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HOTAIR participates in hepatic insulin resistance via regulating SIRT1

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effect of long non-coding RNA (lncRNA) HOTAIR on hepatic insulin resistance and to explore the possible underlying mechanism.

PATIENTS AND METHODS: Liver tissues of type 2 diabetes mellitus (T2D) patients, healthy controls, C57BL/6J mice fed with a high-fat diet and db/db mice were harvested. Meanwhile, the expressions of HOTAIR and SIRT1 were detected. Subsequently, We HepG2 cells were treated with tumor necrosis factor α (TNF-α), and the insulin resistance model was constructed *in vitro*. The mRNA expression levels of HOTAIR and SIRT1 in the insulin resistance model were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Insulin sensitivity in HepG2 cells transfected with lentivirus was evaluated by relative commercial kits. In addition, protein expression levels of key factors in the AKT/GSK pathway were detected by Western blot.

RESULTS: HOTAIR was significantly upregulated in T2D patients, C57BL/6J mice fed with a high-fat diet and db/db mice. However, SIRT1 expression presented an opposite changing trend. Moreover, upregulated HOTAIR remarkably promoted hepatic insulin resistance via the AKT/GSK pathway, which could be reversed by SIRT1 overexpression.

CONCLUSIONS: Upregulated HOTAIR promotes hepatic insulin resistance by inhibiting SIRT1 expression and AKT/GSK pathway.

Key Words
HOTAIR, SIRT1, Hepatocyte, Insulin resistance.

Introduction

Insulin resistance is a pathophysiological condition in which insulin effectors such as liver, skeletal muscle, adipose tissue, vascular endothelial cells and arterial smooth muscle cells fail to respond to normal doses of insulin[1]. Insulin, as an important endocrine hormone in the body, binds to the corresponding receptors and regulates gene expression *via* a series of signal pathways. Recent studies have shown that insulin mainly acts on the liver, muscle and adipose tissue. It also participates in the metabolic regulation of various substances in the body. Meanwhile, insulin resistance is the basis of multiple metabolic disorders, such as diabetes mellitus, hypertension, obesity, atherosclerosis and others[2,3]. Previous studies have suggested that lower levels of insulin and corresponding receptors, abnormal expression levels of adipose-derived cytokines and alteration of insulin signaling pathway all exert crucial roles in the process of insulin resistance. However, the specific mechanism of insulin resistance still remains to be further elucidated[4,5]. In addition, the exploration of insulin resistance will contribute to the diagnosis, treatment and prevention of various metabolic diseases.

Long non-coding RNAs (lncRNAs) are a class of endogenous, non-coding RNAs with over 200 nucleotides in length. They may regulate gene expression at transcriptional, post-transcriptional and epigenetic level, with no protein-coding function[6,7]. LncRNA was previously considered as meaningless by-products of transcription. However, current studies have found that lncRNAs are involved in the process of organism growth, development and death *via* various regulations, such as oncogenes activation, transcriptional alteration, genomic imprinting and nuclear transport[8-10]. Meanwhile, a large number of studies have proved that abnormally expressed lncRNAs are closely related to the proliferation of hepatoma carcinoma cells[11]. In addition, MEG3 has been demonstrated to promote the development of hepatic insulin resistance by upregulating FoxO1 expression[12].

HOTAIR is located on chromosome 12q13 between the coding regions of HOXC11 and

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HOXC12. HOTAIR consists of 5 short exons and 1 long exon, with a total length of 2158 bp[13]. Highly conserved HOTAIR participates in the regulation of multiple biological behaviors, such as cell proliferation, apoptosis, invasion, metastasis, autophagy and others. Upregulated HOTAIR has been observed in various malignancies, including breast cancer, pancreatic cancer and liver cancer. More importantly, HOTAIR is closely related to the invasion and metastasis of tumor cells, as well as tumor recurrence and prognosis[14,15]. However, the exact role of HOTAIR in hepatic insulin resistance has not been elucidated yet. The primary purpose of this study was to investigate the effect of long non-coding RNA (lncRNA) HOTAIR on hepatic insulin resistance and to explore the possible underlying mechanism.

Patients and Methods

Sample Collection

10 T2D patients who underwent weight-loss surgery were enrolled in this study. At the same time, 10 healthy people were included as normal controls. Liver tissues of all subjects were collected and preserved in liquid nitrogen for the following experiments. Liver samples were all healthy without any inflammation or fibrosis. Meanwhile, included subjects did not have a chronic liver disease and history of drug use for hepatotoxicity. Basic characteristics of the enrolled subjects were listed in Table I. This study was approved by the Ethics Committee of the 6th Affiliated Hospital of Xinjiang Medical University. The informed consent was obtained from each subject before the study.

Experimental Animals

6-week-old C57BL/6J and db/db mice were obtained from the Model Animal Research Center of Nanjing University. After 8 weeks of feeding, db/db mice were sacrificed. Subsequently, liver tissues of db/db mice were harvested and preserved in liquid nitrogen. C57BL/6J mice were randomly assigned into two groups, including the high-fat diet group and the normal diet group. After feeding for 16 weeks, C57BL/6J mice were sacrificed, and the liver tissues were collected and preserved in liquid nitrogen for subsequent experiments.

Cell Culture and Transfection

HepG2 cells were obtained from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin, and maintained in a 37°C, 5% CO_2 incubator. For cell transfection, HepG2 cells in the logarithmic growth phase were first seeded into 6-well plates at a dose of 5×10^5 per well. Subsequently, LV-Vector, LV-HOTAIR or LV-SIRT1 were transfected into HepG2 cells, respectively. Culture medium was replaced 24 h later. After incubation for 48 h, the transfection efficiency was evaluated by quantitative real-time polymerase chain reaction (qRT-PCR). Lentiviruses used in this study were obtained from GenePharma (Shanghai, China).

For insulin resistance induction, HepG2 cells were serum-starved overnight and treated with 10 ng/mL tumor necrosis factor-α (TNF-α) for 24 h. Cells were then stimulated with 100 nM insulin for 30 min for the following detections.

Western Blot

Total protein of transfected cells was extracted by radio-immunoprecipitation assay (RIPA) solution (Yeasen, Shanghai, China). The concentration of each protein sample was determined by the bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China). Briefly, 50 μg total protein was

Table I. Basic description of the subjects.

Variables	Normal subjects (n = 10)	Type 2 diabetic subjects (n = 10)
Age (years)	48.2 ± 4.2	45.6 ± 7.39
Females/males	4/6	5/5
BMI (kg/m2)	21.59 ± 2.47	30.3 ± 1.5*
Glucose (mM)	4.41 ± 0.56	13.86 ± 1.69*
Insulin (mU/L)	4.58 ± 0.72	11.31 ±1.47*
HbA1c (%)	4.49 ± 0.37	8.27 ±0.77*
HOMA-IR	0.96 ± 0.21	5.62 ± 0.51*

*$p<0.05$. BMI, Body mass index; HbA1c, Hemoglobin A1c; HOMA-IR, homeostasis model assessment of insulin resistance

separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk for 1 h, the membranes were incubated with specific primary antibodies at 4 °C overnight. After washing with Tris-Buffered Saline with Tween 20 (TBST) 3 times, the membranes were incubated with the corresponding secondary antibody at room temperature for 1 h. Immuno-reactive bands were exposed by enhanced chemiluminescence method.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA of cells was extracted according to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, extracted RNA was reversely transcribed into complementary deoxyribonucleic acids (cDNAs). The reaction conditions were as follows: denaturation at 95°C for 15 s, followed by annealing at 58°C for 15 s and extension at 72°C for 45 s, for a total of 40 cycles. Each sample was repeated 3 times. Primers used in this study were: GAPDH, F: CCAGATTCCAAGGGCTGATA; R: GATGGAGGCATCTGGT; HOTAIR, F: GCCCTTCTCCTAGCCCACCG, R: GTGGGGACCCGCTAGACCTG; SIRT1, F: TCGCAACTATACCCAGAACATAGACA, F: CTGTTGCAAAGGAACCATGACA; PEPCK, F: GGCCACAGCTGCTGCAG, R: GGTCGCATGGCAAAGGG; G6Pase, F: GGGAAAGATAAAGCCGACCTAC; R: CAGCAAGGTAGATTCGTGACAG.

Determination of Glucose and Hepatic Glycogen

Hepatic glycogen level was detected in strict accordance with the instructions of the anthrone reagent kit (Jiancheng Bioengineering Institute, Nanjing, China). The total protein content was determined by the bicinchoninic acid assay (BCA) method to normalize the content of glycogen.

Glucose level was detected based on the instructions of a glucose assay kit (Applygen, Beijing, China). Briefly, 5 μL supernatant and 195 μL buffer were mixed and incubated at room temperature for 30 min. Absorbance at the wavelength of 490 nm was measured. Subsequently, the cells were lysed to extract total proteins, followed by the determination of protein concentration with the BCA method to normalize the number of cells.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 Software (Chicago, IL, USA) was used for all statistical analysis. Measurement data were expressed as mean ± standard deviation ($\bar{x}\pm s$). *t*-test was performed to compare the differences between the two groups. $p<0.05$ was considered statistically significant.

Results

HOTAIR Was Overexpressed in Liver Tissues of T2D Patients

The levels of fasting blood glucose, fasting insulin, glycosylated hemoglobin, insulin resistance index and body mass index (BMI) in T2D patients were significantly higher than those of healthy controls (Table I). QRT-PCR results indicated that the mRNA expression level of HOTAIR in T2D patients was remarkably higher than that of healthy controls (Figure 1A). Meanwhile, we also found that HOTAIR expression was remarkably increased in C57BL/6J mice fed with a high-fat diet (Figure 1B) and db/db mice (Figure 1C). Hepatic insulin resistance was induced *in vitro* by using TNF-α. Results demonstrated that the expression of p-AKT was significantly decreased, suggesting successful construction of the insulin resistance model in HepG2 cells (Figure 1D). Under the condition of insulin resistance in HepG2 cells, HOTAIR expression increased in a time-dependent manner and reached a peak at 24 h (Figure 1E). These results all suggested that HOTAIR might be involved in hepatic insulin resistance.

Overexpressed HOTAIR Promoted Insulin Resistance

HOTAIR was significantly upregulated in HepG2 cells by lentivirus transfection (Figure 2A). Subsequently, we detected key genes in the insulin pathway, and results showed that overexpressed HOTAIR markedly inhibited the phosphorylation levels of AKT and GSK3β (Figure 2B). PEPCK and G6pase are considered as gluconeogenesis genes. In this study, we found that overexpressed HOTAIR resulted in elevated expressions of PEPCK and G6pase, indicating an increase in gluconeogenesis level (Figure 2C). Moreover, due to the reduction of the insulin sensitivity and elevation of gluconeogenesis level induced by overexpressed HOTAIR, the level of glucose in cell supernatant was highly increased (Figure 2D). However, glycogen level in hepato-

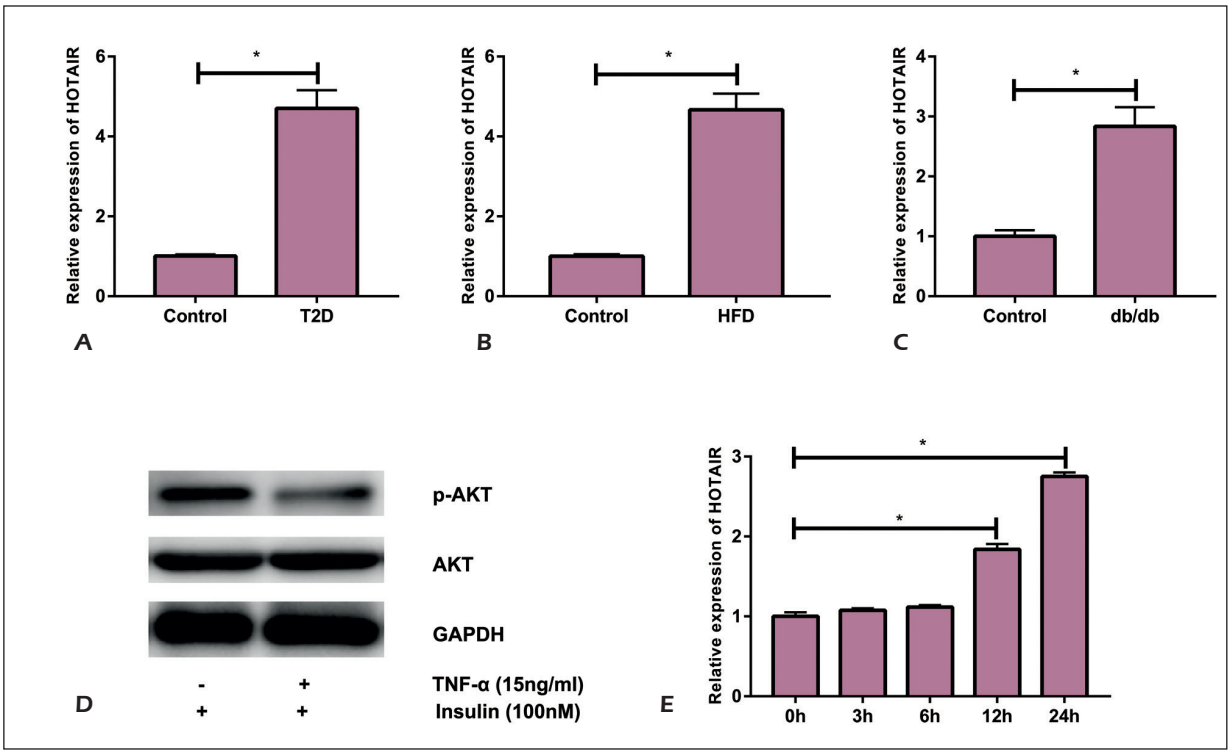

Figure 1. HOTAIR was upregulated in liver tissues of T2D patients. HOTAIR expression levels in liver tissues of T2D patients (**A**), C57BL/6J mice fed with a high-fat diet (**B**), and db/db mice (**C**) were detected by qRT-PCR. **D**, Phosphorylation level of AKT in the insulin resistance model. **E**, HOTAIR expression in the insulin resistance model.

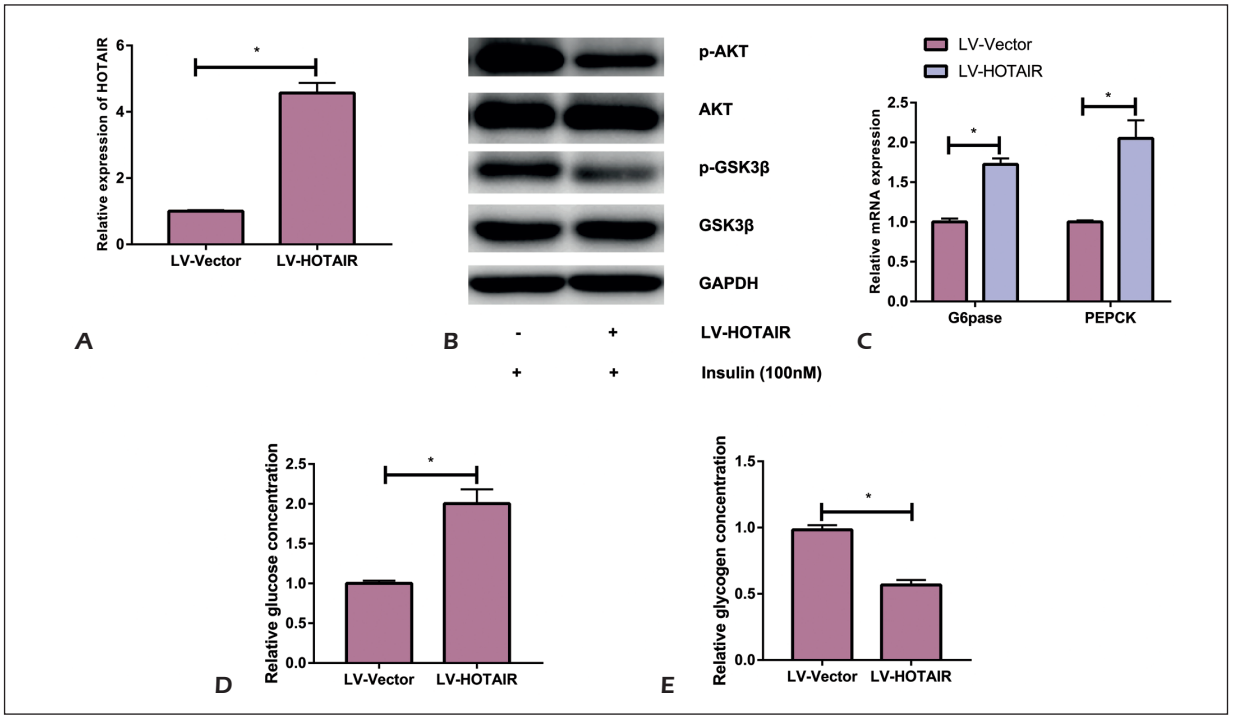

Figure 2. Upregulated HOTAIR promoted insulin resistance. **A**, HOTAIR expression level after lentivirus transfection. **B**, Phosphorylation levels of AKT and GSK3β after HOTAIR overexpression. **C**, Expressions of gluconeogenesis genes after HOTAIR overexpression. **D**, Glucose level in the cell supernatant after HOTAIR overexpression. **E**, Glycogen level in HepG2 cells after HOTAIR overexpression.

cytes was notably decreased after HOTAIR overexpression (Figure 2E). The above results all indicated that upregulated HOTAIR led to insulin resistance.

Overexpressed HOTAIR Inhibited SIRT1 Expression

Given the crucial role of SIRT1 in regulating insulin sensitivity, we explored whether HOTAIR could regulate SIRT1 expression. First, we found that the mRNA expression level of SIRT1 in the liver tissues of T2D patients (Figure 3A), C57BL/6J mice fed with a high-fat diet (Figure 3B) and db/db mice (Figure 3C) were all remarkably decreased when compared with those of negative controls. Similar results were obtained in the protein expression level of SIRT1 (Figure 3D-F). *In vitro* insulin resistance model demonstrated SIRT1 expression gradually decreased in a time-dependent manner (Figure 3G). However, both the mRNA (Figure 3H) and protein (Figure 3I) levels of SIRT1 were greatly decreased after HOTAIR overexpression. This suggested that SIRT1 participated in hepatic insulin resistance and was regulated by HOTAIR.

Overexpression of SIRT1 Reversed HOTAIR-Induced Insulin Resistance

After lentivirus transfection, the intracellular expression level of SIRT1 was remarkably elevated (Figure 4A). Our results showed that the upregulation of gluconeogenesis genes induced by HOTAIR overexpression was reversed to normal level by SIRT1 overexpression (Figure 4B). Moreover, the levels of glucose (Figure 4C) and hepatic glycogen (Figure 4D) induced by HOTAIR overexpression could also be reversed after HOTAIR overexpression.

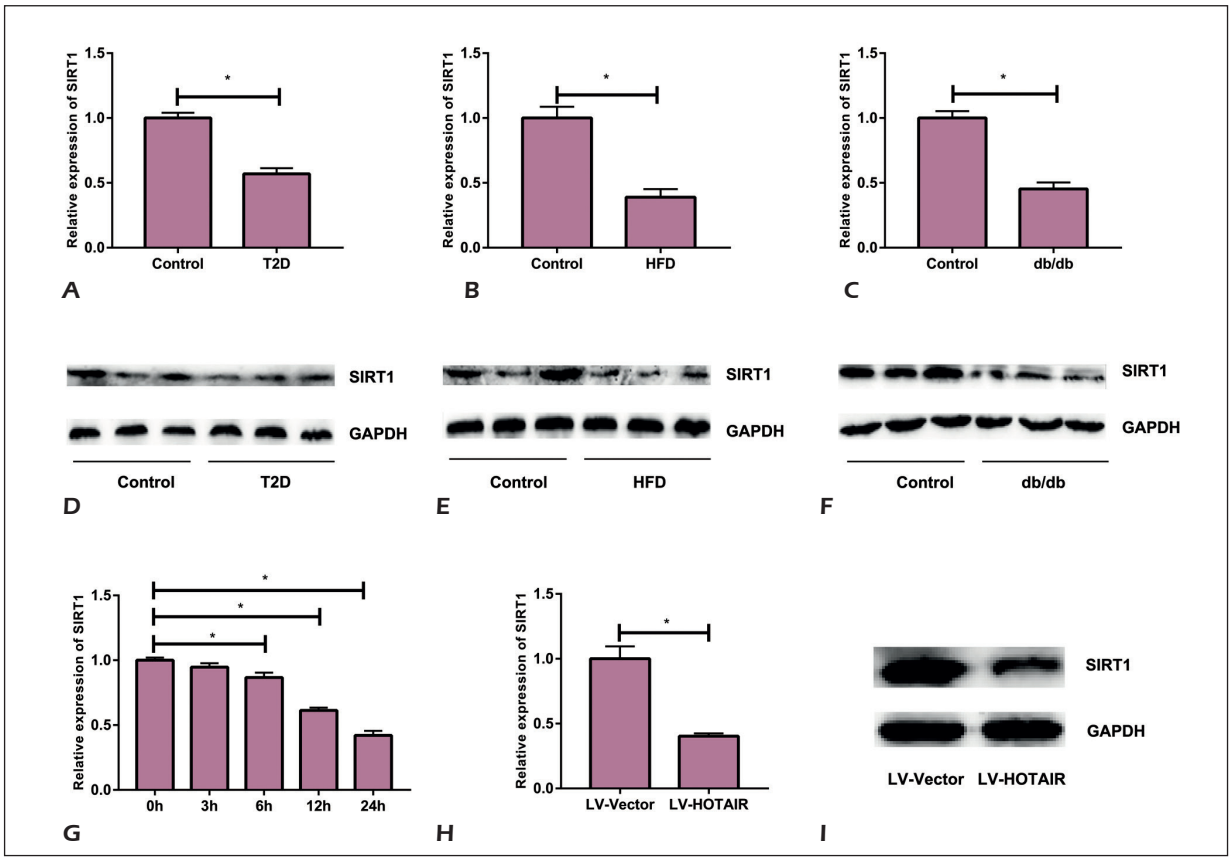

Figure 3. Upregulated HOTAIR inhibited SIRT1 expression. The mRNA expressions of SIRT1 in the liver tissues of T2D patients (**A**), C57BL/6J mice fed with a high-fat diet (**B**), and db/db mice (**C**) were detected by qRT-PCR. Protein expression levels of SIRT1 in the liver tissues of T2D patients (**D**), C57BL/6J mice fed with a high-fat diet (**E**), and db/db mice (**F**) were detected by Western blot. **E**, The mRNA expression of SIRT1 in the insulin resistance model. **F**, The mRNA expression of SIRT1 after HOTAIR overexpression. **G**, Protein expression of SIRT1 after HOTAIR overexpression.

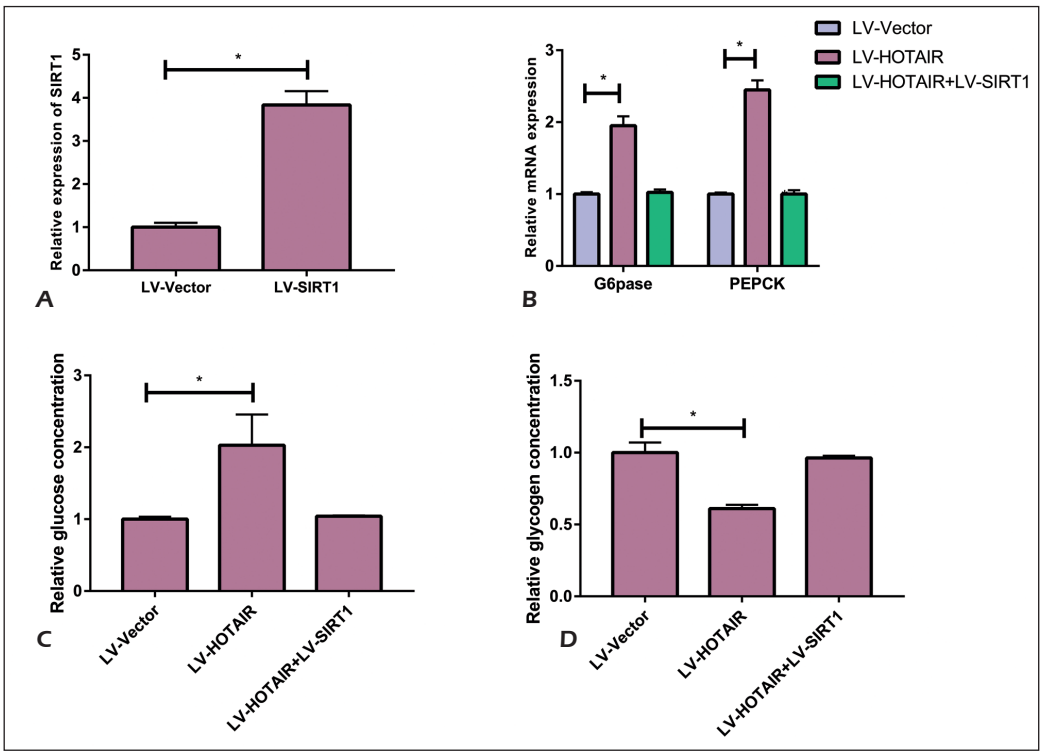

Figure 4. Upregulated SIRT1 increased insulin sensitivity. **A**, The mRNA expression of SIRT1 in HepG2 cells after lentivirus transfection. **B**, Expressions of gluconeogenesis genes after overexpression of HOTAIR and SIRT1. **C**, Glucose level in the cell supernatant after overexpression of HOTAIR and SIRT1. **D**, Glycogen level in HepG2 cells after overexpression of HOTAIR and SIRT1.

Discussion

Insulin resistance is associated with various metabolic diseases, especially diabetes and cardiovascular diseases. Currently, insulin resistance is not only the main pathogenesis of T2D, but also a risk factor for cardiovascular diseases[16]. Prevention and treatment of insulin resistance, as well as relative glucose and lipid metabolism disorders may contribute to better therapeutic effect on diabetes. Therefore, it is of great importance to further explore the pathogenesis of insulin resistance, thereby providing theoretical support for treating diabetes and cardiovascular diseases.

Inflammatory factors are important factors that can induce insulin resistance. Among them, TNF-α induces insulin resistance by inhibiting various related proteins, such as insulin receptor substrate-1 (IRS-1) and glucose transporter 4 (GLUT4)[17,18]. In the present work, we found that TNF-α treatment could reduce the activity of AKT/GSK signaling pathway in HepG2 cells. This indicated that TNF-α could lead to insulin resistance in liver cells. Besides, HOTAIR expression was significantly increased after TNF-α stimulation, suggesting that HOTAIR might be involved in insulin resistance of liver cells.

Currently, there are 3 subtypes of Akt, namely Akt1, Akt2 and Akt3. Akt1 is expressed in most tissues, whereas Akt2 is predominantly found in islet-responsive tissues. Meanwhile, Akt3 is overexpressed in testis and brain. Phosphorylation is the primary mechanism of Akt activation, which is mainly regulated *via* phosphatidylinositol 3 kinase-phosphoinositide-dependent kinase (PDK) signaling pathway[19]. PI3k/Akt signaling pathway is known as the major pathway responsible for regulating insulin expression. Scholars have shown that the activity of the PI3K signaling pathway in the island of Langerhans is decreased under insulin resistance. Furthermore, it is reported[20-22] that the insulin sensitivity in liver and muscle tissues of Akt2 knockout mice is significantly attenuated when compared with that of normal mice. In the present study, our findings demonstrated that overexpression of HOTAIR in liver cells could inhibit the AKT/GSK signaling pathway and increase cellular glycogen level, indicating that HOTAIR might regulate insulin sensitivity of liver cells.

As a NAD^+-dependent protein deacetylase, silent information regulator 1 (SIRT1) belongs to the Sirtuin family. SIRT1 was originally defined as a NAD^+-dependent Sirtuin family that could induce deacetylation of lysine residues in various proteins. Sirtuin is a class of highly conserved proteins involved in multiple biological processes, especially cell survival, lifespan and material metabolism. Meanwhile, SIRT1 is widely expressed in organs and tissues, such as mammalian liver, adipose tissue, muscle, kidney and others. It has been found that SIRT1 is upregulated after energy limitation[23]. In the liver, SIRT1 deacetylates PGClα, resulting in increased expressions of gluconeogenesis genes and decreased expressions of glycolytic genes. This may eventually induce glycogen output[24]. An *in vivo* experiment has found that increased insulin sensitivity and inhibited hepatic glucose production can be observed in genetically obese mice treated with SIRT1 activator[25]. Further studies have shown that SIRT1 is necessary for maintaining the homeostasis of glucose and fat *in vivo*. SIRT1 knockdown in liver tissues can not only increase blood glucose level and insulin resistance, but also leads to higher levels of liver free fatty acids and cholesterol[26]. Meanwhile, *in vitro* experiments have shown that overexpression of SIRT1 in rat liver cells can enhance the activity of AMP-activated protein kinase. This may eventually prevent fatty acid synthase and lipid accumulation induced hyperglycemia[27]. In this study, we found that overexpressed HOTAIR induced insulin resistance in liver cells, which could be reversed by SIRT1 upregulation.

Conclusions

We showed that upregulated HOTAIR promotes hepatic insulin resistance *via* inhibiting SIRT1 expression and the AKT/GSK pathway.

Conflict of Interests:

The authors declared no conflict of interest.

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