

Assessment of Cell-Free Long Non-Coding RNA-H19 and miRNA-29a, miRNA-29b Expression and Severity of Diabetes

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Mohammed Alfaifi^{1,*}
Amit Kumar Verma^{2,*}
Mohammad Yahya Alshahrani¹
Prakash C Joshi²
Ali G Alkhatami¹
Irfan Ahmad¹
Abdulrahim Refdan Hakami¹
Mirza Masroor Ali Beg^{3,4}

¹Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Khalid University, Abha, Saudi Arabia; ²Department of Zoology and Environmental Sciences, GKV, Haridwar, India; ³Department of Medical Elementology and Toxicology, Jamia Hamdard, New Delhi, India; ⁴Department of Biochemistry, Maulana Azad Medical College, New Delhi, India

*These authors contributed equally to this work

Background: Type 2 diabetes mellitus [T2DM] has been one of the common diseases and is characterized by increased blood glucose levels and suggested that cell-free non-coding RNAs and microRNAs (miRNAs) have been demonstrated to serve as important diagnostic/prognostic biomarkers in diabetes.

Materials/Methods: The present study included clinically confirmed newly diagnosed 200 cases of T2DM and 200 healthy subjects, and all the parameters were taken care in diagnosis. Blood samples collected in plain vials were used for cell-free total RNA extraction and after that 100ng of total RNA was used to synthesize the cDNA for cell-free lncRNA H19, miRNA-29a, and miRNA-29b expression using quantitative real-time PCR method. Serum Biochemical parameters were analyzed after collection of the sample to observe the changes among T2DM cases and healthy controls.

Results: It was observed that type 2 diabetic patients had decreased [0.59 fold] lncRNA H19 expression while increased miRNA-29a [5.62 fold] and miRNA-29b [5.58 fold] expression. Decreased expression of lncRNA H19 was observed to be associated with gender [p=0.004], hypertension [p<0.0001], weight loss [p=0.02] and fatigue [p=0.02]. Increased miRNA-29a expression was linked with hypertension [p<0.0001], alcoholism [p=0.04], and smoking [p<0.0001] as well as miRNA-29b expression was associated with hypertension [p=0.0001], weight loss [p=0.002], smoking [p=0.0002], alcoholism [p<0.0001]. Low [≤1 fold] and high [>1 fold] expression of lncRNA H19 expression was linked with miRNA-29a [p=0.005] and miRNA-29b [p<0.0001] expression. lncRNA H19 expression showed negative correlation with miRNA-29a expression [r= -27, p<0.0001] and miRNA-29b [r= -47, p<0.0001].

Conclusion: The present study concluded that lower lncRNA H19 expression, and increased miRNA-29b, a miRNA-29b expression associated with the severity of T2DM patients. Decreased lncRNA H19 expression, and increased miRNA-29b, miRNA-29b expression observed to be interrelated with clinicopathological findings of T2DM patients could involve in pathogenesis disease.

Keywords: diabetes, lncRNA, microRNA, gene expression, cell-free system

Introduction

Type 2 diabetes mellitus [T2DM] is strongly associated with impaired glucose level, which developed due to the inability of the pancreas to secrete enough insulin as well as insulin resistance. Hyperglycemia causes injury to major organs such as blood vessels, liver, brain, heart, kidneys, eyes, and can heavily contribute to the health and financial burden associated with the disease. The liver is particularly

Correspondence: Mirza Masroor Ali Beg
Department of Medical Elementology and Toxicology, Jamia Hamdard, New Delhi, India
Tel +917827655616
Email mirzamasroor1986@gmail.com

involved in maintaining blood glucose levels and excessive hepatic glucose production [HGP] contributing hyperglycemia in T2DM patients.^{1,2} Numerous long non-coding RNAs [lncRNAs] have been identified, and projects like ENCODE have focused on the identification and characterization of lncRNAs. ENCODE project revealed that there are at least 9640 human loci lncRNAs, and the number grows continuously.³ lncRNAs play a significant role in regulation at transcriptional and, post-transcriptional levels, and the organization of chromatin.⁴ Studies suggested that lncRNAs also play a vital role in tumorigenesis.⁵ lncRNAs and microRNAs [miRNAs] have different functions and participate in translational repression by targeting the genes, thereby shifting the nature of disease states. lncRNAs have been involved in multiple cellular processes and emerged as potent genetic regulators. lncRNAs serve as a negative regulator of miRNAs and contribute to inhibit the level of expression.⁶ lnc RNA H19 is positioned on chromosome number 11p15.5, about 100 kb distal of insulin-like growth factor 2 [IGF2], and simultaneously H19 and IGF2 are transcribed from a conserved embossed gene cluster.⁷ In T2DM patients, approximately 5 times lower H19 levels were observed in skeletal muscle compared to healthy individuals.⁸ Considerable attention received on multiple genes linked to epigenetic regulation of path-physiology of diabetes. miRNAs are a group of short sequence about 22 nucleotides and target the multiple mRNAs that regulate protein-coding genes expression⁹ and have been predicted that miRNAs regulate over 60% of all coding genes in mammals.¹⁰ Several miRNAs have been associated with impairment of glycemic homeostasis and the diabetes progression.^{11,12}

The human miRNA-29 family abides miRNA-29a, miRNA-29b, and located and transcribed at chromosome 7 [7q32.3], whereas miRNA-29c is transcribed from chromosome 1 [1q32.2].¹³ Clusters of miRNA-29 transcribed together as polycistronic primary transcripts.¹⁴ Increased miRNA29 family was observed in diabetic rodent models tissues such as liver, β -cells, kidney cells, skeletal muscle cells, and adipose tissue.¹⁴

lncRNA H19 could bind to miRNA-29a competitively following miRNA response elements (MREs) of miRNA-29a.¹⁵ Zhong et al revealed that that lncH19 sponge the miRNA-29b and affect its expression¹⁶ and Lv et al also have been stated that H19 could directly bind to miR-29b reduces its expression.¹⁷ Therefore, the present research work aimed to investigate the clinical relevance of cell-

free lncRNA H19, miRNA-29a, miRNA-29b expression level, and their association with clinical features/variables in T2DM patients in Indian population.

Materials and Methods

Sample Collection

This study included a total of 400 study subjects, 200 were newly diagnosed untreated type 2 diabetes mellitus patients and, 200 were healthy subjects. Three milliliters of blood samples were withdrawn from all the study subjects and collected in plain vials. Fasting as well as post-prandial samples collected in fluoride vials from all the 200 T2DM subjects and 1 mL blood in EDTA vials for HbA1c. All the criteria were followed, such as fasting blood glucose [glucose level ≥ 126 mg/dL] and postprandial glucose [2-hour blood glucose ≥ 200 mg/dL] were monitored for diagnosis of T2DM disease. T2DM patients with Hypertension (BP ≥ 140 or ≥ 90 mm Hg), Nephropathy (polyurea by physical examination as well as 8, 12 and 24hrs urine test), weight loss (>4 kg weight loosed in last 3 to 6 months), fatigue, wound healing, retinopathy (Fundus/fluorescein angiography), loss of appetite, smoking, alcoholism were subjective data recorded for analysis.

Blood samples were centrifuged at 1500 rpm to collect the serum and stored at -80°C for further process. The ethical committee board ethically approved this research study and informed written consent was obtained from all the participants before the study commenced. The present study was ethically approved and the study was conducted at the Department of Zoology, Gurukula Kangri University, Haridwar, India.

Total RNA Extraction

Stored serum samples of T2DM cases and healthy controls were thawed, and total cell-free RNA extraction was done by kit [mirVana total RNA isolation kit, Thermo Fisher Scientific]. All samples are then stored at -70°C in 2mL nuclease-free Eppendorf tubes. The concentration and quality of cell-free RNA were checked by the A260/280 ratio using Nano-drop.

cDNA Synthesis and QRT-PCR for lncH19 Expression Study

A 100 ng of the total cell-free extracted RNA from T2DM patients and healthy controls were used to synthesize cDNA using kit [Verso, Thermo scientific, USA]

following kit provided protocol. Expression of cell-free lncRNA H19 was done by quantitative real-time PCR using SYBR green dye using forward primer sequence 5'-ATCGGTGCCTCAGCGTTCGG-3' and reverse primer sequence 5'-CTGTCCTCGCCGTCACACCG-3' and β -actin were used as internal control, and the forward primer sequence 5'-CGACAACGGCTCCGGCATGTGC-3', reverse primer sequence 5'-GTCACCGGAGTCCATCACGATGC-3'. The program followed as qRT-PCR for lncRNA H19 and β -actin was performed for 40 cycles; initial denaturation was at 94°C for 40 seconds, annealing temperature was at 60°C for 40 seconds, extension at 72°C for 40 seconds and 20 μ l reaction volume was used. Ending additional step at 72°C for 5 minutes to end up reaction and melting curve examined between the ranges 35°C to 90°C to confirm the target amplification. A control without cDNA was included in each experiment, and every reaction was done in duplicate. The relative quantification by $2^{-[\Delta\Delta Ct]}$ method was used to compute the lncRNA H19 level expression.

Polyadenylation, cDNA Synthesis for miRNA-29a, and miRNA-29b Expression Study

A total of 100ng RNA was taken to polyadenylate and cDNA synthesis by miRNA Reverse transcription kit [TaqMan, Thermo Fisher Scientific] by following kit protocol. Essential reagents such as reverse transcriptase enzyme and other reagents were added to switch in poly [A] – tail and then into cDNA using a universal RT primer provided with the kit.

QRT-PCR for miRNA-29a, and miRNA-29b Expression

Quantitative real-time PCR [qPCR] was performed to compute the expression level of cell-free miRNA-29a and miRNA-29b. qPCR was done by advanced Taqman master mix [4,444,556], Taqman probes for miRNA-29a [478587_mir], and miRNA-29b [478369_mir] for quantification and miRNA-16 [477860_mir] were used as internal control as normalizer to calculate the level of expression.

Statistical Analysis

All the statistical testing was done using Graph Pad Prism software version 6.05 and SPSS 20.0 version. Mann Whitney *U*-test was used to calculate the differences in

biochemical parameters among T2DM subjects and healthy controls. QRT-PCR outcome analysis was done by the relative cycle threshold [Ct] method to compute the lncRNA H19, miRNA-29a, and miRNA-29b expression levels by the relative quantification method using the $2^{-[\Delta\Delta Ct]}$. The Mann–Whitney *U*-test was done to calculate the differences in lncRNA H19, miRNA29a, miRNA29b expression level among the subjective variables. Results more than or less than one were considered to specify up-regulation or down-regulation of expression.

Spearman correlation analysis was performed to check the degree of association by calculating the *r*-value of lncRNA H19 with miRNA-29a and miRNA-29b. Regression analysis was performed to calculate the R^2 and beta coefficient to check the effect of lncRNA H19 and biochemical parameters on miRNA-29a and miRNA-29b expression.

All values were normalized relative to the control values, which were depicted as a value of 1. P-value of less than 0.05 was considered statistically significant.

Results

Demographic and Clinical Characteristics of T2DM Patients and Healthy Controls

In brief, the present study included 200 cases of type 2 diabetes mellitus and 200 healthy control subjects [Table 1]. Among the T2DM cases, 68% of patients were males, and 32% were females while in healthy controls, 70% were males, and 30% were females, more details are mentioned in Table 1.

Association of Anthropometric Data and Biochemical Parameters Among T2DM and Healthy Subjects

Study recorded anthropometric data and biochemical data and depicted in Table 2. In T2DM, Hip, waist, and body mass index (BMI) were 35.97, 32.61, and 29.0 while in healthy subjects, 34.87, 31.65, and 24.82 and differences among them were observed to be statistically significant ($p=0.004$, $p=0.02$, $p<0.0001$) respectively. Blood glucose parameters such as fasting, postprandial, and HbA1c were tested and differences among T2DM and healthy subjects were observed to be significant ($p<0.0001$, $p<0.0001$, $p<0.0001$).

Systolic and diastolic blood presser differences among T2DM and healthy subjects were statistically significant ($p<0.0001$, $p=0.0007$). Lipid profile analysis such as high-

Table 1 Demographic and Clinical Characteristics of Enrolled Patients with Type 2 Diabetes Mellitus and Healthy Controls

Variables	T2DM Patients (%)	Controls Subjects (%)
Total no.	200 (100)	200 (100)
Gender		
Males	136 (68)	140 (70)
Females	64 (32)	60 (30)
Age at diagnosis (years)		
≤ 40	94 (47)	100 (50)
> 40	106 (53)	100 (50)
Mean age (in years)	46.32±7.18	47.63±6.76
Hypertension		
Yes	122 (61)	
No	78 (39)	
Increased urination		
Yes	97 (48.5)	
No	103 (51.5)	
Weight loss		
Yes	94 (47)	
No	106 (53)	
Fatigue		
Yes	95 (47.5)	
No	105 (52.5)	
Wound healing		
Yes	84 (42)	
No	116 (58)	
Blur vision		
Yes	67 (33.5)	
No	133 (76.5)	
Loss of appetite		
Yes	91 (45.5)	
No	109 (54.5)	
Smoking status		
Yes	92 (46)	
No	108 (54)	
Alcoholism		
Yes	102 (51)	
No	98 (49)	

density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides (TG), very low-density lipoprotein (VLDL), cholesterol showed significant differences among T2DM and healthy control subjects ($p<0.0001$, $p<0.0001$, $p<0.0001$, $p<0.0001$) respectively.

Table 2 Comparison of Anthropometric Data and Biochemical Parameters Among T2DM and Healthy Subjects

Parameters	T2DM Cases (Mean ±SD)	Healthy Control (Mean±SD)	p-value
Age (years)	46.32±7.18	47.63±6.76	0.13
Hip (cm)	35.97±3.59	34.87±1.87	0.004
Waist (cm)	32.61±3.84	31.65±2.50	0.02
Weight (kg)	71.64±13.63	70.74±7.79	0.53
Body mass index	29.0±5.51	24.82±2.33	<0.0001
Fasting blood glucose	240.0±18.8	90.22±7.10	<0.0001
Postprandial blood glucose	349.0±75	136.0±5.0	<0.0001
HbA1c	7.39±0.77	5.22±0.58	<0.0001
Blood pressure (systolic)	129.4±13.99	118.32±10.78	<0.0001
Blood pressure (diastolic)	86.76±13.31	81.90±6.18	0.0007
High density lipoprotein (HDL)	42.42±12.9	49.78±6.92	<0.0001
Low density lipoprotein (LDL)	192.2±27.0	124.54±14.20	<0.0001
Triglycerides (TG)	375.4±117.7	140.98±5.52	<0.0001
Very low density lipoprotein (VLDL)	35.92±5.18	26.52±2.53	<0.0001
Cholesterol	248.5±13.45	152.63±18.82	<0.0001

lncRNA H19 Expression in T2DM Patients

It was found that T2DM patients had an overall 0.59-fold expression of lncRNA H19 compared to healthy controls [Table 3]. Among the T2DM patients, a significant difference in lncRNA H19 expression was observed between males and females [$P=0.004$]. Males showed 0.70-fold lncRNA H19 expression while females had 0.56-fold lncRNA H19 expression. Hypertensive T2DM patients, had 0.41 fold changes in lncRNA H19 expression, while non-hypertensive patients showed 0.88 fold lncRNA H19 expression, and statistical differences between them were observed to be significant [$P<0.0001$]. T2DM patients with weight-loss showed 0.41 fold lncRNA H19 expression, while patients without weight loss showed 0.79 fold lncRNA H19 expression [$P=0.02$]. Besides, T2DM

Table 3 Long Non-Coding RNA H19 Expression in Type 2 Diabetes Mellitus Subjects

Variables	LncH19 Relative Fold Change in Expression [Mean±SD]	P-value
Overall expression	0.59±0.89	-
Age [in years]		
≤40 years	0.70±1.07	0.82
>40 years	0.56±0.82	
Gender		
Male	0.41±0.68	0.004
Female	0.81±1.04	
Hypertension		
Yes	0.41±0.85	<0.0001
No	0.88±0.87	
Nephropathy		
Yes	0.62±0.92	0.46
No	0.56±0.82	
Weight loss		
Yes	0.41±0.74	0.02
No	0.79±0.99	
Fatigue		
Yes	0.47±0.78	0.02
No	0.73±0.98	
Wound healing		
Yes	0.59±0.86	0.68
No	0.59±0.91	
Retinopathy		
Yes	0.50±0.74	0.87
No	0.64±0.95	
Loss of appetite		
Yes	0.55±0.83	0.41
No	0.64±0.93	
Smoking		
Yes	0.49±0.78	0.13
No	0.68±0.96	
Alcoholism		
Yes	0.48±0.75	0.52
No	0.70±1.0	

Table 4 miR-29a Expression in Subjects with Type 2 Diabetes Mellitus

Variables	miRNA-29a Relative Fold Change in Expression [Mean±SD]	P-value
Overall expression	5.62±1.92	-
Age [in years]		
≤40 years	5.57±1.55	0.64
>40 years	5.63±2.03	
Gender		
Male	5.69±1.84	0.14
Female	5.53±2.03	
Hypertension		
Yes	5.96±1.84	<0.0001
No	5.09±1.95	
Nephropathy		
Yes	5.58±1.97	0.64
No	5.65±1.89	
Weight loss		
Yes	5.81±2.14	0.21
No	5.40±1.63	
Fatigue		
Yes	5.62±1.92	0.88
No	5.62±1.93	
Wound healing		
Yes	5.67±1.95	0.70
No	5.57±1.92	
Retinopathy		
Yes	5.68±2.0	0.65
No	5.49±1.78	
Loss of appetite		
Yes	5.74±2.15	0.75
No	5.52±1.72	
Smoking		
Yes	5.98±1.90	0.04
No	5.24±1.58	
Alcoholism		
Yes	6.17±2.09	<0.0001
No	5.04±1.55	

Table 5 miR-29b Expression in Type 2 Diabetes Mellitus Patients

Variables	miR-29b Relative Fold Change in Expression [Mean±SD]	P-value
Overall expression	5.58±1.83	-
Age [in years]		
≤40 years	5.39±1.73	0.41
>40 years	5.65±1.86	
Gender		
Male	5.41±1.79	0.25
Female	5.69±1.97	
Hypertension		
Yes	5.85±1.68	0.0001
No	5.17±1.97	
Nephropathy		
Yes	5.71±2.03	0.80
No	5.47±1.62	
Weight loss		
Yes	5.91±1.86	0.002
No	5.21±1.72	
Fatigue		
Yes	5.47±1.87	0.20
No	5.69±1.79	
Wound healing		
Yes	5.69±1.84	0.41
No	5.51±1.82	
Retinopathy		
Yes	6.01±2.13	0.09
No	5.37±1.62	
Loss of appetite		
Yes	5.58±1.79	0.97
No	5.59±1.87	
Smoking		
Yes	6.16±2.07	0.0002
No	5.10±1.43	
Alcoholism		
Yes	6.11±2.04	<0.0001
No	5.04±1.39	

patients who had fatigue showed 0.47 fold lncRNA H19 expression, while patients with no fatigue showed 0.73 fold lncRNA H19 expression [P=0.02].

Association of microRNA-29a Among T2DM Patients

T2DM patients showed an overall 5.62 mean fold expression of miRNA-29a compared to healthy controls [Table 4]. T2DM patients who were hypertensive showed 5.96-fold miRNA-29a expression, whereas non-hypertensive reported having 5.09-fold miRNA-29a expression. Besides, the differences between diabetic smoker and non-smoker patients in H19 expression were observed to be statistically significant [P<0.0001]. We also studied whether cigarette smoking has an impact on miRNA-29a expression. We observed that the T2DM patients with smoking habit showed 5.98-fold miRNA-29a expression, and non-smokers showed 5.24-fold miRNA-29a expression [P=0.04]. T2DM subjects with the habit of alcohol consumption showed 6.17-fold miRNA-29a expression, while non-alcoholic T2DM patients showed 5.04-fold miRNA-29a expression. Furthermore, a statistically significant correlation was observed in H19 expression between alcoholic and non-alcoholic diabetic patients [P<0.0001]. Our observations illustrated that miRNA-29a expression in T2DM patients is directly associated with hypertension and alcohol consumptions.

Association of microRNA-29b Expression Among T2DM Patients

T2DM patients showed an overall 5.58 mean fold expression of miRNA-29b compared to healthy controls [Table 5]. T2DM patients who were hypertensive showed 5.85-fold miRNA-29b expression while non-hypertensive showed 5.17-fold miRNA-29b expression.

Table 6 Association of lncRNA H19 with miRNA29a and miRNA29b

Parameters	R ²	P-value
Dependent Variable: miRNA29a	0.13	0.003
Dependent Variable: miRNA29b	0.14	0.001
Predictors: (Constant), H19, VLDL, PPG, FPG, BP (Diastolic), Cholesterol, BP (systolic), Hba1c, HDL, TG, LDL.		

Table 7 Association of lncRNA H19 and Biochemical Parameters with miRNA29a and miRNA29b

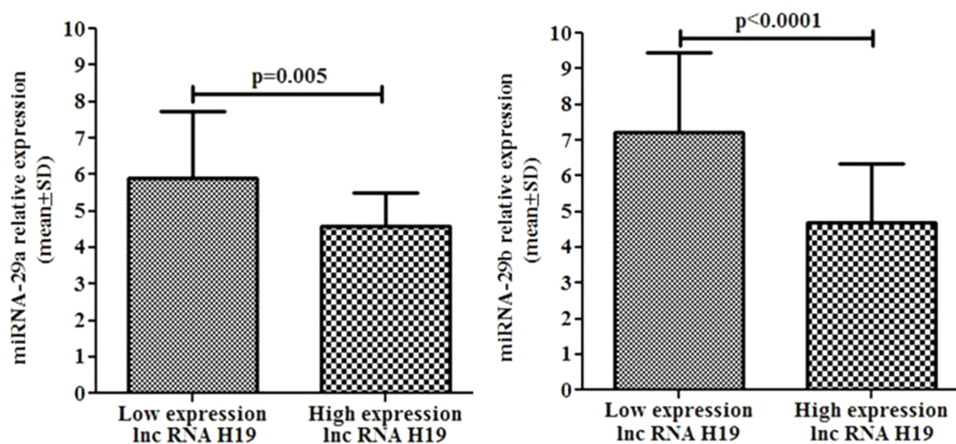
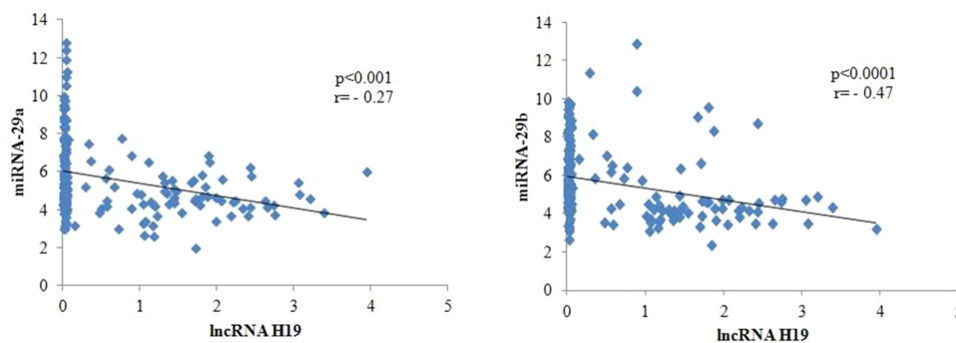
Variables	Standardized Coefficients	P-value	Standardized Coefficients	P-value
	Beta		Beta	
H19	-0.298	0.0001	-0.285	0.0001
FPG	0.075	0.284	0.042	0.549
PPG	-0.015	0.848	-0.124	0.124
Hba1c	0.110	0.130	0.134	0.062
BP (systolic)	0.115	0.111	0.122	0.090
BP(Diastolic)	-0.109	0.120	-0.073	0.293
HDL	-0.106	0.189	0.102	0.202
LDL	-0.129	0.190	0.099	0.312
TG	0.089	0.354	0.013	0.893
Cholesterol	-0.015	0.856	-0.070	0.404
VLDL	-0.019	0.785	-0.026	0.702

Dependent variable: miRNA29a

Dependent variable: miRNA29b

Statistical analyses revealed differences between analyzed groups [P=0.0001]. Furthermore, T2DM patients who had weight-loss showed 5.91-fold miRNA-29b expression, while those who did not show weight loss

had 5.21-fold miRNA-29b expression [P=0.002]. Smoker T2DM patients showed 6.16-fold miRNA-29b expression, while non-smokers showed 5.10-fold miRNA-29b expression [P=0.0002]. Alcoholic T2DM

**Figure 1** Association of lncRNA H19 expression with miRNA29a and miRNA29b expression.**Figure 2** Correlation of lncRNA H19 with miRNA-29a and miRNA-29b.

patients showed 6.11-fold miRNA-29b expression, while non-alcoholic T2DM patients showed 5.04-fold miRNA-29b expression [$P < 0.0001$].

lncRNA H19, miRNA-29a, and miRNA-29b Relative Fold Changes in Expression and Biochemical Parameters with Among T2DM Patients

Regression analysis was done to check the effect of all the parameters such as H19, VLDL, PPG, FPG, BP (Diastolic), Cholesterol, BP (systolic), HbA1c, HDL, TG, LDL with miRNA29a, miRNA29b relative fold changes in expression, and the observed R^2 value was 0.13 ($p = 0.003$) and 0.14 ($p = 0.001$) (Table 6). The beta coefficient was also calculated and the observed beta value for lncH19 to miRNA-29a was -0.298 ($p < 0.0001$) and concerning miRNA-29a was -0.28 ($p < 0.0001$) (Table 7).

Association of lncRNA H19 with miRNA-29a and miRNA-29b

A significant association of low [≤ 1 fold] and high [> 1 fold] expression of lncRNA H19 with miRNA29a and miRNA29b is observed [Figure 1]. It was analyzed that the T2DM patients who had low expression of lncRNA H19 showed 5.91-fold miRNA-29a expression and high expression of lncRNA H19 showed 4.60 fold changes in miRNA-29a expression and difference among them was found to be statistically significant [$P = 0.005$]. T2DM patients who had low expression of lncRNA H19 showed 7.21-fold miRNA-29b expression and high expression of lncRNA H19 showed 4.69-fold miRNA-29b expression was observed [$P < 0.0001$].

Correlation of lncRNA H19 with miRNA-29a and miRNA-29b

Spearman correlation was done to check the association of lncRNA H19 with miRNA-29a and miRNA-29b [Figure 2]. Analysis showed a negative correlation of lncRNA H19 expression with miRNA-29a [$r = -0.27$, $P < 0.0001$] as well as negative correlation with miRNA-29b [$r = -0.47$, $P < 0.0001$] was observed. Suggesting a possible interaction that decreased in lncRNA H19 expression may influence an increased expression of miRNA-29a and miRNA-29b.

Discussion

Diabetes mellitus is a metabolic disorder that developed due to insufficient insulin secretion from β -cells of the pancreas.¹⁸ The frequency of T2DM has increased in a few decades and to date affects more than 300 million lives worldwide due to increased sedentary life and easy access to fast foods.¹⁹ The pathogenesis of T2DM is associated with a genetic disposition, environmental exposures, and multiple causes.²⁰ Long non-coding RNA H19 is a multifunctional long noncoding RNA that is functionally involved in the nucleus and the cytoplasm.⁷ In T2DM patients, five times lower H19 expression was observed in skeletal muscle compared to healthy individuals.⁸ Zhuo et al have shown that diabetic rats had decreased expression of H19 as well as in high glucose exposed to neonatal cardiomyocytes.²¹

A downregulation of cell-free lncRNA H19 was observed in diabetes,²² and in the same way, the present study revealed decreased cell-free lncRNA H19 expression associated with gender, hypertension, weight loss, and fatigue. Male T2DM patients showed 0.40-fold down-regulation, hypertensive T2DM patients showed 0.47-fold down-regulation, T2DM patients who had weight-loss showed 0.38-fold down-regulation, T2DM patients who had fatigue showed 0.26-fold down-regulation of lncRNA H19 expression compared to its counterpart. Increased cell-free miRNA-29a and miRNA-29b expressions were observed among T2DM patients. It has revealed that the cell-free miRNA-29a expression was linked with hypertension and alcoholism. miRNA-29b expression was linked with hypertension, weight loss, smoking, and alcoholism. Increased expression of the miRNA-29 family recorded in different tissues such as the liver²³ and β cells of diabetic rodent models and humans.²⁴

Essentially, an increase in miRNA-29 expression level in the serum of children diagnosed with T1DM and adult T2DM patients was observed.²⁵ Hyperglycemia is the primary situation of diabetes mellitus and leads to up-regulates the expression of miRNA-29a and miRNA-29b.²⁶ Roggli et al revealed that patients treated with proinflammatory cytokine induce the miRNA-29 family members' expression level in human beings and mouse pancreatic cells. Moreover, an increase in the expression of miRNA-29 family miRNAs is associated with the early step of diabetes in mice.²⁷ It has been shown that miRNA-29 suppression by using anti-miRNA-29 oligomers

decreases the expression and protects the patients against diabetic nephropathy.²⁶

Pancreatic islets showed upregulation of miRNA-29a and miRNA-29b and involved in β -cell death, initiation of diabetes, and suggested to be the reliable diabetic marker.²⁴ Dahlman has revealed that the T2DM patient's skeletal muscles showed increased expression of miRNA-29 family members compared to the non-diabetic person.²⁸ miRNA-29b was over-expressed in diabetic rat's muscle cells and correlated with the negative GLUT4 expression, and miRNA-29a has previously been validated as repressors of GLUT4, increased miRNA-29a expression was observed in vastus lateralis muscle T2DM, and GLUT4 protein expression was observed to decrease in mice skeletal muscle over-expressing miRNA-29a,²⁹ as well as in C2C12 muscle cells.³⁰ Expression of GLUT4 level has been reported to decrease in 3T3-L1 adipocytes over-expressing miRNA-29a, miRNA-29b.³¹

This study observed the T2DM patients who had expression level ≤ 1 fold of lncRNA H19 showed higher expression while >1 fold of lncRNA H19 had lower expression comparatively. It was also found that the lncRNA H19 expression had a negative correlation with miRNA-29a and miRNA-29b. This suggested that decreased lncRNA H19 expression showed to increased expression of miRNA-29a and miRNA-29b. In support of the present study, Hee et al in 2007 and Karolina et al in 2011 also revealed that the miRNA-29 family was increased in several tissues, skeletal muscle, derived from diverse rat models of T2DM.^{32,33} It has also been revealed that miRNA-29a and miRNA-29b were up-regulated in T2DM patients' skeletal muscle tissue²⁸ study done by Zhang et al in 2020, indicated that lncRNAs are important regulators of DM and are thus likely to serve as important diagnostic and therapeutic targets for this disorder.³⁴ Increased miRNA-29a expression was observed in T2DM and the present study provides support for the exploration of miR-29a antagonists as a potential therapeutic approach for T2DM treatment and the associated metabolic complications.³⁵ Huang et al revealed that lncRNA H19 could bind to miRNA-29a competitively following miRNA response elements (MREs) of miRNA-29a and affects the expression.¹⁵ Zhong et al said that lncH19 sponge the miRNA-29b,¹⁶ and Lv et al also stated that H19 could directly bind to miR-29b and reduces its expression.¹⁷ Over-expression of miR-29a levels was positively associated with metabolic disorders and demonstrated to have a potential value as

biomarkers in disease prognosis³⁶ as well as a study by Ying et al in 2018, stated that increased miRNAs miR-29b was observed in T2DM patients.³⁷ A study was done by Massart et al in 2017 and revealed that miR-29 acts as an important regulator of insulin-stimulated glucose metabolism and showed relevance in type 2 diabetes.²⁹

Conclusions

The present study observed decreased expression of cell-free lncRNA H19 and increased expression of miRNA-29a as well as miRNA-29b in T2DM patients, Decreased expression was linked with hypertension, weight loss, and fatigue. Increased cell-free miRNA-29a expression was linked with hypertension, smoking, and alcoholism while miRNA-29b expression was linked with hypertension, weight loss, smoking, and alcoholism. This suggested that decreased expression of cell-free lncRNA H19, increased expression of miRNA-29a and miRNA-29b may be associated with the worseness of disease and involved in the pathogenesis of the disease. miRNA-29a and miRNA-29b expression alteration observed to link with mainly smoking and alcoholism and this would be a factor to increase the severity of the disease. This data shed light on this gene interaction may be an essential factor for disease occurrence and worseness of disease. lncRNA H19, miRNA-29a, and miRNA-29b expression pattern information for the diagnosis/prognosis and management of T2DM cases. Further investigations are warranted to derive the difference in gene expression among T2DM cases.

Data Sharing Statement

The datasets used and analyzed during the present study would be available from the corresponding author.

Ethics Approval and Consent to Participate

The study has been conducted only after the due clearance and approval from the Ethics committee of GKV (vide Proposal No. 14/08/2015/GKC/IEC/2015) has. As part of a mandatory standardized ethical norm, a written with signed informed consent was taken from the particular person before inclusion in the research work, and all the ethical principles regarding human experimentation were followed and the study was conducted in accordance with the Declaration of Helsinki.

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Disclosure

The authors report no conflicts of interest for this work.

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