Medical Microbiology and Immunology

An investigation of the utility of plasma Cytomegalovirus (CMV) microRNA detection to predict CMV DNAemia in allogeneic hematopoietic stem cell transplant recipients

--Manuscript Draft--

Manuscript Number:	MMIM-D-19-00123R1
Full Title:	An investigation of the utility of plasma Cytomegalovirus (CMV) microRNA detection to predict CMV DNAemia in allogeneic hematopoietic stem cell transplant recipients
Article Type:	Original Article
Keywords:	Cytomegalovirus (CMV); CMV DNAemia; plasma CMV miRNA; allogeneic hematopoietic stem cell transplantation (allo-HSCT)
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Abstract:	Precise identification of patients at highest risk for developing Cytomegalovirus (CMV) DNAemia may improve CMV infection management in the allogeneic hematopoietic stem cell transplantation (allo-HSCT) setting. Here, we studied the potential use of detecting free CMV micro(mi)RNAs circulating in plasma for predicting CMV DNAemia in this clinical scenario. A total of 62 adult allo-HSCT recipients were included in this prospective observational multicenter study. Plasma CMV DNA load was monitored

	using the CMV RealTime CMV PCR (Abbott Molecular, Des Plaines, IL, USA). Detection of mature CMV miRNAs in plasma drawn by days +7, +14 and +30 after allo-HSCT was performed using the miScript PCR System (Qiagen, Hilden, Germany). Assays could be optimized for five out of the seven targeted CMV miRNAs: UL36-5p, US33-5p, UL148D, UL22A-5p and UL112-3p. Of the 62 patients included in the study, 42 developed a first episode of CMV DNAemia at a median of 35 days after allo-HSCT. All targeted CMV miRNA were detected early after transplantation, with CMV miRNA US33-5p and UL112-3p the most commonly found species at any time point; nevertheless, neither the detection rate of CMV miRNAs nor their abundance allowed discrimination between patients with subsequent CMV DNAemia and those with no CMV DNAemia. The data presented herein do not support any predictive utility of these CMV miRNAs for first episodes of CMV DNAemia in a cohort consisting primarily of allo-HSCT patients receiving haploidential allografts.

Reviewer #1: This is a concise clinical study to determine whether serum levels of CMV expressed miRNAs can be used as predictive diagnostic markers for CMV DNAemia. The major concern is the specificity and sensitivity of the assays used. miRNA assays tend to have higher background levels of signal compared to RT-PCR assays for full length transcripts due to the constraints of the relatively short miRNA resulting in the use of only a single specific forward primer and a generic reverse primer. It is therefore critical to determine whether any signal detected is due to specificallyamplification of the target miRNA or due to background signal. It was not clear from the study what was defined as a positive signal based on CT levels over background. Table 5 indicates that CT levels for positive samples are very high (greater than 35). Without any kind of negative control, ideally samples from seronegative donor and recipient, or at least RNA from uninfected cells it isimpossible to determine whether the signal detected is real or background. It would also be useful to include more details on the validation of the assays including sensitivity levels and specificity of the assays.

We fully agree with the reviewer. As for the specificity issue, we set the cut-off for "positivity" (Ct 40 cycles) by determining background signals in consecutive plasma specimens (n=3) from 6 CMV D-/R- patients, drawn within the same time window than that in study patients. Also, melting curve analyses were performed for each miRNA in every run. In addition, we included an endogenous control (miRNA hsa-let-7i) to normalize and to ensure miRNA yield across samples. We also included the C.elegans spike-in control (10^8 copies/microliter) and the retrotranscription control miRTC in each sample. The commercially-available miScript miRNA system has been optimized for the detection and quantification of very low amounts of mature miRNA in biological fluids; nevertheless, no standard curves using synthetic oligonucleotides for LOD analyses were built, as synthetic oligonucleotides diluted in water may perform differently than endogenous miRNAs found in plasma. In any case whatever the sensitivity of the PCR assays was, it had to be the same for specimens from patients either developing or not CMV DNAemia. We nevertheless acknowledge this as a limitation in the revised version of the manuscript. We have also extended the material and methods section as requested.

MMIM-D-19-00123. REVISED VERSION

An investigation of the utility of plasma Cytomegalovirus (CMV) microRNA detection to predict CMV DNAemia in allogeneic hematopoietic stem cell transplant recipients

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Running Title: Plasma CMV miRNA and prediction of CMV DNAemia in Allo-HSCT.

Abstract

Precise identification of patients at highest risk for developing Cytomegalovirus (CMV) DNAemia may improve CMV infection management in the allogeneic hematopoietic stem cell transplantation (allo-HSCT) setting. Here, we studied the potential use of detecting free CMV micro(mi)RNAs circulating in plasma for predicting CMV DNAemia in this clinical scenario. A total of 62 adult allo-HSCT recipients were included in this prospective observational multicenter study. Plasma CMV DNA load was monitored using the CMV Real*Time* CMV PCR (Abbott Molecular, Des Plaines, IL, USA). Detection of mature CMV miRNAs in plasma drawn by days +7, +14 and +30 after allo-HSCT was performed using the miScript PCR System (Qiagen, Hilden, Germany). Assays could be optimized for five out of the seven targeted CMV miRNAs: UL36-5p, US33-5p, UL148D, UL22A-5p and UL112-3p. Of the 62 patients included in the study, 42 developed a first episode of CMV DNAemia at a median of 35 days after allo-HSCT. All targeted CMV miRNA were detected early after transplantation, with CMV miRNA US33-5p and UL112-3p the most commonly found species at any time point; nevertheless, neither the detection rate of CMV miRNAs nor their abundance allowed discrimination between patients with subsequent CMV DNAemia and those with no CMV DNAemia. The data presented herein do not support any predictive utility of these CMV miRNAs for first episodes of CMV DNAemia in a cohort consisting primarily of allo-HSCT patients receiving haploidential allografts.

Key words: Cytomegalovirus (CMV), CMV DNAemia, plasma CMV miRNA, allogeneic hematopoietic stem cell transplantation (allo-HSCT).

Introduction

Cytomegalovirus (CMV) DNAemia develops frequently following allogeneic hematopoietic stem cell transplantation (allo-HSCT) [1]. Administration of antivirals with intrinsic anti-CMV activity upon detection of a pre-defined CMV DNA load in blood, known as preemptive antiviral therapy, is currently the upfront strategy to prevent CMV end-organ disease in this clinical setting [1]. The advent of new antivirals with potent anti-CMV effect and good safety profiles has renewed interest in anti-CMV prophylaxis as a first-line strategy for minimizing the impact of CMV-related clinical events on allo-HSCT recipient survival [2,3]. In this context, the availability of predictive biomarkers for CMV DNAemia may help personalize antiviral prophylaxis prescription, targeting only patients at highest risk.

MicroRNAs (miRNAs) are short (~22 nucleotide in length) non-coding RNAs incorporated within the multi-protein RNA-induced silencing complex (RISC), that direct the RISC complex to messenger RNAs (mRNAs) through partially complementary sequences usually located within the 3' untranslated region (3' UTR) and nucleotides 2–8 (the seed sequence) of the miRNAs [4,5]. Interaction between the RISC complex and the target mRNA leads to translational repression through several mechanisms [4,5]. CMV encodes at least 26 mature miRNAs (www.mirbase.org, Release-21) [see 6 for review], which are expressed during the lytic virus cycle as well

as in latently-infected cells, and target both host and viral mRNA transcripts [6]. CMV miRNAs are thought to regulate viral gene expression and manipulate cellular signaling pathways, likely playing a critical role in modulating the latent and lytic viral cell cycles and promoting virus immune evasion [7-9].

Evidence has been provided that free circulating CMV miRNA levels may be associated with certain virologic and clinical outcomes in both solid organ transplant recipients and in the context of congenital CMV infection [10,11]. To our knowledge, the potential clinical value of CMV miRNA detection for CMV infection management in allo-HSCT recipients has not been explored.

CMV viremia in immunosuppressed individuals is thought to follow virus replication in organ and tissue sites [1,12,13]. In turn, productively CMV-infected cells are known to generate and release viral miRNAs soon after infection, even before viral DNA synthesis occurs [14], which may gain access to the blood compartment. On the basis of this evidence, we hypothesized that the presence of CMV miRNA in plasma may anticipate viral DNA (CMV DNAemia) in allo-HSCT patients.

Material and methods

Patients

A total of 62 consecutive adult allo-HSCT recipients were included in this prospective observational multicenter study. The patients underwent T-cell replete allo-HSCT at the Hematology Service of the Hospital Regional Universitario, Málaga (n=29), Hospital Clínico Universitario, Valencia (n=14), Hospital General Universitario, Salamanca (n=11) and Hospital General Universitario Gregorio Marañón, Madrid (n=8), between December 2015 and May 2018. The cohort included mostly patients undergoing

haploidentical allo-HSCT with post-transplant cyclophosphamide. The median age of patients at the time of allo-HSCT was 56 years (range, 24 to 73 years). Relevant patient demographic, baseline and post-transplant clinical data are shown in Table 1. The study period comprised the first 100 days after allo-HSCT. This study was approved by the Hospital Clínico Fundación INCLIVA Ethics Committee. Informed consent was signed by all participants.

Management of active CMV infection

Plasma CMV DNA load was monitored using the CMV Real*Time* CMV PCR (Abbott Molecular, Des Plaines, IL, USA) at all participating centers. The limit of detection and quantitation of the assay was approximately 31.5 IU/ml (95% C.I.) [15]. CMV DNA monitoring was conducted on an approximately weekly basis through day +100, as per participating center protocol.

Detection of CMV miRNA in plasma

Detection of mature CMV miRNAs in plasma was performed using the miScript PCR System (Qiagen, Hilden, Germany), following the manufacturer's instructions. Cryopreserved (-80 ºC), never-thawed, leftover plasma specimens primarily used for CMV DNA quantitation at days +7, +14 and +30, were used for the analyses. Briefly, total miRNA from plasma specimens (200 µl) was extracted using the miRNeasy Serum/Plasma Kit. Reverse transcription of mature CMV miRNA was performed using Hispec Buffer. CMV miRNA-specific primers (miScript primer Assay, from Qiagen) were designed using nucleotide sequences annotated on the v11.0 release from miRBase (Supplementary Table 1). CMV miRNAs were then quantified by SYBR Green-based real-time PCR using the miScript SYBR Green PCR Kit on the ABI Prism 7500 real-

time PCR system (Applied Biosciences). Each sample was spiked with 1.6 x10^8copies/µL of *C.elegan* smiR-39 prior to extraction for normalization across samples and to monitor RNA recovery and reverse transcription efficiency. miRNA hsa-let-7i was used as endogenous miRNAfor controlling miRNA yield across samples, as it can be detected in plasma from healthy patients. This endogenous control served as well for relative quantification of target miRNA. In addition, a reverse transcription control (miRTC) was included in each sample. We set the cut-off for "positivity" of each assay by determining background signals in consecutive plasma specimens from 6 CMV D-/R- patients (3 specimens per patient), drawn within the same time window than that in study patients. Cycle threshold values (C_TS) ≥40 were deemed to represent inespecific signals. In addition, melting curve analyses were performed for each miRNA in every run to prove the specificity of the amplifications.

Definitions

CMV DNAemia was defined as detection of CMV DNA at any level in one or more plasma specimens. Acute graft-versus-host disease (aGvHD) was diagnosed and graded as previously reported [16].

Statistical analysis

Differences between medians were compared using the Mann–Whitney U test (for two independent variables). Qualitative variables were compared using the chi-square test. Two-sided exact P values were reported. A *P* value <0.05 was considered statistically significant. These statistical analyses were performed using SPSS version 20.0 (SPSS, Chicago, IL, USA).

Results

CMV DNAemia in the study population

Of the 62 patients included in the study, 42 developed a first episode of CMV DNAemia at a median of 35 days after allo-HSCT (range, 15 to 90 days). Median initial and peak CMV DNA loads were 152 IU/ml (range, 30.5 to 1,707 IU/ml) and 1,224 IU/ml (range, 46 to 82,864 IU/ml), respectively. In this cohort, patients with or without CMV DNAemia did not differ regarding sex, underlying disease, type of allograft, source of hematopoietic stem cells, conditioning regimen, acute GvHD prophylaxis regimen, paired donor/recipient CMV serostatus or cumulative incidence of aGvHD (Table 2).

Plasma CMV miRNAs in patients with or without subsequent CMV DNAemia

A total of 163 plasma specimens were available for CMV miRNA detection. These were obtained by day +7 (58 patients), day +14 (54 patients) and day +30 (46 patients) following allo-HSCT. For patients who eventually developed CMV DNAemia only specimens drawn before documentation of this event were considered. Assays could be optimized for five out of the seven targeted CMV miRNA (CMV miRNA UL36-5p, CMV miRNA US33-5p, CMV miRNA UL148D, CMV miRNA UL22A-5p and CMV miRNA UL112-3p). Assays targeting CMV US25-1-5p and CMV miRNA US4-5p failed to meet the predefined validation criterion (specificity) and were thus excluded from the study without further refinement.

US33-5p and UL112-3p were the most frequently detected CMV miRNAs species at any time point, both in patients with subsequent CMV DNAemia (in 66.6% and 69.0% of patients respectively) and without (in 75% and 65% of patients, respectively). As shown in Table 3, the overall detection rate of CMV miRNAs (any number of species at

any time point) was not significantly different in patients who went on to develop CMV DNAemia throughout the study period compared to those who did not; interestingly, this was irrespective of donor/recipient paired CMV serostatus (P=0.395). Neither were there differences with respect to individual CMV miRNA species at different evaluable time points between patients with or without subsequent CMV DNAemia (Table 4).

CMV miRNA load, as inferred by the PCR C_T, did not vary between patients with subsequent CMV DNAemia and those with no documented CMV DNAemia at any of the evaluable time points throughout the study period. Data for CMV miRNAs PCR C_Ts at different time points across comparison groups (each group including at least five patients) are shown in Table 5.

Of interest, among patients who developed CMV DNAemia, detection of CMV miRNA US33-5p and UL112-3 at any time point was associated with higher initial CMV DNA loads (for US33-5p miRNA species, median 2.18 $\log_{10}$ IU/ml vs. 1.98 $\log_{10}$ IU/ml in patients with or without detectable miRNA; P=0.019; for UL112-3, median 2.19 $\log_{10}$ IU/ml and 1.97 $\log_{10}$ IU/ml, respectively; P=0.036).

Discussion

Accurate identification of allo-HSCT recipients at highest risk for developing CMV DNAemia may improve CMV infection management in this clinical scenario; in particular, it may help to rationalize the use of new prophylactic antivirals (targeted prophylaxis) [1-3]. We previously showed that host genetic and immunological traits, as well as several metabolomic, oxidative stress-related and cytokine biomarkers may permit risk stratification with variable predictive values [1, 17-19]. Here, we investigated whether qualitative detection and/or quantitation of free circulating CMV

miRNAs in plasma could anticipate the occurrence of CMV DNAemia in the allo-HSCT setting.

Among the diverse range of CMV miRNAs characterized thus far [see for 6-9 for review], we selected a few known to be abundantly expressed in productively infected cells soon or very soon after infection [14,20-25], including UL36-5p, US33-5p, UL148D, UL22A-5p, UL112-3p, US25-1-5p US4-5p. Among these, detection of CMV miRNA UL 22A-5p at baseline was shown to independently predict recurrence of CMV viremia in solid organ recipients with preceding symptomatic CMV disease upon discontinuation of antiviral therapy [10]. As assays targeting CMV miRNA US25-1-5p US4-5p could not be standardized in the current study, only data on the remaining miRNA species are presented and discussed here.

In our study all targeted CMV miRNAs were detected soon after transplantation, irrespective of the donor/recipient paired CMV serostatus (not shown), although with variable frequency. CMV miRNA US33-5p and UL112-3p were the most commonly detected CMV miRNAs species at any time point, most notably by day +15 after allo-HSCT (more than two-thirds of patients); nevertheless, neither the detection rate of CMV miRNAs nor their plasma load allowed us to discriminate between patients with subsequent CMV DNAemia and those with no CMV DNAemia. As most patients in our series were CMV seropositive at the time of transplantation, these data support the assumption that CMV reactivation in tissues or mucosal sites occurs frequently irrespective of whether or not viremia ensues.

Interestingly, detection of CMV miRNA US33-5p and UL112-3 predicted higher virus doubling times during CMV DNAemia episodes, as inferred by the magnitude of the CMV DNA load at the time of the first positive PCR test [26]. The limited number of

spontaneously resolving episodes in this series (n=9) and the use of different CMV DNA thresholds for initiation of antiviral therapy across participating centers (not shown) precluded meaningful analysis on whether detection or quantitation of CMV miRNAs could predict other features of CMV DNAemia episodes (i.e. CMV DNA peak levels within episodes or their duration).

In addition to its relatively small size, the current study has certain limitations which, as yet, preclude ruling out plasma CMV miRNA detection/quantitation as a useful tool for anticipating CMV DNAemia. First, we focused on detecting free circulating plasma CMV miRNAs, and thus no attempt was made to screen for ones potentially carried within extracellular vesicles [27,28], which could also be released from productively infected cells early after virus penetration [6-9]. Second, frozen leftover specimens were used for the analyses, but although miRNA degradation in some specimens could not be ruled out, in our view, cryopreservation is unlikely to have had any impact on the results. Third, a number of CMV miRNA species, whose detection may be proven useful in the future, were not targeted in the current study. Fourth, the time elapsed between the last CMV miRNA analysis and CMV DNAemia diagnosis might have been too long in a number of patients (only samples obtained by days +7, +14 and +30 after allo-HSCT were screened for presence of viral CMV miRNAs). Fifth, the sensitivity (limit of detection) of the different assays was not determined, so that miRNA species present at very low amounts could have been missed. Nevertheless, the commercially-available miScript miRNA system has been optimized for the detection and quantification of trace amounts of mature miRNA in biological fluids.

To our knowledge no previous study has investigated the potential clinical value of CMV miRNA detection/quantitation for predicting the occurrence of CMV DNAemia

in any clinical setting. The data presented herein are inconsistent with the hypothesis that the targeted CMV miRNA species might help predict first episodes of CMV DNAemia in a cohort consisting primarily of allo-HSCT patients receiving haploidential allografts. Further studies must be conducted to determine whether this can be extrapolated to recurrent episodes of CMV DNAemia and to all allo-HSCT modalities.

Acknowledgements

Estela Giménez holds a Juan Rodés research contract from the Carlos III Health Institute. Eliseo Albert holds a Río Ortega research contract from the Carlos III Health Institute.

Compliance with ethical standards

Conflict of interest. The authors declare no conflict of interest.

Funding. This work was supported by a Fondo de Investigaciones Sanitarias (FIS) grant from Carlos III Health Institute (15/0060).

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Table 1. Demographic and clinical characteristics of study patients.	
Parameter	**no. of patients (%)**
Sex	
Male	39 (62.9)
Female	23 (37.1)
Underlying disease	
Acute leukemia	24 (38.7)
Chronic leukemia	4 (6.5)
Lymphoma	17 (27.4)
Myelodysplastic syndrome / Myelofibrosis	17 (27.4)
HLA-matching	
Matched	15 (24.2)
Mismatched	47 (75.8)
Haploidentical	44 (71)
Donor type	
Related	52 (83.9%)
Unrelated	10 (16.1%)
Stem cell source	
Peripheral blood	56 (90.3%)
Bone marrow	6 (9.7%)
Conditioning regimen	
Reduced intensity	43 (69.4%)
Standard intensity	19 (30.6%)
Graft vs. Host Disease prophylaxis	
Regimen with Cyclophosphamide	53 (85.5%)
Regimen with Cyclosporin A	5 (8.1%)
Regimen with Sirolimus	4 (6.5%)
CMV serostatus	
D+/R+	33 (53.2%)
D+/R-	6 (9.7%)
D-/R+	23 (37.1%)
Acute Graft vs Host Disease	
Grade 0-I	40 (64.5%)
Grade II-IV	22 (35.5%)
CMV, Cytomegalovirus; HLA, Human Leukocyte Antigen; D, Donor; R, Recipient.	

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Table 2. Demographic and clinical characteristics of patients with or without CMV DNAemia			
Parameter	**CMV DNAemia (% of patients)**		
	Yes (n = 42)	**No (n =20)**	***P*-value**
Sex			
Male	25 (64.1)	14 (35.9)	0.425
Female	17 (73.9)	6 (26.1)	
Underlying disease			
Acute leukemia	15 (62.5)	9 (37.5)	0.147
Chronic leukemia	3 (75.0)	1 (25.0)	
Lymphoma	9 (52.9)	8 (47.1)	
Myelodysplastic syndrome / Myelofibrosis	15 (88.2)	2 (11.8)	
HLA-matching			
Matched	11 (73.3)	4 (26.7)	0.371
Haploidentical	28 (63.6)	16 (36.4)	
Mismatched	3 (100)	0 (0)	
Donor type			
Related	35 (67.3)	17 (32.7)	0.686
Unrelated	7 (70.0)	3 (30.0)	
Stem cell source			
Peripheral blood	36 (64.3)	20 (35.7)	0.075
Bone marrow	6 (100)	0 (0)	
Conditioning regimen			
Reduced intensity	29 (67.4)	14 (32.6)	0.939
Standard intensity	13 (68.4)	6 (31.6)	
Graft vs. Host Disease prophylaxis			
Regimen with Cyclophosphamide	34 (64.2)	19 (35.8)	0.248
Regimen with Cyclosporin A	5 (100)	0 (0)	
Regimen with Sirolimus	3 (75.0)	1 (25.0	
CMV serostatus			
D+/R+	24 (72.7)	9 (27.3)	0.160
D+/R-	2 (33.3)	4 (66.7)	
D-/R+	16 (69.6)	7 (30.4)	
Acute Graft vs. Host Disease			
Grades 0-I	28 (70.0)	12 (30.0)	0.608

Grades II-IV	14 (63.6)	8 (36.4)	
CMV, Cytomegalovirus; HLA, Human Leukocyte Antigen; D, Donor; R, Recipient.			

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Table 3. Overall rate of detection of CMV miRNA species in patients with or without subsequent CMV DNAemia

Number of CMV miRNAs detected at one or more time points	**CMV DNAemia (number of patients)**		***P*-value**
	Yes (42)	No (20)	
≥1	37	19	0.390
≥2	28	12	0.608
≥3	17	8	0.971
≥4	6	4	0.567
CMV, Cytomegalovirus; miRNA, microRNA.			

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Table 4. Detection of individual CMV miRNA species at different time points in patients with or without subsequent CMV DNAemia

CMV miRNA species/day after allo-HSCT	**CMV DNAemia (number of patients)**		***P*-value**
	Yes (total number of patients evaluated)	No (total number of patients evaluated	
UL36-5p			
+7	1 (41)	1 (17)	0.513
+14	3 (35)	3 (19)	0.420
+30	0 (28)	0 (18)	1.0
At any time point	4 (42)	3 (20)	0.624
US33-5p			
+7	14 (41)	8 (17)	0.356
+14	20 (35)	13 (19)	0.417
+30	1 (28)	1 (18)	0.747
At any time point	28 (42)	15 (20)	0.506
UL148D			
+7	13 (41)	9 (17)	0.129
+14	0 (35)	0 (35)	1.0
+30	0 (28)	1 (18)	0.207
At any time point	13 (42)	9 (20)	0.564
UL22A-5p			
+7	7 (41)	5 (17)	0.291
+14	6 (35)	3 (19)	0.899
+30	0 (28)	0 (18)	1.0
At any time point	12 (42)	5 (20)	0.629
UL112-3p			
+7	18 (41)	9 (17)	0.530
+14	20 (35)	9 (19)	0.492

+30	1 (28)	0 (18)	0.418
At any time point	29 (42)	13 (20)	0.750
CMV, Cytomegalovirus; miRNA, microRNA.			

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Table 5. CMV miRNA load at different time points in patients with or without subsequent CMV DNAemia			
CMV miRNA species/day after allo-HSCT	**CMV miRNA PCR cycle threshold (C_T); median (range)**		***P*-value**
	Patients with CMV DNAemia	Patients without CMV DNAemia	
US33-5p			
+7	35.2 (30.9-36.1)	35.2 (33.0-35.8)	0.631
+14	35.8 (32.8-37.4)	35.7 (33.0-37.0)	0.825
UL148D			
+7	36.3 (34.8-37.3)	36.2 (34.8-36.6)	0.825
UL22A-5p			
+7	36.9 (35.9-38.7)	36.2 (34.8-36.6)	0.227
UL112-3p			
+7	36.9 (35.9-38.7)	36.3 (35.4-37.2)	0.192
+14	36.4 (35.9-38.4)	35.9 (34.9-36.7)	0.291
CMV, Cytomegalovirus; miRNA, microRNA.			

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Tables