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Downregulation of HOTTIP regulates insulin secretion and cell cycle in islet β cells via inhibiting MEK/ERK pathway

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Abstract. – OBJECTIVE: To investigate the effect of long non-coding RNA (lncRNA) HOTTIP on islet β cells and its underlying mechanism.

MATERIALS AND METHODS: The expressions of HOTTIP in different organs of db/db mice and C57BL/6J mice were detected by quantitative Real-time polymerase chain reaction (qRT-PCR). Effects of HOTTIP on the proliferation, insulin secretion and apoptosis of islet β cells transfected with lentivirus were detected by cell counting kit-8 (CCK-8) assay, enzyme-linked immunosorbent assay (ELISA) and flow cytometry, respectively. We also assessed the protein expressions of key genes in MEK/ERK pathway by using Western blot.

RESULTS: HOTTIP was upregulated in normal islet tissues of C57BL/6J mice but downregulated in islet tissues of diabetic mice. Inhibition of HOTTIP attenuated insulin secretion and reduced expressions of Pdx1 and MafA. Downregulation of HOTTIP also inhibited cell proliferation and reduced expressions of CyclinDl, CyclinD2, CyclinE1 and CyclinE2. Moreover, islet β cells were arrested in G0/G1 phase after HOTTIP knockdown. Our data showed that the biological function of HOTTIP in regulating insulin secretion and cell cycle in islet β cells might be related to the MEK/ERK pathway.

CONCLUSIONS: Downregulation of HOTTIP inhibits insulin secretion and cell cycle in islet β cells via MEK/ERK pathway.

Key Words:
HOTTIP, Insulin secretion, MER/ERK pathway, Cell cycle.

Introduction

Diabetes mellitus is a type of metabolic disease characterized as genetic or immune dysfunction induced by insulin resistance or insufficient insulin secretion, which is usually accompanied by a variety of serious complications[1]. Islet β cells are the most important source of insulin in the body, which are capable of regulating insulin secretion and maintaining blood glucose balance[2]. Diabetes is usually caused by autoimmune destruction of β cells or impaired function of pancreatic β cells due to harsh environments, such as hyperglycemia and hyperlipidemia[3]. It is of great importance to investigate the islets function because of the complicated pancreatic structure and pancreatic islet physiology, as well as the high incidence of diabetes.

About 5-10% of the human genome sequences are stably transcribed, whereas the vast majority of these transcriptional sequences are non-coding transcripts. One of the most transcribed non-protein coding sequences is the long non-coding RNA (lncRNA). LncRNAs are a class of non-coding RNAs without open reading frames with over 200 nt in length[4]. Abnormal expression of lncRNAs is associated with many diseases such as cardiovascular diseases, tumors and endocrine diseases[4-6]. However, the role of lncRNAs in islet β cells function and diabetes development is not clear.

LncRNA HOTTIP (HOXA distal transcript) is transcribed from the 5'-end of the HOXA gene and is located on chromosome 7p15.2 with a total length of 3764 nt. Evidence has shown that HOTTIP participates in chromatin spatial conformation, histone modification and composition of nuclear complexes, thus affecting gene expressions[7]. HOTTIP is closely related to the pathological process of many diseases, such as rectal cancer and lung adenocarcinoma[8,9]. However, the role of HOTTIP in pancreatic β cells has not been reported. Thus, the aim of this study was to investigate

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the effect of HOTTIP on islet β cells and its underlying mechanism.

Materials and Methods

Experimental Animals

The db/db mice (experimental group) and C57BL/6J mice (control group) with 6-week-old used in this study were purchased from the Animal Model Research Center of Nanjing University (Nanjing, China). All mice were fed for six weeks prior to the experiment. After overnight fasting, the abdominal cavity and chest of the mouse were cut and opened under anesthetic conditions. After collagenase V was injected into the common bile duct, the pancreatic tissue was peeled off alongside the intestine. Tissues were digested in a 37°C water bath for islets harvesting. The remaining heart, brain, lung, spleen, liver and muscle tissue were harvested and preserved in liquid nitrogen immediately. This study was approved by the Animal Ethics Committee of Medical School of Jiangsu University Animal Center.

Cell Culture

MIN6 cells (mouse islet β cell line) were cultured in Dulbecco's modified eagle medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin, and cultured in a 5% CO_2 incubator at 37°C (Gibco, Rockville, MD, USA). Cells were treated with 5.5 mM, 11 mM, 25 mM or 33 mM glucose for another 24 h, respectively at the confluence of 80%. Total RNA was extracted to detect the effect of different glucose concentrations on HOTTIP expression.

Cell Transfection

Cells were inoculated in antibiotic-free medium one day prior to transfection according to the instructions of Lipofectamin 2000 (Invitrogen, Carlsbad, CA, USA). LV-Vector and LV-shHOTTIP (GenePharma, Shanghai, China) were diluted in serum-free medium and incubated at room temperature for 5 min. The medium was replaced after 6 h incubation.

Cell Cycle and Apoptosis

Cells in logarithmic growth phase were digested and seeded in 6-well plates at a dose of 1×10^5/well. After 24 h incubation, cells were harvested for relative detections.

For cell apoptosis detection, 500 μL of Binding Buffer were added to prepare single cell suspension. 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI) (Thermo Fisher Scientific, Waltham, MA, USA) were mixed with cell suspension and incubated in the dark for 10 min, followed by cell apoptosis detection using the flow cytometry. For cell cycle detection, the cells were washed once with phosphate-buffered saline (PBS) and adjusted to 1×10^6/mL after centrifugation. Cells were fixed with 70% ethanol and preserved at 4°C. Fixed cells were then added 100 μL of Nase A and stored in a water bath for 30 min. After that, 400 μL PI was added to stain the cells for 30 min at 4°C in the dark. Flow cytometry was used to determine the cell cycle distribution.

Cell Proliferation

The cells transfected with LV-Vector or LV-shHOTTIP were collected and seeded into 96-well plates at a density of 1×10^3 cells/well. Totally, 100 μL of 10% Cell Counting Kit-8 (CCK-8) solution (Dojindo, Kumamoto, Japan) was added to each well for 1 h incubation. The absorbance at 450 nm of each sample was measured by a microplate reader.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted from each group of cells by TRIzol method (Invitrogen, Carlsbad, CA, USA) and then transcribed into complementary Deoxyribose Nucleic Acid (cDNA). The reverse transcription reaction was carried out in strict accordance with the instructions of SYBR Green Real-Time PCR Master Mix (TaKaRa, Otsu, Shiga, Japan), with a total reaction volume of 20 μL. The parameters of the thermal cycling were as follows: pre-denaturation at 95°C for 15 s, denaturation at 94°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, for a total of 40 cycles. Primer sequences used in this study were as follows: HOTTIP, F: GTGGGGCCCAGACCCGC, R: AATGATAGGGACACATCGGGGAACT; MafA, F: GAGGCCTTCCGGGGTCAGAGCTTCGCGG, R: TCTGTTTCAGTCGGATGACCTCCTCCTTGC; PDX1, F: CGGAAGAAAAAGAGCCATTG, R: GCCAGAGGAAGAGGAGGACT; Nkx6.1, F: GGACCTGTTCCTCGAGGAAATGTGACACTTTAC, R: GTAAAGTGTCACATTTCCTCGAGGAACAGGTCC; Cyclin D1, F: TGAACTACCTGGACCGCT,R:GCCTCTGGCATTTTGGAG; Cyclin D2, F: TACTTCAAGTGCGTGCAGAAGGAC, R: TCCCACACTTCCAGTTGCGA-

TCAT; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), F: CCTGCACCACCAACTGCTTA, R: GGCCATCCACAGTCTTCTGAG; CyclinE1, F: TCCACGCATGCTGAATTATC, R: TTGCAAGACCCAGATGAAGA; CyclinE2, F: AAAAAGTCTTGGGCAAGGTAAA, R: GCATTCTGACCTGGAACCAC.

Western Blot

Radioimmunoprecipitation assay (RIPA) solution (Beyotime, Shanghai, China) was used to extract the total protein. The cell lysates were centrifuged at 12,000 r/min for 5 min, and the supernatant was collected. Protein sample was separated by electrophoresis on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After membranes were blocked with blocking solution (1×TBS, 0.5% Tween-20, 5% w/v skim milk) for 1 h, the membranes were incubated with primary antibodies (dilution at 1:1000) for 2 h at room temperature. The membranes were then washed with Tris-buffered saline-tween (TBST) (Beyotime, Shanghai, China) and followed by the incubation of the horseradish-peroxidase (HRP)-labeled secondary antibody (dilution at 1: 5000). The protein blot on the membrane was exposed by chemiluminescence.

Glucose-Stimulated Insulin Secretion Assay (GSIS)

The transfected cells were collected and seeded into 96-well plates at a density of 5×10^3 cells/well. After culturing overnight, cells were continued to maintain in glucose-free condition for 30 min. Subsequently, the cells were further incubated for 30 min in Krebs-Ringer buffer containing 3 mM and 30 mM glucose. The supernatants and cellular proteins were collected and the insulin content was measured.

Enzyme-Linked Immunosorbent Assay (ELISA)

The supernatants of MIN6 cells from different treatment groups were collected. According to the instructions of ELISA kit (EBiosciences, San Diego, CA, USA), we used insulin standard curve to detect the expressions of different samples of insulin. Briefly, cells were blocked in 5% bovine serum for 40 min. After washing for three times, the sample and enzyme-labeled antibody were added to each well. Substrate solution was used to terminate the reaction and ELISA results were determined within 20 min.

Statistical Analysis

Measurement data were expressed as mean ± standard deviation. Independent-sample *t*-test was used to compare the differences between two groups. $p<0.05$ was considered statistically significant. All statistical analysis was conducted on Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA).

Results

HOTTIP was Upregulated in Pancreatic Islets

We examined HOTTIP expressions in different tissues of C57BL/6J mice by qRT-PCR. The results of qRT-PCR showed that HOTTIP had the highest abundance in pancreatic tissues (Figure 1A). Further, HOTTIP was found to be upregulated in isolated islets than that of exocrine glands (Figure 1B). In addition, HOTTIP expression in the islets of db/db mice with diabetes was markedly downregulated compared with that of C57BL/6J mice (Figure 1C). These results suggested that HOTTIP might regulate islet β cells function and is involved in diabetes development.

Downregulation of HOTTIP Inhibited Insulin Secretion

To explore the factors that regulate HOTTIP expression, we detected the expression level of HOTTIP in MIN6 cells after treatment with different concentrations of glucose for 24 h. The results showed that HOTTIP expression was decreased in a dose-dependent manner (Figure 2A). Transfection of LV-shHOTTIP in MIN6 cells remarkably decreased HOTTIP expression (Figure 2B). Functionally, downregulated HOTTIP significantly inhibited high glucose-stimulated insulin secretion (Figure 2C).

Additionally, our data exhibited that the mRNA levels of Pdxl and MafA were remarkably decreased after transfection with LV-shHOTTIP in MIN6 cells. However, no significant difference in Nkx6.1 expression was found (Figure 2D). MEK/ERK pathway is convinced to exert a crucial role in insulin secretion[10]. Our results indicated that inhibited HOTTIP significantly decreased the phosphorylation of MEK/ERK pathway (Figure 2E). The above results demonstrated that HOTTIP regulates insulin secretion *via* MEK/ERK pathway.

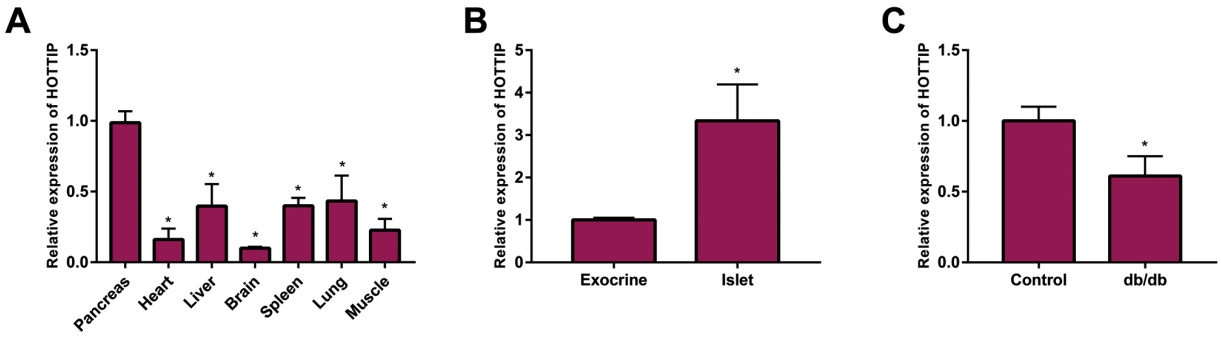

Figure 1. HOTTIP was upregulated in pancreatic islets. *A,* Detection of HOTTIP expressions in different organs of mice. *B,* HOTTIP expressions in mouse pancreatic exocrine gland and islet. *C,* Detection of HOTTIP expression in islets of C57BL/6J mice and db/db mice.

Downregulation of HOTTIP Attenuated β Cells Proliferation

To explore whether HOTTIP affects the biological functions of β cells, we examined the effect of HOTTIP on apoptosis, proliferation and cell cycle of β cells, respectively. No significant changes were observed in cell apoptosis after HOTTIP knockdown (Figure 3A). However, a remarkable increase of proliferation in MIN6 cells was observed when HOTTIP was downregulated (Figure 3B). Cell cycle test revealed that downregulation of HOTTIP arrested cells in the G0/G1 phase (Figure 3C). Besides, we found that protein expressions of key genes that are related to cell cycle, such as CyclinDl, CyclinD2, CyclinE1 and CyclinE2, were remarkably decreased after HOTTIP knockdown (Figure 3D). These data suggested that downregulated HOTTIP could lead to cell arrestment in G0/G1 phase, thereby affecting cell proliferation.

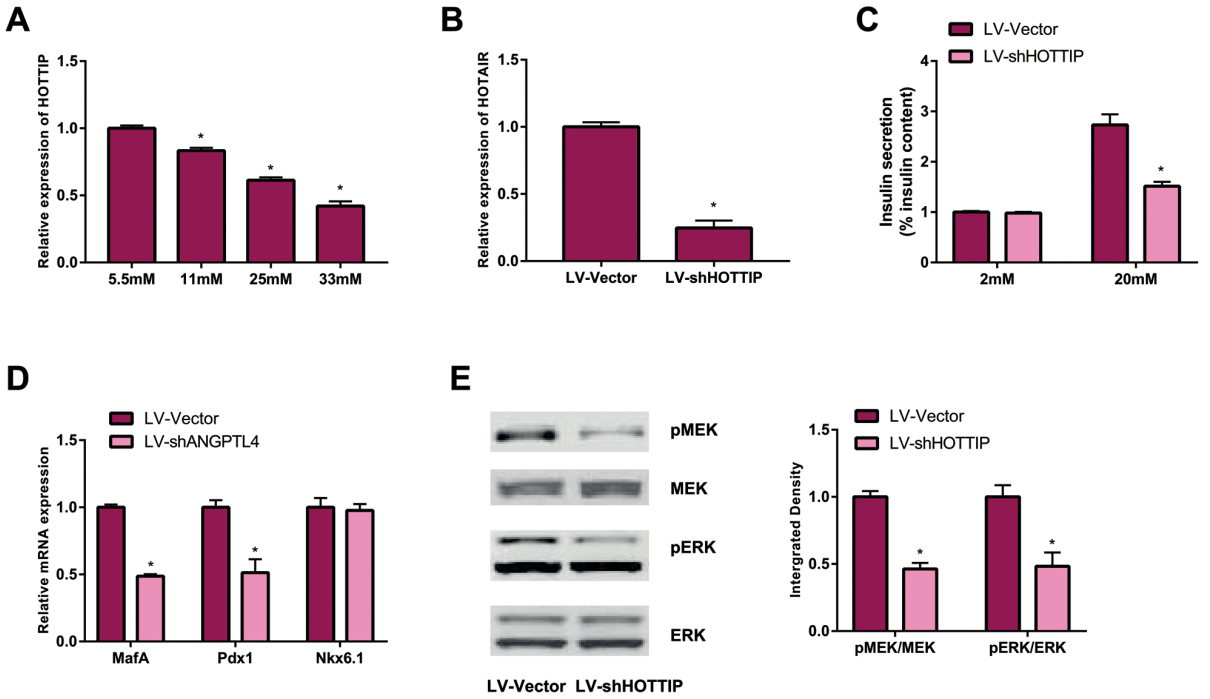

Figure 2. Downregulation of HOTTIP inhibited insulin secretion. *A,* HOTTIP expression was detected after 24 h stimulation with different glucose concentrations. *B,* Detection of HOTTIP expression in cells transfected with LV-shHOTTIP. *C,* Detection of insulin secretion by high glucose-stimulated cells transfected with LV-shHOTTIP. *D,* Expressions of key genes in insulin transcription were detected after LV-shHOTTIP transfection. *E,* Phosphorylation levels of MEK and ERK were detected after LV-shHOTTIP transfection.

Discussion

With the development of sequencing technology, many lncRNAs have been identified to be related to islet β cells, such as MEG3, H19, etc. These lncRNAs play crucial roles in the development of diabetes and its complications[11]. In the present study, HOTTIP knockdown in MIN6 cells led to the reduction of insulin secretion and cell proliferation stimulated by glucose, as well as cell arrestment in the G0/G1 phase.

ERK, an extracellular signal-regulated kinase, is a member of the MAPK family. The ERK family includes 5 members: ERK-1 to ERK-5. Activated ERK mainly regulates the cell growth, development and division. ERK pathway consists of the Ras-Raf-MEK-ERK signal axis[12,13]. MEK is divided into MEK1 and MEK2, with molecular weight of 44kD and 45kD, respectively. MEK is a type of special double-specific kinase that can phosphorylate Thr and Tyr, thus activating ERK[14,15]. ERK is centrally located in the MEK signaling pathway and has long-term effects on the biological activities of cells. Abnormal activation or suppression of the MEK/ERK signaling pathway has been observed in many diseases, while the role of MEK/ERK pathway in insulin secretion has not been explored[16]. Youl et al[10] reported that quercetin regulated the insulin secretion of β cells in rats through ERK1/2 pathway. We also confirmed the remarkable decrease in insulin secretion induced by high glucose was regulated by HOTTIP *via* MEK/ERK pathway.

In view of the important contribution of ERK1/2 to the regulation of cell proliferation, we continued to investigate the effect of HOTTIP on islet β cells[17]. It was found that downregulated HOTTIP significantly inhibited cell proliferation. ERK1/2 regulates cell proliferation *via* several mechanisms, one of which is the regulation of cell cycle[18, 19]. Cell cycle is divided into G1 phase, S phase, G2 phase and M phase, which is regulated

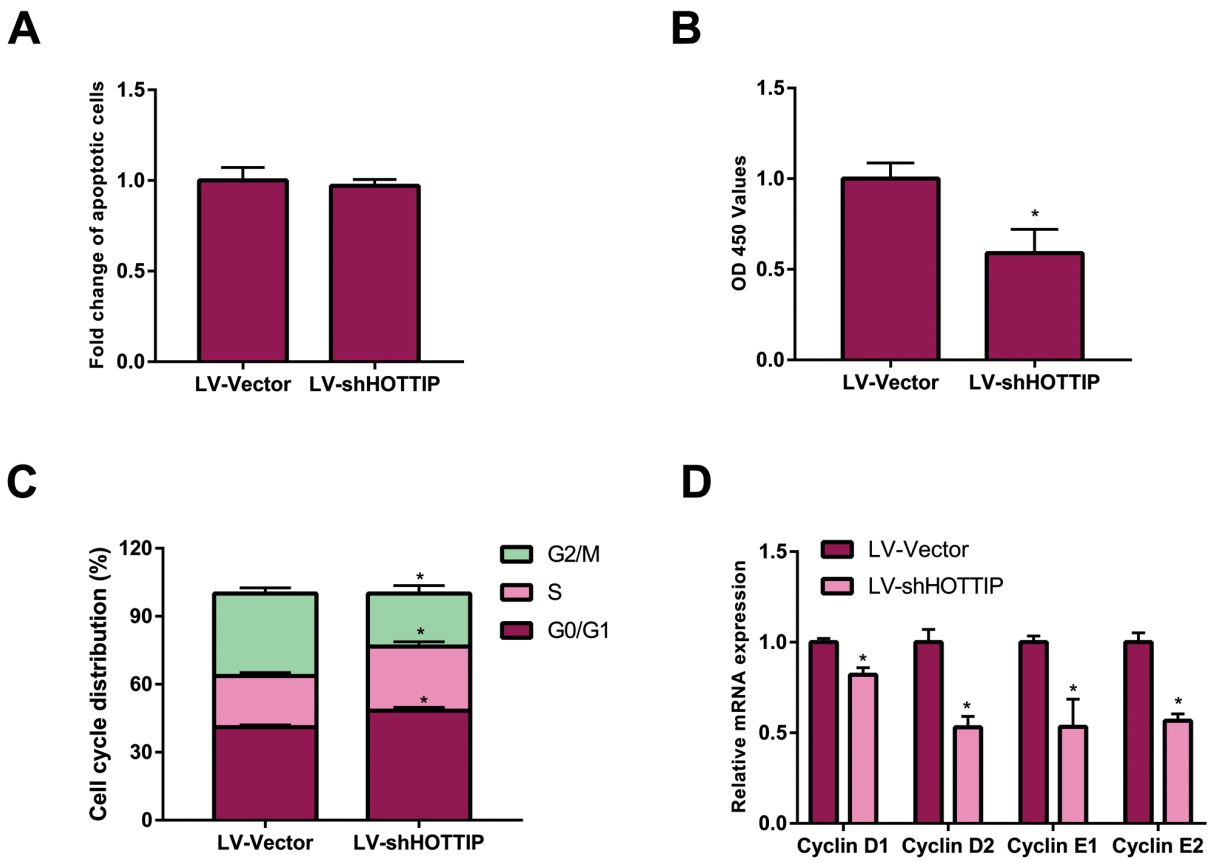

Figure 3. Downregulation of HOTTIP attenuated β-cell proliferation. *A,* Apoptosis was detected by flow cytometry after transfection of LV-shHOTTIP. *B,* Cell proliferation was detected after LV-shHOTTIP transfection. *C,* Cell cycle was detected by flow cytometry after transfection with LV-shHOTTIP. *D,* Expressions of key genes in cell cycle were detected after LV-shHOTTIP transfection.

by cell cycle-related proteins. The conversion of the eukaryotic cell cycle requires the phosphorylation and dephosphorylation of specific substrates. These substrates promote specific events, such as DNA replication (S phase), chromosomal segregation (mitosis), and plasmolysis[18,20,21]. Each cell cycle is controlled by a unique set of serine/threonine protein kinases that are involved in the regulation of important cellular events[22]. These protein kinases, also known as cyclin-dependent protein kinases (CDKs), are capable of forming cycle-regulated complexes that aggregate and activate CDKs at specific time point of cell cycle. ERK1/2 phosphorylates the CDK proteins family, and activated CDK in turn phosphorylates RB and promotes the separation of RB from E2F, which is responsible for encoding proteins during DNA replication. ERK1/2 is rapidly activated by stimulation of mitogenic signals, which efficiently promotes the conversion from G1 phase to S phase[17,23]. At the same time, the alteration of cell cycle is also related to the activation of CDKs and their corresponding cyclin subunits[24,25]. In this study, we found that expressions of CyclinDl, CyclinD2, CyclinE1 and CyclinE2 were significantly decreased after HOTTIP knockdown, and the cells were arrested in G0/G1 phase, which finally led to the inhibition of cell proliferation.

Conclusions

We observed that downregulation of HOTTIP inhibits insulin secretion and cell proliferation *via* MEK/ERK pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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