Serum MiRNA-23a as a Diagnostic and Prognostic Biomarker of Hepatitis C Related Hepatocellular Carcinoma

Marwa M. Fekry 1, Rasha I. Farrag 2, Heba S. Selim 1, Sarah L. Asser 3, Nermeen Abdeen 4

1 Department of Microbiology, High Institute of Public Health, Alexandria University, Egypt
2 Department of Microbiology, Alexandria Central Laboratories, Alexandria, Egypt
3 Department of Microbiology, Faculty of Medicine, Alexandria University, Egypt
4 Department of Microbiology, Faculty of Medicine, Alexandria University, Egypt

Abstract

Background: Micro-ribonucleic acids (MiRNAs) are small, non-coding RNA molecules which regulate gene expression. Several miRNAs including miR-23a were found to be frequently deregulated in hepatocellular carcinoma (HCC).

Objective: This study aimed to evaluate serum miR-23a as a biomarker of hepatitis C related HCC.

Methods: This study was conducted on 60 hepatitis C virus (HCV) infected patients (group I: without cirrhosis, group II with cirrhosis and group III with HCV associated HCC) and a control group of 20 healthy volunteers. All patients were submitted to history taking, clinical examination in addition to categorization and staging of HCC patients. Following extraction of RNA from serum samples, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed. Calculation of serum miR-23a was done using the comparative cycle threshold (Ct) method (2-ΔΔCt).

Results: Serum miR-23a levels (2-ΔΔCt) were significantly higher in cirrhotic and HCC patients compared to chronic hepatitis C patients (CHC). However, no significant difference was noted between cirrhotic and HCC patients. The sensitivity and specificity of miR-23a levels for discriminating HCC patients from cirrhotic patients were 55% and 65%, respectively. MiR-23a levels had sensitivity of 90% and specificity of 70% for discriminating metastatic from non-metastatic HCC patients.

Conclusion: Higher miR-23a levels were detected among metastatic HCC patients than among those without metastasis. The sensitivity and specificity of miR-23a levels for discriminating HCC patients from cirrhotic patients were lower than those of alpha fetoprotein (AFP).

Keywords: hepatocellular carcinoma, microRNA, hepatitis C virus, cirrhosis, quantitative reverse transcription polymerase chain reaction

INTRODUCTION

HCV is an infectious pathogen causing great damage to the liver that can lead to cirrhosis and HCC (1). HCC is an aggressive tumor with a very poor prognosis worldwide. HCC is more frequent in men and it is a leading cause of tumor related death of men globally (2).

Low survival of HCC patients is mainly due to late diagnosis (3, 4). Available diagnostic techniques such as imaging techniques and AFP are inadequate for early detection of HCC. Early diagnosis significantly improves HCC prognosis and survival rates. Thus, new strategies for the early diagnosis of HCC need to be evaluated (3, 5).

AFP is a kind of glycoprotein, derived from embryonic endoderm tissue cells. Patients with chronic active hepatitis or liver cirrhosis may have high levels of AFP whereas some liver nodules may not release AFP. False negative AFP level may reach up to 40% in patients with early stage. Even in patients with advanced HCC, the AFP may remain normal in
MiRNAs are special class of small non-coding RNAs that play critical roles in the regulation of gene expression. It is well-known that each natural tissue harbors peculiar profiles of miRNAs expression (6). They are characterized by their remarkable tissue specificity and are nearly involved in the regulation of all aspects of cellular activity including metabolism, cell proliferation, apoptosis, differentiation, cellular response to viral infection, and oncogenesis (7). MiRNAs can behave as oncogenes or tumor suppressor genes, according to the target genes they regulate (8). In addition, characteristic miRNA patterns have been described in different liver diseases, ranging from chronic hepatitis to cirrhosis and HCC (6).

Importantly, miRNAs were found in human serum and plasma. They are stable and can be used as potential markers for different diseases’ diagnosis and prognosis including cancer (9-11). The human miR-23a gene is located at chromosome 19 and was transcribed as a part of miR-23a-27a-24-2 cluster (12). Up and down regulation of miR-23a has been demonstrated in different diseases, as coronary heart diseases, ischemia-reperfusion injury and cancer (13). MiR-23a was the recent focus of study among the cancer-associated miRNAs (12). It is upregulated or downregulated according to the type of cancer (14). Although various studies have tackled the role of miRNA in HCV and HCC patients however limited data are available on specific role of miR-23a. Thus, this study aimed to evaluate serum miR-23a as a diagnostic and prognostic biomarker in hepatitis C associated HCC.

**METHODS**

This cross-sectional study was carried out from December 2018 to December 2019. The minimal sample size was calculated based on a study aimed to assess the role of some circulating miRNAs as tumor markers for diagnosis of HCC (15). Thirty-nine HCV infected patients were the minimum required sample size to detect an area under the curve (AUC) of 0.75, relative to a null value of 0.5, as statistically significant with 80% power and at a significance level of 0.05 with a minimum event rate equal to 20 patients. Medcalc Program version 14.8.1 was used to calculate the sample size.

The total sample size was increased to eighty individuals. Sixty HCV infected patients (positive for anti-HCV antibodies and HCV-RNA for at least six months) attending the Tropical Medicine Department in Alexandria Main University Hospital were included in this study and divided into 3 groups: Group one: Twenty patients without cirrhosis. Group two: Twenty patients with cirrhosis. Group three: Twenty patients with HCV related HCC. In addition, a control group of twenty healthy volunteers with normal liver enzymes, normal hepatic ultrasonography (US) and negative for hepatitis B virus (HBV), HCV and human immunodeficiency virus (HIV) were included in the study.

All patients were submitted to detailed history and clinical assessment. Routine lab investigations: complete blood count (CBC), liver function tests [alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum bilirubin, serum albumin, prothrombin activity (PA) - international normalized ratio (INR), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT)], C-reactive protein (CRP) and renal function tests (serum creatinine - blood urea). AFP, HCV antibodies and viral load were obtained from patients records. Imaging techniques: abdominal ultrasonography revealed cirrhosis and/or suspicious focal lesion that confirmed by triphasic CT was obtained from patients records. Child-Pugh score was used to categorize cirrhotic and HCC patients (15) while model for end-stage liver disease (MELD) score (16) was used to assess liver disease severity. HCC patients were categorized by Barcelona Clinic Liver Cancer (BCLC) staging system (17), tumor, lymph node and metastasis (TNM) staging system (18) and Okuda score (16) to assess staging of the tumor.

Exclusion criteria included patients with chronic hepatitis B (CHB) infection, any other cause for chronic hepatitis other than HCV, any malignancies other than HCC and organ transplantation.

Three ml blood were obtained from each patient using sterile needles, sera were separated by centrifugation and stored at −80°C until processed.

**Serological testing**

A. **Total RNA isolation**

Extraction of total RNAs was conducted using Qiagen® miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. (ID 217004)

The miRNeasy Mini Kit uses phenol/guanidine-based lysis of samples and silica-membrane-based purification of total RNA. QIAzol Lysis Reagent is a monophasic solution of phenol and guanidine thiocyanate, designed to facilitate lysis, to denature protein complexes and RNases, and to remove most of the residual DNA and proteins from the lysate by organic extraction.

After addition of chloroform, the lysate is separated into aqueous and organic phases by centrifugation. RNA partitions to the upper, aqueous phase, while DNA partitions to the interphase and proteins to the lower, organic phase or the interphase. The upper, aqueous phase is extracted, and ethanol is added to provide appropriate binding conditions for all RNA molecules from approximately 18 nucleotides (nt)
upwards. The sample is then applied to the RNEasy MinElute spin column, where the total RNA binds to the membrane and phenol and other contaminants are efficiently eluted away. High-quality RNA is then eluted in a small volume of RNase-free water.

### B. Reverse Transcription

The Single-stranded cDNA of miR-23a and RNU6B were synthesized from purified RNA samples using miRNA specific primers according to the TaqMan® MicroRNA Reverse Transcription Kit. (Applied Biosystems, Foster City, CA, USA). The 15-μl reaction volumes were incubated in Applied Biosystems Cycler (Bio-Rad Laboratories, Hercules, CA, USA) for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and then held at 4°C.

### C. Quantitative Real time Polymerase Chain Reaction

PCR amplicons were amplified from cDNA samples using the TaqMan miRNA assay with the TaqMan® Universal PCR master mix. Real time PCR was carried out using Applied Biosystems StepOne™ Real-Time PCR System. RNU6B was used as internal control.

Comparative cycle threshold (CT) method was used to calculate the relative expression of miRNA. \( \Delta CT \) was obtained by calculating the difference between CT values of RNU6B and the CT values of the target miRNA. \( \Delta \Delta CT \) was then calculated by subtracting mean \( \Delta CT \) of the control samples from \( \Delta CT \) of tested samples. Fold change (relative-quantitative levels) of target miRNA within each group was then calculated using the equation \( 2^{-\Delta \Delta CT} \), using healthy controls as calibrator. Thus, the relative quantification of miR-23a expression was introduced as the fold change normalized to an endogenous reference (RNU6B) and relative to the reference (control) group. If relative quantification of miR-23a >1, this was considered as high expression in cancer relative to the control, while relative quantification of miR-23a <1 was regarded as Low expression in cancer patients compared to the control (19).

### Statistical analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 21 (Armonk, NY: IBM Corp) (20). Number and percent were used to describe qualitative data. The Kolmogorov-Smirnov test was used to demonstrate the normality of distribution, quantitative variables were described using range (minimum and maximum), mean±standard deviation when normally distributed. Not normally-distributed data were expressed as median and inter quartile range (IQR). For comparing quantitative data between two groups, unpaired t test and Mann-Whitney test were used for parametric and nonparametric data, respectively. Comparison of quantitative data between two groups was made by using Comparison of quantitative data between more than two groups was made by using one way ANOVA for parametric data and Kruskal-Wallis test for nonparametric data followed by Post Hoc (Dunn's multiple comparisons test). Spearman and Pearson correlations were used to test correlations of abnormally distributed quantitative variables.

Receiver operating characteristic (ROC) analysis curves and the corresponding area under the curve were calculated for providing the accuracy of the microRNAs and AFP, in diagnosis of HCC. ROC curve was used for estimation of sensitivity (i.e., true positive rate), specificity (i.e., true negative rate), positive predictive value (PPV), negative predictive value (NPV) and cut-off values showing the best equilibrium between sensitivity and specificity were evaluated. Significance was evaluated at the 5% level.

### Ethical considerations

The study was conducted in compliance with the Declaration of Helsinki (2013) and was approved by the “Ethics Committee” of the High Institute of Public Health, Alexandria University. An informed written consent was obtained from each patient. after explaining the objectives of the study and assuring the confidentiality of the collected data.

### RESULTS

#### The demographic and laboratory features

The studied groups show no significant difference regarding age and sex distribution. Hemoglobin concentration, platelet counts and serum albumin were significantly lower in HCC and cirrhotic patients compared to control and CHC patients. CRP, ALT, ALP and AFP were significantly higher in HCC patients compared to the other groups. Total bilirubin and AST were significantly higher in HCC and cirrhotic patients when compared to control and CHC patients. However, no significant difference was noted between HCC and cirrhosis patients. PA was significantly lower in HCC and cirrhotic patients when compared to control and CHC patients. INR was also significantly higher in HCC and cirrhosis when compared to the other 2 groups (Table 1).

#### Serum miR-23a expression level and its relation to other parameters

The median miRNA was significantly higher in cirrhotic (18.22) and HCC patients (10.58) compared to chronic hepatitis group (0.84). However, no significant difference was noted between HCC and cirrhotic patients with a p value of 0.11 (Table 2, Figure 1). Analysis of the median serum miR-23a levels among HCC patients with and without metastasis revealed higher level in metastatic HCC patients than those without metastasis (20.26 vs 1.0, respectively). However, this result was not statistically significant (Table 3, Figure 2).
Table 1: Demographic and laboratory data of the studied groups

|                          | Control (n = 20) | CHC (n = 20) | Cirrhosis (n = 20) | HCC (n = 20) | Test of Sig. | p    |
|--------------------------|------------------|--------------|-------------------|-------------|-------------|------|
| **Age (years)**          |                  |              |                   |             |             |      |
| <55                      | 11(55%)          | 14(70%)      | 9(45%)            | 13(65%)     | $\chi^2$ = 3.478 | 0.398 |
| ≥55                      | 9(45%)           | 6(30%)       | 11(55%)           | 7(35%)      |             |      |
| **Sex**                  |                  |              |                   |             |             |      |
| Male                     | 14(70%)          | 10(50%)      | 9(45%)            | 13(65%)     |             |      |
| Female                   | 6(30%)           | 10(50%)      | 11(55%)           | 7(35%)      |             |      |
| **Total bilirubin (mg/dl)** |                |              |                   |             |             |      |
| Min. – Max.              | 41.0 – 60.0      | 45.0 – 60.0  | 50.0 – 75.0       | 48.0 – 69.0 | F= 2.648   | 0.055 |
| Mean ± SD.               | 53.90 ± 5.99     | 52.30 ± 4.90 | 56.55 ± 6.72      | 56.90 ± 6.38 |             |      |
| **CRP (mg/dl)**          |                  |              |                   |             |             |      |
| Min. – Max.              | 12.0 – 15.50     | 9.0 – 12.40  | 6.40 – 12.40      | 6.70 – 12.50 | F=37.841*  | <0.001'|
| Mean ± SD.               | 13.55 ± 0.95     | 11.42 ± 0.86 | 9.91 ± 1.92       | 8.94 ± 1.79 |             |      |
| p<                      |                  | <0.001'      | <0.001'           | <0.001'     |             |      |
| **Platelets (Thousands/mm³)** |           |              |                   |             |             |      |
| Min. – Max.              | 240.0 – 402.0    | 180.0 – 400.0| 45.0 – 130.0      | 37.0 – 162.0| F=132.957' | <0.001'|
| Mean ± SD.               | 297.8 ± 54.34    | 280.75±58.02 | 91.40±28.15       | 86.25±31.26 |             |      |
| p<                      |                  | 0.632        | <0.001'           | <0.001'     |             |      |
| **ALT (u/l)**            |                  |              |                   |             |             |      |
| Min. – Max.              | 14.0 – 30.0      | 15.0 – 47.0  | 11.0 – 126.0      | 15.0 – 580.0| F= 6.174'  | 0.001' |
| Mean ± SD.               | 19.25 ± 3.97     | 22.90 ± 7.70 | 43.65 ± 27.54     | 103.15±136.6|             |      |
| p<                      |                  | 0.998        | 0.688             | 0.002'      |             |      |
| **AST (u/l)**            |                  |              |                   |             |             |      |
| Min. – Max.              | 11.0 – 25.0      | 10.0 – 26.0  | 26.0 – 205.0      | 23.0 – 480.0| F=13.372'  | <0.001'|
| Mean ± SD.               | 15.30 ± 3.92     | 17.40 ± 4.44 | 83.75 ± 52.97     | 129.3±124.29|             |      |
| p<                      |                  | 1.000        | 0.011'            | <0.001'     |             |      |
| **Hb (gm/dl)**           |                  |              |                   |             |             |      |
| Min. – Max.              | 50–110           | 40–110       | 40–110            | 40–110      | H=63.848'  | <0.001'|
| Mean ± SD.               | 83.75 ± 6.25     | 83.75 ± 6.25 | 83.75 ± 6.25      | 83.75 ± 6.25|             |      |
| p<                      |                  | <0.001'      | <0.001'           | <0.001'     |             |      |

p: p value for comparing between Control and each other group
p<: p value for comparing between CHC and Cirrhosis
p<: p value for comparing between CHC and HCC
p<: p value for comparing between Cirrhosis and HCC
*

\(\chi^2\): Chi square test
MC: Monte Carlo
F: F for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)
H: H for Kruskal Wallis test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Dunn’s for multiple comparisons test)
p: p value for comparing between the studied groups
p<: p value for comparing between Control and each other group
p<: p value for comparing between CHC and Cirrhosis
p<: p value for comparing between CHC and HCC
p<: p value for comparing between Cirrhosis and HCC
*

Statistically significant at \(p \leq 0.05\)
Table 1: Demographic and laboratory data of the studied groups “Continued”

|                  | Control (n = 20) | CHC (n = 20) | Cirrhosis (n = 20) | HCC (n = 20) | Test of Sig. | p   |
|------------------|-----------------|--------------|-------------------|-------------|-------------|-----|
| **Albumin (gm/dl)** |                 |              |                   |             |             |     |
| Min. – Max.      | 3.50 – 4.80     | 3.0 – 4.50   | 1.50 – 3.0        | 1.60 – 4.70 | F= 67.140* | <0.001* |
| Mean ± SD.       | 4.06 ± 0.44     | 3.84 ± 0.48  | 2.11 ± 0.43       | 2.42 ± 0.74 |             |     |
| Sig. bet. grps.  |                 |              |                   |             | p1<0.001*  |     |
|                  |                 |              |                   |             | p2<0.001*  |     |
| **ALP (u/l)**    |                 |              |                   |             | F= 45.903* | <0.001* |
| Min. – Max.      | 50.0 – 63.0     | 50.0 – 70.0  | 35.0 – 250.0      | 83.0 – 266.0|             |     |
| Mean ± SD.       | 56.75 ± 5.06    | 60.85 ± 6.42 | 104.20 ± 57.63    | 177.15 ± 45.35|             |     |
| Sig. bet. grps.  |                 |              |                   |             | p1<0.001*  |     |
|                  |                 |              |                   |             | p2<0.001*  |     |
| **PA (%)**       |                 |              |                   |             | F= 117.462*| <0.001* |
| Min. – Max.      | 85.0 – 100.0    | 80.0 – 100.0 | 21.50 ± 59.10     | 39.0 – 89.0 |             |     |
| Mean ± SD.       | 94.85 ± 5.61    | 92.30 ± 6.49 | 38.49 ± 13.16     | 54.75 ± 16.86|             |     |
| Sig. bet. grps.  |                 |              |                   |             | p1<0.001*  |     |
|                  |                 |              |                   |             | p2<0.001*  |     |
| **INR**          |                 |              |                   |             | F= 31.715* | <0.001* |
| Min. – Max.      | 1.0 – 1.14      | 1.0 – 1.30   | 1.32 – 3.49       | 1.15 – 1.93 |             |     |
| Mean ± SD.       | 1.03 ± 0.04     | 1.05 ± 0.08  | 2.19 ± 0.82       | 1.53 ± 0.26 |             |     |
| Sig. bet. grps.  |                 |              |                   |             | p1<0.001*  |     |
|                  |                 |              |                   |             | p2=0.039*  |     |
| **AFP levels (ng/dl)** |             |              |                   |             | H= 64.236* | <0.001* |
| Min. – Max.      | 1.0 – 9.0       | 1.50 – 9.20  | 8.0 – 80.0        | 40.0 – 3500 |             |     |
| Median           | 3.60            | 4.15         | 37.50             | 570.0       |             |     |
| Sig. bet. grps.  |                 |              |                   |             | p1<0.001*  |     |
|                  |                 |              |                   |             | p2=0.018*  |     |

F: F for ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)
H: H for Kruskal Wallis test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Dunn’s for multiple comparisons test)
p: p value for comparing between the studied groups
p1: p value for comparing between Control and each other group
p2: p value for comparing between CHC and Cirrhosis
p3: p value for comparing between CHC and HCC
p4: p value for comparing between Cirrhosis and HCC
*: Statistically significant at p ≤ 0.05

Table 2: Comparison between HCV infected groups according to miR-23a levels (2-∆∆Ct)

|                  | CHC (n = 20) | Cirrhosis (n = 20) | HCC (n = 20) | H     | p     |
|------------------|-------------|-------------------|-------------|-------|-------|
| **miR-23a levels (2-∆∆Ct)** |             |                   |             |       |       |
| Min. – Max.      | 0.26 – 23.70 | 0.20 – 1385.9     | 0.0 – 75.41 |       |       |
| Median           | 0.84        | 18.22             | 10.58       |       |       |
| Sig. bet. Grps   | p<0.001*    | p=0.039*          | p=0.111*    |       |       |

F: H for Kruskal Wallis test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Dunn’s for multiple comparisons test)
p: p value for comparing between the studied groups using Kruskal Wallis test
p1: p value for comparing between CHC and Cirrhosis
p2: p value for comparing between CHC and HCC
p3: p value for comparing between Cirrhosis and HCC
*significant
Table 3: MiR-23a levels in the HCC group with and without metastasis (n=20)

| MiRNA-23a levels | Without Metastasis (n=10) | With Metastasis (n=10) | U   | p     |
|------------------|---------------------------|------------------------|-----|-------|
| Min. – Max.      | 0.0 – 75.41               | 1.0 – 44.53            |     |       |
| Mean ± SD.       | 13.17 ± 25.24             | 19.57 ± 13.47          | 25.0| 0.059 |
| Median           | 1.0 (0.06 – 19.05)        | 20.26 (8.63 – 28.83)   |     |       |

U: Mann Whitney test
p: p value for comparing between the studied subgroups
Regarding tumor character, miR-23a was associated significantly with the size of focal lesion, tumor infiltration and TNM staging. However, no significant association was demonstrated with number of focal lesions, portal vein (PV) thrombosis, metastasis, BCLC and Okuda staging (Table 4).

In order to verify the correlation between the expression levels of miR-23a with all studied parameters (Table 5), spearman correlation was performed. It was found that miR-23a levels had significant positive correlation with total and direct bilirubin, CRP, AST, GGT, INR, AFP levels, MELD, Child Pugh, TNM and Okuda staging and size of focal lesion, while they had significant negative correlation with albumin and PA. Other tested parameters showed no correlation with the levels of miR-23a.

**Diagnostic power of miR-23a**

The receiver operator characteristic (ROC) analysis was used to evaluate the potential of miR-23a to differentiate HCC patients from cirrhotic patients. The ROC curve of miR-23a had an AUC of 0.64 ($p=0.126$) at a cut-off value of $\leq 11.06$, with sensitivity of 55% and specificity of 65%. The PPV and the NPV had been estimated to be 61.1% and 59.1%, respectively (Figure 3). The AUC of AFP was 0.99 ($p<0.001$) at the cut-off value of $>80$, with 80% sensitivity and 100% specificity. The PPV and the NPV had been estimated to be 100% and 83.3%, respectively. The potential of miR-23a to differentiate metastatic HCC patients from non-metastatic patients was evaluated using ROC analysis. ROC curve in Figure 4 shows that the sensitivity and specificity of miR-23a were 90% and 70%, respectively at the cut-off value of $>1.76$ with AUC of 0.75 ($p=0.059$). The PPV and the NPV had been estimated to be 75% and 87.5%, respectively. The sensitivity and specificity of AFP were 60% and 70%, respectively at the cut-off value of $\leq 50$ with an AUC of 0.69 ($p=0.151$). The PPV and the NPV had been estimated to be 66.7% and 63.6%, respectively (Data not shown).

**Table 4: Relation between miR-23a levels and tumor-related characteristics among the HCC group (n=20)**

| miR-23a | n | Min. – Max. | Median | Test of Sig. | p  |
|----------|---|-------------|--------|-------------|----|
| **Number of Focal lesions** | | | | | |
| Single | 10 | 0.0 – 75.41 | 1.68 | U=39.0 | 0.436 |
| Multiple | 10 | 0.40 – 31.93 | 14.02 | | |
| **Size** | | | | | |
| $\leq 5$ | 9 | 0.0 – 27.79 | 0.40 | U=15.0’ | 0.007’ |
| $>5$ | 11 | 1.0 – 75.41 | 23.53 | | |
| **PV Thrombosis** | | | | | |
| No | 12 | 0.0 – 75.41 | 1.68 | U= 36.0 | 0.384 |
| Yes | 8 | 1.0 - 31.93 | 14.02 | | |
| **Infiltrative** | | | | | |
| No | 9 | 0.0 – 40.41 | 0.40 | U= 15.0’ | 0.007’ |
| Yes | 11 | 1.0 - 75.41 | 23.53 | | |
| **BCLC** | | | | | |
| A | 6 | 0.0 – 75.41 | 15.12 ± 29.87 | | |
| B | 1 | 0.40 | H=2.479 | 0.479 |
| C | 2 | 1.0 – 27.79 | 14.40 ± 18.94 | | |
| D | 11 | 0.07 – 44.53 | 18.86 ± 15.52 | | |
| **TNM** | | | | | |
| I | 5 | 0.0–11.93 | 2.42 ± 5.31 | | |
| II | 4 | 0.40–75.41 | 29.45 ± 35.83 | | |
| III | - | - | - | | |
| IVa | - | - | - | H=7.088’ | 0.029’ |
| IVb | 11 | 1.0– 44.53 | 17.95 ± 13.86 | | |
| **Okuda staging** | | | | | |
| 1 | 1 | 1.60 | | | |
| 2 | 7 | 0.0 – 27.79 | 6.0 ± 10.54 | H=5.006 | 0.082 |
| 3 | 12 | 0.07 – 75.41 | 23.65 ± 21.95 | | |

U: Mann Whitney test  \ H: H for Kruskal Wallis test  \ p: p value for comparing between different categories
Table 5: Correlation between miR-23a levels and different parameters in each group

|                          | CHC | Cirrhosis | HCC | Total Cases |
|--------------------------|-----|-----------|-----|-------------|
| MELD score               |     |           |     |             |
| rs                       | -   | -0.24     | 0.167 | 0.002       |
| p                        |     | 0.921     | 0.481 | 0.993       |
| Child-Pugh score         |     |           |     |             |
| rs                       | -   | -0.181    | 0.300 | 0.101       |
| p                        |     | 0.445     | 0.198 | 0.537       |
| Total bilirubin          |     |           |     |             |
| rs                       | 0.408 | -0.004   | 0.296 | 0.391*      |
| p                        | 0.074 | 0.987     | 0.206 | 0.002*      |
| Direct bilirubin         |     |           |     |             |
| rs                       | -0.143 | 0.065   | 0.385 | 0.343*      |
| p                        | 0.549 | 0.786     | 0.093 | 0.007*      |
| CRP                      |     |           |     |             |
| rs                       | 0.232 | -0.105   | 0.169 | 0.284*      |
| p                        | 0.325 | 0.659     | 0.477 | 0.028*      |
| HCV viral load           |     |           |     |             |
| rs                       | -0.108 | -0.009   | -0.103 | 0.043      |
| p                        | 0.649 | 0.970     | 0.665 | 0.747       |
| ALT                      |     |           |     |             |
| rs                       | -0.338 | -0.019   | 0.257 | 0.224       |
| p                        | 0.145 | 0.937     | 0.274 | 0.086       |
| AST                      |     |           |     |             |
| rs                       | -0.348 | -0.121   | 0.407 | 0.392*      |
| p                        | 0.132 | 0.610     | 0.075 | 0.002*      |
| ALP                      |     |           |     |             |
| rs                       | -0.336 | -0.023   | 0.376 | 0.249       |
| p                        | 0.147 | 0.925     | 0.103 | 0.055       |
| GGT                      |     |           |     |             |
| rs                       | -0.192 | 0.008    | 0.162 | 0.354*      |
| p                        | 0.418 | 0.975     | 0.495 | 0.006*      |
| Albumin                  |     |           |     |             |
| rs                       | 0.301 | -0.225   | 0.034 | -0.382*     |
| p                        | 0.197 | 0.341     | 0.887 | 0.003*      |
| Urea                     |     |           |     |             |
| rs                       | 0.192 | -0.117   | -0.014 | 0.156      |
| p                        | 0.418 | 0.623     | 0.955 | 0.233       |
| Creatinine               |     |           |     |             |
| rs                       | -0.087 | 0.049   | 0.046 | 0.106       |
| p                        | 0.714 | 0.837     | 0.847 | 0.422       |
| PA                       |     |           |     |             |
| rs                       | 0.391 | 0.012    | -0.172 | -0.358*    |
| p                        | 0.088 | 0.960     | 0.468 | 0.005*      |
| INR                      |     |           |     |             |
| rs                       | 0.060 | -0.060   | 0.043 | 0.359*      |
| p                        | 0.802 | 0.801     | 0.858 | 0.005*      |
| AFP                      |     |           |     |             |
| rs                       | 0.250 | 0.084    | 0.189 | 0.340*      |
| p                        | 0.289 | 0.726     | 0.424 | 0.008*      |
| Size of focal lesions    |     |           |     |             |
| rs                       | -   | -        | 0.543* | 0.543*      |
| p                        | -   | -        | 0.013* | 0.013*      |
| Okuda score              |     |           |     |             |
| rs                       | -   | -        | 0.505* | 0.505*      |
| p                        | -   | -        | 0.023* | 0.023*      |
| BCLC staging system      |     |           |     |             |
| rs                       | -   | -        | 0.313 | 0.313       |
| p                        | -   | -        | 0.179 | 0.179       |
| TNM staging system       |     |           |     |             |
| rs                       | -   | -        | 0.511* | 0.511*      |
| p                        | -   | -        | 0.021* | 0.021*      |

rs: Spearman coefficient  
*: Statistically significant at p ≤ 0.0

Figure 3: ROC curve for miR-23a levels to predict HCC patients from cirrhotic patients

Figure 4: ROC curve for miR-23a levels to discriminate metastatic from non-metastatic HCC patients
DISCUSSION

MiRNAs can act as regulators of many host and viral genes expression. Circulating miRNAs have been found in many biological fluids. Their high stability, accessibility as well as their remarkable tissue specificity make them ideal potential non-invasive biomarkers for different diseases including cancer. Recently, many researches have focused on diagnosis of HCC through evaluation of disease-specific circulating miRNAs.

The present study demonstrated that the median of serum miR-23a levels was significantly elevated in cirrhotic and HCC patients compared to chronic hepatitis patients (18.22 and 10.58 vs. 8.85, \( P=0.001 \)). However, no significant difference was noted between cirrhotic and HCC patients in this study (18.22 and 10.58, respectively). In contrast, Mohamed et al. conducted a study to assess the role of some circulating miRNAs including miR-23a in diagnosis of HCC. They found that miR-23a level was significantly higher in the HCC patients compared to cirrhotic patients. This upregulation suggested a role of miR-23a in the pathogenesis of HCC. In addition, Bao et al. reported that MiR-23a was upregulated in HCC by over twice in most HCC samples. Therefore, they concluded that miR-23a may have a role in the development of HCC. The differences of the results reported by various authors may be contributed to different sampling, procedures, different sample size or patient selection.

In the present study, imaging revealed that miR-23a levels were significantly higher with infiltrated tumors and with focal lesion size \( \geq 5 \text{ cm} \). On the other hand, no significant relation was noted between miR-23a and number and site of focal lesions and PV thrombosis. The same finding was reported by Bao et al. as miR-23a was significantly higher in HCC patients with focal lesion size \( \geq 7 \text{ cm} \) and there was no significant relation between miR-23a levels and number of focal lesions. In agreement with the above results, Mohamed et al. found that there was no significant relation noted between miR-23a and PV thrombosis and that miR-23a was significantly higher in HCC patients with focal lesion size \( \geq 7 \text{ cm} \). However, they found that it was also significantly higher in patients with multiple focal lesions when compared with patients with less advanced HCC disease. Thus it could be used as a prognostic biomarker.

In the present work the serum miR-23a levels were significantly higher in TNM stage II and IVb compared to stage I. The same finding was reported by Bao et al. as they found that there was significant relation between expression of miR-23a in HCC tissue and TNM staging. In this study, the serum miR-23a levels were higher in Okuda stage III compared to stage I and stage II but this result was not statistically significant. On the other hand, Mohamed et al. reported that, 47.37% of HCC patients presented in stage III and that miR-23a levels were significantly higher in Okuda stage III patients when compared with patients with less advanced HCC disease. Thus it could be used as a prognostic biomarker.

The mean level of ALT was significantly higher in HCC patients compared to control group, CHC and cirrhotic patients. However, there was no significant difference between CHC and cirrhotic patients. Pratedrat et al. and Mohammed et al. found the same results where ALT levels was significantly higher in HCC patients than patients without HCC. On the other hand, a study by Weis et al. reported significantly higher ALT levels in cirrhotic patients than HCC patients and mild fibrotic patients.

The levels of serum albumin and PA were significantly lower in cirrhotic and HCC patients compared to CHC patients and control group which could be explained by decreased synthesis of albumin and coagulation factors by the diseased liver. Other studies reported that serum albumin was significantly lower in HCC than other studied groups.

In the present study, ROC curve analysis using serum miR-23a expression level to discriminate HCC patients from cirrhotic patients at the cut-off value of \( \leq 11.06 \) revealed moderate sensitivity and specificity (55% and 65%, respectively) with PPV and NPV of 61.1% and 59.1%, respectively. So miR-23a with such results, could not serve as an ideal biomarker to predict HCC among cirrhotic patients. In contrast, Mohamed et al. concluded that serum miR-23a can be used as a screening test to diagnose HCC. At cut off value \( \geq 2^{10} \text{ Ct} \), miR-23a showed accuracy of 79.3% to differentiate HCC patients from cirrhotic patients and healthy control with high sensitivity about 90%, specificity about 65%, PPV 56% and NPV 92.9.

In the present study, the median of serum miR-23a levels was higher in HCC patients with metastasis than those without metastasis (20.26 versus 1.0). However, this result was not statistically significant. Ahmed et al. reported that the median of miRNA-210 and miRNA-1246 was significantly higher in metastatic HCC than primary HCC.

In the present study, ROC curve analysis using serum miR-23a expression level discriminate metastatic from non-metastatic HCC patients revealed that the sensitivity of miR-23a levels was 90% while its specificity was 70% at the cut-off value of \( >1.76 \) with AUC of 0.750 (\( p=0.059 \)). The PPV and the NPV had been estimated to be 75% and 87.5%, respectively. This high sensitivity and moderate specificity showed that miR-23a could be used as a good biomarker for prediction of metastasis in HCC patients. These values are better than those elicited by AFP. The latter at a cut off level of \( \leq 50 \text{ ng/dl} \) had
60% sensitivity, 70% specificity, 66.7% PPV, and 63.6% NPV for the prediction of metastasis in HCC cases. These values of miR-23a in the current work were even better than those obtained for other miRNAs analyzed by other researchers (14).

CONCLUSION AND RECOMMENDATIONS

In conclusion, serum miR-23a alone cannot be used as a screening tool for detection of HCC among cirrhotic cases. However it can be used for prediction of metastasis among HCC cases. However it can be used for prediction of metastasis among HCC cases. In addition, miR-23a was positively correlated with the size of focal lesions, Okuda scoring and TNM staging system, therefore it can be used as a prognostic marker among HCC cases. However, further studies on larger scales are required to confirm these results.

LIMITATIONS OF THE STUDY

Small sample size as well as inability to choose only early HCC patients in the studied population may explain to some extent the inadequate role of miR-23a as a sole screening marker for early detection of HCC patients.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

Abbreviations

HCV (Hepatitis C virus), HCC (hepatocellular carcinoma), PCR (polymerase chain reaction), miRNA (MicroRNA), qRT-PCR (Quantitative reverse transcription polymerase chain reaction)

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