Different Expression Pattern of Human Cytomegalovirus-Encoded microRNAs in Circulation from Virus Latency to Reactivation

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Research

Keywords: human cytomegalovirus, serum microRNA, HCMV DNA copies, latency, reactivation, switch

Posted Date: August 25th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-60062/v1

Version of Record: A version of this preprint was published on December 9th, 2020. See the published version at https://doi.org/10.1186/s12967-020-02653-w.

Abstract

Background: Human cytomegalovirus (HCMV) is a beta-hersvirinae that has a high latent infection rate worldwide and can cause serious consequences in immunocompromised patients when reactivation; however, the mechanism of how HCMV convert from latent to reactivation has rarely been investigated. In the present study, we aimed to perform a comprehensive analysis of the HCMV-encoded microRNA (miRNA) profile in serum of patients upon HCMV reactivation from latency and to further evaluate its clinical significance for the disease monitoring and preventing usefulness.

Methods: Serum samples from 60 viremia patients and 60 age-gender matched controls were enrolled in this study for screening and validation of different expression of HCMV miRNAs. Serum concentrations of 22 known HCMV miRNAs were determined by a hydrolysis probe-based stem-loop quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay. HCMV DNA was measured by quantitative real-time PCR (qPCR) with the whole blood sample. Serum HCMV IgG and IgM were assessed using enzyme linked immunosorbent assay (ELISA). Another 47 samples from 5 patients at different time points were collected to evaluate the monitoring effectiveness and disease prediction ability of differential expression HCMV-miRNAs during the antiviral treatment.

Results: The RT-qPCR analysis revealed that the serum levels of 16 of the 22 examined HCMV miRNAs were elevated in HCMV viremia patients compared with controls, and a profile of 8 HCMV miRNAs including hcmv-miR-US25-2-3p, hcmv-miR-US4-5p, hcmv-miR-US25-2-5p, hcmv-miR-US25-1-3p, hcmv-miR-US25-1, hcmv-miR-UL36, hcmv-miR-UL148D, hcmv-miR-US29-3p were markedly elevated (fold change > 2, $P < 0.01$). Receiver operating characteristic curve (ROC) analysis were performed on the selected HCMV-miRNAs in all of the patients and controls that enrolled in this study, and which ranged from 0.74 to 0.79 in the autoimmune patients. In addition, hcmv-miR-US25-1-3p levels were significantly correlated with HCMV DNA copies ($r = 0.297$，$P = 0.022$), and were obviously higher in the reactivation set than the latency set in the autoimmune patients, which could be a predictor for the monitoring of the antiviral treatment.

Conclusions: HCMV miRNAs profile showed markedly shift-switch from latency to reactivation in circulation from HCMV infected patients and hcmv-miR-US25-1-3p may be served as a predictor for the switch upon reactivation from latency in patients suffered with autoimmune diseases.

Background

Human cytomegalovirus (HCMV) is a member of the herpesviridae family, beta-hersvirinae subfamily that latently infects approximately 70 ~ 100% of the population worldwide in their lifetime [1]. Initial infection and reactivation of HCMV usually does not result in morbidity in healthy individuals, whereas reactivation of HCMV from the latency in immunocompromised people, such as AIDS patients, solid organ transplant recipients and neonates can lead to severe morbidity and mortality [2–4]. However, the mechanisms involving in HCMV latency and reactivation remain poorly understood.

MicroRNAs (miRNAs) are a subset of non-coding RNA molecules (19–23 nucleotides in length) that mediate post-transcriptional gene silencing. HCMV encodes at least 26 mature miRNAs and which have been implicated in the regulation of viral replication, immune modulation, and immune evasion [5, 6]. Differential HCMV encoded miRNAs expression was observed in the latency and activation infection by HCMV *in vitro*. For instance, hcmv-miR-UL148D facilities latent viral infection by modulating the IER5-CDC25B axis in host cells [7]. Hcmv-miR-UL112-1 can attenuate replication of HCMV and implicates in latency control of HCMV by targeting HCMV IE1, UL112/113, UL120/121 and UL144 [8, 9]. However, all of the HCMV miRNAs that discovered currently were detected in infected fibroblast cells [10, 11], which due to the lack of appropriate cell-lines or animal models for studying HCMV latency.

Circulating miRNAs could be novel biomarker for the diagnosis of virous diseases, including viral infection diseases [6, 12, 13]. *In vivo* evidence of the link between HCMV miRNAs and diseases processes is now emerging, with the description of hcmv-miR-UL112-3p as a biomarker of essential hypertension, diabetes mellitus and glioblastoma [14, 15], and hcmv-miR-UL22A-5p as a biomarker in solid organ transplantation [16]. Our group also demonstrated a distinct expression pattern of HCMV-encoded miRNAs in oral lichen planus (OLP) [17]. Moreover, one recent study showed that there was a different HCMV-miRNAs pattern between latent and lytic *in vitro* [11]. Nevertheless, no report about the relationship between the HCMV DNA titers and HCMV-miRNAs expression levels in *vivo*. In addition, expression patterns of HCMV miRNAs and their roles in the transformation from latency to reactivation have not yet been examined *in vivo*.

Since understanding the HCMV-miRNA expression pattern during latency phase, the reactivation phase and the shift expression between the above two phases will offer great benefit for HCMV associated diseases therapy, and may also provide clues for preventing reactivation of the virus from latency. In the present study, we assessed the *in vivo* expression pattern of HCMV miRNAs in the patients which with the detection of HCMV IgG seropositive, IgM seronegative, differ by the HCMV DNA copies up and below for the 500 IU/mL (viremia or not which equivalent to reactivation or latency phase), and examined their potential as predictors of clinical and virological endpoints. We found a panel of HCMV-encoded miRNAs that showing different expression level between the latency and the reactivation, and some may be used as HCMV indicators, especially in the patients who suffered with autoimmune diseases which co-infected with the latency HCMV virus.

Methods

Participants and study design

A total of 3,986 subjects with high risk HCMV infection were recruited in this study. All the patients were hospitalized in Nanjing Drum Tower Hospital between January 2016 and March 2017. After HCMV DNA titers and HCMV serological examination, a training cohort that containing 24 patients with HCMV viremia (HCMV DNA > 500 IU/mL), HCMV IgG seropositive and HCMV IgM seronegative as the case set (defined as reactivation infectious), and another 24 patients with HCMV DNA copies less than 500 IU/mL,

HCMV IgG seropositive and HCMV IgM seronegative as the control set (defined as latency infectious) was used for screening the differential expression pattern of HCMV miRNAs. A validation cohort that containing 36 patients for the case set and 36 patients for the control set with the same above criterial was used to confirm the results of the training cohort. An additional independent cohort including 47 samples from 5 patients with leukemia (2 severe aplastic anemia patients, 1 myelodysplastic syndromes patient, 1 acute myeloid leukemia M2a patient and 1 acute lymphoblastic leukemia patient) who underwent bone marrow transplantation (the samples were collected at different time points during the antiviral therapy with ganciclovir) were also collected. The overall study design is shown in Fig. 1. All clinical data and blood samples were obtained from participants who had given written informed consent, according to protocols approved by the Ethics Committee of Nanjing Drum Tower Hospital. For all the patients, the age, gender, diagnosis, white blood cell count, C reactive protein (CRP), HCV, EBV and HIV status were recorded and used for the study (Table 1).

Table 1
Demographic and clinical features of training set and validation set in the present study

	Training set			Validation set		
Variable	Viremia (n = 24)	Control (n = 24)	P values	Viremia (n = 36)	Control (n = 36)	P values
Age, years†	43.3 ± 3.9	52 ± 3.6	0.11‡	52.8 ± 3.3	49.7 ± 3.2	0.50 ‡
Sex, n			0.25§			0.48 §
male	10 (41.7%)	14 (58.3%)		19 (52.8%)	16 (44.4%)	
female	14 (58.3%)	10 (41.7%)		17 (47.2%)	20 (55.6%)	
HCMV DNA Copies¶	1755 (1048,3450)	-		1995 (796,9995)	-	
Anti-HCMV IgG (IU/mL) †	1.58 ± 0.16	1.74 ± 0.19	0.53 ‡	1.90 ± 0.15	1.63 ± 0.15	0.22 ‡
Anti-HCMV IgM, n						
positive	0 (0%)	0 (0%)		0 (0%)	0 (0%)	
negative	24 (100%)	24 (100%)		36 (100%)	36 (100%)	
HCV, n						
positive	0 (0%)	0 (0%)		0 (0%)	0 (0%)	
negative	24 (100%)	24 (100%)		36 (100%)	36 (100%)	
EB, n			0.24 §			0.47 §
positive	12 (50%)	16 (66.7%)		13 (36.1%)	16 (44.4%)	
negative	12 (50%)	8 (33.3%)		23 (63.9%)	20 (55.6%)	
HIV, n						
positive	0 (0%)	0 (0%)		0 (0%)	0 (0%)	

†, Age data and anti-HCMV IgG are presented as the mean ± SD; ‡, Student *t* test; §, Two-sided χ^2 test; ¶, HCMV DNA copies are presented as median (25% Percentile, 75% Percentile).

	Training set			Validation set		
negative	24 (100%)	24 (100%)		36 (100%)	36 (100%)	
Diagnostic						
Leukemia, n			0.76 §			1 §
yes	8 (33.3%)	9 (37.5%)		13 (36.1%)	13 (36.1%)	
no	16 (66.7%)	15 (62.5%)		23 (63.9%)	23 (63.9%)	
Autoimmune diseases, n			0.75 §			0.44 §
Yes	6 (25%)	7 (29.2%)		9 (25%)	12 (33.3%)	
no	18 (75%)	17 (70.8%)		27 (75%)	24 (66.7%)	

†, Age data and anti-HCMV IgG are presented as the mean ± SD; ‡, Student *t* test; §, Two-sided χ^2 test; ¶, HCMV DNA copies are presented as median (25% Percentile, 75% Percentile).

HCMV DNA Titers

The HCMV DNA titers in peripheral blood leukocytes were determined by quantitative real-time PCR (qPCR). In brief, DNA was extracted from 200 μL peripheral blood leukocytes using QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Two microliters of DNA were tested with TaqMan PCR assays using diagnostic kit for quantification of human cytomegalovirus DNA (DAAN GENE, Guangzhou, China) on a Roche LightCycler® 96 PCR System (Roche diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Ten-fold diluted recombinant plasmid that contained the HCMV target sequence was used to construct calibration curve. The absolute HCMV DNA titers of each sample were calculated through the calibrator. Results were expressed as IU per 1 mL blood.

Anti-HCMV IgG and IgM antibodies determination

Serum anti-HCMV IgG and IgM were tested using a commercially available ELISA kit (MEDSON, NJ, USA) according to the manufacturer's instructions. In brief, serum (1:100) was added to the 96-well plate which containing HCMV antigen and incubated at 37 ℃ for 1 h, the mixture was then washed for four times and incubated with a horse-radish peroxidase conjugated at 37 ℃ for 1 h. After four times washing, reactivity was determined using o-phenylenediamine and the reaction was blocked with 2.5 M sulfuric acid. For the IgG-ELISA, a calibration curve, calibrated against the 1st WHO international standard, was

used to quantitatively determine IgG antibody concentrations in each sample. For the IgM-ELISA, the test results were calculated using the optical density (OD) value at 450 nm, and the cut-off value for positivity was OD > 0.25.

Serum RNA isolation and RT-qPCR assay

Total RNA was extracted from 100 μL of serum using a 1-step phenol/chloroform purification method and precipitated using isopropyl alcohol as previously described [17]. In brief, 100 μL of serum was mixed with 300 μL deionized water, 200 μL acid phenol, and 200 μL chloroform. The mixture was vortex-mixed vigorously and incubated at room temperature for 15 min. After phase separation, the aqueous layer was mixed with 1.5 volumes of isopropyl alcohol and 0.1 volumes of 3 mol/L sodium acetate (pH 5.3). The solution was stored at -20 ℃ for 1 h. The RNA pellet was obtained by centrifugation at 16, 000 g for 20 min at 4 ℃. The resulting RNA pellet was washed once with 750 mL/L ethanol and dried for 10 min at room temperature. The pellet was dissolved in 20 μL of RNase-free water and stored at -80 ℃. To control the variability in RNA extraction and purification procedures, an exogenous plant small molecular RNA named MIR2911 (5'-GGCCGGGGGACGGGCUGGGA-3'), was spiked into each sample with a final concentration of 10^6 fmol/L during RNA isolation as a synthetic external reference for the normalization of serum miRNAs [18]. Hydrolysis probe-based quantitative reverse transcription polymerase chain reaction (RT-qPCR) was carried out using a TaqMan miRNA PCR kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions with a minor modification as previously described [18]. Briefly, 2 μL of total RNA was reverse transcribed to cDNA using AMV reverse transcriptase (TaKaRa, Dalian, China) and the stem-loop RT primer (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using hydrolysis miRNA probes on a Roche LightCycler® 96 PCR System (Roche diagnostics, Mannheim, Germany). All reactions, including no-template controls, were performed in triplicate. The Cq values were determined using the fixed threshold settings. Relative levels of HCMV miRNAs were then normalized to exogenous MIR2911 and were calculated using comparative Cq method ($2^{-\Delta Cq}$).

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6.0 software or SPSS statistical software (version 16.0). The miRNA concentrations were represented as means and standard errors (Mean ± SEM) and other clinical variables were showed as Mean ± SD or Median (interquartile range). The data analyses were performed using the non-parametric Mann-Whitney tests, Chi-square test and Pearson correlation analyses. Statistically significant was defined as a $P < 0.05$. For each miRNA, a receiver operating characteristic (ROC) curve was generated. The area under curve (AUC) values and 95% confidence interval (CI) were calculated to determine the specificity and sensitivity of diagnosis of HCMV reactivation.

Results

HCMV DNA viral load and serological results

We examined the HCMV viral load in 5,664 samples of peripheral blood leukocytes that collected from 3,986 patients by quantitative real-time PCR. Among which 145 whole blood samples were defined as viremia with the HCMV DNA titers upper than 500 IU/mL and others with HCMV DNA titers lower than 500 IU/mL. Among which, 120 samples from 120 patients with HCMV-IgG seropositive and HCMV-IgM seronegative including 60 samples with HCMV DNA titers over 500 IU/mL and 60 samples with HCMV DNA titers below 500 IU/mL were selected in our study (Fig. 1). The mean levels of HCMV DNA viral load for total 60 viremia patients were 1 925 (840.3, 6 503) IU/mL, 1 755 (1 048, 3 450) IU/mL, in the training cohort and 1 995 (795.5, 9 995) IU/mL, in the validation cohort, respectively (Table 1). For the concentrations of anti-HCMV IgG, there was no significant difference between the total HCMV viremia patients (n = 60) and the controls (n = 60) (t = 0.6013, P = 0.5488). Similar results were also observed in the training cohort and the validation cohort (t = 0.6351, P = 0.5285 and t = 1.245, P = 0.2174), respectively.

Expression profiles of HCMV-encoded miRNAs by RT-qPCR analysis

In the training cohort, 22 HCMV-encoded miRNAs were measured using RT-qPCR assay in individual serum samples from 24 patients with a HCMV DNA > 500 IU/mL (referred as case set) and 24 patients with a HCMV DNA < 500 IU/mL (referred as control set) (Fig. 1). Sixteen of the 22 HCMV-encoded miRNAs were up-regulated in case set when compared with control set ($P < 0.05$), among which 8 miRNAs were significantly up-regulated with a fold change > 2, and $P < 0.01$, including hcmv-miR-US25-2-3p, hcmv-miR-US4-5p, hcmv-miR-US25-2-5p, hcmv-miR-US25-1-3p, hcmv-miR-US25-1, hcmv-miR-UL36, hcmv-miR-UL148D and hcmv-miR-US29-3p (Table 2).

Table 2
Expression profile of HCMV-encoded miRNAs in the training set

HCMV encoded miRNAs	Viremia (n = 24)	Control (n = 24)	Fold change	P values†
US25-2-3p	$6.41 \pm 2.07 \times 10^{-2}$	$2.66 \pm 0.57 \times 10^{-2}$	2.41	0.0028
US4-5p	$9.51 \pm 6.55 \times 10^{-3}$	$1.25 \pm 0.33 \times 10^{-3}$	7.62	0.0035
US-25-2-5p	$6.38 \pm 1.69 \times 10^{-2}$	$2.54 \pm 0.69 \times 10^{-2}$	2.50	0.0037
US25-1-3p	$1.98 \pm 0.75 \times 10^{-2}$	$6.71 \pm 1.59 \times 10^{-3}$	2.95	0.0039
US25-1	$2.46 \pm 0.67 \times 10^{-3}$	$9.53 \pm 2.28 \times 10^{-4}$	2.58	0.005
UL36	$2.19 \pm 0.55 \times 10^{-2}$	$9.97 \pm 2.90 \times 10^{-3}$	2.20	0.0055
UL148D	$1.96 \pm 0.50 \times 10^{-1}$	$7.73 \pm 1.62 \times 10^{-2}$	2.54	0.0081
US29-3p	$2.00 \pm 0.57 \times 10^{-3}$	$7.51 \pm 1.67 \times 10^{-4}$	2.66	0.0091
UL69	$1.34 \pm 0.35 \times 10^{-3}$	$6.09 \pm 1.46 \times 10^{-4}$	2.20	0.0117
US5-1	$1.69 \pm 0.53 \times 10^{-2}$	$8.74 \pm 2.54 \times 10^{-3}$	1.93	0.0117
US22-5p	$2.01 \pm 0.66 \times 10^{-1}$	$8.19 \pm 1.80 \times 10^{-2}$	2.46	0.0132
US33-3p	$2.29 \pm 0.52 \times 10^{-3}$	$1.07 \pm 0.25 \times 10^{-3}$	2.15	0.0132
UL22a	$1.12 \pm 0.29 \times 10^{-2}$	$4.79 \pm 1.07 \times 10^{-3}$	2.34	0.0171
UL70-5p	$1.20 \pm 0.36 \times 10^{-2}$	$5.29 \pm 1.71 \times 10^{-3}$	2.28	0.0262
UL36*	$2.32 \pm 0.74 \times 10^{-3}$	$6.76 \pm 1.79 \times 10^{-4}$	3.45	0.0267
US22-3p	$4.38 \pm 1.29 \times 10^{-3}$	$2.24 \pm 0.62 \times 10^{-3}$	1.95	0.038
US5-2-3p	$3.89 \pm 1.09 \times 10^{-4}$	$1.35 \pm 0.35 \times 10^{-4}$	2.87	0.068
UL112	$2.91 \pm 0.82 \times 10^{-2}$	$1.23 \pm 0.34 \times 10^{-2}$	2.37	0.0889
UL22a*	$4.18 \pm 1.24 \times 10^{-3}$	$1.53 \pm 0.44 \times 10^{-3}$	2.72	0.1147
UL112-5p	$4.53 \pm 1.36 \times 10^{-3}$	$1.95 \pm 0.59 \times 10^{-3}$	2.32	0.1254
US4-3p	$6.10 \pm 1.96 \times 10^{-3}$	$4.54 \pm 1.87 \times 10^{-3}$	1.34	0.2399
UL59	$2.56 \pm 0.61 \times 10^{-2}$	$1.40 \pm 0.35 \times 10^{-2}$	1.83	0.2977

miRNAs are presented as mean ± SEM.†, Mann-Whitney U test.

Confirmation of the up-regulated HCMV-encoded miRNAs

Subsequently, the 8 up-regulated HCMV-encoded miRNAs were confirmed in an additional cohort including 36 cases and 36 controls (refereed as validation cohort). The 8 miRNAs exhibited consistent alterations as the results from the training cohort (Fig. 2a-h). Moreover, when combined the results of the training set and validation set (Fig. 2i-p), consistent with our expectations, the concentrations of all the eight HCMV-miRNAs were significantly increased in the viremia patients as compared with control group.

HCMV miRNAs in the autoimmune disease patients

We next analyzed the 8 up-regulated hcmv-miRs in the only antoimmune disease patient subgroup, and found that seven HCMV-miRNAs were significantly up-regulated except for hcmv-miR-US29-3p. Notably, four miRNAs including hcmv-miR-US25-2-3p, hcmv-miR-US25-2-5p, hcmv-miR-US25-1-3p and hcmv-miR-UL148D were markedly increased with a P value of < 0.01 (Fig. 3a-g). Receiver operating characteristic curve (ROC) analysis on the seven selected HCMV-miRNAs yielded areas under ROC curve (AUCs) ranged from 0.74 to 0.79 (Fig. 3h). Using the optimal cutoff value, we obtained the following sensitivity and specificity values: hcmv-miR-US25-2-3p (AUC: 0.794, sen: 85.7%, spe: 72.2%, 95%CI: 0.629, 0.958), hcmv-miR-US4-5p (AUC: 0.766, sen: 85.7%, spe: 66.7%, 95%CI: 0.597, 0.935), hcmv-miR-US25-2-5p (AUC: 0.786, sen: 78.6%, spe: 72.2%, 95%CI: 0.628, 0.943), hcmv-miR-US25-1-3p (AUC: 0.794, sen: 78.6%, spe: 77.8%, 95%CI: 0.632, 0.955), hcmv-miR-US25-1 (AUC: 0.766, sen: 85.7%, spe: 77.8%, 95%CI: 0.589, 0.942), hcmv-miR-UL36 (AUC: 0.738, sen: 85.7%, spe: 66.7%, 95%CI: 0.562, 0.914) and hcmv-miR-UL148D (AUC: 0.794, sen: 85.7%, spe: 77.8%, 95%CI: 0.629, 0.958).

Viral miRNAs expression pattern in the blood of patients with HCMV disease

In combined samples of the training set and validation set of patients (n = 60), we found that 91.67% patients had detectable expression of at least one HCMV-miRNA (10% with one, 5% with two, 6.67% with three, 3.33% with four, 3.33% with five, 6.67% with six, 6.67% with seven and 50% with eight) (Fig. 4a). Analysis of individual HCMV-miRNA showed that hcmv-miR-US4-5p was the most commonly detected in 81.67% of the patient, followed by hcmv-miR-US29-3p with 76.67%, hcmv-miR-UL36 and hcmv-miR-US25-1-3p with 73.33%, hcmv-miR-UL148D with 61.67%, hcmv-miR-US25-2-5p with 58.33%, hcmv-miR-US25-2-3p and hcmv-miR-US25-1 with 56.67% (Fig. 4b).

Association of serum hcmv-miRNAs' levels with clinical parameters

We subsequently wonder whether serum hcmv-miRNAs' levels were correlated with clinical parameters. We evaluated the associations between the clinical features and miRNA abundance using Pearson correlation analysis in all of the studied individuals. Hcmv-miR-US25-1-3p levels were significantly correlated with HCMV DNA copies (r = 0.2970, P = 0.022) (Fig. 5a). HCMV DNA copies was significantly correlated with CRP (r = 0.3007, P = 0.0246) (Fig. 5b) but not with WBC count (r = 0.0497, P = 0.80). The concentrations of anti-HCMV IgG was significantly correlated with hcmv-miR-US25-1 (r = 0.3071, P = 0.018) (Fig. 5c) but not with other miRNAs. There was no significant difference in WBC count (t = 0.9167, P = 0.3612), CRP (t = 0.6005, P = 0.5494), PCT (t = 1.229, P = 0.2249), and ESR (t = 0.6939, P = 0.4908) between the viremia and the control group.

Independent cohorts or antiviral treatment

Five patients received antiviral therapy, including 2 patients with severe aplastic anemia, one with myelodysplastic syndromes, one with acute myeloid leukemia M2a and acute lymphoblastic leukemia of one case respectively which underwent bone marrow transplantation. 47 serum samples were collected at different time points during the antiviral therapy, and hcmv-miR-US25-1-3p levels was significantly correlated with HCMV DNA copies during the antiviral therapy (Fig. 6a-e).

Discussion

Understanding HCMV shift from latency to reactivation offers great potential for therapy, as it may prevent reactivation of the virus from latency. However, there were only few reports about the HCMV infection related to the isolation of peripheral blood mononuclear cells from HCMV IgG positive donor [19, 20]. The present study is aim to explore the expression characteristic of HCMV-miRNAs in the process of HCMV infection from latency to reactivation *in vivo*, and also to discover the possible function of specific HCMV-miRNAs in such process of viral infection. In this study, sixteen of 22 screened HCMV-miRNAs were found higher in viremia group than in DNA negative group, which was in consistent with studies assessing *in vitro* HCMV-miRNAs expression in HCMV lytic infection models [20] and in solid organ transplant recipients *in vivo* [16].

The latest studies of the HCMV-miRNAs as diagnostic indicator were found in patients with glioblastoma, rheumatoid arthritis (RA), diabetes mellitus and essential hypertension detected with circulating hcmv-miR-UL112-3p [14, 15]. In those studies, HCMV-miRNAs expression was more common and abundant among hypertensive and diabetes patients [14, 15]. Comparatively, detection of hcmv-miR-UL22A-5p in transplant recipients with HCMV infections had the highest sensitivity for the prediction of subsequent virologic recurrence [16]. Our research group also demonstrated different expression profile of HCMV-encoded miRNAs in plasma sample from patients suffered with OLP, and 5 of the miRNAs including hcmv-miR-UL112-3p, hcmv-miR-UL22a-5p, hcmv-miR-UL148D, hcmv-miR-UL36-5p and hcmv-miR-UL59 were significantly upregulated in OLP samples compared with normal samples [17]. All these results indicate that these HCMV-encoded-miRNAs may share similar physiological and pathological roles in

HCMV infection related diseases mentioned above, as well as in this study, even though much further study is needed to confirm this. And also, these HCMV-encoded-miRNAs may be potential biomarkers for indication of the switch of HCMV from latency to reactivation.

In warfare terms, HCMV is in a 'stand-off' relationship with immune system, poised to replicate rapidly if the established immune response becomes impaired, which can happen in patients given immunosuppressive drugs [2]. Increasing evidence suggests that HCMV constitute an important trigger of systemic lupus erythematosus (SLE) and can further aggravate disease progression [21]. Specifically, immunopathogenic mechanisms by which HCMV could contribute to the course of autoimmune disease have been indicated, for example, molecular mimicry by the UL94 antigen in systemic scleroderma (SSc) patients [22] and UL83/pp65 in SLE patients [23], UL44 antigen in SLE [24], US31 in inducing NF-kB mediated mono-macrophage inflammation in the pathogenesis and development of SLE [21], as well as aggravation of joint inflammation by induction and expansion of $CD4^+/CD28^-$ T-cells in HCMV infected RA patients [23, 25]. Chronic inflammation in the autoimmune patients is a driving force for reaction if latent HCMV [26],which results in a vicious cycle. To the best of our knowledge, there was no report about the relationship between HCMV-encoded miRNAs and autoimmune diseases. We found that some hcmv-encoded-miRs may act as a predictor of reactivation, especially for hcmv-miR-US25-1-3p, which showed obviously higher in the reactivation patients than the latency patients in the autoimmune patients' group. Compared with the ROC analysis on the selected hcmv-miRNAs in the patients suffered with autoimmune diseases, the AUCs were ranged from 0.74 to 0.79 (Fig. 3h), and hcmv-miR-US25-1-3p showed the greatest diagnostic performance, which suggests that hcmv-miR-US25-1-3p may play an immunomodulatory role or be a potential biomarker for the switch of HCMV from latency to reactivation.

The origination and function of HCMV miRNAs in circulation was not fully promulgated currently; however, most organs and tissues can be infected with HCMV *in vivo* due to the broad cell tropism of the virus [19], such as fibroblasts and smooth muscle cells are fully permissive to lytic replication [19], endothelial and epithelial cells of many organs undergoes a more protracted replication cycle that results in persistent low-level release of virus, and less differentiated $CD34^+$ hematopoietic progenitor cells in the bone marrow as well as $CD14^+$ monocytes being maintained latency infection [27–29]. Thus, all of the above-mentioned cell types may be the potential source of HCMV miRNAs. On the other hand, latency necessarily requires a careful balance between host and virus, and a successful reactivation from latency under the correct conditions such as following mobilization of stem cells from the bone marrow and myeloid differentiation into macrophages should be the full viral gene expression [19]. The majority of studies investigating the expression of HCMV encoded miRNAs during latency have been performed by HCMV infected THP-1 cells which making an experimental latency [20]. Among different infection stages such as lytic and latent infection, different expression pattern of HCMV miRNAs was a feature of changes, which may play an important role in regulating the expression of latent and lytic-related genes [20]. For example, hcmv-miR-US25-1 was demonstrated to inhibit HCMV DNA replication through the reduction of IE72 and pp65 expression [30], and hcmv-miR-US25-2 can reduce HCMV replication by targeting not only the above targets but also eukaryotic translation initiation factor 4A1 (eIF4A1) [31]. But

in these cells, reactivation is very ineffective, and so may not accurately represent a natural latent infection [29]. In this study, we analyzed the difference of HCMV-encoded-miRNAs in the circulation of clinical patients who suffered with HCMV latency and reactivation, which can be truly reflected *in vivo*. We found 16 of 22 HCMV-encoded-miRNAs detected showed increased expression in the reactivation set compared to the latent set (Table 2). These results suggest that, similar to lytic gene transcription, most HCMV miRNAs are strongly induced expression, which was similar with the *in vitro* study [10]. In a recent report, eight HCMV-encoded-miRNAs were found in latently infected cells, with hcmv-miR-UL112-3p and hcmv-miR-US22-5p being the two most abundant miRNAs during latency [20], which were not detected significant increasing upon reactivation in our study. In addition, a subset of HCMV-encoded-miRNAs show remarkable increase upon reactivation of lytic infection including hcmv-miR-US25-2-5p, hcmv-miR-US25-1-5p, and hcmv-miR-UL112-3p *in vitro* [20]. Whether or not the virus is trying to express certain HCMV-encoded-miRNAs as the regulatory molecules in order to maintain its latent state remains to be further verified. Similar to the expression pattern of hcmv-miR-US25-1, hcmv-miR-UL112-1 was expressed early in infection and gradually increased as the infection progressed which act to attenuate replication of HCMV and implicate in latency control of HCMV by targeting HCMV IE1, UL112/113, UL120/121 and UL144 [8, 9]. However, in our study, the levels of hcmv-miR-UL112-1 in patients who suffered the reactivation infection with HCMV were not significantly higher than that in latent infection patients. Perhaps it just plays a role in inhibiting viral replication in the early stages of infection. Hcmv-miR-US29-5p and hcmv-miR-US29-3p were expressed at different stage of HCMV infection pattern. During lytic infection, hcmv-miR-US29-5p was expressed and undetectable during latent phase, while the opposite occurred with hcmv-miR-US29-3p [20]. This was inconsistent with the results in this study that the level of hcmv-miR-US29-3p was significantly higher than that of latent infection after reactivation infection. Many results suggest that hcmv-miR-US25-1, or hcmv-miR-US25-1-5p, is involved in diverse process; not only cell cycle control, but also virus replication inhibition as well as apoptosis regulation [5, 6, 32]. It has been reported that hcmv-miR-US25-1-3p has the ability to downregulate CDK6 gene associated with suppress cell cycle progression [19, 33]. Here, we identified highly HCMV-miRNA expression pattern upon reactivation from latency *in vivo*, especially for hcmv-miR-US25-1-3p in autoimmune disease patient, which provides a key foothold for the study of HCMV virology, even though HCMV-miRNAs' function remain to be study further in natural diseases such as in autoimmune disease. We also found that hcmv-miR-US25-1-3p expression levels was consistent with HCMV DNA copies in the HCMV antiviral therapy period of patients with lymphocytic leukemia who accepted with hematopoietic stem cell transplantation, which suggested HCMV miRNAs expression be efficient for the diagnosis, evaluation and prediction for HCMV infection.

Conclusion

To the best of our knowledge, we are the first to report a change characterization profile of HCMV encoded miRNA in circulation of HCMV infected patients from latency to reactivation. We identified that hcmv-miR-US25-1-3p may be used as a potential predictor for the switch upon reactivation from latency

in patients suffered with autoimmune diseases. These findings may reveal important insights into the pathogenesis of HCMV infection.

Abbreviations

HCMV: human cytomegalovirus; RT-qPCR: quantitative reverse transcription polymerase chain reaction; miRNAs: microRNAs; qPCR: quantitative real-time PCR; ELISA: enzyme linked immunosorbent assay; ROC: receiver operating characteristic curve; OLP: oral lichen planus; AUC: area under curve; OD: optical density; CI: confidence interval; RA: rheumatoid arthritis; SLE: systemic lupus erythematosus.

Declarations

Acknowledgments

We would like to acknowledge the reviewers for their helpful comments on this study.

Authors' Contributions

(I) Conception and design: C Zhang, C-Y Zhang and C Wang; (II) Administrative support: C Zhang, C Wang, C-Y Zhang and J Wang; (III) Provision of study materials or patients: W Zhou and H Shen; (IV) Collection and assembly of data: W Zhou and M Ding; (V) Data analysis and interpretation: W Zhou, Y Zhong and Y Bian; (VI) Manuscript writing: W Zhou, C Zhang, and C Wang; (VII) Final approval of manuscript: All authors.

Funding

This study was supported by grants from National Natural Science Foundation of China (no. 81472021, 81672102, and 81772282), Fund of State Key Laboratory of Analytical Chemistry for Life Science (no. 5431ZZXM1601, and 5431ZZXM1907), and Foundation of Jiangsu Provincial Medical Youth Talent (QNRC2016893).

Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Nanjing Drum Tower Hospital. All patients signed the informed consent form.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest.

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Figures

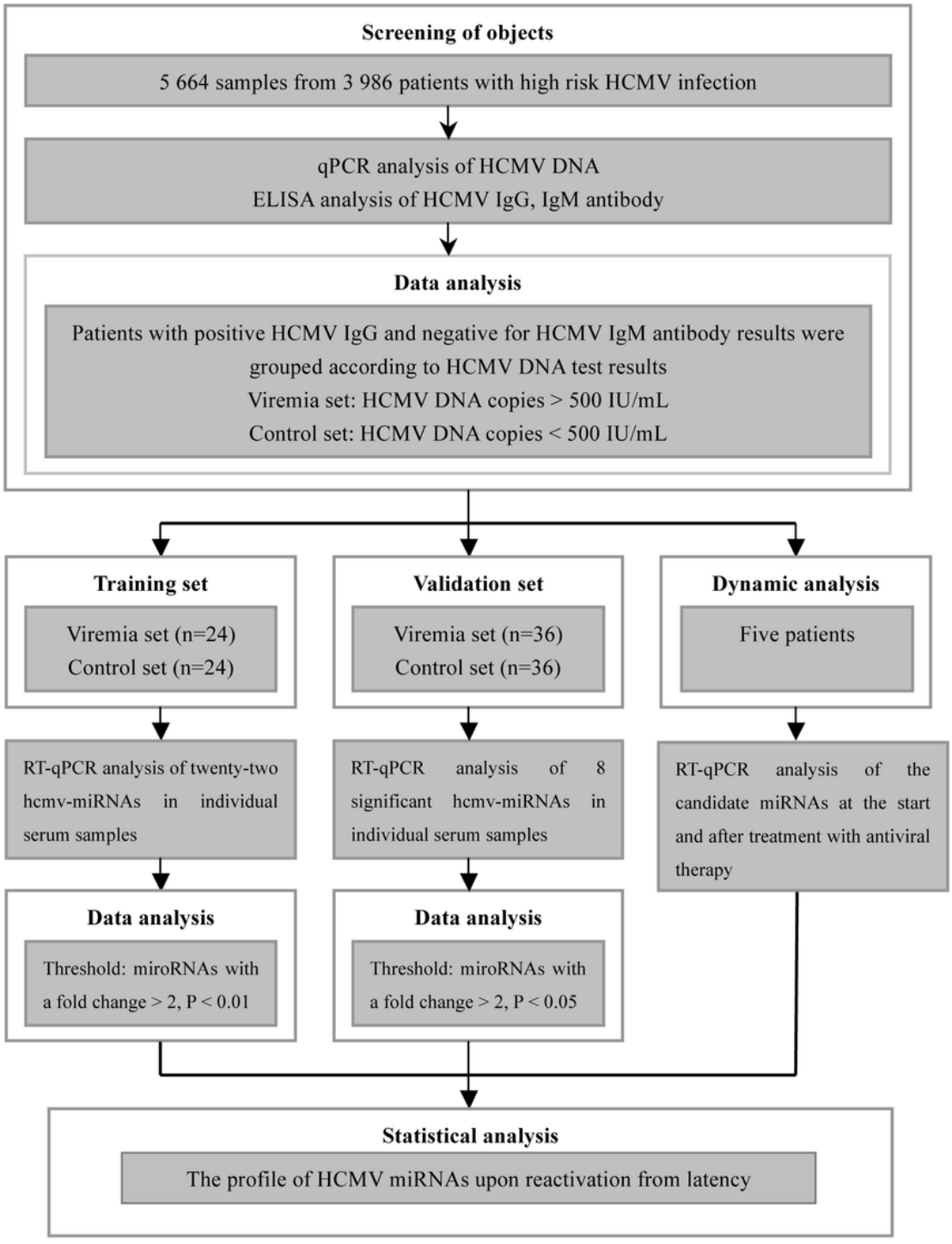

Figure 1

Study design. HCMV, human cytomegalovirus; qPCR, quantitative real-time PCR; ELISA, enzyme linked immunosorbent assay; RT-qPCR, quantitative reverse transcription polymerase chain reaction.

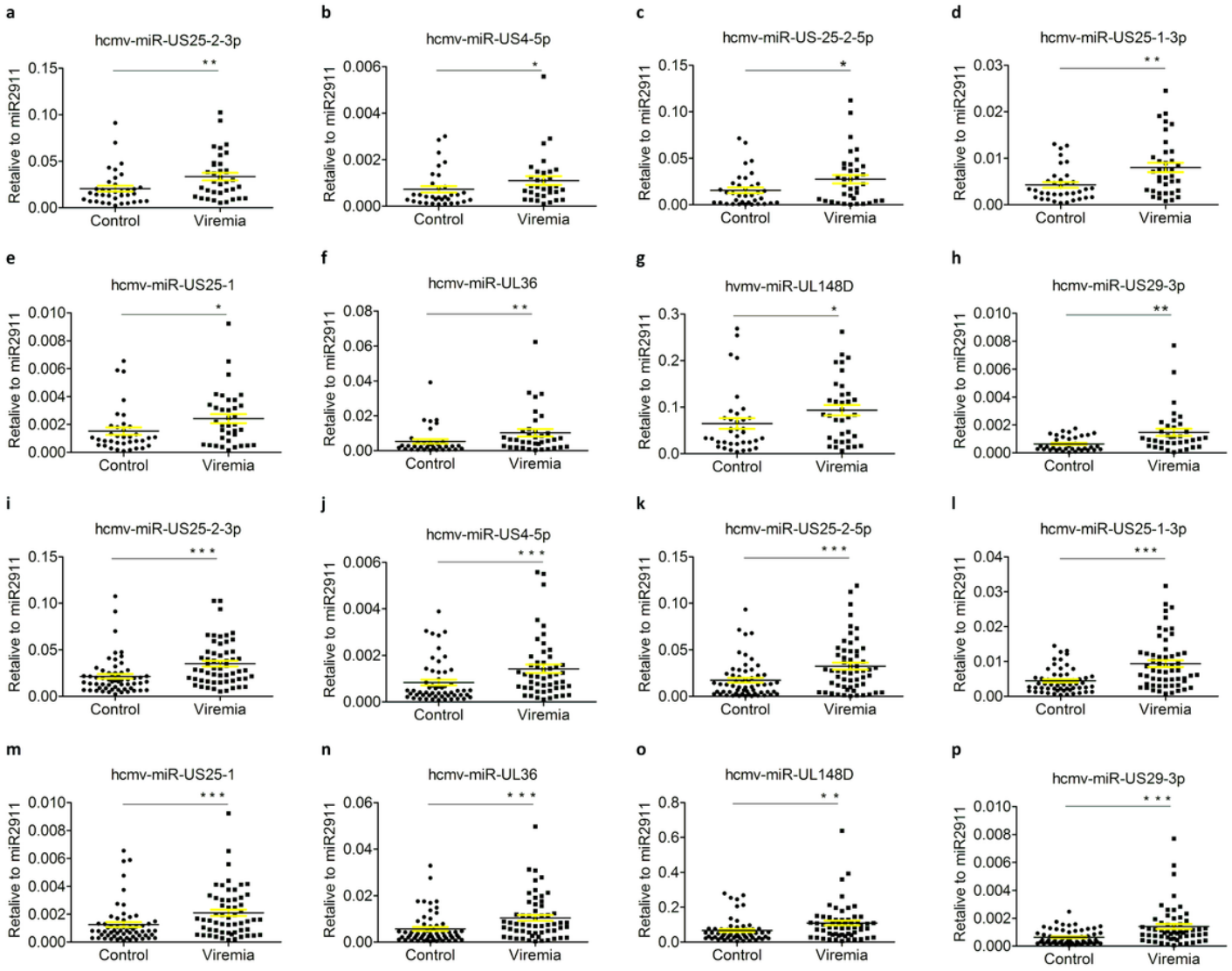

Figure 2

Serum levels of the 8 upregulated hcmv-miRNAs in the validation set and the combination set. a, i hcmv-miR-US25-2-3p; b, j hcmv-miR-US4-5p; c, k hcmv-miR-US25-2-5p; d, l hcmv-miR-US25-1-3p; e, m hcmv-miR-US25-1; f, n hcmv-miR-UL36; g, o hcmv-miR-UL148D; h, p hcmv-miR-US29-3p. Cq values were converted to relative concentrations normalized to MIR2911 values, and were calculated using the comparative Cq method (2-ΔCq). Each point represents the mean of triplicate sample. □, P < 0.05; □□, P < 0.01; □□□, P < 0.001.

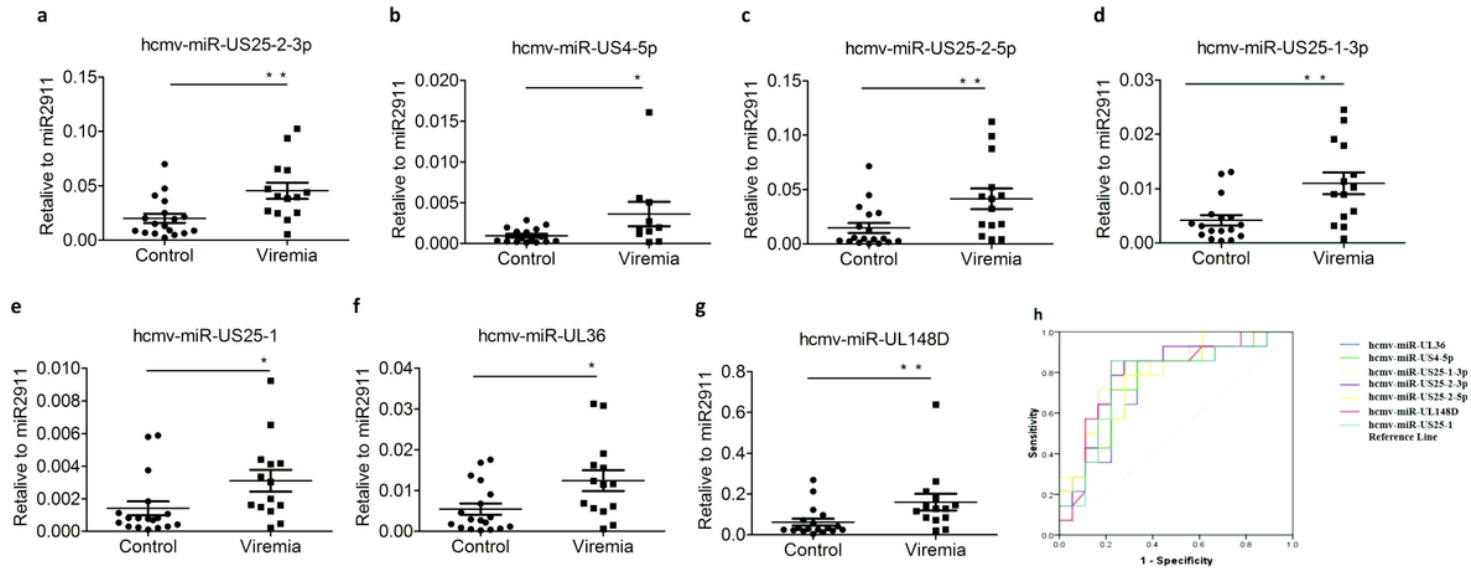

Figure 3

Serum levels of the 7 upregulated hcmv-miRNAs and ROC in the autoimmune disease patient subgroup. a hcmv-miR-US25-2-3p; b hcmv-miR-US4-5p; c hcmv-miR-US25-2-5p; d hcmv-miR-US25-1-3p; e hcmv-miR-US25-1; f hcmv-miR-UL36; g hcmv-miR-UL148D; h ROC analysis of the 7 upregulated hcmv-miRNAs in the autoimmune disease patients. Areas under ROC curve (AUCs) ranged from 0.74 to 0.79. Cq values were converted to relative concentrations normalized to MIR2911 values and were calculated using the comparative Cq method (2-ΔCq). Each point represents the mean of triplicate sample. □, P < 0.05; □□, P < 0.01.

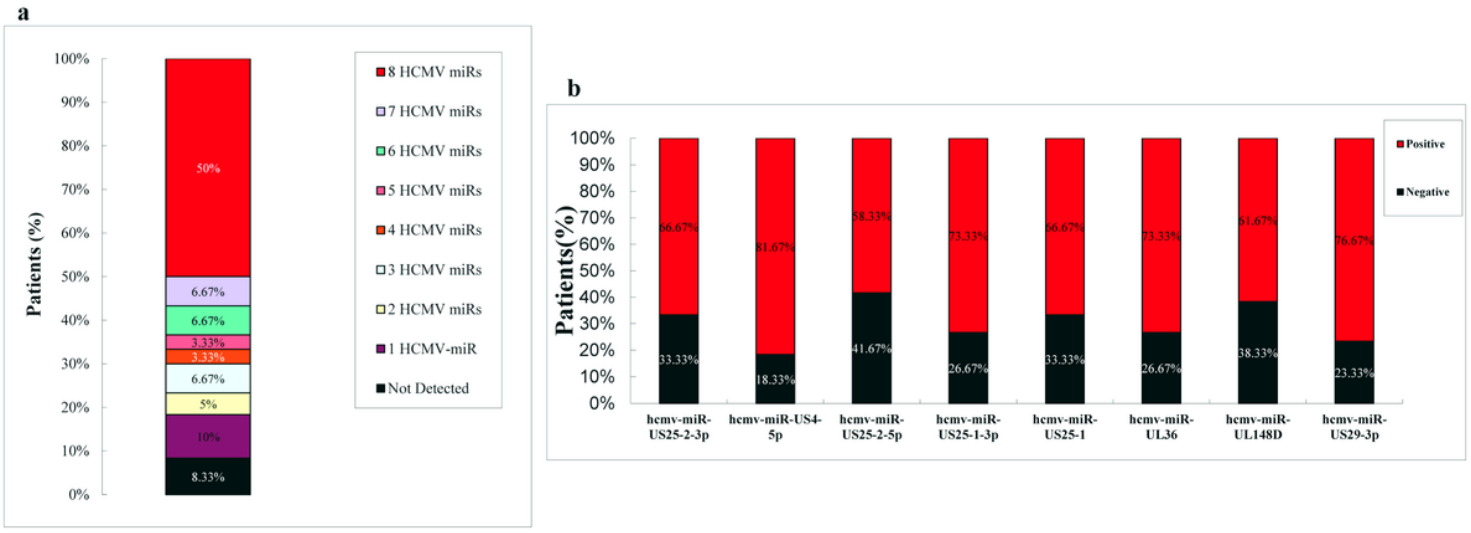

Figure 4

Hcmv-miRNAs as serum markers for HCMV infection. a Percentage of patients' positive for the serum expression of one, two, three, four, five, six, seven and eight hcmv-miRs (hcmv-miR-UL36, hcmv-miR-US4-

5p, hcmv-miR-US25-1-3p, hcmv-miR-US25-2-3p, hcmv-miR-US29-3p, hcmv-miR-US25-2-5p, hcmv-miR-UL148D and hcmv-miR-US25-1); b Positive rates of each hcmv-miRs in the combined samples.

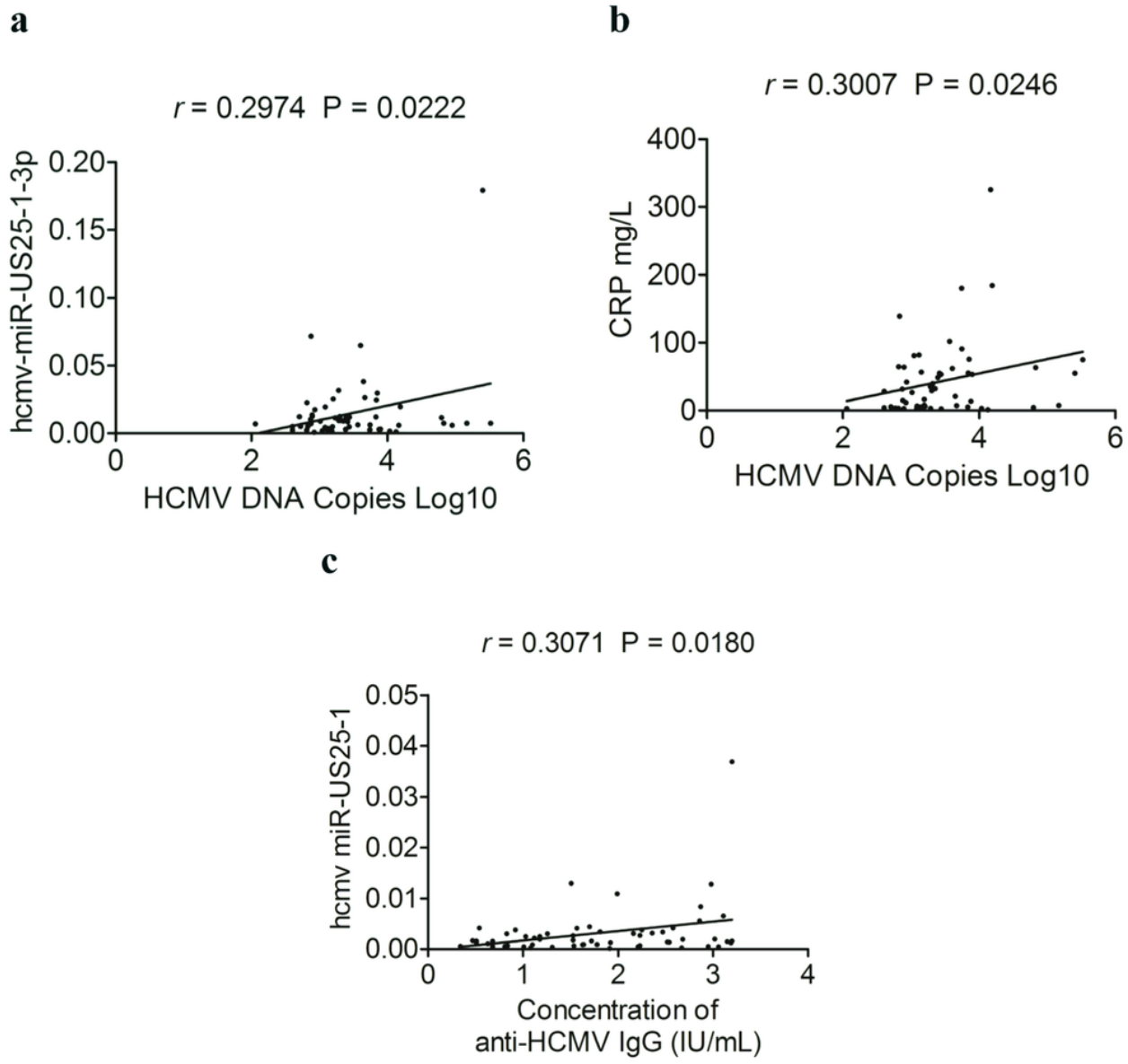

Figure 5

Associations between the clinical features and miRNA abundance. a Correlation between HCMV DNA copies and hcmv-miR-US25-1-3p; b Correlation between HCMV DNA copies and CRP; c Correlation between the concentration of anti-HCMV and hcmv-miR-US25-1. Pearson correlation analysis.

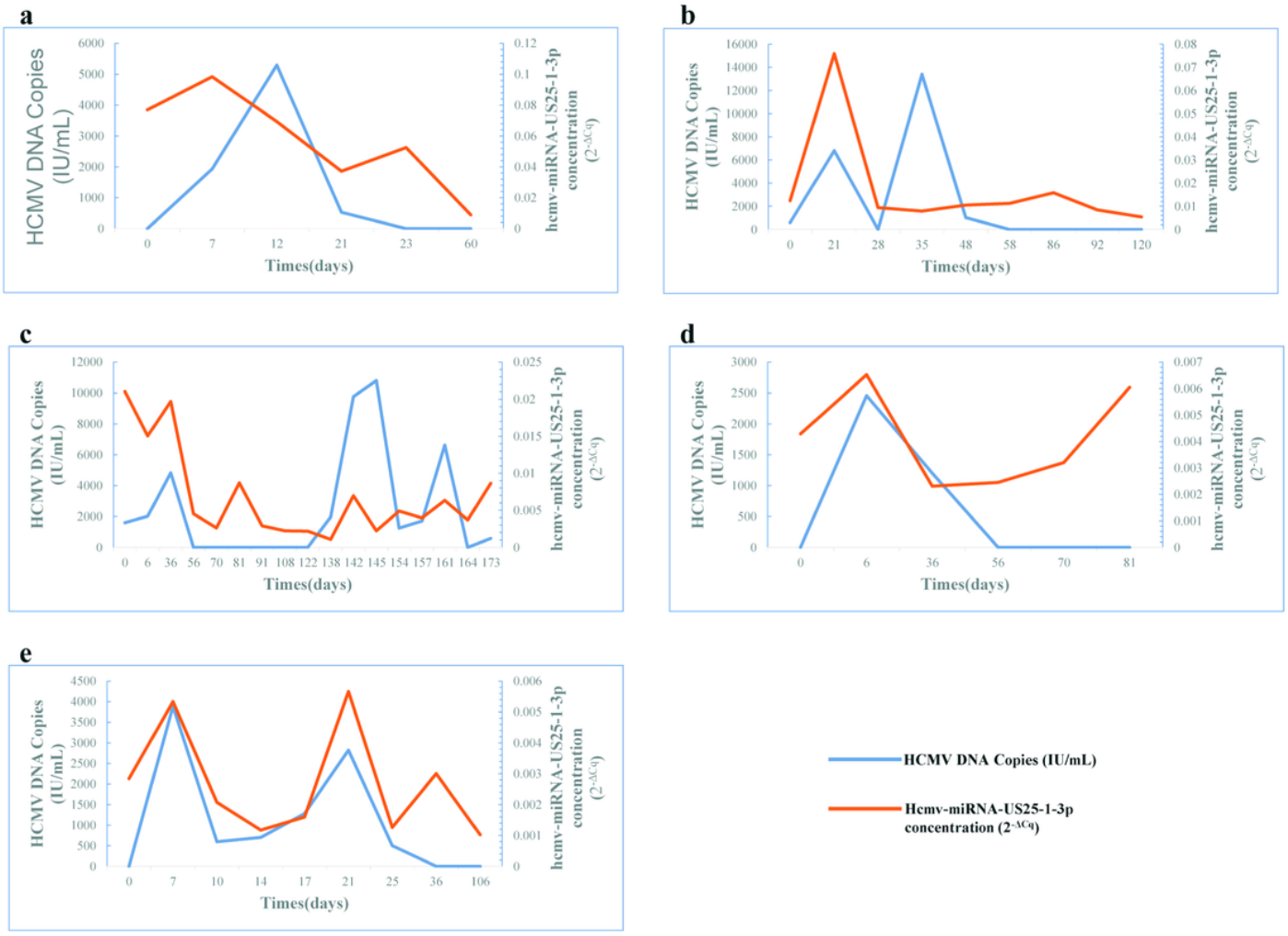

Figure 6

Dynamic monitoring of changes in HCMV DNA and hcmv-miR-US25-1-3p during the treatment of anti-HCMV virus in five patients. a Patient no. 1 suffered with severe aplastic anemia; b Patient no. 2 suffered with severe aplastic anemia; c Patient no. 3 suffered with AML M2a; d Patient no. 4 suffered with myelodysplastic syndromes; e Patient no. 5 suffered with ALM. ALM, acute lymphoblastic leukemia; AML M2a, acute myeloid leukemia M2a.