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The Egyptian Journal of Internal Medicine (2024) 36:16
https://doi.org/10.1186/s43162-024-00282-z

The Egyptian Journal of Internal Medicine

RESEARCH

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Role of serum micro-RNA 486-5p in Egyptian cirrhotic hepatitis C patients with hepatocellular carcinoma

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Abstract

Background Hepatocellular carcinoma (HCC) is the prevailing primary liver tumor. To pick HCC at the initial stages is quite strenuous, despite the advent of serum biomarkers, mainly alpha-fetoprotein (AFP), to predict the development and progression of HCC. One proposed biomarker for the diagnosis of HCC is micro-RNA 486-5p (miRNA 486-5p). Hence, the current study was conducted to interrogate the role of miRNA 486-5p in the diagnosis of HCC in a cohort of Egyptian patients with hepatitis C virus (HCV) related liver cirrhosis (LC). This case–control study included twenty-five patients with HCC as studied cases and twenty-five patients with LC as controls. Patients in both groups were classified according to the Child–Pugh score. HCC patients were further classified according to the Barcelona Clinic Liver Cancer (BCLC) classification.

Results MiRNA 486-5p was found to be statistically notably elevated in patients with HCC than in those with LC. It was found to significantly correlate with portal vein invasion.

Conclusions Serum miRNA 486-5p could be a particularly sensitive biomarker in the diagnosis of HCC as well as prediction of portal vein invasion, as firmly advocated by this study.

Keywords Liver cirrhosis, HCV, HCC, miRNA 486-5p, Child–Pugh stage, BCLC

Background

Chronic hepatitis C virus (HCV) infection is often clinically ominous and eventually yields liver cirrhosis (LC) [1]. The survey carried out in 2015 concluded that 7% of the Egyptian population aged between 19 and 65 years old had chronic HCV infection. The eradication campaign that began in 2014 screened more than 50 million individuals and profitably decreased prevalence from 7 to 2% in 7 years [2].

Based on clinical outcomes, LC can be categorized into compensated and decompensated stages, with hepatocellular carcinoma (HCC) being the most unfavorable consequence of LC [3]. HCC ranks as the sixth most prevalent cancer globally and is the fourth most common cause of death attributed to cancer. Moreover, it is the most frequent primary malignancy of the liver [4].

HCC occurrence in patients with LC ranges from 2 to 4% per year [5]. Notably, the presentation of HCC has exponentially risen over the past few decades with patients presenting at relatively advanced disease stages [6].

The reliable diagnosis of HCC principally in the early stage of the disease is strenuous even with the use of AFP as a predictor of HCC development [7]. Its conclusive utility is bounded by a lack of sensitivity as well as specificity, in addition to the inconsonance among the diverse methods of its evaluation [7].

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Micro-RNAs (miRNAs) are small noncoding RNAs, which exert their effect post-transcriptionally by regulating gene expression. Innumerable studies have endorsed that miRNAs could be implicated in various cell biological processes, including cell growth, differentiation, and cell apoptosis along with moderation of gene expression in cancers [8].

MiRNA 486-5p is a chief member in the branch of oncology. It is encrypted by the MIR 486–1 gene in the human genomic map and is based on chromosome 8 [9]. MiRNA 486-5p is abundant in the cytoplasm and the nucleus of cells [10]. Moreover, it circulates unbound or within exosomes in plasma where it stays highly stable [11] and is highly resistant to nuclease digestion [12].

Under physiological conditions, miRNA 486-5p is immensely produced in myoblasts as well as in skeletal muscles, while myogenesis is taking place [12]. However, under pathological conditions, it was found to have a non-constant expression in several solid malignancies through being involved in diverse signaling pathways, such as HCC, non-small cell lung cancer, breast cancer, esophageal squamous cell carcinoma, and pancreatic cancer. In some cancer lines, it could function as a tumor-suppressive miRNA. However, it was concluded to have a probable oncogenic role in other cancers such as pancreatic cancer [13].

Considering the several biological functions of miRNA 486-5p implicated in oncogenesis [10], its high abundance in plasma [11], and the paucity of studies as well as contradicting conclusions regarding its diagnostic role in HCC, its role in HCC is yet to be explored.

Aim

This study aimed to evaluate the role of miRNA 486-5p in the diagnosis as well as the prediction of the prognosis of HCC in a cohort of Egyptian patients with HCV-related LC.

Subjects

The study design is case–control, with twenty-five patients with chronic HCV LC-related HCC as studied cases and twenty-five patients with chronic HCV-related LC without HCC as controls. To achieve a power of study of 90% at 5% significance, the minimum sample size was estimated to be thirty-eight patients (nineteen patients in each group) based on Weis et al. which was increased to fifty (twenty-five patients in each group) to increase the accuracy of the results [14]. The calculation was done using MedCalc® Statistical Software version 19.6 (MedCalc Software Ltd, Ostend, Belgium; http://www.medcalc.org;2020).

All the patients were recruited from the Tropical Medicine Department, Alexandria University, during the period from June 2021 to December 2021, after the local ethical committee has approved the study and according to the Declaration of Helsinki. Child–Pugh A patients were recruited from the outpatient clinic during routine follow-up by US and AFP following successful HCV eradication, while Child–Pugh B and Child–Pugh C patients were recruited from the inpatient ward during hospitalization owing to signs of decompensated LC. All patients had given an informed consent regarding both the nature and the aim of the study.

Inclusion criteria were patients with HCV-related LC with or without HCC who were negative for HCV ribonucleic acid (RNA) at the time of the study, aged between 18 and 70 years old. All recruited patients have been previously successfully treated with combined sofosbuvir 400 mg and daclatasvir 90 mg twice daily with or without ribavirin for 12 weeks, according to the national Egyptian guidelines for treatment of HCV [15].

Exclusion criteria

Patients with no history of HCV, chronic hepatitis B (HBV) by hepatitis B surface antigen (HBsAg), autoimmune hepatitis diagnosed by positive autoimmune markers including anti-nuclear antibody (ANA), anti-smooth muscle antibody (ASMA), and anti-liver kidney microsomal antibody (anti-LKMA), a positive family history of cancer colon, positive carcinoembryonic antigen (CEA), increased serum level of prostatic surface antigen (PSA) or increased prostatic volume by US, females with palpable breast masses proven to be malignant by mammogram, patients with chronic kidney diseases, sepsis as well as cardiac fibrosis, diabetes mellitus, and hypertension were all excluded from the study.

Methods

All the study participants were subjected to detailed history taking and thorough clinical examination for signs as ascites which was classified according to the International Ascites Club (IAC) classification [16]. Examining the patients for hepatic encephalopathy (HE) and subclassification of the studied patients according to the West Haven Criteria was done [17]. In addition, laboratory investigations including complete blood picture (CBC), fasting blood glucose (FBG), serum levels of alanine transaminase (ALT), aspartate transaminase (AST), prothrombin time (PT), international normalized ratio (INR), total and direct bilirubin, serum albumin, urea, and creatinine as well as potassium were carried out to all studied patients. LC was diagnosed based on clinical and laboratory findings in coherence with chronic liver disease, FIB-4 which is a combination of four simple variables: AST, ALT, age, and platelet count. FIB-4 of more than 3.35 was the cutoff diagnostic value for advanced fibrosis and cirrhosis. It was

calculated with the following formula: [age (years) × AST (IU/L)]/[platelet count (109/L) × ALT (IU/L)]1/2 [18], as well as radiological evidence of LC by the US. LC patients were staged according to the Child–Pugh grading and classification into groups A, B, and C [19].

HCC was diagnosed based on characteristic arterial phase hyperenhancement (APHE) and non-peripheral washout in venous and delayed phases as shown by contrast-enhanced triphasic computer tomography (CECT) scan [20]. HCC patients were classified according to the degree of severity according to the Barcelona Clinic Liver Classification (BCLC), 2018 [21].

Measuring of serum miRNA 486-5p by RT-PCR [22, 23]

For each subject, 10 ml of venous blood was obtained and were centrifuged for 10 min at 3000 rpm at 4 °C. Serum

Table 1 Demographic, clinical and laboratory findings of the studied patients

	Without HCC ($n=25$)	HCC ($n=25$)	Test of sig.	p
Gender				
Male	15 (60.0%)	20 (80.0%)	$\chi^2=2.381$	0.123
Female	10 (40.0%)	5 (20.0%)		
Age (years) (mean ± SD)	60.72 ± 10.60	59.64 ± 7.54	$t=0.415$	0.680
Ascites				
Absent	3 (12.0%)	4 (16.0%)	$\chi^2=4.378$	$^{MC}p=0.367$
Grade 1	7 (28.0%)	8 (32.0%)		
Grade 2	6 (24.0%)	9 (36.0%)		
Grade 3	9 (36%)	4 (16%)		
Encephalopathy				
Grade 0	15 (60.0%)	17 (68.0%)	$\chi^2=2.329$	$^{MC}p=0.581$
Grade 1	5 (20.0%)	5 (20.0%)		
Grade 2	0 (0.0%)	1 (4.0%)		
Grade 3	5 (20.0%)	2 (8.0%)		
Hb (mean ± SD) (g/dl)	9.26 ± 2.04	10.53 ± 2.36	$t=2.033^*$	0.048^*
RBCs (mean ± SD) (million/mm$^{3)}$	3.34 ± 0.65	3.61 ± 0.97	$t=1.163$	0.250
WBCs (microliters)				
Min.–Max	1.90–14.06	0.02–44.19	$U=259.5$	0.304
Median (IQR)	5.79 (3.95–9.12)	7.14 (5.54–9.48)		
Platelets (thousand/microliters)				
Min.–Max	9–308	20–273	$U=270.0$	0.410
Median (IQR)	96 (65.0–120.0)	110 (60.0–178.0)		
SGOT (U/L)				
Min.–Max	16–130	16–430	$U=110.5^*$	$<0.001^*$
Median (IQR)	45 (35.0–51.0)	97 (58.0–130.0)		
SGPT (U/L)				
Min.–Max	12–85	20–449	$U=189.5^*$	0.017^*
Median (IQR)	34 (29.0–37.0)	47 (31.0–80.0)		
Bilirubin (mg/dl)				
Min.–Max	0.50–16.0	0.70–20.0	$U=163.0^*$	0.004^*
Median (IQR)	1.40 (0.80–2.20)	2.40 (1.80–6.90)		
INR (mean ± SD)	1.37 ± 0.21	1.45 ± 0.30	$t=1.101$	0.276
Albumin (mean ± SD) (g/dl)	2.50 ± 0.52	2.62 ± 0.71	$t=0.683$	0.498
Child score (mean ± SD)	9.28 ± 1.46	9.24 ± 1.79	$t=0.087$	0.931
Child grade				
A	1 (4.0%)	2 (8.0%)	$\chi^2=0.638$	0.824
B	10 (40.0%)	11 (44.0%)		
C	14 (56.0%)	12 (48.0%)		

Hb hemoglobin, *sGOT* serum glutamic oxaloacetic transaminase, *RBCs* red blood cells, *sGPT* serum glutamic pyruvic transaminase, *WBCs* white blood cells, *INR* international normalization ratio, *SD* standard deviation, *p p* value for comparing the two studied groups

* Statistically significant at $p \leq 0.05$

devoid of cells was then stored at − 80 °C until RNA was extracted.

Total RNA extraction

Total RNA was separated from serum samples with the aid of the Qiagen® miRNA easy Mini Kit (Qiagen, CA) according to the manufacturer's instructions.

Real-time quantitative reverse transcription polymerase chain reaction (QRT-PCR)

The quantification of miRNA-486-5p expression was performed using the TaqMan mRNA assay using a two-step RT-PCR.

a. Reverse transcription (RT) step: RT of cDNA from purified RNA samples was performed by using specific miRNA stem-loop primers from the TaqMan miRNA assays and reagents from the TaqMan® miRNA reverse transcription kit (Applied Biosystems, USA).
b. Quantitative real-time PCR (qPCR) step: PCR products were amplified from cDNA samples using the aid of TaqMan miRNA assays together with the TaqMan® Universal PCR Master Mix (Applied Biosystems, USA). Thermocycling was done using Applied Biosystems Step One™ real-time PCR System. The $2^{-\Delta\Delta CT}$ method was used to assess the relative expression [23].

Syncel-miR-39 mi script miRNA mimic was used as a control to normalize the data.

Statistical analysis of data

Data was analyzed statistically using IBM SPSS software package version 20.0 (Armonk, NY: IBM Corp). The Shapiro–Wilk test was utilized for testing the normality of the distribution of variables. Comparisons between groups for categorical variables were evaluated using the chi-square test (χ^2) and Monte Carlo test (MC). Student *t*-test (*t*-test) was used to compare two groups for normally distributed quantitative variables. Kruskal–Wallis and Mann–Whitney (*U*) tests were used to compare groups for abnormally distributed quantitative variables.

Results

Patient characteristics

Demographic, clinical, and laboratory findings of the studied patients are summarized in Table 1.

Tumor characteristics

Tumor characteristics are summarized in Table 2.

Table 2 CT findings and BCLC staging of HCC group ($n = 25$)

	No. (%)
No. of lesions	
Single	8 (32.0%)
Multiple	17 (68.0%)
Size (cm)	
2 to 3	3 (12.0%)
> 3	22 (88.0%)
Portal vein invasion	9 (36.0%)
Lymph node metastasis	1 (4.0%)
BCLC	
A	1 (4.0%)
B	7 (28.0%)
C	5 (20.0%)
D	12 (48.0%)

The role of miRNA 486-5p and AFP in diagnosis of HCC

MiRNA 486-5p was significantly upregulated in HCC patients compared to HCV-related LC patients (*p* value < 0.001*). Moreover, AFP was significantly elevated in HCC patients compared to LC patients (*p* value 0.010*) (Table 3).

At a cutoff value of 1.22 ng/ml, miRNA 486-5p was found to diagnose HCC with a sensitivity and specificity of 60% and 76%, respectively. However, AFP had a sensitivity of 36% and specificity of 76% at a cutoff value of 100 ng/ml, as shown in Table 4.

Correlation between miRNA 486-5p, demographic, clinical and laboratory parameters as well as tumor characteristics

MiRNA 486-5p was found to be higher in patients with tumor size > 3 cm than smaller sized tumors, but the difference did not reach the level of significance. Moreover, serum miRNA 486-5p was found to be significantly higher in HCC patients with portal vein thrombosis (PVT) (*p* 0.049*), as shown in Table 5.

Table 3 Comparison between the two studied groups according to serum miRNA 486-5p and AFP

	Without ($n = 25$)	HCC ($n = 25$)	*U*	*p*
miRNA 486-5p				
Min.–Max	0.01–18.64	0.16–1147	110.0*	< 0.001*
Median (IQR)	0.66 (0.14–0.97)	2.59 (1.13–68.59)		
AFP				
Min.–Max	1.60–300	4.40–1210	180.0*	0.010*
Median (IQR)	8.6 (3.4–12.3)	68 (9.1–155)		

Min minimum, *Max* maximum

* Statistically significant at $p \leq 0.05$

Table 4 Validity (AUC, sensitivity, specificity) for miRNA 486-5p and AFP to diagnosis HCC ($n=25$) from cirrhosis ($n=25$)

	AUC	*p*	95% CI	Cutoff	Sensitivity	Specificity	PPV	NPV
Micro-RNA 486-5p	0.667	0.043*	0.512–0.822	> 1.22[a]	60.0	76.0	71.4	65.5
Alpha-fetoprotein	0.712	0.010*	0.565–0.859	> 100	36.0	76.0	60.0	54.3

CI confidence interval, *NPV* negative predictive value, *PPV* positive predictive value

* Statistically significant at $p \leq 0.05$

[a] Cutoff was chosen according to the Youden index

Only female gender and WBCs were found to be independent risk factors for elevated miRNA 486-5p levels in patients with HCC, as shown in Table 6.

Discussion

Despite the abundance of highly efficacious treatment regimens to the different HCV genotypes, HCV-related LC continues to persist with its elaborate and morbid complications. It is of urgent importance to differentiate patients with LC from those with chronic hepatitis C to determine those with a need for long-term surveillance for evolving complications, most dangerously, HCC. To our mischief, in patients with LC, the risk of HCC is not completely abolished even after HCV elimination by DAADs therapy.

The current study evaluates the potential diagnostic role of miRNA 486-5p in the diagnosis of HCC in patients with HCV-related LC. The serum level of miRNA 486-5p was found to be significantly higher in patients with HCC than in patients with HCV-related LC with a higher sensitivity than AFP. Its level was found to increase in patients with larger tumor sizes; however, the difference did not reach the level of significance. Moreover, it was found to be significantly upregulated in patients with PVT.

Conflicting studies regarding the role of miRNA 486-5p in previous studies have been found. Findings by Weis et al. [14] support our conclusion since they found that miRNA 486-5p was markedly overexpressed in HCC over patients with LC. In this study, seven micro-RNAs were investigated for their potential role in HCC diagnosis, and only miRNA 486-5p was found to be upregulated in patients with HCC. Moreover, Huang et al. demonstrated that higher serum miRNA 486-5p was associated by early HCC recurrence post resection [24].

One factor that influences the miRNA 486-5p profile is the pathophysiological state of the source cell. For instance, hypoxia induced by growing cancers including prostatic cancer triggers the transcription of the miRNA 486-5p as its promoter contains a binding site for hypoxia-inducible factor α (HIF α) [25]. Also, in mesenchymal stem cells, miRNA 486-5p was found to increase the expression of the vascular endothelial growth factor messenger RNA (VEGF mRNA), thus stimulating the growth of new blood vessels and angiogenesis [26]. Similar mechanisms could be the contributing factors to the increase in the serum expression of miRNA 486-5p in HCC patients; however, the exact explanation needs further validation.

Other research has concluded that miRNA 486-5p is primarily HCC suppressive and that its expression is related to favorable tumor outcome and diminished invasiveness. For instance, Younes et al. postulated that miRNA 486-5p works by blocking of the insulin growth factor 1 downstream signaling (IGF-R1) and hence is primarily suppressive to cancers, and highlighted their immune enhancing nature in terms of augmenting natural killer immune cells (NK) and strengthening their destructive potential against HCC lines [9]. Moreover, Sun et al. discovered that miR-486 is down-expressed in the serum of HCC relative to that of healthy subjects [27].

In the current study, WBC count and female gender were the only two independent risk factors for miRNA 486-5p elevation. Gender differences regarding miRNA 486-5p expression in patients with HCC have not been explored in former research. However, obesity-related chronic inflammation may a contributing factor to this finding. Previous work by Sun and Guo proved that miRNA 486-5p was markedly

Table 5 Relation between miRNA 486-5p and the different parameters in the HCC group ($n=25$)

		Micro-RNA 486-5p		*U*	*p*
		Mean ± SD	Median (Min.–Max.)		
No. of lesions					
Single	8	181.1 ± 397.2	7.52 (0.59–1147)	47.0	0.238
Multiple	17	81.99 ± 214.4	2.12 (0.03–867)		
Size (cm)					
2 to 3	3	25.99 ± 42.97	2.35 (0.03–75.58)	29.0	0.783
> 3	22	125.7 ± 297.9	2.17 (0.08–1147)		
Portal vein invasion					
Absent	16	105.5 ± 219.5	9.60 (0.08–867)	37.0*	0.049*
Present	9	128.3 ± 382	0.59 (0.03–1147)		

* Statistically significant at $p \leq 0.05$

Table 6 Univariate and multivariate linear regression analysis for the parameters affecting micro-RNA 486-5p for HCC group ($n=25$)

	Univariate		Multivariate	
	p	***B* (95% CI)**	***p***	***B* (95% CI)**
Age (years)	0.914	0.848 (−15.220–16.915)		
Female	0.002*	407.86 (168.87–646.85)	0.013*	314.12 (74.369–553.878)
Ascites	0.143	−226.46 (−535.16–82.25)		
Encephalopathy	0.605	64.188 (−188.77–317.15)		
WBCs	0.006*	18.273 (5.829–30.716)	0.041*	12.443 (0.543–24.343)
Albumin	0.081	140.58 (−18.884–300.04)		
INR	0.643	−90.807 (−490.98–309.37)		
Bilirubin	0.682	−4.065 (−24.312–16.181)		
Child score	0.230	−39.155 (−104.851–26.541)		
Multiple of lesions	0.422	−99.090 (−349.95–151.77)		
Size of lesions > 3	0.575	99.675 (−263.07–462.42)		
Portal vein invasion	0.850	22.79 (−224.314–269.897)		

* Indicates significant difference

upregulated in patients with sepsis and had significant positive correlation with procalcitonin and C-reactive protein [28]. Moreover, miRNA 486-5p was found to be upregulated in patients with chronic inflammatory conditions including knee arthritis and obstructive pulmonary fibrosis [29, 30]. The proposed mechanism of miRNA 486-5p upregulation is through constant stimulation by inflammatory mediators by phagocytes during infections and over expressed cytokines during chronic inflammation.

Our study focused on HCC related to HCV-induced LC. Elevation of miRNA 486 5p could also be explained by persistent inflammation and necrosis before virus eradication. Moreover, miRNA biogenesis is a complex process involving synthesis of both cytosolic and nuclear components during various stages of carcinogenesis and hence provides an area of continuous interest for ongoing exploitation for their diagnostic and prognostic roles in both benign and malignant conditions.

Conclusions

This study supports the hypothesis that miRNA 486-5p is upregulated in patients with HCV-related HCC; however, the exact mechanism needs to be further elucidated. It strongly suggests that serum miRNA 486-5p could be an extremely sensitive biomarker for the diagnosis of HCC in patients with HCV-induced LC, and that its level correlates positively with increased tumor size and strongly predicts portal vein invasion.

Limitations of the study

This study has a few limitations. First, a group of normal participants was not included to show the serum level of miRNA 486-5p in normal individuals as well as the effect of LC on its serum level. Moreover, this study focused on HCC owing to HCV-induced LC and no comparison was done between HCC secondary to other etiologies. Furthermore, the duration since treatment was not taken into consideration.

Abbreviations

HCV	Hepatitis C virus
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
miRNA	Micro-RNA
PA	Prothrombin activity
CBC	Complete blood count
FBG	Fasting blood glucose
PVT	Portal vein thrombosis
AFP	Alpha-fetoprotein
DAADs	Direct acting antiviral drugs
BCLC	Barcelona Clinic Liver Classification
CECT	Contrast-enhanced computerized tomography
APHE	Arterial phase hyperenhancement
LC	Liver cirrhosis
SVR	Sustained virological response
AASLD	American Association for the Study of Liver Diseases
AUC	Area under curve
RNA	Ribonucleic acid
ROC	Receiver operating characteristics
SD	Standard deviation
χ^2	Chi-square
MC	Monte Carlo
t-test	Student's *t*-test

U	Mann-Whitney test
IQR	Interquartile range
Min	Minimum
Max	Maximum
CI	Confidence interval
H	Kruskal-Wallis

Acknowledgements
The authors present sincere gratitude to the staff of tropical medicine department for facilitating patients' data collection as well as clinical biochemistry department for providing the lab work, at Alexandria Faculty of Medicine.

Authors' contributions
Amany N. Abbasy: conceptualization, planning study design, major writing of the manuscript. Rasha Saeed: biochemical work, statistical analysis of results and contributed to writing of the manuscript. Mohamed M. El Shafei: diagnosis of HCC based on radiological findings. Mohamed A. Abdel Aziz: patient's data collection and contributed to writing of the manuscript. All authors revised and approved the manuscript.

Funding
The authors declare that they did not receive any funding for this study.

Availability of data and materials
All data analyzed and generated during this study are available upon request from the valued editors. The corresponding author "Amany Nabil Abbasy" will oversee sending data when required.

Declarations

Ethics approval and consent to participate
This study has been approved by the Faculty of Medicine, Alexandria University Ethics Committee. All procedures were strictly conducted in accordance with the Declaration of Helsinki. The serial registration number for this study is 0305692. All patients had given an informed written consent stating the title, procedure, and purpose of the study.

Consent for publication
Not applicable.

Competing interests
The authors of this manuscript declared no competing interests.

Received: 17 October 2023 Accepted: 27 January 2024
Published online: 06 February 2024

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