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miRNA Expression and HCC Occurrence in HCV Cirrhotic Patients Treated with Direct Acting Antivirals

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Abstract: Background: The risk of hepatocarcinoma in HCV cirrhotic patient responders after treatment with DAAs decrease, but HCC still occurs. A correlation between specific miRNAs and the development of hepatocarcinoma have been highlighted. **Aim:** To investigate miRNA expression in HCV-infected cirrhotic patients treated with DAAs, regarding whether or not they developed HCC at follow-up. **Methods:** A total of 73 outpatients with HCV-related cirrhosis treated with DAAs were enrolled, 28 of which had HCC. Samples were collected at the start and at the end of treatment. In the screening phase, 172 miRNAs were analyzed at baseline. Differentially expressed miRNAs were validated in the entire cohort. **Results**: In the validation phase, at baseline and in patients treated for 12 weeks, miR-28-5p was confirmed to be more highly expressed in the HCC group compared to the non-HCC group. In all of the patients treated for 12 weeks, at end of the treatment we found a significant downregulation in miR-132-3p, miR-133b-3p, miR-221-3p and miR-324-3p. In the HCC group, miR-28-5p was significantly downregulated after DAA therapy as well as in HCC patients treated for 24 weeks. **Conclusion:** In the HCC group, miR28-5p was differently expressed both at baseline and at the end of therapy with DAAs. This difference in expression should suggest its involvement in HCC development.

Keywords: advanced liver disease; liver cancer; hepatitis; biomarkers

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1. Introduction

Hepatocellular carcinoma (HCC) is the third cause of cancer-related death in the world. Hepatitis C virus (HCV) is still one of the leading causes of hepatocellular carcinoma

(HCC) [1–3]. New direct-acting antiviral drugs (DAAs) have completely changed the treatment of HCV by having high efficacy and tolerability, even in patients with advanced liver disease [4,5]. Before the extensive use of DAAs, the risk of HCC in cirrhotic patients who responded to interferon therapy was reduced but not completely eliminated [6]. In this new DAA era, a prospective study suggested that in cirrhotic patients achieving SVR by DAA treatment reduces HCC incidence over time. Despite this, the risk of HCC after SVR with DAA remains high, and this residual risk is a relevant clinical problem [7–10].

In this scenario, early diagnosis of HCC is crucial for improving treatments and patient survival. Nowadays, ultrasound every six months, alone or in combination with AFP serum levels, is considered the gold standard for HCC screening [11]. Both techniques have suboptimal sensitivity and specificity in the diagnosis of early-stage HCC [12]. For this reason, markers for the early diagnosis of HCC are needed.

Micro-RNAs (miRNAs) have been investigated as potential biomarkers in patients with viral hepatitis, liver fibrosis and HCC [13–16]. miRNAs are a class of non-coding RNAs that are circulating, detectable and stable in different body fluids. They are known to negatively regulate gene expression by binding to complementary sequences of specific mRNA targets or through post-transcriptional regulation [17,18]. Some studies have shown a correlation between serum and tissue levels of specific miRNAs and the development of HCC in patients with chronic hepatitis C, and have demonstrated that miRNAs modulate multiple targets in HCC-associated signaling pathways [17–20]. In the early stage of HCC, there is abnormal miRNA expression, and this characteristic suggests that circulating miRNAs could have a potential role as a new type of HCC biomarker. Moreover, targeting candidate miRNAs can be considered a new approach for HCC therapy.

The aim of this study was to characterize miRNA profiles in HCV-related cirrhotic patients treated with DAAs, regarding whether or not HCC develops after short-term SVR.

2. Patients and Methods

Seventy-three patients with HCV-related cirrhosis (52 (71.2%) with compensated cirrhosis and 21 (28.8%) decompensated) treated with DAAs between January 2015 to March 2016 and without a previous history of HCC were retrospectively enrolled at the University Hospital of Padova, Bologna, and Hospital Clinic in Barcelona. Up to 28 patients developed an HCC during the 24 weeks of follow-up after the end of therapy with DAAs (HCC group), whereas 45 patients had no HCC at follow-up (non-HCC group). Samples for miRNA analysis were collected prior to starting therapy (baseline) and at the end of DAA treatment. Clinical data, liver imaging data and laboratory results were registered at 3 points: baseline, end of therapy (EOT) and 24 weeks after the end of therapy (SVR24). Patients received different DAA combinations according to the current national/regional guidelines and availability of treatments. Treatments available were as follows: sofosbuvir (SOF), sofosbuvir + ledipasvir (SOF + LDV), sofosbuvir + daclatasvir (SOF + DCV), sofosbuvir + simeprevir (SOF + SMV) and ombitasvir + paritaprevir + dasabuvir (OMV + PTV + DSV) alone or in combination with ribavirin (RBV). Seven patients from the HCC group and eight from the non-HCC group from the Padova cohort were selected for a previous screening phase of miRNAs at baseline. Afterwards, for the validation phase, baseline differentially expressed miRNAs from the screening phase were tested on patients from Padova, Bologna, and Barcelona (patients considered for the validation phase did not include patients from the screening phase). In this phase, patients were grouped into HCC or non-HCC groups (depending on whether or not HCC developed after DAA treatment) and according to the duration of DAA exposure (12 weeks vs. 24 weeks) (Figure 1). This allowed for a comparison between groups with the same duration of therapy and comparable follow-up time, and an evaluation of micro-RNA expression at the same time after the end of exposure to DAAs. Furthermore, this subdivision took into account the possibility that patients with longer therapy could have a more advanced liver disease. miRNA expression was then analyzed at the end of therapy (EOT) and compared to baseline expression in the HCC and non-HCC groups.

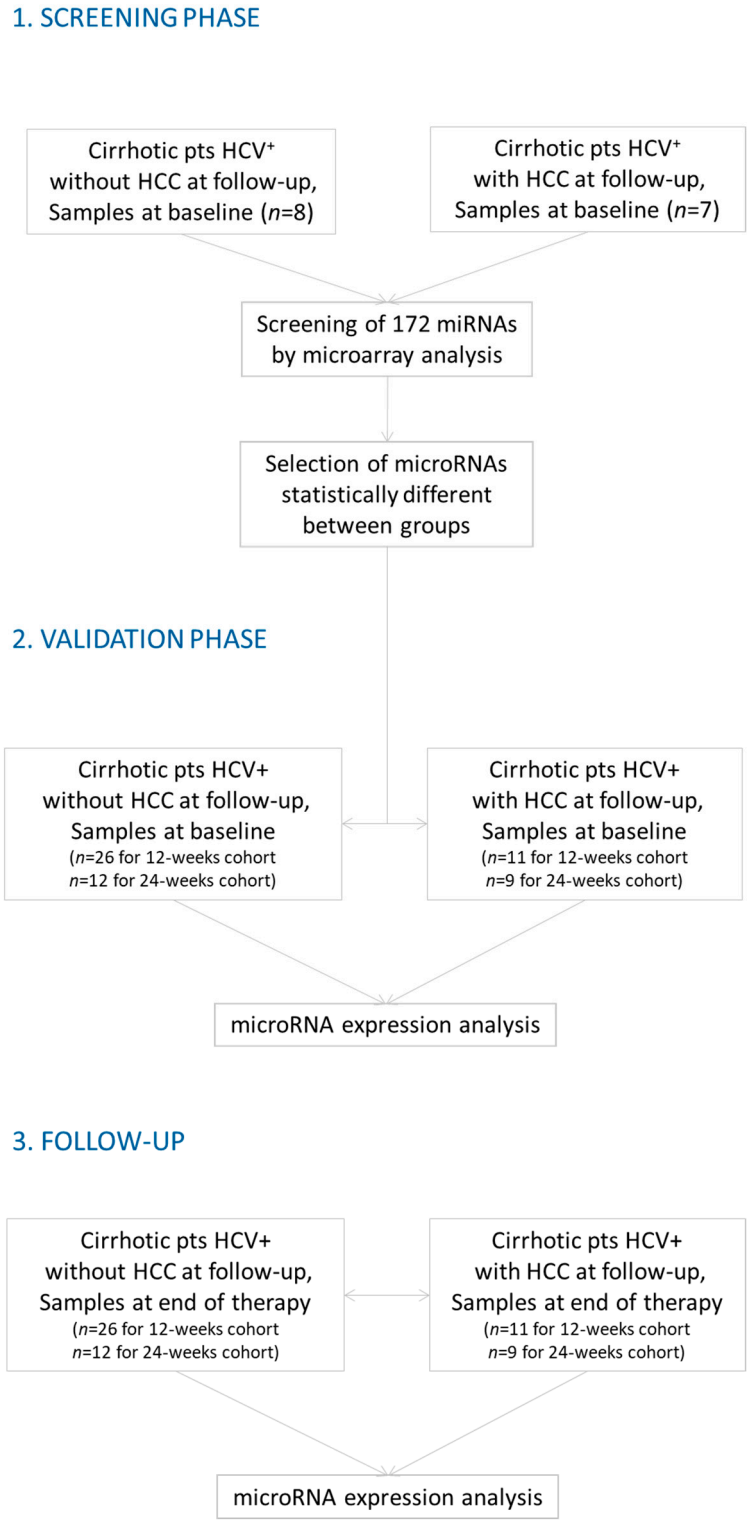

Figure 1. Study design. In the screening phase, miRNAs that were differently expressed at baseline between HCC and non-HCC groups were selected. These miRNAs were validated in a bigger population at baseline and at the end of therapy. The same analysis was conducted in patients treated with DAAs for 12 and 24 weeks.

This study was conducted according to the ethical guidelines of the 2013 Declaration of Helsinki and was approved by the Local Ethics Committee (4045/AO/17).

2.1. Inclusion and Exclusion Criteria

Inclusion criteria were as follows: (a) Patients with HCV-related cirrhosis evaluated by clinical, radiological, elastographic (values of transient elastography > 14 Kpa) or histological criteria [21,22]; (b) patients receiving DAAs between January 2015 to March 2016 for 12 or 24 weeks; (c) absence of HCC at baseline, evaluated with abdominal ultrasound or with a second level radiological examination (computed tomography (CT), magnetic resonance imaging (MRI), contrast-enhanced ultrasound (CEUS) if evidence of nodules on ultrasound; (d) available follow-up of at least 6 months after the EOT; and (d) in the HCC group, HCC occurrence between the start of therapy and 24 weeks after the end of therapy. Exclusion criteria were as follows: history or current HCC at the start of DAAs, previous liver transplantation, HBV or HIV co-infections, and <18 years old.

2.2. Sample Collection, miRNAs Isolation and Analysis

Serum and plasma samples were obtained from 10 mL of whole blood, collected in sterilized tubes and centrifuged at 1500 g/min in a refrigerated centrifuge. Supernatants were transferred to 1 mL Eppendorf tubes and stored at −80 °C. Total miRNAs were isolated from 200 µL of serum or plasma via the Exiqon's miRCURYTM RNA Isolation Kit (Copenhagen, Denmark) according to the manufacturer's protocol [23]. All purified RNA was eluted in 40 µL RNase-free water and stored at −80 °C. To monitor RNA isolation, we used the RNA Spike-in Kit, UniRT (UniSp2, UniSP4, UniSp5 and UniSp6) (Exiqon, Copenhagen, Denmark).

miRNAs were reverse-transcribed using miRCURY LNA™ Universal RT mi-RNA PCR and Universal cDNA Synthesis Kit II (Exiqon, Denmark) according to the manufacturer's instructions. cDNA was diluted 40 times and assayed in 10 µL PCR reactions. In the screening phase (Figure 2), each miRNA was assayed once via qPCR using the miRNA Ready-To-Use Serum/Plasma Focus microRNA PCR panels V.3 (Exiqon, Denmark). Each set of panels comprised 172 LNA miRNA primer sets, 5 spike-in control primer sets and candidate reference miRNAs. Negative controls were included. Amplifications were performed via a Bio-Rad CFX96 Real-Time PCR detection system using the ExiLENT SYBR® Green master mix (Exiqon, Denmark). Amplification curves were analyzed using the CFX Manager software (version 1.1) for the determination of quantification cycle (Cq) values.

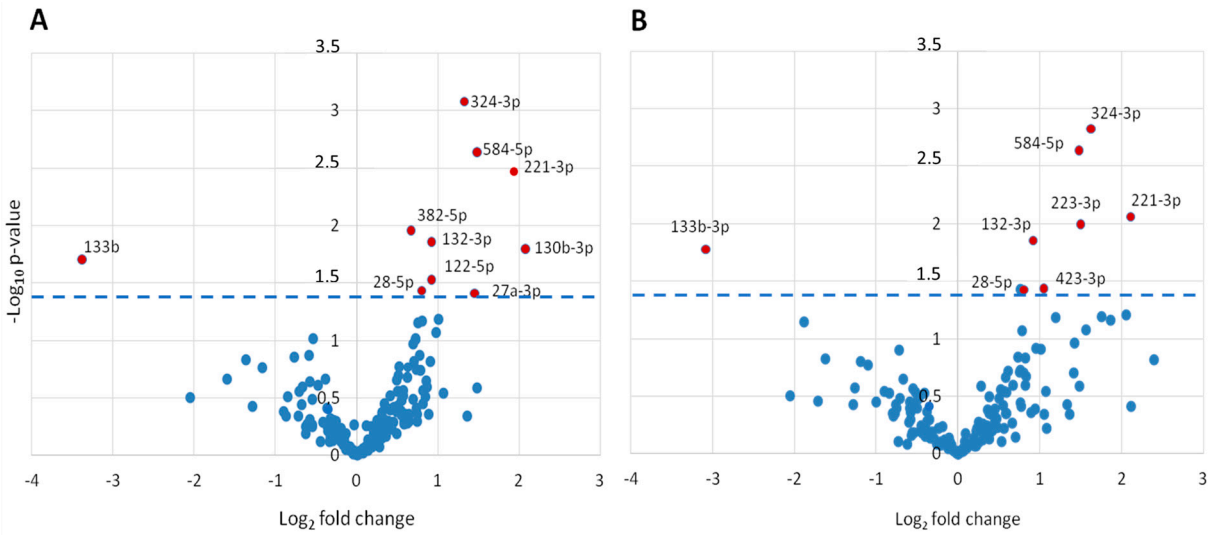

Figure 2. Volcano plot of miRNAs expression data in the screening phase. Differentially expressed miRNA analysis showed the relationship between fold change and statistical significance. Points above the dashed line in the plot represent differentially expressed miRNAs with statistical significance. (**A**): analysis considered all screened patients; (**B**): analysis considered only patients with SVR.

In the validation phase (Figure 1), individual qPCR assays were made for the detection of selected miRNAs in samples. qPCR was performed with a ExiLENT SYBR® Green master mix using LNA™ primers (Exiqon; Denmark), and hsa-miR-92a-3p as an miRNA reference for plasma samples, according to the manufacturer's protocol [24]. All reactions were run in triplicate in a 10 µL reaction. Cq values > 45 were considered negative, and melting point curves were used to verify primer specificity. Primers for selected miRNAs were Exiqon (Denmark).

Potential differences, due to the analysis of plasma and serum samples, had been averted by performing preliminary tests in seven different miRNAs.

2.3. Statistics

Comparisons between more than two groups were performed using the Kruskal–Wallis test. In the case of a positive test ($p < 0.05$), a comparison analysis between pairs of groups was performed using the Mann–Whitney test (non-parametric samples). The Mann–Whitney test was also used for comparison between two independent groups. For paired sample comparison, we used the Wilcoxon test. Statistical significance accepted corresponds to a p-value < 0.05. Statistical analyses were performed using the MedCalc™ software (vers. 15.6 MedCalc Software, Mariakerke, Belgium) and GraphPad Prism 5.01.

3. Results

3.1. Screening Phase: Selection of Relevant miRNAs

At baseline, 172 miRNAs were analyzed in 8 patients from the non-HCC group and 7 patients from the HCC group (screening cohort). Baseline characteristics are extensively reported in Table S1 and summarized in Table 1.

To identify differentially expressed miRNAs, we performed volcano plot filtering between the two groups of the experiment. The threshold used to screen upregulated or downregulated miRNAs is a fold change ≥ 1.5 and a p-value ≤ 0.05 (Figures 2 and S1). In order to avoid the effect of treatment failure, one further analysis was conducted considering only patients who achieved SVR. Two different volcano plots were made, the first with miRNA expression of all patients selected (8 HCC and 7 non-HCC) (Figures 2A and S1A) and the second only with patients with SVR (8 of non-HCC group and 4 of HCC group) (Figures 2B and S1B)).

The volcano plot of all the screened patients identified 10 miRNAs differently expressed in the two groups at baseline (Figures 2A and S1A). Nine miRNAs were upregulated in the HCC group compared to the non-HCC group (hsa-miR-382-5p, hsa-miR-28-5p, hsa-miR-132-3p, hsa-miR-122-5p, hsa-miR-130b-3p, hsa-miR-27a-3p, hsa-miR-324-3p, hsa-miR-584-5p and hsa-miR-221-3p), while hsa-miR133b was downregulated in the HCC group compared to the non-HCC group. The volcano plot of only patients with SVR (Figures 2B and S1B) confirmed the upregulation of miR-221-3p, miR-132-3p, miR-28-5p, miR-584-5p and miR-324-3p and the downregulation of miR-133b-3p in the HCC group. Furthermore, in patients who reached SVR, two new miRNAs (miR-223-3p and miR 423-3p) were upregulated in patients from the HCC group compared to the non-HCC group.

Based on these results, miRNAs that presented a significant difference in expression (in the HCC and non-HCC groups), both in all patients included in the screening and in patients with SVR, were further analyzed in the validation phase.

3.2. Validation Phase

Forty-eight patients were included in the validation phase and were divided into two groups. The first group had a treatment duration of 12 weeks; 26 patients were "non-HCC" and 11 patients were "HCC". The second group had a treatment duration of 24 weeks; 12 were "non-HCC" and 9 were "HCC". We compared the baseline characteristics of patients treated for 12 weeks and 24 weeks and no significant differences were found.

Table 1. Baseline characteristics of patients included in the screening phase.

	Non-HCC (*n* = 8)	**HCC (*n* = 7)**	***p*-Value**
Age, (y) median (Q1–Q3)	68 (55–73)	57 (50–74)	0.6943
Gender (M/F), *n*	4/4	5/2	0.4142
HCV Genotype, *n* (%)			0.2898
Genotype 1	8 (100)	5 (71)	
Genotype 2	0	1 (14)	
Genotype 3	0	1 (14)	
HCV-RNA log(U/mL), median (Q1–Q3)	6.03 (5.71–6.28)	5.48 (5.38–5.67)	0.0289
CTP class, *n* (%)			0.4533
CTP A	7 (88)	5 (71)	
CTP B	1 (12)	2 (29)	
MELD, median (Q1–Q3)	8.5 (7–10.5)	8 (7.3–10.8)	0.7681
Cirrhosis complications, *n* (%)	5 (63)	2 (29)	0.1882
AFP, median (Q1–Q3)	9.1 (5–20.9)	11.7 (8.4–26.7)	0.6126
Previous Therapy, *n* (%)	2 (25)	3 (43)	0.4439
IFN + RBV	2 (100)	1 (33)	
IFN + RBV + Telaprevir	0	1 (33)	
SOF + RBV	0	1 (33)	
Type of DAAs therapy, *n* (%)			0.0443
SOF + RBV	0	3 (43)	
SOF + LDV ± RBV	8 (100)	3 (43)	
SOF + DCV ± RBV	0	1 (14)	
Ribavirin, *n* (%)	6 (75)	6 (86)	0.6171
SVR	8 (100)	4 (57)	0.0455

Legend: CTP: Child–Turcotte–Pugh score; MELD: model for end-stage liver disease; AFP: alpha-fetoprotein; SOF: sofosbuvir; RBV: ribavirin; LDV: ledipasvir; DCV: daclatasvir; SVR: sustained virological response. Cirrhosis complications: ascites, bacterial infection, gastrointestinal bleeding and encephalopathy.

3.2.1. Patients Treated for 12 Weeks

HCC and non-HCC groups of patients did not differ in the baseline characteristics evaluated and all patients from this cohort achieved SVR (Table 2). HCC characteristics of the HCC group are reported in Supplementary Table S2.

In the HCC group, 91% of patients underwent ultrasound before the start of HCC screening, with a time of 1.7 months from the last imaging to the start of DAA treatment (Q1–Q3: 0.5–3.1). HCC occurred after the end of therapy in 10 out of 11 patients (Table S2).

The expression of miRNAs at baseline was compared between the HCC and non-HCC groups, as reported in Figure 3 and Supplementary Table S3, showing no significant difference between the two, except for miR-28-5p, which was significantly upregulated in patients from the HCC group.

3.2.2. Patients Treated for 24 Weeks

Twenty-one patients were treated for 24 weeks. Twelve patients were in the non-HCC group and nine patients were in the HCC group. Demographic and clinical features are reported in Table S4. The two groups of patients did not significantly differ in the baseline characteristics evaluated and there were no differences in SVR. In the HCC group, 67% of patients developed HCC after the end of therapy (Table S5).

None of the miRNAs selected in the screening phase and validated in this group of patients at baseline were differentially expressed in the HCC group compared to the non-HCC group (data shown in Table S6).

Table 2. Baseline characteristics of patients included in validation phase and treated for 12 weeks.

	Non-HCC Group (*n* = 26)	**HCC Group (*n* = 11)**	***p*-Value**
Age, median (Q1–Q3)	67 (58–75)	73 (69–78)	0.06
Gender M/F	15/11	6/5	0.86
BMI kg/m^2 *n* (%)			
x $\leq$ 25	10 (38)	4 (36)	0.91
25 < x < 30	9 (35)	6 (55)	0.27
x $\geq$ 30	7 (27)	1 (9)	0.23
HCV Genotype *n* (%)			
Genotype 1a	1 (4)	0	0.52
Genotype 1b	23 (88)	11 (100)	0.25
Others	2 (8)	0	0.35
Transient elastography at baseline (Kpa)	17.1 (14.4–28.6)	26.7 (18.1–32.7)	0.25
HCV-RNA log (UI/mL) median (Q1–Q3)	5.78 (5.47–6.34)	6.23 (5.85–6.30)	0.31
CTP Class *n* (%)			
CTP-A	23 (88)	9 (82)	0.59
CTP-B	3 (12)	2 (18)	0.59
MELD, median (min–max)	7 (6–13)	7 (6–11)	0.80
APRI-score, median (min–max)	1.8 (0.4–8.1)	3.9 (0.7–6.8)	0.05
Platelets ($\times 10^9$/L)	131 (108–182)	67 (55–120)	0.08
Cirrhosis complications, *n* (%)	4 (15)	3 (27)	0.83
Previous therapy, *n* (%)	14 (54)	6 (55)	0.97
INF +/− RBV	13 (93)	5 (83)	0.80
INF +/− RBV + DAAs	1 (7)	1 (17)	0.52
Type of DAAs therapy, *n* (%)			
SOF + LDV ± BV	9 (35)	3 (27)	0.67
OMV + PTV + DSV ± BV	14 (54)	4 (36)	0.34
SOF + SMV ± RBV	3 (12)	4 (36)	0.08
RBV use, *n* (%)	21 (81)	11 (100)	0.12
Time from last liver imaging and start of DAAs therapy (months) median (min–max)	3.4 (0.0–7.2)	1.7 (0.0–6.1)	0.13
SVR, *n* (%)	26 (100)	11 (100)	-

Legend: CTP, Child–Turcotte–Pugh score; MELD: model for end-stage liver disease; APRI-score: aspartate aminotransferase to platelet ratio index-calculation tool; INF: interferon; RBV: ribavirin; SOF: sofosbuvir; LDV: ledipasvir; OMV: ombitasvir; PTV: paritaprevir; DSV: dasabuvir; SMV: simeprevir; SVR: sustained virological response. Cirrhosis complications: ascites, bacterial infection, gastrointestinal bleeding and encephalopathy.

3.3. miRNAs at EOT Samples

miRNA expression was also determined at the end of therapy and compared to baseline expression (Table 3 and Figure S2). At the end of 12 weeks of therapy, the data showed significant downregulation in miR-132-3p, miR-133b-3p, miR-221-3p and miR-324-3p ex-

pression in the non-HCC group. The same results were observed in the HCC group, where miR-28-5p was also significantly downregulated after DAA therapy. In both groups, the expression of miR-584-5p was lower after DAA therapy, but the difference from baseline expression was not statistically relevant.

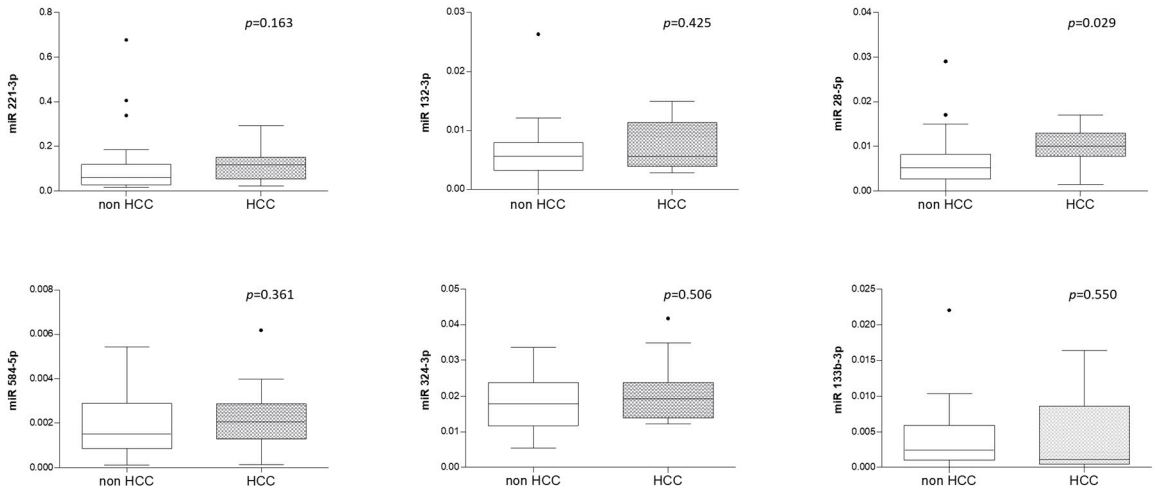

Figure 3. Baseline miRNA expression in non-HCC group compared to HCC group in patients treated for 12 weeks. Y-axis represents relative expression of miRNA.

Table 3. Relative expression of miRNAs at baseline vs. end of therapy in patients treated with DAAs for 12 weeks (HCC and non-HCC group).

Non-HCC Group (*n* = 26)				
	Relative miRNA Expression at Baseline (Median)	**Relative miRNA Expression at the End of Therapy (Median)**	**Fold Change**	***p*-Value**
miR132-3p	0.005864	0.003279	0.559175	0.0051
miR133b-3p	0.002003	0.0004567	0.228008	0.0017
miR221-3p	0.06400	0.03850	0.601563	0.0019
miR28-5p	0.005239	0.005078	0.969269	0.9612
miR324-3p	0.01957	0.008679	0.443485	0.0001
miR584-5p	0.001530	0.0003747	0.244902	0.2491
HCC Group (*n* = 11)				
	Relative miRNA Expression at Baseline (Median)	**Relative miRNA Expression at the End of Therapy (Median)**	**Fold Change**	***p*-Value**
miR132-3p	0.007849	0.003188	0.406166	0.0195
miR133b-3p	0.001114	0.0003831	0.343896	0.0391
miR221-3p	0.1266	0.05219	0.412243	0.0117
miR28-5p	0.01243	0.006539	0.526066	0.0391
miR324-3p	0.01987	0.009663	0.486311	0.0039
miR584-5p	0.002323	0.0002347	0.101033	0.1289

Regarding the patients treated for 24 weeks, no differences were found in miRNA expression in the non-HCC patients compared to baseline. Similar results were observed in the HCC group, except for the expression of miR-28-5p, which was downregulated at the end of therapy (Table S7).

3.4. miRNAs and Relevance of DAA Duration and Follow-Up Time

Patients with a different DAA treatment duration had a different follow-up time and a different pattern of miRNA expression. At baseline, patients with HCC treated for 12 weeks showed a significant upregulation in miR-28-5p expression ($p = 0.029$), while this upregulation was not present in patients with HCC treated for 24 weeks. In the same way, at the end of therapy, patients treated for 12 weeks showed significant downregulation in miR-132-3p, miR-133b-3p, miR-221-3p and miR-324-3p expression both in the HCC group and in the non-HCC group. Furthermore, in patients with HCC treated for 12 weeks, there was also a significant downregulation in miR-28-5p expression after DAA therapy. The downregulation in miR-132-3p, miR-133b-3p, miR-221-3p and miR-324-3p expression was not present in patients treated for 24 weeks, while in the HCC group, the downregulation of miR-28-5p after DAAs therapy was confirmed. To explain a possible relationship between follow-up time and miRNA expression, we studied the correlation between miR-28-5p at baseline and the time of HCC occurrence (from the start of DAA therapy). We found a significant inverse correlation between the relative expression levels of miR-28-5p and the time of HCC occurrence (Figure S3).

4. Discussion

The risk of HCC after SVR with DAA treatment remains a relevant clinical problem. In this scenario, it is crucial to identify patients at risk of developing HCC, as well as finding early biomarkers of HCC. The aim of our study was to analyze the profile of miRNAs in cirrhotic patients treated with DAAs who either developed HCC or did not. In a preliminary screening phase, we identified miRNAs differentially expressed between the HCC and non-HCC groups and then validated them in a larger population.

In the validation phase, baseline levels of miR-28-5p in cirrhotic patients treated for 12 weeks with DAAs and developed HCC were higher compared to the non-HCC group. This confirmed the results of the screening phase, where miR28-5p expression was significantly higher in the HCC group, with a fold change of 1.74 (both for patients with and without SVR). In the HCC group ($n = 11$) treated with DAAs for 12 weeks, HCC occurrence was diagnosed between the start of therapy (baseline) and a maximum of 6 months after the end of therapy, with 91% of patients developing HCC after the end of therapy (between 3 and 9 months after the start of DAAs) and 9% during therapy.

In the smaller group of patients treated with DAAs for 24 weeks, the expression of miR28-5p at baseline was not different between the HCC and non-HCC groups. HCC occurrence was diagnosed between the start of therapy and 12 months from the beginning of the therapy, with 67% of patients developing HCC after the end of therapy (between 6 and 12 months after the start of DAA treatment) and 33% during therapy. In this group, it was expected that the longer time between baseline and HCC occurrence and the smaller number of patients would result in no significant difference in the expression of miR28-5p. Indeed, we showed that the miR-28-5p expression at baseline is inversely correlated with the time from the start of DAA treatment to HCC diagnosis. (Figure S3).

miR28-5p has been associated with the development of several tumors, including gastrointestinal tumors and ovarian tumors. The expression of miR28-5p was lower in patients with colorectal cancer and prostate cancer, suggesting its possible role as a tumor suppressor. Meanwhile, miR28-5p expression was shown to be increased in brain and ovarian cancer, suggesting an oncogenic role [24,25]. Recently, miR28-5p was also shown to be correlated with the development of HCC [26]. The expression of miR28-5p has been tested on HCC biopsy samples of patients who underwent a liver resection. These patients showed a reduction in the expression of miR28-5p, which was associated with poor survival, tumor metastasis and tumor recurrence [27]. An in vitro study revealed that the knockdown of miR-28-5p promotes liver cancer stem cell (CSC) self-renewal and tumorigenesis; insulin-like growth factor-1 (IGF-1) is supposed to be a direct target of miR-28-5p in liver CSCs, and the correlation between miR-28-5p and IGF-1 was confirmed in human HCC tissues [28].

In the last part of this study, we analyzed miRNA expression in samples collected at the end of therapy compared to baseline. In the group treated for 12 weeks, we observed a relevant downregulation in four of the six miRNAs, in both the HCC and non-HCC groups. Considering that the change in miRNA expression was the same in both groups, this might be related to HCV therapy, as other studies have shown the influence of DAA treatment on the circulatory miRNome [29]. Furthermore, it is important to consider, by analyzing these downregulated micro-RNAs in detail, that the majority of miRNAs (miR-132-3p, miR-221-3p and miR-324–3p) have been shown to be implicated in the mechanisms of liver carcinogenesis. The level of miR-132-3p in liver cancer tissue was significantly lower than that found in non-tumor tissue. It has been proven that the expression of miR-132-3p inhibits tumor growth through cell cycle pathways such as PI3K, TGFβ and hippo signaling pathways, or through oncogenes such as Ras, AKT, mTOR and glycolysis [30–32]. miR-221-3p upregulation was found to enhance the proliferation, migration, invasion and clonogenicity in cell lines of human hepatocellular carcinoma (targeting a key tumor suppressor), and high levels of miR-324–3p have been markedly associated with a worse HCC prognosis (in HCC samples) [33,34]. It should be hypothesized that this specific downregulation in microRNA expression after DAA therapy could be related to a modification of the balance of liver carcinogenesis mechanisms. This balance not only involves cancer cells, but also stromal cells, endothelial cells and immune cells, and the whole immunological response. All the factors involved in the mechanisms of carcinogenesis can be influenced by the virological response to therapy [35,36].

Instead, miR28-5p was differently expressed at the baseline in the HCC group compared to the non-HCC group, and after DAA therapy, was downregulated only in the HCC group. Therefore, in patients treated for 24 weeks who developed HCC, miR28-5p was downregulated at the end of therapy. At the end of treatment, miR28-5p was downregulated both in patients treated for 12 and 24 weeks but was not downregulated in patients without HCC. The significant difference in expression of miR28-5p in the HCC group, both at baseline and at the end of therapy, does not seem to be related to the effects of therapy but might be related to the development of HCC.

Our study has some important limitations: the bias of selection, a small and uncontrolled population and the lack of a translational component using cell lines. Despite these limitations, miR28-5p appears to be related to the development of HCC, and its significantly different expression in patients treated with DAAs who develop HCC should be correlated with pathophysiological mechanisms underlying the onset of HCC. miR28-5p should be considered a possible early biomarker and therapeutic target for HCC, although the limitations of this study do not allow for a further definition of its pathogenetic role. Further investigations are required to better clarify the role of miR28-5p in liver carcinogenesis.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/livers4020020/s1: Table S1: Demographic and clinical parameters of patients selected for screening phase in this study. Figure S1: miRNA expression data in screening phase. Table S2: HCC characteristics in HCC group treated for 12 weeks. Table S3: Baseline miRNA expression in validation phase in patients treated with DAAs for 12 weeks. Table S4: Baseline characteristics of patients treated with DAAs for 24 weeks and selected for validation phase. Table S5: HCC characteristics in HCC group treated for 24 weeks. Table S6: baseline miRNAs expression in validation phase in patients treated with DAAs for 24 weeks. Figure S2: Relative expression of miRNAs between baseline (start of therapy) and end of therapy in patients treated for 12 weeks in HCC and non-HCC groups. Table S7: Relative expression of miRNAs at baseline vs. end of therapy in patients treated with DAAs for 24 weeks (in HCC and non-HCC groups). Figure S3: Correlation between relative expression of miR28-5p at baseline and the time to HCC occurrence in all HCC patients ($r = -0.5057$, p-value = 0.0037).

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