


miRNA expression profiles in liver grafts of HCV and HIV/HCV-infected recipients, 6 months after liver transplantation

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Abstract

In hepatitis C virus (HCV)/human immunodeficiency virus (HIV) co-infected patients, HIV enhances HCV replication and liver damage. Several microRNAs (miRNAs), active in pro-fibrotic and inflammatory pathways, have been implicated in the pathogenesis of this phenomenon. However, these miRNAs have been tested only in explanted cirrhotic livers, when the liver damage has become chronic and irreversible. No data are available on the early phase of viral infection, such as early after liver transplantation (LT). In the present study, the expression of miR-101, miR-122, miR-155, miR-192, miR-200c, miR-338, and miR-532 was determined by quantitative real-time polymerase chain reaction in liver biopsies of HCV ($n = 19$) and HCV/HIV-infected ($n = 20$) LT recipients, as well as in a control group ($n = 18$) of noninfected patients, transplanted for alcoholic cirrhosis. The timing of liver biopsy was 6 months post-LT. None of the patients was treated with direct-acting anti-HCV drugs. All co-infected recipients had suppressed HIV viral load. Grading and staging were assessed according to the Ishak Classification. HCV and HIV viral load were measured in the sera. miR-101 ($p = .03$), miR-122 ($p = .012$), and miR-192 ($p = .038$) were significantly downregulated in HCV/HIV co-infected and HCV mono-infected recipients when compared with noninfected recipients, and such downregulation was more pronounced in co-infected ones. Moreover, in co-infected recipients but not in mono-infected ones, miR-101 inversely correlated with the peripheral HCV-RNA levels ($r = .41$, $p = .04$) and miR-122 inversely correlated with peripheral HCV-RNA levels ($r = .49$, $p = .03$) and with the

Abbreviations: BMI, body mass index; DAA, direct acting anti-HCV drug; ESLD, end-stage liver disease; FFPE, formalin-fixed paraffin-embedded; HAART, highly active antiretroviral therapy; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIV, human immunodeficiency virus; LT, liver transplantation; MELD, model for end-stage liver disease; miRNA, microRNA; mRNA, messenger RNA; RNA, ribonucleic acid; RT-PCR, real-time polymerase chain reaction.

Michela Bulfoni and Riccardo Pravisani contributed equally to this study.

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histological grading ($r = .51$, $p = .02$). In conclusion, as early as 6 months after LT, the presence of HIV-HCV co-infection enhanced a significant down-regulation of certain miRNAs that showed a direct correlation with HCV viral load and liver inflammation.

KEYWORDS

hepatitis C virus, human immunodeficiency virus, liver transplantation, microRNA, real-time polymerase chain reaction

1 | INTRODUCTION

The pathogenesis of end-stage liver disease (ESLD) in human immunodeficiency virus (HIV)-infected patients is multifactorial. Between alcohol abuse and antiretroviral therapy-related toxicity, hepatitis C virus (HCV) co-infection has been so far the major underlying cause, with a reported prevalence of 70%.¹⁻³ Liver transplantation (LT) has been demonstrated to be a feasible and effective treatment for ESLD HIV-positive patients, even in the presence of hepatocellular carcinoma (HCC) diagnosis, reaching outcomes comparable to non-HIV patients.¹⁻³ Nonetheless, HCV/HIV co-infected LT recipients have been characterized by a dismal outcome, mainly due to a severe HCV recurrence on the liver allograft, that is accelerated by HIV co-infection.⁴ Nowadays, direct-acting anti-HCV drugs (DAAs) have been effectively controlling such risk. However, the pathogenic mechanisms that sustain the liver damage in HCV infection, and that are enhanced by HIV co-infection, may underlie even other liver diseases, thus potentially becoming new effective diagnostic markers and/or therapeutic targets even in noninfected patients. Recent studies have identified a crucial role of miRNAs in the biological interaction between HIV, HCV, and host cells.⁵ microRNAs are small, noncoding RNAs that control gene expression of about 60% of the human genome by regulating mRNA translation and stability in the cytoplasm.⁵ So far, all investigations in the LT setting have analyzed miRNA expression profiles in cirrhotic livers, showing a significant miRNA dysregulation in HCV mono and HCV/HIV co-infected patients.⁶ However, liver cirrhosis represents the terminal stage of a chronic, irreversible pathologic process and miRNA dysregulation at this stage might not be specifically induced by a viral infection but rather by chronic inflammation itself or might be the biological feature of an irreversible disease with limited possibility of therapeutic interventions. Therefore, the aim of the present study was to analyze the miRNA expression profiles in HCV and HIV/HCV LT recipients in the early post-LT period (6 months), when the liver allograft was already HCV-infected but had not developed yet any clinically significant damage. Such analysis was performed on liver biopsies using a Real-Time Polymerase Chain Reaction (RT-PCR) technology. A potential correlation of miRNA profiles and post-LT HCV viral load and histological HCV-related liver injury was assessed as well.

2 | MATERIALS AND METHODS

2.1 | Patient and FFPE sample selection

During the 2007–2014 period, 42 HIV/HCV co-infected and 83 HCV mono-infected patients were treated with LT from DBD donors at the Liver Transplant Unit of the University Hospital of Udine. Indications to LT in HIV-infected patients have been already reported elsewhere⁷ and did not differ between mono-infected and co-infected cases. Exclusion criteria comprised split liver graft, HBV positivity, coexisting autoimmune hepatitis, post-LT surgical or immunologic complications, treatment with DAA before LT or within 6 months post-LT, unavailability of a specimen of a 6 ± 1 months post-LT protocol liver biopsy, a biopsy core containing less than 11 portal tracts, pathologic features of graft rejection, or cholangiopathy on liver biopsy. Thus, 19 HCV mono-infected and 20 HCV/HIV co-infected recipients were selected. Moreover, 18 recipients without any viral infection and transplanted for alcoholic liver cirrhosis, were included as controls. The selection of these patients was based on the same exclusion criteria applied to the study groups and on a matching with the study groups for model for end-stage liver disease (MELD) score, HCC diagnosis, graft steatosis, and total ischemia time. Recipient age was not considered for the matching because the epidemiology of ESLD due to alcohol abuse, HCV infection, and HIV infection, respectively, is significantly different in terms of age.^{1,8}

The liver biopsies were formalin-fixed paraffin-embedded (FFPE). HCV-related liver injury was retrospectively re-evaluated on the liver specimens according to the Ishak classification,⁹ by an expert pathologist, blinded to clinical information. The staging measures fibrosis (score 0–6) while grading measures inflammation (score 0–18). Donor and recipient characteristics were retrospectively reviewed from prospectively maintained databases. Immunosuppression was based in all cases on tacrolimus twice daily plus steroids and possible mycophenolate mofetil introduction for renal sparing. In HIV recipients, HAART therapy was maintained unmodified after LT. The presence of an active viral replication and the peripheral viral load were tested as per protocol in the preliminary blood tests before the liver biopsy. In all cases, the HIV viremia was suppressed and the CD4 lymphocyte count was >200 cells/mm³. Written informed consent was obtained from all participants at the time of LT for the use of FFPE samples for

scientific purposes. The present study was approved by the local Institutional Review Board.

2.2 | RNA extraction and quantification

Total RNA was extracted using the RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion), according to the manufacturer's instructions. The FFPE area containing the tissue was removed by manual dissection using a sterile scalpel and harvested in an Eppendorf tube. Then, the tissue was deparaffinized using a series of xylene and ethanol washes and then incubated with proteinase K at 50°C for 30 min. RNA was purified using glass-fiber columns and washed with ethanol-based solutions. Lastly, RNA was recovered with 30 µl of nuclease-free water. Nanodrop 2000 (Thermo Fisher Scientific) spectrophotometer was used to quantify the amount of total RNA recovered and to assess its quality.

2.3 | miRNA reverse transcription and TaqMAN qPCR assay

miRNA reverse transcription reaction was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Each reaction included 10 ng of total RNA extracted from FFPE samples, 10× RT Buffer, 5× RT Primers, MultiScribe Reverse Transcriptase, RNase Inhibitor, and nuclease-free water in a final reaction volume of 7 µl. RT-mixtures were incubated with the following thermal profile: 16°C for 30 min, 42°C for 30 min and, lastly, 85°C for 5 min. 1.33 µl of the RT product was used for the real-time qPCR assay. The reagents required for quantitative PCR were combined in a master mix (TaqManUniversal PCR Master Mix, with no UNG; Applied Biosystems) containing enzymes, specific primers, and TaqMan probes to evaluate miR-101, miR-122, miR-192, miR-338 3p, miR-200c, miR-155, and miR-532 expression profiles.

These specific miRNAs were selected due to their established biological involvement in liver fibrosis, in HCV and HIV viral infection pathways, in hepatic metabolism, and in the immune response. RNU6 small-nucleolar RNA was employed as endogenous control. The amplification protocol was carried out using the LightCycler 480 (Roche) instrument. The reactions were incubated in a 96-well plate at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. For each miRNA, the qPCR was run in duplicate. The threshold cycle (C_t) signal was collected for each amplification reaction.

2.4 | miRNA expression analysis

The miRNA relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method. First, all C_t values of target miRNAs, obtained from the amplification analysis, were normalized through the RNU6B endogenous control (ΔC_t). Then, the $\Delta\Delta C_t$ was calculated as the difference

between the normalized C_t values of the target miRNA of each HCV/HIV or HCV-infected patient with the normalized C_t values of the target miRNA of transplanted noninfected patients. Samples with undetermined C_t values were excluded from the analysis.

2.5 | Statistics

The continuous variables were expressed as mean \pm standard deviation (SD) or median and range, as appropriate. The normal distribution of data was evaluated by Kolmogorov-Smirnov's test. The comparison between the three groups of patients was conducted with the Kruskal-Wallis test, followed by pairwise comparisons between groups using the pairwise Wilcoxon rank-sum test, corrected by the Benjamini-Hochberg test for multiple comparisons. Correlation tests were conducted with the Pearson test. p was considered significant if less than .05. The analysis was conducted with the Prism (version 5.0), SPSS (version 21.0.0.0), and R/Bioconductor (version 3.6.2) software.

3 | RESULTS

3.1 | Patients characteristics

The demographic and clinical data of the recipients and the donor/graft characteristics are summarized in Table 1. As expected, the HIV/HCV patients were significantly younger and with a significantly lower body mass index (BMI), compared to the mono-infected ones. Nonetheless, the severity of ESLD at LT, as evaluated by the MELD score, was comparable among the groups. Due to the different ages of the two patient groups and as a consequence of the donor/recipient matching policy, the donor age was statistically lower in the HCV/HIV group. The study groups were otherwise homogeneous in terms of donor and graft characteristics and HCV serotypes. At the 6 months post-LT follow-up control, the liver function blood tests were within the normal range for all the patients. In co-infected patients, HIV viremia was suppressed and the CD4 lymphocyte count was >200 cells/mm³. The prevalence of active HCV infection was not significantly different between the groups, although the HCV peripheral viral load was higher at a nearly significant level in co-infected patients (Table 2). In liver biopsies, the staging and grading of HCV-related liver damage according to the Ishak classification were comparable between the groups (Table 2). None of the liver biopsies in the control group showed fibrosis or inflammation.

3.2 | Correlation between miRNA expression levels and recipient's virological status

Compared to the noninfected controls, the expression levels of miR-101, miR-122, miR-192, and miR-200c were significantly downregulated in the HCV and HCV/HIV-infected patients, while miR-338-3p showed a nearly significant trend of downregulation (Figure 1).

TABLE 1 Demographic and clinical data, graft characteristics, and surgical details of recipient patients and donors

	Controls (n = 19)	HCV/HIV co-infected recipients (n = 22)	HCV-infected recipients (n = 19)	Controls versus HCV/HIV p value	Controls versus HCV p value	HCV/HIV versus HCV p value
Recipient characteristics						
Gender (M:F)	15:3	18:2	17:2	.653	.660	>.999
Age (years)	61.2 ± 4.9	46.0 ± 3.5	55.2 ± 8.7	<.001	.147	<.001
BMI	24.3 ± 3.3	22.9 ± 3.5	25.7 ± 3.5	.139	.231	.018
HCC diagnosis (%)	8 (44.4%)	9 (45%)	10 (52.6%)	.973	.618	.634
MELD score	14 (12–22)	15 (12–19)	15 (10–22)	.953	.915	.827
HCV genotype (%)						.893
– 1	-	10 (45.4%)	10 (52.6%)			
– 3	-	7 (31.8%)	5 (26.3%)			
– 4	-	5 (22.8%)	4 (21.1%)			
Donor characteristics						
Donor age (years)	56.1 ± 16.6	45.1 ± 14.1	55.1 ± 15.7	.032	.834	.043
Donor sex (M:F)	10:8	10:10	12:7	.732	.638	.408
Mild graft steatosis (5%–30%) (%)	4 (22.2%)	4 (20%)	5 (26.3%)	>.999	>.999	.716
Total ischemia time (warm + cold) (min)	515 ± 110.1	487.6 ± 139.8	468 ± 106.5	.509	.208	.638

Abbreviations: BMI, body mass index; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIV, human immunodeficiency virus; MELD, model for end-stage liver disease.

TABLE 2 Virologic status and HCV-related liver damage according to the Ishak classification at 6 months post-LT

	HCV/HIV co-infected recipients (n = 20)	HCV-infected recipients (n = 19)	p value
Post-LT HCV-RNA serum positivity (%)	17 (85)	12 (63.1)	.215
Post-LT HCV peripheral viral load (UI/ml)	6.1×10^6 (0– 10^8)	2.4×10^5 (0– 7.9×10^7)	.06
Grading, according to Ishak classification (%)			.92
0	1 (5)	1 (5.3)	
1	8 (40)	6 (31.6)	
2	4 (20)	2 (10.5)	
3	3 (15)	3 (15.8)	
4	2 (10)	4 (21.1)	
5	0 (0)	0 (0)	
6	2 (10)	3 (15.8)	
Staging, according to Ishak classification (%)			>.99
0	9 (45.0)	9 (47.4)	
1	8 (40.0)	7 (36.8)	
2	1 (5.0)	1 (5.3)	
3	3 (10.0)	2 (10.5)	

Abbreviations: HCV, hepatitis C virus; LT, liver transplantation.

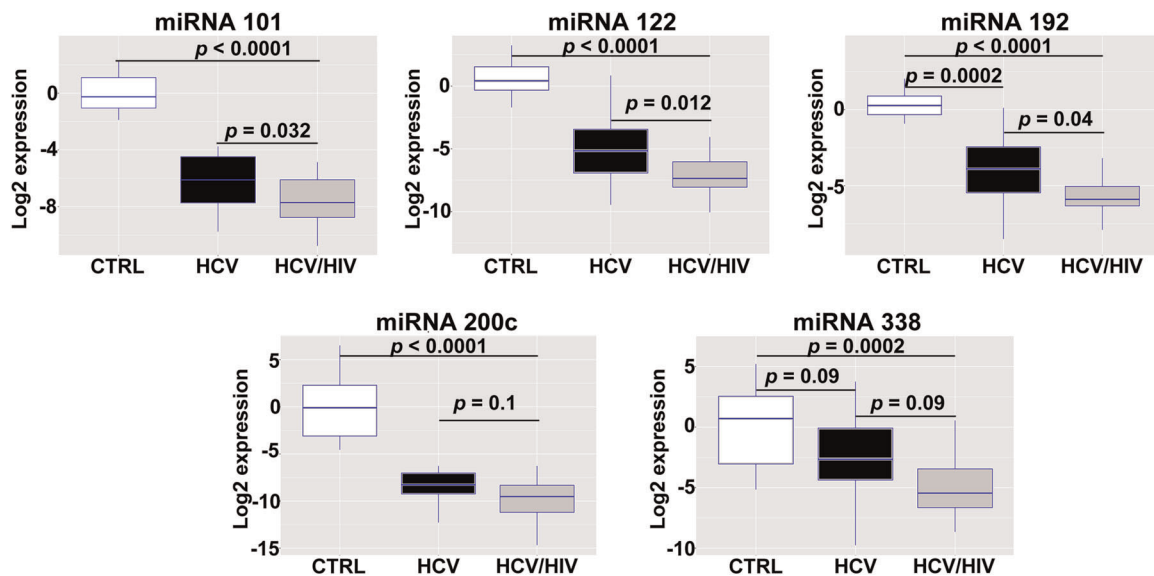


FIGURE 1 Boxplots representing the miRNA expression levels in liver biopsies of noninfected (controls, open bar), HCV mono-infected (black bar), and HCV/HIV co-infected (gray bar) LT recipients as evaluated by qRT-PCR. The statistical significance of the differences is shown for every boxplot. HCV, hepatitis C virus; HIV, human immunodeficiency virus; LT, liver transplantation; miRNA, microRNA; qRT-PCR, quantitative real-time polymerase chain reaction

In the confront between HCV/HIV co-infected and HCV mono-infected patients, the expression levels of miR-101, miR-122, and miR-192 were significantly downregulated in HCV/HIV co-infected (Figure 1). A similar trend of downregulation in co-infected patients was also observed for miR-338-3p and miR-200c expression profiles, although without reaching statistical significance. On the contrary, miR-155 and miR-532 were not differentially expressed between HCV mono-infected and HCV/HIV co-infected patients (Supplementary Figure 1).

3.3 | Correlation among miRNA expression levels, staging, grading, and HCV peripheral viral load

The grading and staging on liver graft biopsies 6 months post-transplant were comparable between HCV and HIV/HCV-infected recipients (Table 2). In HCV mono-infected recipients, the targeted miRNA did not show any significant correlation with either histological staging, grading, and HCV peripheral viral load (Figure 2). Conversely, in the HCV/HIV-co-infected group (Figure 3), miR-101 inversely correlated with the peripheral HCV-RNA levels ($r = -0.41$, $p = .04$) and miR-122 inversely correlated with peripheral HCV-RNA levels ($r = -0.49$, $p = .03$) and with the histological grading ($r = -0.51$, $p = .02$). In the control group of non-infected transplanted patients, no significant correlations between miRNA expression, grading, and staging emerged (Figure S2).

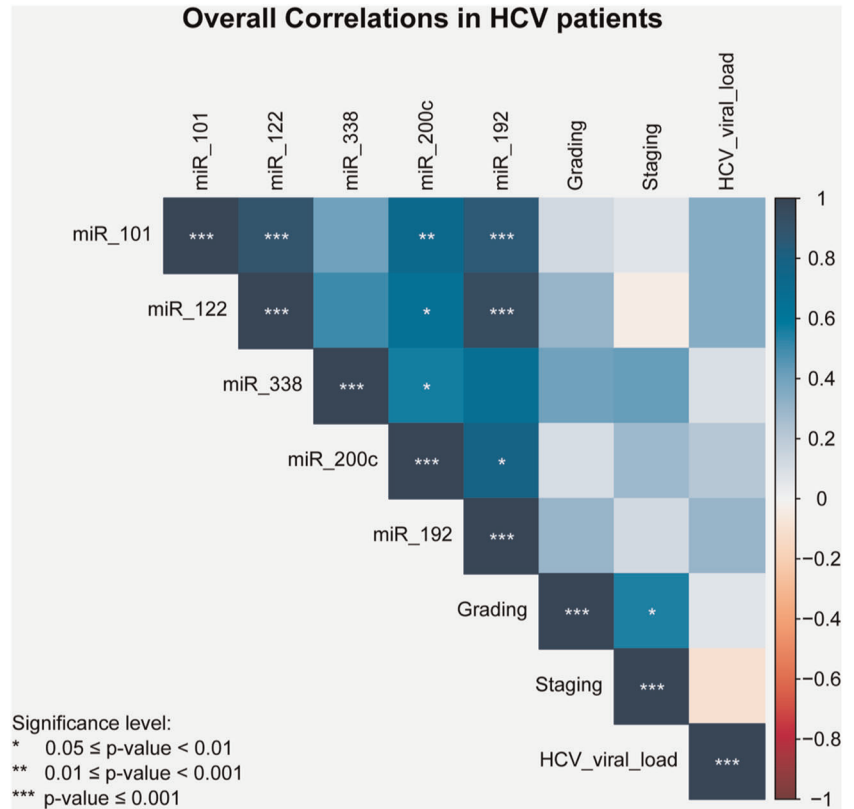
4 | DISCUSSION

Clinical data have provided solid evidence that HIV increases HCV replication, decreases the rate of HCV clearance during acute infection, and accelerates liver fibrogenesis with resulting increased rates of hepatic

decompensation, liver-related mortality, and HCC.¹ These effects were confirmed even by the present study since co-infected patients underwent LT at a significantly younger age than mono-infected due to a more rapidly progressed liver disease. In the exploration of the pathogenesis of HIV/HCV co-infection, several cytokines, receptors, or viral components have been implicated as potential signaling pathways, and several cell types have been identified as potential targets or effectors.¹⁰⁻¹² However, these pathogenic aspects mainly represent biological effects rather than biological mechanisms of virus-host cell interaction. Conversely, miRNAs seem to be one of these mechanisms.⁵ The interactions between the activated miRNAs complex and the target m-RNA are based on a partial complementarity of miRNA with the target mRNA and results in an inhibitory effect by translation repression and destabilization or degradation.⁵ However, there are several other non-conventional mechanisms of action where miRNAs can alternatively target coding genes or regulatory proteins with both up or downregulation effects.⁵ Viruses such as HCV and HIV do interfere and interact with the host-cell miRNAs to develop the infection. They can block or impair the host miRNA pathways by interacting with some key proteins, synthesize their miRNAs to regulate their own mRNAs (this is just for HCV, not for HIV), or they can make use of cellular miRNAs to enhance their replication cycle.⁵ Such multimodal mechanism of miRNA function and viral interaction makes the understanding of the underlying pathogenic process still very complex. At present, several microRNAs have been found to be potentially involved in the molecular mechanisms of liver damage, HCC development, and viral infection.⁶

However, to the best of our knowledge, this is the first investigation on miRNA profiling in naive liver grafts transplanted to HCV and HCV/HIV-infected patients. Such aspect may at least partially explain the difficulty and heterogeneity in comparing the present results with previous reports. We detected that HIV/HCV

FIGURE 2 Correlation plot summarizing the existing relationships between specific miRNA expression, stage, grade, and HCV peripheral viral load referred to the HCV mono-infected patients. Each box represents the result of the correlation analysis between two factors. The color indicates the type of correlation (blue, direct; red, inverse), the color intensity correlation coefficient (color bar on the right), and the asterisks number the statistical significance (legend on bottom left). HCV, hepatitis C virus; miRNA, microRNA



co-infected recipients, showed significant downregulation of miR-101, miR-122, miR-192 when compared to HCV mono-infected patients. Miyaaki et al.⁶ analyzed the miRNA expression profiles on explanted livers in HCV mono-infected and HIV/HCV co-infected LT patients. In line with the present findings, they also reported in co-infected patients a significant downregulation of miR-101. One of the biological activities of this miRNA is to suppress the TGF- β signaling, which has a potent pro-fibrotic effect by activating hepatic stellate cells (HSCs) for extracellular matrix remodeling and by enhancing the release of pro-fibrogenic cytokines by the hepatocytes.¹³ Therefore, miR-101 downregulation associated with HCV/HIV infection may represent one of the mechanisms sustaining accelerated fibrosis noted in these patients. In the present study, no significant correlation was noted between miR-101 and Ishak staging, probably because fibrosis severity was relatively low. On the other hand, we noted that miR-101 levels inversely correlated with the peripheral HCV viral load. To the best of our knowledge, this is the first report on the potential role of miR-101 in the viral replication cycle of HCV.

miR-122 is one of the most expressed miRNAs in the liver, accounting for about 52% of the whole hepatic miRNome in humans.¹⁴ It plays a central role in hepatocytes replication, differentiation, and homeostasis, as well as in the regulation of cholesterol and fatty acid metabolism.^{5,14} The genetic deletion of miR-122 has been shown not only to severely impact lipid metabolism but also to drive microsteatosis and inflammation, which progressed to steatohepatitis and fibrosis.¹⁴ However, miR-122 is also used by HCV to enhance its viral translation and genome stabilization.⁵ Our results confirmed these

functions of miR-122. As a matter of fact, in HCV/HIV co-infected recipients, miR-122 levels were significantly downregulated and were inversely correlated with HCV peripheral viral load and grading. However, some studies have reported even an upregulation of miR-122 in co-infected patients.^{5,15}

miR-192 has been associated with relevant signaling pathways in different types of liver injury.^{16,17} In HCV infection, it has been reported that miR-192 is upregulated, with a secondary expression of transforming growth factor- β 1 but heterogeneous effect on HCV replication.^{17,18} No data are available on HCV/HIV-co-infected recipients.

All components of the miR-200 family, including miR-200c, are functionally involved in the regulation of liver damage and inflammatory response.^{19,20} Moreover, miR-200c is highly expressed in chronic HCV patients and may represent an early biomarker of reinfection after transplantation.²¹ Even miR-338 3p could represent a key clinical biomarker for the identification of new therapeutic strategies or in the prevention of liver rejection. Under normal conditions, miR-338 3p has a tumor-suppressive function and contributes to liver homeostasis.²² However, its downregulation promotes cell proliferation,²³ hepatic fibrosis, and hepatic stellate cells activation.²⁴⁻²⁸ The present investigation aimed at further exploring the potential role of miRNA in the pathogenesis of liver fibrosis, inflammation, and virus reactivation in HCV-HIV transplanted patients, and these data altogether support the selection of miRNA performed in the present study.

The demonstration that, as early as 6 months after an uneventful LT, the grafts of HCV and HCV/HIV recipients had a significant

Overall Correlations in HCV-HIV patients

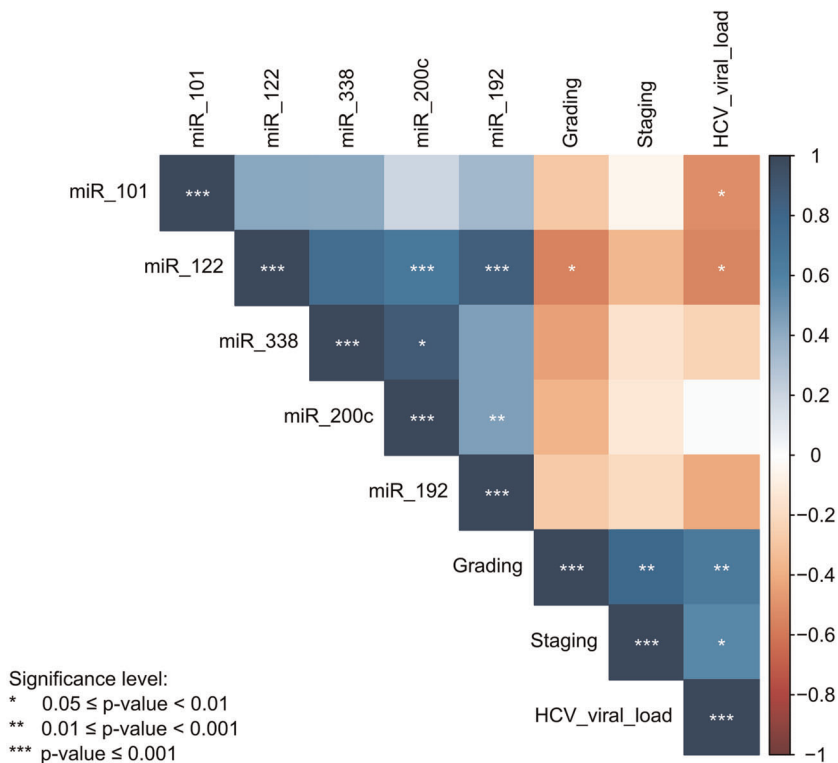


FIGURE 3 Correlation plot summarizing the existing relationships between miRNA expression, stage, grade, and HCV peripheral viral load referred to the HCV-HIV co-infected patients. Each box represents the result of the correlation analysis between two factors. The color indicates the type of correlation (blue, direct; red, inverse), the color intensity the correlation coefficient (color bar on the right), and the asterisks number the statistical significance (legend on the bottom left). HCV, hepatitis C virus; HIV, human immunodeficiency virus; miRNA, microRNA

difference in the expression of clinically relevant miRNAs when compared to noninfected control, was an impactful finding with crucial clinical implications. miRNAs could potentially be used as markers of early liver damage or be the target of new therapeutic strategies. Of notice, none of the patients were treated with DAAs before or within 6 months after LT, thus no therapy-related confounding effect was present. Meanwhile, these results enforce the importance of treating HCV with DAA, particularly in HCV/HIV co-infected patients, before LT rather than waiting the post-LT phase, as the risk of very early or immediate graft damage due to HCV recurrence is significant. Overall, a correlation among some of the targeted miRNAs and Ishak staging, grading or HCV peripheral viral load was found only in HCV/HIV co-infected recipients but not in mono-infected ones. A possible explanation might be found in the timing of the liver graft biopsies. At 6 months after LT and in the absence of the hyper-replicative trigger by HIV, the isolated HCV graft infection might have been at a too early stage to be detectable.

Surely, HCV infection can nowadays be treated with DAAs and does no more constitute a major clinical concern. Nonetheless, it does still represent a relevant model of liver damage with profibrotic, pro-inflammatory, and pro-oncogenic effects. Moreover, some miRNAs involved in HCV and HCV/HIV co-infection have been identified as biological biomarkers and/or potential therapeutic targets even for other nonviral liver diseases, HCC, and other non-hepatic tumor types.¹⁸ For example, the downregulation of miR-101 seems to be implicated in the proliferation, apoptosis, angiogenesis, drug resistance, invasion, and metastasis of HCC, gastric cancer,

intrahepatic cholangiocarcinoma, osteosarcoma, non-small-cell lung cancer, oral squamous cell carcinoma, bladder transitional cell carcinoma, cervical cancer, intraductal and ER α -positive breast cancer.^{29,30} Moreover, several studies have shown its prognostic value not only in terms of correlation with clinicopathological features of the tumors but also in terms of prediction of patient overall survival and risk of tumor recurrence.³¹ miR-122 has been investigated as a biomarker of drug-induced liver injury³¹ as well as endometriosis,³² metabolic syndromes, and type 2 diabetes.³³ Moreover, it has been identified as a prognostic marker for patients with HCC,³⁴ colorectal cancer,³⁵ and glioma.³⁶ Therefore, the investigation and understanding of the precise pathogenic mechanism underlying HIV/HCV co-infection may be considered still clinically relevant not only in infected patients but also in patients with tumor or severe liver injury. Lastly, the present investigation has also demonstrated the feasibility of analyzing miRNA profiles with RT-PCR from FFPE samples, rather than relying just on plasma circulating miRNAs. At present, several liver-deregulated miRNAs have been found to be potentially involved in the molecular mechanisms of viral infections and liver damage. However, considering the poor quality of FFPE-extracted nucleic acids and the low amount of liver tissue available from a post-transplantation needle biopsy, we decided to explore the feasibility of studying miRNA in our experimental setting by adopting a hypothesis-driven approach, selecting miRNAs whose possible role in liver infections was supported by the literature. For this reason, a targeted approach by RT-qPCR was employed to better characterize the expression profile of selected miRNA with a known functional

role in the inflammatory process, in the hepatic fibrogenesis, and in the viral replication cycle.

This study presents several limitations: a small study population; the unavailability of data on the impact of DAA therapy on the miRNA expression profiles; the unavailability of biopsies obtained at a later period after LT, possibly characterized by greater fibrosis, and thus useful to corroborate the pathogenic link between miRNA dysregulation and fibrosis.

The feasibility of analyzing miRNAs from post-transplant liver biopsies as well as the evidence of an miRNA expression profile dysregulation underlying early liver graft injury bring new research perspectives. Further studies may better characterize gene networks and pathways involved in viral reinfection, by taking advantage of high-throughput technologies, such as next generation sequencing. An overview of miRNAs expression profile using an untargeted approach can assess more targets simultaneously, giving a more comprehensive analysis of biological processes. Furthermore, intracellular signaling, regulatory pathways, and gene networks could be associated with differences in mRNA expression by transcriptional analysis of targeted genes.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Riccardo Pravisani, Michela Bulfoni, Umberto Baccarani, Daniela Cesselli, and Susumu Eguchi, designed the study. Michela Bulfoni and Daniela Cesselli performed the analysis. Emiliano Dalla performed the statistical analysis. Riccardo Pravisani, Umberto Baccarani, Michela Bulfoni, Daniela Cesselli, and Masaaki Hidaka wrote the paper. Carla Di Loreto, Umberto Baccarani, and Susumu Eguchi supervised the research and reviewed the paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author on reasonable request. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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