Check for updates

Journal of Viral Hepatitis

WILEY

JOURNAL OF VIRAL HEPATITIS JVH

SHORT COMMUNICATION OPEN ACCESS

MiRNome Profiling of Circulating Extracellular Vesicles in Patients With Chronic Hepatitis D Undergoing Pegylated Interferon Alpha Treatment

Gian Paolo Caviglia[1] | Elisabetta Casalone[2] | Antonella Olivero[1,3] | Giovanni Birolo[2] | Alessia Ciancio[1,3] | Giuseppe Matullo[2,3] | Mario Rizzetto[1,3]

[1]Liver Unit, Department of Medical Sciences, University of Turin, Turin, Italy | [2]Medical Genetics Unit, Department of Medical Sciences, University of Turin, Turin, Italy | [3]A.O.U. Città della Salute e della Scienza, Molinette Hospital, Turin, Italy

Correspondence: Gian Paolo Caviglia (gianpaolo.caviglia@unito.it)

Received: 12 February 2024 | **Revised:** 24 July 2024 | **Accepted:** 31 July 2024

Funding: The study was funded by University of Turin local research grant (OLIA_RILO_20_02).

Keywords: biomarkers | hepatitis D virus | microRNA | treatment

ABSTRACT

Micro-RNAs (miRNAs) are involved in the modulation of viral replication and host immune antiviral response. Using next-generation sequencing, we investigated the miRNome profile of circulating extracellular vesicles in 20 patients with chronic hepatitis D virus (HDV) infection undergoing pegylated interferon alpha (Peg-IFNα) treatment. Circulating miRNAs' expression was analysed according to virologic response (i.e., HDV RNA clearance maintained at least 6 months after the end of therapy). Overall, 8 patients (40%) achieved a virologic response to Peg-IFNα treatment. At baseline, 14 miRNAs were differentially expressed between responders and non-responders; after 6 months of Peg-IFNα treatment, 7 miRNAs (miR-155-5p, miR-1246, miR-423-3p, miR-760, miR-744-5p, miR-1307-3p and miR-146a-5p) were consistently de-regulated. Among de-regulated miRNAs, miR-155-5p showed an inverse correlation with HDV RNA (at baseline: $r_s = -0.39$, $p = 0.092$; at 6 months: $r_s = -0.53$, $p = 0.016$) and hepatitis B surface antigen (HBsAg) (at baseline: $r_s = -0.49$, $p = 0.028$; at 6 months: $r_s - 0.71$, $p < 0.001$). At logistic regression analysis, both miR-155-5p (at baseline: OR = 4.52, $p = 0.022$; at 6 months: OR = 5.30, $p = 0.029$) and HDV RNA (at baseline: OR = 0.19, $p = 0.022$; at 6 months: OR = 0.38, $p = 0.018$) resulted significantly associated to virologic response. Considering that Peg-IFNα still has a relevant role in the treatment of patients with chronic hepatitis D infection, the assessment of EV miR-155-5p may represent an additional valuable tool for the management of HDV patients undergoing Peg-IFNα treatment.

1 | Introduction

Chronic hepatitis D virus (HDV) infection is responsible for the most severe form of chronic hepatitis in humans. Though the epidemiological scenario of HDV has changed in the last decades following the implementation of hepatitis B virus (HBV) vaccination programmes, the medical burden caused by chronic HDV infection is still relevant; paradigmatic are the data recently reported by our group on the disproportionate ratio of HDV-to-HBV liver transplants in spite of a prevalence of HDV much lower than HBV in the general population [1]. Since the 1980s, interferon-α (IFNα) was the only therapy empirically adopted for the treatment of chronic hepatitis D despite the suboptimal response rates (10% of hepatitis B surface

Abbreviations: EVs, extracellular vesicles; FC, fold change; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HDV, hepatitis D virus; miRNAs, micro-RNAs; NGS, next generation sequencing; NR, non-responders; Peg-IFNα, pegylated interferon alpha; R, responders.

Gian Paolo Caviglia and Elisabetta Casalone should be considered joint first authors.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2024 The Author(s). *Journal of Viral Hepatitis* published by John Wiley & Sons Ltd.

antigen [HBsAg] loss and 25% of HDV RNA clearance) and not negligible safety concerns. Furthermore, more than 50% of patients with undetectable HDV RNA at the end of therapy developed a virologic relapse within 10 years after IFNα treatment [2]. In order to identify predictors of response or to establish appropriate stopping rules, several virologic (i.e., HDV RNA) and serologic (i.e., HBsAg) biomarkers have been investigated, but results are still far from being conclusive [3]. Micro-RNAs (miRNAs) are short non-coding RNA involved in the epigenetic regulation of multiple signalling pathways among which the modulation of viral replication and host immune antiviral response. Most of circulating miRNAs are released in the circulation packaged in extracellular vesicles (EVs); host miRNAs can be transferred to the recipient cells to regulate viral infection and modulate cellular functions. Recent studies have shown an association between circulating miRNAs and the progression of liver fibrosis in patients with chronic HBV infection [4]. Moreover, specific serum miRNAs' profiles have been associated both with the different phases of chronic HBV infection and with the response to IFN-based treatment [4]. At present, data in patients with chronic HDV undergoing pegylated (Peg)-IFNα are missing. Therefore, the aim of the present pilot study was to evaluate the role of EV miRNAs as potential biomarkers for the prediction of Peg-IFNα treatment response in patients with chronic HDV infection.

2 | Materials and Methods

We retrospectively enrolled 20 patients with chronic HDV infection (median age: 44, IQR 34–56 years; males: $n = 16$; 80%) that underwent Peg-IFNα with available plasma samples collected at baseline and 6 months of therapy and stored at −80°C. Virologic response was defined as HDV RNA clearance maintained at least 6 months after the end of treatment. All patients signed written informed consent; the study was conducted according to the guidelines of the Declaration of Helsinki as revised in 2008 and approved by the Institutional Ethics Committee (approval no. CEI-690).

For HDV RNA quantitation, viral RNA was isolated from 400 μL of plasma by automated extractor (Qiagen EZ1 Advanced extractor) using Qiagen EZ1 DSP Virus kit (QIAGEN GmbH, Germany) and amplified by in-house real-time quantitative PCR calibrated against the 1st WHO International Standard for HDV RNA (PEI code 7657/12; Paul-Ehrlich Institute, Germany) (95% limit of detection = 9.7 IU/mL). HBsAg was measured by a chemiluminescent enzyme immunoassay (Lumpilse, Fujirebio, Japan). Circulating EVs were isolated from plasma samples collected at T0 and at 6 months of Peg-IFNα treatment; miRNAs expression was determined by next-generation sequencing (NGS) approach using NEBNext Multiplex Small RNA Library PrepSet (NewEngland Biolabs) on NextSeq550 (Illumina Inc.) platform. Differential expression analysis was performed using DESeq2 v1.22.2 adjusting for age and sex. Differentially expressed miRNAs were defined as having a $p < 0.05$ with at least ±2.0-fold change (FC) (Log_2 FC ±1.0) between the groups. MultiMiR Bioconductor's package was used to retrieve miRNAs validated target genes from Mirecords, Mirtarbase and Tarbase databases [5]. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis (ShinyGO v. 0.80) was performed to identify gene-signalling pathways regulated by the differentially expressed miRNAs. The Mann–Witney test or Wilcoxon test were used to compare continuous variables between independent or paired samples, respectively. Correlation analyses were performed by non-parametric Spearman's correlation test while association between variables was investigated by logistic regression analysis.

3 | Results

At baseline, median HDV RNA and HBsAg were 4.98 (IQR 3.65–5.52) Log IU/mL and 3.82 (IQR 3.26–4.26) Log IU/mL, respectively. Eight patients (40%) cleared HDV RNA during treatment and were still negative at 6 months after the end of treatment (responders, R); among them, two patients cleared the HBsAg and one became anti-HBs positive. Twelve patients (60%) were non-responders (NR).

Overall, 144 miRNAs were identified by NGS and 14 miRNAs resulted differentially expressed between R and NR at baseline (4 miRNAs were upregulated while 10 were downregulated in R vs. NR) (Figure 1). Log_2 FC values and the corresponding levels of significance are reported in Table 1. Functional enrichment analysis revealed 1650 experimentally validated target genes (Table S1); the significantly enriched terms in KEGG for each miRNA-target mRNA are reported in Table S2.

After 6 months of Peg-IFNα treatment, 7 of the 14 miRNAs (namely miR-155-5p, miR-1246, miR-423-3p, miR-760, miR-744-5p, miR-1307-3p and miR-146a-5p) were consistently de-regulated. Comparing miRNA expression levels between baseline and 6 months plasma samples, we observed distinct expression profiles according to response to Peg-IFNα treatment; miR-4433b-3p and miR-155-5p were significantly de-regulated in R but not in NR, while miR-1-3p resulted differentially expressed in NR but not in R. However, only for miR-155-5p the variation observed during Peg-IFNα further increased the difference in miR-155-5p expression between R and NR at 6 months of therapy.

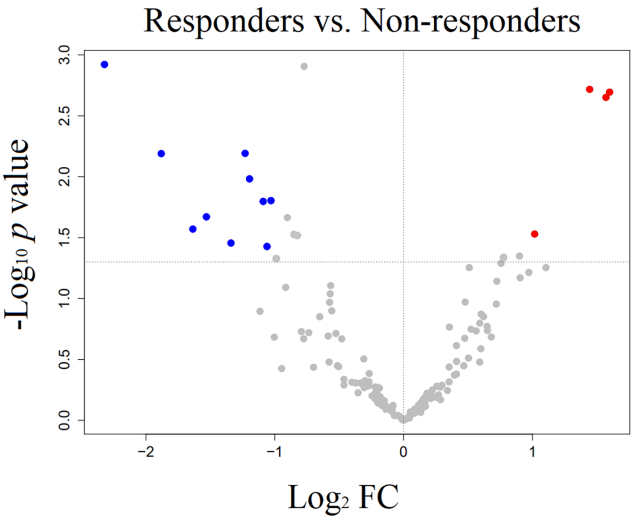

FIGURE 1 | Volcano plot depicting the 144 miRNAs identified by NGS at baseline. Blue dots represent the 10 downregulated miRNAs, while red dots represent the 4 upregulated miRNAs.

13652893, 2024, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/jvh.14000 by Universita Di Torino, Wiley Online Library on [20/02/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 | Principal EV miRNAs differentially expressed according to treatment response at baseline and at 6 months of therapy.

	T0		6 months		R		NR	
	R versus NR		R versus NR		T0 versus 6 months		T0 versus 6 months	
miRNAs	Mean Log_2 FC	*p*	Mean Log_2 FC	*p*	Mean Log_2 FC	*p*	Mean Log_2 FC	*p*
miR-4433b-3p	−2.32	0.001	−0.86	0.139	1.66	0.037	−0.15	0.750
miR-155-5p	1.44	0.002	2.51	<0.001	1.22	0.021	0.26	0.508
miR-1-3p	1.60	0.002	0.31	0.601	−0.45	0.389	1.20	0.013
miR-1246	1.57	0.002	1.41	0.015	0.16	0.684	0.26	0.633
miR-423-3p	−1.23	0.006	−0.98	0.016	−0.16	0.707	−0.62	0.075
miR-760	−1.88	0.006	−1.77	0.001	0.80	0.293	0.13	0.765
miR-744-5p	−1.20	0.010	−1.82	<0.001	0.03	0.954	−0.47	0.257
miR-361-3p	−1.03	0.016	−0.80	0.140	−0.54	0.364	−0.56	0.110
miR-140-3p	−1.09	0.016	−0.34	0.456	0.65	0.146	−0.41	0.269
miR-342-5p	−1.53	0.021	0.72	0.113	0.92	0.036	−0.63	0.101
miR-625-3p	−1.64	0.027	−1.18	0.080	0.07	0.923	−0.65	0.281
miR-224-5p	1.02	0.030	0.51	0.322	0.28	0.642	0.30	0.367
miR-1307-3p	−1.34	0.035	−1.29	0.012	−0.11	0.864	−0.43	0.365
miR-146a-5p	−1.06	0.037	−1.37	0.001	0.10	0.798	−0.14	0.728

Note: $p < 0.05$ were considered significant.
Abbreviations: FC, fold change; miRNAs, micro RNAs; NR, non-responders; R, responders; T0, baseline.

Given the unique de-regulation profile of miR-155-5p, we further investigated the relationship miR-155-5p expression levels and virologic features as well as with virologic response to Peg-IFNα therapy. At baseline, miR-155-5p was inversely correlated with HBsAg ($r_s = -0.49$, 95% CI −0.77 to −0.06; $p = 0.028$) while showed just a trend with HDV RNA ($r_s = -0.39$, 95% CI −0.71 to 0.07; $p = 0.092$). At 6 months of therapy, miR-155-5p showed a strong inverse correlation with both HBsAg ($r_s = -0.71$, 95% CI −0.88 to −0.39; $p < 0.001$) and HDV RNA ($r_s = -0.53$, 95% CI −0.79 to −0.12; $p = 0.016$). At logistic regression analysis, both miR-155-5p (at baseline: OR = 4.52, 95% CI 1.25–16.38; $p = 0.022$; at 6 months: OR = 5.30, 95% CI 1.19–23.65; $p = 0.029$) and HDV RNA (at baseline: OR = 0.19, 95% CI 0.05–0.79; $p = 0.022$; at 6 months: OR = 0.38, 95% CI 0.17–0.84; $p = 0.018$) resulted significantly associated to virologic response.

4 | Discussion

To the best of our knowledge, this is the first study that investigated the differences of plasma EVs miRNA profiles in patients with chronic HDV infection who have received IFN-based treatment. By analysing the whole miRNA profile, we identified 14 differentially expressed miRNAs that distinctly characterised R and NR to treatment. Notably, miR-155-5p was significantly de-regulated at baseline, after 6 months of therapy, and showed different kinetics between R and NR, suggesting a potential role for miR-155-5p not only as biomarker but also as potential therapeutic target.

MiR-155 is a master regulator of immune response and inflammation; upregulation of miR-155 induces an increase of cytokine release during the inflammatory process and it is a positive regulator of tumour necrosis factor (TNF)-α [6]. As a matter of fact, type 1 cytokines such as TNF-α are significantly increased both in patients with chronic HBV mono-infection as well as chronic HDV coinfection compared to healthy subjects [7]. Furthermore, in hepatitis B e antigen (HBeAg)-positive chronic hepatitis B patients, Peg-IFNα monotherapy induced TNF-α expression only in virologic responders while no TNF-α increased was observed in non-responders [8]. Even though no mechanistic data are available in patients with chronic HDV infection, a previous in vitro study showed that miR-155 was able to elicit innate antiviral immunity through promoting JAK/STAT signalling pathways by targeting SOCS1, while inhibiting HBV replication in human hepatoma cells [9]. In addition, patients with chronic HCV infection exhibited altered intrahepatic miR-155 expression as compared to healthy controls, while among HCV patients undergoing IFN-based treatment, serum miR-155 expression was significantly lower in NR than in those achieving a sustained virologic response, both at baseline and at the end of therapy [10]. Consistently, KEGG enrichment analysis identified target genes and pathways related to TNF signalling, hepatitis B, hepatitis C and HCC.

Recently, it has been observed that serum miRNA profile was significantly different between patients with chronic hepatitis B and those with HBeAg-negative-HBV infection independently from HDV-co-infection [11]. Specifically, circulating miRNAs revealed a highly similar pattern in patients with chronic hepatitis B and D, while 31 miRNAs were significantly deregulated

13652893, 2024, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/jvh.14000 by Universita Di Torino, Wiley Online Library on [20/02/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

in HBV carriers with HBeAg-negative infection as compared to both chronic hepatitis B and D patients, further supporting the hypothesis of a shared pathogenic mechanism of liver disease progression in HBV mono-infected and HDV co-infected patients. Given that miR-155-5p is involved in a number of viral infections other than chronic viral hepatitis [12], it is reasonable to suppose that this miRNA is critical for the induction of an effective anti-virus immune response and viral clearance, paving the way to potential combination approaches using miR-155-5p modulators to conventional therapies.

Finally, results from the MYR204 trial showed that 46% of chronic hepatitis D patients treated with bulevirtide (BLV) at a daily dose of 10 mg plus Peg-IFNα2a achieved undetectable HDV RNA at 48 weeks after the end of treatment; interestingly, the rate of undetectable HDV RNA was distinctly higher compared to Peg-IFNα2a alone (25%), BLV at a daily dose of 10 mg alone (12%) and BLV at a daily dose of 2 mg plus Peg-IFNα2a (26%) [13]. However, should be noted that the majority of grade ≥3 adverse events occurred in the IFN-based treatment arms [13]. In this scenario, miR-155-5p may improve the selection of patients that could benefit from combination treatment and allow the definition of appropriate stopping rules.

We are aware of the limitations of this study. First of all the small sample size may limit the scope of the analysis, leading to less accurate results. Furthermore, we investigated the expression of miRNAs packaged in EVs, thus restricting the analysis to those miRNAs actively released into the circulation. Therefore, our results may not be directly comparable to the results from other studies that investigated miRNAs expression in the whole serum compartment.

In conclusion, patients with chronic HDV infection showed different profiles of EV miRNAs expression according to the virologic response to Peg-IFNα treatment. Noteworthy are the results observed for miR-155-5p that is involved in the modulation of inflammatory pathways, host immune response and tumour development. Considering that Peg-IFNα still has a relevant role in the treatment of chronic hepatitis D, further studies are required to confirm the diagnostic usefulness of circulating miR-155-5p for the management of patients with chronic hepatitis D undergoing Peg-IFNα treatment.

Author Contributions

Concept and design: G.P.C., E.C. and A.O. Data collection: G.P.C., A.O. and A.C. Performing experiments: G.P.C., E.C. and A.O. Data analysis: G.P.C., E.C. and G.B. Drafting of the manuscript: G.P.C. and E.C. Critical revision of the manuscript: A.C., G.M. and M.R. Supervision: G.M. and M.R.

Acknowledgements

The study was funded by University of Turin local research grant (OLIA_RILO_20_02). Open access publishing facilitated by Universita degli Studi di Torino, as part of the Wiley - CRUI-CARE agreement.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. G. P. Caviglia, S. Martini, A. Ciancio, et al., "The Hepatitis D Virus in Italy. A Vanishing Infection, Not Yet a Vanished Disease," *Journal of Advanced Research* 33 (2021): 183–187.

2. T. Asselah and M. Rizzetto, "Hepatitis D Virus Infection," *New England Journal of Medicine* 389, no. 1 (2023): 58–70.

3. L. Sandmann and H. Wedemeyer, "Interferon-Based Treatment of Chronic Hepatitis D," *Liver International* 43 (2023): S69–S79.

4. D. Loureiro, I. Tout, S. Narguet, S. M. Benazzouz, A. Mansouri, and T. Asselah, "miRNAs as Potential Biomarkers for Viral Hepatitis B and C," *Viruses* 12, no. 12 (2020): 1440.

5. Y. Ru, K. J. Kechris, B. Tabakoff, et al., "The MultiMir R Package and Database: Integration of MicroRNA-Target Interactions Along With Their Disease and Drug Associations," *Nucleic Acids Research* 42, no. 17 (2014): e133.

6. L. A. O'Neill, F. J. Sheedy, and C. E. McCoy, "MicroRNAs: The Fine-Tuners of Toll-Like Receptor Signalling," *Nature Reviews. Immunology* 11, no. 3 (2011): 163–175.

7. E. C. Townsend, G. Y. Zhang, R. Ali, et al., "The Balance of Type 1 and Type 2 Immune Responses in the Contexts of Hepatitis B Infection and Hepatitis D Infection," *Journal of Gastroenterology and Hepatology* 34, no. 4 (2019): 764–775.

8. S. L. Chen, H. Xiao, G. J. Li, and Y. J. Shen, "Expression Pattern of Cytokines in Patients With Chronic Hepatitis B Receiving PEGinterferon Therapy," *International Journal of General Medicine* 16 (2023): 1771–1782.

9. C. Su, Z. Hou, C. Zhang, Z. Tian, and J. Zhang, "Ectopic Expression of MicroRNA-155 Enhances Innate Antiviral Immunity Against HBV Infection in Human Hepatoma Cells," *Virology Journal* 8 (2011): 354.

10. X. Xue, J. Wang, K. Fu, et al., "The Role of miR-155 on Liver Diseases by Modulating Immunity, Inflammation and Tumorigenesis," *International Immunopharmacology* 116 (2023): 109775.

11. D. Cavallone, E. D. B. Ornos, G. Ricco, et al., "The Circulating miRNA Profile of Chronic Hepatitis D and B Patients is Comparable but Differs From That of Individuals With HBeAg-Negative HBV Infection," *Viruses* 15, no. 11 (2023): 2257.

12. A. Jafarzadeh, A. Naseri, L. Shojaie, et al., "MicroRNA-155 and Antiviral Immune Responses," *International Immunopharmacology* 101 (2021): 108188.

13. T. Asselah, V. Chulanov, P. Lampertico, et al., "Bulevirtide Combined With Pegylated Interferon for Chronic Hepatitis D," *New England Journal of Medicine* 391 (2024): 133–143.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.

13652893, 2024, 11, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/jvh.14000 by Universita Di Torino, Wiley Online Library on [20/02/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License