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The Expression Pattern and Diagnostic Capability of Hsa_Circ_0051732 Plasma Biomarker in Hepatocellular Carcinoma

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Abstract: The most prevalent primary liver cancer that originates in hepatocytes has the name hepatocellular carcinoma (HCC). It is a serious global health concern due to high incidence of morbidity and death rates, making early diagnosis crucial for better patient outcomes. Therefore, conducting an investigation of non-invasive biomarkers is crucial as it aids in diagnosis and measures susceptibility in different types of HCC. The aim of this manuscript is to examine the clinical role of Hsa_circ_0051732 in terms of diagnosis and susceptibility as circular RNAs (circRNAs) play crucial role in diagnosis and prognosis in different types of cancer. The expression levels of hsa_circ_0051732 in plasma of HCC patients vs. healthy control group were measured using Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The results of this research revealed that Hsa_circ_0051732 was significantly elevated in HCC indicating its concomitant with the advancement of HCC, moreover Hsa_circ_0051732 was considerably upregulated in metastatic group of HCC vs. un-metastasis group of HCC, however, it didn't reach the statistical significance. Thus, the studied marker could be a differentiator between HCC stages in larger population. In addition, Hsa_circ_0051732 showed higher ACU than AFP suggesting that the combination between AFP as a standard marker for HCC diagnosis with Hsa_circ_0051732 could act as a robust panel for diagnosis of HCC. In conclusion, as far as we know hsa_circ_0051732 possibly act as a novel prospective biomarker for diagnosis of HCC, playing a significant role in differentiation between early and advanced stages of HCC.

Keywords: Biomarkers; CircRNA; Diagnosis; Liver cancer; qRT-PCR.

1. INTRODUCTION

The The Global Cancer Statistics for 2020 show that liver cancer ranks fourth in terms of the number of deaths from cancer and sixth in terms of the frequency of all cancer types [1]. Of all primary liver cancer casesbetween 75 and 85 percent of cases are caused by hepatocellular carcinoma (HCC) [1]. Due to the missing early symptoms, most patients are often identified with HCC after they have already reached the intermediary or terminal stages of the disease [2]. Nevertheless, none of the routine indicators, such as Alpha-fetoprotein (AFP), can be used to predict the early diagnosis of HCC [3, 4]. Finding sensitive, non-invasive molecular indicators unique to early prediction of the presence of HCC is of utmost importance.

Circular RNAs (circRNAs) are a recently discovered group of many intrinsic non-coding RNAs (ncRNAs) that are significantly involved in several forms of cancer, such as gastric, lung, and HCC [5, 6]. They create continuous, covalently closed loop patterns lacking polarity and polyadenylated tails. This characteristic makes them more resilient and prominent than the linear transcripts produced by similar cell genes [7, 8]. In addition to being present in tissues, circRNAs may also be detected in other bodily fluids, including saliva, blood, and urine.

This indicates that they have the potential to be used as noninvasive diagnostic tools for cancer [9].

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Many studies have shown that circRNAs can control gene expression at each transcription, post-transcription, and translation stage [10, 11].

Exonic circRNAs, found chiefly in the cytoplasm, harbor microRNA (miRNA) response elements (MREs) [12]; they may act as miRNA sequesters by competitively attaching to them, which decreases their expression level and activity [13]. Exon-intron circRNAs or Circular intronic RNAs mostly concentrate in the nucleus and can potentially control gene transcription and post-transcriptional processes [14, 15]. These findings indicate that circRNAs could influence cancer advancement by playing a role in multiple biological functions, including cell proliferation, invasion, migration, metastasis, and apoptosis [16]. The previously mentioned features make them ideal molecular markers of hepatocellular carcinoma [17]. This study has demonstrated the' significance of circRNAs in diagnosing HCC and their possible biological functions in its development.

2. METHODS

2.1. Patients and sample collection

One hundred newly diagnosed HCC patients were recruited from the National Cancer Institute (NCI) inpatient and outpatient clinics from January 2022 to August 202. The other group included fifty healthy volunteers. Both groups were matched with age, gender, and ethnicity. HCC patients were categorized based on the Child-Pugh staging system, Barcelona Clinic Liver Cancer (BCLC) staging system, degree of fibrosis, tumor size, and presence of metastasis.

The research followed the guidelines and principles outlined in the Declaration of Helsinki, and written agreement was obtained from each patient to participate in the study. The research received approval from the Committee on Human Research Ethics at Al-Azhar University, with the Institutional Review Board (IRB) number 327. No radiation or chemotherapy was administered before the sample was collected.

The inclusion criteria include patients with newly diagnosed hepatocellular carcinoma, while the exclusion criteria for the study include hepatitis B or C infection, another solid-organ malignancy, liver transplant or a liver transplant candidate, or any terminal condition, alcohol abuse, and any metabolic diseases.

Sample collection

Samples of blood were drawn from the antecubital vein. These samples were collected in EDTA containing vacutainers. Using a cooled centrifuge, cells are extracted from plasma by centrifuging at 1,000–2,000 x g for 10 minutes. In the plasma sample, centrifugation at 2,000 x g for 15 minutes depletes the platelets. The supernatant that is left over is called plasma. After centrifugation, the plasma was pipetted using a clean microcentrifuge tube, and those separated plasma aliquots were then frozen at −80°C until needed for RNA extraction.

2.2. Methods

2.2.1. Selection of circular RNA

Previously reported microarrays in HCC, GSE94508, and GSE97332 were taken from the Gene Expression Omnibus (GEO) dataset at (www.ncbi.nlm.nih.gov/geo/) and were screened individually [18]. **Hsa_circ_0051732** showed statistical significance between hepatocellular carcinoma (HCC) and non-tumor tissues. Based on circular RNA databases [19-21], it was focused on Hsa_circ_0051732 (Alias: 102587). Its gene is located at chr19:48660270-48660397. The spliced sequence length is 127 nt, and its gene symbol is LIG1.

2.2.2. Extraction of whole RNA and reverse transcription

Total RNA was extracted from samples of HCC and healthy controls using the RNeasy® Mini Kit Isolation System *(Qiagen, USA)* (Cat. No. 217004), according to the instructions provided by the supplier. The RNA's concentration and purity were assessed using Nanodrop, POLAR star Labtech. The RNA, held in a solution, was kept at a temperature of -80°C until it was used. The cDNA was synthesized by reverse transcription (RT) using a Revert Aid First Strand cDNA Synthesis; Thermo Scientific (Cat. NO. K1622).

2.2.3. Quantitative estimation of the expression of hsa_circ_0051732 by reverse transcription-quantitative Real-time polymerase chain reaction

SensiFASTTM SYBR No-ROX (Bioline) (Cat.NO BIO-98005) was used to perform the reverse transcription-quantitative Real-time polymerase chain reaction for the Quantitative

estimation of hsa_circ_0051732 on a Real-Time PCR System (BioRad, MiniOpticon, USA). As stated in a recent study [22], the use of divergent primers enables the amplification of circular RNAs exclusively. In contrast, the employment of convergent primers allows for the amplification of linear RNA only.

Sangon Biotech synthesized all primers used in this study in Shanghai, China. The dissociation curve analysis was used to assess the specificity of the PCR products.

A divergent primer was specifically created for hsa_circ_0051732 while using a convergent primer for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control [23]. The sequences of hsa_circ_0051732 and GAPDH primers were 5′-GCTTCCCTCTCTGACACCT-3′ **forward**, 5′-TTGTTCTCAGGAGATGTGGC-3′ **reverse** and for GAPDH 5′-TCGACAGTCAGCCGCATCTTCTTT -3′ **forward,** 5′-ACCAAATCCGTTGACTCCGACCTT-3′ **reverse**.

The primers were tested using primer blast tool.

The samples were standardized using endogenous control (GAPDH), and the fold changes were determined by relative quantification [24-26].

The cycle threshold (Ct) values were recorded for hsa_circ_0051732, and GAPDH was used as an internal control. The real-time PCR data were analyzed using the $\Delta\Delta C_t$ method [27]**.** The data that is not normally distributed was presented as median values accompanied by their corresponding interquartile range (IQR). In contrast, normally distributed data was expressed as mean ± standard error of deviation (SED). The fold change was calculated using $\mathbf{2^{-\Delta\Delta Ct}}$**.** The thermal cycling conditions were as follows: The first step involves heating the sample to 95°C for 2 minutes to initiate the reaction. This is followed by 40 cycles of temperature changes: 95°C for 5 seconds, 60°C for 10 seconds, and 72°C for 10 seconds.

2.3. Statistical analysis

The statistical data were performed and visualized by Graph Pad Prism 9.0 (San Diego, CA, USA). Numbers and percentages were used to express qualitative data. When applicable, the mean ± SD, median, interquartile range (IQR), and range were used to characterize numerical data. Tests of normality, such as the D'Agostino & Pearson test were performed to determine the distribution of the data. Differences in the expression levels of hsa_circ_0051732 in two groups were analyzed by Mann-Whitney t-test, while in the case of more than two groups, the Kruskal-Wallis test with post hoc was performed. Spearmen's Rho correlation and chi-square or Fisher for demographics tests were used when appropriate. $P < 0.05$ was thought to be statistically significant. The diagnostic accuracy of the parameters under study was assessed using a receiver-operating-characteristic (ROC) curve, and the area under the curve (AUC) was calculated. Although AUC > 0.9 was expected to be a major discriminator, it was deemed insignificant when AUC < 0.6. Conversely, between 0.7–0.89, a possible or promising discriminator was taken into consideration. $P < 0.05$ was thought to be statistically significant.

3. RESULTS

3.1. The baseline data on clinical features and demographics

There was significant difference between HCC and the control group in the following parameters: liver function tests and AFP. While demographic data such as age and gender, there was no significant difference between HCC and the control group regarding the test of demographics (Table 1).

3.2. The levels of expression of hsa_circ_0051732.

The study observed a substantial upregulation of hsa_circ_0051732 expression levels in the plasma of patients with HCC patients with fold change 4.380 (2.4- 6.8), compared to the matched healthy control with fold change 0.2037 (0.31- 1.4), with a statistically significant difference (P<0.001) as shown in figure 1.

3.3. Comparing the fold change of hsa_circ_0051732 in patients with HCC based on clinicopathological variables

By comparing the relative expression levels of hsa_circ_0051732 with the clinicopathological factors of patients with HCC, the study examined the possible diagnostic values of this gene. It has been found that there was no association between hsa_circ_0051732 expression levels and BCLC, appearance of cirrhosis, tumor size and metastasis

Table 1. Baseline demographics and clinical pathological data of the studied group

	HCC n=100 (n, % or mean ± SE)	**Control n=50 (n, %) or mean ± SE**	***P* -value**
Gender			
Female n,% **Male n,%**	42,42% 58,58%	23,46% 27,54%	0.64
Age	59.2±0.8	60.7±0.96	0.22
<60 **≥60**	55,55% 45,45%	20,40% 30,60%	0.08
AST (IU/L)	102.2±12.1	18±0.6	**<0.001**
ALT (IU/L)	62.3±5.8	16.6±0.8	**<0.001**
Albumin (gm/dl)	2.67±0.06	4.6±0.12	**<0.001**
Bilirubin (mg/dl)	6.8±1.43	0.52±0.03	**<0.001**
AFP (ng/ml)	139.2±20	3.7±0.2	**<0.001**
Tumor size (cm) **<3** **≥3**	 40, 40% 60, 60%	 -----	 -----
Distant Metastasis **Yes** **No**	 25, 25% 75, 75%	 -----	 -----
BCLC **Early stage** **Late stage**	 49, 49% 51,51%	 -----	 -----

Data are expressed as Mean ± SE, AFP: alpha-fetoprotein, ALT: alanine aminotransferase, AST: aspartate aminotransferase, BCLC staging system: Barcelona Clinic Liver Cancer; Stage A (Early stage), Stage B (Intermediate Stage), Stage C (Advanced stage), and Stage D (severe liver damage).Data were analyzed by independent t-test. Categorical data were compared using the chi-square test. P values < 0.05 are regarded as significant

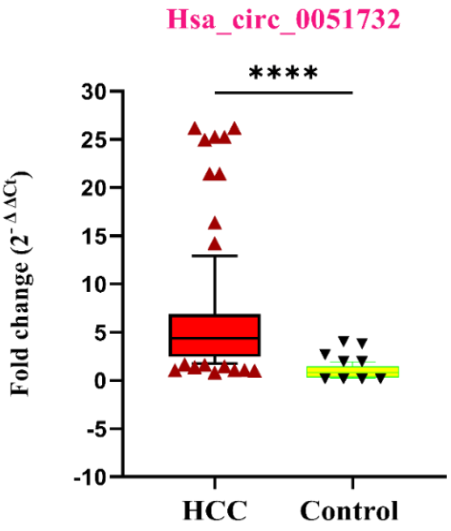

Figure 1. Plasma expression levels of hsa_circ_0051732 in HCC vs. healthy control group.

***fold change of plasma hsa_circ_0051732 levels in HCC (n=100) is 4.380 (2.4- 6.8), and control (n=50) is 0.2037 (0.31- 1.4). The Mann-Whitney test measured the Expression difference between HCC and healthy control.*

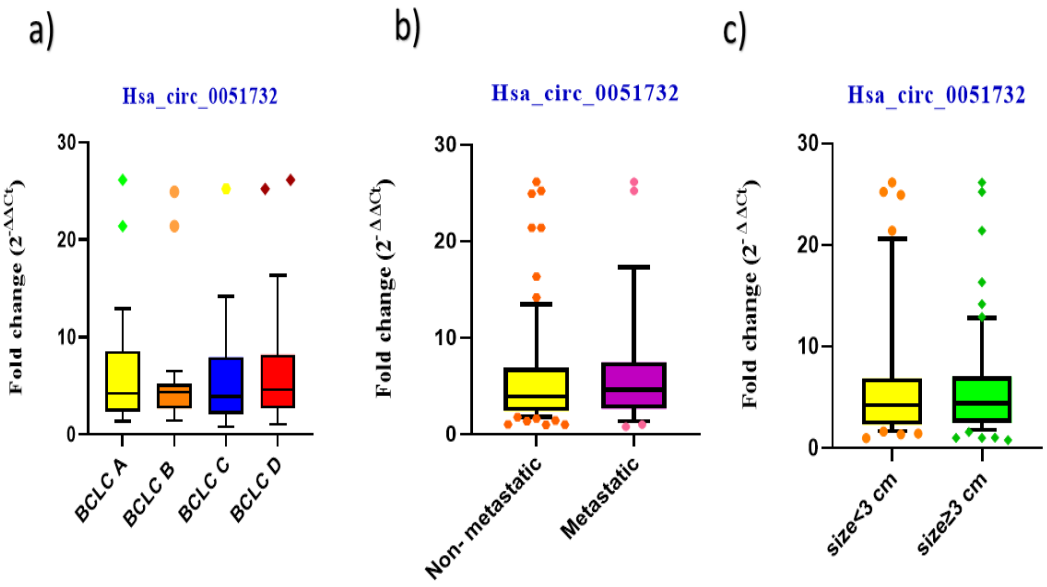

Figure 2. Comparing the plasma expression levels of hsa_circ_0051732 according to BCLC stage system (a), appearance of metastasis (b), and tumor size (c).

***BCLC staging system: Barcelona Clinic Liver Cancer; Stage A (Early stage), Stage B (Intermediate Stage), Stage C (Advanced stage), and Stage D (severe liver damage). Statistical analysis was done by independent t-test.*

Table 2. The Correlation between hsa_circ_0051732 with the clinicopathological factors.

Hsa_circ_0051732	
Parameter	Correlation (r)
Albumin	-0.635**
ALT	0.525**
AST	0.637**
AFP	0.620**
Bilirubin	0.564**

*The correlations were showed by spearman's rho correlation test in 150 samples; * p<0.05, **P<0.01.*

Table 3. The receiver operating characteristics (ROC) curve of hsa_circ_0051732 and AFP

	AUC	Cut off	Sensitivity	Specificity
ROC curve of AFP in HCC vs control group	**87%**	**27.5**	**74%**	**94%**
ROC curve of Hsa_circ_0051732 in HCC vs control group	**95.1%**	**1.53**	**93%**	**90%**
Roc of AFP combined with hsa_circ_0051732 in HCC vs control group	**97.9%**	**0.5**	**93%**	**94%**

AFP: Alpha-fetoprotein, AUC: area under the curve, HCC: hepatocellular carcinoma, ROC: receiver-operating-characteristic, HCC (n=100), and control (n=50).

However, there is an increase in metastatic group versus non-metastatic group but didn't reach the statistical significance (p=0.057).

3.4. The Correlation between hsa_circ_0051732 with the clinicopathological factors.

Table 2 shows the correlations between the expression levels of hsa_circ_0051732 and other liver function markers in 150 samples (HCC=100, matched control=50).

The expression levels of hsa_circ_0051732 was Positevily correlated with ALT(r=0.525**), AST(r=0.637**),AFP (r=0.620**) and bilirubin (r=0.564**) while was inversely correlated with albumin (r = -0.635**).

3.5. The receiver operating characteristics (ROC) curve of hsa_circ_0051732 and AFP

The receiver operating characteristics (ROC) curve was performed to determine the diagnostic performance of hsa_circ_0051732 comparing it with the diagnostic accuracy of AFP. The studied circular RNA showed area under the curve (AUC)=95%, sensitivity=93% and specificity=90%, respectively. HOWEVER, AFP showed AUC=87%, sensitivity=74% and specificity=94%, respectively. By combining AFP with hsa_circ_0051732 as shown in figure 3 and table 3, the ROC curve demonstrated improvement in the diagnostic performance than hsa_circ_0051732 and AFP alone, showing a potential panel with AUC=97.9%, sensitivity=93% and specificity=94%, respectively.

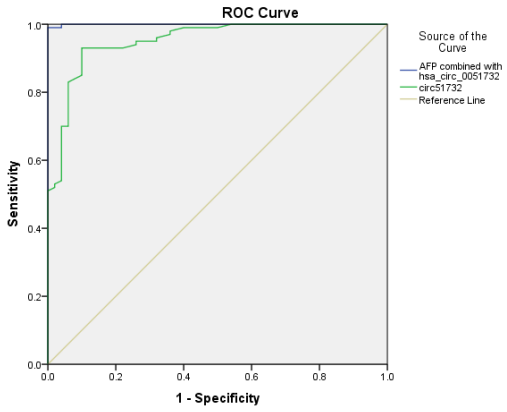

Figure 3: ROC curve for the expression of hsa_circ_0051732 in HCC patients vs healthy control group.

4- DISCUSSION

Hepatocellular carcinoma (HCC) ranks second in terms of cancer fatality rates and is the sixth most frequent kind of cancer globally [28]. The biological mechanism that causes HCC disease is multistep and complex [29]. Although the present diagnostic level and procedures for liver cancer are constantly advancing, the diagnostic strategy and prognosis are still poor [30]. As a result, suitable biomarkers are needed to enhance diagnostic procedures in the early stages of HCC and to determine prognosis rates. AFP is the most often used biomarker for the identification of HCC in clinical practice, yet multiple studies have indicated that the AFP expression level is normal in around 40% of HCC patients, indicating its poor degree of sensitivity [31, 32]. Therefore, investigating sensitive and potentially useful biomarkers is an absolute need.

Mammals are known to possess a kind of non-coding RNAs called circular RNAs, or circRNAs [33]. Circlular RNAs are more resistant to exonucleases than linear non-coding RNAs because of their unique covalently closed circular shape [34]. Because of its abundance, conservation, and tissue specificity, circRNA has the potential to play a pivotal role in several disorders [35]. Circ-RNAs are created via back-splicing, a process distinct from linear RNA's typical splicing mode [36]. Significantly, they have crucial functions in the progression of many tumor ailments, including colorectal cancer, oral cancer, esophageal squamous cell carcinoma, and hepatocellular carcinoma [37-42]. Despite lacking knowledge about the specific processes involved, research indicates that circRNAs interact with endogenous miRNAs [43]. This emphasizes the potential roles of circRNAs as biomarkers in identifying diseases and targets in treatments [44-46]; based on this, it is possible to use circular DNA as an early diagnostic tool for liver cancer. Yongwei Zhang et al. discovered that hsa_circ_0006091 has the potential to serve as a biomarker for HCC [31]. Also, hsa_circ_0068669, circular RNA SMARCA5, and hsa_circ_0001445 serve as potential diagnostic markers for HCC [47-49]. Conversely, circular RNAs have the potential to serve as therapeutic targets in the therapy of HCC [50]. The current research has shown that the expression level of hsa_circ_0051732 was increased in the plasma of patients with HCC compared to the matched healthy control group indicating its

concomitant expression in HCC patients. Besides, the plasma levels of hsa_circ_0051732 was upregulated in HCC patients who have large tumor size and metastasis, their levels didn't reach the statistical significance, which means hsa_circ_0051732 may give such significance in a larger sample size (and/or) in the latest stages of that type of cancer. Furthermore, according to the results of correlation of hsa_circ_0051732 with clinicopathological parameters hsa_circ_0051732 failed to be considered a prognostic biomarker according to the tumor markers guidelines and general principles in clinical practice [51, 52].

The research showed that hsa_circ_0051732 had positive correlations with ALT, AST, AFP, and bilirubin but had a negative correlation with albumin. The findings demonstrate a robust correlation between hsa_circ_0051732 and liver function tests. Nevertheless, it is essential to uncover the intricate molecular pathways behind the involvement of hsa_circ_0051732 in HCC.

The ROC curve was performed to examine if hsa_circ_0051732 may function as a biomarker for differentiating HCC patients from healthy individuals. The analysis revealed that hsa_circ_0051732 exhibited an AUC of 95%. The sensitivity and specificity were also determined to be 93% and 90%, respectively which means that hsa_circ_0051732 act as excellent discriminator between healthy and diseased personnel with HCC. To enhance the diagnostic accuracy and performance of hsa_circ_0051732 as a biomarker for HCC, the authors tested the model that incorporates hsa_circ_0051732 and AFP together. The ACU of the combined model showed an improve in the diagnostic accuracy with high sensitivity and specificity indicates that hsa_circ_0051732 has the potential capacity to serve as a potent biomarker for HCC, with potent diagnostic performance when combined with HCC.

CircRNAs include binding sites complementary to miRNAs and function as "sponges" for miRNAs [43]. For example, CDR1as has 70 binding sites complementary to miR-7 [53, 54]. Recent research indicates that cytoplasmic circRNAs may function by forming a complex with microRNAs, preventing their activity [55]. The features of the 'sponge' resembled those of long non-coding RNAs (lncRNAs), indicating that it may be regulated by binding to miRNAs and targeting functional gene mRNAs [56].

To gain more insight into the internal functioning of circular 0051732 as a miRNA sponge, we used Circinteractome, a bioinformatic tool, to identify potential downstream miRNAs. The online database indicates that circ_0051732 has the potential to interact with miR-576-3p. Furthermore, the miR-576-3p expression was down-regulated in many types of cancer, including hepatocellular carcinoma [57-63]. ZFPM2-AS1, an overexpressed long non-coding RNA, sequesters miR-576-3p, causing HCC to proliferate, migrate, and invade through the miR-576-3p/HIF-1α axis [60]. According to our theory, hsa_circ_0051732 could promote the development of HCC by acting as an oncogene through the sponging of miR-576-3p. To ensure these results are met, larger-scale investigations and more studies are needed. It is also very important to remember that all of the funding for this research comes from private donations. Owing to financing constraints, much follow-up research is required to explore this concept further.

5. CONCLUSIONS

Overall, it was observed that there was a significant increase in the expression levels of hsa_circ_0051732 in the plasma of patients with hepatocellular carcinoma. This finding suggests that hsa_circ_0051732 has the potential to be a new and valuable biomarker for HCC.

Supplementary Materials:

Authors' contributions: M. S. A, A. F. R., O. S. M. A and A. I. conceived and designed the research, M. S., O. E., O. S. M. A and A. F. R. shared in the study design. M. S. and A. F. collected the samples, contributed the reagents, besides carried out the practical work. M. S. A., A. F. R., A. I., and O. E. analyzed the data, conducted the statistical analysis. All authors shared in writing and the revision of the manuscript.

Conflicts of interest: The authors disclose no conflict of interest, including any investment, personal or other relationships with other entities or organizations that may improperly affect the work in this paper or are perceived to affect it.

Compliance with Ethics Requirement: All of the research and experiments were carried out in accordance with the rules and regulations that were in effect at the time. An official informed consent agreement has been given to all patients, controls, or their legal representatives. The informed permission was obtained in accordance with the ethical

guidelines outlined in the Declaration of Helsinki, and the study protocol was approved by the Ethics Committee of the University of Cairo's Faculty of Pharmacy under approval number BC2553.

Availability of data and material

All the data and material used are available in the manuscript.

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List of abbreviations

AFP: Alpha-fetoprotein
AST: Aspartate aminotransferase
ALT: Alanine aminotransferase
AUC: Area under the curve
BCLC: Barcelona Clinic Liver Cancer
circRNAs: Circular RNAs
Ct: Cycle threshold
lncRNAs: lncRNAs
MREs: microRNA response elements
HCC: Hepatocellular carcinoma
IQR: Interquartile range
miRNA: microRNA
ncRNAs: Non-coding RNAs
ROC: Receiver-operating-characteristic
SED: standard error of deviation
qRT-PCR: Real-time quantitative reverse transcription-polymerase chain reaction

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