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Original Research Article

KLF8 enhances acute myeloid leukemia cell growth and glycolysis via AKT/mTOR pathway

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Abstract

Purpose: *To determine the role and mechanisms of Krüppel-like transcription factor 8 (KLF8) in acute myeloid leukemia (AML).*
Methods: *The transcriptional and translational levels of KLF8 in AML cell lines were determined by quantitative real time-polymerase chain reaction (qRT-PCR) and western blotting. Two RNAs targeting KLF8 were transfected into KG-1 and HL-60 cells. The growth and apoptosis of AML cells were determined using CCK-8, 5-ethynyl-2-deoxyuridine (EdU), flow cytometry, and western blot assays. Glycolysis was evaluated by relative glucose consumption, lactate generation, ATP levels, and hexokinase II (HK2), while glucose transporter 1 (GLUT1) protein expression levels. AKT, phosphorylated AKT (p-AKT), mammalian target of rapamycin (mTOR), and p-mTOR expression levels were assessed by western blot.*
Results: *KLF8 mRNA and protein expression levels were elevated in AML cells ($p < 0.01$). KLF8 knockdown in AML cells decreased cell viability, EdU-positivity, relative glucose consumption, lactate generation, ATP levels, and HK2 and GLUT1 protein levels ($p < 0.01$). Apoptosis increased in KG-1 and HL-60 cells, with enhanced Bax and reduced Bcl-2 protein levels, after transfection with sh-KLF8. The relative expression levels of p-AKT/AKT and p-mTOR/mTOR were reduced in KG-1 and HL-60 cells transfected with sh-KLF8.*
Conclusion: *Downregulation of KLF8 inhibits proliferation and glycolysis, and also promotes apoptosis in AML cells via AKT/mTOR pathway.*

Keywords: *Acute myeloid leukemia, KLF8, Proliferation, Apoptosis, Glycolysis, AKT/mTOR*

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INTRODUCTION

Acute myeloid leukemia (AML) is a type of hematological malignancy with high invasiveness and heterogeneity that originates from hematopoietic stem cells [1]. The characteristics of AML are early differentiation arrest and uncontrollable amplification of leukemia cells in the peripheral blood and bone marrow [1]. Conventional chemotherapy, targeted therapy, and allogeneic stem cell transplantation have been shown to be effective therapies for AML [2]. Nevertheless, its clinical outcome is still pessimistic [3]. Moreover, older patients have a higher incidence and show poorer prognoses

[1,3]. Therefore, discovering clinically effective targets for AML is urgently needed.

Krüppel-like transcription factor 8 (KLF8) is a member of the KLF family [4]. As a sequence-specific DNA-binding protein, KLF8 recognizes the CACCC-box element [4]. This KLF8 has been shown to play a crucial role in various processes in diverse tumors. Downregulation of KLF8 inhibits proliferation and elicits cell-phase arrest in pancreatic cancer cells [5]. It enhances the resistance of glioma cells to temozolomide by activating β-catenin, binds and activates FHL2 to enhance tumorigenesis, invasion, and metastasis in colorectal cancer cells [6,7]. Moreover, findings by Mao *et al* [8] showed that upregulated KLF8 in gastric cancer was associated with poor prognosis and that KLF8 modulated glycolysis by targeting GLUT4. Furthermore, bioinformatics results showed that KLF8 was highly expressed in AML, based on the Gene Expression Profiling Interactive Analysis (GEPIA) database, indicating that KLF8 may play a vital role in the development of AML.

In the present study, the role of KLF8 in AML progression was determined in AML cell lines.

EXPERIMENTAL

Cell culture

The normal human bone marrow stromal cell line HS-5 (CRL-11882) and the AML cell lines HL-60 (CCL-240), KG-1 (CRL-8031), MOLM-14 (ACC 777), and MOLM-13 (ACC 554) were obtained from the American Type Culture Collection (Manassas, VA, USA). HS-5 cells were maintained in Dulbecco's modified Eagle medium (Solarbio, Beijing, China), whereas AML cell lines were maintained in RPMI-1640 medium (Solarbio) containing 10 % fetal bovine serum (Gibco, Rockville, MD, USA) and 1 % streptomycin-penicillin (Solarbio) and incubated in a 5 % CO_2 atmosphere at 37 °C.

Cell transfection

Two small hairpin RNAs (shRNAs) targeting KLF8, referred to as sh-KLF8#1 and sh-KLF8#2, and sh-negative control (shNC), were obtained from GenePharma (Shanghai, China). The HL-60 and KG-1 cells were inoculated into 6-well plates (Corning, Corning, NY, USA) and maintained at 37 °C in a 5 % CO_2 atmosphere. When the cells reached 80 % confluence, sh-KLF8#1 and sh-KLF8#2 (50 nM) or sh-NC were co-transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs from cells were harvested with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and then reverse-transcribed with a RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Thereafter, qRT-PCR was conducted on a PIKORed 96 (Thermo Fisher Scientific) using SYBR Green PCR Kit (Takara, Dalian, China). The gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The primers for KLF8 (upstream, 5´-TCATTGGAGGAGATGGTAA-3' and downstream, 5´-GCTGCTGGTTCTTGC TGT-3') and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; upstream, 5´-CAGCTAGCCGCATCTTCTTTT-3' and down-stream, 5-GTGACCAGGCGCCCAATAC-3') were generated by Sangon Biotech (Shanghai, China).

Western blotting

The KG-1 and HL-60 cells were seeded in 6-well plates at a plating density of 1 × 10^6 cells/well and maintained for 24 h. The cells were then lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Protein samples were obtained by centrifugation and quantified using a BCA kit (Thermo Fisher Scientific) to determine protein concentrations. Equal amounts of protein from each sample were then resolved using 10 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electrically transferred onto polyvinylidene difluoride (PVDF) membranes according to standard procedures for western blotting. The primary antibodies used were against KLF8 (1:1,000, ab168527), hexokinase II (HK2, 1:10,000, ab227198), glucose transporter 1 (GLUT1, 1:2,500, ab14683), Bax (1:500, ab53154), Bcl-2 (1:2,000, ab196495), phosphorylated AKT (p-AKT, 1:1,000, ab8933), AKT (1:500, ab8805), phosphorylated mammalian target of rapamycin (p-mTOR, 1:1,000, ab1093), mTOR (1:10,000, ab2732), and GAPDH (1:3,000, ab9485), which were all from Abcam (Cambridge, UK).

The membranes were washed three times and incubated with the appropriate secondary antibodies at 37 °C for 1 h. A 3,3′-diaminobenzidine kit (Sigma-Aldrich, St. Louis, MO, USA) was used to visualize the bands, and gray values were determined using Quantity One software (Bio-Rad, Hercules, CA, USA).

Cell counting kit-8 (CCK-8) assay

The KG-1 and HL-60 cell lines with a density of 1 × 10^5 cells/well were seeded into 96-well plates and maintained in a 5 % CO_2 atmosphere at 37 °C for 24 h. After transfection for 48 h, cell viability was measured using a CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. The absorbance was determined using a microplate reader at 450 nm (Thermo Fisher Scientific).

5-Ethynyl-2′-deoxyuridine (EdU) assay

The KG-1 and HL-60 cells were plated into 96-well plates and cultured for 24 h at 37 °C in a 5 % CO_2 atmosphere. Subsequently, EdU reagent (1:1,000 dilution with culture medium) was added to the cells for 1 h. Following two washes with phosphate-buffered saline (PBS), the cells were fixed with 4 % paraformaldehyde for 0.5 h.

After rinsing twice with PBS, 0.3 % Triton X-100 diluted in PBS was used to permeabilize the cells. The cells were then stained with 4',6-diamidino-2-phenylindole (Solarbio), and images were captured using a fluorescence microscope (Olympus, Tokyo, Japan).

Flow cytometry

Apoptosis in HL-60 and KG-1 cells was detected using a flow cytometry assay. The KG-1 and HL-60 cells were plated into 24-well plates at a density of 2.5 × 10^5 cells/well and cultured overnight. After 48 h of transfection, the cells were enriched, rinsed with PBS (Solarbio), resuspended using 0.5 mL binding buffer, and stained with 5 µL annexin V/fluorescein-isothiocyanate and 5 µL propidium iodide (both from Thermo Fisher Scientific) at room temperature for 15 min. Cell apoptosis was assessed using FACScan flow cytometry with CellQuest software (BD Biosciences, San Jose, CA, USA).

Determination of glycolysis levels

Following 48 h of transfection, culture supernatants were collected to determine the levels of ATP, lactate, and glucose using an ATP detection kit (Beyotime), lactate assay kit (Biovision, Mountain View, CA, USA), and glucose assay kit (Sigma-Aldrich, St. Louis, MO, USA), respectively, based on the manufacturers' instructions. Lactate generation and glucose consumption were assessed using the ratio of glucose/lactate concentrations in the treatment and control groups.

Statistical analysis

Statistical analysis was conducted using SPSS statistical software for Windows, version 22.0 (IBM, Armonk, NY, USA). Data are presented as the mean ± standard error. Significant differences were detected by unpaired Student's *t*-tests between two groups, and significant differences among multiple groups were detected using a one-way analysis of variance with Dunnett's post hoc test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

KLF8 is highly expressed in AML cells

To determine the effect of KLF8 on the progression of AML cells, the expression level of KLF8 was first examined in AML cell lines. Based on the GEPIA database, KLF8 was overexpressed in AML cells (Figure 1 A). In addition, the transcriptional and translational levels of KLF8 in AML cell lines were determined using qRT-PCR and western blotting, respectively. Both the relative mRNA and protein levels of KLF8 were significantly increased in the four AML cell lines (HL-60, KG-1, MOLM-14, and MOLM-13) compared with those in HS-5 cells (Figure 1 B and C). Moreover, the relative mRNA and protein expression levels of KLF8 were higher in HL-60 and KG-1 cells than in MOLM-13 and MOLM-14 cells (Figure 1 B and C). This HL-60 and KG-1 cells were, therefore, used for subsequent studies.

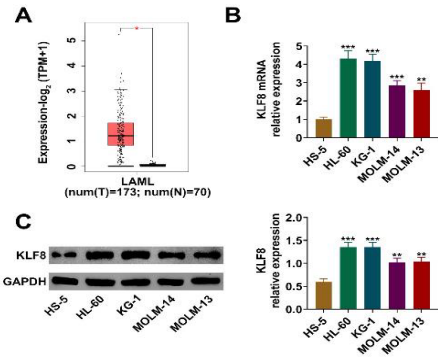

Figure 1: The expression level of KLF8 in AML cells. (A) Based on data extracted from the GEPIA database ($^{*}p < 0.05$), (B) The relative mRNA expression levels of KLF8 in HS-5 and four AML cell lines (HL-60, KG-1, MOLM-14, and MOLM-13). The data are expressed after normalization against *GAPDH* expression levels ($^{**}p < 0.01$ and $^{***}p < 0.001$) and relative to HS-5 expression levels. (C) The relative protein expression levels of KLF8 in HS-5 and four AML cell lines. The data are expressed after normalization against GAPDH ($^{**}p < 0.01$, $^{***}p < 0.001$ compared to HS-5)

KLF8 promotes the proliferation of AML cells

The role of KLF8 in the growth of AML was determined by CCK-8 and EdU studies. Because the levels of KLF8 were enhanced in AML cell lines, two sh-RNAs targeting KLF8, sh-KLF8#1 and sh-KLF8#2, were used to downregulate the levels of KLF8 in HL-60 and KG-1 cells. The results of the CCK-8 assay showed that the viabilities of both HL-60 and KG-1 cells were significantly decreased by transfection using both sh-KLF8#1 and sh-KLF8#2 compared with those of the control groups, whereas no significant difference in viability was found between the sh-NC and control groups (Figure 2 A). Similar results were also found using the EdU assay (Figure 2 B and C). Together, these results showed that KLF8 enhanced the proliferation of AML cells.

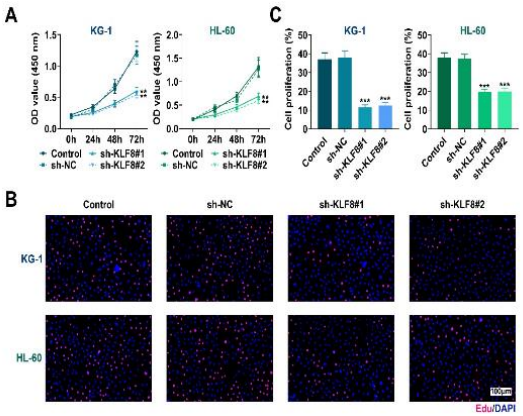

Figure 2: Effect of inhibition of KLF8 on the proliferation of AML cells, (A) The viabilities of both HL-60 and KG-1 cells, (B and C) The proliferation capacities of both HL-60 and KG-1 cells ($^{**}p < 0.01$, $^{***}p < 0.001$ compared to the control group)

KLF8 accelerates glycolysis in AML cells

The effect of KLF8 on glycolysis in AML cells was also determined. Transfections using sh-KLF8#1 and sh-KLF8#2 decreased relative glucose consumption, lactate generation, and ATP levels in both KG-1 and HL-60 cells compared to those in the control group (Figure 3 A - C). The relative protein levels of HK2 and GLUT1 in both HL-60 and KG-1 cells transfected with sh-KLF8#1 and sh-KLF8#2 were also remarkably diminished relative to those in the control group (Figure 3 D). No significant differences were found in relative glucose consumption, lactate generation, ATP levels, or protein expression levels of HK2 or GLUT1 in either KG-1 or HL-60 cells between the sh-NC and control groups (Figure 3). Together, these results indicated that KLF8 increased glycolysis in AML cells.

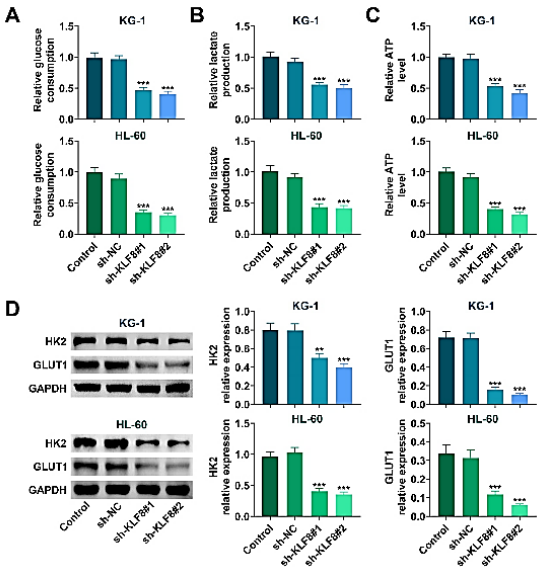

Figure 3: Effect of suppression of KLF8 on glycolysis in AML cells. (A–C) The relative glucose consumption (A), lactate generation (B), and ATP levels (C) in both HL-60 and KG-1 cells, (D) The relative protein expression levels of HK2 and GLUT1 in HL-60 and KG-1 cells. The data are expressed after normalization against GAPDH levels ($^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the control group)

KLF8 suppresses apoptosis in AML cells

The effect of KLF8 on apoptosis in AML cells was determined by flow cytometry and western blotting. Apoptosis was significantly increased, accompanied by an increase in the relative protein expression level of Bax and a decrease in the relative protein level expression level of Bcl-2 after both KG-1 and HL-60 cells were transfected with both sh-KLF8#1 and sh-KLF8#2 (Figure 4). No significant differences ($p > 0.05$) were found among these parameters between the sh-NC and control groups for either HL-60 or KG-1 cells (Figure 4). Together, these results showed that KLF8 inhibited apoptosis in AML cells.

KLF8 regulates the AKT/mTOR pathway

To further identify the underlying mechanisms mediating the role of KLF8 in the development of AML, the levels of related proteins associated with the AKT/mTOR pathway were determined using western blotting. Both the relative expression levels of p-AKT/AKT and p-mTOR/mTOR were reduced after KG-1 and HL-60 cells were transfected with both sh-KLF8#1 and sh-KLF8#2 (Figure 5). However, this decrease disappeared following transfection with

sh-NC into both HL-60 and KG-1 cells (Figure 5), suggesting that the role of KLF8 in the development of AML involved the AKT/mTOR pathway.

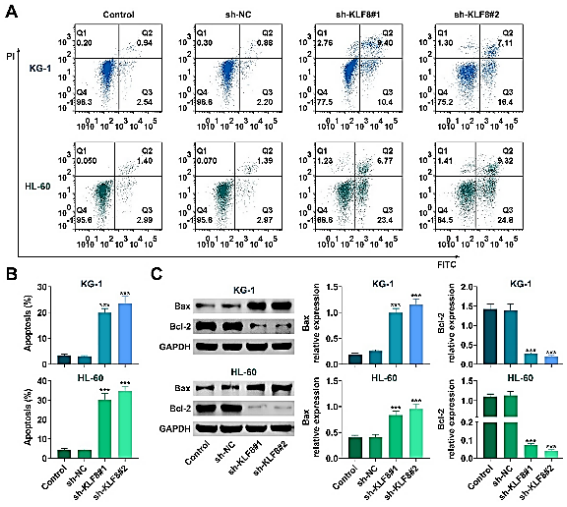

Figure 4: Effect of repression of KLF8 on apoptosis of AML cells. (A and B) The apoptosis of HL-60 and KG-1 cells, (C) The relative protein expression levels of Bax and Bcl-2 in both HL-60 and KG-1 cells. The data are expressed after normalization against GAPDH expression (***p < 0.001 compared to the control group)

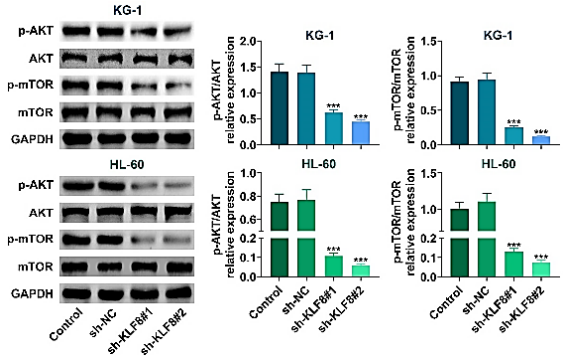

Figure 5: Effect of interference with KLF8 on the expression levels of related proteins associated with the AKT/mTOR pathway. The data are expressed after normalization against GAPDH levels (***p < 0.001 compared to the control group)

DISCUSSION

Acute myeloid leukemia is a type of malignant syndrome characterized by poor survival and prognosis [3]. Accumulated evidence has suggested that abnormal glucose metabolism is a hallmark of AML [9]. Therefore, strategies targeting glucose metabolites may be effective treatments for AML. Upregulated KLF8 in gastric cancer has been shown to modulate glycolysis via targeting GLUT4 [8]. Based on the GEPIA database, it was observed that KLF8 was also upregulated in AML, which prompted the need to investigate the detailed role and mechanism of KLF8 in the progression of AML.

It has been shown in this study that upregulated KLF8 promoted proliferation and glycolysis and inhibited apoptosis in AML cells, which involved the AKT/mTOR pathway. The upregulation of KLF8 has been reported for various tumors. In this case, KLF8 was also overexpressed in AML cells. Moreover, upregulated KLF8 generally led to the detrimental development of tumors. The KLF8 overexpression in pancreatic cancer promotes growth and tumorigenicity, both *in vivo* and *in vitro* [5]. Upregulation of KLF8 enhances the progression of the epithelial–mesenchymal transition in lung adenocarcinomas [10].

Moreover, KLF8 is required for the growth, migration, invasion, and metastasis of colorectal cancer [7]. Furthermore, other members of the KLF family, including KLF 2, 3, 4, 5, and 6, are also dysregulated in AML [11]. Downregulated KLF4, found in AML patients and cells, accelerates proliferation and decreases differentiation by regulating miR-150, MYC, and CDKN1A [11]. Consistent with these reports, the CCK-8 and EdU experiments revealed that knockdown of KLF8 inhibited the growth of both KG-1 and HL-60 cells. In addition, down-regulation of KLF8 increased apoptosis and the level of pro-apoptotic Bax but decreased the level of anti-apoptotic Bcl-2, indicating that suppression of KLF8 enhanced apoptosis in AML cells. Taken together, these results showed that KLF8 overexpression in AML enhances growth and inhibits apoptosis in AML cells.

Tumor cells metabolize glucose to generate ATP through glycolysis rather than mitochondrial oxidative phosphorylation, even in the presence of sufficient oxygen. Thus, glycolysis and glucose uptake are enhanced in tumor cells. The high-affinity glucose transporter GLUT1 is expressed in nearly every tissue that modulates glucose transmembrane transport, thereby acting as a rate-limiting step in glycolysis. In addition, glucose requires modification with a phosphate group by HK2 to modulate glucose flux within cells during glycolysis, which is relevant during the initiation and development of tumors. Many studies have reported that glycolysis increases in AML cells; therefore, strategies inhibiting glycolysis are widely used for the treatment of AML. The R-2-hydroxyglutarate targets the FTO/m6A/PFKP/LDHB signaling pathway to decrease aerobic glycolysis in AML [12]. Knockdown of the long non-coding RNA

(lncRNA) UCA1 inhibits chemoresistance by decreasing glycolysis in pediatric AML [13].

Downregulation of the lncRNA TUG1 also suppresses glycolysis in AML by targeting miR-185 [14]. This study consistently showed that glycolysis increased in AML cells. Moreover, KLF8 has been reported to modulate glycolysis by targeting GLUT4 in gastric cancer [8]. In the present study, downregulating KLF8 also counteracted the increase in glycolysis observed in AML, as transfection with shRNAs targeting KLF8 decreased relative glucose consumption, lactate generation, ATP levels, and protein expression of HK2 and GLUT1 in both KG-1 and HL-60 cells. These findings, therefore, showed that suppression of KLF8 decreased glycolysis in AML cells.

The AKT/mTOR pathway is involved in the regulation of cell growth, apoptosis, metabolic glycolysis, and other physiological processes [15,16]. Wu *et al* [17] reported that deoxyshikonin attenuated growth and glycolysis by inhibiting the AKT/mTOR signaling pathway in AML cell lines. Upregulated programmed death-ligand 1 enhances glycolysis, reduces apoptosis, and induces S phase arrest mediated by the AKT/mTOR/HIF-1α pathway in AML [18].

In addition, it has been shown that KLF8 modulates angiogenesis and the expression of vascular endothelial growth factor A (VEGFA), which is involved in AKT signaling in hepatocellular carcinomas. In the present study, knockdown of KLF8 reduced the relative levels of p-AKT/AKT and p-mTOR/mTOR in both HL-60 and KG-1 cells, indicating that the effects of KLF8 are mediated by the AKT/mTOR pathway in AML. More importantly, AKT signaling modulated cell proliferation by GLUT1 and controlled hexose metabolism by HK2. Thus, a direct relationship between the AKT/mTOR pathway and AML progression, involving cell proliferation, glycolysis, and apoptosis, needs more study.

CONCLUSION

The findings of this study show that expression of KLF8 increases in AML cells. Knockdown of KLF8 inhibits proliferation and glycolysis, and also promotes apoptosis in AML cells, which might involve AKT/mTOR pathway. Characterization of clinical samples and *in vivo* experiments should therefore be the next focus of studies. Overall, results also provide a theoretical basis for the development of possible therapies for AML patients.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the contents of this article will be borne by the authors. Tingting Lu and Qiurong Zhang designed and conducted the experiments. Xiao Wu analyzed and interpreted the data, and Senjun Liu prepared the manuscript with contributions from all co-authors.

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