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Large-scale screening of circulating microRNAs in individuals with HIV-1 mono-infections reveals specific liver damage signatures

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1)-induced inflammation and/or long-term antiretroviral drug toxicity may contribute to the evolution of liver disease. We investigated circulating plasma microRNAs (miRNAs) as potential biomarkers of liver injury in patients mono-infected with HIV-1. We performed large-scale deep sequencing analyses of small RNA level on plasma samples from patients with HIV-1 mono-infection that had elevated or normal levels of alanine aminotransferase (ALT) or focal nodular hyperplasia (FNH). Hepatitis C virus (HCV) mono-infected patients were also studied. Compared to healthy donors, patients with HIV-1 or HCV mono-infections showed significantly altered (fold change > 2, adjusted $p < 0.05$) level of 25 and 70 miRNAs, respectively. Of the 25 altered miRNAs found in patients with HIV-1, 19 were also found in patients mono-infected with HCV. Moreover, 13 of the 14 most up-regulated miRNAs (range: 9.3–3.4-fold increase) in patients with HCV mono-infections were also up-regulated in patients with HIV-1 mono-infections. Importantly, most of these miRNAs significantly and positively correlated with ALT and aspartate aminotransferase (AST) levels, and liver fibrosis stage ($p < 0.05$). MiR-122-3p and miR-193b-5p were highly up-regulated HIV-1 mono-infected patients with elevated ALT or FNH, but not in HIV-1 patients with normal levels of ALT. These results reveal that HIV-1 infections impacted liver-related miRNA levels in the absence of an HCV co-infection, which highlights the potential of miRNAs as biomarkers for the progression of liver injury in HIV-1 infected patients.

1. Introduction

After the advent of highly active antiretroviral therapy (ART), liver disease became a leading cause of morbidity and mortality in patients with human immunodeficiency virus type 1 (HIV-1) infections (Ford et al., 2015). HIV-1 infections may cause liver disease through several mechanisms; HIV-1 can directly infect stellate and Kupffer cells, or it can cause chronic inflammation, microbial product translocation, and low-grade disseminated coagulopathy (Deeks et al., 2013). Consequent to these complex interactions, end-stage liver disease and hepatocellular carcinoma (HCC) are often complications in patients with HIV-1 infections. This effect is exacerbated in patients with chronic hepatitis C virus (HCV) infections and/or drug-induced hepatotoxicity. Remarkably, abnormal liver enzymes are common in patients with HIV-1, even in the absence of viral hepatitis or alcohol abuse (Rockstroh, 2017). End-stage liver disease and cirrhosis were also associated with bacterial translocations (Wiest and Garcia-Tsao, 2005), immune system activation and inflammation (Montes-de-Oca et al., 2011), and heart failure (So-Armah et al., 2017). Consequently, we need biomarkers of liver disease progression to assess for advanced fibrosis and to identify patients that should be enrolled in HCC screening programs.

MicroRNAs (miRNAs) are 19–22-nucleotide-long, non-coding RNAs that operate as negative regulators of translation; they are involved in many cellular processes (Ameres and Zamore, 2013). Numerous

Abbreviations: HIV-1, Human immunodeficiency virus type 1; miRNA, microRNA; ALT, alanine aminotransferase; FNH, focal nodular hyperplasia; HCV, Hepatitis C virus; AST, aspartate aminotransferase; HCC, hepatocellular carcinoma; QC, quality control; LNA, Locked Nucleic Acid; RT, Reverse Transcription; qPCR, quantitative PCR; PCA, principal component analysis; IQR, interquartile range; FC, fold change

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miRNAs are in the circulation and in tissues. Importantly, many circulating miRNAs regulate gene expression in other tissues (Thomou et al., 2017). Dysregulated levels of specific miRNAs have been associated with a variety of diseases, including cancer (Mitchell et al., 2008), diabetes (Guay et al., 2011), obesity (Capobianco et al., 2012), and cardiovascular disease (Caroli et al., 2013). The relationship between liver injury and miRNA biology has been studied profusely (Anadol et al., 2015; Bandiera et al., 2015; Hatziapostolou et al., 2013; Hung et al., 2016; Huo et al., 2017; Jopling et al., 2005; Kitano and Bloomston, 2016; Koenig et al., 2016; Li et al., 2014, 2015; Liang et al., 2010; Liu et al., 2017; Mazzu et al., 2017; Petrelli et al., 2012; Roy et al., 2017; Shaker and Senousy, 2017; van der Ree et al., 2017; Wang et al., 2012, 2015, 2017). Those studies have demonstrated the relevance of miRNAs in liver function and diseases of diverse etiologies.

Some reports have shown that HIV-1 can modify the miRNA profile in plasma (Yahyaei et al., 2016), peripheral blood mononuclear cells (Egana-Gorrono et al., 2014), and gastrointestinal mucosa (Fulcher et al., 2017). The circulating miRNA profile associated with liver damage in HIV-1 has been recently explored in patients with HIV-1/HCV co-infections (Anadol et al., 2015). However, knowledge is lacking about the association between liver injury and peripheral miRNA profiles in patients with HIV-1 mono-infections. In the present study, we conducted a large-scale genome-wide screen to construct a profile of circulating plasma miRNAs related to liver injury in patients with HIV-1 that were not co-infected with hepatotropic viruses. Our results showed that the HIV-1 infection could dysregulate circulating liver-associated miRNAs.

2. Materials and methods

2.1. Study patients

Stored human plasma samples from patients and healthy donors were obtained with written informed consent under Institutional Review Board-approved protocols. For this study, we cross-sectionally analyzed 21 healthy volunteers (control group), 54 patients with HIV-1 mono-infections and 22 patients with HCV mono-infections. Table 1 shows the characteristics of these 3 groups. Patients with HIV-1 infections were recruited from patients that attended the outpatient HIV Clinical Unit at Hospital Universitari Germans Trias i Pujol. Out of 53 patients with HIV -1 mono-infections, 22 had normal ALT levels, 20 had ALT levels above the upper limit of normal, and 11 had biopsy-proven nodular regenerative hyperplasia (Table 2). Patients with HCV mono-infections were recruited from the Liver Unit at Hospital Universitari Germans Trias i Pujol. In this group, liver fibrosis was evaluated with transient elastometry.

2.2. RNA extraction and RT-qPCR-based miRNA assays

Plasma samples from 97 individuals were processed to isolate RNA with the miRCURY RNA Isolation kit-Biofluids (Exiqon), according to the manufacturer's protocol, with slight modifications. We included a pre-clearing step by centrifuging 300 µl of plasma for 5 min at 3000 $\times g$ to pellet cellular debris. Prior to extraction, we added 3 µg glycogen carrier (Ambion) and 2 fmol synthetic RNA from a spike-in mix (spike-in kit, Exiqon) to 200 µl of cleared plasma supernatant. RNA was eluted in 50 µl of RNase-free water. Total RNA extracted from each plasma sample was subjected to quality control (QC) to analyze the robustness of the plasma RNA isolation procedure and the quality of biological samples for assaying miRNA expression. We amplified all samples with the miRNA QC PCR panel (Exiqon) and the miRCURY Locked Nucleic Acid (LNA™) Universal Reverse Transcription (RT) microRNA PCR system. Expression levels of the red blood cell-specific miR-451 and the stable miR-23a were used to monitor hemolysis in all plasma samples. A ΔCt (miR-23a-miR-451) value < 5 indicated no hemolysis; a value ≥ 7 implied an increased risk of hemolysis. We chose samples with the lowest miR-451/miR-23a-3p ratios in each clinical group for subsequent sequencing-based miRNA profiling (ΔCt values ranged from −0.72 to 6.3; average 3.15). We performed RT, followed by quantitative PCR (RT-qPCR), according to the manufacturer's protocol. Plasma RNA was reverse transcribed, followed by real-time PCR amplification with LNA enhanced primers in the ExiLENT SYBR Green master mix (Exiqon) on a Lightcycler 480 real-time PCR system (LC480, Roche). RT-qPCR Cp values were determined with the second derivative method provided in LC480 software.

2.3. Small RNA library generation and sequencing

Small RNA sequencing libraries were prepared according to the TruSeq® Small RNA Sample Preparation Guide (Illumina). Briefly, we

Table 1
Clinical and biochemical characteristics of the whole study population according to infection status.

	Healthy	HCV monoinfected	p-value[a]	HIV-1 monoinfected	p-value
N	21	17	–	53	–
Age, median [IQR], yr	37 (35–44.5)[b]	54 (44.5–70.5)	0.0011	55 (50–60)	< 0.0001
Gender, Male (%)	6 (28)	7 (41)	–	38 (71)	–
Fibroscan (kPa)	–	7.9 (5.3–10.65)	–	–	–
Alkaline Phosphatase (U/L), median (IQR)	54 (46–65)	75 (60–115.5)	0.001	85 (66–111)	< 0.0001
ALT, (U/L) median (IQR)	15 (12–19.5)	36 (29.5–51)	< 0.0001	31 (19–70)	< 0.0001
AST, (U/L) median (IQR)	15 (15–20.5)	35.5 (25.5–46)	< 0.0001	31 (21–50)	< 0.0001
GGT, (U/L) median (IQR)	–	39 (21–128)	–	47 (27–105)	–
Serum Albumin, (g/L) median (IQR)	–	42 (38.5–43)	–	46.1 (42.1–47.65)	–
Total protein, (g/L) median (IQR)	73.4 (71.1–76.6)	71.7 (68.2–74.6)	0.1727	71.9 (68.6–74.4)	0.0765
Total bilirrubin, (umol/L) median (IQR)	8 (6.4–12.65)	10 (8–17)	0.1421	8 (5.3–13.5)	0.7852
HDL, (mmol/L) median (IQR)	1.45 (1.3–1.8)	–	–	1.2 (1–1.5)	–
LDL, (mmol/L) median (IQR)	2.55 (1.95–3.35)	–	–	3.35 (2.6–4.05)	–
Cholesterol, (mmol/L) median (IQR)	4.5 (3.6–5.45)	4.1 (3.55–4.85)	0.2645	5.2 (4.5–6)	0.0374
Triglycerides, (mmol/L) median (IQR)	0.9 (0.5–1.05)	0.9 (0.75–1.1)	0.4359	1.5 (1.2–2)	< 0.0001
Creatinin, (umol/L) median (IQR)	69 (60–79.5)	69 (61.4–84.5)	0.5971	73.5 (65–86.5)	0.1139
Glucosa (mmol/L), median (IQR)	–	5.5 (4.85–6.55)	–	4.8 (4.5–5.5)	–
Platelet counts (×109), median (IQR)	253.5 (189.5–348.5)	190 (144.5–223)	0.0039	198 (149–232)	0.0042
CD4 cell counts (cell/mm3), median (IQR)	–	–	–	454 (285–750)	–
HCV RNA Viral load log10 (IU/ml), median (IQR)	–	6.05 (5.75–6.71)	–	–	–
Undetectable HIV viral load, N (%)	–	–	–	42 (79.2)	–
Patients on ART, N (%)	–	–	–	48 (90.6)	–

[a] Mann Whitney test.
[b] Median, IQR.