Serum microRNA-26, microRNA-122 and microRNA-192 expressions in hepatocellular carcinoma

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Abstract

Introduction: There is not yet an ideal biomarker to detect hepatocellular carcinoma (HCC) in early stage. MicroRNA (miRNA) dysregulation in HCC results in inhibition of cellular apoptosis, increased angiogenesis, invasion and development of metastasis. The aim of the present study was to evaluate the efficacy of serum miRNA-26, miRNA-122 and miRNA-192 levels in diagnosis of HCC compared to other prognostic criteria.

Materials and Methods: The study included newly diagnosed HCC patients (n=42) and patients with non-alcoholic fatty liver disease (NAFLD) as controls (n=45). We measured serum miRNA-26, miRNA-122 and miRNA-192 levels for each patient.

Results: Serum levels of miRNA-26 were significantly lower in HCC patients (29.03) than those in control group (30.34) (p<0.001). There was no significant difference in serum levels of miRNA-122 between the two groups (p=0.181). MiRNA-192 was not expressed as assessed by the real-time PCR method. For diagnosis of HCC, cut-off value was set at 29.38 for miR-26. With this cut-off value, sensitivity was 71.4%, and specificity was 82.2%, while positive and negative predictive values were 78.9% and 75.5%, respectively (p<0.0001). We also compared the miRNA-26 and miRNA-122 levels of patients with other diagnostic, clinical and prognostic data, which are significant for HCC. No statistically significant correlation was found between alpha-fetoprotein (AFP) and miRNA-122 and miRNA-26 gene expressions.

Conclusions: Serum levels of miRNA-26 may contribute to early diagnosis of HCC when used together with other diagnostic, clinical and prognostic markers.

Keywords: Hepatocellular carcinoma, microRNA-26, microRNA-122, microRNA-192.

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Introduction

Hepatocellular carcinoma (HCC) is a malignant neoplasm of the liver characterized with nodular lesions, generally developing in a background of cirrhotic liver. HCC is the fifth most prevalent malignancy worldwide, and the second most common cause of cancer mortality[1]. As a primary liver cancer, HCC accounts for 85-90% of all primary liver cancers[2]. Each year 250,000-1,000,000 people die from HCC all over the world[3]. The prevalence of HCC varies by geographic region, ethnic group and gender.

The most known major risk factors of HCC include hepatitis B virus (HBV), hepatitis C virus (HCV), chronic alcohol intake, non-alcoholic steatohepatitis (NASH), autoimmune hepatitis, primary biliary cirrhosis and aflatoxin-contaminated food intake[4]. The three most common serum markers used for diagnosis of HCC are alpha-fetoprotein (AFP), Lens culinaris agglutinin-reactive alpha-fetoprotein-L3 (AFP-L3) and Des gamma carboxy protrombin (DCP). For diagnosis of HCC, the sensitivity of AFP-L3, DCP, and AFP are 61%, 72%, and 67%, respectively, and it increases to 85.9% when all three markers are combined[5]. In HCC, the role of liver biopsy remains controversial. Biopsy is recommended for lesions smaller than 2 cm in the absence of high quality imaging methods and qualified experts who are competent to perform assess-
ment of these methods. However, studies found a false negative biopsy rate of approximately 30 to 40%\(^6\). Therefore, a false negative biopsy result does not conclusively exclude diagnosis of hepatocellular cancer.

Many studies have been conducted and further studies are required to elucidate the genomic instability pathway, host-virus relationship, micro processes (inflammation, cirrhosis) of HCC, and cellular origin of hepatocarcinogenesis, and identify those with higher risk of developing HCC by biomarkers in early stage\(^7\).

Recently, microRNAs (miRNA) that can be assessed in serum, plasma or other body fluids are being increasingly used for diagnosis and monitoring of many diseases including carcinomas\(^8\). In genetics, miRNA is a single-stranded RNA molecule, which is approximately 21–23 nucleotides in length, and involved in regulation of gene expression. In normal tissues, regular miRNA transcription and binding to target mRNA inhibit protein translation, resulting in normal rate of growth, proliferation, differentiation and cell apoptosis\(^9\). In HCC, dysregulation of miRNA has been associated with inhibition of cellular apoptosis, increased angiogenesis, invasion and development of metastasis\(^10\).

In HCC, miRNA-122, miRNA-192, miRNA-26a, miRNA-21, miRNA-223 and miRNA-801 have been demonstrated to be associated with regulation of the cell cycle\(^11\). A study by Wang et al. showed that tissue level of miRNA-26 was downregulated in patients with HCC compared to the level in healthy individuals\(^12\).

Any disruption in the regulation of miRNA under the control of these genes provides background for development of HCC. It has been shown that miR-122 suppresses tumor necrosis factor-alpha-converting enzyme (TACE) expression in patients with HCC, and inhibits angiogenesis and intrahepatic metastasis\(^13\). It has been suggested that miRNAs may also be used for treatment of HCC.

A relevant mice study found that tumor-specific apoptosis is induced in treatment of HCC when miRNAs are replaced or repaired, and it has a good prognosis following treatment\(^14\).

Based on these studies, it is suggested that miRNAs may be a novel marker for early diagnosis, treatment and prognosis of HCC. We aimed to demonstrate the efficacy of serum miRNA-26, miRNA-192 and miRNA-122 levels in 42 patients with HCC at different stages, who were follow-up in different clinics, and compare them with the diagnostic, clinical and prognostic data of these patients.

### Materials and methods

#### Patients

The study included a total of 42 patients with HCC and liver cirrhosis, who presented to or already hospitalized at the Gastroenterology Outpatient Clinic of the Medical Faculty Hospital of Gaziantep University between December 2014 and February 2016, and received a new radiological and/or histological diagnosis as well as 45 patients with non-alcoholic fatty liver disease (NAFLD) as a control group. NAFLD patients were included as a control group since they might have had liver cirrhosis and progression to HCC. Any patient with a hepatic mass associated with other etiology and hepatic metastatic lesion was excluded. All patients in both groups underwent physical examination. Hepatic markers (HBsAg, antiHBs, anti-HBc IgG, anti-HCV) were tested. Serum AFP levels were assessed. The patients underwent ultrasonography (USG), color Doppler ultrasonography of portal vein, Dynamic Magnetic Resonance (MRI) / Dynamic Computed Tomography (CT) imaging. Their Child-Pugh and the Model for End-stage Liver Disease (MELD) scores were calculated. The patients were classified according to presence of portal vein thrombosis, tumor size, etiology and treatment status. A 2.5 mL of peripheral blood was drawn from each patient into a tube containing RNaNalT solution. The peripheral blood samples obtained from patient and control groups were kept at -80ºC until use.

#### Diagnostic criteria

The diagnosis of HCC was made by pathologically or non-invasive imaging methods in cirrhotic patients. The number of patients who were diagnosed with HCC pathologically was 4 while the number of patients diagnosed by a non-invasive imaging method was 38. The diagnostic criteria of the non-invasive imaging methods were as follows:

- Evidence of a typical HCC manifestation (hypervascular in the arterial phase with washout in the portal venous or delayed phases) with nodules in size of 1-2 cm in at least two dynamic imaging (4-phase multidetector CT scan or dynamic contrast-enhanced MRI).
- Evidence of a typical HCC manifestation
hypervascular in the arterial phase with washout in the portal venous or delayed phases) with nodules in size larger than 2 cm in only one dynamic imaging modality (4-phase multidetector CT scan or dynamic contrast-enhanced MRI)\(^{(15)}\).

NAFLD was diagnosed clinically by presence of obesity, diabetes and hyperlipidemia, as well as ultrasonographic findings of increased diffuse echogenicity in the liver.

**Circulatory miRNA expression**

This analysis was performed at the Molecular Genetic Laboratory at the Şahinbey Hospital of the Medical Faculty of Gaziantep University. We used Real-Time PCR method. Blood samples were drawn into a 5 mL serum gel separator tube (SST), and centrifuged at 4,000 rpm for 15 minutes. The supernatant was carefully collected, and transferred into a clean microcentrifuge tube. We used a Qiagen miRNeasy Serum/Plasma Kit (Hilden, Germany) to isolate miRNA from serum samples. The tissue was turned upside down, and homogenized by QIAzol Lysis Reagent of almost 5 times the serum amount, and incubated at room temperature for 5 minutes. Then, it was mixed with miRNeasy Serum/Plasma control solution. After adding chloroform at the same amount, it was agitated with a vortex for 15 seconds and incubated at room temperature for 4 minutes, and then centrifuged at 12,000 g 4°C for 15 minutes. The supernatant of the phase was transferred into a clean tube after being centrifuged. 100% ethanol was added approximately 1.5 times of the total amount, and it was mixed by pipetting. This mixture containing miRNA was transferred into a RNeasy MinElute spin column tube, and centrifuged at room temperature for 15 seconds, and this procedure was repeated until the sample was finished. RWT (15sec), RPE Buffer (15sec) and 80% ethanol (2 min) solutions were used, and added to spin columns sequentially, and then centrifuged at 8000 xg. RNeasy MinElute spin column was transferred into a fresh tube, and 14µl RNase-free water was added. For cDNA synthesis from the resultant miRNA, we followed the standard protocol for Qiagen miScript Reverse Transcription (RT) Kit II (Hilden, Germany).

The amount of RNA was adjusted to be 200ng/µl within a volume of 20 µl, adding onto cDNA synthesis mixture, and RT-PCR was performed at 37°C for 60 minutes based on the reaction conditions at 95°C for 5 minutes. We used the Qiagen miScript SYBR Green PCR kit to adjust the amount of cDNA to 50 ng/µl on preparations, and performed qRT-PCR at reaction conditions of 95°C for 15 minutes, 94°C for 15 minutes, 55 °C for 30 minutes and 70°C for 30 minutes (a total of 40 cycles). The results were evaluated by statistical analysis.

**Statistical analysis**

Statistical analyses were performed using a licenced Statistical Package for the Social Sciences (SPSS) software [SPSS V22.0 (IBM Corporation, Armonk, NY, USA)]. We used Kolmogorov Smirnov test to evaluate normal distribution of continuous variables. For comparison of normally distributed variables in 2 independent groups, we used Student’s t test, and for comparison of abnormally distributed variables in two independent groups, we used Mann Whitney U-Test. The numerical data from more than two independent groups were analysed by the Kruskal Wallis test. A cut-off value was determined for numerical variables by means of a ROC curve analysis. A p value < or =0.05 was considered to be significant.

**Results**

The study included 42 patients diagnosed with HCC, and 45 patients diagnosed with NAFLD (control group). The number of male patients was significantly higher in HCC group than in control group (p=0.001). The mean age was 60.26 ± 12.80 years in HCC group, and 48.98 ± 13.21 years in control group. A statistically significant difference was found in mean age between the groups (p=0.001).

All of the patients in HCC group were previously diagnosed with liver cirrhosis. The patients were divided into 3 groups based on the Child-Pugh classification. The number of patients in groups A, B and C was 13 (31%), 20 (48%) and 9 (21%), respectively. The patients were divided into 3 groups based on tumor size. The number of patients with a tumor size <2 cm, between 2-3 cm, and >3 cm was 9 (21%), 13 (31%) and 3 cm 20 (48%), respectively. Thirteen (31%) patients had portal vein thrombosis. The number of patients with ascites was 25 (60%). An analysis of cirrhotic etiology in HCC showed that 29 (69%) patients had HBV, 8 (19%) HCV, and 1 (2%) tyrosinemia. No etiology was found in remaining 4 (10%) patients, and they were diagnosed with HCC associated with
cryptogenic liver cirrhosis. Three of these patients with HCC associated with cryptogenic cirrhosis had (75%) anti-HBc IgG(+), HBsAg(-) levels. Among all patients with HCC, 33 (79%) had anti-HBc IgG positivity.

We couldn’t perform miRNA analysis and statistical analysis since MiR-192 gene expression was under the normal level.

The miRNA-122 and miRNA-26 gene expressions in HCC group were compared to control group. The median level of miRNA-122 was 28.24 ng/µL in control group vs. 27.99 ng/µL in HCC group. The median level of miR-26 was 30.34 ng/µL in control group vs. 29.03 ng/µL in HCC group. There was no statistically significant difference between the HCC and control groups in serum miRNA-122 levels (p=0.181).

The serum miRNA-26 levels were significantly reduced in HCC group compared to control group (p<0.001). The demographic, clinical and laboratory characteristics of patient and control groups are shown in Table 1. Fig. 1 shows a comparison of miRNA gene expressions between HCC and control groups.

We used Pearson’s correlation test to show the correlation between miRNA-122 and AFP variables in HCC patients. There was no statistically significant correlation between AFP and miRNA-122 gene expression (r=0.093, p=0.558). Similarly, no statistically significant was found between AFP and miRNA-26 gene expression (r=0.095, p=0.549) (Fig. 2).

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We used Pearson’s correlation test to show the correlation between miRNA-122, miRNA-26 and AFP variables in HCC patients. There was no statistically significant correlation between AFP and miRNA-122 gene expression (r=0.093, p=0.558). Similarly, no statistically significant was found between AFP and miRNA-26 gene expression (r=0.095, p=0.549) (Fig. 2).

We also used Pearson’s correlation test to demonstrate the correlation between tumor size and gene expression levels in patients with HCC. There was no statistically significant correlation between
tumor size and miRNA-122 a ($r=0.261$, $p=0.103$). And no statistically significant correlation was found between tumor size and miRNA-26 ($r=0.266$, $p=0.097$) (Table 2 and Fig. 3).

In patients with HCC, we compared portal vein thrombosis and miRNAs gene expression. No significant relationship was found between portal vein thrombosis and miR-122 gene expression ($p=0.648$). In HCC patients with portal vein thrombosis, the miR-26 gene expression was significantly higher ($p=0.046$) (Table 3).

We compared ascites and miRNAs gene expression levels in patients with HCC. There was no significant relationship between presence of ascites and miRNA-122 gene expression ($p=0.76$). Similarly, no significant relationship was found between presence of ascites and miRNA-26 gene expressions ($p=0.086$) (Table 4).

We calculated sensitivity and specificity for miR-26 in HCC and control groups, and a cut-off value was determined for diagnosis of HCC by evaluating the area under the ROC curve. The cut-off value was 29.38 ng/$\mu$L in the patient group for miRNA-26 based on the area under the ROC [AUC:0.748, 95% CI (0.679-0.920), sensitivity 71.4%, and specificity 82.2%]. The 95% confidence interval was 55.4%-84.3% for sensitivity, and 67.9%-92.0% for specificity.

Table 2: A comparison between tumor size and miRNA-122 and miRNA-26 gene expression levels.

<table>
<thead>
<tr>
<th>Tumor size (cm)</th>
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<tbody>
<tr>
<td>0-2</td>
<td>0.103</td>
</tr>
<tr>
<td>3-Feb</td>
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<td>&gt;3</td>
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Table 3: A comparison between portal vein thrombosis and miRNA gene expressions.

<table>
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<tr>
<th>Portal vein thrombosis</th>
<th>p value</th>
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<tbody>
<tr>
<td>Yes</td>
<td>0.648</td>
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Table 4: A comparison between ascites and miRNA gene expressions.

<table>
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<tr>
<th>Ascites</th>
<th>p value</th>
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<tr>
<td>Yes</td>
<td>0.76</td>
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Fig. 3: a) Correlation between miRNA-122 and tumor size in hepatocellular carcinoma patients ($r=0.261$, $p=0.103$), b) Correlation between miRNA-26 and tumor size in hepatocellular carcinoma patients ($r=0.266$, $p=0.097$).

Fig. 4: The efficacy of serum miRNA-26 levels in diagnosis of hepatocellular carcinoma patients. The area under the curve (AUC) is 0.748 (95% CI (0.679-0.920)) in ROC (receiver operating characteristic) analysis ($p<0.0001$).

For diagnosis of HCC, when serum miRNA-26 cut-off value was taken as 29.38 ng/$\mu$L, the sen-
sitivity was 71.4%, specificity was 82.2%, with a positive predictive value of 78.9% and a negative predictive value of 75.5% (p<0.0001) (Fig. 4).

Discussion

HCC is a type of cancer still with a high mortality and poor prognosis(15). Early diagnosis and early surgical therapy may contribute to improvement in prognosis. Many studies have been conducted to identify people with a high risk of developing HCC by biomarkers in early stage. However, we still do not have a reliable biomarker to detect hepatocellular carcinoma early. Alpha-fetoprotein has been used to detect hepatocellular carcinoma, but its sensitivity and specificity were found to be lower(16). A study showed that the sensitivity increases to 85.9% when AFP, AFP-L3 and DCP, each with a lower sensitivity individually, are used together(5).

MiRNAs are a series of gene regulators with a single-chain and no protein code, having broad biological effects in cell proliferation and apoptosis. Since they account for 1-4% of all genes, miRNAs represent one of the largest groups of gene regulators(17). Many studies on miRNA and malignancies showed distinct miRNA expression profiles in development and of malignancies(18). In HCC, dysregulation of miRNA results in inhibition of cellular apoptosis, increased angiogenesis, invasion and development of metastasis(19). MiRNA-122, miRNA-192, miRNA-26a, miRNA-21, miRNA-223 and miRNA-801 have been associated with cell cycle function in HCC(10). Measureable in serum or plasma, these miRNAs are claimed to be a novel biomarker in diagnosing and determining prognosis of cancer(10).

A study by Xu et al. examined the serum miRNA-122 levels in patients with chronic hepatitis and HCC, and found that the serum miRNA-122 level was higher in patients with HCC compared to healthy control patients(20). Another case-controlled prospective study examined the serum miR-122 levels in 48 patients with HCC, 48 non-HCC patients with HBV and 24 healthy individuals, and found that serum miRNA-122 level was significantly higher in both patients with hepatitis and HCC compared to healthy controls(21).

Another study comparing tissue miRNA-26 levels of patients with HCC and healthy individuals showed that tissue miRNA-26 level was reduced in patients with HCC(12). A study examining the miRNA expression in peripheral blood mononuclear cells, miRNA-26b expression was significantly lower in patients with HCV-associated non-Hodgkin lymphoma and HCV-associated HCC compared to patients with chronic hepatitis C (CHC) and healthy individuals(22).

In the present study, comparison of serum miRNA-122 levels between patients with HCC and control group diagnosed with NAFLD showed no statistically significant difference. MiRNA-122 is a liver specific miRNA, and its serum level is increased with hepatocyte injury(13). Therefore, it is likely that patients in control group with NAFLD might also have had hepatocyte injury, with an effect on the miRNA-122 level, resulting in no significant difference between the two groups.

In the present study, the serum miRNA-26 levels were statistically significantly lower than in control group (p<0.0001). Considering this statistical significance, we developed a ROC curve for miRNA-26 levels in patients with HCC. Based on the area under the ROC curve in the patient group, the cut-off value for miRNA-26 was 29.38 ng/µL. For diagnosis of HCC, when serum miRNA-26 cut-off value was taken as 29.38 ng/µL, the sensitivity was 71.4%, specificity was 82.2%, with a positive predictive value of 78.9% and a negative predictive value of 75.5% (p<0.0001).

A study by Wang et al. showed that plasma miRNA-122 levels are increased while miR-26 levels are reduced as inflammation and damage in tissue is increased with administration of acetaminophen(23). Therefore, we examined the relationship between tumor size and serum miRNA-122, and miRNA-26 levels. However, no statistically significant difference was found between tumor size and serum miRNA-122 and miRNA-26 levels. We found no study which examined the relationship between increasing or decreasing tumor size and serum miRNA-122 and miRNA-26 levels in patients with HCC.

In the present study, the groups were not homogeneous with respect to age and gender. HCC develops more frequently in males(24). In our study, the number of male patients was also higher. There was a significant difference between the patient and control groups in gender (p=0.001). The mean age was 60.26 ± 12.80 years in patient group vs. 48.98 ± 13.21 years in control group. There was a significant difference in age between the groups (p=0.001). A correlation analysis was performed for miR-122, miR-26, age and gender variables in
patient and control groups. We found no statistically significant correlation in miRNA-122, miRNA-26, age and gender between the groups.

It is known that portal vein thrombosis is associated with poor prognosis in HCC (25). A study by Liu et al. showed that tissue miRNA-135 level is increased in HCC patients with portal vein thrombosis (26). We compared portal vein thrombosis and serum miRNA-122, miRNA-26 gene expressions, and found no significant relationship between portal vein thrombosis and miRNA-122 gene expression in patients with HCC. In patients with portal vein thrombosis, miRNA-26 gene expression levels were significantly higher (p=0.046). Therefore, serum miRNA-26 levels may contribute to prediction of poor prognosis in HCC.

We also compared ascites and miR gene expression levels, and found no significant relationship between ascites and miRNA-122 and miRNA-26 gene expressions.

A study showing the relationship between miRNAs and AFP found that miRNA-122 increased AFP expression (27). The present study found no statistically significant correlation between AFP and serum miRNA-122 and miRNA-26 levels. We didn’t perform statistical analysis since we failed to show miRNA-192 gene expression in serum.

Anti-HBc IgG (+) may persist for a long period of time, even for life in people who experienced HBV infection (28). Covalently closed circular DNA (cccDNA) associated with seroconversion secondary to prior HBV virus has a high risk for development of HCC. Therefore, the risk for developing HCC is increased in both anti-HBc IgG positive, and HBsAg negative patients (29). We had 4 patients with HCC associated with cryptogenic cirrhosis. Three out of 4 patients diagnosed with cryptogenic cirrhosis had anti-HBc IgG positivity (75%). Presence of anti-HBc IgG positivity in patients with HCC associated with cryptogenic cirrhosis might have resulted in development of HCC in these patients. Larger studies are needed to clarify it.

One of the limitations of our study was inclusion of patients with NAFLD as control group and absence of any healthy volunteers. Secondly, the number of cases was small both in patient and control groups. We need further studies in which patients with HCC are compared to patients with chronic hepatitis and healthy individuals, and sufficient number of cases is included.

In conclusion, it is most likely that miRNAs will be used routinely in the near future for diagnosing and monitoring HCC. Particularly miRNA-26 gene expression may serve as a good biomarker in diagnosis of HCC and prediction of prognosis. However, we need large prospective studies examining both serum and tissue miRNA-26 gene expressions.

References


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Competing interests
We declare that we have no conflict of interests.

Ethical approval
The study was carried out in accordance with the principles of the Helsinki Declaration of 1975 as revised in 2008 and with Approval No. 24.08.2015/243 of the Clinical Research Ethics Committee of Faculty of Medicine, University of Gaziantep.

Informed consent
The blood samples were collected from the study and control groups based on a volunteer basis according to the ethical codes of conduct. Both patients and volunteers in control group were clearly informed about the objective and content of the study before their participation.

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