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Circulating Levels of HOTAIR- lncRNA Are Associated with Disease Progression and Clinical Parameters in Type 2 Diabetes Patients

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Abstract

Background: Recent studies have implicated dysregulated long non-coding RNA (lncRNA) levels in the pathogenesis of type 2 diabetes (T2D). This study aimed to assess the expression of circulating HOTAIR and uc.48+, examining their correlation with clinical and biochemical variables in T2D patients, pre-diabetic individuals, and healthy controls.

Methods: Peripheral blood levels of lncRNAs were quantified using QRT-PCR in 65 T2D patients, 63 pre-diabetic individuals, and 63 healthy subjects. Pathway enrichment analysis was conducted to explore the functional enrichment of lncRNA-miRNA targets.

Results: Analysis revealed a significantly elevated circulating level of HOTAIR in both T2D ($P < 0.0001$) and pre-diabetic patients ($P = 0.04$) compared to controls. ROC analysis demonstrated that, at a cutoff value of 9.1, with a sensitivity of 80% and specificity of 62%, HOTAIR could distinguish T2D patients from controls (AUC = 0.723, 95% CI 0.637-0.799, $P < 0.0001$). Spearman correlation analysis identified a significant positive correlation between HOTAIR expression, HbA1c, and insulin resistance ($P < 0.005$). MiRNA enrichment analysis indicated significant enrichment of diabetes-related pathways among HOTAIR's miRNA targets. Conversely, no significant difference in uc.48+ circulating levels between groups was observed, but a significant positive correlation emerged between uc.48+ and systolic blood pressure.

Conclusions: This study provides evidence that elevated HOTAIR expression levels are associated with T2D progression, suggesting their potential as biomarkers for early diagnosis and prognosis.

Keywords: Diabetes Mellitus, HOTAIR, Long Non-Coding RNA, MicroRNAs.

Introduction

The global prevalence of diabetes is estimated to be 463 million subjects in 2019, rising to 578 million by 2030 (1). Type 2 diabetes (T2D) accounts for approximately 90% of diabetic subjects, and its prevalence has increased rapidly over the past three decades. Emerging studies have revealed that the deregulation of non-coding RNA (ncRNA) levels is tightly correlated with several human disorders (2-5). In this regard, enormous investigations have reported that deregulated levels of long non-coding RNAs (lncRNAs) could be associated with T2D susceptibility and related complications (6, 7).

HOX antisense intergenic RNA (HOTAIR, Gene ID: 100124700) is a 2.2kb lncRNA residing in 12q13.13, between HOXC11 and HOXC12 genes (8). HOTAIR could interact

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with various chromatin-modifying complexes and DNA-binding proteins, and act as a molecular sponge for miRNAs, thereby regulating the target gene transcripts (9). Studies suggest that deregulated levels of HOTAIR could be associated with shorter overall survival, metastasis, and resistance to therapy in different malignancies (9, 10). Furthermore, recent investigations point out the role of HOTAIR in T2D pathogenesis. Li et al. reported that HOTAIR is upregulated in the liver of T2D murine models and could affect insulin resistance by modulating SIRT1 expression (11).

The uc.48+ belongs to a novel class of lncRNAs named transcribed ultra conserved region (T-UCR), which has been shown to have a role in adipogenesis, cartilage degeneration, and myogenic differentiation (8). An experimental study showed a marked increase in uc.48+ expression in the posterior root ganglia of the mouse model of diabetic neuropathy (9). However, little is known about evaluating HOTAIR and uc.48+ circulating levels in T2D patients. Accordingly, we aimed here to determine and compare the circulating levels of HOTAIR and uc.48+ in peripheral blood samples of T2D patients, pre-diabetic cases, and control subjects. Moreover, we evaluated the correlation of lncRNA expression levels with biochemical and clinical parameters.

Materials and Methods

Subjects and laboratory measurements

A total of 191 age- and sex-matched subjects, including 65 cases with T2D, 63 pre-diabetes, and 63 non-diabetic subjects, were included in this case-control study. Subjects who had either of the following criteria were diagnosed as having T2D: fasting blood sugar (FBS) ≥ 126 mg/dL; 2-hour plasma glucose (2-h PG) ≥ 200 mg/dL; or hemoglobin A1c (HbA1c) ≥ 6.5%. Pre-diabetes was defined as FBS levels between 100-125 mg/dL, 2-h PG levels between 140-199 mg/dL, and HbA1c between 5.7-6.4%. The controls were selected from the healthy volunteers with an FBS level of < 100 mg/dL or HbA1c <5.7% and no history of diabetes. Subjects with inflammatory disorders, malignancies, and endocrine diseases were excluded.

Interviewer-administered questionnaires and medical records collected the demographic characteristics of the study subjects. All anthropometric measurements were performed by a nutritionist using standardized methods. Determination of FBS, HbA1c%, total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-c), and low-density lipoprotein cholesterol (LDL-c) levels were measured using usual colorimetric methods. Insulin resistance is determined using the homeostasis model assessment of insulin resistance (HOMA-IR) equation.

The study was confirmed by the Yasuj University of Medical Sciences Ethics Committee (IR.YUMS.REC.1400.107). Informed consent was obtained from all subjects before participating in the study.

Determination of circulating levels of lncRNAs

Total RNA was extracted from the peripheral blood samples using a Hybrid-RTM Blood RNA kit (GeneAll, Seoul, Korea). The quality of the extracted RNA was determined using a NanoDrop Lite spectrophotometer (NanoDrop Technologies, Inc., Wilmington, USA). Real-time PCR analysis was done by the Applied Biosystems StepOnePlus System using forward 5′-GGAAAGATCCAAATGGGACCA-3′ and reverse primers 5′-CTAGGAATCAGCACGAAGCAAA-3′ for HOTAIR and forward 5′-GCAAACTGGATGAGGAT-3′ and reverse primers 5′-GTAGTGCCACAAGGAGA-3′ for uc.48+. The specificity of PCR amplification was evaluated by the melting curves containing single peaks and visualized PCR product size by electrophoresis. The relative expression of lncRNAs was estimated using the ΔΔCt method and normalized using GAPDH.

Pathway enrichment analysis

Experimentally validated miRNA targets of HOTAIR lncRNA were extracted from NPInter.V4 (10). Subsequently, a list of experimentally validated mRNA targets of

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miRNAs related to HOTAIR was obtained from the miRTarBase 2020 database (11). The KEGG pathway enrichment analysis was performed by the DAVID and miEAA 2.0 databases to clarify the functional annotation and pathway enrichment of selected mRNAs (12, 13).

Statistical analysis

All statistical analysis was performed by the SPSS Statistical Software Package (version 20.0). The assessment of normality was done by the Kolmogorov-Smirnov test. Accordingly, quantitative parameters were presented as mean±SD (standard deviation) or median (interquartile range). The comparisons of the quantitative variables between groups were carried out using the one-way ANOVA for the normally distributed data or the Kruskal-Wallis test for the nonparametric data. The correlation between molecular and biochemical parameters was determined by Spearman's correlation analysis. A receiver operating characteristic (ROC) curve was used to evaluate the possibility of using lncRNAs as a diagnostic biomarker for T2D. MedCalc (version 18.2.1, MedCalc Software Ltd, Ostend, Belgium) was used to perform the ROC analysis and sample size estimation.

Results

Features of the study subjects

The clinical features of a total of 191 age- and sex-matched subjects, including 65 cases with T2D, 63 pre-diabetes, and 63 non-diabetic subjects (N = 191), are presented in Table 1. The mean age of control, pre-diabetic, and patients was 53.98 ± 8.05, 53.71 ± 7.7, and 53.49 ± 9.1 respectively. There were no significant differences in age (P = 0.74) and gender (P = 0.85) distributions between groups, as confirmed by the one-way ANOVA and chi-square test, respectively. The results revealed that diabetic subjects had significantly elevated levels of BMI (P = 0.016), systolic blood pressure (P = 0.012), FBS (P < 0.0001), HbA1c (P < 0.0001), and HOMA-IR (P < 0.0001) compared to pre-diabetic and control subjects. There were no significant differences for diastolic blood pressure (P = 0.11), LDL-c (P = 0.53), HDL-c (P = 0.71), TG (P = 0.19), and TC (P = 0.48) between groups.

Comparison of lncRNA circulating levels between T2D and control subjects

The Kruskal-Wallis test analysis revealed a significant elevation in the circulating level of HOTAIR in T2D (P < 0.0001) and pre-diabetes (P = 0.04) compared to controls (Fig. 1). However, no significant difference was observed between the pre-diabetic and T2D groups (P > 0.05). To assess the diagnostic value of HOTAIR for detecting T2D and pre-diabetes, a ROC curve was employed. The results showed that at the cutoff value of 9.1 (Δct) and with a sensitivity and specificity of 80% and 62%, respectively, HOTAIR could effectively differentiate T2D patients from controls (AUC = 0.723, 95% CI 0.637-0.799, P < 0.0001) (Fig. 2A). Additionally, this lncRNA could serve as a potential diagnostic marker to distinguish pre-diabetic patients from healthy subjects (AUC = 0.6, 95% CI 0.51-0.69, P = 0.03) at a cutoff value of 10.33 (Δct) with 77.8% sensitivity and 42.9% specificity (Fig. 2A). The Kruskal-Wallis test reported no significant difference in uc.48+ levels among the three groups (Fig. 3).

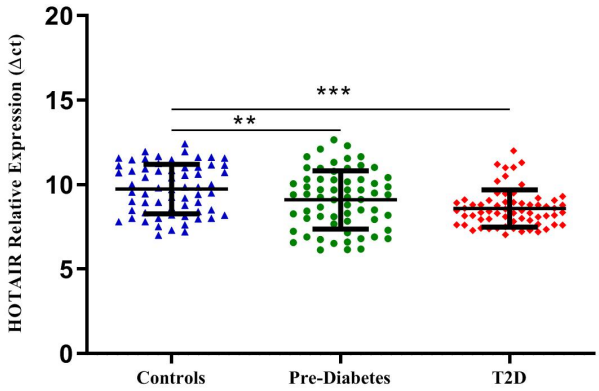

Fig. 1. Scatterplots illustrating the relative expression of HOTAIR in T2D (N= 65), pre-diabetic patients (N= 63), and control (N= 63) subjects. The analysis employed the Wilcoxon signed-rank test. *** P-value < 0.0001, ** P value <0.05. The y-axis represents ΔCT values. HOTAIR circulating levels were markedly elevated in T2D and pre-diabetic individuals compared to controls, with no significant difference observed between the pre-diabetic and T2D groups.

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Table 1. Demographic and clinical characteristics of the study subjects.

Parameter	Groups			*P* value
	T2D patients (N=65)	pre-diabetes (N=63)	Control (N=63)	
Sex, male (%)/female (%)	36(55.4%)/29(44/6%)	30(47.6%)/33(52.4%)	33(52.4%)/30(47.6%)	0.67
Age (years)	53.49 ± 9.1	53.71 ± 7.7	53.98 ± 8.05	0.94
Body mass index (kg/m2)	26.23 (24.48 - 28.77)	25.2 (22.4 - 27.14)	25.43 (22.04 - 27.3)	0.016*
Systolic blood pressure (mmHg)	120 (110 - 130)	120 (110 - 130)	120 (110 - 124)	0.012*
Diastolic blood pressure (mmHg)	80 (73 - 90)	80 (70 - 90)	80 (70 - 84)	0.11
LDL-C (mg/dL)	84 (74 - 97.5)	86 (74 - 100)	83 (68 - 95)	0.53
HDL-C (mg/dL)	42 (36.5 - 47.5)	43 (36- 52)	42 (38 - 47)	0.71
TG (mg/dL)	145 (104.5 - 197)	129 (98 - 164)	129 (123 - 158)	0.19
TC (mg/dL)	163 (134 - 190.5)	161 (142- 184)	158 (131 - 177)	0.48
FBG (mg/dL)	141 (131 - 152)	120 (110 - 123)	89 (83 - 92)	<0.0001*
HbAlc (%)	7.3 (6.7 – 7.8)	6.1 (6 – 6.2)	5.1 (5 – 5.2)	<0.0001*
HOMA-IR	1.47 (0.97-2.1)	1.03 (0.77-1.39)	0.7 (0.52 - 1.32)	<0.0001*

The data are presented as mean ± SD (standard deviation) or median (interquartile range), depending on whether the data were normally distributed. LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; TG: triglycerides; TC: total cholesterol; FBG: fasting blood glucose; HbA1c: hemoglobin A1c; HOMA-IR: homeostatic model assessment of insulin resistance.

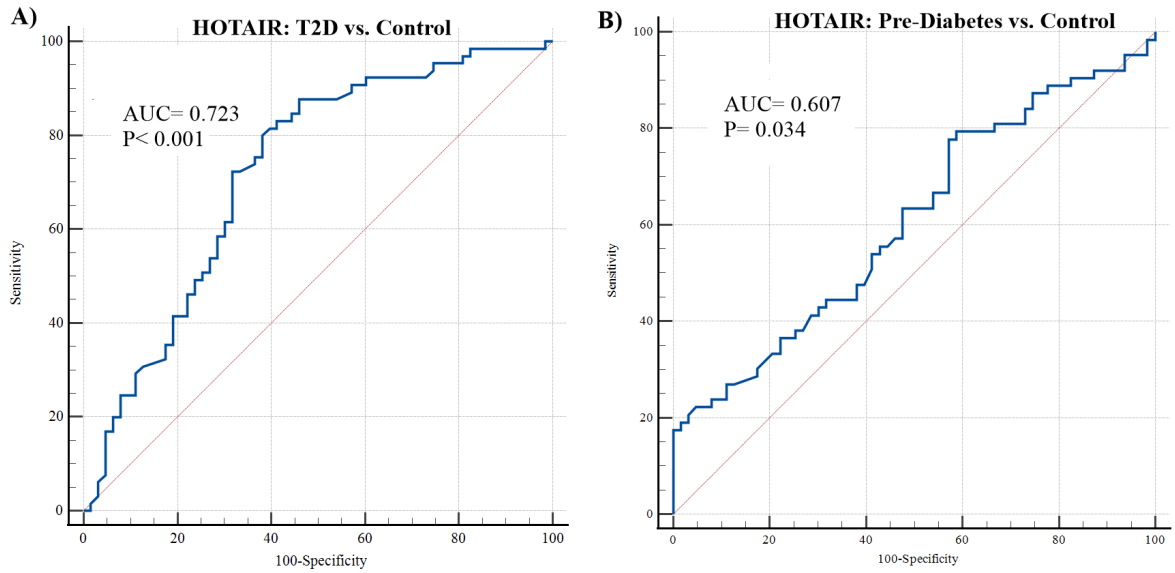

Fig. 2. ROC curve analysis and AUC for HOTAIR: T2D vs. control and HOTAIR: Pre-diabetes vs. controls. HOTAIR effectively differentiates T2D patients from controls with a sensitivity and specificity of 80% and 62%, respectively (A). Additionally, this lncRNA distinguishes pre-diabetic patients from healthy subjects with a sensitivity of 77.8% and specificity of 42.9% (B).

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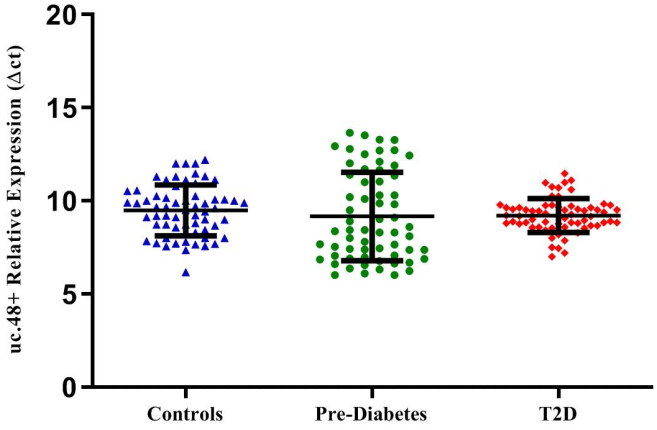

Fig. 3. Scatterplots depicting the relative expression of uc.48+ in T2D (N= 65), pre-diabetic patients (N= 63), and control (N= 63) subjects.
The data were analyzed using a Wilcoxon signed-rank test. No significant difference was observed among the T2D, pre-diabetic, and control groups.

Correlation of lncRNA levels and laboratory parameters

The Spearman correlation analysis revealed a significant positive correlation between the HOTAIR expression level (ΔCt values) and systolic blood pressure (R = 0.21, P = 0.01), FBS (R = 0.40, P = 0.000), HbA1c (R = 0.31, P = 0.000), and HOMA-IR (R = 0.25, P = 0.004). Furthermore, a significant positive correlation was observed between uc.48+ and systolic blood pressure (R = 0.17, P = 0.05) (Table 2).

Table 2. Correlation of lncRNA expression levels with biochemical and anthropometrical parameters.

Parameters	HOTAIR expression level		uc.48+ expression level	
	R value	*P* value	R value	*P* value
Body Mass Index (kg/m^2)	0.152	0.08	0.03	0.73
Systolic blood pressure (mmHg)	**0.21**	**0.01***	**0.17**	**0.05***
Diastolic blood pressure (mmHg)	0.10	0.23	0.02	0.75
LDL-Cholesterol (mg/dl)	- 0.03	0.7	- 0.07	0.37
HDL-Cholesterol (mg/dl)	- 0.01	0.86	0.01	0.84
Triglycerides (mg/dl)	0.123	0.16	- 0.02	0.77
Total Cholesterol (mg/dl)	- 0.02	0.81	- 0.002	0.98
Fasting blood glucose (mg/dl)	**0.40**	**0.000***	0.12	0.14
HbAlc (%)	**0.31**	**0.000***	0.07	0.42
HOMA-IR	**0.25**	**0.004***	0.11	0.2

R and *P* values are presented from Spearman's correlation analysis.

Pathway Enrichment Analysis

Twelve experimentally validated miRNA targets for HOTAIR were identified by the NPInter database. The outputs of the miRNA pathway enrichment analysis highlighted that miRNA targets of HOTAIR were significantly enriched in diabetes- related pathways, including HIF-1, FoxO, PI3K-Akt, Ras, and mTOR signaling pathways (Table 3). Most of these miRNAs were found to be significantly enriched in the KEGG categories of "insulin resistance" and "insulin signaling pathway" (Fig. 4).

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Table 3. KEGG pathway enrichment analysis for genes regulated by the HOTAIR-miRNA regulatory network.

KEGG pathways	P	Fold Enrichment	Bonferroni	Benjamini	FDR	Gene targets (N)
PI3K-Akt signaling pathway	2.25E-15	4.079322	5.68E-13	4.79E-14	2.53E-14	44
FoxO signaling pathway	1.09E-14	6.764427	2.79E-12	1.74E-13	9.18E-14	27
MAPK signaling pathway	4.08E-09	3.460612	1.04E-06	2.99E-08	1.57E-08	31
TNF signaling pathway	5.73E-09	5.567679	1.47E-06	3.86E-08	2.03E-08	19
TGF-beta signaling pathway	2.07E-08	5.873053	5.31E-06	1.26E-07	6.66E-08	17
Wnt signaling pathway	1.46E-05	3.475059	0.003723	5.65E-05	2.98E-05	18
HIF-1 signaling pathway	2.37E-05	4.215413	0.006041	8.53E-05	4.50E-05	14
Type II diabetes mellitus	6.17E-05	6.421304	0.015684	2.08E-04	1.10E-04	9
AMPK signaling pathway	7.19E-05	3.797355	0.018234	2.32E-04	1.23E-04	14
Insulin resistance	9.84E-05	3.950556	0.024873	3.03E-04	1.60E-04	13
JAK-STAT signaling pathway	1.49E-04	3.163373	0.037498	4.50E-04	2.37E-04	16
Rap1 signaling pathway	2.10E-04	2.813143	0.05223	6.24E-04	3.29E-04	18
Ras signaling pathway	2.87E-04	2.642288	0.070931	8.17E-04	4.31E-04	19
Toll-like receptor signaling pathway	2.97E-04	3.786923	0.073219	8.26E-04	4.36E-04	12
mTOR signaling pathway	0.020201	2.314231	0.994616	0.045363	0.023922	11
Insulin signaling pathway	0.023355	2.39562	0.997642	0.049824	0.026274	12
PPAR signaling pathway	0.077001	2.6256	1	0.143916	0.077001	6

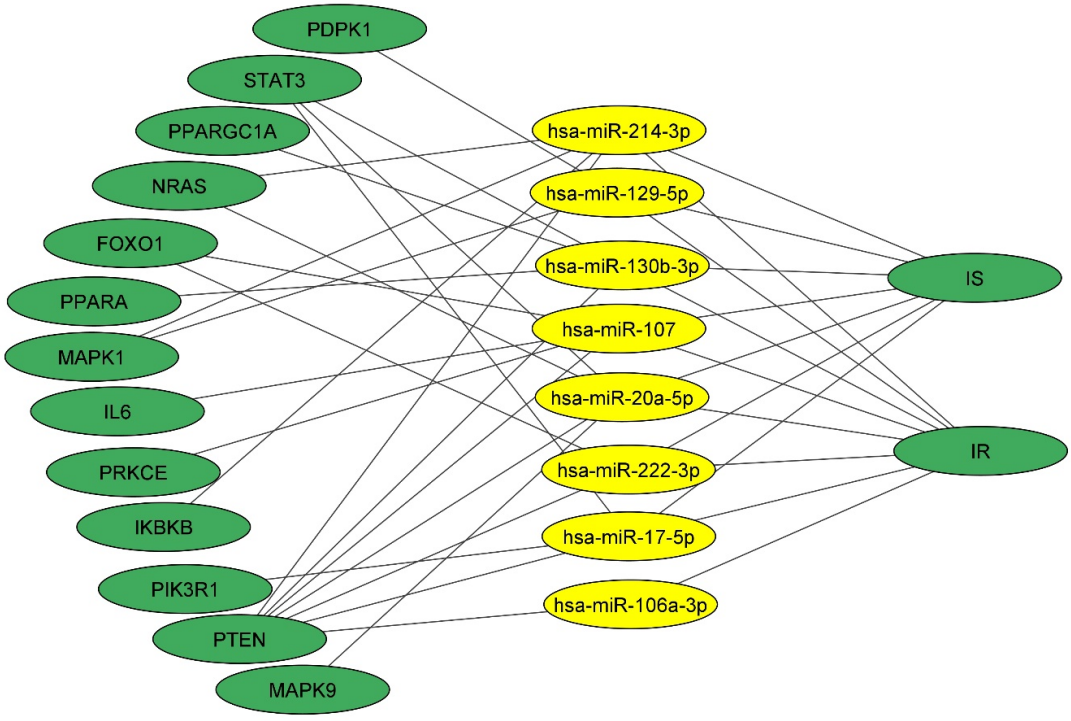

Fig. 4. HOTAIR lncRNA-miRNA-mRNA Interactions. HOTAIR functions as a "molecular sponge" for miRNAs, modulating their inhibitory effects on the expression of target genes associated with insulin resistance and the insulin signaling pathway. IS - Insulin Sensitivity, IR - Insulin Resistance, IKBKB - Inhibitor of nuclear factor kappa B kinase subunit beta, MAPK1 - Mitogen-activated protein kinase 1, MAPK9 - Mitogen-activated protein kinase 9, PIK3R1 - Phosphoinositide-3-kinase regulatory subunit 1, PRKCE - Protein kinase C epsilon, PDPK1 - 3-phosphoinositide-dependent protein kinase 1, PPARGC1A - PPARG coactivator 1 alpha, FOXO1 - Forkhead box O1, IL6 - Interleukin 6, PPARA - Peroxisome proliferator-activated receptor alpha, PTEN - Phosphatase and tensin homolog, STAT3 - Signal transducer and activator of transcription 3, NRAS - NRAS proto-oncogene, NRAS - GTPase.

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Discussion

Our results revealed elevated circulating levels of HOTAIR lncRNA in T2D and pre-diabetes patients compared to healthy subjects, indicating its potential to distinguish diabetic and pre-diabetic patients from controls. Consistent with our findings, previous studies reported increased levels of HOTAIR lncRNAs in PBMC samples from T2D cases compared to control subjects (14). Wang et al. demonstrated elevated expression of HOTAIR in the plasma samples of T2D patients, enabling the discrimination of diabetic patients from controls (15). Moreover, elevated HOTAIR levels were associated with hepatic insulin resistance by suppressing SIRT1 expression (16). Notably, Sargazi et al. identified HOTAIR polymorphisms, such as rs1899663 G/T and rs12826786 C/T, influencing T2D susceptibility (17).

A crucial factor contributing to high mortality in diabetic patients is the delayed diagnosis of the disease and its complications(18) (19). Traditional markers are insufficient for predicting diabetes complications. Our findings showed a gradual increase in HOTAIR expression levels from the control to the pre-diabetic group, reaching the highest values in T2D patients. ROC curve analysis demonstrated that HOTAIR could effectively distinguish T2D and pre-diabetic patients from controls. We propose that the elevated expression of HOTAIR is associated with T2D progression, suggesting its potential as a biomarker for early diagnosis and prognosis of T2D.

While little is known about the evaluation of HOTAIR circulating levels in T2D patients, its association with T2D complications has been investigated in several studies. HOTAIR has been identified as an independent biomarker for T2D patients developing long-term sequelae like diabetic retinopathy and nephropathy (15). Overexpression of HOTAIR has been linked to inducing angiogenesis, mitochondrial aberrations, and oxidative damage in retinal endothelial cells, contributing to diabetic retinopathy (20).

Additionally, patients with diabetic retinopathy exhibited increased HOTAIR serum levels compared to healthy subjects (19). HOTAIR has been suggested to ameliorate diabetic cardiomyopathy by enhancing cardiomyocyte vitality (21) and acting as a competing endogenous RNA to modulate inflammation and pyroptosis in diabetic cardiomyocytes (22). Furthermore, HOTAIR overexpression has been shown to inhibit the Wnt/β-catenin signaling pathway in human aortic smooth muscle cells (23).

Our results revealed a positive correlation between HOTAIR and FBS, insulin resistance, and systolic blood pressure levels. This aligns with two previous investigations that reported a positive correlation between HOTAIR and FBS levels (24, 25). Another study demonstrated that altered expression of HOTAIR is positively associated with poor glycemic control and insulin resistance (15). Additionally, in line with our findings, HOTAIR expression has been suggested to have a favorable correlation with systolic blood pressure (SBP) (26). HOTAIR has been implicated in facilitating glucose uptake in LPS-triggered macrophages by regulating genes involved in glucose metabolism (27). Elevated levels of HOTAIR may also regulate glucose levels by promoting the expression of genes involved in the regulation of insulin transcription (28). Finally, upregulation of HOTAIR expression could induce hepatic insulin resistance by inhibiting SIRT1 expression (16).

Research has shown that lncRNAs can competitively bind to miRNAs, reducing the number of miRNAs binding to mRNAs and thus regulating the expression of miRNA-target genes (29). Our enrichment analysis highlighted that most miRNA targets of HOTAIR are enriched in diabetes-related pathways, including HIF-1, FoxO, PI3K-Akt, Ras, and mTOR signaling pathways. Four miRNAs—miR-17-5p, miR-214-3p, miR-20a-5p, and miR-222-3p—were identified as targeting the phosphatase and tensin homolog

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(PTEN) gene. Overexpression of PTEN has been reported to inhibit the PI3K pathway and glucose uptake, leading to insulin resistance (30). FOXO1, another target, plays a protective role for beta cells against damage caused by elevated oxidative markers associated with increased glucose concentrations (31). Lack of FOXO in beta cells has been linked to a lack of insulin secretion capacity in T2D cases (32).

Regarding uc.48+ lncRNA, there is limited information on its presence in whole blood samples and its association with T2D pathogenesis. Studies have shown that uc.48+ siRNA could improve diabetic neuropathy in a rat model of T2D by suppressing the release of CGRP, IL-1β, and TNF-α (33). Additionally, the downregulation of uc.48+ could influence immune and inflammatory responses in a diabetic monophagocyte system through the P2X7 receptor. Our results revealed no significant difference in uc.48+ circulating levels between T2D and control groups. However, a significant positive correlation was found between its expression level and systolic blood pressure. Consistent with our findings, Wu et al. reported that uc.48+ plays a crucial role in the pathological changes of blood pressure in T2D by interacting with the P2X7 receptor. They demonstrated a decrease in blood pressure in DM model mice following uc.48+ siRNA injection (34).

In conclusion, our study demonstrated that HOTAIR expression levels increased in T2D and pre-diabetic patients compared to control subjects, suggesting its potential as a biomarker to distinguish these groups. Furthermore, positive correlations were identified between HOTAIR expression, FBS levels, and insulin resistance. In silico analysis suggested that HOTAIR deregulation could be involved in T2D pathogenesis through lncRNA-miRNA-mediated regulation of signaling pathways related to diabetes. Further in vitro and in vivo investigations are necessary to elucidate and confirm the roles of HOTAIR and uc.48+ in T2D pathogenesis.

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Conflict of interest

The authors declare no conflicts of interest.

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