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Profiles of serum microRNAs; miR-125b-5p and miR223-3p serve as novel biomarkers for HBV-positive hepatocellular carcinoma

Burcu Gurer Giray · Gurol Emekdas · Seda Tezcan · Mahmut Ulger · Mehmet Sami Serin · Orhan Sezgin · Engin Altintas · Eyup Naci Tiftik

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Abstract Recently, circulating miRNAs have been reported as promising biomarkers for various pathologic conditions including cancer. Certain microRNAs (miRNAs) have been shown early diagnostic potential for many types of cancer. The objective of this study was to investigate the potential of certain serum/plasma miRNAs as novel non-invasive biomarkers for early diagnosis of hepatitis B virus (HBV) related hepatocellular carcinoma (HCC). For this reason, the expression levels of 24 miRNA (let-7c, miR-92a-3p, 423-5p, 150-5p, 223-3p, 125b-5p, 342-3p, miR-206, 122-5p, 375, 223-5p, 10a-5p, 23b-5p, 99a-5p, 23a-5p, 10a-3p, 122-3p, 125b-1-3p, 23b-3p, 125b-2-3p, 23a-3p, 92a-1-5p, 92a-2-5p, 99a-3p) were analyzed in plasma of patients with chronic hepatitis B, HBV-positive cirrhosis and HBV-positive HCC and compared with control group samples. Totally 94 plasma samples; 28 control and 66 patient plasma (24 CHB, 22 HBV-positive cirrhosis, 20 HBV-positive HCC) and were included in this study. The expression levels of 24 miRNAs were detected for all control and patient group plasma samples by qRT-PCR using BioMark^{TM} 96.96 Dynamic Array (Fluidigm Corporation) system. The expression levels of miR-125b-5p were detected 2.85 fold, 2.46 fold and 1.89 fold ($p = 0.01513$, $p = 0.0009440$, $p = 0.0001446$) up regulated in CHB, HBV-positive cirrhosis and HBV-positive HCC, respectively when compared versus control group individually by Mann–Whitney U test. The expression levels of miR-223-3p were detected 5.55 fold, 13.88 fold and 12.65 fold ($p = 0.01513$, $p = 0.0009440$, $p = 0.0001446$) down regulated in same comparisons. When all groups were compared versus control group by one-way ANOVA test, the expression levels of miR-223-3p were also found statistically significant ($p < 0.05$). Although not statistically significant, miR-125b-5p tended to be upregulated. ($p = 0.07192$). These results significantly imply that miR-125b-5p and miR223-3p could be used as novel non-invasive biomarkers of HBV-positive HCC in very early, even at CHB stage of liver disease.

Keywords Hepatocellular carcinoma · miRNA · HBV · Early detection · Tumor biomarker · Diagnosis

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer-related mortality [1]. It is rarely detected at its early stage, resulting in a short survival of few months. About 90 % of HCC cases arise from cirrhosis, which can be attributed to a wide range of factors including chronic viral hepatitis B or C (HBV or HCV) infections, highly alcohol consuming, nonalcoholic steatohepatitis (NASH), autoimmune hepatitis, primary biliary cirrhosis (PBC), and carcinogens exposure [2].

Chronic hepatitis is recognized as an important risk factor for HCC. The mechanisms involved in chronic

B. G. Giray · G. Emekdas · S. Tezcan · M. Ulger
Department of Medical Microbiology, Faculty of Medicine, Mersin University, Mersin, Turkey

M. S. Serin (✉)
Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Mersin University, Yenisehir, 33169 Mersin, Turkey
e-mail: serinmss@yahoo.com; serinm@mersin.edu.tr

O. Sezgin · E. Altintas
Department of Gastroenterology, Faculty of Medicine, Mersin University, Mersin, Turkey

E. N. Tiftik
Department of Hematology, Faculty of Medicine, Mersin University, Mersin, Turkey

hepatitis include a combination of several, complementary effects involved in liver cell necrosis, inflammation and thus, cytokine synthesis and fibrosis. Cirrhosis is the histological end point of this chronic inflammatory and fibrotic process, and liver cell DNA synthesis is indeed increased in cirrhotic, as compared to normal, livers. Hepadna viruses such as HBV infection have provided fundamentally important information on these issues [3].

The incidence of HCC in chronically HBV infected individuals is approximately 100-fold higher than in the uninfected population and the lifetime HCC risk of males infected with HBV at birth is estimated to be approximately 40 %. Most cases of HCC occur after many years of chronic hepatitis which can provide the mitogenic and mutagenic environment to precipitate random genetic alterations and lead to the development of HCC [4].

This deadly cancer affects more than 500,000 people worldwide and it is quite resistant to conventional chemo- and radiotherapy [5]. The poor prognosis of this disease is partially due to the lack of an effective means of early diagnosis. Discovery of an effective and reliable tool for early diagnosis of HCC would play a pivotal role in improving the prognosis of patients with HCC. In fact, the high mortality rate is due to its detection at late stage with limited therapeutic options [6].

For these reason, much attention paving the way to the early detection and treatment of HCC [2]. Ideally, biomarkers should be easily accessible such that they can be sampled non-invasively. Therefore biomarkers that can be sampled from body fluids, such as serum or urine, are particularly desirable. Circulating nucleic acids are extracellular nucleic acids found in cell-free serum, plasma and other body fluids from healthy subjects as well as from patients. The ability to detect and quantitate specific DNA and RNA sequences has opened up the possibility of diagnosis and monitoring of diseases, especially in the field of cancer. Furthermore, it has been suggested a kind of non-coding RNA—microRNA (miRNA), also exist in cell-free serum and plasma, highlighting the field of using circulating nucleic acids to diagnose cancer [7].

miRNA is an endogenous, small, single-strand, non-coding RNA consisting of 20–25 bases and regulates gene expression of various cell types. It plays an important role in various biological processes, including organ development and differentiation as well as cellular death and proliferation, and is also involved in infectious diseases and cancer [8]. Aberrant expression of several miRNAs was found to be involved in a large variety of neoplasms including HCC. Investigation of cancer-specific miRNAs in the circulation is an emerging and exciting field of study [7].

In this study, we determined the expression profile of 24 miRNA in chronic hepatitis B, HBV-positive cirrhosis and HBV-positive HCC patient's sera. The expression profiles of these miRNAs had been studied previously in HBV-positive HCC patient's samples [7, 9]. However, in our study, we aimed to discover biomarkers possibly capable to diagnose all progress from chronic hepatitis to HCC; may be in very early stages, other than HCC, even in chronic hepatitis and/or cirrhosis and/or HCC stage of HBV related liver disease.

Materials and method

Patients and samples

28 control blood samples of healthy individuals (7 Female, 21 Male), 24 CHB (7 Female, 17 Male; 49.58 ± 2.002, mean ± SEM years old), 22 HBV-positive cirrhosis (1 Female, 21 Male; 56.52 ± 2.011 years old) and 20 HBV-positive HCC blood samples of patients (6 Female, 14 Male; 60.40 ± 2.570 years old) were obtained from department of gastroenterology and blood banking unit of Mersin University hospital. Blood samples of patients with chronic hepatitis B, cirrhosis and HCC were HBV DNA and HBsAg positive when tested by PCR and ELISA. Cirrhosis and HCC was diagnosed histopathologically. HCC stages of patients were stage IIIc and IVa. All control blood samples were obtained from blood donors and were negative for HBV DNA and HBsAg when tested by PCR and ELISA.

RNA isolation, reverse transcription and qPCR

RNA isolations, reverse transcriptions and qPCR were performed as described by Gorur et al. [10], with minor modifications.

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 TaqMan 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 10X dNTPs with dTTP 100 mM, MultiScribe Reverse Transcriptase 50 U/µl, 10X 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 TaqMan 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 TaqMan 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). Preamplified cDNA samples were diluted with Low EDTA (0.1 mM) TE Buffer (1:5). About 490 μl TaqMan 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 24 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 B group

Eleven miRNAs were detected deregulated in chronic hepatitis B group when compared versus control group. However, only the expression levels of miR-125b-5p and miR-223-3p were found statistically significant ($p < 0.05$). All data were summarized at Fig. 1a.

HBV-positive cirrhosis group

Ten miRNAs were detected disregulated in HBV-positive cirrhosis group when compared vs control group. However, the expression levels of miR-223-3p, miR-122-5p and miR-125b-5p were found statistically significant ($p < 0.05$). All data were summarized at Fig. 1b.

HBV-positive hepatocellular carcinoma group

Eleven miRNAs were detected deregulated in HBV-positive HCC group when compared versus control group. However, the expression levels of miR-223-3p, miR-92a-3p, miR-122-5p and miR-125b-5p were found statistically significant ($p < 0.05$). All data were summarized Fig. 1c.

When all groups were compared versus control group by one-way ANOVA test, the expression levels of miR-223-3p were also found statistically significant ($p < 0.05$). miR-125b-5p was also showed similar deregulations in same comparisons and found almost significant ($p = 0.07192$). All data were summarized at Table 1 and Fig. 1d.

Discussion

HCC represents an extremely poor prognostic cancer that remains one of the most common and aggressive human malignancies worldwide. The early diagnosis of HCC is of great clinical desirable and the improved prognosis of HCC if the patients could get surgical treatment early. Up to now, alpha-fetoprotein (α-AFP) has mainly been used in clinic for diagnosis of primary HCC; however, its

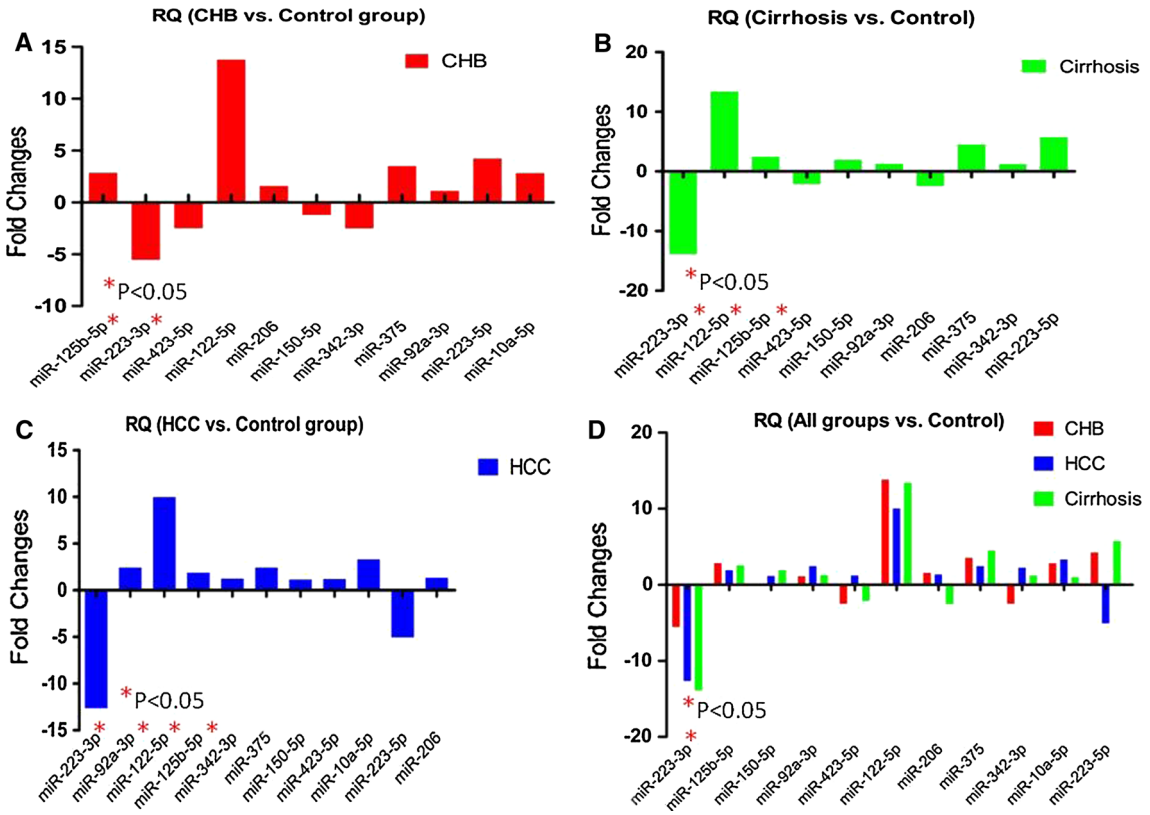

Fig. 1 **a–c** demonstrate relative quantities of individual comparisons of each patient group versus control; **d** demonstrates relative quantities of all patient groups versus control groups. "*" demonstrates $p < 0.05$

sensitivity and specificity are not satisfying [11], novel biomarkers for early HCC diagnosis are greatly needed.

Discovery of an effective and reliable tool for early diagnosis of HCC would play a pivotal role in improving the prognosis of patients with HCC.

We test the hypothesis that expression profiles of miRNAs in serum can serve as biomarkers for diagnosis of HBV infection to HBV-positive HCC.

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 (CHB, HBV-positive cirrhosis and HBV-positive HCC) versus control group. All 24 miRNAs were chosen from the research articles of Qi et al. [7], and Li et al. [9], who they had studied on serum miRNA profiles of HCC patients with HBV. However, in our study we included three patients groups as described above.

Several miRNAs were found significant expression deregulations in every patient group when comprised versus control group (Fig. 1a–c).

In chronic hepatitis B group, the expression level of miR-125b-5p was detected up regulated and the expression level of miR-223-3p was detected down regulated. These alterations were found statistically significant when compared versus control group by Mann–Whitney U test. In this patient group some certain miRNAs such as miR-423-5p and miR-122-5p were shown deregulations (Fig. 1a). However, no significant result was found even their p values are close to 0.05 level ($p = 0.06147$, $p = 0.08824$ respectively).

In HBV-positive cirrhosis group the expression levels were detected deregulated and found statistically significant of miR-223-3p, miR-122-5p and miR-125b-5p by Mann–Whitney U test ($p = 0.0009440$, $p = 0.02778$ and $p = 0.03670$ respectively). miR-122-5p and miR-125b-5p were found up regulated while miR-223-3p was down regulated. These three miRNAs may be used a non-invasive prognostic marker for cirrhosis. The expression levels were also found deregulated of miR-423-5p and miR-150-5p (Fig. 1b). However, no statistically significant result was found even their p values are close to 0.05 level ($p = 0.06586$ and $p = 0.06777$ respectively).

In HBV-positive HCC group the expression levels were found deregulated and statistically significant of miR-223-3p, miR-92a-3p, miR-122-5p and miR-125b-5p when tested by Mann–Whitney U test versus control group ($p = 0.0001446$,

Table 1 All patient groups versus Control group comparisons, fold changes and "*p*" values

miRNA	Comparisons	Mean	Fold changes	*p*
miR-223-3p	CHB	0.18	5.550 ↓	0.0006652*
	HCC	0.079	12.65 ↓	
	Cirrhosis	0.072	13.87 ↓	
	Control	1.000	1.000 –	
miR-125b-5p	CHB	2.854	2.854 ↑	0.07192*
	HCC	1.904	1.904 ↑	
	Cirrhosis	2.549	2.549 ↑	
	Control	1.000	1.000 –	
miR-150-5p	CHB	0.821	1.218 ↓	0.1192
	HCC	1.159	1.159 ↑	
	Cirrhosis	1.934	1.934 ↑	
	Control	1.000	1.000 –	
miR-92a-3p	CHB	1.110	1.110 ↑	0.1549
	HCC	2.453	2.453 ↑	
	Cirrhosis	1.280	1.280 ↑	
	Control	1.000	1.000 –	
miR-423-5p	CHB	0.400	2.500 ↓	0.2659
	HCC	1.221	1.221 ↑	
	Cirrhosis	0.474	2.109 ↓	
	Control	1.000	1.000 –	
miR-122-5p	CHB	13.831	13.831 ↑	0.288
	HCC	10.027	10.027 ↑	
	Cirrhosis	13.407	13.407 ↑	
	Control	1.000	1.000 –	
miR-206	CHB	1.575	1.575 ↑	0.2907
	HCC	1.344	1.344 ↑	
	Cirrhosis	0.392	2.550 ↓	
	Control	1.000	1.000 –	
miR-375	CHB	3.532	3.532 ↑	0.6316
	HCC	2.444	2.444 ↑	
	Cirrhosis	4.523	4.523 ↑	
	Control	1.000	1.000 –	
miR-342-3p	CHB	0.399	2.506 ↓	0.6356
	HCC	2.257	2.257 ↑	
	Cirrhosis	1.208	1.208 ↑	
	Control	1.000	1.000 –	
miR-10a-5p	CHB	2.825	2.825 ↑	0.8144
	HCC	3.318	3.318 ↑	
	Cirrhosis	NaN	NaN –	
	Control	1.000	1.000 –	
miR-223-5p	CHB	4.232	4.232 ↑	0.9106
	HCC	0.197	5.076 ↓	
	Cirrhosis	5.742	5.742 ↑	
	Control	1.000	1.000 –	

*$p < 0.05$

$p = 0.02434$, $p = 0.02778$ and $p = 0.04186$ respectively). miR-92a-3p, miR-122-5p and miR-125b-5p were detected up regulated, miR-223-3p was detected down regulated.

In many studies, the expression levels were found deregulated of miR-223-3p, miR-92a-3p, miR-122-5p and miR-125b-5p in HCC [6, 12–22].

miR-223-3p was found down regulated by in HCC tissues or HBV-positive HCC cases [6, 12–14].

Karakatsanis et al. [12], were found miR-21, miR-31, miR-122, miR-221, miR-222 were significantly up-regulated in HCC tissues, whereas miR-145, miR-146a, miR-200c, and miR-223 were found to be down regulated. In our study, the expression level of miR-122 was found up-regulated whereas miR-223 was down-regulated. Our these findings are in agreement with this results.

Wong et al. [13], reported that frequent incidences of up regulation of miRNAs, including miR-99a, miR-184, miR-224, and miR-222, were detected, whereas common down regulation was suggested for miR-325, miR-199a, miR-199a, and miR-223 in HCC tumors and cell lines when compared with normal liver controls. Our miR-223 finding is in agreement with this study. However, other mirNAs were not studied in our study.

Zhou et al. [6], reported that low expression levels of miR-122, miR-223, miR-26a, and miR- 27a were observed in patients with HCC compared with those in the control group. High expression levels of miR-192, miR-21, and miR-801 were observed in patients with hepatitis B related HCC compared with those in the control group. In our study, the expression level of miR-122 was found high in HCC group. However, our miR-223 finding is in agreement with this study.

Han et al. [14], reported that they have identified down-regulated miRNAs including miR-223, miR-122 as recurrence related mirNAs in patients with HCC following liver transplantation. In our study groups we identified miR-122 as up-regulated miRNA. However, our miR-233 finding is also in agreement with this study.

miR-92 is also found deregulated in many studies. Li et al. [15], reported that they have detected the expression level of miR-92 low in HCC without metastasis whereas high in HCC with metastasis. In our samples we found the expression profile of miR-92a-3p high when compared with control group.

Murakami et al. [16], have also found the level of miR-92a was high in HCC. Qi et al. [17], have also been the expression level of miR-92a high in HBV related HCC. All these findings are in agreement with ours.

Li et al. [15], reported that they have identified miR-92a specific for HBV infection. In our study, we found the

expression profile of miR-92a was up-regulated in all groups. However, statistically significant result was only found in HCC group ($p = 0.02434$). These workers also showed that over expression of miR-125b in HCC cell line could obviously suppress the cell growth and phosphorylation of Akt. In conclusion, the authors have demonstrated the diagnostic miRNA profile for HCC, and for the first time, identified the miR-125b with predictive significance for HCC prognosis.

Among the most important miRNA families, miR-125 family has been reported to be implicated in a variety of carcinomas and other diseases as either repressors or promoters [18]. Members of the family play crucial roles in many different cellular processes like cell differentiation, proliferation and apoptosis by targeting many different transcription factors [19], matrix-metalloprotease [20, 21], growth factors [22].

In our study, miR-125b-5p was identified over-expressed in all patients group when compared versus control and found statistically significant by Mann–Whitney *U* test as mentioned before. Our these findings are in agreement with these findings completely.

When we compared all groups versus control by one-way ANOVA test, only miR-223-3p was found statistically significant ($p = 0.0006652$). miR-125b-5p was also showed similar deregulations in same comparisons and found almost significant ($p = 0.07192$). There is no statistically significant alteration was detected for control RNU48 within the patient groups ($p = 377.1$).

In conclusion, we can clearly say that we identified 4 miRNAs; miR-223-3p, miR-92a-3p, miR-122-5p and miR-125b-5p could be used non-invasive biomarker by a simple sampling procedure such as taking blood at early HCC stage of liver disease. However, miR-223-3p is a potentially very early diagnostic biomarker of HBV related HCC even at chronic hepatitis B stage of liver disease. miR-125b-5p has also a high potential for a very early diagnostic biomarker even at chronic hepatitis B stage too. These two miRNAs should be studied in further and larger volume patient populations in CHB, HBV-positive cirrhosis and HBV-positive HCC in the future.

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