Materials and Methods

Patient serum and information collection

We conducted a retrospective observational study on all patients with confirmed EVD who were admitted for care at the Freetown China–Sierra Leone friendship hospital The standard case definition established by the WHO [1] was used for this study. Eighty-three patients were enrolled in this study. Laboratory confirmation of EVD was made on the basis of the results of real-time polymerase chain reaction. Treatment was conducted in line with the protocols for viral hemorrhagic fever under the urgent interim guidance for case management established by the WHO [2]. The protocol for this study was approved by the Ethics Committee of the Department of Health in Freetown. Written informed consent was given to every patient enrolled prior to the start of this study. All of the clinical blood samples were collected from samples sent to our mobile laboratory for EVD diagnosis. The blood samples were heat-inactivated at 60°C for 1 h, and then, the sera were isolated and stored at -20°C before use. Epidemiological information, including age, sex, and symptom onset date, was recorded using a questionnaire sent together with the blood samples.

Computational prediction of viral miRNAs

Reference Genomic sequences of EBOV (Zaire ebolavirus isolate Ebola virus/H.sapiens-tc/COD/1976/Yambuku-Mayinga, NC002549) were downloaded from the National Center for Biotechnology Information (NCBI) database (Bethesda, MD, USA) and scanned for stretches of the typical hairpin-like stem-loop structure of miRNA precursors (pre-miRNA) by using VMir[3], a computational analyzer program for the prediction of putative pre-miRNAs encoded by virus. Then, the MiPred[4] was used to distinguish real and pseudo miRNA precursors from the obtained sequences with a prediction confidence equal to or greater than 70%. Subsequently, mature miRNA sequences were predicted from the pre-miRNA stem-loops by using the MatureBayes tool[5]. To confirm the specificity of the EBOV miRNAs, we used the online nucleotide blast tool in NCBI to scan for highly similar sequences against the human genomic and transcript database with default setting. We also search for highly similar sequences against other ebola virus subtypes and marburg viruses

genome by using NCBI online nucleotide blast tool with default settings. The default conditions were used for the MatureBayes tool. To show the conservation, we retrieved full genome sequences from the genome browser at NCBI Database. A total of 125 human EBOV genomes were derived from the 2014 outbreak [6]. The multiple sequence alignment tools ClustalW and MUSCLE were applied for the alignment of the EBOV genomes. The alignments were then analyzed to identify the characteristic sites as potential signatures to distinguish different virus genomes and proteins.

Plasmid construction

Mammalian expression plasmids encoding pre-miR-16 and pre-miR-VP (pcDNA6.2-GW-miR) were purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA). An empty plasmid served as a negative control. The expression plasmids were transfected into HEK293A cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The total RNA was isolated at 24 h post-transfection.

Cells

HEK293A cells were purchased from the China Cell Culture Center (Shanghai, China) and were maintained at 37°C in a humidified 5% CO_2 incubator in Dulbecco's modified Eagle medium (Gibco, CA, USA) containing 10% fetal bovine serum (FBS, Gibco), 100 units/ml penicillin, and 100 μg/ml streptomycin.

Reagents

Synthetic miRNA standards were obtained from Invitrogen. The miRCURY LNA miRNA detection probes were obtained from Exiqon. The DIG Luminescent Detection Kit was purchased from Roche Applied Science (Indianapolis, IN, USA).

Exosome isolation

Exosomes were isolated from 100 μl of pooled serum samples of EVD patients by using Total Exosome Isolation (from serum) Kit (Invitrogen) according to the manufacturer's instructions. Briefly, 100 μl of serum was centrifuged at 2000 × g for 30 minutes to remove cells and

debris. Then 100 μl of serum was transferred to a new tube and added with 0.2 volumes of the Total Exosome Isolation (from serum) reagent. The serum and reagent were mixed by either vortexing or pipetting up and down until there is a homogenous solution. The sample was incubated at 4°C for 30 minutes. After incubation, the sample was centrifuged at 10,000 × g for 10 minutes at room temperature. The exosome-free supernatant was transferred to a new tube, and the exosomes that were contained in the pellet at the bottom of the tube were resuspended in 100 μl of 1 × PBS.

RNA isolation

The total RNA was extracted from 100 μl of serum via the RNA isolation kits provided by the Nanjing MicroMedMark Biotech Company (Nanjing, China) according to the manufacturer's instructions. Briefly, 100 μl of serum was mixed with 300 μl of RNase-free water, 200 μl of phenol, and 200 μl of chloroform. The sample was vortexed and incubated at room temperature for 15 min. The mixture was centrifuged at 12,000 g for 10 min, and the upper aqueous layer was collected. Subsequently, a 1/10 volume of 3 M sodium acetate and a two-fold volume of isopropyl alcohol were added to the aqueous phase. The total RNA was precipitated via incubation at -20°C for 1 h. The RNA pellet was collected via centrifugation at 16,000 g for 20 min, washed once with 75% ethanol, dried for 10 min at room temperature, and then dissolved in 20 μl of RNase-free water.

Northern blot analysis

A sensitive non-radioactive Northern blot assay was performed to detect miRNAs [7]. The total RNA (including miRNAs) was isolated from 100 μl of watermelon juice or 4 ml of serum using a one-step phenol/chloroform purification protocol. The Northern blot analysis was performed using the miRCURY LNA™ microRNA Detection Probes with DIG-labeling (Exiqon) and a DIG Luminescent Detection Kit (Roche), according to the manufacturer's instructions. Briefly, the total RNA isolated from 10^6 HEK293A cells or 5 ml of serum was dissolved in Gel Loading Buffer II (Ambion), heated at 95°C for 5 min, loaded on a 15% TBE-urea denaturing gel and transferred to a Zeta Probe Plus Membrane (Bio-Rad, Hercules, CA, USA). After UV crosslinking at 1200 mJ, the membranes were equilibrated with 2× SSC

and prehybridized at 42°C for 1 h in ULTRAhyb-Oligo buffer (Ambion). Prior to the hybridization, the miRCURY™ LNA detection probes were labeled using a 2nd Generation DIG Oligonucleotide Tailing Kit (Roche Applied Science, Indianapolis, IN, USA). The DIG-labeled LNA probes were hybridized to the membranes overnight at 37°C in ULTRAhyb-Oligo buffer (Ambion). Following the hybridization, the membranes were washed twice for 30 min in NorthernMax Low Stringency Wash Solution No. 1 (Ambion) at 42°C, rinsed for 5 min in 1× Wash Buffer from the DIG Wash and Block Buffer Set (Roche), blocked for 1 h in 1× Blocking Solution (Roche), incubated for 1 h in an antibody solution (anti-DIG-AP 1:10,000 in 1× Blocking solution; Roche), washed twice for 15 min in 1× Wash Buffer and equilibrated by rinsing twice for 5 min each with 1× Detection Buffer (Roche). Then, as recommended in the DIG Luminescent Detection Kit instructions (Roche), the blots were incubated in a chemiluminescent substrate for alkaline phosphatase CSPD (Roche) and exposed to Amersham Hyperfilm ECL (GE Healthcare Life Sciences, Piscataway, NJ, USA). The DIG-labeled LNA probes for miR-VP-3p (5'-GCCCCAAAGTGCTAATGAAGCA-3') and miR-16 (5'- CGCCAATATTTACGTGCTGCTA-3') were purchased from Exiqon (Exiqon A/S, Vedbaek, Denmark). Synthetic miR-VP-3p (5'-UGCUUCAUUAGCACUUUGGGGC-3') was purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA).

qRT-PCR for Ebola virus genomes

The qRT-PCR procedure for Ebola virus genomes was performed using a one-step qRT-PCR assay (Puruikang Biotech Co. Ltd, Shenzhen, China) according to the manufacturer's instructions [8]. Briefly, tests for the presence of Ebola virus were performed on site by using a qRT-PCR assay targeting the glycoprotein gene of Ebola virus subtype Zaire with primers 5'-TGGGCTGAAAAYTGCTACAATC-3' (forward) and 5'-CTTTGTGMACATASCGGCAC-3' (reverse) and probe FAM-5'-CTACCAGCAGCGCCAGACGG-3'-TAMR). RT-PCR was conducted by using the Light Cycler 96 System (Roche Co. LTD, Basel, Switzerland) in a 25 µl mixture containing 3 µl of viral RNA, 20 µl of PCR buffer A, and 2 µl of PCR buffer B. The reaction was performed for 5 min at 42°C, followed by 10 s at 94 °C, with a subsequent 40 cycles of

amplification (94°C for 5 s, 55°C for 30 s). Fluorescence was recorded at 55°C. A calibration standard was generated by diluting virus-like particles containing glycoprotein gene Zaire subtype Ebola virus based on the published sequence in GenBank. The dilutions (5×10^6 copies/ml) were subjected to RT-PCR analyses as a positive control test. The cycle threshold (C_T) value of the positive control was used to estimate the absolute concentration of the RNA, which was representative of the original virus load. The approximate virus load (copies/ml) of the original sample was calculated and converted as follows: $5\times10^6\times2^{(C_{T1}-C_{T2})}$, where C_{T2} represents the C_T value of samples and C_{T1} is the C_T value of the dilutions of the positive control each test.

qRT-PCR assay for miR-VP-3p

The qRT-PCR procedure for miR-VP-3p was performed using a TaqMan miRNA PCR Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Briefly, 2 μl of total RNA was reverse-transcribed to cDNA using the AMV reverse transcriptase (TaKaRa, Dalian, China) and stem-loop RT primers (Applied Biosystems). The reaction conditions were as follows: 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. Real-time PCR was performed using TaqMan miRNA probes (Applied Biosystems) and an Applied Biosystems 7300 Sequence Detection System (Applied Biosystems). The reactions were incubated in a 96-well optical plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All of the reactions, including the no-template controls, were run in triplicate. After the reactions, the cycle threshold (C_T) values were determined using fixed threshold settings, and the mean C_T values were determined from triplicate PCR reactions. To calculate the expression levels of the target miRNAs, a series of synthetic miRNA oligonucleotides at known concentrations that were diluted in water were also reverse-transcribed and amplified. The quantity of each miRNA was then calculated according to the standard curve.

Statistical analysis

All of the data from the qRT-PCR analyses were obtained from at least three independent experiments. The data shown are presented as the means ± SD of at least three independent

experiments. Differences were considered to be significant at $P < 0.05$ using Student's *t*-test.

References

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