Role of mir-33a, mir-203b, mir-361-3p, mir-424 in hepatocellular carcinoma

ABSTRACT

Background/Aim: Hepatocellular carcinoma (HCC) is one of the most aggressive cancer. MicroRNAs (miRNAs), are small noncoding regulatory RNAs, that function post-transcriptionally. MiRNA deregulation was observed in the development and progression of HCC. In this study we aimed to investigate the expression levels of four miRNA (mir-33a, mir-203b, mir361-3p and mir-424) in HCC patients in comparison to healthy individuals.

Materials and methods: Venous blood samples were collected from both HCC patients and healthy individuals. In order to determine relative expression levels of mir-33a, mir-203b, mir361-3p and hsa-mir-424 in HCC patients using probe-based Quantitative Real Time PCR (qRT-PCR) is performed. The cycle threshold (Ct) results were analyzed according to the $2^{-\Delta\Delta\text{Ct}}$ method and statistical analyses was performed by IBM SPSS Statistics for Windows, version 15.

Results: qRT-PCR analysis revealed that the expression level of mir-33a (fold change: 7.3 and $p<0.001$), mir-203b (fold change: 4.6 and $p<0.001$), mir361-3p (fold change: 5.1 and $p<0.001$) were down regulated compared to healthy individuals and mir-424 did not show any significant change between the HCC patients and controls.

Conclusion: Our results indicated that mir-33a, mir-203b and mir-361-3p, may significantly contribute to tumor pathogenesis in HCC and would have potential to be used as a non-invasive biomarker for cancer therapy.

Key words: Mir-33a; mir-203b; mir361-3p; mir-424; HCC; qRT-PCR
1. **Introduction**

HCC is the leading cause of cancer-related deaths worldwide. It caused by hepatocytes is one of the most frequently diagnosed of primary liver cancer and constitutes 80-90% of primary liver tumors [1,2]. Infection with Chronic Viral Hepatitis B and Hepatitis C are leading etiological factor for the developing HCC. It has many different risk factors including hereditary, exposure to chemical agents, such as aflatoxin B1, smoking, and chronic alcohol abuse [3]. miRNAs are single stranded comprised of ~20 long, small non-coding post transcriptional regulator RNAs and have important role cancer progression [4].

Analysis of miRNA expression levels in blood provides critical information about prognosis of the diseases such as cancer, and monitoring of treatment. Tissue-specific miRNA expression level analysis can detect both tumor origin and allow early diagnosis of carcinogenesis. Numerous studies shown that circulating miRNAs have clinical importance as diagnostic and prognostic biomarkers in HCC [5–7].

Mir-33a is a highly conserved member of the mir-33, which is located in the intronic region, plays a role in lipid metabolism and provides glucose and cholesterol regulation. miR-33a has been shown to have growth, apoptosis, epithelial-mesenchymal transition and tumor suppressor effects in cancer cells [8].

Mir-203 serves as a tumor suppressor in many types of cancer including hepatocellular carcinoma, prostate, esophageal cancers and breast cancer. MiR-203 has been reported to play an important role in the carcinogenesis and progression of HCC, and there are also studies suggesting that miR-203 may be a prognostic factor in HCC [9,10].

The effects of miR-361-3p on proliferation, invasion, migration and colony formation have been reported. Mir-361-3p, which has been shown to play an active role in body
fluids, has been shown to decrease levels of expression in prostate secretion in prostate

Mir-424, which has been found to have decreased expression in many cancer types, has
been reported to play a role as tumor suppressor. It has been found to induce cell
migration and play an important role cancer development and progression in HCC [5].
In the literature, the mir-33a, mir-203b, mir361-3p and mir-424 expression levels has
been reported to be either increased or decreased in various malignancies. A limited
number of studies were reported for these circulating miRNAs in cancer types.
In the current study, this is the first time we examined the expression level of mir-33a,
mir-203b, mir361-3p and mir-424 of peripheral blood serum in order to determine
potential biomarkers for early diagnosis, treatment and prognosis in HCC.

2. Materials and Methods

Sample collection

Venous blood samples were collected from 34 patients who were admitted to University
of Health Sciences Haydarpasa Numune Training and Research Hospital
Gastroenterology Outpatient Clinic and Istanbul University Oncology Institute. They had
been newly diagnosed with HCC and patients who agree to participate in the study over
the age of 18. Patients who were not included the study are who had received
radiotherapy and chemotherapy and, cases with confirmed infection and, chronic
inflammatory diseases, as well as those with tumors in another organ, those who used
antibiotics and anti-inflammatory drugs and corticosteroids.

As a control group, blood samples were derived from healthy individuals who have not
related to any type of cancer. All venous blood samples were centrifuged at 3000 rpm
for 10 minutes and the extracted serum samples were stored at -80°C freezer.
Drinking status, smoking status, and family history of cancer were recorded according to declarations of patient and healthy individuals. Presence of chronic hepatitis B and C, and cirrhosis were recorded according to laboratory and radiological test results. All epidemiologic variables and clinical data were collected by physicians. (Table 1). The study was approved by the Ethics Committee of the Istanbul University. Document number 2016/1297.
RNA isolation

RNA from 500 µL fresh serum samples were isolated with mirVana™ miRNA Isolation Kit (Thermo Fisher Scientific, Catalog No AM1560) according to the manufacturer's protocol. The concentration and purity of isolated RNAs were measured at 230, 260 and 280 nm with NanoDrop Spectrophotometer (ND-1000 Thermo Fisher Scientific).

Reverse transcription and Real Time Quantification of miRNA Expression

From each samples of HCC patients and healthy individuals, 20 ng of total RNA was reverse-transcribed to cDNA using specific miRNA TaqMan Assays and TaqMan MicroRNA Reverse Transcription Kit (Cat no: 4366596, Thermo Fisher Scientific) according to the manufacturer's protocol [12]. TaqMan™ MicroRNA Assays hsa-miR-33a, hsa-miR-203b, hsa-miR-361-3p, hsa-miR-424, and RNU6B (Cat No: PN4427975) were obtained from Applied Biosystems Thermo Fisher Scientific. RNU6B was used for endogenous control of miRNA expression analyses.

The relative expression levels of miR-33a, miR-203b, miR-361-3p and miR-424 were evaluated with qRT-PCR using TaqMan™ Universal PCR Master Mix (Cat no: 4364338, Thermo Fisher Scientific) with LightCycler 480® (Roche).

The RT-qPCR was performed as follows: 1 cycle of 95°C for 5 minutes, followed by 40 cycles of 95°C for 10 seconds, 60°C for 20 seconds, and 72°C for 25 seconds. All samples were run in duplicate. The relative quantification analysis was performed by delta-delta-Ct method, as described previously [13,14].

Statistical analysis

All data were analyzed using IBM SPSS Statistics for Windows, version 15. Two-sided Student's t-test was used to determine the significance of the difference between the expression levels of the analyzed microRNAs. The threshold p-value <0.05 was considered of statistical significance [13]. Online tools were used for analyzing the power
of the microRNAs in distinguishing HCC patients from healthy individuals and Receiver operating characteristic (ROC) curves plotted [15].

3. Results

Epidemiologic variables and clinical data of HCC patients and healthy individuals are summarized in Table 1.

The mean female patient age was 51±9; 29% and 41% of the healthy individuals were female with a mean age of 48±10 years. More patients had family history of cancer than healthy individuals (p<0.05) which it was determined as 32% and 12%, respectively.

In the patients 24%, were ever smoker and 21%, of them were ever drinker. In the healthy individuals 32%, were ever smoker and 12%, of them were ever drinker. As expected, most of the patients (71%) had cirrhosis and 41% had chronic hepatitis B, and 32% had chronic hepatitis C of the patients and none of the healthy individuals had chronic hepatitis B or C also none of them had cirrhosis. There was no significant difference between patients and healthy individuals in terms of gender, age, smoking and drinking status (all p>0.05).

The relative expression levels of cell free miR-33a, miR-203b, miR-361-3p and miR-424 from serum samples were evaluated with RT-qPCR. The Δ-ΔCt analysis of the RT-qPCR data showed that mir-33a, 7.3 fold less expressed in HCC patients in comparison to the healthy individuals (p=,000002), and mir-203b less expressed like mir33a, it was determined that 4.6 fold decreased in patients when compared to the control group. Also, mir361-3p showed that 5.1-fold less expressed in patients than healthy individuals (p=,000032). These results indicated that relative mir-33a, mir-203b, mir361-3p expression level was significantly decreased in HCC patients in comparison to the control group (p<0.05) (Figure 1;A,B,C). When the mir-424 RT-qPCR data
evaluated, it was shown that, there is not any significant change between the patient and control group (Figure 1;D).

When RT-qPCR results were compared, it was found that some HCC patients' miRNA have significantly expression levels of other status of patients. It was found that mir361-3p and mir424 expression level was significantly decreased in female HCC patients in comparison to the male HCC patients. Also, chronic hepatitis B and smokers upregulated, and cirrhosis had downregulated than other HCC patients (p<0.05) (Table 2).

We plotted ROC curves for analyzing the power of the microRNAs in distinguishing HCC patients from healthy individuals. It was found that mir-361-3p had the highest area under curve (AUC; 0.848), mir-33a, mir-203b, and mir-424 had 0.847, 0.83, 0.43 showed AUC values respectively, in comparison to healthy individuals (Figure 2; A,B,C,D).

4. Discussion

In this study, we demonstrated that mir-33a mir-203b mir361-3p and mir-424 had variable but significantly reduced expression compared to HCC tumor samples and healthy control serum. It is thought that the causes of different miRNA expression profiles in cancer patients may be passive or active release of miRNAs by tumor cells. Expression levels of these 4 miRNAs in serum of HCC patients were observed to be significantly reduced. It is known that miRNAs in body fluids can survive without degradation for a long time and are not affected by changing pH and temperature levels [16].

miR 33a plays a crucial role in regulation of cholesterol metabolism, on the cellular phenotypes associated with carcinoma progress. MiR-33a suppresses some cancer types
cell proliferation and metastasis by targeting oncogenes [17,18]. Down regulation of miR-33a activity in HCC increases the proliferative and invasive potential of HCC cells. Data obtained from the study also show that miR-33a plays a tumor suppressor role in HCC. The mean 7.3 fold decrease in miR-33a was observed in HCC patients. Studies of miRNA 203 related to various types of cancer as a tumor suppressor. It was shown that mir-203 have critical role in cell proliferation and invasion in prostate carcinoma [19]. Also it was demonstrated that miR-203 expression levels was significantly lower in colorectal cancer tissues in comparison to non-tumor tissues and it has over expression in ovarian cancer [9,20]. These results shown that mir-203 differs expression levels in each carcinoma. Our results show that mir-203 expression levels 4.6 fold decreased in patients in comparison to the control group. The result shows similarity with colorectal carcinoma.

The effects of miR 361-3p on proliferation, invasion, migration and colony formation have been reported. Mir-361-3p, which has been shown to play an active role in body fluids, has been shown to decrease levels of expression in prostate secretion in prostate cancer patients. Wei Chen et al. reported that miR-361-3p upregulated in NSCLC and it was shown that it inhibits cell proliferation, migration and invasion [11]. The other study indicated that overexpression of miR-361-5p suppressed lung cancer proliferation and invasion and it acts as a tumor suppressor in lung cancer [21]. Our results show that miR-361-3p 5.1 down regulated fold decreased in patients in comparison to the control group.

Decreased expression of miR-424 in HCC cell lines and primary tumors has been shown, while decreased mir-424 has been reported to accelerate cell proliferation migration and invasion. In addition, the important role of c-Myb, an important invasive molecule in HCC, is a direct target of miR-424 in tumorigensis [5]. In our study, there is no
significant expression levels of mir-424 expression level in serum samples obtained from
with HCC patients in comparison to healthy individuals.
There are significant gender differences in the risks and outcomes of cancer between
females and males [22]. Our study shown that there are differences in microRNA
(miRNA) expression in adult male and female in HCC patients. Mir-361-3p and mir-424
expression level was significantly decreased in female in comparison to the male. Also
drinking and smoking status, chronic hepatitis B and, cirrhosis significantly associated
with circulating miRNA expression [6].
According to our results there is no correlation with mir-203b, mir-361-3p and mir-424
expression levels in patients of chronic hepatitis C. However mir-33a, mir-203b, mir-
361-3p and mir-424 expression levels decreased in patients of chronic hepatitis B. It was
shown that mir-33a, mir-203b, mir361-3p and mir-424 upregulated in smokers, and mir-
203b, mir361-3p downregulated in cirrhosis patients compared to other HCC patients.
Despite these results, when all data of HCC patients were compared with healthy
individuals, it was obvious that mir-33a, mir-203b and mir361-3p expression levels were
less expressed (p<0.05).
The ROC curve results of mir-361-3p, mir-33a, and mir-203b, show that their sufficiency
to have the power to differentiate the HCC patients from healthy individuals. Since the
AUC value of mir-414 is 0.43, it was determined that there was no significant difference
between the groups.

5. Conclusions
In conclusion, this is the first study that examined the expression level of mir-33a, mir-
203b, mir-361-3p and mir-424 of peripheral blood serum in order to determine potential
biomarkers for early diagnosis, treatment and prognosis in HCC. It was determined that,
mir-33a (fold change: 7.3 and p<0.001), mir-203b (fold change: 4.6 and p<0.001), mir-361-3p (fold change: 5.1 and p<0.001) less expressed than healthy individuals and mir-424 did not show any significant change between the patient and control group. mir-33a, mir-203b and mir-361-3p, would have potential to be used as a non-invasive biomarker for HCC diagnosis and treatment in the future.

**Declaration of interest**

The authors declare no conflicts of interest that pertain to this work.


8. Xie RT, Cong XL, Zhong XM, Luo P, Yang HQ et al. MicroRNA-33a downregulation is associated with tumorigenesis and poor prognosis in patients with hepatocellular


17. Kostopoulou F, Malizos KN, Papathanasiou I, Tsezou A. MicroRNA-33a regulates
cholesterol synthesis and cholesterol efflux-related genes in osteoarthritic chondrocytes.


Table 1. Epidemiologic variables and clinical data of HCC patients and healthy individuals

<table>
<thead>
<tr>
<th>Epidemiologic variables /Clinical data</th>
<th>Patients (n=34, %)</th>
<th>Control (n=34, %)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Years) (Mean ±SD)</td>
<td>51±9</td>
<td>48±10</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Female/Male</td>
<td>10(29)/24 (71)</td>
<td>14 (41)/20 (59)</td>
<td>p&gt;0.05</td>
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<tr>
<td>Family history of cancer</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>11 (32)</td>
<td>4 (12)</td>
<td>p&lt;0.05</td>
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<tr>
<td>Drinking Status</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Never</td>
<td>27 (79)</td>
<td>30 (88)</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Ever</td>
<td>7 (21)</td>
<td>4 (12)</td>
<td>p&gt;0.05</td>
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<tr>
<td>Smoking Status</td>
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<td></td>
<td></td>
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<tr>
<td>Never</td>
<td>26 (76)</td>
<td>23 (68)</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Ever</td>
<td>8 (24)</td>
<td>11 (32)</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Cirrhosis</td>
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<td></td>
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<tr>
<td>Yes</td>
<td>24 (71)</td>
<td></td>
<td></td>
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<tr>
<td>Chronic hepatitis B</td>
<td>14 (41)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic hepatitis C</td>
<td>11 (32)</td>
<td></td>
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<tr>
<td>Tumour size</td>
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</tr>
<tr>
<td>&lt;5 cm</td>
<td>24 (71)</td>
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<td></td>
</tr>
<tr>
<td>≥ 5 cm</td>
<td>10 (29)</td>
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<td>TNM stage</td>
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<tr>
<td>III+IV</td>
<td>12 (35)</td>
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<td>α-fetoprotein (ng/mL)</td>
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</tr>
<tr>
<td>&lt;100</td>
<td>26 (76)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥100</td>
<td>8 (24)</td>
<td></td>
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</table>
Table 2. Fold change in expression level of miRNA’s with epidemiological status of HCC patients

<table>
<thead>
<tr>
<th>Patients/healthy individuals</th>
<th>HCC Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male/Female</td>
</tr>
<tr>
<td>Mir-33a</td>
<td>7.3↓*</td>
</tr>
<tr>
<td>Mir-203b</td>
<td>4.6↓*</td>
</tr>
<tr>
<td>Mir-361-3p</td>
<td>5.1↓*</td>
</tr>
<tr>
<td>Mir-424</td>
<td>↔</td>
</tr>
</tbody>
</table>

*; p<0.05, ↑; upregulate, ↓; downregulate, ↔; no difference
Figure 1. A. Expression of mir-33a between HCC patients (Cancer) and control group. B. Expression of mir-203 between Cancer and control group. C. Expression of mir-361-3p between Cancer and control group. D. Expression of mir-424 between Cancer and control group. ***; p<0.001.
Figure 2. A. Receiver operating characteristic (ROC) analysis curve of mir-33a between HCC patients (Cancer) and control group. B. ROC curve of mir-203 between cancer and control group. C. ROC curve of mir-361-3p between cancer and control group. D. ROC curve of mir-424 between cancer and control group. Curves to see the power of miRNAs in distinguishing HCC patients from healthy individuals.