Human microRNA responses predict cytomegalovirus replication following solid organ transplantation

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\textbf{Running title}: hsp-miRNA 200 family with CMV in SOT

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\textbf{Abbreviations}

<table>
<thead>
<tr>
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<tr>
<td>CFU</td>
<td>colony forming unit</td>
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<tr>
<td>CI</td>
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<td>D/R</td>
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<td>MOI</td>
<td>multiplicity of infection</td>
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PBMC: peripheral blood mononuclear cells
PBS: phosphate buffered saline
PCR: polymerase chain reaction
qRT-PCR: quantitative RT-PCR
RT: reverse transcription
SOT: solid organ transplantation
3′UTR: 3’ untranslated region
Abstract

Background. Homo sapiens mature microRNA-200b-3p and -200c-3p are predicted to bind to 3’ UTR of mRNA encoding human cytomegalovirus (HCMV) immediate early protein 2 (IE2). We hypothesized that expression of these microRNAs pre-transplant could predict HCMV replication after solid organ transplantation (SOT).

Methods. 272 SOT recipients were HCMV-seropositive pre-transplant and were managed using pre-emptive therapy. Pre-transplant PBMCs were stimulated with HCMV followed by collection of RNA one day post-stimulation. MicroRNAs were quantified using real-time RT-PCR. Human foreskin fibroblasts were transfected with 200b-3p and 200c-3p and infected with HCMV one hour post-transfection. Protein was collected at 3- and 7-dpi and underwent immunoblotting for IE2.

Results. Medians of 200b-3p and 200c-3p were significantly lower in recipients with HCMV replication (n = 144) (361.6 vs. 552.6, P = .035; 3586.8 vs. 12986.8 copies/μL, P = .03, respectively). Multivariate regression revealed that 200b-3p ≥100 copies/μL (OR: 0.53, P = .02), D-/R+ HCMV serostatus (OR: 0.55, P = .02) and graft rejection (OR: 1.86, P = .03) were independently associated with HCMV replication. Transfection with 200b-3p resulted in 2.7- and 2.5-fold decreased IE2 at 3- and 7-dpi, respectively, compared to mock cells.

Conclusions. MicroRNAs may play a biologically relevant role in controlling HCMV replication post-transplant.

Keywords. cytomegalovirus; solid organ transplant; microRNA; immediate early protein
Introduction

Human cytomegalovirus (HCMV) is an opportunistic pathogen causing significant morbidity and potential mortality in solid organ transplantation (SOT) recipients [1-5]. The majority of HCMV infection after SOT is due to replication of latent virus due to immunosuppressive drugs [6-8]. Immediate early protein 2 (IE2) encoded by the HCMV UL 122-123 region, plays an essential role in initiating and regulating viral early (E) gene activation, as well as propagating the subsequent steps of the HCMV lytic replication cycle [9-11]. These findings underscore the potential utility in targeting HCMV IE2 as a novel therapy aimed at preventing HCMV replication.

The replication of HCMV post-transplantation involves a complex interaction between the host immune response (both innate and adaptive), immunosuppressive drugs, and the virus. HCMV-specific T-cell responses have been shown to be important for controlling HCMV replication [12]. Another important aspect of host-pathogen interaction may be through the microRNA (miRNA) response. MiRNAs are small non-coding RNA molecules composed of 18-22 nucleotides that can alter the process of protein synthesis, mainly by suppressing gene expression via translational suppression. This process occurs when the mature miRNA (miR) binds the 3’ untranslated region (UTR) of specific messenger RNA (mRNA) transcripts; the miRNA binding site on the mRNA transcript is termed the seed sequence [13, 14]. In silico analysis is capable of predicting which of the 2,500+ known miRNA molecules binds the 3’ UTR of mRNA molecules corresponding to proteins of interest [15]. Several studies have suggested that the host miRNA response is capable of binding specific viral targets thereby affecting viral replication [16]. However, there are less data on the biological significance of this response in a relevant clinical population.

The hsp-miRNA-200 family, with highly conserved seed sequences, is composed of five members divided into two clusters: hsp-miR-200a, -200b, and -429 are found at a locus
on chromosome 1p36, while hsp-miR-200c and -141 are located on chromosome 12p13 [17].
The BiBiServe RNA hybrid algorithm predicts that hsp-miR-200b-3p, -200c-3p and -429 may bind the 3’UTR of mRNA encoding HCMV IE2 and recent in vitro data has supported this prediction [18] (Supplementary Figure 1). Based on this, we hypothesized that the host miRNA response may play a biologically relevant role in preventing HCMV replication post-transplant. To test this hypothesis, we evaluated whether the expression of hsp-miR-200b-3p, -200c-3p or -429 in peripheral blood mononuclear cells (PBMCs) could predict HCMV replication in SOT recipients.

Materials and Methods

Patient cohort
PBMCs were collected from 272 SOT recipients prospectively enrolled in the Swiss Transplant Cohort Study (STCS) from May 2008 to March 2011. The details and unique features including clinical data collection for the STCS were previously described [19-22]. For the current study, clinical data during the first year of follow-up was used. Characteristics of our cohort included the following: (a) baseline whole blood was drawn pre-transplantation [21], (b) HCMV pre-transplant D+/R+ or D-/R+ serostatus, and (c) no patient received antiviral prophylaxis (preemptive monitoring strategy). The detailed protocol of the preemptive approach was previously described [22]. HCMV disease (which includes both viral syndrome and tissue-invasive disease) was defined according to international guidelines [22, 23]. HCMV replication was classified as asymptomatic HCMV viremia, or HCMV disease, either viral syndrome or tissue-invasive disease [23].
PBMC Stimulation and miRNA measurement

All PBMCs were collected pre-transplant and samples were transferred for processing in a single centre. PBMCs were separated using the density-gradient centrifugation method using Ficoll®-Plaque media (Sigma-Aldrich, Oakville, ON, CA). Next, $3 \times 10^5$ PBMCs were stimulated with HCMV Towne strain at an MOI of 0.03 for 24 hours in 1.5 mL of complete RPMI-1640 with 5% CO$_2$ at 37°C [24]. After stimulation, PBMCs were washed twice with PBS, lysed using QIAzol™ (QIAGEN Inc., Toronto, ON, CA) and 30 µL of total RNA was eluted using a miRNeasy kit (QIAGEN Inc.), according to the manufacturer’s instructions. All RNA samples contained an RNA Integrity Number (RIN) ≥ 7, as measured by fluorescent on-chip micro-fluidic capillary electrophoresis using the RNA 6000 Pico Assay (Agilent Technologies, Inc., Waldbronn, Germany) [25-27]. All RNA was stored at -80°C until needed.

Real-time quantitative RT-PCR

To quantify miRNA, we performed TaqMan® MicroRNA real-time qRT-PCR for hsp-miRNA-200b-3p (Assay ID: 002251), hsp-miRNA-200c-3p (Assay ID: 002300) and hsp-miRNA-429 (Assay ID: 001024) (Life Technologies, Burlington, ON, CA) as per the manufacturer’s instructions. Using 2 ng/µL of total RNA, the RT reaction with stem-loop primers was performed in a thermal cycler (Eppendorf Mastercycler EP Gradient S, NY, USA) with the following cycling conditions: 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. For the quantitative real time PCR reaction, we combined 1.33 µL of the RT product, 10 µL of TaqMan® Universal PCR master mix, forward and reverse primers and nuclease-free water to bring the total volume to 20 µL per reaction. The reaction was completed using the StepOne™ Real-Time PCR System (Thermo-Fischer Scientific, Mississauga, ON, CA) with the following cycling conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec
and 60°C for 60 sec. To quantify, we utilized a standard curve consisting of mirVana™ miRNA mimics (Life Technologies) corresponding to each of the three miRNA of interest, ranging from 10 to 10⁶ copies/µL per PCR test. We expressed the amount of each miRNA as copies per µL of input RNA. The limit of detection of the assay was 100 copies/µL of input RNA and anything under this threshold was considered as zero for continuous variable analysis.

PBMC Culturing, Cell Lines and Virus Strains

Primary PBMCs were maintained in RPMI-1640 (Life Technologies). Three cell lines were used in this study: human foreskin fibroblast-1 (HFF-1), HeLa, and the human fetal lung fibroblast cell line MRC-5 (ATCC®, Gaithersburg, MD, USA). Dulbecco’s Modified Eagle’s Medium (Life technologies) was used to maintain HFF-1, MRC-5 and HeLa cells. All complete growth media were supplemented with 10% heat-inactivated FBS, 4.5 g/L of glucose, 4 mM of L-glutamine, 1 mM of sodium pyruvate, 1.5 g/L of sodium bicarbonate, 100 U/mL of penicillin and 100 µg/mL of streptomycin (Sigma-Aldrich). HCMV strain AD169 and Towne (ATCC) was propagated in MRC-5 cells and collected according to a previously published protocol [28].

Transfection of microRNA mimics and plasmid DNA

3 x 10⁴ HFF-1 cells were transfected with synthetic oligonucleotides of precursor miRNA (pre-miRNA) with double-strand stem-loop structure, corresponding to the target miRNA and recombinant pmirGLO plasmid DNA simultaneously, and mixed with Lipofectemine 3000 (Life Technologies) and OptiMEM (Life Technologies), according to the manufacturer’s protocol. Transfections were performed at 80-90% cell confluence. We used the mirVana miRNA negative control mimic (Life Technologies) as a negative control for transfection
experiments. This mimic consists of a pre-miRNA with a double-stranded stem-loop structure, and is composed of chemically modified random, scrambled sequence that has been extensively tested in human cell lines and validated to not produce any identifiable biological responses [29]. To evaluate the transfection efficiency, a mirVana miRNA-1 positive control mimic was used (Life Technologies), which has been shown to down-regulate histone deacetylase 4 (HDAC4) expression [30].

Immunoblotting

10^6 HFF-1 cells were seeded in 6-well plates. After overnight incubation, cells were infected with HCMV AD169 strain at an MOI of 0.1. Next, cells were transfected with one of the miRNA mimics at one hour post-infection. To evaluate whether the delayed transfection of mimics after infection could change the expression of IE2, cells were infected and then transfected 1 and 3 dpi. For mock experiments, we did not transfect the cells with miRNA mimics, but only added Lipofectemine RNAiMAX (Life technologies). We collected total protein after cell lysis using radioimmunoprecipitation assay buffer (Bio-Rad Laboratories, Inc., CA, USA) at 3 and 7 dpi in experiments evaluating effectiveness of transfection 1 hour after infection and at 3 day after transfection in experiment of delayed transfection, and stored protein at -20°C.

Mouse anti-human primary monoclonal antibodies were used at 1:200 for the HCMV IE2 (pp86) (clone 12E2), 1:500 for β-actin (clone C4) and 1:100 for HDAC4 (clone D-1) detection, and the secondary antibody (goat anti-mouse IgG-HRP) was used at 1:2,000 (Santa Cruz Biotechnology, Inc., CA, USA). We captured the chemiluminescent signal using a charge coupled device camera-based imager (MicroChemi, DNR Bio-Imaging Systems Ltd., Jerusalem, Israel). All experiments were completed in triplicate. We obtained the quantitative values for signal intensity in captured bands using Image J software (Center for Information
Statistical analysis

Statistical analysis was performed using SPSS V20.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism V6 (GraphPad Software, Inc. La Jolla, CA, USA). Differences in categorical and continuous variables between groups was analyzed by $\chi^2$-test and Mann-Whitney $U$-test, respectively. To assess the independent predictive factors associated with the development of HCMV replication after SOT, we performed multivariate logistic regression analysis using all statistically significant variables with a $P$ value of $\leq .05$ obtained from univariate analyses. All $P$ values were two-tailed and a $P$ value of $\leq .05$ was considered statistically significant.

Results

Patient clinical characteristics

The basic clinical characteristics of all 272 patients are summarized in Table 1. The majority of patients were kidney or liver transplant recipients (92%). HCMV replication developed in 144 (52.9%) recipients. The most common presentation of HCMV replication was asymptomatic viremia in 48.5% (132/272), followed by viral syndrome in 6.3% (17/272) and tissue-invasive disease in 3.7% (10/272). Thirty-nine (27.1%) of 144 patients experienced $\geq$ 2 episodes of HCMV replication. Median duration from first SOT to all episodes of first HCMV replication in all patients was 1.2 months (IQR, 0.9-2.2 months).

Graft rejection in the HCMV replication group occurred with significantly higher frequency than in the group that did not develop HCMV replication (36.1% vs. 24.2%, $P = .036$). Furthermore, HCMV replication was more common among individuals with a D+/R+ serostatus compared to those with D-/R+ serostatus (68.1% vs. 31.9%, $P = .045$).
Expression levels of miRNAs in PBMCs

Among all 272 SOT recipients, the median expression level of hsp-miR-200c-3p (7,353.7 copies/µL input RNA [155.4-37853.2]) was 17-fold higher than -200b-3p (432.0 copies/µL [48.8-2582.2], $P < .001$) and significantly higher than that of -429 (0 copies/µL [0-115.7], $P < .001$) (Figure 1A). The frequency of samples with a negative result (<100 copies/µL) for hsp-miR-429 (199, 73.2%) was significantly higher than that for -200b-3p (77, 28.3%, $P < .001$) or -200c-3p (65, 23.9%, $P < .001$).

SOT recipients with HCMV replication had significantly lower levels of hsp-miR-200b-3p compared to those who did not develop HCMV replication (361.6 [26.2-1999.2] vs. 552.6 [128.5-3054.5] copies/µL, $P = .035$) (Figure 1B). Similarly, expression levels of hsp-miR-200c-3p were also significantly lower in the HCMV replication group than in the group that did not show evidence of HCMV replication (3,586.8 [64.1-27052.6] vs. 12,986.8 [440.5-52531.6] copies/µL, $P = .03$) (Figure 1C). Similar results were observed when groups were separately analyzed for symptomatic disease and asymptomatic viremia (Figures 2A and B). No significant differences were measured between groups with respect to hsp-miR-429(0 [0-113.0] vs. 0 [0-116.0] copies/µL, $P = .870$) (Figure 1D). When analyzed as a categorical variable (less than vs. greater than 100 copies/µL), hsp-miR-200b-3p levels below the limit of detection were more common among SOT recipients that developed HCMV replication vs those that did not (34.0% vs. 21.9%, $P = .026$), and in those that developed HCMV disease vs. no disease (57.7% vs. 25.2%, $P < .001$). No differences were observed for hsp-miR-200c-3p and -429. Expression of hsp-miR-200b-3p at ≥100 copies/µL was a significant independent predictive factor for HCMV replication after SOT with consistent statistical values in three models of multivariate regression analysis (OR, 0.53; 95% CI, 0.30-0.92; $P = .02$) (Table 3; data shown for multivariate model including HCMV serostatus and graft...
In vitro miRNA target validation

HeLa cells were transfected with miRNA mimics or control (scrambled) miRNA mimics and subsequently transfected with a recombinant pmirGLO plasmid containing the target sequence of interest. Luciferase activity was significantly lower in cells transfected with hsp-miR-200b-3p and -200c-3p, relative to cells transfected with control scrambled miRNA mimics (Figure 3; methods in supplementary material). We next performed immunoblotting to assess whether miRNA transfection in HFF-1 cells plays a role in targeting and decreasing IE2 production post-HCMV infection. First, we evaluated the antiviral role of miRNA in targeting IE2 when cells were transfected one-hour post-infection. Transfection with hsp-miR-200b-3p one hour after infection resulted in reduced IE2 protein expression by days 3 (Figure 4A) and 7 (Figure 4B) post-infection, with 2.7- and 2.5-fold lower signal densities compared with mock-transfected cells, respectively. Transfection with hsp-miR-200c-3p one hour after infection did not result in significant changes to IE2 protein expression at 3 dpi, but induced a 1.5-fold lower signal density at 7 dpi in comparison.

Next, we assessed the antiviral role of miRNA in targeting IE2 when cells were transfected 1- or 3 dpi. With regard to cells infected and subsequently transfected 1 dpi, we saw a significant decrease in IE2 expression among cells transfected with hsp-miR-200b-3p and -200c-3p, with the later resulting in the greatest reduction in IE2 protein expression (Figure 4C). With respect to cells infected with HCMV and transfected with miRNA 3 dpi, IE2 protein levels were also decreased among hsp-miR-200c-3p-transfected cells (Figure 4D), but to a lesser degree than when cells were transfected one hour post-infection. At both time points, the greatest reduction in IE2 protein expression occurred in cells transfected with hsp-miR-200c-3p. Cells transfected with hsp-miR-200b-3p or -200c-3p did not have an effect
on HDAC4 expression levels, but transfection with HDAC4-specific positive control (hsp-miRNA-1), resulted in significant decrease of HDAC4 expression with 2.7-fold lower signal densities than that the mock-transfected cells (Supplementary Figure 2).

**Discussion**

Our study provides evidence that the host miRNA response to HCMV, specifically hsp-miR-200b-3p, could have relevant biological role in controlling viral replication post-transplant. Control of HCMV replication may occur through suppression of the production of the HCMV IE2 protein, thereby directly inhibiting replication of latent virus. We demonstrate that the *ex-vivo* production of hsp-miR-200b-3p in response to viral stimulation was associated with a lower incidence of asymptomatic HCMV replication and disease. This association remained significant in a multivariate model controlling for other factors associated with HCMV replication. While the potential utility of a single miRNA measurement as a biomarker is unclear, this study provides a first proof of concept that miRNAs likely have biologically relevant effects post-transplant. Measurements of these and other miRNAs, possibly in conjunction with T-cell and other immunologic assessments, could be explored as potential biomarkers. The clinical data we present is supported by *in-vitro* validation of IE2 as a target of miRNA 200 family and by previous work published by O'Connor et al. [18].

Current strategies for prevention of HCMV after transplantation rely on antiviral prophylaxis or regular viral load monitoring with subsequent pre-emptive antiviral therapy in patients with early evidence of replication. Antiviral prophylaxis is effective in HCMV disease prevention, but is costly and may result in significant toxicity. Similarly, pre-emptive strategies are effective but require frequent monitoring and viral load thresholds for initiation of therapy are poorly defined. Recently, significant interest in assessing the host immune
response as a biomarker to predict HCMV replication, and further refine prevention has gained significant interest. The most common parameters that have been evaluated include HCMV-specific T-cell responses [31]. Examples include interferon-γ release assays that measure HCMV-specific T-cell-mediated immunity like Quantiferon-CMV assay, as well as ELISPOT, and intracellular cytokine staining using flow cytometry [4, 7, 32, 33]. For example, in a multicenter study evaluating the predictive value of CD8+ T-cell responses to HCMV, a total of 127 HCMV D+/R- patients were assessed with serial CD8+ T-cell measurements using the Quantiferon-CMV assay [12]. The incidence of symptomatic HCMV disease was significantly higher in patients with undetectable IFN-γ responses at the end of prophylaxis vs. those with positive responses. In another study, CD4+ and T-reg responses in patients with early evidence of viral replication allowed prediction of spontaneous viral clearance vs. progression [34].

The potential role of miRNAs as another regulatory layer that may affect HCMV replication represents an exciting and novel area of study. HCMV infection likely results in a complex host miRNA response that may influence viral replication. For example, using high throughput sequencing, Fu et al. evaluated host miRNA expression in a model of latent HCMV infection [35]. They found 49 cellular miRNAs that were differentially expressed. Functional annotation of the target genes suggested that most were involved in endocytosis, and certain signaling pathways. The miR-200 family was not among the 49 differentially expressed ones, but this may be due to differences in the model used including the specific lab-adapted strain of HCMV (Toledo strain in a monocytic cell line). In a study by Zawislak et al., murine CMV infection resulted in an up-regulation of miR-155, which in turn affected distinct stages of NK cell activation and homeostasis [36].

There are minimal data on miRNAs and HCMV in the transplant setting. Egli et al. used miRNA microarray data from PBMCs of transplant recipients with and without HCMV
viremia, and showed that 142 of 847 hsp-miRNAs were differentially expressed between the two patient groups. Based on further in vitro analysis, a subset of miRNAs were then evaluated in a cohort of 242 transplant patients with active HCMV viremia. Significant dynamic changes of some miRNAs were observed coincident with antiviral therapy although no clear predictive biomarkers were observed [16]. Again, the miR-200 family was not among those found to be differentially expressed. Methodical differences (stimulated vs. unstimulated PBMCs, microarray method vs. real-time PCR) and assessment at a post-transplant vs. pre-transplant time point may account for some of these differences. Gambarino et al. evaluated hsp-miR-146a expression in 22 kidney transplant recipients with high, low and negative HCMV viral loads. However, they found no association between expression level and viral replication [37]. Another layer of complexity involves viral production of miRNAs. For example, detection of hcmv-miR-UL22A-5p in the whole blood of transplant recipients has been associated with recurrence of HCMV viremia after discontinuation of therapy [38].

The role of the miR-200 family in HCMV following transplant is particularly interesting. Based on seed sequences of the miRNA family (Supplementary Figure 1), three members are predicted to bind to the 3’UTR of HCMV UL122 (hsp-miR-200b-3p, -200c-3p, and -429). O’Connor et al. demonstrated that miR-200 family members target the UL122 (IE2) protein of HCMV using a combination of in vitro validation methods. These included luciferase reporter assays, as well as demonstrating that the overexpression of miR-200 family miRNAs in fibroblasts resulted in decreased viral titers. They found higher levels of miR-200 family member miRNAs in monocytes, associated with HCMV latency, as compared to cells permissive for lytic infection such as macrophages [18]. Our in vitro data support that hsp-miR-200b-3p is capable of targeting IE2 and downregulating its expression. Previously, O’Connor et al. evaluated the change of IE2 protein levels at the early time
intervals of 6 and 9 hours post-infection [18]. In contrast to the study by O’Connor et al., we measured IE2 expression at 3 and 7 dpi, and furthermore evaluated the in vitro suppressive effect of hsp-miR-200b-3p and -200c-3p when administered 1 hour post-infection, as well as 1 or 3 dpi. Interestingly, both hsp-miR-200b-3p and -200c-3p were still able to decrease IE2 expression.

Limitations of the study include small number of recipients who experienced HCMV disease. In addition, only a single time point pre-transplant was evaluated. Assessment of further post-transplant samples to look at dynamic changes in miRNA may be useful. Quantitative viral load data were not available for this cohort, in part due to differences in viral load assays across centers. An analysis of viral load vs. miRNA levels would provide further useful data. In addition, we assessed miRNA response in PBMCs after stimulation with live virus. The optimal stimulant in this setting is unknown but live virus may reflect the most accurate physiologic stimulus. Another approach may be to assess basal levels of miRNA in the absence of specific ex vivo stimulation. Finally, the absolute log-difference in miRNA expression level between patients with and without HCMV (approximately 0.5 log_{10}-difference or about 3-fold) was modest but we believe within the range of reproducibility of the assay. For each sample steps were taken to ensure high RNA integrity of the input sample; as well, the stem-loop PCR design used in our assay contributed enhanced sensitivity and specificity of detection, providing us with more confidence in the validity of the findings.

Strengths of our study include the large sample size, and the lack of antiviral prophylaxis in this cohort of patients, which allows a real assessment of the impact of miRNA responses on the development of HCMV replication. Our findings need to be evaluated in various clinical settings including high-risk groups for HCMV infection such as D+/R- SOT recipients. In addition, miRNA expression may be assessed in different types of samples such as bronchoalveolar lavage fluid for lung transplant recipients and urine for kidney transplant
recipients.

In conclusion, our study suggests that miRNA responses may play a biologically relevant role in controlling HCMV replication after transplantation. Exploring their role as biomarkers or therapeutic targets may open new avenues to prevent and treat replication of HCMV.
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Conflict of interest.
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Table 1. Clinical characteristics of total solid organ transplant recipients and comparisons of the characteristics in groups with and without HCMV replication

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<td>188 (67.9)</td>
<td>103 (70.1)</td>
<td>85 (65.4)</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>67 (24.2)</td>
<td>33 (22.4)</td>
<td>34 (26.2)</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>12 (4.3 )</td>
<td>9 (6.1)</td>
<td>3 (2.3)</td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>8 (2.9 )</td>
<td>1 (0.7)</td>
<td>7 (5.3)</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>2 (0.7 )</td>
<td>1 (0.7)</td>
<td>1 (0.8)</td>
<td></td>
</tr>
<tr>
<td>Re-Tx after enrollment(^f), yes</td>
<td>45 (16.5)</td>
<td>22 (15.3)</td>
<td>23 (18.0)</td>
<td>0.625(^b)</td>
</tr>
<tr>
<td>HCMV serostatus</td>
<td></td>
<td></td>
<td></td>
<td>0.045(^b)</td>
</tr>
<tr>
<td>D+/R+</td>
<td>170 (62.5)</td>
<td>98 (68.1)</td>
<td>72 (56.2)</td>
<td></td>
</tr>
<tr>
<td>D-/R+</td>
<td>102 (37.5)</td>
<td>46 (31.9)</td>
<td>56 (43.8)</td>
<td></td>
</tr>
<tr>
<td>Anti-HCV in recipients, (+)</td>
<td>33 (12.1)</td>
<td>20 (13.9)</td>
<td>13 (10.2)</td>
<td>0.360(^b)</td>
</tr>
<tr>
<td>Induction IS drugs</td>
<td></td>
<td></td>
<td></td>
<td>0.214(^c)</td>
</tr>
<tr>
<td>Basiliximab</td>
<td>174 (64.0)</td>
<td>87 (60.4)</td>
<td>87 (68.0)</td>
<td></td>
</tr>
<tr>
<td>ATG</td>
<td>25 (9.2 )</td>
<td>17 (11.8)</td>
<td>8 (6.2)</td>
<td></td>
</tr>
<tr>
<td>Rituximab</td>
<td>6 (2.2 )</td>
<td>2 (1.4)</td>
<td>4 (3.1)</td>
<td></td>
</tr>
<tr>
<td>Plasmapheresis</td>
<td>1 (0.4 )</td>
<td>0 (0)</td>
<td>1 (0.8)</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>66 (24.3)</td>
<td>38 (26.4)</td>
<td>28 (21.9)</td>
<td></td>
</tr>
</tbody>
</table>
Graft rejection\textsuperscript{g}, yes & 83 (30.5) & 52 (36.1) & 31 (24.2) & 0.036\textsuperscript{b} \\
HCMV replication\textsuperscript{h} \\
  HCMV disease & 26 (9.6) & — & — & — \\
  Tissue-invasive disease & 10 (3.7) & — & — & — \\
  Viral syndrome & 17 (6.3) & — & — & — \\
  Asymptomatic viremia & 132 (48.5) & — & — & — \\

Data were expressed as mean ± SD or number (percent). Independent two-sample Student’s \textit{t}-test\textsuperscript{a}, \textit{χ}\textsuperscript{2}-test\textsuperscript{b}, Fisher’s exact test\textsuperscript{c}, at that time of first transplantation\textsuperscript{d}, included five cases in which two independent organs were transplanted simultaneously\textsuperscript{e}, included 41 (15.1%), 3 (1.1%) and 1 (0.4%) of twice, third and fourth transplantation\textsuperscript{f}, respectively. acute and/or chronic graft rejection proven by histopathologic examination\textsuperscript{g}, included multiple episodes in 14 recipients\textsuperscript{h}. Aberrations: Tx, transplantation; D, donor; R, recipient; HCV, hepatitis C virus, IS, immunosuppressive; ATG, anti-thymocyte globulin.
Table 2. Comparison of negative results for hsp-miR-200b-3p, -200c-3p and -429 according to the development of HCMV replication

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hsp-miR-200b-3p</th>
<th>Hsp-miR-200c-3p</th>
<th>Hsp-miR-429</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>P value</td>
<td>Negative</td>
</tr>
<tr>
<td>All episodes of HCMV replication</td>
<td>0.026</td>
<td>0.111</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>34.0%</td>
<td>27.8%</td>
<td>73.6%</td>
</tr>
<tr>
<td>No</td>
<td>21.9%</td>
<td>19.5%</td>
<td>72.7%</td>
</tr>
<tr>
<td>HCMV disease</td>
<td>&lt;0.001</td>
<td>0.067</td>
<td>0.166</td>
</tr>
<tr>
<td>Yes</td>
<td>57.7%</td>
<td>38.5%</td>
<td>84.6%</td>
</tr>
<tr>
<td>No</td>
<td>25.2%</td>
<td>22.4%</td>
<td>72.0%</td>
</tr>
<tr>
<td>Asymptomatic viremia</td>
<td>0.074</td>
<td>0.121</td>
<td>0.696</td>
</tr>
<tr>
<td>Yes</td>
<td>33.3%</td>
<td>28.0%</td>
<td>74.2%</td>
</tr>
<tr>
<td>No</td>
<td>23.6%</td>
<td>20.0%</td>
<td>72.1%</td>
</tr>
</tbody>
</table>

The negative result was defined as expression of < 100 copies/µL of input RNA.
**Table 3.** Multivariate logistic regression analysis to reveal the predictive factors for the development of HCMV replication after solid organ transplantation

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>OR</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graft rejection</td>
<td>1.856</td>
<td>1.079-3.194</td>
<td>0.026</td>
</tr>
<tr>
<td>HCMV serostatus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D+/R+</td>
<td>—</td>
<td>—</td>
<td>0.020</td>
</tr>
<tr>
<td>D-/R+</td>
<td>0.545</td>
<td>0.327-0.909</td>
<td></td>
</tr>
<tr>
<td>Hsp-miR-200b-3p, ≥ 100 copies/µL of input RNA</td>
<td>0.526</td>
<td>0.302-0.917</td>
<td>0.023</td>
</tr>
</tbody>
</table>

Aberrations: D, donor; R, recipient; OR, odds ratio; CIs, confidential interval
Figure 1. Comparison of miRNA expression levels of hsp-miR-200b-3p (B), -200c-3p (C) and -429 (D) in pre-transplant PBMCs of 272 SOT recipients with and without HCMV replication. Figure 1(A) showed the difference of expression levels of three miRNAs in all 272 patients. Total RNA was extracted from pre-transplant PBMCs that were stimulated by HCMV Towne strain at an MOI of 0.03 for 24 hours. Quantitative real-time RT-PCR was performed with 2 ng/µL of total RNA in each sample. A standard curve consisting of miRNA mimics for each of the miRNAs used in this study was used for quantitation, with concentrations ranging from 10 to 10^6 copies/µL. Each dot corresponds to the miRNA expression level expressed as log_{10} copies/µL in input RNA. Bars indicate median values and the interquartile range.

Figure 2. Expression levels of hsp-miR-200b-3p and -200c-3p in pre-transplant PBMCs of transplant patients for the development of all episodes of HCMV replication (A) or asymptomatic viremia (B) post-transplant. Total RNA was extracted from pre-transplant PBMCs and were stimulated with HCMV Towne strain at an MOI of 0.03 for 24 hours. Then, quantitative real-time RT-PCR was performed. Bars represent median miRNA levels with the interquartile range.

Figure 3. Luciferase reporter assay to evaluate the binding ability of hsp-miR-200b-3p and -200c-3p with the 3’UTR target mRNA from the HCMV UL122-123 region of IE2. Firefly and Renilla luciferase were measured at 2 days after simultaneous transfection of both recombinant pmirGLO plasmid and each miRNA mimic. The mirVana™ miRNA negative control mimic was used as negative control of miRNAs. The mock experiment was performed with only transfecting agents without any miRNA. Each experiment was done in
triplicate. The upper line of each bar and error bar indicate the median and interquartile range, respectively. The $p$-value in each mimic was obtained from the comparison with mock experiment. *$P < .05$, **$P < .001$. The materials and methods were described in supplementary material.

**Figure 4.** Expression of IE2 protein in various time intervals between HCMV infection and transfection of each miRNA. miRNA mimics were transfected 1 hour after HCMV infection and then proteins were extracted at 3 dpi ($A$) and 7 dpi ($B$). Each miRNA mimic was transfected 1 day ($C$) and 3 days ($D$) after HCMV infection and then proteins were extracted 3 days post-transfection. The mock experiment was performed with only transfecting agents without any miRNA. The mirVana™ miRNA negative control mimic was used as scrambled oligonucleotide for negative control of miRNAs. The upper line of each bar and error bar indicate the median and interquartile ranges, respectively. The $P$ value in each mimic was obtained from the comparison with mock experiment. *$P < .05$, **$P < .001$. Abbreviations: MOI, multiplicity of infection; NC, negative control.
Reference


10. Petrik DT, Schmitt KP, Stinski MF. The autoregulatory and transactivating functions


Figure 1
Figure 2

**Hsp-miR-200b-3p**

- Yes (n = 26)
- No (n = 246)

**Hsp-miR-200c-3p**

- Yes (n = 26)
- No (n = 246)

*P = .002*

**Hsp-miR-200b-3p**

- Yes (n = 132)
- No (n = 140)

**Hsp-miR-200c-3p**

- Yes (n = 132)
- No (n = 140)

*P = .047*

*P = .040*

*P = .011*
Figure 3

![Bar graph showing normalized relative firefly/Renilla luciferase luminescence for Mock, 200b-3p, 200c-3p, and NC conditions.](image-url)
Figure 4

IE2
β-actin

Transfection
No No Mock 200b-3p 200c-3p NC

HCMV infection
No AD169 at an MOI of 0.1

Relative IE2/β-actin signal intensities

Mock 200b-3p 200c-3p NC

* * * *