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Original Article

Serum-derived hsa_circ_101555 as a Diagnostic Biomarker in Non-hepatocellular Carcinoma Chronic Liver Disease: A Phase II Cross-sectional Study

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Abstract

Background and objectives: Circular RNAs (circRNAs) are non-coding RNAs characterized by a strictly closed-loop covalent structure. They are abundantly detected in various cells due to their conserved nature. Studies have reported their potential association with chronic liver disease (CLD), including hepatitis, cirrhosis, and hepatocellular carcinoma (HCC), with possible roles as diagnostic and prognostic markers. This study aimed to analyze the potential use of serum-derived hsa_circ_101555 as a diagnostic tool for CLD without HCC, and to compare it with other known non-invasive parameters for liver disease severity and inflammation. Additionally, it aimed to evaluate its expression among non-HCC CLD patients, CLD with HCC cases reported in our published (phase I) study, and healthy controls.

Methods: A cross-sectional study (phase II) was conducted involving 30 clinically, laboratory, and radiologically diagnosed Egyptian non-HCC CLD patients and 30 healthy subjects. The serum expression level of hsa_circ_101555 was measured using real-time polymerase chain reaction. The diagnostic accuracy was assessed through receiver operating characteristic curve analysis, calculating the area under the curve to determine sensitivity and specificity. The study also compared hsa_circ_101555 levels with established non-invasive parameters such as the Child-Turcotte-Pugh and model for end-stage liver disease scores, as well as inflammatory markers like the neutrophil-to-lymphocyte ratio and platelet-to-lymphocyte ratio.

Results: hsa_circ_101555 demonstrated high diagnostic accuracy (area under the curve of 0.970) at a cutoff point of 2.088 for differentiating non-HCC CLD patients from healthy controls. Elevated circRNA levels were noted in patients with hepatic encephalopathy and ascites, correlating with advanced liver disease scores (Child-Turcotte-Pugh/model for end-stage liver disease scores). Mean circRNA values were highest in HCC cases, followed by non-HCC CLD patients, and lowest in healthy controls.

Conclusions: Serum-derived hsa_circ_101555 demonstrates high diagnostic accuracy in differentiating non-HCC CLD patients from healthy controls. These findings suggest that hsa_circ_101555 has the potential to serve as a reliable non-invasive biomarker for the early diagnosis of CLD, correlating with disease severity and inflammation markers. Further research with larger sample sizes is warranted to validate its clinical utility and enhance the management of CLD.

Keywords: Serum circRNAs; Chronic; Liver disease; Non-invasive biomarkers; Diagnosis; Hepatocellular carcinoma.

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Introduction

As is known, persistent parenchymal injury and inflammation in chronic liver diseases (CLDs) may lead to fibrosis, cirrhosis, and eventually hepatocellular carcinoma (HCC). Advanced fibrosis and cirrhosis are among the common causes of liver-related mortality and morbidity. Hence, diagnosing patients with CLD is a vital step for better management. Liver biopsy has long been considered the gold standard for diagnosing cirrhosis and advanced fibrosis; how-

ever, it has gained low popularity among physicians and patients due to being an invasive procedure that can cause pain and bleeding. Therefore, non-invasive methods have been developed, including laboratory tests and radiological examinations such as ultrasonography, magnetic resonance imaging, and elastography.[1–3]

Recently, various studies have shown that serum non-coding RNAs (microRNAs (miRNAs), long non-coding RNAs, and circular RNAs (circRNAs)) play vital roles in the diagnosis of CLD and advanced fibrosis.[4,5] circRNAs represent a novel class of non-coding RNAs that are abundantly observed in many species. They are characterized by a covalently closed continuous loop structure, lacking 5′ caps and 3′ polyadenylated ends, making them more stable than linear RNAs. Several studies have identified the main functional roles of circRNAs as molecular sponges for miRNAs and RNA-binding proteins, in addition to regulating gene transcription and expression. Moreover, they are highly expressed with characteristic tissue- and developmental stage-dependent patterns across various species, as revealed by high-throughput sequencing and bioinformatics analyses. These features support their potential role in critical biological and pathological processes, particularly in malignancies of various origins.[6–10]

In the hepatic field, various studies have identified several forms of circRNAs, either upregulated or downregulated, in diseased liver tissues that play vital roles in disease progression and carcinogenesis, making them promising biomarkers. Our phase I study observed significant upregulation of hsa_circ_101555 in Egyptian CLD patients with HCC, demonstrating significant diagnostic accuracy in differentiating HCC cases from healthy subjects.[10,11] Subsequently, our current phase II study aimed to investigate the potential use of serum-derived hsa_circ_101555 as a diagnostic tool for CLD at an earlier stage, without HCC, and to compare its effectiveness with other known non-invasive parameters for liver disease severity and inflammation. Additionally, it evaluated the expression of hsa_circ_101555 among non-HCC CLD patients, those with HCC reported in our published phase I study, and healthy controls. Therefore, this study addressed the current gap in large-scale clinical validation of circRNAs in non-HCC cirrhosis populations. By doing so, it highlighted the critical need for reliable non-invasive biomarkers that can aid in the early diagnosis and monitoring of CLD, underscoring the significance of hsa_circ_101555 in contributing to improved clinical outcomes for patients with non-HCC cirrhosis.

Materials and methods

Study subjects

Our controlled cross-sectional study was designed to adhere to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines[12] and was approved by the Research Ethics Committee based on the Helsinki ethical guidelines at the Faculty of Medicine, Ain Shams University (FWA 000017585; FMASU MD 234/2022). The study was conducted at the Tropical Medicine Department, Ain Shams University hospitals, over one year, to evaluate the serum expression level of hsa_circ_101555 among CLD patients with HCC, those without HCC, and healthy controls (Fig. 1).

The study was performed in two phases: Phase I demonstrated the role of serum circRNA hsa_circ_101555 as a diagnostic and potential prognostic marker in CLD patients with HCC, as reported by Gado *et al*.[11] Our current phase II study evaluated its potential clinical utility in CLD patients at an earlier stage without HCC, as follows: the serum expression level of hsa_circ_101555 was measured in 30 clinically, laboratory, and radiologically diagnosed CLD patients without HCC compared to 30 healthy subjects using real-time polymerase chain reaction (PCR). This aimed to assess its value as a diagnostic tool for CLD and to correlate it with other known non-invasive parameters of liver disease severity. Lastly, hsa_circ_101555 levels were analyzed among non-HCC CLD cases, those with HCC (from the published phase I study), and healthy controls to explore its potential clinical application in differentiating these groups and monitoring liver disease progression.

Exclusion criteria included non-HCC malignancies, immune-related illnesses (autoimmune diseases or immunocompromised status), patients undergoing radiotherapy or chemotherapy (immunosuppressive therapies), infections, and individuals unwilling or refusing to participate.

Identification of unique circRNAs involved in CLD

Unique circRNAs were obtained from circRNA databases using a bioinformatics pipeline involving several steps:

Sample and data preparation

RNA-sequencing (RNA-seq) data for liver disease (both with and without HCC) and normal liver tissues were retrieved from various databases, including The Cancer Genome Atlas, Gene Expression Omnibus, and the European Nucleotide Archive. Quality control of RNA-seq data was conducted using FastQC to assess read quality.

circRNA identification

circRNAs were identified from the RNA-seq data using circRNA prediction tools. This involved mapping RNA-seq reads to the reference genome using splice-aware aligners such as BWA, STAR, or Bowtie2. CircExplorer2 was then used to detect potential circRNAs from the mapped data.

High-confidence circRNA filtration

Identified circRNAs were filtered based on criteria such as read support, back-splice junction reads, and minimum read counts to ensure reliability for further analysis.

Selection of hsa_circ_101555

After filtering, the specific circRNA hsa_circ_101555 was chosen for measurement in recruited subjects due to its relevance in CLD.

Differential expression identification

The expression of hsa_circ_101555 was quantified in diseased liver tissues compared to normal tissues. This involved normalizing expression data using DESeq2's normalization method and performing differential expression analysis to detect significant differences between diseased and normal samples.

Database validity

Detected hsa_circ_101555 was confirmed by comparison with known circRNA databases and studies, including circBase, circAtlas, and circBank, validating its presence and relevance in diseased liver tissue.

Designing primers for hsa_circ_101555-related protocols

Designing specific primers for hsa_circ_101555 involved several detailed steps to ensure effective and specific amplification of the target circRNA:

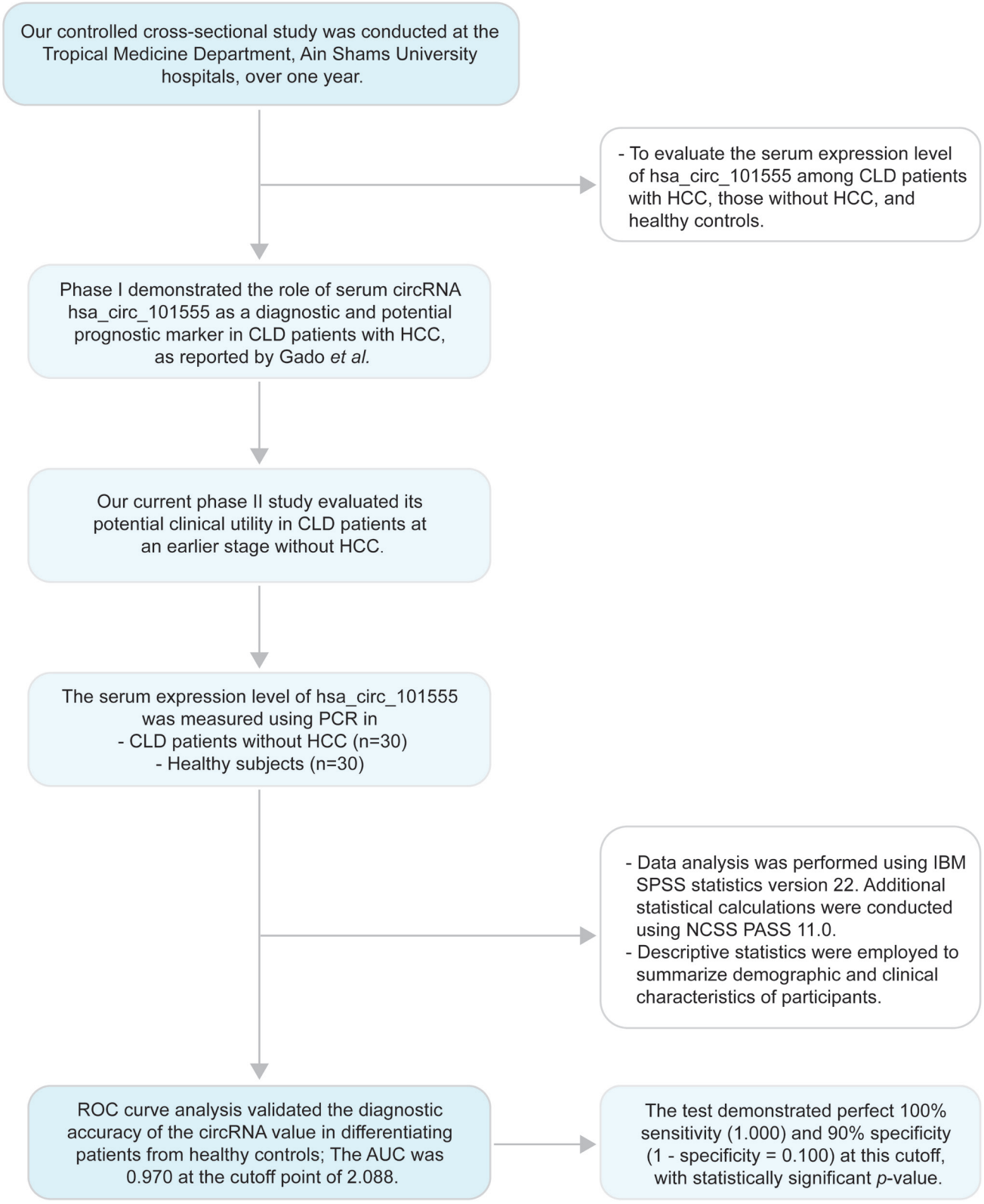

Fig. 1. A flowchart illustrates the sequential steps demonstrating the diagnostic value of circRNA in differentiating diseased patients from healthy controls. AUC, area under the curve; circRNA, circular RNA; CLD, chronic liver disease; HCC, hepatocellular carcinoma; PCR, polymerase chain reaction; ROC, receiver operating characteristic.

Sequence retrieval

The nucleotide sequence of hsa_circ_101555 was obtained from the circBase database, including regions surrounding the back-splice junction, a characteristic feature of circRNAs.

Back-splice junction identification

The back-splice junction connects the downstream exon to the upstream exon in a circRNA. Identifying this junction is critical for designing primers specific to the circRNA form rather than linear transcripts.

Extraction of the back-splice junction sequence

The sequence around the back-splice junction was extracted, typically including 15–20 nucleotides upstream and downstream, ensuring primers bind specifically to the circRNA.

Primer design

Primers were designed to amplify the back-splice junction of hsa_circ_101555 using NCBI Primer-BLAST. This tool aids in selecting sequences specific to the target circRNA while minimizing amplification of linear mRNA or non-target sequences.

Specificity validation

Primer specificity was validated using Primer-BLAST to confirm amplification exclusivity to the target circRNA.

Evaluation of secondary structures

Potential secondary structures (e.g., hairpins, dimers) were assessed using tools like OligoAnalyzer, since such structures can interfere with PCR efficiency.

Final primer sequences

The final designed primer sequences for hsa_circ_101555 were:
- Forward Primer: 5′-AGTCTGACGTGACGTCGAGT-3′
- Reverse Primer: 5′-TCGACGTACTGAGCTCAGTA-3′

These primers specifically target the unique back-splice junction of hsa_circ_101555, allowing accurate amplification and quantification in subsequent real-time PCR experiments. This rigorous design process ensures reliability and specificity of the results.

Measurement of circ_101555 in serum using real-time PCR

Measuring the levels of hsa_circ_101555 in serum samples using real-time PCR involves several key steps to ensure accurate quantification of the circRNA. Below is a detailed description of the process:

Sample collection

Venous blood (3 mL) was collected from participants in gel vacutainer tubes. The blood samples were then processed to separate the serum, which contains the RNA of interest.

Serum processing

The collected blood was centrifuged at 4,000 rpm for 15 m at 37°C to separate serum from cellular components. The serum was preserved at −80°C until analysis to prevent RNA degradation.

RNA extraction

Total mRNA and miRNAs were extracted from serum using the miRNeasy serum advanced kit, following the manufacturer's protocol. This kit efficiently isolates RNA from serum samples, ensuring high-quality RNA suitable for downstream applications.

Complementary DNA (cDNA) synthesis

Extracted RNA was converted into cDNA via reverse transcription using the miRCURY LNA reverse transcription kit. The reaction mix was incubated at 42°C for 60 m to allow the reverse transcriptase enzyme to synthesize cDNA from RNA. The reaction was then inactivated by heating at 95°C for 5 m, followed by cooling to 4°C for storage.

Real-time PCR amplification

The levels of hsa_circ_101555 were amplified using the designed primers in a real-time PCR setup. The RT2 SYBR Green qPCR Mastermix Kit was used for amplification. This kit contains SYBR Green dye, which binds double-stranded DNA and emits fluorescence, allowing quantification of PCR products in real time.

Housekeeping gene

The Hs_ACTB_1_SG QuantiTect Primer Assay was used as the housekeeping gene to normalize hsa_circ_101555 expression levels. Normalization accounts for variations in RNA quantity and quality across samples.

PCR analysis

Samples were analyzed using the 5-plex Rotor Gene PCR Analyzer. This instrument detects fluorescence emitted during PCR, which correlates with the amount of amplified product.

Data analysis

The delta-delta Ct (ΔΔCt) method was employed to analyze PCR results. This method compares hsa_circ_101555 expression levels in experimental samples to positive controls. Relative gene expression changes were calculated using the formula 2-ΔΔCt, providing a quantitative measure of circRNA levels in serum.

By following these steps, the study accurately measured hsa_circ_101555 levels in serum samples, providing valuable data for assessing its potential as a biomarker for CLD.

Data management and statistical analysis

This section outlines the methods used to analyze the collected data:

Data analysis was performed using IBM SPSS Statistics version 22, a widely used software for statistical analysis offering comprehensive data management and analysis tools. Additional statistical calculations were conducted using NCSS PASS 11.0, which provides advanced statistical methods. The analysis framework was based on studies by Sun *et al*.[13] and Bedair *et al*.[14]

Descriptive statistics were employed to summarize demographic and clinical characteristics of participants. This included calculating frequencies and percentages for categorical variables, as well as means and standard deviations for continuous variables. Descriptive statistics provide a clear overview of the study population.

Comparative analyses:
- The independent sample t-test compared means of continuous variables between two independent groups (e.g., non-HCC CLD patients vs. healthy controls).
- The paired t-test assessed within-group changes, comparing measurements taken from the same subjects under different conditions or times.
- The chi-square test analyzed associations between categorical variables to determine significant relationships.

Analysis of variance: Analysis of variance was used to compare means across more than two groups, identifying statistically significant differences among groups.

Fisher's exact test: Applied when expected cell counts were less than 5 in 20% or more of cells. This test is suitable for small sample sizes to evaluate nonrandom associations between categorical variables.

Correlation analysis: Pearson's correlation coefficient (r) was used to evaluate linear relationships between continuous variables, such as between circRNA levels and liver function markers.

Receiver operating characteristic curve analysis: Receiver operating characteristic curve analysis validated the diagnostic accuracy of biomarkers. The area under the curve (AUC) was used as an indicator of discriminatory ability, with p-values < 0.05 considered statistically significant.

By employing these statistical methods, the study rigorously analyzed data, drew meaningful conclusions, and assessed the potential of hsa_circ_101555 as a diagnostic biomarker for CLD.

Results

Demographic, clinical, and laboratory characteristics of CLD patients

The demographic and clinical characteristics revealed several key findings. Females constituted the majority (60.0%), and most patients were overweight (60.0%). Half of the patients (50.0%) had at least one comorbidity, with diabetes mellitus being the most prevalent (26.7%). Causes of liver disease include SLD steatotic liver disease, bilharzial infections (60.0%), and viral causes, primarily hepatitis C virus (40.0%). Advanced disease features such as hepatosplenomegaly (60.0%), ascites (60.0%), jaundice (40.0%), and hepatic encephalopathy (10.0%) are illustrated in Table 1.

The clinical, laboratory, and fibrosis score parameters provided valuable insights into patients' health status (Supplementary Tables 1 and 2). The mean age of the cohort was 55.50 ± 13.32 years, representing a middle-aged to elderly population. The average body mass index (BMI) was 27.33 ± 2.97, indicating an overweight status. Hematological parameters showed anemia (mean Hb: 10.95 ± 1.63 g/dL) and thrombocytopenia (mean platelets: 161.80 ± 107.68 × 10^3/μL), consistent with CLD effects. Elevated total and direct bilirubin levels (3.01 ± 2.63 mg/dL and 1.53 ± 1.37 mg/dL, respectively) suggested significant liver dysfunction. Liver enzyme levels showed an elevated aspartate aminotransferase/alanine aminotransferase ratio (2.17 ± 1.70), often associated with advanced fibrosis. Child-Turcotte-Pugh (CTP) scores indicated that most patients were classified as Late B (40.0%) or Class C (30.0%), reflecting severe liver dysfunction. The model for end-stage liver disease (MELD) and MELD-Na scores averaged 15.80 ± 4.14 and 19.80 ± 7.04, respectively, indicating moderate disease severity with a substantial risk of complications. The mean albumin-bilirubin score of −1.60 ± 0.45 showed that most patients (70.0%) were in Grade 2, reflecting moderate liver impairment.

Inflammatory markers such as neutrophil-to-lymphocyte ratio (NLR) (8.40 ± 5.25) and platelet-to-lymphocyte ratio (PLR) (210.07 ± 118.50) indicated ongoing inflammation that may have correlated with disease severity. Fibrosis scores, including FIB-4 (4.85 ± 3.16), APRI (1.13 ± 0.82), and NFS (1.52 ± 2.85), highlighted significant hepatic fibrosis or cirrhosis in most patients. The mean circRNA value was 5.52 ± 2.49, suggesting its potential as a biomarker in this population.

Overall, these findings underscore the significant burden of liver dysfunction and systemic inflammation among CLD patients, emphasizing the need for close clinical monitoring and targeted interventions.

Evaluation of serum-derived circRNA in non-HCC CLD patients and healthy subjects

The role of circRNA value in differentiating non-HCC CLD patients from healthy controls

The diagnostic accuracy of the circRNA value in differentiating patients from healthy controls is presented in Table 2 and Figure 2. At the cutoff point of 2.088, the test demonstrated perfect sensitivity (1.000), which was critical for early diagnosis and intervention. The specificity at this cutoff was 90% (1 - specificity = 0.100).

The AUC was 0.970, indicating excellent diagnostic performance, as it was close to the maximum value of 1.0. The standard error of 0.019 reflected a precise estimate, while the highly significant p-value (0.000) confirmed that the observed AUC was unlikely to be due to chance. The 95% confidence interval (0.933–1.000) further supported the robustness of this diagnostic tool, as the lower bound remained well above 0.5, which indicated no discriminatory ability.

Overall, these findings suggested that the circRNA value was a reliable biomarker for distinguishing CLD patients from healthy subjects. The optimal cutoff point balanced high sensitivity with acceptable specificity, making the cutoff of 2.088 particularly effective for clinical use by prioritizing the identification of all diseased cases while maintaining a relatively low false-positive rate.

Relationship between circRNA and CLD patients' demographic, clinical, and laboratory data

The relationship between circRNA values and various demographic and clinical characteristics in these patients (Table 3 and Supplementary Table 3) highlighted the following: The mean circRNA value was higher in females (5.86 ± 2.68), although the difference was not statistically significant (p = 0.375). Similarly, circRNA levels varied across BMI categories, with the highest mean value observed in overweight patients (5.97 ± 2.47), followed by obese patients (5.54 ± 2.50), but without a statistically significant difference (p = 0.124). Patients with comorbidities showed a slightly higher mean circRNA value (5.76 ± 2.50), though this association was not significant (p = 0.606).

Regarding etiology, the highest mean circRNA level was observed in patients with SLD (8.21 ± 0.00), followed by hepatitis C virus (5.15 ± 2.91), and bilharzial patients (4.70 ± 0.62); however, these differences were not statistically significant (p = 0.221). Overall, the results suggested no significant relationship between circRNA values and the demographic characteristics assessed in this patient cohort.

On the other hand, higher circRNA levels were observed in cases with hepatic encephalopathy (8.21 ± 0.00, *p = 0.047) and ascites (6.53 ± 2.68, **p = 0.005), suggesting an association between elevated circRNA and these conditions. Additionally, higher circRNA values were noted in patients with jaundice (6.55 ± 3.01, *p = 0.065) and hepatosplenomegaly (6.28 ± 2.86, *p = 0.123), although these differences were not statistically significant, indicating a potential trend worthy of further investigation. These results highlighted specific clinical features that may have influenced circRNA levels, particularly those reflecting disease severity and complications.

Regarding the correlation between circRNA levels and various laboratory measurements (Supplementary Table 4), significant

Table 1. Demographic characteristics of chronic liver disease patients

Variables	No.	%
Gender		
Male	12	40.0%
Female	18	60.0%
BMI categories		
Normal	3	10.0%
Overweight	18	60.0%
Obese	9	30.0%
Comorbidities		
No	15	50.0%
Yes	15	50.0%
Etiology		
HCV	12	40.0%
HBV	0	0.0%
HCV + HBV	0	0.0%
SLD	12	40.0%
Bilharzial	6	20.0%
Etiology categories		
Viral	12	40.0%
Non-viral	18	60.0%
HTN		
No	27	90.0%
Yes	3	10.0%
DM		
No	22	73.3%
Yes	8	26.7%
Renal impairment		
No	27	90.0%
Yes	3	10.0%
HSM		
No	6	20.0%
Splenomegaly only	3	10.0%
Hepatosplenomegaly	18	60.0%
Hepatomegaly only	3	10.0%
Jaundice		
No	18	60.0%
Yes	12	40.0%
Ascites		
No	12	40.0%
Yes	18	60.0%
HE		
No	27	90.0%
Yes	3	10.0%
Performance score		
ECOG 0	27	90.0%
ECOG 1	3	10.0%
ECOG 2	0	0.0%
ECOG 3	0	0.0%

BMI, body mass index; DM, diabetes mellitus; ECOG, eastern cooperative oncology group; HBV, hepatitis B virus; HCV, hepatitis C virus; HE, hepatic encephalopathy; HSM, hepatosplenomegaly; HTN, hypertension; SLD, steatotic liver disease.

findings included: A strong positive correlation with total bilirubin (r = 0.622, p = 0.000), Direct bilirubin (r = 0.473, p = 0.008), NLR (r = 0.774, p = 0.000), and PLR (r = 0.727, p = 0.000). These findings suggested a potential link between elevated circRNA and markers of liver dysfunction and inflammation. Other variables showed a moderate positive correlation, such as aspartate aminotransferase (r = 0.463, p = 0.010), while the albumin-bilirubin score (r = 0.360, p = 0.051) approached significance. Additionally, circRNA levels were significantly higher in patients with elevated inflammatory markers such as NLR and PLR (p = 0.001* for both), indicating its potential role as a biomarker for inflammation (Supplementary Table 5). Higher circRNA levels were also observed in patients with advanced liver disease, reflected by significant associations with CTP scores (p = 0.000*) and MELD categories (p = 0.023**), suggesting relevance to liver function severity. These results highlighted the association of circRNA with liver disease severity, emphasizing its potential as a biomarker for liver disease complications and immune-inflammatory activity. However, further research is needed to confirm these findings and clarify the underlying mechanisms.

The role of circRNA values in differentiating non-HCC CLD cases, CLD with HCC patients, and healthy controls (supported by published phase I study)

Comparison of circRNA values among non-HCC CLD cases, CLD with HCC patients, and healthy controls showed a statistically significant difference (p = 0.000). The mean circRNA value was highest in CLD with HCC cases (7.66 ± 3.74), followed by CLD patients without HCC (5.52 ± 2.49), and lowest in healthy controls (1.21 ± 0.96). This significant elevation of circRNA values in HCC cases compared to both non-HCC cirrhotic cases and healthy controls suggested a potential role for circRNA as a biomarker for distinguishing HCC from cirrhosis without HCC and healthy states. These findings highlighted the utility of circRNA in identifying and potentially monitoring liver disease progression, particularly HCC. Further research could explore its diagnostic and prognostic applications (Table 4, Fig. 3).

Additionally, the comparison between CLD with and without HCC cases regarding biochemical and prognostic markers suggested that while non-HCC cirrhotic cases exhibit greater liver dysfunction and fibrosis, those with HCC are characterized by elevated tumor markers and increased systemic inflammation, underscoring the distinct disease progression pathways in each group (Table 5).

Table 2. Optimal cutoff points and corresponding diagnostic performance of circRNA values for differentiating diseased patients from healthy controls

Area under the curve				
Test result variable(s): circRNA value				
Area	Std. error[a]	Asymptotic Sig.[b]	Asymptotic 95% confidence interval	
			Lower bound	Upper bound
.970	.019	.000**	.933	1.000

Coordinates of the curve		
Test result variable(s): circRNA value		
Positive if greater than or equal to[a]	Sensitivity	1 - Specificity
-.5310	1.000	1.000
.5095	1.000	.900
.6375	1.000	.800
.7485	1.000	.700
.8420	1.000	.600
.9515	1.000	.500
1.0340	1.000	.400
1.0805	1.000	.300
1.3930	1.000	.200
2.0880	1.000	.100
2.6385	.900	.100
3.0340	.800	.100
3.5605	.700	.100
3.9940	.700	.000
4.4230	.600	.000
4.9905	.500	.000
5.5965	.400	.000
7.0670	.300	.000
8.4120	.200	.000
9.2225	.100	.000
10.8290	.000	.000

**Highly statistically significant. [a]Under the nonparametric assumption. [b]Null hypothesis: true area = 0.5. [c]The smallest cutoff value is the minimum observed test value minus 1, and the largest cutoff value is the maximum observed test value plus 1. All other cutoff values are the averages of two consecutive ordered observed test values. circRNA, circular RNA.

Discussion

Diagnosing CLD is crucial for managing the condition and preventing its progression and related health complications. However, many hepatologists find it challenging to use non-invasive markers for early diagnosis.[1–3] Nowadays, circRNAs are emerging as promising diagnostic markers for liver disease and its complications, including HCC.[6,10,11] Recently, our published phase I study identified serum-derived hsa_circ_101555 as a potential diagnostic and prognostic biomarker for CLD patients with HCC compared to healthy individuals.[11] Subsequently, in this current phase II study, we evaluated the role of hsa_circ_101555 in 30 CLD patients without HCC compared to 30 healthy subjects. We aimed to assess its clinical utility as a diagnostic biomarker and to correlate it with other standard non-invasive measures of liver dysfunction and inflammation, while also evaluating circRNA levels among non-HCC CLD patients, those with HCC, and healthy controls.

Recent studies using high-throughput sequencing techniques have shown that circRNAs are a promising new class of non-coding RNAs that can serve as biomarkers. These circRNAs have been found in human serum, tissues, and more recently, in exosomes. Their potential role in the development of various diseases, particularly cancers such as lung, breast, urinary tract, gastric, pancreatic, and liver cancers, has attracted significant research interest.[13–18] In hepatology, circRNAs have been observed to have dual effects on the development of liver fibrosis,

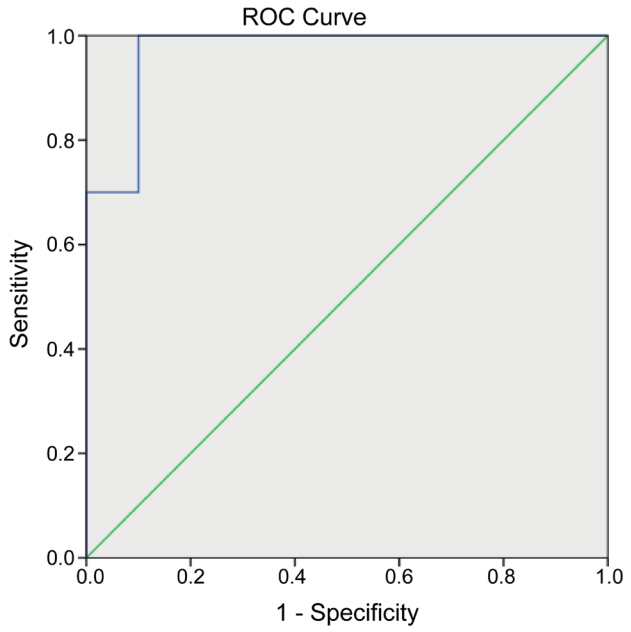

Fig. 2. ROC curve displaying the diagnostic value of circRNA to differentiate between diseased patients and healthy controls. circRNA, circular RNA; ROC, receiver operating characteristic.

depending on their ability to interact with miRNAs. For instance, *in vitro* studies have demonstrated that circUbe2k can promote liver fibrosis by enhancing the TGF-β2 pathway through sponging miR-149-5p, contributing to the progression of liver fibrosis induced by carbon tetrachloride (CCl4).[19,20] Additionally, abnormal levels of circRNAs have been linked to tumor progression, including disruptions in cell cycle regulation and proliferation, suggesting their potential as early detection biomarkers. A growing number of circRNAs, whether upregulated or downregulated, have been identified as playing significant roles in the pathophysiology of advanced liver disease and HCC, which may help guide treatment strategies. Notable examples include hsa_circ_101555, hsa_circ_000996, hsa_circ_101094, hsa_circ_100053, and hsa_circ_104760.[11,21–26]

Regarding hsa_circ_101555, our phase I study by Gado *et al.*[11] reported its upregulation in CLD patients with HCC, demonstrating significant diagnostic accuracy for HCC. Furthermore, high expression levels of hsa_circ_101555 were linked to tumor progression, as indicated by the RECIST/mRECIST response categories, suggesting its potential as a predictor of disease response. Notably, Gu *et al.*[27] highlighted the bioinformatic role of hsa_circ_101555 in diseased liver tissue with HCC, showing it was conserved and significantly upregulated in three GSE datasets from the Gene Expression Omnibus database, ranking among the top 25 upregulated differentially expressed circRNAs. This circRNA has been identified as an oncogenic promoter, facilitating HCC cell proliferation, migration, and invasion via the miR-145-5p/CDCA3 signaling axis. However, the precise mechanisms by which circRNAs contribute to advanced fibrosis, carcinogenesis, and metastasis remain to be fully elucidated and warrant further investigation.[11,22,27]

Our phase II research demonstrated the potential of hsa_circ_101555 as a diagnostic tool for identifying early CLD in patients without HCC. We found that serum levels of this circRNA can effectively differentiate between diseased patients and healthy individuals, with a specific cutoff point of 2.088. The diagnostic accuracy was indicated by an AUC of 0.970, suggesting that hsa_circ_101555 is a reliable biomarker for clinical use, maintaining a low false-positive rate. Additionally, our findings revealed a significant correlation between elevated levels of hsa_circ_101555 and markers of liver inflammation, such as the NLR and the PLR. We also observed associations with non-invasive parameters as-

Table 3. Relationship between circRNA and patients' demographics

Variables	circRNA value		Independent sample t-test	*p*-value
	Mean	±SD		
Gender				
Male	5.02	2.18	0.811	0.375
Female	5.86	2.68		
BMI categories				
Normal	2.80	.00	2.262#	0.124
Overweight	5.97	2.47		
Obese	5.54	2.50		
Comorbidities				
No	5.28	2.54	0.272	0.606
Yes	5.76	2.50		
Etiology				
HCV	5.15	2.91	1.568#	0.221
SLD	8.21	.00		
Bilharzial	4.70	.62		

#A one-way ANOVA test was used to compare more than two independent group means. ANOVA, analysis of variance; BMI, body mass index; circRNA, circular RNA; HCV, hepatitis C virus; SD, standard deviation; SLD, steatotic liver disease.

Table 4. Comparison between healthy controls, CLD with HCC cases, and non-HCC cirrhotic cases regarding circRNA value

circRNA value	Type						One-way ANOVA	*p*-value
	Healthy controls		HCC cases		Non-HCC cirrhotic controls			
	Mean	±SD	Mean	±SD	Mean	±SD		
	1.21	.96	7.66	3.74	5.52	2.49	47.230	0.000**

**Highly statistically significant at $p < 0.01$. ANOVA, analysis of variance; circRNA, circular RNA; CLD, chronic liver disease; HCC, hepatocellular carcinoma; SD, standard deviation.

sessing liver disease severity, including CTP and MELD scores. This indicates that hsa_circ_101555 may serve as a biomarker not only for diagnosing CLD but also for evaluating liver inflammation and disease severity. Finally, when comparing circRNA values among different groups, healthy controls, patients with HCC, and those with non-HCC CLD, we found statistically significant differences ($p = 0.000$). The highest circRNA values were observed in HCC cases (7.66 ± 3.74), followed by non-HCC CLD patients (5.52 ± 2.49), with the lowest values in healthy controls (1.21 ± 0.96). This further supports the potential of hsa_circ_101555 as a valuable biomarker in distinguishing these groups.

In summary, the understanding of the mechanisms and clinical applications of serum circRNA hsa_circ_101555 in liver disease and HCC is still evolving. Notable research includes the study by Gu *et al.*,[27] which explored the bioinformatic role of hsa_circ_101555 in diseased liver tissue and HCC, and our own phase I study by Gado *et al.*,[11] which demonstrated its clinical utility in CLD patients with HCC. These initial findings motivated our team to further investigate hsa_circ_101555 as a biomarker in patients with CLD at earlier stages, particularly those without HCC. However, limitations of our current research include a small sample size and lack of longitudinal data to track changes in hsa_circ_101555 levels over time and their correlation with disease progression. Additionally, comparisons with non-invasive elastography tools could enhance the assessment of its diagnostic accuracy. These limitations highlight the need for further studies with larger cohorts to confirm the clinical utility of hsa_circ_101555 as a biomarker for CLD.

Conclusions

hsa_circ_101555 upregulates in CLD patients, showing potential diagnostic accuracy in differentiating diseased patients from healthy subjects. Moreover, its high expression correlates with markers of liver inflammation and dysfunction. Hence, our findings suggest that hsa_circ_101555 may serve as a valuable auxiliary biomarker for identifying and potentially monitoring the progression of liver diseases. Further research with larger cohorts is needed to validate its clinical utility and establish its role alongside other non-invasive parameters for CLD.

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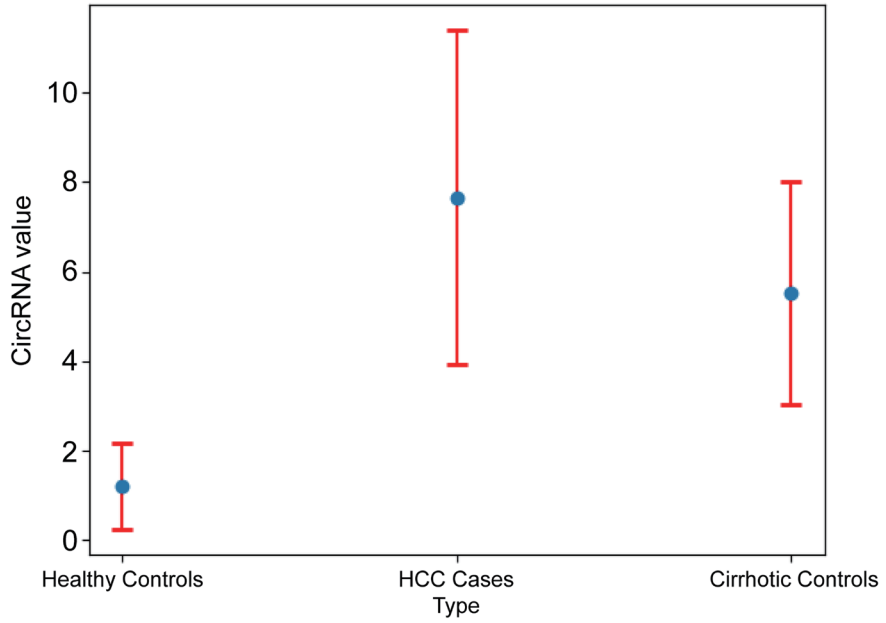

Fig. 3. Comparison between healthy controls, CLD with HCC cases, and non-HCC cirrhotic cases regarding circRNA value. circRNA, circular RNA; CLD; chronic liver disease, HCC; hepatocellular carcinoma.

Table 5. Comparison of biochemical and prognostic markers between CLD with and without HCC cases

Variables	Type				Chi square	*p*-value
	CLD with HCC cases		Non-HCC cirrhotic cases			
	No.	%	No.	%		
AFP categories						
Normal (0–19)	10	16.1%	30	100.0%	59.662 FE#	0.000**
Mildly elevated (20–100)	10	16.1%	0	0.0%		
Moderately elevated (101–200)	10	16.1%	0	0.0%		
Severely elevated (201–1,000)	20	32.3%	0	0.0%		
Extremely elevated (>1,000)	12	19.4%	0	0.0%		
ALBI categories						
Grade I (≤−2.60)	20	32.3%	0	0.0%	17.851	0.000**
Grade 2 (>−2.60-≤−1.39)	38	61.3%	21	70.0%		
Grade 3 (>−1.39)	4	6.5%	9	30.0%		
CRP categories						
Normal (≤6)	18	29.0%	24	80.0%	21.168	0.000**
Elevated (>6)	44	71.0%	6	20.0%		
NLR categories						
Normal (1–5)	22	35.5%	12	40.0%	45.170	0.000**
Mild (6–8)	16	25.8%	3	10.0%		
Moderate (9–18)	0	0.0%	15	50.0%		
Severe (>18)	24	38.7%	0	0.0%		
PLR_categories						
Low levels (<106)	6	9.7%	6	20.0%	2.082	0.353
Normal levels (106–300)	32	51.6%	15	50.0%		
High levels (>300)	24	38.7%	9	30.0%		
ALT AST ratio categories						
Low (≤1)	16	25.8%	6	20.0%	0.375	0.540
High (>1)	46	74.2%	24	80.0%		
FIB 4 categories						
Stage 0–1 (<1.45)	12	19.4%	6	20.0%	7.178	0.028*
Stage 2–3 (1.45–3.25)	22	35.5%	3	10.0%		
Stage 4–6 (stage 4–6)	28	45.2%	21	70.0%		
APRI categories						
No significant fibrosis (<0.07)	30	48.4%	9	30.0%	7.067	0.029*
Significant fibrosis (0.7–1)	18	29.0%	6	20.0%		
Severe fibrosis/Cirrhosis (>1)	14	22.6%	15	50.0%		
NFS categories						
F0-F2 (<−1.455)	12	19.4%	6	20.0%	7.178	0.028*
Indeter. score (−1.455–0.675)	22	35.5%	3	10.0%		
F3-F4 (>0.675)	28	45.2%	21	70.0%		
CTP score						

(continued)

Table 5. *(continued)*

Variables	Type				Chi square	*p*-value
	CLD with HCC cases		Non-HCC cirrhotic cases			
	No.	%	No.	%		
A	47	75.8%	9	30.0%	31.697 FE#	0.000**
Early B	5	8.1%	0	0.0%		
Late B	10	16.1%	12	40.0%		
C	0	0.0%	9	30.0%		
MELD categories						
Low risk (<10)	48	77.4%	0	0.0%	57.944 FE#	0.000**
Moderate risk (10–19)	14	22.6%	27	90.0%		
High risk (20–30)	0	0.0%	3	10.0%		
Very high (>30)	0	0.0%	0	0.0%		

AFP levels showed a highly significant difference ($p = 0.000$), with over half of the CLD with HCC cases exhibiting markedly elevated levels (>200 ng/mL). Markers of systemic inflammation, such as CRP and NLR, were significantly higher in CLD with HCC cases ($p = 0.000$), highlighting a potential link between inflammation and tumor progression. Fibrosis scores (FIB-4, APRI, NFS) were significantly higher in non-HCC cirrhotic controls, indicating more advanced liver fibrosis in this group. In addition, non-HCC cirrhotic cases were predominantly in the more severe CTP B/C and moderate-to-high MELD risk categories. *Statistically significant at $p \leq 0.05$. **Highly statistically significant at $p < 0.01$. #Fisher's Exact test was used as 20.0% or more of the cells had an expected count less than 5. AFP, alpha-feto protein; ALBI, Albumin-Bilirubin score ; ALT, aspartate aminotransferase; APRI, AST to Platelet Ratio Index; AST, alanine aminotransferase; CLD, chronic liver disease; CRP, C. reactive protein; CTP, Child-Turcotte-Pugh; FE, Fisher's Exact test; FIB-4, Fibrosis-4; HCC, hepatocellular carcinoma; MELD, Model for End-Stage Liver Disease score; NFS, Nonalcoholic Fatty Liver Disease Fibrosis Score; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio.

Conflict of interest

The authors declare no competing interests. The authors declare no use of generative artificial intelligence or artificial intelligence-assisted technologies in the writing process.

Author contributions

Data collection (NB, ATAM, NE, MSG), study design, writing of the manuscript, revision, editing, supervision, validation, and visualization (NB, ATAM, MSG). All authors have approved the final version and publication of the manuscript.

Ethical statement

Our study was reviewed and approved by the Research Ethics Committee based on the Helsinki ethical guidelines, at the Faculty of Medicine, Ain Shams University (FWA 000017585; FMASU MD 234/2022). The study conformed to the ethical guidelines of the Helsinki Declaration (as revised in 2024). All subjects were informed and provided written informed consent before the start of the study, preserving the rights and privacy of patients' data. Written informed consents were obtained from the participants for the publication of this study.

Data sharing statement

All data generated or analyzed during this study are included in this published article. Additional data will be available from the corresponding author upon reasonable request.

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