**Circulating MiR-150 and miR-130b as promising novel biomarkers for hepatocellular carcinoma**

Nevine F. Shafik, MD1; Reham A. A. Elshimy, MD1; Mohamed Rahouma, MD2; Ahmed M Rabea, MD3

1Clinical and Chemical Pathology Department, National Cancer Institute, Cairo University, Egypt.

2Surgical Oncology Department, National Cancer Institute, Cairo University, Egypt.

3Medical Oncology Department, National Cancer Institute, Cairo University, Egypt.

nevinegad123@yahoo.com

**Abstract: Background**: Hepatocellular carcinoma (HCC) is known to cause significant public health problems; treatment is effective in the early phases of the disease. Unfortunately, HCC is still diagnosed at an advanced stage. Recently, circulating microRNAs (miRNAs) have been accounted to be promising biomarkers for diagnosing cancers. This study was conducted to evaluate the feasibility of using serum miR-150 and miR-130b in the early diagnosis of HCC. **Methods**: The expression of miR-150 and miR-130b was evaluated using a quantitative real-time RT-PCR in 203 serum samples (60 healthy individuals, 43 cirrhotic and 100 HCC patients). **Results**: Serum miR-150 levels were significantly reduced in HCC patients, compared to healthy controls (P < 0.0001) and cirrhotic patients (P <0.0001), while miR-130b was significantly increased in HCC patients, compared to healthy controls (P < 0.0001) and cirrhotic patients (P < 0.0001). Serum miR-150 levels were down-regulated in cirrhotic group compared with healthy control (P=0.334) while miR-130 was significantly up-regulated in cirrhotic patients group compared with healthy control (P <0.0001). Receiver operating characteristic curve (ROC) analyses suggested that serum miR-150 and miR-130b had significant diagnostic value for HCC. **Conclusion**: Serum miR-150 and miR-130b can serve as a minimally invasive biomarker for the possible early diagnosis of HCC, and to differentiate it from the cirrhotic patients.

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**Keywords**: miR-150; miR-130b; serum; hepatocellular carcinoma

**1. Introduction**:

HCC is a noteworthy medical issue in Egypt and its rate is expanding [1] In the National Cancer Institute (NCI) of Egypt, Cairo University, liver cancer ranked third most common cancer after lymphoma in both genders (8.1%), first in males (12.1%) and fifth in females (4.0%) [2]*.* In 2014, Ibrahim et al published a paper that showed HCC to be the most common cancer in males and 2nd most common in females [3].

Worldwide, it is the second most common cause of cancer deaths [4]. Therefore, HCC is becoming one of the major health threats; however, the mechanism of HCC development is not well understood. Both the immune response to hepatitis viral infection and oxidative stress are involved in the pathogenesis of *HCC*[5]. Egypt has the highest pervasiveness of HCV in the world (14.7%)[6].

The importance of miRNAs in oncogenesis has also been recognized. Dysregulation of miRNA expression assumes a critical part in malignancy advancement through different instruments, for example, cancellations, intensifications, epigenetic quieting, or transformations in miRNA loci [7].

Aside from their tissue-particular starting point and expression, miRNAs are additionally appeared to be steady and discernible in many body liquids including serum and plasma [8]*.* Increasing evidence has shown that circulating miRNAs can possibly be biomarker for HCC [9, 10].

Over-expression of miR-150 added to the concealment of activated hepatic stellate cells (HSCs) in liver fibrosis, leading to the reduction of cell proliferation. [11]

Additionally, miR-130b was distinguished as a vigorous biomarker of HCC with high positive predictive value [12].

To explore the clinical applicability of miRNAs asminimally invasive circulating HCC biomarker, we examined the expression profile ofmiR-150 and miR-130b. They were evaluated in a set of serum samples to validate the use of these miRNA biomarkers for the possible early diagnosis of HCC, and to differentiate it from the cirrhotic patients.

**2. Patients and Methods**:

This study included 203 subjects collected during a period of 2 years. They were divided into three groups; the first group included 100 patients who were recruited from the oncology outpatient clinic at the National Cancer Institute – Cairo University. These 100 patients were preliminary diagnosed as HCC according to noninvasive diagnostic criteria obtained by imaging modalities [13–15] and, whenever possible, were confirmed by the gold standard histopathological examinations. Patients with any cancer other than HCC, as well as those with acute hepatitis, chronic inflammatory disorders or chronic heart disease were excluded from this study. The second group included 43 patients who were recruited from the National Liver Institute – Menofia University and were suffering from liver cirrhosis as confirmed by their clinical examinations, radiological findings. The third group included 60 apparently healthy normal subjects who were considered as the control group. These subjects had normal liver function tests and were sero-negative for hepatitis B and C markers.

This study was approved by the local institutional review board (ethics committee) as this work was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Written informed consents were obtained from all participating subjects before they were enrolled into the study. The clinical data and laboratory results (like serum AFP levels and PCR for viral hepatitis B and C) were all collected from the patient’s medical files.

 **Methods*:***

Four ml of venous blood samples were collected in serum vacutainer tubes under complete aseptic precautions. Immediately after sample collection, the blood samples were centrifuged at 5000 rpm for 10 minutes at 4°C to spin down the blood cells; the supernatant serumwas transferred into fresh tubes and stored at −80°C until further processing.

RNA extraction was done using miRNeasy Serum/Plasma Kit (cat. no. 4427975) Applied Biosystem according to the manufacturer’s instruction. All serum RNA preparations were quantified by NanoDrop.

Serum miR-150 level and miR-130b were quantified by qRT-PCR (Reverse transcription polymerase chain reaction) using TaqMan microRNA assay kit® (Applied Biosystems) and Step one Real time PCR Apparatus.

The reverse transcription reaction was performed in a 20 μl reaction volume; cDNA was reversely transcribed from total RNA samples using specific primer for miR-150 and miR-130b respectively contained in the TaqMan MicroRNA Reverse Transcription kit containing (Applied Biosystems, Foster City, CA). For synthesis of cDNA, the reaction mixtures were sequentially incubated at 16 °C for 30 minutes, 42 °C for 30 minutes, and 85 °C for 5 minutes.

In the PCR step, PCR products were amplified from cDNA samples using the TaqMan MicroRNA Assay together with the TaqMan® Universal PCR Master Mix. with the following cycle: 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15seconds and 60 °C for 60seconds. Each PCR mixture (20 μl) included the reverse transcription products, TaqMan 2X Universal PCR Master Mix without UNG Amperase, miRNA-specific TaqMan probes, and primers supplied by Applied Biosystems.

The expression of the target genes were calculated and normalized to miR-16 as endogenous normalization control by setting appropriate thresholds to obtain accurate Ct value that were provided from the real-time PCR instrumentation. The relative expression for the investigated miRNA was calculated using the 2-ΔΔCT method. The fold change in expression was analyzed using the equation the2-ΔΔCT where ΔΔCT= (CT target miR- CT miR-16) patient sample - (CT Target miR- CT miR-16) control sample.

**Statistical Methods .**

Different clinico-pathological variables included in our study including; age, gender, stage (TNM stage for HCC 7th edition [16] (with stage I and II compared to stage III and IV), tumor size, number of tumor masses, presence of lymph node or distant metastasis, liver cirrhosis, portal vein thrombosis, splenomegaly, ascites, presence of hepatitis B or C viral infection, alpha fetoprotein (AFP) level; (expressed as mean ± standard deviation).

Pearson’s chi (X2) test and student t-test were used to compare categorical and continuous variables respectively. Values less than 0.05 were considered statistically significant. Continuous variables were expressed as median and range while categorical ones expressed as frequency and percentages.

Receiver operating characteristic (ROC) curves were used to for each of miR-150 and miR-130b to identify its value in differentiation between each of the 3 group (A) HCC patients versus healthy controls, B) HCC patients versus cirrhotic and C) cirrhotic patients versus healthy controls); Area under the curve (AUC), 95% confidence interval, slandered error were reported. Cut-off values of miR-150 and miR-130b that were associated with the highest sensitivity, specificity were chosen. Positive and negative and predictive values (PPV and NPV) were calculated for each miR-150 and miR-130b cut-off value. All analyses were performed using SPSS version 22.0 (IBM, Armonk, NY).

**3. Results*:***

The Patient’s characteristics are shown in table 1.

Using qRT-PCR analysis, serum miR-150 and miR-130b expression levels were measured in HCC patients, cirrhotic patients and healthy controls. Serum miR-150 levels were significantly down-regulated in HCC patients compared with the cirrhotic patients (P <0.0001) and healthy controls (P < 0.0001). In addition, Serum miR-150 levels were reduced in cirrhotic patients group compared with healthy control but this statistically insignificant (P = 0.334). Serum miR-130b levels were significantly up-regulated in HCC patients compared with the cirrhotic patients (P <0.0001) and healthy controls (P < 0.0001) and were significantly up- regulated in cirrhotic patients group compared with healthy control (P <0.0001).

To confirm whether serum miR-150 and miR-130b levels could be served as a potential diagnostic marker for HCC, ROC curve analysis was performed. Our results were shown in table 2.

The Relation between of miR-150 and 130b with clinico-demographic data of the HCC patients are shown in table 3, indicating a statistical significant relationship between miR-130b and late stages (III & IV) (p= 0.001) and tumor size >3 (p=0.029). Also, there was a trend toward significance regarding miR-150 with late stages in HCC patients with a p value= 0.070.

**Table 1: Characteristics of Patients with Hepatocellular Carcinoma**

 (No. of patients; %)

**Age**

 ≤54 51 (51%)

 >54 49 (49%)

**Gender (M/F)** 75/25

**Number of Masses**

≤3 71 (71%)

>3 29 (29%)

**Stage Categories**

 Early (Stage I/II) 32 (32%)

 Late (Stage III/IV) 68 (68%)

**Distant metastasis**

 Negative 83 (83%)

 Positive 17 (17%)

**Liver Cirrhosis**

Negative 9(9%)

 Positive91 (91%)

**Tumor size**

≤3 36(36%)

>3 63 (63%)

**Splenomegaly**

Negative 32 (32%)

Positive 68(68%)

**Ascites**

Negative 55 (55%)

Positive 45 (45%)

**Lymph Node metastasis**

Negative 67 (67%)

Positive 33 (33%)

**Hepatitis C Virus**

Negative 31 (31%)

 Positive 69 (69%)

**Hepatitis B Virus**

Negative 97 (97%)

 Positive 3 (3%)

**AFP (mean ±SD)** 5778.5810±20426.566

Early stage I & II, late stages III & IV

**Table 2:** miR-150 and 130b expression in the HCC, cirrhotic patients and the control group:

|  |  |  |
| --- | --- | --- |
|  | miR-150 | miR-130b |
|  | HCC patients versus healthy controls | HCC patients versus cirrhotic  | Cirrhotic patients versus healthy controls | HCC patients versus healthy controls | HCC patients versus cirrhotic | Cirrhotic patients versus healthy controls |
| AUC | 0.947 | 0.923 | 0.444 | 0.946 | 0.740 | 0.918 |
| 95 % confidence interval | 0.903-0.991 | 0.880-0.967 | 0.315-0.573 | 0.905-0.987 | 0.659-0.821 | 0.844-0.991 |
| Std error | 0.022 | 0.022 | 0.066 | 0.021 | 0.041 | 0.038 |
| Cut off | 0.0052 | 0.0978 | 1.2210 | 1.57745 | 10.9730 | 1.5315 |
| Sensitivity | 98.3% | 97.7% | 34.9% | 94% | 78% | 90.7% |
| Specificity | 90% | 64% | 90% | 95% | 48.8% | 95% |
| P value | <0.001 | 0.000 | 0.334 | <0.001 | 0.000 | <0.001 |
| PPV | 88.89% | 98.46% | 71.43% | 96.91% | 71.43% | 92.86% |
| NPV | 39.07% | 53.85% | 65.85% | 90.48% | 30.15% | 93.44% |

\*Significant P value ≤ 0.05.

**Table 3:** Association of Mir- Rna 130 and 150 with some prognostic factors of HCC:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **miR- 150**Median value of relative expression | **P value** | **miR- 130b**Median value of relative expression | **P value** |
| **Age(median)**<54≥54 | 0.05690.0325 | 0.163 | 41.605431.9814 | 0.279 |
| **Gender** MaleFemale | 0.3860.0428 | 0.937 | 23.37039.4358 | 0.426 |
| **Number of Masses** ≤3>3 | 0.0582 | 0.401 | 39.4358 | 0.257 |
| **Stage Categories**Early(Stage I/II)Late (Stage III/IV) | 0.02800.0569 | 0.070 | 14.937957.3844 | **0.001** |
| **Distant metastasis**NegativePositive | 0.03860.0674 | 0.741 | 38.726056.4020 | 0.455 |
| **Liver Cirrhosis**NegativePositive | 0.08050.0432 | 0.725 | 39.029238.7960 | 1.00 |
| **Tumor Size**≤3>3 | 0.05550.0307 | 0.188 | 46.552119.2480 | **0.029** |
| **Splenomegaly**NegativePositive | 0.04480.0407 | 0.631 | 22.020641.3205 | 0.103 |
| **Ascites**NegativePositive | 0.03070.0594 | 0.171 | 25.236850.3435 | 0.113 |
| **Lymph Node Metastasis**NegativePositive | 0.3420.0555 | 0.484 | 23.832158.3668 | 0.095 |
| **Hepatitis C Viral infection**NegativePositive | 0.03400.0555 | 0.526 | 38.865938.7260 | 0.498 |
| **Hepatitis B Viral infection**NegativePositive | 0.038600.11840 | 0.746 | 38.726084.8355 | 0.275 |



Figure 1: A) Comparison of miR-150 levels among the healthy control, Cirrhotic group, and HCC group. B) Comparison of miR-130b levels among the healthy control, Cirrhotic group, and HCC group.



Figure 2: 1) ROC curve analysis of serum miR-150 for discriminating; (a) HCC patients from healthy controls, (b) HCC patients from cirrhotic patients, (c) Cirrhotic patients from healthy controls. 2) ROC curve analysis of serum miR-130b for discriminating; (a) HCC patients from healthy controls, (b) HCC patients from cirrhotic patients, (c) Cirrhotic patients from healthy controls.

**4. Discussion*:***

Many reviews have demonstrated that unusual miRNAs expression was related with the development and progression of numerous types of human cancer, which showed that miRNAs can be reliable biomarkers for cancers [17–19]. Tissue-specific miRNAs cannot be used on a wide scale in light of the fact that the procedure is invasive; however, use of serum miRNAs is negligibly obtrusive and thus more practical. Strikingly, serum miRNAs are steady and expression designs appear to be tissue-particular, which makes it a decent contender for insignificantly intrusive disease testing [20].

For example, Xie et al [10] found that serum miR-101 level was significantly down-regulated in the HBV-HCC patients and could differentiate HBV-HCC form HBV related liver cirrhosis. Eminently, changed serum/ plasma miRNAs levels were additionally connected with the development of HCC [7]*.* Li et al. identified miR-18a as a potential marker for hepatitis B infection related HCC screening [21].

In the present study, we analyzed the levels of two miRNAs (miR-150, miR-130b) in serum from 100 HCC patients, 43 cirrhotic and 60 healthy controls. We found that serum miR-150 levels were significantly reduced in HCC patients when compared to healthy controls, and cirrhotic group. Serum miR-150 levels were reduced in cirrhotic patients group compared with healthy control (P = 0.334). It yielded an area under the curve (AUC) of ROC of 0.947 with 98.3 % sensitivity and 90 % specificity in discriminating HCC from healthy controls, and an AUC of ROC of 0.923with 97% sensitivity and 64% specificity in discriminating HCC from cirrhotic group. Also, it yields AUC of ROC of 0.444with 34.9% sensitivity and 90 % specificity indiscriminating cirrhotic patients from healthy controls. This was consistent with Yu et al [22] that indicated the reduction of serum miR-150 level in HBV related HCC patients when compared to those in healthy controls with AUC of 0.931, with the sensitivity of 82.5 % and the specificity of 83.7 %. That was reliable with past reviews that showed the reduction of miR-150 levels in HCC tissue and cell lines [23-25].

Zheng J et al[11] reported that over-expression of miR-150 contributed to the suppression of activated hepatic stellate cells (HSCs) in liver fibrosis, resulting in the reduction of cell expansion.

Studies showed that miR-150 was abnormally expressed in various types of cancer. Ma et al.2012found that miR-150 expression was down-regulated in colorectal cancer compared with paired non-cancerous tissue. Low expression of miR-150predicted a shorter survival and a worse response to adjuvant chemotherapy in colorectal malignancy[[26]](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4280173/#pone.0115577-Ma1)*.* MiR-150 suppresses colorectal cancer cell migration and invasion through directly targeting MUC4[[27]](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4280173/#pone.0115577-Wang4). The expression of miR-150 was also lower in esophageal squamous cell carcinoma (ESCC) compared with normal esophageal mucosa [[28]](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4280173/#pone.0115577-Yokobori1)*.* Low miR-150 expression in ESCC contributes to malignant potential, for example lymph node metastasis, venous invasion and humble prognosis.

Serum miR-130b levels were significantly up-regulated in our HCC patients compared with the cirrhotic patients (P =0.0001) and healthy controls (P < 0.0001) and were significantly up- regulated in cirrhotic patients group compared with healthy control (P <0.0001). It yielded an AUC of ROC of 0.946 with 94 % sensitivity and 95 % specificity in discriminating HCC from healthy controls, and an AUC of ROC of 0.740with 78% sensitivity and 48.8% specificity in discriminating HCC from cirrhotic group. Also, it yielded AUC of ROC of 0.918 with 90.7% sensitivity and 95% specificity indiscriminating cirrhotic patients from healthy controls. That was in consistency with Liu et al that identifiedmiR-130b as a robust biomarker of HCC with a high positive predictive value, the high expression of serum miR-130b level in HCC patients when compared to those in non-cancerous controls with AUC of 0.913, with the sensitivity of 87.7 % and the specificity of 81.36 % [12]. Also, Wang et al [29] reported that miR-130b expression level was significantly higher in HCC tissues compared with normal adjacent liver tissues (P < 0.0001). Kutay et a reported that miR-130b was elevated in HCC tissues [30].

The oncogenic role of miR-130b in hepatocarcinogenesis is noticeable and is considered as a cancer stem cell miRNA in HCC. First, it has been demonstrated highly expressed in CD133+ tumour-initiating cells in HCC, in addition to transduction of miR-130b into CD133-negative cells could support tumorigenesis and induce opposition to chemotherapy.[31] Second, miR-130b specifically focuses on an outstanding tumor silencer, RUNX3, and regulates expression of proapoptotic Bim, in this way enhancing cell viability[32].

In this study, we identified the association of miR-150 and miR-130b with some prognostic factors of HCC, indicating a statistical significant relationship between miR-130b and late stages (III & IV) (p= 0.001) and tumor size >3 cm(p=0.029). Also, there was a trend toward significance regarding miR-150 with late stages in HCC patients with a p value= 0.070. Circulating miRNAs association with some clinicopathological parameters have been reported in other studies[12,22] **s**uggesting its potential use as a clinical prognostic value and carry the possibility of a serologic test that can expand the histologic information of a tumor without the requirement for biopsy.

MicroRNA 150 and 130b in serum could be a minimally-invasive biomarker for screening and early detection of HCC that could be translated to better disease control, to differentiate it from cirrhosis and to follow up cirrhotic patients to pick up progression to HCC.

**Conflicts of interest**:

The authors declare that they have no conflict of interest. The study was not sponsored by any organization.

**Correspondence:**

Nevine F. Shafik

Postal work address: National Cancer Institute, Fom El-Khalig square, Kornaish-El Nil, Cairo, Egypt

Post code: 11796

E-mail: nevinegad123@yahoo.com

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