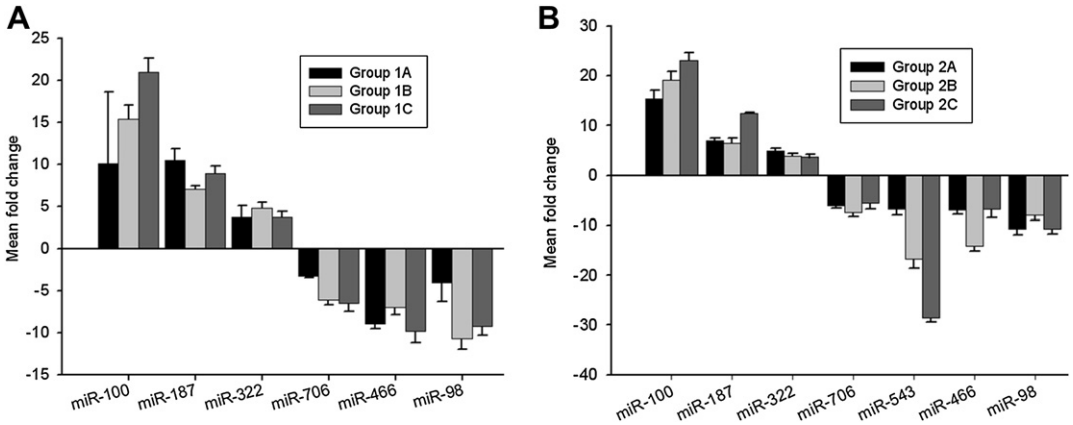

Figure 2. Mean change of serum miRNAs in the inoculated groups compared with that of the uninoculated control group. Mice were assigned to (A) Group 1 or (B) Group 2 based on the results of serologic assays and nested RT-PCR.

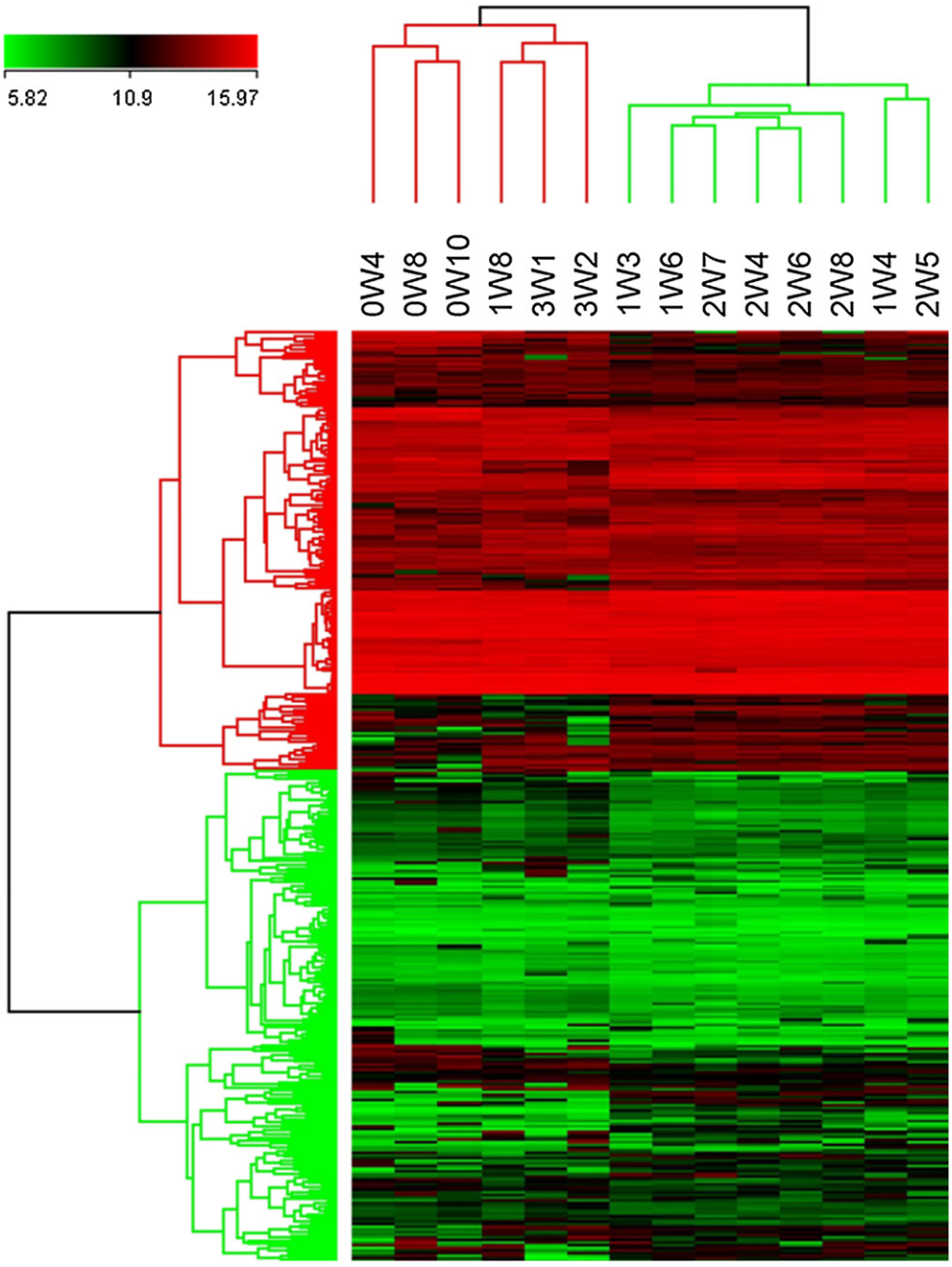

Figure 3. Hierarchical clustering analysis of miRNA expression using euclidean method and complete linkage. The degree of relatedness of expression profiling is represented at the top of the panel. The color and intensity in each cell indicate the expression level of the corresponding miRNAs. The increased intensity of red and green indicates higher and lower expression of miRNAs, respectively.

antibody and virus, clustered separately from the other mice.

4. Discussion

miRNAs play pivotal roles in gene regulation. The expression of a third of human genes is predicted to be controlled by miRNAs [12]. Upregulated or down-regulated expression of miRNAs is related to several diseases including cancers, cardiovascular diseases, neurologic diseases, and immunologic diseases [9,13]. Because of the correlation of miRNA expression levels with disease, miRNAs have been explored as biomarkers for the diagnosis of diseases and as therapeutic targets [14,15].

Circulating cell-free nucleic acids were first detected in plasma of healthy individuals and patients and also from patients with pancreatic cancer and rheumatoid arthritis [16]. The release of nucleic acids into the general circulation has been suggested to be associated with blood-brain barrier rupture, necrosis, apoptosis, and

lysis of cells, placental and fetal cells, and secretory exosomes [17,18]. However, the origin of serum nucleic acids including miRNAs remains incompletely understood.

Rabies virus causes neuronal dysfunction, which may be mediated by impairment of neurotransmitter release or ion homeostasis, rather than by neuronal death or pathological damage of organs [19,20]. We hypothesized that miRNAs may be involved in the regulation of genes that encode neurotransmitter proteins and that the expression profiles of miRNAs differ in different infection stages. In this study, mouse groups had distinct expression patterns depending on the stage of infection. Mouse 1W8 was negative to both rabies antibody and virus. However, the mouse had a different miRNA expression pattern from uninoculated mice of the control group. The pattern was closely related to mice 3W1 and 3W2. In mouse 1W8, of the 352 miRNAs, 35 had more than a twofold change of expression level. These results indicate that mouse 1W8 was infected with rabies virus. Whether miRNA patterns in mouse 1W8 indicate the early stage of infection was not clear in the current study due to a limited number of mice examined. New research approaches will be critical to determine miRNA expression profiles in the early stage of infection.

The miRNAs upregulated or downregulated in one group had the same pattern in the other group, regardless of the inclusion of mouse 1W8. This result suggested that there was a specific miRNA expression profile in infected mice. The expression of six miRNAs was specific to the infected mice, regardless of infection stage. These miRNAs may have potential as diagnostic biomarkers for rabies. The current study investigated serum miRNA expression patterns of mice infected with rabies virus. miRNAs that displayed upregulated or downregulated expression could have potential as biomarkers for the development of additional diagnostic assays of rabies.

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