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Serum microRNAs; miR-30c-5p, miR-223-3p, miR-302c-3p and miR-17-5p could be used as novel non-invasive biomarkers for HCV-positive cirrhosis and hepatocellular carcinoma

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Abstract Recently, serum miRNAs have been evolved as possible biomarkers for different diseases including hepatocellular carcinoma and other types of cancers. Investigating certain serum miRNAs as novel non-invasive markers for early detection of HCV-positive cirrhosis and hepatocellular carcinoma (HCC). The expression profiles of 58 miRNA were analyzed in patient's plasma of chronic hepatitis C (CHC), HCV-positive cirrhosis and HCV-positive HCC and compared with control group samples. Totally 94 plasma samples; 64 patient plasma (26 CHC, 30 HCV-positive cirrhosis, 8 HCV-positive HCC) and 28 control group plasma, were included. The expression profiles of 58 miRNAs were detected for all patient and control group plasma samples by qRT-PCR using BioMarkTM 96.96 Dynamic Array (Fluidigm Corporation) system. In CHC group, expression profiles of miR-30a-5p, miR-30c-5p, miR-206 and miR-302c-3p were found significantly deregulated ($p < 0.05$) when compared versus control group. In HCV-positive cirrhosis group, expression profiles of miR-30c-5p, miR-223-3p, miR-302c-3p, miR-17-5p, miR-130a-3p, miR-93-5p, miR-302c-5p and miR-223-3p were found significantly deregulated ($p < 0.05$). In HCV-positive HCC group, expression profiles of miR-17-5p, miR-223-3p and miR-24-3p were found significant ($p < 0.05$). When all groups were compared versus control, miR-30c-5p, miR-223-3p, miR-302c-3p and miR-17-5p were found significantly deregulated for cirrhosis and HCC. These results imply that miR-30c-5p, miR-223-3p, miR-302c-3p and miR-17-5p could be used as novel non-invasive biomarkers of HCV-positive HCC in very early, even at cirrhosis stage of liver disease.

Keywords Cirrhosis · Hepatocellular carcinoma · miRNA · HCV · Tumor biomarker

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Introduction

Approximately 3 % of world population have been infected by hepatitis C virus (HCV). Hepatitis C virus infection generally induces chronic liver diseases including chronic hepatitis C, liver cirrhosis and hepatocellular carcinoma [1].

Hepatocellular carcinoma (HCC) is known one of the most common cancer and cause of cancer-related mortality worldwide [2]. It is difficult to be detected at early stages, therefore the survival rate is low about few months. Approximately 90 % of HCC develops from cirrhosis. A wide range of factors such as chronic viral hepatitis B or C infections, alcohol consuming, nonalcoholic steatohepatitis (NASH), autoimmune hepatitis, primary biliary cirrhosis

(PBC), and exposures of carcinogens may play critical roles for developing of HCC [3]. Hepatitis C virus is a major cause of acute and chronic hepatitis. Hepatitis C virus infection frequently induces chronic liver inflammation, which is one of the major reasons of liver cancer. However, the molecular mechanisms underlying are not clear [4, 5].

Several mechanisms and complementary effects such as inflammation thus, cytokine synthesis and fibrosis involved in chronic hepatitis and liver cell necrosis. Cirrhosis is the histological end point [4]. HBV and HCV infections have fundamentally important roles on these issues [4, 6].

The possible reason of poor prognosis of HCC is the lack of an effective early diagnosis. Development of an effective and reliable tool for early diagnosis, would play an important role in improving the prognosis of HCC patients. Its detection at late stages raises the mortality rate and limits the therapeutic options [7].

Therefore, much efforts paying for the discovery of a tool for the early diagnosis and treatment of HCC [3]. An ideal biomarker should be monitored by a clinical sample obtained non-invasively such as serum or urine. Circulating nucleic acids such as microRNA (miRNA) are found in cell-free serum, plasma and other body fluids of individuals. The ability to detect and quantitate specific miRNA sequences offers a great advantage for diagnosis and monitoring of many important diseases including cancer. [8].

microRNAs are a class of noncoding RNA consisting of 20 to 25 bases. They regulate gene expression and play important roles in organ development and differentiation, cellular death and proliferation. They are also involved in development of many diseases including infectious diseases and cancer [9]. Development of many neoplasms including HCC was associated with aberrant expression of several miRNAs. Although miRNAs are endogenous, they are found not just within cells but also in body fluids including serum. Probably they released from the cells during tissue damages as a result of chronic inflammations or in response to drug treatment. Many diseases have been associated with alterations in serum miRNA profiles. Their stability is high in body fluids. Therefore, they may be potential biomarkers and used for diagnosis by quantitative PCR with easy sampling procedures. Moreover, profiling of serum miRNAs may also be used for studying of pathogenesis, tissue damage or cell–cell communication [10, 11]. Therefore, investigation of miRNAs in the serum is an emerging field of molecular biology study [8].

In the present study we have examined the expression profiles of 58 serum miRNA profiles of patients with chronic hepatitis C, HCV-positive cirrhosis and HCV-positive HCC as compared to healthy individuals. The expression profiles of several miRNAs had been studied in HCC patient's samples [5, 12, 13]. However, in our study, we aimed to discover biomarkers possibly capable to diagnose all progress from chronic hepatitis C to HCC; may be in early stages, other than HCC, even in chronic hepatitis C and/or cirrhosis and/or HCC stage of HCV related liver disease.

In this study, we studied the expression profiles of 58 miRNAs in chronic hepatitis C, HCV-positive cirrhosis and HCV-positive HCC patient's sera. The expression profiles of several miRNAs had been studied in HCC patient's samples [5, 12, 13]. However, in our study, we aimed to discover biomarkers possibly capable to diagnose all progress from chronic hepatitis C to HCC; may be in very early stages, other than HCC, even in chronic hepatitis C and/or cirrhosis and/or HCC stage of HCV related liver disease.

Materials and method

Patients and samples

Twenty six CHC, 30 HCV-positive cirrhosis, 8 HCV-positive HCC and 28 control blood samples were obtained from department of gastroenterology and blood banking unit of Mersin University hospital. Blood samples of patients with chronic hepatitis C, cirrhosis and HCC were HCV RNA positive when tested by RT-PCR. Cirrhosis and HCC was diagnosed histopathologically. All control blood samples were obtained from blood donors and were negative for HCV RNA when tested by RT-PCR.

RNA isolation, reverse transcription and qPCR

RNA isolations, reverse transcriptions and qPCR were done as described earlier [14].

Blood samples drawn into EDTA containing tubes and centrifuged at 4,000×*g* for 15 min for plasma separation. Plasma transferred into a clean micro centrifuge tube and centrifuged again at 12,000×*g* for 5 min and 200 µl of plasma was transferred to a new micro centrifuge tube and stored at −80 °C until analysis. RNA was isolated using High Pure miRNA Isolation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions and then stored at −80 °C until the experiment.

Reverse transcription reaction

Isolated RNA samples were reverse-transcribed into cDNA in 5 µl final reaction volumes using *Taq*Man MicroRNA Reverse Transcription Kit (catalog number: 4366596; Applied Biosystems, Foster City, CA, USA). All reactions were performed as specified in the manufacturers protocol:

2 µl total RNA were added to 3 µl of the RT reaction mix (Megaplex RT Primers 109, dNTPs with dTTP 100 mM, MultiScribe Reverse Transcriptase 50 U/µl, 109 RT Buffer, MgCl2 25 mM, RNase Inhibitor 20 U/µl and Nucleasefree water). Reverse transcription was performed using a GenePro Thermal Cycler TC-E-3846 (Hangzhou, P.R. China). Reaction conditions: 16 °C for 120 s, 42 °C for 60 s, 50 °C for 1 s, and these three steps repeated for 40 cycles. Finally, 85 °C for 300 s and 4 °C for at least 600 s until further processing or storage. cDNA samples were kept at −80 °C until PCR analysis. Pre-amplification We performed a pre-amplification after the reverse transcription using the *Taq*Man PreAmp Master Mix 29 (PN 4391128; Applied Biosystems, Foster City, CA, USA) as well as the Megaplex Human Primer Pools Set v3.0 (PN 4444750; Applied Biosystems, Foster City, CA, USA). All reactions were performed as specified in the protocols of the manufacturer. For pre-amplification 2 µl 1/5 diluted RT product were added to 3 µl of the PreAmp mix. The reaction volume was 5 µl. miRNA *Taq*Man PreAmp Thermal Protocol was performed using a GenePro Thermal Cycler TC-E-3846 (Hangzhou, P.R. China) as follows: 95 °C for 600 s, 55 °C for 120 s and 72 °C for 120 s, followed by 18 cycles with 95 °C for 15 s, 60 °C for 240 s, finally 600 s at 99.9 °C; rest period at 4 °C.

qRT-PCR

Quantitative real-time PCR reactions (qRT-PCR) were performed using the high-throughput BioMark Real-Time PCR system (Fluidigm, South San Francisco, CA). Pre-amplified cDNA samples were diluted with Low EDTA (0.1 mM) TE Buffer (1:5). About 490 µl *Taq*Man Universal PCR Master Mix, No AmpErase UNG, (Applied Biosystems, Foster City, CA, USA), and 49 µl 209 GE Sample Loading Reagent (Fluidigm, PN 85000746) mixed and pipetted into a 96 well plate as 3.85 and 3.15 µl of 1:10 diluted PreAmplified cDNA pipetted into each well and mixed then 5 µl of this mixture pipetted into sample inlets of a 96.96 Dynamic Arrays (Fluidigm, South San Francisco, USA) 4.0 µl 1:1 diluted 209 Assays pipetted into assay inlets of a 96.96 Dynamic array (Fluidigm). The BioMark IFC controller HX (Fluidigm, San Francisco, CA) was used to distribute the assay mix and sample mix from the loading inlets into the 96.96 Dynamic array reaction chambers for qRT-PCR by Fluidigm's Integrated Fluidic Circuit Technology. Real-time PCR step performed by using BioMark System by using this protocol; firstly thermal mix protocol is followed by 50 °C for 120 s, 70 °C for 1,800 s, 25 °C for 600 s. Then UNG and Hot start protocol is followed by 50 °C for 120 s and 95 °C for 600 s. Finally, PCR cycle is followed by 40 cycles with 95 °C for 15 s (denaturation) and 60 °C for 60 s (annealing).

Statistical analysis

All statistical analyses were performed using the Biogazelle's qbase PLUS 2.0 software which uses global means normalization method in order to troubleshoot the house keeping gene problem in circulation. RNU48 was used as endogenous control. This qPCR profiling platform that consists of 58 miRNAs were analyzed together by using global mean normalization. Mann–Whitney U test was performed to compare differences in miRNA levels between patients and controls and the one-way ANOVA test for three or more groups. $p < 0.05$ was considered statistically significant.

Results

Chronic hepatitis C group

Twenty-two miRNAs were detected deregulated in chronic hepatitis C group when compared versus control group. However, only the expression levels of miR-30a-5p, miR-30c-5p miR-206 and miR-302c-3p were found statistically significant ($p < 0.05$). All data and fold changes were summarized at Table 1 and Fig. 1a.

HCV-positive cirrhosis group

Seventeen miRNAs were detected deregulated in HCV-positive cirrhosis group when compared versus control group. However, the expression levels of miR-30c-5p, miR-223-3p, miR-302c-3p, miR-17-5p, miR-130a-3p, miR-93-5p and miR-302c-5p were found statistically significant ($p < 0.05$). All data and fold changes were summarized at Table 2 and Fig. 1b.

HCV-positive hepatocellular carcinoma group

Seventeen miRNAs were detected deregulated in HCV-positive HCC group when compared versus control group. However, the expression levels of miR-17-5p, miR-223-3p and miR-24-3p were found statistically significant ($p < 0.05$). All data and fold changes were summarized at Table 3 and Fig. 1c.

When all groups were compared versus control group by one-way ANOVA, the expression levels of miR-30c-5p, miR-223-3p, miR-302c-3p and miR-17-5p were found significantly deregulated ($p < 0.05$) for cirrhosis and HCC. All data and fold changes (only statistically significant data) were summarized at Table 4 and Fig. 1d.

Table 1 Chronic hepatitis B versus control group comparisons, fold changes and "p" values

miRNA	Comparisons	Mean	Fold changes	p
miR-30a-5p	CHC/Control	0.352/1.000	2.83 ↓	0.006159*
miR-30c-5p	CHC/Control	1.814/1.000	1.82 ↑	0.007034*
miR-206	CHC/Control	0.307/1.000	3.66 ↓	0.03524*
miR-302c-3p	CHC/Control	3.719/1.000	3.72 ↑	0.04117*
miR-223-3p	CHC/Control	0.440/1.000	2.27 ↓	0.05528
miR-181a-5p	CHC/Control	2.193/1.000	2.20 ↑	0.05708
miR-130a-3p	CHC/Control	8.598/1.000	8.60 ↑	0.6277
miR-15b-5p	CHC/Control	2.520/1.000	2.52 ↑	0.06876
miR-29a-3p	CHC/Control	2.352/1.000	2.35 ↑	0.1333
miR-29c-3p	CHC/Control	0.026/1.000	38.46 ↓	0.1333
miR-17-5p	CHC/Control	1.681/1.000	1.72 ↑	0.1408
miR-155-5p	CHC/Control	0.246/1.000	4.65 ↓	0.1667
miR-93-3p	CHC/Control	0.346/1.000	2.86 ↓	0.2
miR-93-5p	CHC/Control	1.321/1.000	1.32 ↑	0.2067
miR-146a-5p	CHC/Control	0.802/1.000	1.24 ↓	0.2313
miR-221-3p	CHC/Control	1.269/1.000	1.27 ↑	0.239
miR-302c-5p	CHC/Control	2.461/1.000	2.46 ↑	0.25
miR-30b-5p	CHC/Control	1.208/1.000	1.20 ↑	0.3649
miR-24-3p	CHC/Control	1.720/1.000	1.72 ↑	0.4462
miR-106a-5p	CHC/Control	1.190/1.000	1.19 ↑	0.4727
miR-122-5p	CHC/Control	2.210/1.000	2.21 ↑	0.5
miR-128	CHC/Control	0.792/1.000	1.26 ↓	0.6

* $p < 0.05$

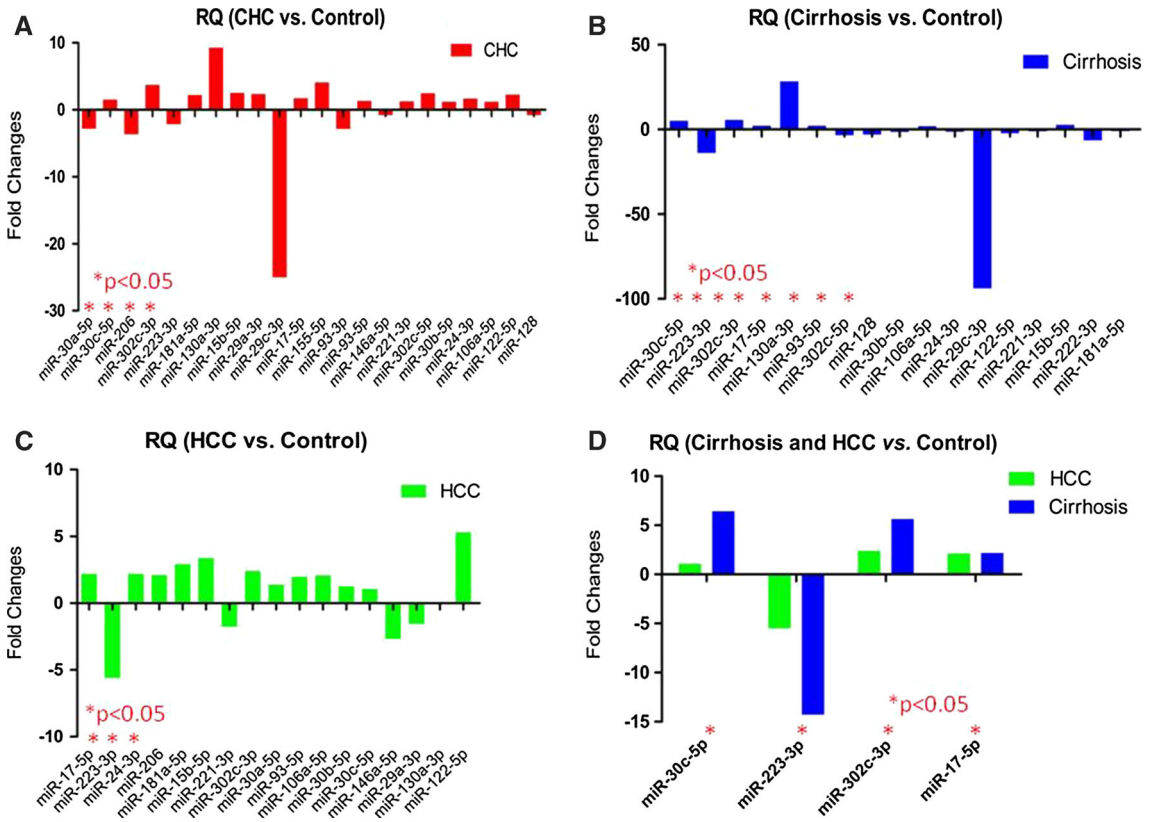

Fig. 1 **a**, **b**, **c** demonstrate relative quantities of individual comparisons of each patient group versus control; **d** demonstrates relative quantities of cirrhosis and HCC groups versus control groups for only statistically significant miRNAs, "*" demonstrates $p < 0.05$

Table 2 Cirrhosis versus control group comparisons, fold changes and "p" values

miRNA	Comparisons	Mean	Fold changes	p
miR-30c-5p	Cirrhosis*/Control	6.429/1.000	6.40 ↑	0.00005649**
miR-223-3p	Cirrhosis*/Control	0.070/1.000	14.00 ↓	0.0005693**
miR-302c-3p	Cirrhosis*/Control	5.629/1.000	5.63 ↑	0.002969**
miR-17-5p	Cirrhosis*/Control	2.178/1.000	2.18 ↑	0.01369**
miR-130a-3p	Cirrhosis*/Control	26.078/1.000	26.00 ↑	0.01587**
miR-93-5p	Cirrhosis*/Control	2.210/1.000	2.21 ↑	0.0176**
miR-302c-5p	Cirrhosis*/Control	0.295/1.000	3.38 ↓	0.02381**
miR-128	Cirrhosis*/Control	0.329/1.000	3.04 ↓	0.09524
miR-30b-5p	Cirrhosis*/Control	0.652/1.000	1.53 ↓	0.1293
miR-106a-5p	Cirrhosis*/Control	1.947/1.000	1.94 ↑	0.1583
miR-24-3p	Cirrhosis*/Control	0.562/1.000	1.50 ↓	0.1795
miR-29c-3p	Cirrhosis*/Control	0.338/1.000	2.95 ↓	0.25
miR-122-5p	Cirrhosis*/Control	0.722/1.000	1.38 ↓	0.30
miR-221-3p	Cirrhosis*/Control	0.110/1.000	9.09 ↓	0.3294
miR-15b-5p	Cirrhosis*/Control	2.328/1.000	2.32 ↑	0.3333
miR-222-3p	Cirrhosis*/Control	0.804/1.000	1.24 ↓	0.5
miR-181a-5p	Cirrhosis*/Control	0.374/1.000	2.67 ↓	0.4773

* HCV-positive cirrhosis

** $p < 0.05$

Table 3 Hepatocellular carcinoma versus control group comparisons, fold changes and "p" values

miRNA	Comparisons	Mean	Fold changes	p
miR-17-5p	HCC*/Control	2.111/1.000	2.18 ↑	0.01561**
miR-223-3p	HCC*/Control	0.182/1.000	5.60 ↓	0.03996**
miR-24-3p	HCC*/Control	2.180/1.000	2.18 ↑	0.04731**
miR-206	HCC*/Control	2.091/1.000	2.09 ↑	0.09608
miR-181a-5p	HCC*/Control	2.910/1.000	2.91 ↑	0.1045
miR-15b-5p	HCC*/Control	3.357/1.000	3.37 ↑	0.1111
miR-221-3p	HCC*/Control	0.558/1.000	1.77 ↓	0.1477
miR-302c-3p	HCC*/Control	2.382/1.000	2.40 ↓	0.1793
miR-30a-5p	HCC*/Control	0.427/1.000	1.37 ↓	0.2176
miR-93-5p	HCC*/Control	1.390/1.000	1.39 ↑	0.2205
miR-106a-5p	HCC*/Control	1.969/1.000	1.96 ↑	0.2231
miR-30b-5p	HCC*/Control	2.086/1.000	2.08 ↑	0.267
miR-30c-5p	HCC*/Control	0.795/1.000	1.06 ↓	0.31
miR-146a-5p	HCC*/Control	1.069/1.000	1.06 ↑	0.3192
miR-29a-3p	HCC*/Control	0.372/1.000	2.67 ↓	0.4
miR-130a-3p	HCC*/Control	0.649/1.000	1.54 ↓	0.4286
miR-122-5p	HCC*/Control	5.220/1.000	5.22 ↑	0.50

* HCV-positive hepatocellular carcinoma

** $p < 0.05$

Discussion

Hepatocellular carcinoma is a poor prognostic cancer and still remains an aggressive malignancy worldwide. The early diagnosis of HCC is a valuable clinical advantage. It offers surgical treatment early. Therefore, it improves prognosis of patients with HCC. Usually, alpha-fetoprotein has been used as a biomarker for diagnosis of primary HCC. However, the sensitivity and specificity of alpha-fetoprotein is not good enough. Therefore, novel biomarkers are urgently needed for early diagnosis of HCC [15].

The prognosis of patients with HCC would be improved with discovery of an effective and reliable biomarker.

In this study, the hypothesis that the expression profiles of miRNAs in serum can serve as biomarkers for early diagnosis of HCV-positive HCC was tested.

This study was performed in order to discover valuable non-invasive biomarkers which could be obtained without any invasive manipulations such as biopsy for the earliest diagnosis of HCC. For these reasons the study was performed in 3 patients groups (CHC, HCV-positive cirrhosis and HCV-positive HCC) versus control group. Several

Table 4 Cirrhosis and hepatocellular carcinoma versus control group comparisons, fold changes and "p" values

miRNA	Comparisons	Mean	Fold changes	p
miR-30c-5p	HCC	1.069	1.069 ↑	0.0001308*
	Control	1.000	–	
	Cirrhosis	6.429	6.429 ↑	
miR-223-3p	HCC	0.182	5.5 ↓	0.004137*
	Control	1.000	–	
	Cirrhosis	0.070	14.28 ↓	
miR-302c-3p	HCC	2.382	2.382 ↑	0.01111*
	Control	1.000	–	
	Cirrhosis	5.629	5.629 ↑	
miR-17-5p	HCC	2.111	2.111 ↑	0.05335*
	Control	1.000	–	
	Cirrhosis	2.178	2.178 ↑	

* $p < 0.05$

miRNAs were found significant expression deregulations in every patient group when comprised versus control group (Tables 1, 2 and 3).

In chronic hepatitis C group, the expression profiles of miR-30a-5p, miR-30c-5p, miR-206 and miR-302c-3p were found deregulated. These alterations were found statistically significant when compared versus control group by Mann–Whitney U test (Table 1).

The expression alterations of miR-30 family members such as miR-30a-5p and miR-30c-5p were associated with interferon-beta therapy [16, 17]. Our samples were obtained from patients under interferon beta therapy. Therefore, our findings are in agreement with these previous studies.

The expression level was detected 3.66 fold down-regulated of miR-206 in chronic hepatitis C group. The expression deregulations of miR-206 were reported in several types of cancer such as lung cancer, rhabdomyosarcoma, breast cancer and endometrial carcinoma [18]. In our study, it was found down-regulated in CHC and HCC groups. As we know, our finding about miR-206 is novel for such patient groups.

Another significantly deregulated miRNA is miR-302c-3p in CHC patient group. miR-302c-3p was reported up-regulated in patient with acute hepatitis B and HCV-positive fibrosis [19, 20]. This miRNA was also detected up-regulated in HCV-positive cirrhosis group in our study.

In HCV-positive cirrhosis group, the expression profiles of miR-30c-5p, miR-223-3p, miR-302c-3p, miR-17-5p, miR-130a-3p, miR-93-5p and miR-302c-5p were found deregulated when compared versus control and the difference was statistically significant (Table 2).

The expression level of miR-30c was reported up-regulated during liver development [21, 22]. However, the expression profiles of miR-30c were reported down-regulated in HCV infection and fibrosis [23–27]. As we know, deregulation of miR-30c-5p has not been reported at cirrhosis stage. In our study, its expression profile was detected 6.4 fold up-regulated and the difference was very significant ($p = 0.00005649$, $p < 0.0001$). Probably, our finding for miR-30c-5p is also novel for HCV-positive cirrhosis.

The expression level of miR-223-3p was detected 14 fold down-regulated in HCV-positive cirrhosis group. miR-223-3p was found down regulated by in HCC tissues or HBV-positive HCC cases [7, 28–30]. However, deregulation was not reported in CHC and HCV-positive cirrhosis. This miRNA was detected down-regulated in all patient groups in our study.

The expression level of miR-17-5p was also found up-regulated in both of HCV-positive cirrhosis and HCV-positive HCC groups in our study. This miRNA was reported up-regulated in HCC and a novel biomarker in a recently published study [31]. Our finding is also in agreement with this study. And as we know this alteration has not been reported at cirrhosis stage of liver disease.

The expression level of miR-130a-3p was reported up-regulated in liver regeneration [19]. However, its expression level was detected up-regulated in only HCV-positive cirrhosis patient group significantly.

The expression level of miR-93-5p was reported up-regulated in fibrosis and could be a biomarker of early diagnosis of fibrosis [32]. This miRNA was detected significantly up-regulated in HCV-positive cirrhosis group in our study. Our finding seems to be in agreement with this study.

In HCV-positive HCC group, the expression levels of miR-17-5p, miR-223-3p and miR-24-3p were detected deregulated and found significant ($p < 0.05$). miR-17-5p and miR-223-3p were discussed above.

The expression level was detected of miR-24-3p more than 2 folds in HCC patients and reported that a novel biomarker and a therapeutically important target in recently published studies [30, 33]. The expression profile was detected significantly up-regulated (2.18 folds, $p = 0.047$) in our study. This finding is in agreement with previous study mentioned above [30].

When all groups were compared versus control group by one-way ANOVA, the expression levels of miR-30c-5p, miR-223-3p, miR-302c-3p and miR-17-5p were found significantly deregulated ($p < 0.05$) for cirrhosis and HCC. In one of our earlier study miR-223-3p had been found deregulated in HBV related HCC patients (unpublished data). These data could be considered that these two different chronic viral liver infection stimuli the same ways of HCC developing.

In conclusion, we can clearly say that we identified 4 miRNAs; miR-30c-5p, miR-223-3p, miR-302c-3p and

miR-17-5p could be used non-invasive biomarker by a simple sampling procedure such as taking blood at very early of HCC, even at cirrhosis stage of liver disease. These four miRNAs should be studied in further and larger volume of patient populations in HCV-positive cirrhosis and HCV-positive HCC in the future.

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