Original Article

Role of circulating microRNA-21 and microRNA-215 in the diagnosis of hepatitis C related hepatocellular carcinoma

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Abstract

Introduction: Several micro ribonucleic acids (miRNAs) are deregulated in hepatocellular carcinoma (HCC). Others are linked to clinical pathological features of HCC. The goal of this study was to investigate whether miRNA-21 and miRNA-215 gene expression could be used as a non-invasive diagnostic tool to diagnose HCC.

Methodology: The gene expression of mature miRNA -21 and miRNA -215 in serum was analysed retrospectively using singleplex TaqMan two-step stem-loop quantitative real-time reverse-transcription PCR in 40 patients with HCC, 40 with chronic hepatitis C virus (HCV) with cirrhosis and 40 apparently healthy controls.

Results: Expression of miRNA -21 was significantly more down regulated in patients with HCC than in those with non-cirrhotic HCV ($P = 0.007$; odds ratio= 5; 95% confidence interval 1.6–15.4). The receiver operating curve analysis of the ability of miRNA-21 expression to discriminate between HCC and non-cirrhotic HCV revealed an area under the curve of 0.712 with 70% sensitivity and 68% specificity at a cut-off of ≤ 1.4468. Thus, the expression level of miRNA -21 could discriminate HCC from non-cirrhotic HCV. Significant positive correlation was observed between expression levels of microRNA-21 and miRNA -215 ($r = 0.783$, $p < 0.001$), but no association was observed between expression level of miR-215 and HCC or chronic HCV ($p = 0.474$).

Conclusions: MiRNA-21 may be a useful, non-invasive tool for diagnosing HCC. Non-cirrhotic HCV patients have five times the risk of developing HCC when the miRNA -21 level ≤ 1.4468.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most frequent malignancy in the world and the third most frequent cause of cancer mortality [1]. Prevalence of HCC has increased in both developed and developing countries. In Egypt, HCC is the most serious complication of hepatic cirrhosis [2]. The World Health Organization estimates that about 3 to 4 million people globally become infected with hepatitis C virus (HCV) each year [3]. Chronic HCV increases susceptibility to serious sequelae, such as cirrhosis, HCC and liver failure [4,5]. In 2008, Egypt had the world's highest HCV prevalence, with 90% of patients infected with HCV genotype 4 [6,7]. In 2015, the national HCV prevalence decreased to 6.3% [8] a reduction of 30% [9, 10]. Still, HCC remains the second most prevalent cancer, sixth most frequent cancer in women and

second most frequent cancer-related cause of death in Egypt [11].

Early screening, detection and diagnosis of HCC are crucial for optimal treatment outcomes. However, the current diagnostic tools for detecting early stages of HCC are insufficient. Sensitive and specific biomarkers are therefore needed. Micro ribonucleic acids (miRNAs) are small molecules with principal roles in the cell cycle, haematopoiesis, apoptosis, proliferation, differentiation and metabolism [12]. Altered expression of many miRNAs may have regulatory, tumour suppressor and oncogenic potential in many human cancers. Circulating MiRNAs, which are stably expressed in serum and urine, have emerged as novel diagnostic and prognostic markers for early detection and diagnosis of HCC [13]. MicroRNAs are frequently deregulated in HCC, and specific miRNAs are

correlated with clinical pathological features of the disease, such as vascular or distant metastasis, prognosis and recurrence [14]. Numerous techniques have been established for high-performance and sensitive miRNA profiling, including microarrays [15], quantitative reverse transcription PCR (qRT-qPCR) [16] and next-generation sequencing [17].

In a meta-analysis of studies using novel diagnostic biomarkers to detect hepatocellular carcinoma, altered miRNA expression was found to have several effects on tumour cells [18]. In several solid tumours of the breast, colon, lung, pancreas, prostate and stomach and in cholangiocarcinoma cell lines, miRNA-21 and miRNA-215 have been shown to be overexpressed. As such, they likely have major roles in tumour cell behaviour and malignancy [18]. Inhibition of miR-21 promotes cell growth in other types of cancer cells. Furthermore, miR-21 has shown anti-apoptotic properties in glioblastoma and cholangiocarcinoma [18]. The goal of this study was to determine if gene expression of miR-21 and miR-215 could be used to distinguish HCC from chronic HCV to establish a non-invasive tool for HCC diagnosis.

Methodology

Patients and samples

This case-control study was conducted on 120 subjects (40 with HCC and HCV-related liver cirrhosis, 18 with HCV-related cirrhosis, 22 with chronic HCV and 40 apparently healthy controls). Patients with HCC were recruited at Kasr Alainy Hospital, Cairo University, from a multidisciplinary HCC clinic. Other patients were recruited from Kasr Alainy Medical School Hospital. Patients were recruited between January 2016 and January 2018. Patients with non-HCC malignancies, recurrent HCC, chronic hepatitis B diseases, alcoholic liver diseases, autoimmune liver diseases or presence of distant metastasis were excluded. The local ethical committee of the Faculty of Medicine at the University of Cairo approved the study. We obtained informed consent from all participants. All patients were diagnosed and treated in compliance with the Helsinki Declaration on Good Clinical Practice guidelines. HCC was diagnosed according to the practice guidelines of the American Association for the Study of Liver Diseases [19]. Further evaluation of focal liver lesions was conducted using abdominal ultrasound. HCC was diagnosed using Triphasic computed tomography or contrast enhanced magnetic resonance imaging. AFP was measured using a solid phase-two sequential immunometric assay kit on the Immulite 2000 System Analyzer (cat#L2KAP2, Siemens Healthcare Diagnostics, United States) [20]. Clinical, laboratory and imaging with and without hepatic decompensation or portal hypertension were used to diagnose liver cirrhosis. HCV was confirmed via serum tests for 40 chronic cases [21]. The 40 controls were recruited and matched with included patients by gender and age (within 5 years), after excluding controls with clinical liver diseases or positive serum hepatitis markers. All subjects underwent a complete history and clinical examination.

RNA extraction

Following the serum sample manufacturer protocols, the total RNA for profiling miRNA expression was isolated from the serum using the miRNeasy Mini kit (cat.no. 217004) following the manufacturer's protocol (QIAGEN GmbH, Hilden, Germany). Total RNA yield was around 25 ng per 200 µl (microliter) of a single serum sample.

Analysis of Circulating Micro RNA Using Singleplex TaqMan Two Step Stem-Loop qRT-PCR

The two steps qRT-PCR process consisted of: 1-Reverse Transcription step (RT): cDNA is reverse transcribed from total RNA samples using a small RNA-specific, stem-loop RT primer and TaqMan ® MicroRNA Reverse Transcription Kit. 2-PCR step: using the Taqman ® Small RNA Assay and TaqMan ® Universal PCR Master Mix II, cDNA samples are amplified to PCR products.

Real-time PCR quantification for miRNA expression

In this study, two mature miRNAs were detected (miRNA-21 and miRNA-215) using TaqMan® miRNAs Assay. A synthetic control (Celmir-39) was used for normalization using TaqMan® Small RNA control. On the StepOne real-time PCR system (Applied Biosystems, Foster City, CA, USA), all these steps were run. To determine the relative number of copies (RQ) of miRNA, the $\Delta\Delta CT$ method was used.

Table 1. Target microRNA and control sequences analyzed and amplified using quantitative reverse transcription real-time PCR (qRT-PCR).

Target miRNA	MiRbase No.	Mature sequence
miR-21	MIMAT0000076	UAGCUUAUCAGACUGAUGUUGA
miR-215	MIMAT0000272	AUGACCUAUGAAUUGACAGAC
Cel miR-39	MIMAT0000010	UCACCGGGUGUAAAUCAGCUUG

The levels of miRNA expression were calculated using the cycle threshold (CT). The difference between its CT value and the average CT value of the reference genes, per sample, is calculated for every miRNA expression. Synthetic control (Cel-miR-39) was used as a reference gene. The relative expression (fold change) for each candidate miRNA of each group was then calculated by equation

$$2(-\Delta\Delta\ CT)\qquad [22].$$

1. In each sample, the ΔCT for each miRNA was calculated as follows:

ΔCT sample = CT miRNA − CT CelmiR 39

2. Then ΔΔCT was calculated:

ΔΔ CT = (CT miRNA − CT CelmiR 39) patients
− (CT miRNA
− CT CelmiR 39) controls

The miRNA base was used to identify sequences of mature miRNAs and synthetic control (http://www.mirbase.org). Table 1 shows the miRNAs mature sequence in addition to TaqMan® Small RNA controls sequence.

Statistical analysis

The data were analysed using IBM SPSS version 22 (Armonk, NY, USA). All tests were two-tailed. The median and interquartile ranges were used to express numerical data, as needed. Frequency and percentage were used to express qualitative data. The relationship between qualitative variables was tested via the chi-square or Fisher exact test. For quantitative data, two types of non-normally distributed data were compared, using either Student's *t* test or the Mann-Whitney test (non-parametric t-test). A one-way analysis of variance analysis (ANOVA) or Kruskal-Wallis (non-parametric ANOVA) was used to compare three or more groups, and a post-hoc test was used for pair-wise comparison. The Spearman-rho method was used to verify the correlation between numerical variables. A *P* value < 0.05 was regarded as significant. An odds ratio (OR) with 95% confidence interval (CI) was used for risk calculation. To determine the serum miRNAs' diagnostic value for HCC, a receiver operating curve analysis was conducted with predictions of the best cut-

Table 2. Demographic, Clinical and laboratory characteristics of the studied groups.

| Variables | HCC (N = 40) | Chronic hepatitis C | | Controls | P-value |
		Cirrhotic (N = 18)	Non-cirrhotic (N = 22)		
Age (years) mean ± SD	57 ± 7.7	58 ± 7.3	47±7.2	31 ± 7.6	
Male N (%)	26 (65)	14 (78)	8 (36)	34 (85)	
ALT (U/L)	41 (31-63)[a]	33 (29-47)[a]	14 (12-22)[b]	12 (10-32)	**< 0.001**
AST (U/L)	66 (42-80)[a]	50 (38-64)[a]	23 (19-27)[b]	10 (5-35)	**< 0.001**
AST/ALT	1.4 (1.2-1.6)	1.4 (1.2-1.9)	1.5 (1.0-1.6)		0.810
T. Bilirubin (mg/dl)	1.0 (0.7-1.6)[a]	1.2 (0.9-4.8)[a]	0.9 (0.7-1.1)[b]	0.4 (0.1-0.5)	**0.045**
Albumin (g/dl)	3.2 (2.9-3.6)[a]	2.55 (2-3.1)[d]	4.5 (4.1-4.8)[b]	4.6 (4.0-4.8)	**< 0.001**
Creatinine (mg/dl)	0.9 (0.8-1.2)	1.0 (0.8-1.5)	0.8 (0.7-0.9)	0.5 (0.3-1.2)	0.226
Prothrombin concentration (%)	80 (67-86)[a]	61 (49-75)[b]	88 (80-92)[a]	95 (90-99)	**< 0.001**
Prothrombin Time (seconds)	14 (13-16)[a]	17 (14-20)[b]	13 (12-13)[a]	12 (11-13)	**< 0.001**
INR	1.2 (1.1-1.3)[a]	1.4 (1.2-1.8)[b]	1.0 (1.0-1.1)[a]		**< 0.001**
Child-Pugh Class n (%)					
A	24 (60)	6 (33)			0.15
B	12 (30)	8 (45)			
C	4 (10)	4 (22)			
Diabetes mellitus	13 (32.5)	9 (50)	4 (27)	-	0.63
Cigarette Smoking habits n (%)	13 (32.5)	11 (61)	10 (45.5)	-	0.813
Family history of HCC n (%)	5 (12.5)	0 (0)	0(0)	-	-
HCC characteristics n (%)					
Site: right lobe	34(85)				
-Number of tumors: Single	23(57.5)				
-Size of main tumor:					
< 3 cm	12(30)				
3-5cm	19(48)				
≥ 5cm	9 (22)				
-Portal vein thrombosis: Yes	3(7.5)				
Alpha fetoprotein (AFP) (ng/ml)	75 (25- 213)				
AFP <200 n (%)	28 (70)				
AFP ≥200 n (%)	12 (30)				
miRNA -21	0.7 (0.3-2.4)a	0.7 (0.3-1.4)a	1.8(1.3-6.5)b	0.8 (0.4-1.5)a	**0.007**
miRNA-215	2.0 (0.4-5.8)a	0.8 (0.4-6.7)a	0.9(0.4-2.1)a	0.9 (0.4-2.2)a	0.474

Data are presented as median (25th -75th percentiles) otherwise indicated. N: number, %: percent.

off values. Sensitivity was plotted on the Y-axis and specificity on the X-axis.

Results

Table 2 summarizes the demographic, clinical and laboratory characteristics of the groups. The median levels of serum alanine transaminase and aspartate transaminase were significantly higher in the HCC ($p < 0.001$) and cirrhotic HCV ($p < 0.5$) groups than in the non-cirrhotic HCV group. The median level of serum total bilirubin was higher in the HCV cirrhosis group than in the HCC and non-cirrhotic HCV groups ($p = 0.045$). Regarding the coagulation profile, the prothrombin concentration was significantly lower in the cirrhotic HCV group than in the HCC and non-cirrhotic HCV groups ($p < 0.001$). No statistically significant difference was found between the HCV group and HCC group regarding the Child-Pugh score, the frequency of DM and smoking habit. In comparison with non-cirrhotic HCV patients, the median fold-change values indicated significantly lower miRNA-21 gene expression in patients with HCC ($p = 0.007$). However, the median expression of miRNA-21 was not statistically different from the HCC, cirrhotic HCV and control groups. For miRNA-215, no significant difference was detected among the studied groups ($p = 0.474$). When comparing the median fold-change values from the studied miRNAs as a marker to differentiate HCC with small tumour (< 3 cm) from cirrhotic HCV and non-cirrhotic HCV, the HCC group showed significantly higher downregulation of miRNA-21 than the non-cirrhotic HCV group (0.7 versus 1.8, $p = 0.004$). However, miRNA-21 expression was not statistically different from the HCC with small tumour size (< 3 cm) and cirrhotic HCV groups. No statistically significant difference was found between the three groups regarding miRNA-215 expression levels ($p = 0.394$) (Table 3).

The utility of serum miRNA-21 and miRNA-215 as potential diagnostic markers for discriminating between HCC and non-cirrhotic HCV was evaluated using a receiver operating curve (Figure 1). The analysis revealed an area under the curve of 0.712 with 70% sensitivity, 68% specificity, 80% positive predictive

Figure 1. Receiver Operating Curve (ROC) of miRNA-21 and miRNA-215 expression levels in differentiating hepatocellular carcinoma (HCC) from hepatitis C virus (HCV) infection without cirrhosis.
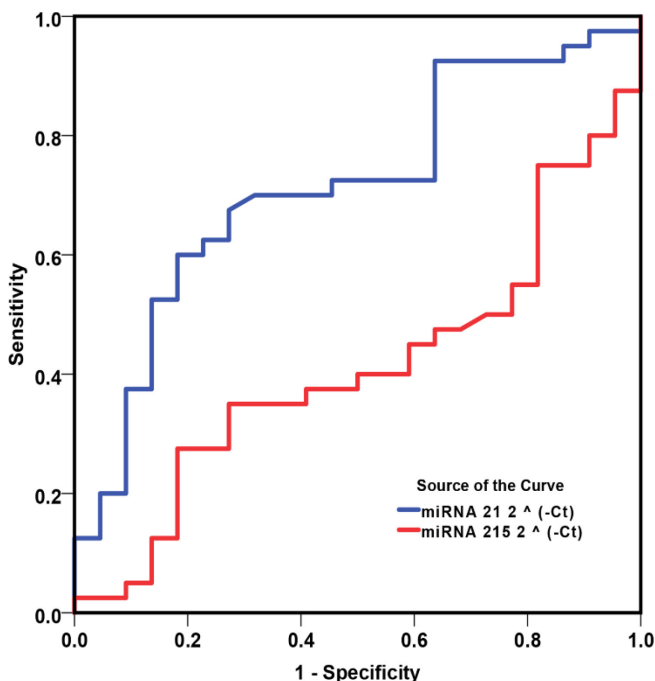
Figure 2. Scatter diagram of the positive correlation between miRNA-21 and miRNA-215 expression levels among HCC group.
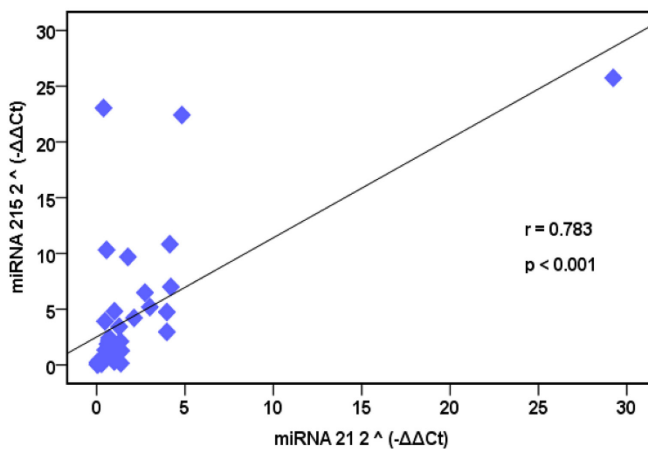
Table 3. Comparison of the Median values of fold change of miRNA-21, miRNA-215 expressions as indicators to distinguish small HCC (< 3 cm) and HCV infection.

Variables	HCC (Tumor size < 3cm) (N = 12)	Cirrhotic HCV (N = 18)	Non-cirrhotic HCV (N = 22)	p-value
miRNA-21	0.7 (0.5 - 1.2) [a]	0.7 (0.3 - 1.4) [a]	1.8 (1.3 - 6.5) [b]	**0.004**
miRNA-215	1.8 (0.8 - 4.2) [a]	0.8 (0.4 - 6.7) [a]	0.9 (0.4 - 2.1) [a]	0.394

All data are presented as median (25th -75th percentiles). Groups bearing the same initials are not significantly different from each other at P value=0.05.

value, 56% negative predictive value and 69% diagnostic accuracy at a cut-off of ≤ 1.4468 (*p* = 0.014; 95% CI 0.58–0.84). However, the area under the curve for miR-215 was 0.410 (*p* = 0.24; 95% CI 0.26–0.55). A logistic regression analysis was done to measure the power of circulating miRNA-21 expression levels in diagnosing HCC. A serum miRNA-21 level ≤ 1.446 had a 5-fold potential to differentiate HCC from non-cirrhotic HCV (OR = 5; *p* = 0.004). The miRNA-21 and miR-215 gene expression levels were positively correlated among HCC group (r = 0.78; *p* < 0.001) (Table 4, Figure 2). No other significant correlations were found.

Discussion

The serum expression levels of two miRNAs, miR-21 and miR-215, were analysed in this study of HCC patients, HCV patients with or without cirrhosis and healthy control subjects. MiR-21 gene expression was significantly more down regulated in the HCC, cirrhotic HCV and control groups than in the non-cirrhotic HCV group, suggesting a possible role of this miRNA in the pathogenesis of HCC. No statistically significant difference in miR-215 gene expression was observed among the three groups. A possible explanation for this finding is the fact that hepato-carcinogenesis is associated with continuous oxidative stress, caused by reactive oxygen species (ROS), due to environmental factors or mitochondrial dysfunction. A distinction of the pathological characteristics of HCC is the dramatic down regulation of oxido-reductive enzymes represented by the main free radical scavenger systems: catalase, superoxide dismutase and glutathione peroxidase. Together, these factors result in down regulation of miR-21 expression, which in turn results in overexpression of key genes that are involved in cell cycle regulation and proliferation, such as PLAG1 and SATB1.

The results of our study align with another study concluding that miR-21 is substantially more down regulated in patients with HCC and cirrhotic HCV than in patients with non-cirrhotic HCV [23]. However, Alnoanmany *et al.* (2015) asserted that miR-21 is significantly higher in an HCC group than in an HCV or normal control group [24]. Further, Elghoroury *et al.* (2017) showed significantly higher miR-21 up-regulation in hepatic patients versus controls and in HCC patients versus HCV patients [25]. The identification of miR-21 as a key regulator of tumour cell behaviour (e.g. migration, invasion and proliferation) emphasizes its essential role in hepato-carcinogenesis.

The results are in partial agreement with another study demonstrating significantly up-regulated gene expression of miRNA-21, miRNA-122 and miRNA-223 in patients with HCC or chronic hepatitis B, compared with healthy controls [26]. That study also finds significantly higher mean expression levels of miRNA-21 genes in patients with chronic hepatitis type B than in patients with HCC, although all three miRNAs levels indicate possible HCC or chronic hepatitis diagnostic values. Serum levels of miRNA-21 and miRNA-223 increase significantly in patients with other cancer [27, 28], which lead authors to conclude that elevated levels in patients' sera may indicate liver damage not primarily due to hepatic tumor itself.

Few studies reported the relation between miRNA-215 and HCC diagnosis. One study [29] using miRNA array analysis has shown highly increased expression of miRNA-215 after HCV infection. Moreover, the results of the current study contradict to the results of another study showing significantly up-regulated miR-215

Table 4. Correlation analysis between the expression levels of the studied miRNAs and laboratory parameters among HCC group.

	miRNA-21		*miRNA-215*	
	r	**p - value**	**R**	**p value**
Age	0.15	0.33	0.11	0.48
ALT (U/L)	0.11	0.48	0.06	0.67
AST (U/L)	0.06	0.70	-0.0	0.81
T. Bilirubin (mg/dl)	0.05	0.74	0.17	0.28
Albumin (g/dl)	0.23	0.14	0.04	0.78
PC (%)	0.13	0.41	-0.09	0.60
INR	-0.06	0.67	0.09	0.80
PT (second)	-0.07	0.64	0.15	0.33
Creatinine (mg/dl)	0.04	0.77	0.06	0.67
miRNA-21	-	-	0.78	**< 0.001**
miRNA-215	0.78	**< 0.001**	-	-
AFP (ng/ml)	0.08	0.61	0.07	0.63

r: Correlation Coefficient; r < 0.3: no correlation; r = 0.3 – < 0.5: weak correlation; r = 0.5: fair correlation; r = > 0.5- 0.75: good correlation; r > 0.75: very good correlation.

expression levels in both HCC and non-cirrhotic HCV patients compared with cirrhotic patients and a control group. Authors also revealed a significant positive correlation between serum viral load and miRNA-215 expression levels [30]. MiRNA-215 thus appears to work either as a tumor suppression gene or as an oncogene in a context-dependent pattern.

The current study used the ROC analysis to assess the utility of measuring serum expression levels of miRNA-21 as a potential diagnostic marker for discriminating HCC from non-cirrhotic HCV infection. At a cutoff value of ≤ 1.4468, the serum expression level miRNA-21 showed 70% sensitivity, 68.2% specificity and 69.4% diagnostic accuracy. The difference between our results and other studies might be due to different HCV genotype infection other than genotype 4, which causes more than 90 % of cases in Egypt. Additional risk factors for HCC besides HCV also should be considered, such as HBV and alcoholism [31]. Moreover, the complexity of miRNA regulation depends on a cell type and environment. Most HCC-related miRNAs change continuously during disease development, such as in the progression from acute hepatitis to chronic fibrotic disorder to cirrhosis [32].

The cases and the controls were not completely matched in regard to the age, controls were younger, and this is a limitation of the current study. As a non-invasive method of cancer diagnosis, the circulation of nucleic acids in serum and plasma is promising. Compared with DNA and miRNAs, circulating miRNAs are more stable at room temperature and over several freezing/ thawing processes after extended incubation. In recent years, more studies have combined diagnostic and prognostic applications of miRNAs in diseases, including cancer. Their applications in different tissue types and specimens may be particularly useful in clinical practice [33].

Conclusions

Our study could conclude that the gene expression level of miRNA-21 was significantly down regulated in HCC compared with HCV non-cirrhotic group which is surely clinically useful in follow up periods of HCV patients, it can predicts early developing HCC in those patients. However, miRNA-21 expression level showed no difference between HCC and cirrhotic HCV groups which are easily differentiated by ultrasonography. Also, miRNA-21 downregulation was useful as a diagnostic tool for HCC where patients carry 5 times risk of HCC susceptibility among HCV non-cirrhotic patients. The gene expression level of miRNA-215

reported no statistically significant difference among studied groups.

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