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The Emerging Role of Circular RNA Homeodomain Interacting Protein Kinase 3 and Circular RNA 0046367 through Wnt/Beta-Catenin Pathway on the Pathogenesis of Nonalcoholic Steatohepatitis in Egyptian Patients

Marwa Abdelgwad[1], Reem Zakaria*[1], Samar Marzouk[1], Dina Sabry[1], Rasha Ahmed[2], Hedy Ayman Badary[2], Mai Samir[1]

Abstract

Background: Non-alcoholic fatty liver disease is a major problem worldwide that needs non-invasive biomarkers for early diagnosis and treatment response assessment. We aimed to assess the correlation between circRNA-HIPK3 and miRNA-29a expression and its role as miRNA-29a sponge, as well as the correlation between circRNA-0046367 and miRNA-34a expression and its role as miRNA-34a sponge and their effect on regulation of the Wnt/β catenin pathway, which may provide a new target for treatment of non-alcoholic steatohepatitis.

Methods: the research was performed on 110 participants: group (I): fifty-five healthy donors served as controls and group (II): fifty-five patients with fatty liver pattern on abdominal ultrasound. Lipid profile and liver functions were assessed. RT-PCR was performed to assess the RNAs: circRNA-HIPK3, circRNA-0046367, miRNA-29a, miRNA-34a and *Wnt* mRNA gene expression. ELISA was performed to determine β-catenin protein levels.

Results: miRNA-34a and circRNA-HIPK3 expression were significantly greater, while miRNA-29a and circRNA-0046367 expression were significantly less, in patients than in controls. Wnt/β-catenin regulated by miRNA-29a and miRNA-34a showed a significant decrease that leads to its abnormal effect on lipid metabolism.

Conclusions: our results imply that miRNA-29a can be investigated as a target for circRNA-HIPK3, while miRNA-34a can be investigated as a target for circRNA-0046367, and that circRNA-HIPK3 and circRNA-0046367 may have emerging roles that can affect the pathogenesis of nonalcoholic steatohepatitis through the Wnt/β-catenin pathway and thus be used as therapeutic targets for the disease.

Keywords: Circrna-HIPK3, circRNA-0046367, miRNA-29a, miRNA-34a, Wnt/β-catenin NASH.

Introduction

Nonalcoholic fatty liver disease (NAFLD), the commonest cause of chronic liver disease, affects 25% of the world's population. Nonalcoholic steatohepatitis (NASH), a subclass of NAFLD, can advance to liver cirrhosis, hepatocellular carcinoma (HCC), and death (1). Studying the metabolic basis of NASH pathogenesis showed that accumulation of fat in liver induces lipotoxicity leading to oxidative and endoplasmic reticulum stress, mitochondrial damage, and hepatocellular dysfunction. Finally, the ensuing injury to the

1: Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Cairo University, Egypt.
2: Endemic Medicine Department, Faculty of Medicine, Cairo University, Egypt.
***Corresponding author: Reem Zakaria; Tel: +20 1004865515; E-mail: Reem.Z.Ebrahim@kasralainy.edu.eg.**
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cells causes immune-mediated hepatic damage and cell death, leading to activation of hepatic stellate cells (HSC) and fibrogenesis, then cirrhosis and HCC (2).

MicroRNAs (miRNAs) are small endogenous RNAs varying in length from 21–25 nucleotides. They have crucial regulatory roles in plants and animals by targeting various mRNAs for degradation or repressing their translation. miRNA regulation is involved in disease etiology and many preclinical/clinical trials have been performed to identify miRNA-dependent therapeutics (3). Increasing corroboration revealed that some miRNAs regulate pathways that control lipid metabolism, inflammation, and oxidative stress in the liver, thus having vital roles in the pathophysiology of NASH. For example, miRNAs-34a, -33, -21, -122, and -29 were recognized for their roles in regulating hepatic functions and their therapeutic capacities (4).

Unlike linear RNAs, circular RNAs (circRNAs) make a covalently fastened continuous loop in which the 3' and 5' ends are joined together. CircRNAs participate in epigenetic, transcriptional, and posttranscriptional regulation (5). Many studies have examined their role as efficient miRNA sponges, where most circRNA sponges are provided with miRNA-binding sites; thus, overexpression of circRNA leads to miRNA suppression and upregulation of miRNA target gene expression (6). Many studies reported the role of circRNAs in reducing lipotoxicity and oxidative stress in NASH. Examples include circRNA-0046367, circRNA-0046366, circRNA-0071410, and circRNA-homeodomain interacting protein kinase 3 (circHIPK3) (7,8,9). CircHIPK3 has been shown to be aberrantly expressed in a variety of diseases, contributing to disease initiation and progression (10).

β-catenin is part of the cadherin protein complex that acts as an intracellular signal transducer in the wingless related integration site (Wnt) signaling pathway. It interacts with and modulates the activity of many transcription factors, thus regulating gene expression that regulates glucose and lipid metabolism. Liver injury causes changes in signaling and localization of β-catenin. The resulting changes can affect reparative responses and disease development. Changes in β-catenin signaling can cause activation of HSCs and contribute to the pathogenesis of NASH (11).

The Wnt pathway was investigated in many cancers, with many cell signaling components and pathways being controlled by microRNAs (12).

Materials and Methods

Ethics statement

the research was accepted by the local ethical committee in Kasr Al-ainy Hospitals (Approval number: MD-82-2020). Written informed consent was obtained from all the participants.

Subjects

the study was conducted on 110 subjects of matched age and sex; the patients were enrolled from Outpatient Clinics of Hepato-Gastroenterology and Endemic medicine Department, Faculty of Medicine, Cairo University. Detailed medical histories were obtained and all the subjects were clinically examined.

Participants were classified into two groups: Group (I): fifty-five healthy donors volunteered for the study and served as controls. They had normal liver function tests and lipid profiles, and their livers appeared normal on abdominal ultrasounds. Group (II): fifty-five patients with fatty liver pattern on abdominal ultrasound showed hepatomegaly and decreased caliber of the intrahepatic vessels with increased echogenicity. Patient inclusion criteria: adults > 18 years, both sexes, patients with fatty liver pattern in abdominal ultrasound. Patient exclusion criteria: patients with evidence of decompensated liver cirrhosis, autoimmune disorder, viral hepatitis (HCV Ab + ve, HBsAg + ve, Anti HBc + ve), history of steatogenic drug intake, history of significant alcohol consumption (≥ 30 g/day for males and ≥ 20 g/day for females), or hepatocellular carcinoma.

Sample collection

five mL peripheral venous blood samples were collected from each participant by vacutainer system technique and withdrawn on serum separator tubes, and then centrifuged for 10 min at 4,000 x g. Serum was separated and used for biochemical analyses including lipid profile, liver functions, and ELISA for β-catenin. Another five mL were withdrawn, collected in EDTA vacutainer tubes, and centrifuged for 10 min at 4,000 x g. Plasma samples were collected and stored at -80 °C for later RNA extraction.

Biochemical analysis: the serum samples were used to assess lipid profiles, including HDL, LDL, triglycerides, and total cholesterol and liver functions, including total bilirubin, direct bilirubin, AST, ALT, ALP, GGT, and total proteins.

RNA extraction

was performed with the miRNeasy Mini Kit (Catalog Number# 217004) for purifying total RNA, including small RNAs.

Quantitative real-time PCR assessment

for circular RNA and ordinary mRNA gene expression TransScript® Green One-Step qRT-PCR SuperMix kit (Catalog Number# AQ211-01) was used. The kit was compatible with three-step cycling: reverse transcription (1 cycle) at 55°C for 10 minutes, enzyme activation (1 cycle) at 95 °C for 8 min, denaturation (40 cycles) at 95 °C for 10 sec and annealing and extension (40 cycles) at 60 °C for 60 seconds.

For miRNA gene expression TransScript® Green miRNA Two-Step qRT-PCR SuperMix kit (Catalog Number# AQ202-01) was used for reverse transcription of targeted miRNAs and quantitative RT-PCR. For cDNA synthesis total RNA, TransScript® miRNA RT enzyme mix, 2×TS miRNA reaction mix, and RNase-free water was incubated at 37 °C for one hr and then incubated at 85 °C for 5 sec to inactivate the RT enzyme mix.

Thermal cycling for qPCR reaction: pre-denaturation (1 cycle) at 94 °C for 30 sec, cycling reaction (٤٠ cycles) at 94 °C for 5 sec, then 60 °C for 30 sec, melting curve (١ cycle) at 95 °C for 15 sec, 60 °C for 60 sec, then 95 °C for 15 sec.

The studied gene primer sequences are shown in Table1. The reverse primer of all miRNAs was supplied in the kit as universal reverse primer.

Table 1. Primer sequences used in the study.

Gene	#Accession number	**Primer sequence from 5′- 3′** ***(F: Forward primer, R: Reverse primer)***
wnt 1	NM_005430.4	F: ATGAACCTTCACAACAACGAG
		R: GGTTGCTGCCTCGGTTG
Circ_HIPK3	NM_005734.5	F: TTCAACATATCTACAATCTCGGT
		R: ACCATTCACATAGGTCCGT
Circ_0046367		F: AGATTCCATCCTACGCTCCG
		R: GCTATGGAAGTGCAGGTTGG
miRNA 29a	NR_029503.1	F: GTTATGACTGATTTCTTTTGGTGTT
		R:
miRNA 34a	NR_029610.1	F: GGCCAGCTGTGAGTGTTTCT
		R:
β-Actin	NM_001101.5	F: GGC GGCACCACCATGTACCCT
		R: AGG GGCCGGACTCGTCATACT
RUN U6B	NR_002752	F: GCTTCGGCAGCACATATACTAAAAT
		R:

Calculation of Relative Quantification (RQ)
after the RT-PCR the data were demonstrated in cycle threshold (Ct). The PCR results included Ct values of the studied and housekeeping genes; β actin was used for circular RNA and ordinary mRNA and RUN U6B for miRNA. To measure specific gene expression, a control sample was used. The RQ of each target gene was calculated according to the calculation of delta-delta Ct (ΔΔCt). Each gene RQ was obtained by taking $2^{-\Delta\Delta Ct}$

ELISA for β-catenin protein levels
the kit used was provided by Sunlong Biotech Co., LTD for quantitative determination of human β-catenin in plasma, serum, body fluids, cell culture supernatants, and tissue homogenates (Catalog # SL1870Hu). This kit depends on the sandwich-ELISA technique. The micro-ELISA strip-plate in the kit was pre-coated with a specific antibody to β-catenin. The β-catenin sample concentrations were obtained from the standard curve.

Statistical Analysis
data were coded and entered using the statistical package SPSS (Statistical Package for the Social Sciences) version 23 and GraphPad Prism 7. Data was summarized using mean and standard deviation (SD) in quantitative data and using frequency (count) and relative frequency (percentage) for categorical data. Quantitative non-normally distributed data of the two groups were compared using the U Mann-Whitney test. Spearman's correlation coefficient was used to correlate any two variables. P values less than 0.05 were considered statistically significant (13).

Results

Demographic data
Patient ages were 44.3 ± 9.8 years and control ages were 42.1 ± 9.2 years (p = 0.41) The patient group contained 37 females (67.3%) and 18 males (32.7%), while the control group contained 28 females (50.9%) and 27 males (49.1%) (p = 0.103). No statistical differences for age or gender between the two groups were found.

No statistically significant difference in height was found between the two groups; however, weight, waist circumference (WC), and body mass index (BMI) were all significantly greater in patients than in controls (Table 2).

Table 2. Height, Weight, WC, and BMI of patients and controls.

	Groups				p value
	Patients		Controls		
	Mean	Standard Deviation	Mean	Standard Deviation	
Height (cm)	166	6.8	165	5.8	0.4
Weight (Kg)	92	16	71	8	< 0.0001*
WC (cm)	105	12	81	5.7	< 0.0001*
BMI (Kg/m2)	33	5.9	26	1.6	< 0.0001*

Clinical Data Analysis
Diabetes mellitus was significantly more in patient than the control group while systemic hypertension showed no significant difference between the two groups (Table 3).

Table. 3. Distribution of Hypertension and Diabetes Mellitus in Patients and Controls.

		Groups				p value
		Patients		Controls		
		Count	%	Count	%	
Hypertension	**YES**	13	23.64%	10	18.18%	0.43
	NO	42	76.36%	45	81.82%	
Diabetes Mellitus	**YES**	18	32.73%	4	7.27%	0.0015*
	NO	37	67.27%	51	92.73%	

Biochemical analysis

patients had serum lipid abnormalities with significantly greater total cholesterol, LDL-c, and triglycerides, and significantly less HDL-c than controls. Serum fasting blood glucose levels were significantly greater in patients than in controls. Similar results were seen with two-hour post-prandial blood glucose, which was significantly greater in patients than in controls (Table 4).

Table 4. Lipid Profile and Blood Glucose Levels in Patients and Controls.

mg/dl	Groups				p value
	Patients		Controls		
	Mean	Standard Deviation	Mean	Standard Deviation	
Total Cholesterol	205	45	130	28	< 0.0001*
HDL-C	43	11	65	4	< 0.0001*
LDL-C	127	43	91	18	< 0.0001*
Triglycerides	169	81	88	19	< 0.0001*
Fasting Blood Glucose	101	30	85	10	0.002*
2HPP Blood Glucose	163	46	143	17	0.013*

Liver function tests

serum hepatic transaminases ALT and AST, total bilirubin, and GGT and ALP were significantly greater in patients than in controls. Total protein and prothrombin were significantly less in patients than in controls. No statistically significant differences were found between patients and controls for albumin, direct bilirubin, or INR (Table 5).

Table 5. Liver Function Test Results from Patients and Controls.

	Groups				p value
	Patients		Controls		
	Mean	Standard Deviation	Mean	Standard Deviation	
Total Bilirubin (mg/dl)	0.75	0.30	0.62	0.23	0.03*
Direct Bilirubin (mg/dl)	0.26	0.19	0.20	0.13	0.11
Total Protein (gm/dl)	7.10	0.52	8.00	0.46	< 0.0001*
Albumin (gm/dl)	4.30	0.43	4.40	0.50	0.36
AST (U/L)	37.00	19.00	19.00	4.90	< 0.0001*
ALT (U/L)	39.00	24.00	17.00	6.80	< 0.0001*
GGT (U/L)	26.00	11.00	18.00	6.00	< 0.0001*
ALP (U/L)	93.00	43.00	51.00	19.00	< 0.0001*
PC (%)	96.00	5.70	99.00	2.40	0.0006*
INR	1.00	0.07	1.00	0.03	0.53

β-Catenin protein levels among the studied groups

β- catenin was significantly less in patients than in controls (Fig. 1A).

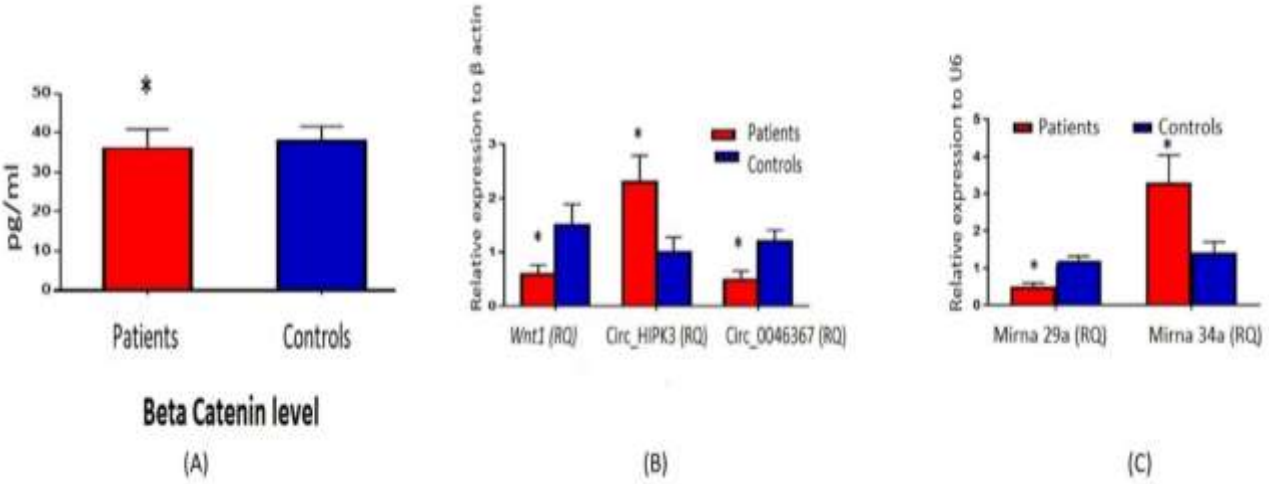

Fig. 1. A: β-Catenin concentrations in patients and controls. **B:** RQ of targeted circular RNAs and *Wnt1* expression in patients and controls. **C:** RQ of targeted miRNA expression in patients and controls.

Gene expression in the studied groups

circRNA-HIPK3 and miRNA-34a expression were significantly greater in patients than in controls (Figs. 1B and 1C, respectively). *Wnt1*, circRNA-0046367, and miRNA-29a expression were significantly less in patients than in controls (Figs. 1B, and 1C, respectively).

Correlations

Correlations between circHIPK3 RNA, miRNA-29a and Wnt1: miRNA-29a and circRNA-HIPK3 expression were negatively correlated while miRNA-29a and *Wnt1* expression were positively correlated (Figs. 2A) and 2C). *Correlations between circRNA-0046367, miRNA-34a, and Wnt1*: miRNA-34a and circRNA-0046367 expression were negatively correlated, as were miRNA-34a and *Wnt1* expression (Figs. 2B and 2D).

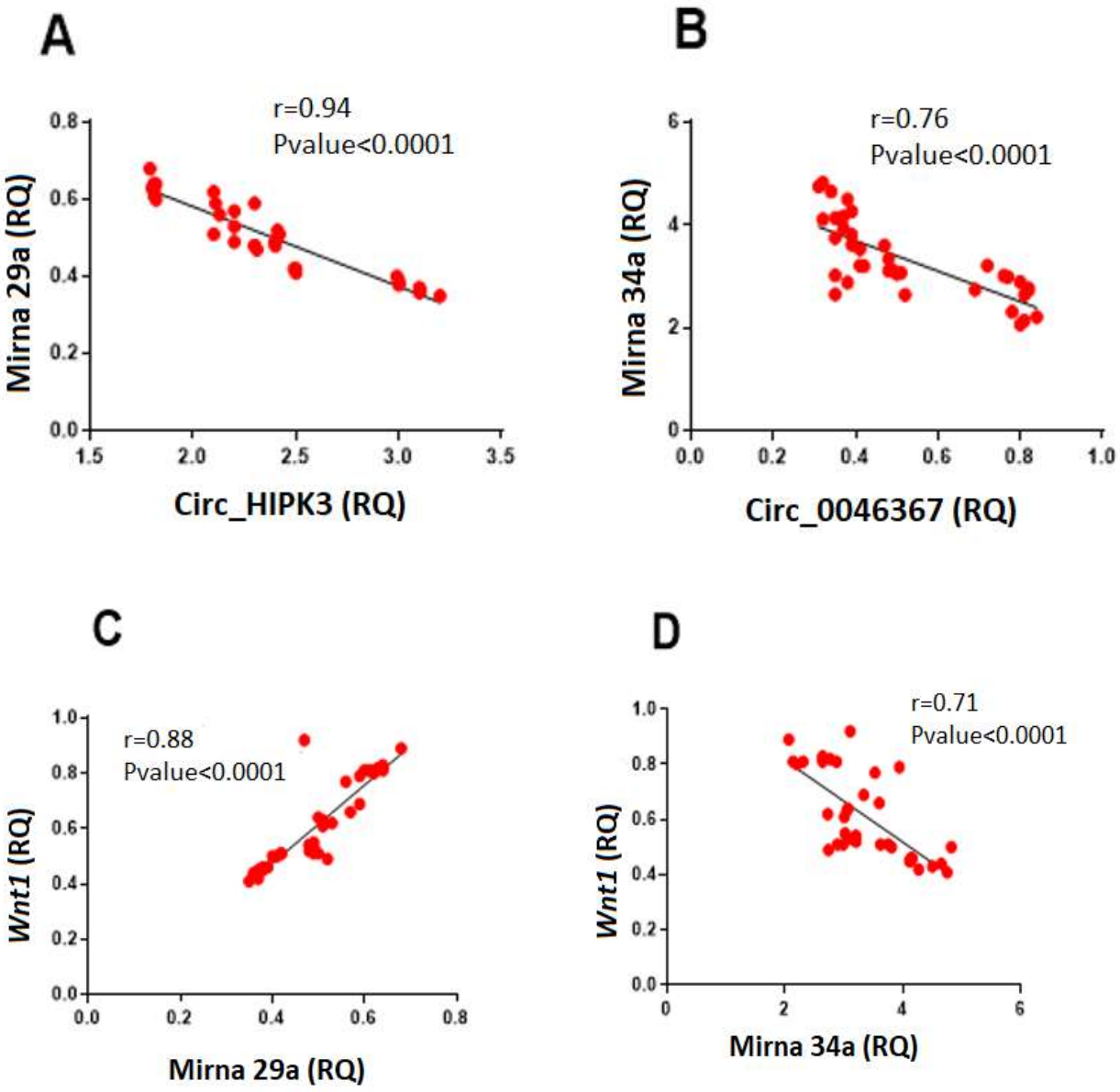

Fig. 2. Variable were correlated using Spearman's correlation coefficient. **A:** miRNA-29a and circRNA-HIPK3 expression were negatively correlated ($r = 0.94$, $p < 0.0001$). **B:** miRNA-34a and circRNA-0046367 expression were negatively correlated ($r = 0.76$, $p < 0.0001$). **C:** miRNA-29a and *Wnt1* expression were positively correlated ($r = 0.88$, $p < 0.0001$). **D:** miRNA-34a and *Wnt1* expression were negatively correlated ($r = 0.71$, $p < 0.0001$).

Discussion

NAFLD is an extensive chronic liver disease that can forefront liver cirrhosis, cancer, and finally death. NAFLD is pathologically categorized as simple non-alcoholic fatty liver (NAFL) and NASH. Both NAFL and NASH appear as hepatic steatosis, but NASH is

distinguished by inflammation with injury to hepatocytes with or without fibrosis (14). New insights on pathogenesis of NASH are crucially needed to help in diagnosis, keep track of disease progression, and develop new treatments. Lately, many studies indicated that circulating levels of microRNAs and circRNAs are correlated with NASH and disease severity. Although studies with these biomarkers were encouraging, their roles in pathogenesis need more extensive studies (15).

Our study found that BMI and WC were significantly greater in NASH cases than in controls. These data agree with the findings of a previous meta-analysis, which showed that the prevalence of NASH increases with obesity, ranging from 34.2-60.9% (16).

Diabetic patients have a greater prevalence of steatohepatitis, hepatic fibrosis, and end-stage liver disease. Genetics entailing adipose tissue dysfunction, lipotoxicity, and glucotoxicity appe ar to have a role. In addition, patients with NAFLD have 2-3 times greater risk for development of type 2 DM (T2DM) (17). In our study the prevalence of DM was significantly greater in NASH patients than in controls. Also, both fasting and two-hour post-prandial blood glucose levels were greater in NASH patients than in controls. These findings are concordant with the findings of previous work reporting that T2DM and NAFLD usually coexist as part of metabolic syndrome, where NAFLD has a prevalence of about 70% in T2DM patients linking insulin resistance, overweight, and obesity with NAFLD (18).

Lipotoxicity enhances progressive liver inflammation and fibrosis in NAFLD patients, causing NASH and even development of liver cirrhosis and HCC (19).

The current study showed that serum total cholesterol, LDL-cholesterol, and triglyceride were greater, and HDL-cholesterol was less, in patients than in controls. These data agree with the results of a meta-analysis, which reported that the overall pooled prevalence estimates of hyperlipidemia/dyslipidemia among NASH patients were 72.13%, and those of hypertriglyceridemia were 83.33% (16).

In our study ALT, AST, and GGT were significantly greater in NASH patients than in controls. Previous studies showed that in up to nearly 60% of patients with NASH, serum ALT can be normal (20). NASH is increasingly recognized as a cause of chronic hepatic disease, usually ending in cirrhosis of the liver, portal hypertension, and hepatocellular carcinoma. ALT and AST are markers of hepatocellular injury and many studies have illustrated that high ALT levels are linked to increased NASH risk (21). Also, GGT $\geq$ 30 IU/L is a predictive marker of NASH (22). The incidence of fatty liver pattern in patients with high GGT levels was greater than that in subjects with normal GGT levels. Thus, elevated GGT increases fatty liver risk, and elevated triglycerides further increase that risk (23).

The relative expression of miRNA-29a and -34a were measured in both patients and controls in the current work. The results showed greater miRNA-34a expression and less miRNA-29a expression in patients than in controls.

miRNAs can affect the onset and course of numerous diseases in a number of different ways by targeting certain genes (24). Hepatocyte miRNA-34a expression regulates the evolution of fatty liver disease and its progression by affecting lipid absorption, lipogenesis, inflammation, apoptosis, and fatty acid oxidation. This indicates that hepatic miRNA-34a may be a good target for fatty liver disease treatment (25).

Certain miRNAs, including miRNA-122, -34a, and -451, are linked to progression from steatosis to NASH, histological abnormalities, reduced markers of hepatic injury, and insulin resistance attenuation in rats fed high fat diets. In the liver, NASH induced by high fat diet increased miR-34a expression and decreased miRNA-122 and -451expression (26).

Concordant with the results of the current study in which miRNA-29a expression was less in patients than controls, a previous study stated that miRNA-29a can mitigate obesity caused by high fat diet, steatosis, and fibrosis, and highlighted the role of miRNA-29a in

pathogenesis of NASH (27). Another study revealed that increased miRNA-29a not only reduced weight gain caused by high fat diet but also subcutaneous, visceral, and intestinal fat accumulation and hepatic steatosis in mice. The protective role of miR-29a on liver damage and its role in epigenetic activity, mitochondrial homeostasis, and immunomodulation may enhance our insight of the pathogenesis of NASH (28). MiRNA-29a has been shown to decrease hepatic damage in NAFLD, NASH, and cholestatic injury. However, the mechanism involved in the protective effect of miRNA-29a in liver in diet-induced NASH remains elusive where miRNA-29a was found to decrease liver inflammation and fibrosis following hepatic injury (29).

In the current study *Wnt1* expression and β-catenin protein levels were assessed. Both *Wnt1* expression and β-catenin were less in patients than controls, indicating down regulation of the Wnt/β-catenin pathway.

Wnt/β-catenin signaling combats adipogenesis and enhances glucose tolerance and insulin sensitivity. The canonical Wnt/β-catenin signaling can have a protective effect on the liver in cases of metabolic stress. Hepatocyte-specific loss of β-catenin is accompanied with increased incidence of steatohepatitis and fibrosis development in mice fed with a methionine and choline deficient (MCD) diet (30). Genes of the Wnt signaling pathway were downregulated in NASH, but upregulated in HCC; thus, Wnt signaling is assumed to mediate the transformation of NASH to HCC and is linked to the development and pathogenesis of both diseases (31). Treatment with pyrvinium pamoate protects against NASH induced by high fat diet and liver fibrosis via downregulating the expression of essential factors in the Wnt/β-catenin signaling pathway (32). However, it is not clear whether upregulation or downregulation of Wnt signaling is responsible for disease progression in the NAFLD spectrum. One reason for this lack of clarity could be the complex interrelationship of canonical and non-canonical Wnt signaling and potential crosstalk of extra- and intracellular components of this pathway with other signaling pathways (33).

Numerous studies have revealed that circRNAs have special expression signatures and play essential roles in many diseases, allowing them to act as diagnostic biomarkers and therapeutic targets (34).

In the current study, circRNA-HIPK3 and circRNA-0046367 expression were assessed. circRNA-HIPK3 was greater and circRNA-0046367 was less in patients than controls.

CircRNA-0046367 can be considered an endogenous modulator of miRNA-34a that participates in hepatic steatosis. Studies of circRNA-0046367 both in vivo and in vitro revealed a decrease in circRNA-0046367 expression during hepatocellular steatosis, and that circRNA-0046367 returning to normal removed miR-34a's inhibitory effect on PPARα by blocking the miRNA/mRNA interaction. The return to normal circRNA-0046367 expression decreased lipid-associated oxidative stress and steatosis. Thus, circRNA-0046367 can be considered a possible target for lipid peroxidative damage treatment (7). Twenty miRNA targets were identified for circRNA-0046367, including both miRNA-34a and miRNA-122 by the use of target prediction on the basis of base complementation, and the results showed direct, high-affinitive targeting of circRNA-0046367 to miRNA-34a (7). Decrease in circRNA_0046366 and circRNA_0046367 was found in NAFLD patients, and the restoration of these circRNAs to their normal levels decreased oxidative stress, lipotoxicity, and disease severity in NAFLD (35).

In this study, circRNA-HIPK3 expression was greater patients than in controls. CircHIPK3 was discovered to sponge nine miRNAs with 18 potential binding sites, with circHIPK3 directly binding to miRNA-29a and miRNA-124, and they act as direct targets circHIPK3 targets (36).

The present work correlates the studied miRNAs with the Wnt/β-catenin pathway in which strong positive correlation was found

between miRNA-29a and *Wnt1* expression and strong negative correlation was found between miRNA-34a and *Wnt1* expression.

MiRNA-29a expression was elevated after Wnt/β-catenin signaling pathway activation; moreover, treatment with Wnt/β-catenin signaling pathway inhibitor decreased miR-29a expression, whereas upregulation of miRNA-29a suppresses the protein that inhibits the Wnt signaling pathway, indicating that miR-29a can regulate the Wnt/β-catenin signaling pathway (37). Activation of the Wnt pathway can lead to upregulation of miR-29a, while in turn miR-29a stimulates Wnt/β-catenin signaling; thus, miR-29a and Wnt pathways can form a positive regulatory feedback loop (38,39).

MiRNA-34a plays essential roles in cell proliferation, differentiation, migration, invasion, and survival by targeting genes involved in the Wnt/β-catenin and phosphatidylinositol 3-kinase/protein kinase B pathways. Up-regulation of miRNA-34a inhibited the phosphatidylinositol 3-kinase/protein kinase B and Wnt/β-catenin signaling pathways (40).

Our research investigated the possible role of each circRNA-HIPK3/miRNA-29a and circRNA- 0046367/ miRNA- 34a axis in the pathogenesis of nonalcoholic steatohepatitis. We concluded that circHIPK3 was upregulated and correlated to down regulated miRNA-29a expression, and circRNA-0046367 was downregulated and correlated to upregulated miRNA-34a. Thus miRNA-29a can be investigated as a target for circRNA-HIPK3, while miRNA-34a can be investigated as a target for circRNA-0046367 and can affect the pathogenesis of nonalcoholic steatohepatitis. Correlations between miRNA-29a and miRNA34a with the Wnt/β-catenin pathway imply that they can target and be responsible for reduced expression of the Wnt/β-catenin pathway sharing in NASH pathogenesis. Learning how these markers might be used as therapeutic targets in NASH needs more research.

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Conflicts of Interest

All authors declare no conflict of interest.

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