miRNA-223-3p and let-7b-3p as potential blood biomarkers associated with the ischemic penumbra in rats

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The present study aimed to identify commonalities in the microRNA (miRNA) expression profiles of the brain ischemic penumbra and the blood after hyperacute ischemic stroke and then to address whether the miRNA profile of blood has potential usefulness as a diagnostic biomarker of hyperacute ischemic stroke. Blood was collected from the jugular vein 4 h after permanent middle cerebral artery occlusion (pMCAO). After venous blood was collected, the rats were decapitated immediately, and brain ischemic penumbra samples were collected. Hematoxylin and eosin staining was used to observe the histopathological changes. Penumbra and blood miRNAs were measured by miRNA microarray and real-time polymerase chain reaction (PCR) analysis. MicroRNA profiles differed between hyperacute ischemic stroke and sham-operated rats. The expression of some miRNAs changed by more than 1.5-fold in the penumbra and blood 4 h after pMCAO; among those miRNAs, several were upregulated and several were downregulated. MiR-223-3p was found to be highly expressed in both the penumbra and the blood 4 h after pMCAO, and let-7b-3p was found to have low expression in both the penumbra and the blood 4 h after pMCAO. Moreover, miR-223-3p and let-7b-3p expression in blood and brain ischemic penumbra were positively correlated. The results show that select blood miRNAs may correlate with miRNA changes in the penumbra in a rat model of hyperacute ischemic stroke. Our findings suggest the potential usefulness of blood miR-223-3p and let-7b-3p as noninvasive biomarkers for the diagnosis of hyperacute ischemic stroke.

Key words: hyperacute ischemic stroke, penumbra, blood, microRNA, miR-223-3p, let-7b-3p, biomarkers

INTRODUCTION

Stroke is a leading cause of death and long-term disability worldwide, and most strokes are of ischemic origin (Goldstein et al., 2001). Intravenous tissue plasminogen activator (t-PA) remains the only U.S. Food and Drug Administration-approved treatment for acute ischemic stroke (National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995). Although thrombolytic treatment within 4.5 h of ischemic stroke onset greatly improves the clinical outcome, only a small number of patients with hyperacute ischemic stroke receive thrombolytics (National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995; Wang et al., 2000). The chief limitation of current thrombolytic treatment for ischemic stroke is the narrow time window for safe and effective treatment. In addition to these time constraints, diagnostic uncertainty limits the use of thrombolytic treatment in many cases of hyperacute ischemic stroke (Barber et al., 2001). At present, the diagnosis of hyperacute ischemic stroke depends on clinical examination and neuroimaging techniques. Blood samples can be easily collected from patients with ischemic stroke in clinical practice and used for clinical diagnosis in general; how-
ever, there are no reliable circulating biomarkers for the diagnosis of hyperacute ischemic stroke.

Previous studies have shown that hundreds of different genes changed in the blood of ischemic stroke patients at both DNA and RNA levels (Tan et al., 2009; Sorensen et al., 2014; Zhang et al., 2014; Li et al., 2015). Therefore, further research is necessary to better understand the molecular biology of hyperacute ischemic stroke and identify potential circulating blood biomarkers for early diagnosis and accurate evaluation.

MicroRNAs (miRNAs) are a novel class of small, non-coding RNAs that are widely expressed in mammalian cells and negatively regulates gene expression at the post-transcriptional level by binding to the 3'-untranslated regions (3'-UTRs) of their target mRNAs (Bartel, 2004; Guo et al., 2010). Over the past decade, many studies have demonstrated that miRNAs are associated with the occurrence and development of various diseases (Lu et al., 2005; Liu et al., 2010; Li and He 2012; Di Gregorio et al., 2017). Some recent studies have demonstrated that the expression patterns of miRNAs are altered in patients with various neurological disorders, including ischemic stroke (Tan et al., 2009; Dewdney et al., 2018).

Changes in miRNA levels are closely correlated with the pathological processes of ischemic stroke – oxidative damage, excitotoxicity, inflammatory reaction, cortical neurogenesis, neuronal cell death, brain edema formation and blood–brain barrier disruption (Jeyaseelan et al., 2008; Nielsen et al., 2009; Yin et al., 2010; Rink and Khanna 2011; Tan et al., 2011; Li et al., 2017). It is generally believed that miRNAs released from circulating cells or damaged cells result in an increase in blood miRNA levels (Mayr et al., 2013). On the basis of the above data, we believe that miRNA expression profiles may be very helpful for earlier diagnoses, as well as for achieving a better understanding of the pathogenesis of hyperacute ischemic stroke.

To date, few studies have focused on the expression levels of miRNAs in blood samples from patients in the hyperacute phase of ischemic stroke. Moreover, it is not clear whether changes in circulating miRNA expression are consistent with those in the brain ischemic penumbra in cases of hyperacute ischemic stroke.

Therefore, we presume that specific blood miRNAs released from the brain ischemic penumbra may reflect certain pathophysiological conditions of hyperacute ischemic stroke, and some of these specific circulating miRNAs may be potential biomarkers for the early noninvasive diagnosis of ischemic stroke in the hyperacute stage, thereby helping clinicians choose efficient interventions and treatments. This study aims to explore the expression profiles of miRNAs in the brain ischemic penumbra and blood of rats 4 h after permanent middle cerebral artery occlusion (pMCAO) in order to investigate their potential value as biomarkers of this condition.

To the best of our knowledge, this is the first study aimed at investigating miRNA expression changes in the penumbra and blood of rats with hyperacute ischemic stroke. We speculated that the rats with hyperacute ischemic stroke would show abnormal expression of circulating miRNAs, which could serve as novel non-invasive biomarkers for the early detection of hyperacute ischemic stroke.

In the present study, the brain ischemic penumbra and blood miRNAs were measured in rats 4 h after pMCAO or a sham operation to test the hypothesis that blood concentrations of penumbra-specific miRNAs are promising biomarkers for hyperacute ischemic stroke.

METHODS

Animals

Male Sprague-Dawley rats (300±20 g) were obtained from the Animal Experiment Center of Guangxi Medical University. The rats were housed in a light and temperature-controlled room (23±0.5°C with 12 h of light and 12 h of dark) and given free access to commercial food and tap water. The rats, matched for weight in each experiment, were randomized into a brain ischemia group (n=12) and a sham group (n=12). Body temperature was maintained at 37.0°C with a heating pad during all surgical procedures. All animals were handled according to the guidelines of the Institutional Animal Ethics Committee of Guangxi Medical University. Ethical approval was obtained from the University Animal Ethics Committee prior to the start of the study.

pMCAO and sham surgeries

The rats assigned to the brain ischemia group (n=12) were anesthetized. The right common carotid artery, internal common carotid artery and main trunk of the external carotid artery were exposed by blunt dissection. The right common carotid artery and external carotid artery were ligated with a silk suture, and a small opening was made in the common carotid artery. A monofilament nylon suture (40 mm in length, Φ0.34–Φ0.36 mm) was inserted into the common carotid artery and then threaded through the right internal carotid artery to occlude the right middle cerebral artery. The suture was anchored in place to cause pMCAO. After the surgery, the rats were returned to their cages to recover from anesthesia at room temperature. At 4 h after surgery, all rats were assessed for neurological
Neurological deficit scores

Neurological functional evaluations were performed after the rats fully awoke from anesthesia. Neurological function was assessed using the following 5-point neurological deficit scale: 0 = no neurological deficit; 1 = failure to fully extend the left forepaw; 2 = contralateral circling or walking; 3 = falling to the left but walking when stimulated; and 4 = unconsciousness, death or other state of unresponsiveness to stimulation (Longa et al., 1989). Scores were tallied separately for each animal. Rats receiving a score of 0, 1 or 4 were excluded from the study. In addition, rats with cerebral hemorrhage were excluded.

Collection of ischemic penumbra tissue and peripheral blood

According to the experimental protocol, rats were anesthetized and their blood and penumbra tissue samples were collected 4 h after pMCAO. Tissue samples from the brain ischemic penumbra were harvested as previously reported (Ashwal et al., 1998) (Fig. 1A) and stored at -80°C. Blood samples were drawn from the left or right jugular vein of each rat and collected in tubes containing ethylenediaminetetraacetic acid (EDTA).

Histopathologic analysis

Hematoxylin and eosin (H&E) staining was used to assess neuronal damage in the brain ischemic penumbra after pMCAO in rats. H&E staining was performed according to standard protocols. The rats were anesthetized at 4 h after pMCAO and transcardially per-

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Fig. 1. H&E staining showing the neuropathological changes in the ischemic penumbra after pMCAO. (A) A schematic representation of brain tissue from the ischemic penumbra. (B) H&E staining in the ischemic penumbra area from the sham group. (C) H&E staining in the ischemic penumbra area from the brain ischemia group. Brain tissue in the ischemic penumbra area was stained with H&E and observed under a microscope (x200 magnification).
fused with 4% formalin. The brains were removed and postfixed in 10% formalin for 24 h. A two-millimeter-thick sample of brain ischemic penumbra tissue, from the optic chiasm to 2 mm posterior, was obtained from each rat. The samples were then embedded in paraffin blocks and cut into successive 3-μm-thick coronal sections. The sections were deparaffinized, stained with H&E and subsequently observed under a microscope.

Total RNA isolation from penumbra and blood samples

At 4 h after pMCAO or the sham procedure, all rats were anesthetized. Blood (2 ml) was drawn from the right or left jugular vein into EDTA-containing tubes, and then the rats were euthanized. The brain was removed from each rat, and the tissue of the ischemic penumbra area was dissected as rapidly as possible, frozen, and stored at -80°C. Total RNA, including miRNAs, was extracted from the ischemic penumbra tissue or blood with TRIzol Reagent (Invitrogen, CA, USA) following the manufacturer’s protocol. Three milliliters of TRIzol was used per 1 ml of blood. The quantity and quality of the total RNA were measured by a Nanodrop ND-2000 spectrophotometer. OD260 was used to calculate the concentration, while the OD260/OD230 and OD260/OD280 ratios were used to determine the purity of total RNA. Specifically, all of the OD260/OD280 ratios were between 1.8 and 2.0, while the OD260/OD230 ratio was greater than 1.6. The integrity of total RNA was assessed by electrophoresis on a 1% denaturing agarose gel and analysis by an Agilent 2200 Bioanalyzer (Agilent Technologies, USA). The Agilent 2200 TapeStation software was used to calculate the RNA integrity number equivalent (RINe), an objective measurement of RNA degradation. The samples were immediately used or stored at -80°C for future use.

miRNA microarray profiling

miRNA microarray experiments were carried out by a service company (Guangzhou Ribobio Co. Ltd., China) according to the method described on the company’s website (http://www.ribobio.com/) using a miRNA array (RiboArray™ miDETECT™ Rat Array 1×12K, Guangzhou Ribobio Co. Ltd., China) composed of 765 probe sets from miRNAs registered in miRBase 21.0. The expression data were subjected to median normalization. After normalization, significant differential expression of miRNAs between the brain ischemia group and the sham group was identified through fold changes (≥1.5) and p-values (≤0.05). Hierarchical clustering was used to determine the distinguishable miRNA expression profiles among samples. A total of twelve samples were used, including three penumbra brain tissue samples, three sham samples from the corresponding area of the rat brain, three blood samples from ischemic rats and three blood samples from sham rats.

Detection of miRNA expression with qRT-PCR

According to the results of the miRNA profiling analysis, the expression level of selected miRNAs in all the pMCAO- and sham-treated rats was further confirmed by quantitative real-time polymerase chain reaction (qRT-PCR). One microgram of total RNA was reverse transcribed into complementary DNA (cDNA) using a Mir-X miRNA First-Strand Synthesis Kit (Clontech Laboratories, Takara, Japan). qRT-PCR was carried out on a LightCycler® 480 RT-PCR system (Roche Applied Science, Boehringer Mannheim, Germany) using SYBR Green master mix (Takara, Japan) according to the manufacturer’s instructions, and the results were analyzed with the LightCycler 480 Software 1.5.0. PCR amplification was conducted in a 25-μl reaction volume. The reactions were incubated in a 96-well optical plate at 95°C for 10 sec, followed by 40 cycles at 95°C for 5 sec and 60°C for 20 sec. Samples were analyzed in triplicate, and all the PCR primers were obtained from TaKaRa Biotechnology (Dalian, China). U6 was used as the internal standard control. The 2-ΔΔCt method was used to analyze miRNA expression.

Statistical analysis

Differential miRNA expression was identified on the basis of the following three conditions: (1) the miRNA was expressed in both penumbra and blood samples; (2) the difference in miRNA expression between the brain ischemia group and the sham group was greater than or equal to a 1.5-fold change in both the penumbra and blood; and (3) a Student’s t-test between the brain ischemia group and the sham group yielded a p-value of less than 0.05 in both the penumbra and blood. The qRT-PCR data were analyzed with the software SPSS 16.0 (IBM, USA). Differences between two independent groups were analyzed for statistical significance using a Student’s t-test (two-tailed). Differential expression was considered statistically significant at a p-value <0.05 between the two groups. The correlation between miRNA expression levels in the penumbra and blood was assessed using Pearson’s linear correlation analysis. Sensiti-
ty, specificity and accuracy of miRNA were compared using receiver operating characteristic (ROC) analyses. The data are expressed as the mean ± standard error of the mean (SEM). All of the experiments were performed in triplicate.

RESULTS

Ischemic brain damage was induced by pMCAO

A total of 18 male rats were selected to establish brain ischemia models by pMCAO. Among these 18 rats, 3 rats died, while the remaining 15 successfully modeled ischemia. Three rats that had a Bederson neurological score of 0 or 4 points were excluded. The other 12 rats with a brain ischemia score between 2 and 3 were selected for the experiment. In addition, 12 rats were subjected to a sham operation. To identify any pathological changes, we examined the neuronal morphology in the ischemic penumbra area of the rat brain. H&E staining was used to observe histopathological changes. Our results showed that, compared with rats in the sham group, rats in the brain ischemia group sustained neuronal damage. Rats receiving sham surgery had normal microscopic features and no inflammatory infiltration from the brain ischemic penumbra area. The neurons and glial cells were normally shaped, had round, lightly stained nuclei and were arranged in an orderly fashion (Fig. 1B). Meanwhile, rats in the brain ischemia group displayed glial cell swelling and vacuolization, neuronal pyknosis and anachromasis, fragmentation, interstitial hyperemia and edema (Fig. 1C).

Identification of differentially expressed miRNAs by microarray analysis

Total RNA was extracted from brain tissues in the ischemic penumbra area and from blood, and spectrophotometry was used to assess RNA yield and quality. All brain and blood samples were of high quality and sufficient concentration (Fig. 2). In total, 765 miRNAs were detected by miRNA microarray analysis. From the miRNA expression profiles, we identified miRNAs that were differentially expressed between the brain ischemia group (n=3) and the sham group (n=3). Hierarchical clustering was used to group miRNAs on the basis of their expression levels across samples (Fig. 3). Each column represents a sample, and each row represents a miRNA. The color scale shown on the right illustrates the relative expression level of a miRNA in a given slide: red indicates a high relative gene expression, and blue indicates a low relative gene expression. The relative expression level of each miRNA in a single sample is depicted according to the color scale provided. We set thresholds of fold change>1.5 and p-value<0.05. In the ischemic penumbra area, 321 miRNAs demonstrated at least 1.5-fold change in expression in the brain ischemia group compared with the sham group. Among these miRNAs, 6 were upregulated, and 315 were downregulated in the penumbra 4 h after pMCAO. In blood, 88 miRNAs demonstrated at least 1.5-fold change in expression in the brain ischemia group compared with the sham group. Among these miRNAs, 80 were upregulated, and 8 were downregulated in blood 4 h after pMCAO.

Unique miRNA expression profiles in penumbra tissue and blood

First, we used Venn diagrams to analyze the common miRNAs and the unique miRNAs between the sham group (n=3) and the brain ischemia group (n=3) in both blood and brain (Fig. 4A, C). As there may be miRNAs that are specifically regulated in hyperacute ischemic stroke, we looked for unique miRNA expression profiles in the penumbra and blood using cluster analysis. This analysis showed that brain ischemic injury had a unique miRNA expression profile in both blood (Fig. 4B) and the penumbra (Fig. 4D). For the expression profiles shown, the expression profiles found in blood and the penumbra were not identical. The differential expression patterns of miRNAs between the sham and brain ischemia groups emphasize the uniqueness of the different miRNA responses of the blood and penumbra to ischemic brain damage. The results showed that most miRNAs in the blood were up-regulated and most miRNAs in penumbra were down-regulated in rats 4 h after pMCAO.

qRT-PCR validation of several differentially expressed miRNAs in the penumbra and blood

We hoped to find a potential blood biomarker for the detection of brain ischemic damage. The biomarker should be simultaneously detectable in the penumbra tissue and blood and should be sensitive to ischemic damage. More importantly, the expression trends of miRNAs in the penumbra and blood should be consistent. Thus, several miRNAs were selected from the miRNA profiling studies. However, some of those miRNAs were not expressed in all samples. Therefore, only miR-223-3p and let-7b-3p were selected for further identification by qRT-PCR. Our results show that in both penumbra and blood, miR-223-3p was confirmed...
to be upregulated (Fig. 5A and 5B), while let-7b-3p was confirmed to be downregulated (Fig. 5C and 5D). The results of the qRT-PCR analysis were essentially consistent with the miRNA microarray results.

Fig. 2. Validation of the quality of total RNA. (A) Agarose gel electrophoresis analysis showed that total RNA from blood had no obvious degradation and had a good degree of purity. (B) Agarose gel electrophoresis analysis showed that total RNA from brain tissue in the ischemic penumbra area had no obvious degradation and had a good degree of purity. (C) Agilent 2200 TapeStation software analysis showed high quality total RNA with RINe scores consistently greater than 7. (D) This RNA ladder is ideal for approximate RNA quantification on gels.
Correlation analysis and ROC curve analysis was performed to evaluate the diagnostic value of blood miRNAs

First, in the correlation analysis, miR-223-3p (Fig. 6A) and let-7b-3p (Fig. 6B) expression levels in the penumbra and blood showed a moderate correlation for both the sham and brain ischemia groups. Next, we generated ROC curves to evaluate the potential diagnostic value of blood miR-223-3p and let-7b-3p as biomarkers for hyperacute ischemic stroke. The ROC curve analysis revealed a significant diagnostic value of blood miR-223-3p (Fig. 7A) and let-7b-3p (Fig. 7B) for ischemic stroke in rats. This result suggests that blood miR-223-3p and let-7b-3p may be valuable biomarkers for diagnosing hyperacute ischemic stroke.

DISCUSSION

In the present study, we sought to investigate the expression of miRNAs in hyperacute ischemic stroke by analyzing miRNA expression profiles in the penumbra and blood 4 h postoperatively in pMCAO-treated and sham-operated control rats.

An important finding of this study is that significantly different miRNA expression profiles occurred in both the penumbra and blood after ischemic brain damage. Some of these miRNAs were significantly up- or down-regulated by more than 1.5-fold in the penumbra and blood 4 h after pMCAO. Moreover, the levels of some miRNAs showed parallel patterns of expression in blood and the penumbra. These results suggest the possible use of circulating miRNAs as noninvasive biomarkers for hyperacute ischemic stroke. Our findings specifically identified candidate brain-specific miRNAs that appear to be novel and stable biomarkers for hyperacute ischemic stroke in rat.

A remarkable finding of this study is that miR-223-3p was upregulated in both the penumbra and blood 4 h after pMCAO-induced ischemic stroke in rats. This result is consistent with previous findings that the expression level of miR-223 in the blood of patients with acute ischemic stroke was significantly increased compared with that of the healthy controls (Wang et al., 2014). A recent study also demonstrat-
ed that the expression level of miR-223 was greatly upregulated in the blood of young ischemic stroke patients aged 18 to 49 years (Tan et al., 2009). In addition, correlation analysis demonstrated that the expression level of miR-223-3p in the brain ischemic penumbra was positively correlated with the blood miR-223-3p level in this rat model of hyperacute ischemic stroke.

Moreover, several previous studies have indicated that miR-223 is selectively highly expressed in immune cells and that this miRNA plays a crucial role in regulating cell cycle progression and cell proliferation (Johnnidis et al., 2008; Tsitsiou and Lindsay, 2009). A recent study suggested that miR-223 is closely correlated with acute ischemic stroke and may play a crucial role in stroke by upregulating the insulin-like growth factor.
gene (Wang et al., 2014). Another study demonstrated that platelet-released miR-223 promotes advanced glycation end products, leading to vascular endothelial cell apoptosis by targeting the insulin-like growth factor receptor (Pan et al., 2014). According to the comprehensive analysis above, our findings suggest that miR-223-3p may be involved in the pathological process of hypoxia-ischemia; furthermore, they indicate that circulating blood miR-223-3p levels may represent changes in miR-223-3p levels in the brain ischemic penumbra in response to hyperacute ischemic stroke.

Another important finding of this study was that the expression of let-7b-3p was downregulated in both the penumbra and blood 4 h after pMCAO. This finding is consistent with the results of many previous studies. A recent study showed that the plasma let-7b level was decreased

![Fig. 5. The expression of miRNAs in the penumbra and blood of rats 4 h after pMCAO.](image)

(A) The expression level of miR-223-3p in the blood 4 h after pMCAO. (B) The expression level of miR-223-3p in the penumbra 4 h after pMCAO. (C) The expression of let-7b-3p in the blood 4 h after pMCAO. (D) The expression of let-7b-3p in the penumbra 4 h after pMCAO. (A, B) miR-223-3p was found to be highly expressed in both the penumbra and blood 4 h after pMCAO. (C, D) let-7b-3p was found to have low expression in both the penumbra and blood 4 h after pMCAO. *p<0.01 vs. the sham group.
in ischemic stroke patients with large artery atherosclerosis (Long et al., 2013). Another study showed that the expression of let-7b was significantly decreased in patients with ischemic stroke compared with healthy controls (Sepramaniam et al., 2014). In 2000, the let-7 family was found to play important roles in regulating the cell cycle, apoptosis, proliferation, migration and immunity (Pasquinelli et al., 2000; Reinhart et al., 2000). let-7b was also found to be differentially expressed in the pathophysiological process of ischemic stroke, and it may thus play key roles in angiogenesis, inflammation and neurogenesis (Pasquinelli et al., 2000). Moreover, a previous study demonstrated that let-7b activates toll-like receptor (TLR) 7, which contributes to the spread of central nervous system injury. Specifically, it was found that let-7b is a crucial activator of TLR7 in both neurons and immune cells. That study characterized TLR7 expression in neurons and established a new role for TLR7 as a receptor that regulates neuronal death. The investigators also speculated that let-7b is released during pathological processes and stimulates TLR7, thereby sending a danger signal to neurons and accelerating their decay (Lehmann et al., 2012). In general, members of the let-7 family may function as important regulatory molecules in the pathophysiology of ischemic stroke. Our correlation analysis also demonstrated that the expression level of let-7b-3p in the penumbra
was positively correlated with the level in the blood after ischemic brain damage. Our data suggest that changes in let-7b-3p in the blood represent similar changes in the penumbra. However, the exact reason why let-7b-3p levels are decreased at 4 h after ischemic brain damage is not clear; the cause of this reduction of let-7b-3p in both the penumbra and blood after ischemic brain damage deserves further investigation.

In conclusion, our results show that the blood levels of miR-223-3p and let-7b-3p are correlated with the levels in the penumbra of an ischemic brain lesion, suggesting that changes in blood miR-223-3p and let-7b-3p levels may represent changes in the corresponding penumbra miRNA levels. The diagnostic performance of these miRNAs in rats was measured by ROC analysis, which indicated that miR-223-3p (AUC was 0.9688) and let-7b-3p (AUC was 0.9180) are useful for distinguishing the brain ischemia group from sham group. Therefore, our study provides evidence that circulating miR-223-3p and let-7b-3p might be novel noninvasive biomarkers for the diagnosis of hyperacute ischemic stroke.

However, this study has some limitations. First, although we found that blood miR-223-3p and let-7b-3p levels are associated with the levels of those miRNAs in the brain ischemic penumbra in rats, there is no direct evidence that the circulating miRNAs were initially released from the ischemic penumbra or that the miRNAs caused the outcome. Second, in order for a circulating biomarker to be useful in diagnosing hyperacute ischemic stroke, there must be a very rapid test for the marker. The techniques we used in this study need to be modified and improved to increase their clinical applicability. Finally, our findings need to be validated in a human randomized clinical trial with a large cohort of hyperacute ischemic stroke patients.

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