THE INVESTIGATION OF MICRORNAS AS POTENTIAL BIOMARKERS IN SERUM AND TISSUE FOR HEPATITIS B VIRUS RELATED HEPATOCELLULAR CARCINOMA

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ABSTRACT

Introduction*: Aberrant microRNA expression is associated with the development and progression of hepatocellular carcinoma (HCC). The aim of this study is to investigate of microRNAs as potential biomarkers in serum and tissue for hepatitis B virus related HCC.*

Material and methods*: The expression levels of miR-21- 5p, miR-122a-5p, miR-182-5p, miR-221-5p and miR-223-5p in serum and tissue samples from 35 HCC patients, 35 chronic HBV patients (CHB), and 30 healthy individuals (HC) were analyzed by quantitative real-time PCR test (qRT-PCR).*

Results*: Serum and tissue expression levels of miR-21-5p and miR-221-5p were found upregulated in patients with HCC compared to CHB and HC, while serum and tissue miR-223-5p expression levels were downregulated. Whereas serum miR-122a-5p expression levels were upregulated in patients with HCC compared to CHB and HC, the tissue miR-122a-5p expression levels were downregulated. However, the expression levels of serum and tissue miR-182-5p were not found statistically significant between the working group. In the diagnostic evaluation of serum expression levels were determined in patients with HCC compared to HC by ROC (Reciever Operator Characteristic Curve) analysis. miR-21-5p AUC (Area Under Curve) value of 0.863, miR-122a 5p AUC value of 0.819, miR- 221-5p AUC value of 0.801 and miR 223-5p AUC value of 0.815 were detected. In tissue, miR-21-5p AUC value of 0.810, miR-122a-5p AUC value of 0.794, miR-221-5p AUC value of 0.800 and miR-223-5p AUC value of 0.783 were found.*

Conclusion: *The miR-21-5p, miR-122a-5p, miR-221-5p and miR-223-5p have been shown used as potential non-invasive biomarker in the early diagnosis and prognosis of HCC.*

Keywords: *Biomarker, Hepatocellular carcinoma, microRNA, Serum, Tissue.*

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Introduction

HCC is the most important cause of liver cancer. It is one of the most common fifth malignancy and the second cause of death from cancer all over the world[(1)]. The major causes of HCC can be associated with risk factors, including hepatitis B and C viral factors, excessive alcohol intake, fatty liver disease, diabetes, chemical contamination by aflatoxin in food and water and hereditary liver disease[(2)]

Other physiological factors such as sexuality, age, obesity and different geographic regions may also pose a risk for HCC. Among these risk factors, CHB is the most important etiological factor of HCC development[(3)].

Since HCC is heterogeneous disease, the early diagnosis and monitoring of HCC development is rather significance[(4)]. To assist the diagnosis of HCC, imaging techniques are used in screening. However, in the case of early HCC, the diagnosis of small

lesions is relatively inaccurate and repeated examination is costly. Alpha-fetoprotein (AFP) is the most important convenient and effective serum biomarker, which is widely used for early detection of HCC. However, this biomarker fails to meet the needs for accurate early diagnosis and prognosis of HCC because of low sensitivity and specificity[5]. Therefore, additional biomarkers that can be used complementarily are required, particularly those associated with early HCC[6].

miRNAs are endogenous and small non-coding RNA molecules that negatively alter gene expression by promoting messenger RNA (mRNA) degradation and translation of their target mRNA genes[7]. Accumulating evidence showed that some aberrant expression miRNAs contribute to tumor induction and development. The main targets of miRNAs regulate biological processes associated with cancer development, apoptosis, cell differentiation, metastasis and invasion. The abnormal expression of miRNAs may also contribute to overexpression of oncogenes or inactivation of tumor suppressors by affecting their target genes[8,9].

In our study, we investigated miRNA expression profiling both serum and tissue in patients with HBV related to HCC in order to determine potential biomarkers for early detection in HCC.

Materials and methods

Patients and sample collection

miRNA expression levels in serum samples and liver tissue, pairs of cancerous and adjacent non-cancerous tissue specimens were investigated. From March 2016 to December 2019, A total number of 100 serum samples including 35 samples from individuals with HBV-related HCC, 35 from patients with chronic HBV and 30 healthy individuals were collected from the Departments of Radiology and İnfectious Diseases Faculty of Medicine, Cukurova University, Turkey.

All patients were confirmed as HCC by pathology, tumor tissues and paired normal adjacent tissues were collected after operation. Tissue samples of these patients were obtained from the Department of Pathology. All samples were stored at 80°C prior to analysis.

miRNA extraction and reverse transcription

miRNAs were extracted from serum samples by using miRNeasy Serum/Plasma kit (Qiagen, Germany) and from tissue samples by using miRNeasy Mini kit (Qiagen, Germany) according to the manufacturer's protocol. The concentration and purity of isolated RNAs were measured with NanoDrop Spectrophotometer (ND-1000 Thermo Fisher Scientific).

For cDNA synthesis, miRNA was reverse transcribed using miScript II RT Kit (Qiagen, Germany). RT reactions mix included n4 µl 5x miScript HiSpec Buffer, 2 µl 10x miScript Nükleic acid mix, 2 µl miScript Reverse Transcriptase and 12 µl RNA. The reaction mixtures were incubated for 60 min at 37°C then for 5 min at 95°C in a MJ Mini Thermal Cycler (BioRad). cDNA samples were diluted 90 µl with RNase Free Water. Finally, cDNA samples were stored at 20°C prior to RT-qPCR analyses.

miRNA quantification by qRT-PCR

miRNA expression levels were quantified in qRT-PCR systems by using miScript SYBR Green PCR kit according to the manufacturer's protocols (Qiagen, Germany). RT-qPCR mixture with 20 µl final volume contained 10 µl 2x QuantiTect SYBR Green PCR Master Mix, 2 µl 10x miScript Universal Primer, 2 µl 10x miScript Primer Assay, 5 µl diluted cDNA, and 1 µl RNease free water. All RT-qPCR analyses were performed using Rotor-Gene Q (Qiagen, Germany) with the cycling conditions were 95°C for 15 min followed by 40 cycles of 94°C for 15 sec, 58°C for 30 sec, and 72°C for 30 sec.

Statistical analysis

The expression levels of target miRNAs were normalized to Snord61, the relative expression was detected by $2^{-\Delta\Delta CT}$ relative to control[10]. All analyzes were made on the "QIAGEN GeneGlobe Data Analysis Center" website.

Comparisons of differences in the categorical data between groups were performed using Student's t-test. ROC analysis was used to estimate the diagnostic efficiencies of miRNA levels. The adequacy of the diagnostic test was determined by the AUC value[11]. The ROC analysis was performed using SPSS® statistical software version 16.0 (SPSS Inc., Chicago, IL, USA) p values of < 0.05 were defined as statistical significance.

Results

Demographic and clinical features of patients

The clinical and demographic characteristics (age, gender, liver biochemical tests, viral loads, AFP levels) are summarised in Table 1. Gender was not significantly different between studied groups

(p>0.05). However, there was a male predominance in HCC patients. The levels of HBsAg and HBV loads were significantly higher among CHB compared to HCC patients (P<0.05). Serum levels of AST, ALP, AFP, and total bilirubin (P<0.05 for each) were significantly higher in HCC patients versus the other group.

Characteristics	HC (n=30)	CHB (n = 35)	HCC (n = 35)
Age (years)	38	35	64.7
Male	17	16	30
Female	13	19	5
HBsAg (IU/ml)	NA	5038	4008
HBV load (copies/ml)	NA	$1.2x10^7$	$8x10^4$
ALT (IU/ml)	29,7	60	79,3
AST (IU/ml)	31.2	65	85,7
Total Bilirubin (mg/dl)	0.8	1.2	2.1
AFP <20 ng/ml	NA	30	4
AFP 20-200 ng/ml	NA	NA	11
AFP >200 ng/ml	NA	NA	20
Child-Pugh			
A	NA	NA	30
B	NA	NA	4
C	NA	NA	1
Tumor size (cm)			
< 2	NA	NA	2
2-3 cm	NA	NA	5
3-5 cm	NA	NA	27
> 5	NA	NA	1

Table 1: Characteristics of study paticipants according to clinical presentation.

Comparison of miRNAs expression profiling in the serum samples of HCC, CHB and HC

We were performed to examine the expression levels of 5 miRNAs (miR-21-5p, miR-122-5p, miR-182-5p, miR-221-5p and miR-223-5p). Serum expression levels of miR-21-5p, miR-122a-5p and miR-221-5p were statistically significantly upregulated in patients with HCC compared to CHB (p<0.05), whereas miR-223-5p was statistical significance downregulated (p<0.05).

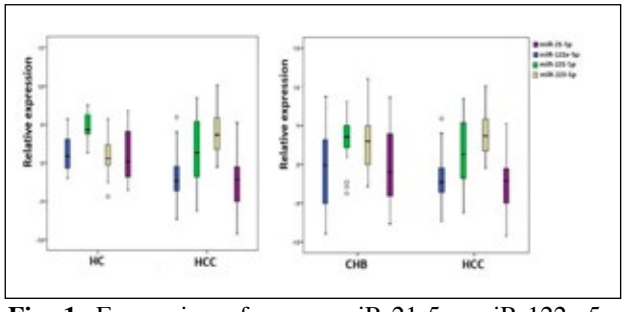

Fig. 1: Expression of serum miR-21-5p, miR-122a-5p, miR-221-5p and miR-223-5p in patient with HCC compared to CHB and HC.

However, no statistically significant difference was found between HCC and CHB serum for the expression level of miR182-5p (p>0.05).

The levels of miR-21-5p, miR-122a-5p and miR-221-5p in serum were statistically significantly upregulated in patients with HCC in comparison with healthy controls (p<0.05), while miR-223-5p was statistically significantly downregulated (p<0.05) (Fig. 1). Although the serum expression level of miR-182-5p was upregulated, this increase was not found to be statistically significant (p>0.05).

Comparison of miRNAs expression profiling in the tissue samples of HCC, CHB and HC

The tissue miRNA profiles (miR-21-5p, miR-122-5p, miR-182-5p, miR-221-5p and miR-223-5p) were analyzed among the three groups. The expression levels of tissue miR-221-5p and miR-21-5p were statistically upregulated in HCC patients compared to CHB (p<0.05), whereas tissue miR-122a-5p and miR-223-5p expression levels were statistically significantly downregulated (p<0.05). Although expression of miR-182-5p in tissue was upregulated, this increase was not found to be statistically significant (p>0.05).

Tissue miR-21-5p and miR-221-5p were significantly overexpressed in patient with HCC compared with HC (p<0.05), while tissue expression levels of miR-122a-5p and miR-223-5p were statistically significant downregulated (p<0.05) (Fig. 2). Although the tissue expression level of miR-182-5p was upregulated, it was not detected statistically significant (p>0.05).

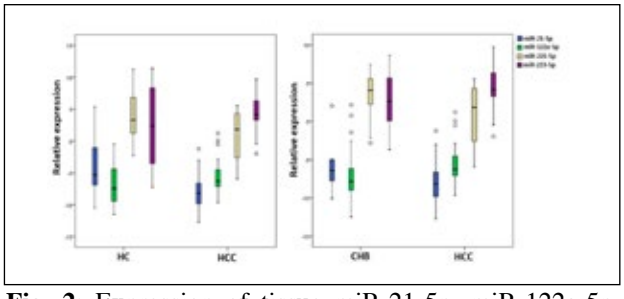

Fig. 2: Expression of tissue miR-21-5p, miR-122a-5p, miR-221-5p and miR-223-5p in patient with HCC compared to CHB and HC.

Diagnostic performance of serum and tissue miRNAs

The ROC curve was performed to assess the possibility of using statistically significant serum and tissue levels of miRNAs as diagnostic biomarkers for early detecting HCC from CHB and HC.

ROC analysis revealed that studied miRNAs could discriminate between HCC and CHB with AUC value 0.819 for miR-21-5p, 0.843 for miR-122a-5p, 0.801 for miR-221-5p and 0.821 for miR-233-5p (Fig. 3). Similarly, It was revealed that the levels of serum miR-21-5p, miR-122a5p, miR-221-5p and

miR-223-5p were potential markers for discriminating HCC patients from HC, with ROC curve areas of 0.863, 0.819 0.801 and 0.815 in turn (Fig. 4).

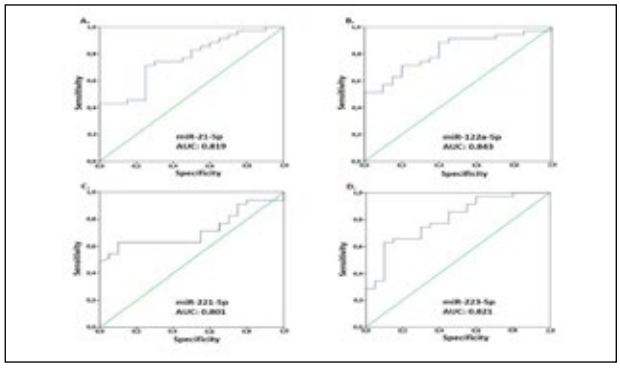

Fig. 3: Diagnostics performance of serum miRNA in patients with HCC compated to CHB. A. miR-21-5p, B. miR-122a-5p, C. miR-221-5p and D. miR-223-5p.

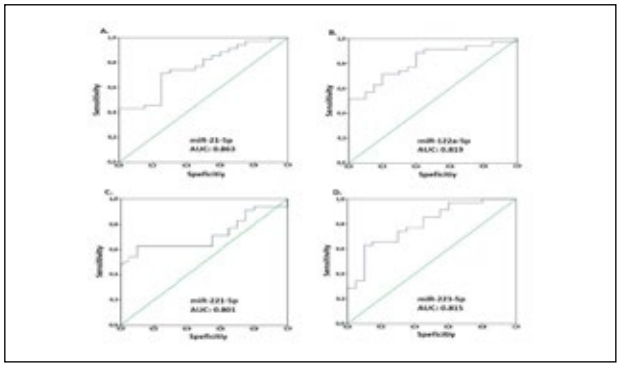

Fig. 4: Diagnostics performance of serum miRNA in patients with HCC compated to HC. A. miR-21-5p, B. miR-122a-5p, C. miR-221-5p and D. miR-223-5p.

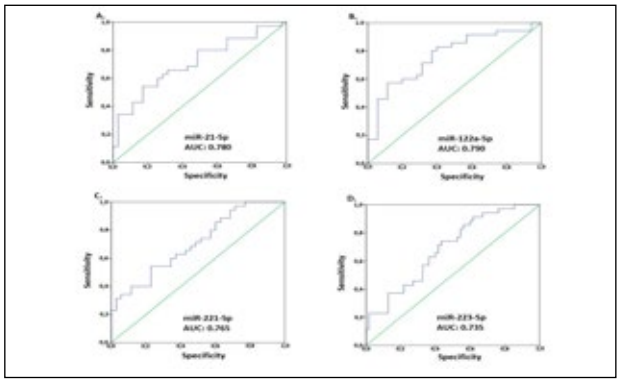

Fig. 5: Diagnostics performance of tissue miRNA in patients with HCC compated to CHB. A. miR-21-5p, B. miR-122a-5p, C. miR-221-5p and D. miR-223-5p.

The tissue expression levels of miR-21-5p, miR-122a-5p, miR-182- 5p, miR-221-5p and miR-223 5p, which we detected statistically significant levels of HCC patients compared to patients with CHB sensitivity and specificity values were determined by ROC analysis. The AUC value of tissue miR-21, miR-122, miR-221 and miR-223 were detected 0.780, 0.790 0.765 and 0.735 respectively (Fig. 5). In addition, ROC curve analyses revealed that these four miRNAs were useful markers for discriminating patients with HCC from HCC. ROC curve areas for miR-21-5p, miR-122a-5p, miR-223-5p and miR-223-5p were 0.810, 0.794, 0.800 and 0.783 in turn (Fig. 6).

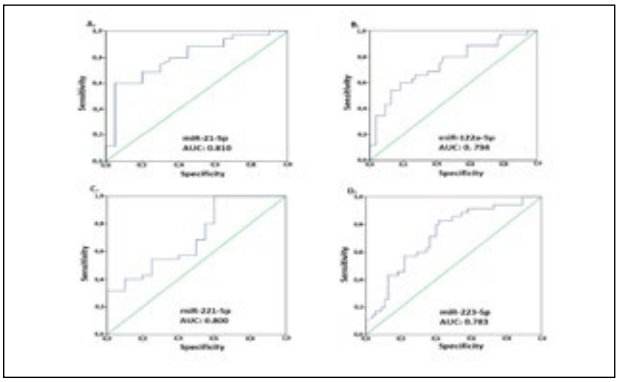

Fig. 6: Diagnostics performance of tissue miRNA in patients with HCC compated to HC. A. miR-21-5p, B. miR-122a-5p, C. miR-221-5p and D. miR-223-5p.

Discussion

miRNAs are highly conserved non-coding RNA molecules miRNAs lead to inhibition of protein translation or destruction of mRNA by binding to mRNA with target genes complementary to their nucleotide sequences via base-pairing feature[12]. Irregularity of miRNA expression has been associated with many cancer diseases. It has been shown that dysregulation of miRNA contritubes to expression levels of oncogenes and tumor suppressor genes. miRNAs can also affect HBV entry and replication in liver cells and many other basic biological events such as tumor development, checkpoints, programmed cell death and determining cell migration[13]. miRNAs are found among preneoplastic and neoplastic lesions in various body fluids, including serum and plasma and normal liver and liver cancer tissues. In addition, miRNAs are subject to extreme conditions such as low pH and resistance to RNAase. It is also considered as an alternative non-invasive biomarker because it shows significant stability under. miRNAs are expressed not only in blood serum but also in liver tissues[14]. The recent study has shown that miRNAs can be applied not only as diagnostic biomarkers but also as prognostic factors for cancer[15].

The role of miR-21, which is often overexpressed, has been analysed in various human malignant cancers, including especially liver cancer[16]. miR-21 is considered an onco miRNA that inhibits the action of the tumor suppressor gene and induce

cell proliferation, invasion, and metastasis. Xu et al shown that the levels of serum miR-21 were significantly upregulated in HCC patients compared to healthy individual[17]. Guo et al. analysed that serum miR-21 levels significantly overexpressed in patients with HCC than healthy donors[18]. Tomimaru et al. shown that serum levels of expression miR-21 in HCC patients were significantly upregulated compared with CHB and HC[19]. In addition, the overexpression of miR-21 has been detected in HCC tissue than non-tumor liver tissues. In our study, we released that serum and tissue expression levels of miR-21-5p were statistically significantly upregulated in patients with HCC compared to CHB and HC ($p<0.05$).

miR-122 has been shown to play vital roles in hepatic functions, including viral infection and hepatocarcinogenesis. The expression levels of miR-122 are upregulated non-tumor liver tissue, however downregulated in approximately 70% of infected HBV with hepatocytes and HCC cell lines[20]. It has been shown that miR-122 is downregulated in HCC tissues[21]. However, Qi et al. found that the serum miR-122 level was upregulated in HCC patients compared with healthy controls[22]. In this study, we analyzed that miR-122a-5p serum levels were statistically significantly upregulated, while miR-122a-5p tissue expression levels were statistically significantly downregulated in HCC when compared CHB and HC ($p<0.05$).

The function of miR-182-5p is complex because it may role as oncogene or tumor suppressor in several cancers[23]. Chen et al. showed that the expression levels of miR-182 were contributed to the processing of HCC by promoting various cellular processes. miR-182 is often upregulated in HCC[24]. In our study, while the expression levels of serum and tissue of miR-182-5p were upregulated HCC compared to CHB and HC, it was not detected statistically significant ($p> 0.05$).

miR-221 that oncogenic miRNA is one of the most studied miRNAs in HCC cases. miR-221 leads to induce tumor formation and development by altering cell proliferation and apoptosis[25]. Li et al. researched that the expression level of serum miR-221 were significantly upregulated in HCC. In addition, miR-221 has been reported overexpression in HCC tissues than non-tumor tissues[26]. Karakatsanis et al. have shown that the expression levels of miR-21, miR-122 and miR-221 were significantly upregulated in HCC tissues, while miR-223 expression level was detected downregulated[27]. Data obtained from the study also show serum and tissue expression of miR-221-5p were statistically significant upregulated in patients with HCC than CHB and HC ($p<0.05$).

miR-223 is contritube to the pathogenesis of HCC by affecting cell growth, cell apoptosis, metabolic signaling pathways, and inflammatory signaling pathways[28]. Bhattacharya et al found that the expression level of serum miR-223 was downregulated in HCC patients compared to healty control[29]. On the contrary, Qi et al. reported that the levels of miR-223 were significantly upregulated in HCC patients compared to healthy controls[22]. Pratedrat et al. shown that miR-223-3p was significantly downregulated in HCC tissue compared to non-tumor liver tissue[30].

Our results indicated that serum and tissue expression levels of miR-223-5p were statistically significant downregulated in patients with HCC when compared CHB and HC ($p<0.05$).

The ROC curve results of mir-21-5p, mir-122a-5p, miR-221-5p and miR-223 show that their sufficiency to have the power to differentiate the HCC patients from CHB and HC. Since the expression level of miR-182-5p was not found to be statistically significant, the AUC value of miR-182-5p was not evaluated between the groups.

In conclusion, our findings suggest that that miR-21-5p, miR-122a-5p, miR-221-5p and miR-223-5p can be used as non-invasive biomarker to distinguish HCC from CHB and healthy individuals in early diagnosis. In addition to the rapid diagnosis of our study, evaluating these miRNAs together with tumor lesion diameter and AFP levels or determining panel miRNAs will greatly benefit us to create high accurate diagnosis and prognostic models for HCC and early diagnosis, tumor monitoring and will shed important light on prognosis prediction.

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