RESEARCH ARTICLE

Editorial Process: Submission:12/21/2023 Acceptance:04/07/2024

Evaluation of Circular RNA SMARCA5 as a Novel Biomarker for Hepatocellular Carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) is the fourth most prevalent type of cancer in Egypt and the sixth globally. Most patients with HCC are typically diagnosed during the advanced stages of the disease due to the absence of biomarkers for early detection. Consequently, these patients miss the optimal timeframe for receiving therapy. **Objective:** we aimed to assess the circular RNA SMARCA5 level and SMARCA5 mRNA gene expression as a potential biomarker for early detection of HCC. **Methods:** The present study utilized a case-control design comprising 159 participants. Participants were selected from both inpatient and outpatient hepatology and gastroenterology clinics at the National Liver Institute Hospital, Menoufia University. They were evenly distributed among three groups: Group I: 53 control subjects, Group II: 53 HCV cirrhotic patients, and Group III: 53 HCC patients. Tumor staging was done using BCLC staging system. Each patient underwent a thorough clinical examination, radiological examination, complete history taking, and serum Alpha-fetoprotein (AFP) assessment and detection of circular RNASMARCA5 and SMARCA5mRNA gene sutilizing quantitative real-time polymerase chain reaction. **Results:** Statistically substantial differences were observed in the examined groups in terms of AFP, SMARCA5, and CircSMARCA5 (P-value = 0.001, 0.001 & 0.001). CircSMARCA5 and SMARCA5mRNA were markedly down regulated in the HCC group compared to HCV cirrhotic patients and controls. ROC analysis for early HCC diagnosis demonstrated that the CircSMARCA5 area under the curve (AUC) at cut-off point 4.55 yielded a specificity of 83.8% and sensitivity of 91.7%. The AUC for AFP at a cut-off point of 515ng/ml yielded a specificity of 89.2% and a sensitivity of 91.3%. **Conclusion:** CircSMARCA5 has the potential to be a more sensitive predictor of HCC disease compared to AFP.

Keywords: Alpha-fetoprotein- CircSMARCA5- Hepatocellular Carcinoma

Asian Pac J Cancer Prev, **25 (4)**, 1411-1417

Introduction

Hepatocellular carcinoma (HCC) is a widespread problem worldwide, and variations in its epidemiological data across different geographical regions have been identified. Furthermore, it ranks as the fourth most prevalent type of cancer in Egypt and the sixth globally [1, 2]. With a population of 15 million, Egypt ranks the 15th globally and the third in Africa [3, 4].

Most HCC patients are typically diagnosed at advanced stages, leading to missed chances for treatment due to the lack of an early diagnostic biomarker. Therefore, early detection of HCC in patients seems to be a potentially effective strategy for enhancing their prognoses [5, 6]. Numerous biomarkers have been documented as diagnostic for HCC in recent decades. However, their sensitivity and specificity continue to be below ideal levels. As a result, there is an urgent need to find new biomarkers that can enhance early HCC detection [7, 8].

Endogenous non-coding RNAs, a newly identified class known as circular RNAs (circRNAs), often exhibit expression patterns specific to particular tissues or developmental stages [9]. Mounting evidence indicates that the dysregulation of circRNAs is implicated in various diseases, including cancer. Moreover, it plays a vital role in determining the clinical outcome, apoptosis, tumor invasion, and growth [10]. Due to their high degree of stability and conservation, circadian RNA scan be considered ideal biomarkers for cancer diagnosis.

CircSMARCA5, a circRNA, is found to be associated with dysregulation in various noninfectious diseases such as atherosclerosis, osteoporosis, tumors, and coronary

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heart disease. Furthermore, circSMARCA5 has been associated with the progression and occurrence of various tumors, such as multiple myeloma, glioblastoma, HCC, gastric cancer, breast cancer, colorectal cancer, and osteosarcoma [11]. We aimed to estimate the level of circular RNA SMARCA5 and CircSMARCA5 mRNA gene expression as novel biomarkers for early HCC detection.

Materials and Methods

The study was conducted as a case-control study involving159 subjects, and they were equally categorized into three groups: Group I: 53control subjects, Group II: 53 HCV-related liver cirrhosis patients, and Group III: 53 HCC patients. Liver cirrhosis and HCC patients were recruited from the Hepatology and Gastroenterology Department inpatient ward and outpatient HCC clinic (National Liver Institute-Menoufia University). In addition, the study was carried out at both Hepatology and Gastroenterology, and Clinical Pathology departments (National Liver Institute-Menoufia University) during the period from August 2022 to May 2023.

Inclusion criteria: Apparently, healthy blood donors with no history of liver disease comprised the control group. Patients diagnosed with hepatitis C-associated cirrhosis have the following characteristics: Ultrasound-confirmed cirrhosis of HCV origin, positive serum anti-HCV antibodies, spiral cycle threshold (CT) scans, and the HCC absence as indicated by the focal liver lack of a mass on ultrasonography or CT scan. High-risk patients satisfied the diagnostic criteria for HCC, including a hepatic focal lesion, serum AFP levels not falling below 200ng/ml, or a triphasic CT scan exhibiting characteristic HCC characteristics (cirrhotic patients). A triphasic CTscan, an AFP level of at least 200ng/ml, or the focal lesion presence measuring at least 1cm indicated the HCC-type criteria presence. When an abdominal triphasic CT scan revealed characteristic features of HCC or alpha-fetoprotein (AFP) level ≥ 200ng/ml in the focal lesion presence ≥ 1cm, a dynamic contrast MRI or targeted liver biopsy was done. Patients who were under the age of 18, those who had other types of liver tumors (such as cholangiocarcinoma or combined cholangiocarcinoma and HCC), or those with tumors in organs other than the liver were excluded from participating in the study.

Valid consents were obtained from patients or caregivers before involvement in the study. All study procedures were conducted following the declaration of Helsinki. In addition, the Ethical Committee (National Liver Institute-Menoufia University; 00003413 FWA0000227 with (IRB No: 00428) approved the study. In addition, all procedures adhered to the declaration of Helsinki. The participants' names have been recorded in the database and linked only with a study identification number for the purpose of this research. Each patient underwent complete history taking, as well as clinical, radiological, and laboratory examinations.

Procedure

Sampling

Through sterile veni-puncture, 9mL blood samples were collected and then divided into four tubes. One tube containing 3mL was used for serum preparation for liver and kidney function tests, and it contained a clot activator. Another tube, containing two mL, contained sodium citrate and was used for the preparation of platelet-poor plasma for coagulation studies. The remaining two tubes, each containing two mL, were withdrawn with K2 EDTA. One tube was used for a complete blood count, while the other tube was used for the separation and detection of circular RNA SMARCA5 and CircSMARCA5 mRNA genes.

The measurement of prothrombin time (PT) and international normalization ratio (INR) was done utilizing the Sysmex CS-1600 Automated hemostasis testing (Sysmex-Kobe-Japan). Complete blood count (CBC): was done utilizing Sysmex XN-1000 Automated Hematology Analyzer using hydrodynamic focusing technologies fluorescent flow cytometry (Sysmex, Kobe, Japan). All parameters of the liver function tests were done on the Cobas 6000 analyzer (c-501 module), an auto analyzer's photometric unit (Roche, Germany).Renal function tests were carried out using a cobasc6000 auto-analyzer (c 501 model) (Roche, Germany). AFP was carried out utilizing [(cobasc-6000 auto-analyzer (c-501 model) (Roche, Germany)].

Quantitative real-time PCR of circular RNA SMARCA5 was done by qRT-PCR utilizing the Rotor gene (Qiagen-Germany).

RNA extraction

Total the extraction of total plasma RNA was performed using the Whole Blood RNA Purification Mini Kit (Thermo Scientific-EU/Lithuania) according to the instructions provided by the manufacturer. Subsequently, 200 µl of blood was centrifuged at 12,000 rpm for 10 minutes at 4°C. The resulting plasma was separated from the cells, which were then mixed with lysis solution (600 µl lysis + 20 µl mercopto ethanol per sample) and vortexed. First, add 450 µl of ethanol to the solution. Then, vortex the mixture to ensure thorough mixing. Afterward, the mixture was transferred into a column with a volume of 700µl before centrifugation at a speed of 12000 rpm at a temperature of 23°C for duration of 10 minutes. After centrifugation, the solution was removed from the collection tube. The previous step was step as necessary. Then, 700µl wash was added and subjected to centrifugation at 12,000 rpm for 10 min at 23°C. We discarded the solution in the collection tube and then added 600µl wash (for centrifugation at 12,000 rpm for 10 min at 23°C). We discarded the solution in the collection tube before adding 250 µl wash and then subjected to centrifugation at 12000 rpm for 1 min at 23°C. We discarded the solution in the collection tube before adding 50µl from Rosny free water. We incubated the mixture for 5 min. Then, it was centrifuged at 12000 rpm at 23°C for 1 min. The RNA sample's yield and purity were assessed utilizing the Nano Drop instrument (Thermo Scientific, USA).

cDNA synthesis

The SensiFASTcDNA Synthesis Kit, manufactured by Bioline in Germany, was utilized for the initial step of cDNA synthesis. This involved the reverse transcription process to generate complementary DNA (cDNA). The reactions were conducted at a low temperature (on ice) with a combined volume of 20µL. The mixture consisted of nuclease-free water (5µL), template RNA (10µL), reverse transcriptase buffer (4µL), and reverse transcriptase enzyme (1µL). The sample was subsequently subjected to incubation in a 2720 thermal cycler (Applied Bio Systems-Singapore) for one cycle with the following conditions: at 42°C for10 min, followed by 5 min at 95°C to halt the reverse transcriptase enzyme, and subsequently, at 4°C for 5 min. Afterward, synthesized cDNA was kept at -20 °C until amplification utilizing real-time PCR.

Quantitative reverse transcriptase real-time PCR (qRT-PCR)

Initially, qRT-PCR was performed using SensiFASTTM SYBR Lo-ROX Kit, USA. Total PCR mix (20µL) contained 10µL of SYBR green Master Mix, 1µL of Nuclease-free water, template cDNA (1µL), and 1.5 µL of each primer (forward and reverse). The National Center for Biotechnology Information (NCBI) confirmed the primer sequence.

The primers utilized to analyze circSMARCA5, SMARCA5 mRNA genes, and the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained from Midland, Texas. These primers were employed as internal controls for circRNA and mRNA analysis. The primer sequences used for qRT-PCR were forward primers; 5′-CTCC AAGATGGGCGAAAG-3′, 5′-TGCAAA CTGACCGGGCAAATA-3′ (forward) and 5′-CAGG AGGCATTGCTGATGAT-3′ directed for circSMARCA5, SMARCA5 and GAPDH, respectively. Reverse primers were; 5′-TGTGTTGCTCC ATGTCTAATCA-3′, 5′-TCGCCAACG GATAGTAAGTTCT-3′ and 5′-GAAGGCTGG GGCTCATTT-3″ directed for circSMARCA5, SMARCA5 and GAPDH, respectively.

The PCR conditions utilized for amplification comprised three distinct phases: an initial activation phase lasting 5minutes at 95°C, 45 cycles of 60°C for 30 seconds, 95°C for 20 seconds, and 72°C for one minute; and a concluding extension phase lasting 10 minutes at 72°C. Data analysis and fluorescence detection were conducted using 7500 ABIPRISM v.2.0.1 (Applied Biosystems, USA). The relative quantification (RQ) of the gene expression was assessed through the utilization of the comparative 2-ΔΔCT technique [12].

The Comparative Ct method is calculated from the following equation:

Relative gene expression= (2)-[Δ] [Δ] Ct

Where; Δ ΔCt = ΔCt sample –ΔCt reference

Here, Δ Ct sample is the Ct value for any sample normalized to the endogenous housekeeping gene and Δ Ct reference is the Ct value for the calibrator also normalized to (the endogenous housekeeping gene) as well.

The software captures and displays the amplification plot, which shows a rise in the reporter signal (Rn) over time. This plot depicts the amount of product amplified (Rn) against CT. Evidently, CT value is utilized as a metric to determine the initial template amount in each sample. This phase is during which the fluorescence exhibits its initial perceptible and substantial rise.

The 21st version of the SPSS software was utilized for data collection and tabulation (IBM Corp., Released 2012). The results were deemed significant when the probability of error was < 5% (P value ≤ 0.05).

Results

There were no substantial differences regarding sex or age (P-value = 0.75 & 0.9, respectively). Mean age ±SD in the studied groups was (52.8±3.6; 56.6±5.7; 57.7±5.8, respectively) (Table 1). With respect to CBC, only platelet count showed statistically substantial differences among the three groups. It was significantly decreased in HCC patients compared to the other two groups p=0.001. There were marked differences regarding liver function tests among the study groups. Albumin was substantially down regulated in the HCC and Cirrhosis groups. In contrast, ALT, AST, GGT, ALP, total, and direct bilirubin were markedly elevated in the HCC group. There were no discernible differences in renal function tests between the groups (Table 2).

There were substantial differences among the study groups regarding AFP, CircSMARCA5 RNA, and CircSMARCA5 mRNA. CircSMARCA5 RNA and CircSMARCA5 mRNA were considerably diminished in the HCC group than in HCV cirrhotic patients and controls. Conversely, AFP level was markedly increased in group HCC when compared to group HCV cirrhotic

Table 1. Comparison of Demographic Data among the Studied Groups

Demographics	Group I (Control) N=53	Group II (Cirrhosis) N=53	Group III (HCC) N=53	Test of sig.	P- value
Gender N (%)					
Male	43 (81.1)	44 (83)	46 (86.8)	χ^2=0.59	0.75
Female	10 (18.9)	9 (17)	7 (13.2)		
Age (years)					
Mean ±SD	52.8±3.6	56.6±5.7	57.7±5.8	F=2.5	0.9
Range	48-66	47-65	48-66		

χ^2, Chi square test; SD, standard deviation; F, One-way ANOVA.

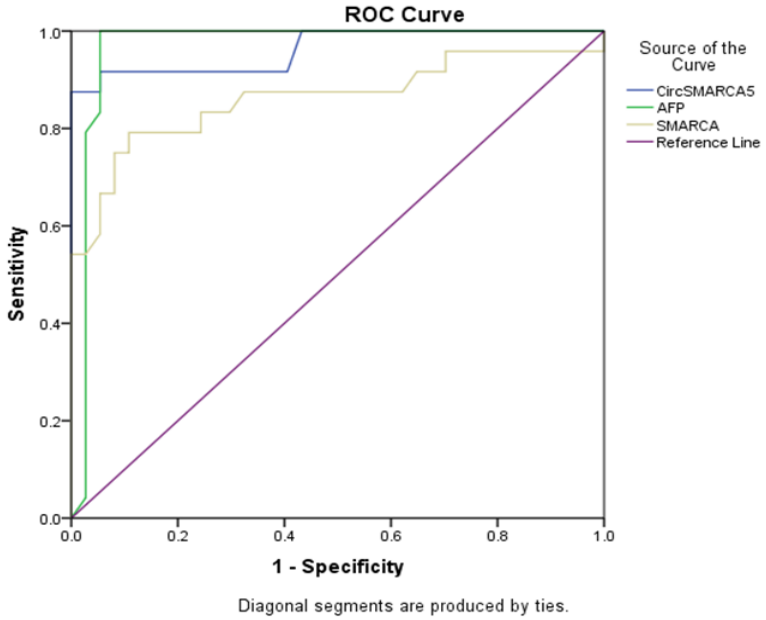

Figure 1. ROC Curve of CircSMARCA5, SMARCA5 and AFP for Prediction of HCC

patients and controls (Table 3).

The AUC of CircSMARCA5 at cut-off point 4.55 yielded a specificity of 83.8% and a sensitivity of 91.7%. Conversely, the AUC for AFP at the 515ng/ml cut-off

Table 2. Comparison between the Studied Groups Regarding Routine Laboratory Investigations

	Group I (Control) N=53	Group II (Cirrhosis) N=53	Group III (HCC) N=53	Test of sig.	p-value
Hb (gm/dl)	8.7±1.9	9.7±1.5	9.3±1.3	F=1.9	p=0.15
WBC (*10^9/L)	5.8±1.5	5.4±1.1	4.9±1.3	F=1.6	p=0.17
Platelet count (*103/mcL)	180 (126-253) 53-315	99 (88-118) 58-251	76 (58-76) 45-316	KW=16.3 p=0.001*	p1=0.04* p2=0.001* p3=0.003*
Albumin (gm/dl)	4.2±0.54	2.9±0.69	2.8±0.56	F=19.1 P=0.001*	p1=0.001* p2=0.001* p3=0.73
AST (IU/L)	21.4±4.8	84.3±28.1	69.9±16.1	F=18.8 P=0.001*	p1=0.01* p2=0.001* p3=0.02*
ALT (IU/L)	20 (19-27) 17-28	88 (68-106) 56-125	49 (45-62) 30-95	KW=30.4 P=0.001*	p1=0.001* p2=0.001* p3=0.001*
ALP (IU/L)	72.6±24.7 34-112	255.3±80.5 137-433	626.8±91.1 492-844	F=221.3 P=0.001*	p1=0.001* p2=0.001* p3=0.001*
GGT (U/L)	27.7±7.9	146.9±41.3	167.9±47.5 54-254	F=39.2 P=0.001*	p1=0.001* p2=0.001* p3=0.34
Total bilirubin (mg/dl)	0.5 (0.5-0.6) 0.4-0.7	1.4 (1.1-1.9) 1.1-1.9	2 (1.6-2.6) 0.6-3.5	KW=26.8 P=0.001*	p1=0.001* p2=0.001* p3=0.01*
Direct bilirubin (mg/dl)	0.2 (0.2-0.4) 0.2-0.5	0.8 (0.5-1.1) 0.3-1.5	0.8 (0.5-1.2) 0.1-2.1	KW=16.8 P=0.001*	p1=0.001* p2=0.001* p3=0.7
Serum creatinine (mg/dl)	1.1±0.15	1.2±0.26	1.2±0.23	F=0.31	0.74
BUN (mg/dl)	24.8±7.4	36.3±15.9	33.4±19.4	F=1.28	0.28

F, One-way ANOVA; KW, Kruskal Wallis test; p1, gp1 and gp2 difference; p2, gp2 and gp3 difference; p3, gp1 and gp3 difference;*, p-value<0.05: Significant.

Table 3. Comparison between the Studied Groups Regarding HCC Biomarkers

Biomarkers	Group I (Control) N=53	Group II (Cirrhosis) N=53	Group III (HCC) N=53	Test of sig.	p-value
AFP (ng/ml)					
Median	2.5	452	2500	KW=36.8	p1=0.01*
(IQR)	(1.9-3.2)	(310-782)	(1200-3650)	p=0.001*	p2=0.01*
Range	1.3-1500	115-1240	512-5300		p3=0.01*
SMARCA5					p1=0.01*
Mean ±SD	8.9±2.5	6.7±2.5	3.9±1.4	F=30.9	p2=0.01*
Range	3.2-12.3	1.3-10.4	1.7-7.8	p=0.001*	p3=0.01*
CircSMARCA5					p1=0.01*
Mean ±SD	11.4±1.8	7.6±2.3	2.7±1.5	F=106.7	p2=0.03*
Range	8.6-13.7	2.8-9.8	0.2-5.9	p=0.001*	p3=0.01*

F, One way ANOVA; KW, Kruskal Wallis test; p1, gp1 and gp2 difference; p2, gp2 and gp3 difference; p3, gp1and gp3 difference;*, p-value<0.05: Significant.

Table 4. Correlation between SMARCA5, CircSMARCA5 and each Demographics and Laboratory Investigations

	SMARCA5		CircSMARCA5	
	r	P	r	P
Age (years)	-0.22	0.09	-0.13	0.31
BMI (kg/m^2)	-0.09	0.45	-0.06	0.63
Hb. (gm/dl)	0.19	0.14	0.09	0.45
WBC (*10^9/L)	0.41	0.001*	0.43	0.001*
Platelet count (*10^3/mcL)	0.51	0.001*	0.42	0.001*
Albumin (gm/dl)	0.44	0.001*	0.37	0.004*
AST (IU/L)	0.009	0.95	0.01	0.9
ALT (IU/L)	0.12	0.39	0.11	0.38
ALP (IU/L)	-0.68	0.001*	-0.71	0.001*
GGT (U/L)	-0.49	0.001*	-0.5	0.001*
Total bilirubin (mg/dl)	-0.47	0.001*	-0.48	0.001*
Direct bilirubin (mg/dl)	-0.35	0.005*	-0.31	0.01*
Serum creatinine (mg/dl)	-0.12	0.35	-0.04	0.76
BUN (mg/dl)	-0.03	0.84	0.04	0.76
PT (seconds)	-0.02	0.87	-0.11	0.41
INR	-0.03	0.79	-0.12	0.42
AFP (ng/ml)	-0.66	0.001*	-0.65	0.001*
BCLC	-0.025	0.88	-0.11	0.54
SMARCA5	1		0.75	0.001*
CircSMARCA5	0.75	0.001*	1	

r, Spearman correlation coefficient; *, p-value<0.05: significant

point yielded a specificity of 89.2% and a sensitivity of 91.3% (Figure 1).

There was a significant positive correlation between both SMARCA5, CircSMARCA5, and each of white blood cells (WBC), platelet count, and albumin, while there was a significant negative correlation between both SMARCA5, CircSMARCA5 mRNA and each ALP, GGT, total bilirubin, direct bilirubin, and AFP (Table 4).

Discussion

In the present study, 86.8% of the HCC group was males, which is consistent with the findings of Ramadan and his colleagues, who demonstrated the predominance of HCC in males. In addition, they illustrated that variations in the level of exposure to risk factors could explain the importance of other genetic factors linked to other X-linked genetic factors and sex hormones [12].

In our study, we found that patients with HCC had significantly increased AFP compared to cirrhotic patients and controls. Our results were in accordance with the findings reported by Stewart and his colleagues. They discovered that HCC is linked to upregulated levels of AFP, with over 90% of patients exhibiting elevated levels ranging from 400 to 500 ng/ml [13].

Chan and his colleagues also discovered that AFP levels are below 200ng/ml in 965 benign liver conditions patients, such as cirrhosis and hepatitis [14]. Elevated serum AFP has been used as a marker for hepatic regeneration after the destruction of hepatocyte in viral hepatitis [6]. This finding is consistent with our research, which revealed that AFP levels are below 200ng/ml in nearly all cirrhotic patients. The present study showed that the ROC for AFP in differentiating suspected HCC cases is 0.92. The optimal cut-off for AFP was found to be 515ng/ml, yielding an overall accuracy of 81.3%. Consistent with our findings, Yvamoto and his colleagues observed that the AFP sensitivity was 28%, and its specificity was 99% when a reference value of 200 ng/ml was utilized [15].

Trevisani F and his colleagues obtained comparable findings by utilizing the same cut-off value (200ng/ml), with a specificity of 99.4%and a sensitivity of 22.4% [16]. In our study, we found that almost all cases of HCC have an AFP level > 200 ng/ml. In contrast, Daniele and his colleagues conducted a review study comparing studies that mainly used a cut-off value of 20ng/mL. They found that the specificity values ranged from 76% to 94%, while the sensitivity values ranged from 39% to 65% [17].

Cai and Chen Zuo X demonstrated that circ-SMARCA5 was diminished and inhibited antitumor effects such as cervical cancer, gastric cancer, non-small-cell lung cancer,

and HCC [18].

Chen et al. also found that the circ-SMARCA5 expression was diminished in HCC tissues. This reduction was associated with aggressive tumor characteristics and poor survival profiles among HCC patients. These findings were supported by in vivo experiments that demonstrated the effects of circ-inhibitory SMARCA5on HCC cell migration and proliferation [19]. Previous research confirmed our findings that plasma levels of SMARCA5 RNA and CircSMARCA5 mRNA were markedly down regulated in the HCC group compared to the other groups. According to these findings, circSMARCA5 may serve as a viable indicator for monitoring the progression of HCC.

According to the findings of Li et al., the plasma circSMARCA5 expression was decreased in HCC relative to the control group, hepatitis, and cirrhosis. Additionally, the circSMARCA5 levels in tissues and plasma decreased progressively from the control healthy group to those with hepatitis, cirrhosis,and HCC [7]. Yu et al. further demonstrated that overexpression of circSMARCA5 prevented metastasis and HCC growth in vivo and in vitro. They also observed a decrease in the levels of has-circ-0001445 (circSMARCA5) in 208 pairs of HCC samples compared to adjacent noncancerous liver tissues [20].

Our study revealed a non-significant negative correlation between the expressions of SMARCA5 and CircSMARCA5gene and the BCLC prognostic staging system. However, there was a significant negative correlation with liver function tests, specifically ALP, GGT, and total and direct bilirubin. This finding indicates that circSMARCA5 expression is negatively correlated with clinic-pathological features. Consistent with our findings, a prior investigation conducted by Yu et al. reported that down regulated circSMARCA5 expression in HCC tumor tissues was markedly linked to microvascular invasion occurrence, larger tumor size, more advanced tumor stage, and poorer tumor differentiation [20]. In contrast to our findings, a few studies showed that circSMARCA5 plays a tumor-promoting role in cervical cancer, prostate cancer, and bladder cancer [21, 22].

In conclusion, CircSMARCA5 may serve as a potential indicator for HCC cases with better accuracy and sensitivity than AFP. These results will contribute to improving patient management and outcomes.

Author Contribution Statement

All authors shared in: design of the work, conceptualization, resources detection, formal analysis, data curation, interpretation of data, creation of new software used in the work, validation and methodology plus revision.

Acknowledgements

All authors in this study participated in a good selection of cases, instructive supervision, continuous guidance, valuable suggestions and good instructions.

Ethics approval and consent to participate

A well informed written consent was obtained from all individuals included in the study. Our study was reviewed, approved by the ethical committee at the National Liver Institute.

Consent for publication

All authors gave consent with manuscript content.

On behalf of all authors, I have the pleasure for communication with your Journal. I wish that our research work is under your kind care and observation.

Availability of data and material

All data is available upon request from the first author dr Hanan M. Bedair.

Conflict of interests

No conflict of interest regarding the publication of this paper.

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