**MiR-140-3p Ameliorates The Inflammatory Response of Airway Smooth Muscle Cells by Targeting HMGB1 to Regulate The JAK2/STAT3 Signaling Pathway**

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**Abstract**

**Objective:** The growth and migration of airway smooth muscle cells (ASMCs) are dysregulated in asthma. MicroRNAs (miRNAs) are associated with the pathogenesis of many diseases including asthma. Instead, the function of miR-140-3p in ASMCs’ dysregulation in asthma remains inconclusive. This study aimed to explore the role and mechanism of miR-140-3p in ASMCs’ dysregulation.

**Materials and Methods:** In this experimental study, ASMCs were stimulated with platelet-derived growth factor (PDGF)-BB to construct an asthma cell model in vitro. MiR-140-3p expression level in the plasma of 50 asthmatic patients and 50 healthy volunteers was measured with quantitative real-time polymerase chain reaction (qRT-PCR). Besides, the enzyme-linked immunosorbent assay (ELISA) was applied to detect the contents of interleukin (IL) -1β, IL-6, and tumor necrosis factor-α (TNF-α) in the cell culture supernatant of ASMCs. Additionally, CCK-8 and transwell assays were adopted to probe the multiplication and migration of ASMCs. In addition, the western blot was employed to examine HMGB1, JAK2, and STAT3 protein expressions in ASMCs after miR-140-3p and HMGB1 were selectively regulated.

**Results:** miR-140-3p expression was declined in asthmatic patients’ plasma and ASMCs stimulated by PDGF-BB. Upregulating miR-140-3p suppressed the viability and migration of the cells and alleviated the inflammatory response while inhibiting miR-140-3p showed opposite effects. Additionally, HMGB1 was testified as the target of miR-140-3p. HMGB1 overexpression could reverse the impact of miR-140-3p upregulation on the inflammatory response of ASMCs stimulated by PDGF-BB. MiR-140-3p could repress the activation of JAK2/STAT3 via suppressing HMGB1.

**Conclusion:** In ASMCs, miR-140-3p can inhibit the JAK2/STAT3 signaling pathway by targeting HMGB1, thus ameliorating airway inflammation and remodeling in the pathogenesis of asthma.

**Keywords:** Asthma, HMGB1, JAK2/STAT3, miR-140-3p

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**Introduction**

Bronchial asthma is a prevalent chronic respiratory inflammatory disease, featuring airway inflammation, airway remodeling, and hyperresponsiveness. It’s been shown that various cells (eosinophils, T cells, neutrophils, mast cells, and airway epithelial cells, etc.) and inflammatory mediators are involved in its pathogenesis (1, 2). It is estimated that 5% of adults and 10% of children suffer from asthma (3, 4). The anti-inflammatory drugs and bronchodilators can effectively control airway inflammation and hyperresponsiveness respectively; however the current treatments are not enough to reverse airway remodeling. Reportedly, the abnormal proliferation and migration of airway smooth muscle cells (ASMCs) are pivotal in airway remodeling (5). Specifically, platelet-derived growth factor BB (PDGF-BB) can promote the multiplication and migration of ASMCs, thus aggravating airway remodeling in asthma (6).

MicroRNAs (miRNAs) are single-stranded non-coding RNAs with about 19-22nt in length, which inhibit the translation process via binding with the 3’-untranslated region (3’-UTR) of mRNAs (7, 8). More and more evidence has shown that miRNAs are vital in modulating the phenotype of ASMCs in the pathogenesis of asthma (9). Up to now, many miRNAs including miR-200a, miR-142, and miR-485 have been reported to be implicated in the regulation of multiplication and migration of ASMCs (10-12). In addition, miR-140-3p is declined in human bronchial smooth muscle cells which are...
stimulated by interleukin (IL-13) (13). Instead, how miR-140-3p modulates the dysfunction of ASMCs in asthma is indeterminate.

High mobility group box 1 (HMGB1), is known as a non-histone chromosome binding protein with a highly conserved structure (14). It is reported that HMGB1 is an important inflammatory mediator, which is related to immune diseases, malignancies, and other diseases (15). HMGB1 is also a pivotal regulator in airway inflammation and remodeling in asthma (16). The aim of this study was to investigate the regulatory effects of miR-140-3p on the proliferation, migration, and inflammatory response of ASMCs after PDGF-BB stimulation and to explore the interplay between miR-140-3p and HMGB1. This study revealed a new molecular mechanism in the process of asthma exacerbation and suggested a new theoretical foundation for its treatment.

Materials and Methods

Clinical samples

This experimental study, with the written informed consent of all patients, healthy volunteers, or guardians of participants, was endorsed by the Ethics Committee of Linyi Central Hospital (2017-0041). Experiments involving human tissue were performed according to the Declaration of Helsinki. Blood samples of 50 patients with acute asthma treated in Linyi Central Hospital from March 2018 to June 2019 were collected. Blood samples of 50 healthy volunteers were used as the negative control. The patients were selected by simple random sampling. Blood samples were collected by vacuum blood collection tubes containing ethylene diamine tetra acetic acid (EDTA) and centrifuged at 1600×g for 15 minutes at 4°C to obtain supernatant and at 16000×g for another 10 minutes at 4°C to separate plasma. Ultimately, the separated plasma was stored at -80°C for the subsequent analysis. 50 patients with acute exacerbation asthma, were included 32 males and 18 females, with a mean age of (35.8 ± 8.76) years. Inclusion criteria were A. Meeting the diagnostic criteria of the Global Initiative for Asthma (GINA) guidelines (2016) (17); B. Being in the acute exacerbation phase, C. Age ≥16 years old. Exclusion criteria: A. Those with autoimmune diseases, hematologic diseases, severe infections, and malignancies, B. Pregnant or lactating women, and C. A recent history of leukotriene receptor antagonists as well as glucocorticoid use.

Cell culture and cell transfection

In this experimental study, human ASMCs were available from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Subsequently, cells were cultivated in DMEM (Corning, Manassas, VA, USA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (Thermo Fisher Scientific, MA, USA) in 5% CO2 at 37°C. When cells reached 90% confluence, subculture was carried out. ASMCs were treated with different concentration of PDGF-BB (0, 1, 10, 20, 40, 60 mg/mL, R&D Systems, Minneapolis, MN, USA) for different times (1-48 hours) to construct the in vitro model of asthma. MiR-140-3p mimics (miR-140-3p), mimic negative control (miR-NC), miR-140-3p inhibitors (miR-140-3p-in), inhibitor negative control (miR-in), pcDNA3.0-HMGB1 (HMGB1) and empty vector pcDNA3.0 were available from Ribobio (Guangzhou, China). Subsequently, ASMCs were transferred into a 24-well cell plate at 3×104 cells/well, and cultured at 37°C in 5% CO2 for 24 hours, and then the cells were transfected by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Quantitative real-time polymerase chain reaction

Total RNA was extracted from plasma and cells by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was reverse transcribed into cDNA by TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and PrimeScript RT Kit (Takara, Dalian, China). According to the manufacturer’s protocol, qRT-PCR was conducted on ABI 7300 system (Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR Master Mix kit (Thermo Fisher Scientific, Carlsbad, CA, USA), with U6 or β-actin as the endogenous control. Ultimately, the relative expressions were estimated by the 2-ΔΔCT method. The primer sequences are detailed in Table 1.

| Gene      | Primer sequences (5’-3’)                               |
|-----------|--------------------------------------------------------|
| miR-140-3p| F: GCGCGTACCACAGGGTAGAA                                 |
|           | R: AGTGCAGGGTAGGTAATT                                   |
| U6        | F: CTGCGTTTCCGCAGCATATACTA                              |
|           | R: AGCAATTTGCCGTGCATTTGC                               |
| HMGB1     | F: AGCGTCTAGCGCCATAGCGAT                                |
|           | R: CCCGTCTGATAGCGCATTTGC                               |
| β-actin   | F: CGTGGGTGACAATAGAGAG                                  |
|           | R: TTGCCTGATAGGACCT                                    |

Table 1: Primer sequences
Enzyme-linked immunosorbent assay

Cells and the medium were collected after 48 hours of continuous culture, and then the cells were under centrifuged at 1000×g for 10 minutes at 4°C to collect the supernatant. ELISA kits (Shanghai Xitang Biotechnology Co., Ltd, Shanghai, China) were adopted to detect the contents of interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α), respectively, according to the manufacturer’s instructions.

Cell proliferation assay

Cell counting kit 8 (CCK-8; Dojindo, Kumamoto, Japan) was adopted to estimate the proliferative capability of ASMCs. Cells were transferred into a 96-well plate (1×10^4 cells/well) and cultured at 37°C for 12 hours, and then 10 μL of CCK-8 solution was added to each well. After incubation of cells for 1 hour, the absorbance was detected at 450nm wavelength by a spectrophotometer reader (Bio-Rad, Hercules, CA, USA). The proliferation was plotted.

5-bromo-2-`deoxyuridine (BrdU) assay

Cell proliferation was also probed by a BrdU kit (Sigma-Aldrich, Louis, MO, USA). Briefly, the cells were transferred into 96-well plates and incubated with BrdU labeling reagent for 2 hours at 37°C. Next, the cells were incubated with FixDenat solution for 30 minutes at ambient temperature. Subsequently, the cells were incubated with the anti-BrdU antibody for 90 minutes in the dark. Thereafter the nucleus was stained with DAPI staining solution for 10 minutes. Subsequently, the ASMCs were washed with phosphate buffered saline (PBS, Beyotime Biotechnology, China) and observed under a fluorescence microscope.

Transwell assay

Cell migration was examined with the transwell chamber (Costar, Cambridge, MA, USA). Briefly, 1×10^5 cells resuspended in 200 μl of serum-free medium were transferred into the upper compartment, with the lower compartment filled with a medium containing 20% FBS. Twenty-four hours later, the ASMCs that failed to migrate were removed from the upper membrane surface with cotton swabs, and the migrating cells fixed in methanol were stained with 0.1% crystal violet. Cells were rinsed in tap water, dried, and then photographed under an optical microscope. Image J software was used for counting the cells.

Dual-luciferase reporter assay

Wild-type (WT) and mutant (MUT) sequences of HMGB1 were subcloned into psi-CHECK2 luciferase reporter vector (Promega, Madison, WI, USA), and HMGB1-WT or HMGB1-MUT reporter vectors were constructed, respectively. Then luciferase reporter vector and miR-140-3p or miR-NC were co-transfected into ASMCs by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Forty-eight hours later, the luciferase activity was examined by a dual luciferase analysis system (Promega) as manufacturer’s instructions.

Western blot

Total proteins in ASMCs were extracted with RIPA lysis buffer (Solarbio, Beijing, China), and their concentration was quantified by a BCA protein detection kit (Solarbio, Beijing, China). An equal amount of protein samples (20 μg per group) was separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA), which was then blocked with 5% skimmed milk for 1 hour. The membrane was firstly incubated with the primary antibody at 4°C overnight, and then with the secondary antibody, goat anti-rabbit IgG H&L (Abcam, ab6721, 1: 3000) at 37°C for 1 hour. Ultimately, the bands were developed with the enhanced chemiluminescence reagent (Pierce Biotechnology, Rockford, IL, USA). The primary antibodies were: anti-HMGB1 (1: 1000, ab79823), anti-p-JAK2 (1: 1000, #8082), anti-p-STAT3 (1: 1000, #9145), anti-JAK2 (1: 1000, #3230), anti-STAT3 (1: 1000, #12640) and anti-β-actin (1: 1000, ab6276). Among them, anti-HMGB1 antibody and anti-β-actin antibody were bought from Abcam (Shanghai, China); anti-p-JAK2 antibody, anti-p-STAT3 antibody, anti-JAK2 antibody, and anti-STAT3 antibody were all purchased from Cell Signaling Technology (Cell Signaling Technology, Danvers, MA, USA).

Statistical analysis

SPSS 21.0 statistical software (SPSS Inc., Chicago, IL, USA) was applied for statistical analysis, with data shown as mean ± standard deviation. Accordingly, the differences between the two groups were analyzed by student’s t test. Besides, one-way ANOVA followed by Tukey post-hoc test was executed for comparing the data of more than two groups. P<0.05 indicated the statistical significance.

Results

miR-140-3p expression is declined in plasma of asthmatic patients and ASMCs stimulated by PDGF-BB

First, we evaluated miR-140-3p expression levels in the plasma of 50 asthmatic patients and 50 healthy volunteers by qRT-PCR and observed that miR-140-3p expressions in the plasma of asthmatic patients were remarkably
lower than that of healthy individuals (Fig.1A). qRT-PCR also suggested that PDGF-BB inhibited miR-140-3p expression in ASMCS dose- and time-dependently (Fig.1B, C). The half-inhibitory concentration (IC_{50}) value of PDGF-BB-induced miR-140-3p expression inhibition was about 22.37 ng/mL (Fig.1B). 24 hours of PDGF-BB treatment, were reduced miR-140-3p expression to less than 50% in ASMCS (Fig.1C). So, in the subsequent experiments, ASMCS were treated with 20 ng/mL PDGF-BB for 24 hours as the in vitro asthma model. It was found that compared with ASMCS in the control group, IL-1β, IL-6, and TNF-α levels in the asthma cell model were up-regulated (Fig.1D).

MiR-140-3p represses the inflammatory response of PDGF-BB-treated ASMCS

Next, we performed ELISA and found that compared with the PDGF-BB+miR-NC group, the contents of IL-1β, IL-6, and TNF-α in the supernatant of ASMCS were markedly decreased subsequent to the transfection of miR-140-3p mimics; as against PDGF-BB+miR-in group, miR-140-3p inhibition restrained the production of IL-1β, IL-6, and TNF-α of PDGF-BB-stimulated ASMCS (Fig.3A-C), suggesting that miR-140-3p may repress the inflammatory response in asthma.
MiR-140-3p targets HMGB1

We then predicted the target genes of miR-140-3p with the StarBase database and observed that there was a complementary binding site between miR-140-3p and HMGB1 mRNA 3’-UTR (Fig.4A). Dual-luciferase reporter gene assay showed that transfection of miR-140-3p mimics impaired HMGB1-WT activity, but that of HMGB1-MUT was not significantly affected (Fig.4B). qRT-PCR and western blot showed that in PDGF-BB-treated ASMCs, miR-140-3p up-regulation restrained HMGB1 mRNA and protein expression, while miR-140-3p down-regulation had the opposite effect (Fig. 4C, D). Collectively, HMGB1 was the downstream target of miR-140-3p in ASMCs.

Fig.3: miR-140-3p inhibits the inflammatory reaction of ASMCs induced by PDGF-BB. ELISA was used to detect the levels of A. IL-1β, B. IL-6, and C. TNF-α in ASMC supernatants stimulated by 20 ng/ml PDGF-BB after transfection of miR-140-3p mimics or inhibitors. Data are expressed as mean ± standard deviation (n=3). **; P<0.01, ***; P<0.001, ASMCs; Airway smooth muscle cells, PDGF-BB; Platelet-derived growth factor, ELISA; Enzyme-linked immunosorbent assay, IL-1β; Interleukin-1β, and TNF-α; Tumor necrosis factor-α.

Fig.4: HMGB1 is the downstream target of miR-140-3p. A. The binding sequence between miR-140-3p and HMGB1 3’-UTR. B. Dual-luciferase reporter gene experiment was used to verify the targeting relationship between miR-140-3p and HMGB1. C. qRT-PCR and D. Western blot were used to detect HMGB1 mRNA and protein expression in ASMCs transfected with miR-140-3p mimics or inhibitors and stimulated by 20 ng/ml PDGF-BB. Data are expressed as mean ± standard deviation (n=3). **; P<0.01, ***; P<0.001, HMGB1; High mobility group box 1, qRT-PCR; Quantitative real-time polymerase chain reaction, ASMCs; Airway smooth muscle cells, and PDGF-BB; Platelet-derived growth factor.
**HMGB1 counteracts the impact of miR-140-3p in asthmatic ASMCs**

To study the function of the miR-140-3p/HMGB1 axis in regulating the dysfunction of ASMCs, we transfected miR-140-3p mimics, HMGB1 overexpression plasmid or co-transfected miR-140-3p mimics and HMGB1 overexpression plasmids in ASMCs, respectively. qRT-PCR and western blot showed that HMGB1 overexpression enhanced HMGB1 mRNA and protein expressions in ASMCs stimulated by PDGF-BB and attenuated the impact of miR-140-3p overexpression on HMGB1 (Fig.5A, B). CCK-8, BrdU, and transwell assays indicated that HMGB1 overexpression markedly accelerated the multiplication and migration of PDGF-BB-treated ASMCs, and greatly counteracted miR-140-3p overexpression’s impacts on the growth and migration of PDGF-BB-stimulated ASMCs (Fig.5C-E). ELISA showed that compared with the PDGF-BB group, the contents of IL-1β, IL-6, and TNF-α in the PDGF-BB+HMGB1 group were up-regulated significantly; compared with those in the PDGF-BB+miR-140-3p group, these inflammatory factors in the PDGF-BB+miR-140-3p+HMGB1 group were also significantly increased (Fig.5F). Besides, Western blot showed that up-regulation of miR-140-3p repressed p-JAK2 and p-STAT3 expression levels in ASMCs, and HMGB1 overexpression significantly raised p-JAK2 and p-STAT3 expression levels, counteracting miR-140-3p overexpression’s impact on p-JAK2 and p-STAT3 (Fig.5G).

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**Fig.5:** miR-140-3p targets HMGB1 through JAK2/STAT3 signaling pathway and inhibits the pathogenesis of asthma. A. qRT-PCR and B. Western blot were used to detect HMGB1 mRNA and protein expression in ASMCs stimulated with 20 ng/mL PDGF-BB after transfection with miR-140-3p mimics, HMGB1 overexpression plasmid or miR-140-3p mimics +HMGB1 overexpression. C. CCK-8 assay. D. BrdU assay. E. Transwell assay were used to detect the proliferation and migration of ASMCs stimulated by 20 ng/mL PDGF-BB after transfection. F. ELISA was used to detect the contents of IL-1β, IL-6, and TNF-α in the supernatant of ASMCs stimulated with 20 ng/mL PDGF-BB after transfection. G. Western blot assay was used to detect the protein expression of p-JAK2, p-STAT3, JAK2, and STAT3 in ASMCs stimulated by 20 ng/mL PDGF-BB after transfection. Data are expressed as mean ± standard deviation (n=3). *; P<0.05, **; P<0.01, ***; P<0.001. HMGB1; High mobility group box 1, JAK2; Janus Kinase 2, STAT3; Signal transducer and activator of transcription 3, qRT-PCR; Quantitative real-time polymerase chain reaction, ASMCs; Airway smooth muscle cells, PDGF-BB; Platelet-derived growth factor, CCK-8; Cell counting kit 8, and BrdU; 5-bromo-2’-deoxyuridine, ELISA; Enzyme-linked immunosorbent assay, IL-1β; Interleukin-1β, and TNF-α; Tumor necrosis factor-α.
Discussion

The abnormal growth and migration of ASMCs are crucial in the pathogenesis of respiratory diseases, like asthma (18). Many stimuli, such as growth factors, contraction agonists, inflammatory cytokines, and extracellular matrix proteins, have been reported to induce the multiplication and migration of ASMCs (19). PDGF-BB-induced growth and migration of ASMCs have been used to study the dysfunction of ASMCs in asthma in vitro (5). Here we observed that the stimulation of PDGF-BB markedly promoted the dysfunction of ASMCs, and when ASMCs were stimulated by PDGF-BB, the secretion of inflammatory cytokines was also significantly promoted, which is coherent with the previous studies (5, 20).

More and more evidence has shown that miRNAs exert crucial functions in regulating cell proliferation, migration, differentiation, and apoptosis, and are closely relevant to the progression of many human diseases, such as tumors, cardiovascular diseases, and asthma (21, 22). Importantly, it is reported that some miRNAs modulate the proliferation and migration of ASMCs (5, 23, 24). For example, miR-638 expression is declined in PDGF-BB-induced ASMCs, and it suppresses the excessive growth and migration of ASMCs via pointing to Cyclin D1 and NOR1 (20); miR-590-5p represses the proliferation of ASMCs induced by PDGF via inhibiting STAT3 (23); miR-375 is down-regulated in ASMCs treated with PDGF, and it can block the proliferation and migration of ASMCs by targeting the JAK2/STAT3 signaling (24). In this work, we demonstrated that the circulating miR-140-3p was down-regulated in the plasma of asthmatic patients. Furthermore, miR-140-3p expression was reduced in PDGF-BB-stimulated ASMCs, consistent with what was found in a previous study (25). Reportedly, miR-140-3p expression is impaired in ASMCs treated with TNF-α, and miR-140-3p can block the activation of p38 MAPK in ASMCs and inhibit the up-regulation of CD38 induced by TNF-α (26). In addition, miR-140-3p inhibits PDGF-BB-induced ASMCs proliferation and promotes apoptosis by targeting C-Myb and BCL-2 (25). Similarly, the present study confirmed that miR-140-3p up-regulation inhibited PDGF-BB-induced proliferation of ASMCs. Additionally, miR-140-3p up-regulation restrained the migration of ASMCs and the secretion of inflammatory cytokines induced by PDGF-BB; on the contrary, inhibition of miR-140-3p facilitated the growth, migration, and inflammation of ASMCs. Collectively, miR-140-3p could modulate the dysfunction of ASMCs in asthma.

To expound on the mechanism by which miR-140-3p regulates the proliferation and migration of ASMCs, we predicted the downstream targets of miR-140-3p. Interestingly, HMGB1 was predicted as a target for miR-140-3p. Our results indicated that miR-140-3p could negatively modulate HMGB1 expression in ASMCs. HMGB1 can serve as a modulator in airway inflammation (27). Reportedly, resveratrol inhibits airway inflammation and remodeling in asthma via blocking the HMGB1/TLR4/NF-κB pathway (28); curcumin E can alleviate the injury and inflammation of bronchial epithelial cells induced by lipopolysaccharide via suppressing HMGB1/TLR4/NF-κB signaling (29). In a mouse model, HMGB1 and TLR4 depletion ameliorate asthmas induced by diisononyl phthalate (DINP) (30). These studies imply that targeting HMGB1 can probably alleviate the symptoms of asthma. In this study, we discovered that HMGB1 overexpression promoted the viability, migration, and the activation of JAK/STAT3 pathway of ASMCs stimulated by PDGF-BB. In addition, the effect of transfection of mir-140-3p mimics on the growth, migration, and inflammation of PDGF-BB-induced ASMCs could be counteracted by HMGB1 overexpression. As reported, the abnormal activation of the JAK/STAT3 pathway aggravates inflammatory diseases (31, 32). These findings suggest that, in the pathogenesis of asthma, the biological function of miR-140-3p in ASMCs is partly mediated by HMGB1.

Conclusion

miR-140-3p expression is suppressed in asthmatic patients’ plasma and ASMCs stimulated by PDGF-BB. miR-140-3p inhibits JAK/STAT3 signaling activation via targeting HMGB1, thus blocking the growth, migration, and inflammatory response of PDGF-BB-stimulated ASMCs. Our findings highlight that the miR-140-3p/HMGB1 axis is pivotal in regulating the pathogenesis of asthma and imply that targeting the miR-140-3p/HMGB1 axis is a promising strategy to treat asthma.

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Authors’ Contributions

Y.L., C.W., J.D.; Designed the study and experiments. J.M., Y.Z., L.Ho., Y.L., M.C.; Collected clinical samples and performed the experiments. J.M., Y.Z., L.Ho., Y.L., L.He.; Conducted the data analysis. J.M., Y.Z., L.Ho., Y.L.; Drafted the manuscript. Y.L., J.M.; Reviewed and revised the manuscript. All authors read and approved the final manuscript.

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