Identification of FBLN5, mir128 and mir532-5p Related to Unstable Plaques in Carotid Atherosclerosis Patients by Single-Cell RNA Sequencing

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Research

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Abstract

Background:

Rupture of the carotid plaque is one of the main causes of cerebral embolism. The molecular mechanisms underlying carotid plaque rupture remain unknown. Many previous studies have studied the histologic instability of plaques after a stroke, but few have predicted microRNA-gene axes of plaque rupture and mapped potential target genes at the cellular level using single-cell RNA sequencing in normal and atherosclerosis samples. The aim of the present study was to systematically identify the gene signature and prediction diagnostic module, as well as hub miRNA-gene axes to explore the potential biomarkers and mechanisms associated with the carotid atherosclerosis.

Results:

Microarray dataset GSE163154, containing advanced atherosclerotic plaques group and early lesions group, was obtained from the Gene Expression Omnibus database. Weighted gene co-expression network analysis (WGCNA) was conducted to identify advanced plaque-related module. Subsequently, we detected eight optimal hemorrhage-associated genes (FBLN5, FMOD, GAL, GEM, SLC14A1, STBN1, TMEM119, and GREM1) using LASSO regression analysis and confirmed by using receiver operating characteristic (ROC) curve. The area under the ROC curve was 0.951 (95% confidence interval [CI]: 0.887, 1.000) for the matched sample. The interaction of thirteen upstream microRNAs and five genes were constructed, in which microRNA-532-3p and microRNA-128 were upstream regulators of FBLN5 by searching for Starbase v3.0 and GEO datasets. Finally, through performing single-cell RNA sequencing in artery samples of 1 normal people and 1 patient with coronary atherosclerosis, we found that in atherosclerotic samples, FBLN5 was highly expressed in smooth muscle cells, derived smooth muscle cells and endothelial cells. Therefore, we speculated that FBLN5 mainly plays a role in atherosclerosis through being regulated by microRNA-532-3p and microRNA-128.

Conclusion:

This finding backs up the popular idea that miRNA-gene axis and FBLN5 are linked to acute plaque disruption and occur before symptoms appear. These genes could become targets for novel medical treatments in the future, or they could help identify patients with histologically unstable plaques who would benefit from surgical intervention if they were validated.

Background

Stroke is a serious health issue. Thromboembolism of carotid atherosclerotic plaques ⁽¹⁾. is responsible for more than a third of strokes. However, little is known about the factors that cause atherosclerotic plaque instability, rupture, and embolism. Therefore, it is critical to clarify the molecular mechanism behind the development and progression of intraplaque hemorrhage, as well as to identify possible new molecular targets for pharmacological therapy to decrease plaque instability and avoid ischemia events.

Instability is characterized by the inflammation of large fatty nuclei and plaques, causing cap rupture, sores on the surface, and thrombi ⁽²⁾. Plaque instability has no known hereditary source, although it is thought to have a role in changing gene expression in both local and systemic inflammation ⁽³⁾.

Previous research used microarray data from carotid atherosclerotic plaques to find differentially expressed genes (DEGs). By analyzing the gene expression profiles and protein makeup of symptomatic and asymptomatic individuals, Han et al. discovered a protein-gene linked multiomics model capable of identifying susceptible, hemorrhaged lesions with high predictive performance ⁽⁴⁾. DEG and protein screening, on the other hand, cannot fully expose gene connections or identify genes with critical biological activities.

In comparison to patients with histologically stable plaques, we expected that individuals with histologically unstable plaques would have a different gene and microRNA expression profile. Our findings reveal that the weighted correlation network analysis (WGCNA) and LASSO regression analysis are conducive for revealing genes and microRNAs that have a strong clinical correlation, finding effective targets and upstream regulators for prevention of intraplaque hemorrhage (IPH), and laying a foundation for the development of genic prevention for stroke onset ^(5, 6).

Results

Flowchart

A flowchart summarizing our study is shown in Fig. 1. We screened 497 DEGs, constructed co-expression networks for the IPH and non-IPH groups and detected gene modules. Then select intersection genes between DEGs and gene modules by LASSO regression analysis, retrieve the upstream microRNA of hub genes from the database of non-coding RNAs and analysis differentially expressed miRNAs (DEMs) from GEO database. The microRNAs that are both differentially expressed and present upstream of hub genes are strongly associated with carotid atherosclerotic plaque instability.

Data preprocessing and identification of DEGs

The expression matrix of the GEO dataset included 27 samples with IPH and 16 samples without IPH. A total of 497 DEGs (276 upregulated and 221 downregulated) were chosen for further investigation since their FDR was less than 0.05 and their fold change was more than two. The volcano plot and heatmap of the DEGs are shown in Fig. 2A and 2B. We identified the five most differentially expressed up-regulated and down-regulated genes. The difference of FBIN5 is the most obvious.

Evaluation of the training set's quality and the creation of a co-expression network

We matched the samples' illness states to their expression matrices. The sample dendrogram and trait heatmap were shown after the 43 samples were grouped (Additional file 1). To design a weighted network based on a scale-free topology criterion, the soft thresholding power was set as $\lambda = 17$, where the curve first approached $R^2 = 0.85$. (Additional file 2).

Identification of clinically significant modules

The dynamic tree cutting method was used to identify two modules, as shown in Fig. 2C. Table 1 shows the number of genes in each module. High correlations were found with the characteristic of disease status (IPH or no IPH) after linking the modules to traits, as illustrated in Fig. 2D. The blue and turquoise modules were declared clinically significant and eligible for further investigation since they had a $p < 0.05$ value.

Table 1. The total number of genes in each of the two modules

Module colors	Number
blue	1189
turquoise	2155

Identification of critical genes and GO annotation and KEGG pathway enrichment analysis

From the clinically significant modules in the co-expression network, 3344 important genes were chosen. The intersection of DEGs and genes in two clinical modules gained 463 genes as shown in Figure 4. To obtain a deeper insight into the biological roles of these critical genes, we conducted the GO annotation and KEGG pathway enrichment analyses. Fig. 2F lists the top 10 enriched GO terms and KEGG pathways of the clinically significant genes. The common hub genes were significantly enriched in the pathways of positive regulation of secretion, signal release, response to steroid hormone, and neutrophil mediated immunity, according to a GO biological processes (BP) analysis. MAPK signaling pathway, Neuroactive ligand-receptor interaction, PPAR signaling pathway, and Calcium signaling pathway were the top four markedly enriched pathways (Fig. 2F). The hub genes may play a crucial role in immunological and inflammatory responses triggered by hormone-mediated factors, according to the results of the GO and KEGG pathway studies.

Establishment predicted model

We eventually found eight optimum prognostic genes by LASSO regression analysis and put them into the prognostic risk model: *FBLN5*, *FMOD*, *GAL*, *GEM*, *SLC14A1*, *SPTBN1*, *TMEM119* and *GREM1*, as shown in Fig. 3A and 3B. We calculated the risk score for each patient using gene mRNA levels and risk

estimate regression coefficients to identify the relevance of hub genes. The formula for the calculation is explained in the Methods section. Risk score = $(-0.31774005 \times \text{expression of } FBLN5) + (-1.58406823 \times \text{expression of } FMOD) + (0.04517572 \times \text{expression of } GAL) + (-2.44920986 \times \text{expression of } GEM) + (0.04109032 \times \text{expression of } SLC14A1) + (-0.12978159 \times \text{expression of } SPTBN1) + (-0.33417587 \times \text{expression of } TMEM119) + (0.25663856 \times \text{expression of } GREM1)$.

Identification of hub genes

ROC curve analysis was performed to evaluate the diagnostic significance of prognostic genes in patients with ruptured or stable human atheromatous lesions and showed that (Fig.3C). TMEM119 was not shown in the ROC curve for the absence in this dataset. The mRNA levels of these genes accurately distinguished IPH tissues and indicated high accuracy of gene prediction. This demonstrates that the genes obtained by LASSO regression analysis expression are potential diagnostic biomarkers for IPH.

Analysis and identification of microRNA of diagnostic genes

We searched for 392 upstream microRNAs of eight hub genes from starBase Version3.0. GSE11794, the dataset of ncRNA of carotid atherosclerosis, was downloaded and conducted difference analysis. 638 up-regulated and 244 down-regulated ncRNAs were obtained (P), and the volcano plot was shown in Fig.3D. We intersected DEMs with the upstream microRNAs and obtained 13 core upstream regulators (Fig.3E), which include hsa-miR-769-5p, hsa-miR-532-3p, hsa-miR-501-3p, hsa-miR-4739, hsa-miR-455-5p, hsa-miR-421, hsa-miR-339-5p, hsa-miR-331-3p, hsa-miR-330-3p, hsa-miR-23c, hsa-miR-193a-3p, hsa-miR-133b and hsa-miR-128. These miRNAs and their closely related hub genes were introduced into Cytoscape software to draw interaction network (Fig.3F). miR-532-3p is linked with the largest number of hub genes. It is concluded that mir-532-3p closely relates to the rupture of the carotid plaque. Among the intersection genes, FBLN5 expression was significantly different after plaque rupture (Fig. 3G). Meanwhile, we used GSE28829 and GSE100927 to draw Box plot after data standardization, in which identify the FBLN5 expression difference between IPH and non-IPH. It is obviously that FBLN5 has high specificity.

Single-Cell Genomics reveals FBLN5 distribution during atherosclerosis

To figure out the farther mechanism, we compared the expression of hub genes between normal and atherosclerotic arteries at the cellular level. Subsequently, we calculated the expression of the hub genes in cells based on scRNA sequence data. All high-quality cells were screened for limitations of number of mitochondrial genes, the number of genes in a single cell, and the number of erythrocyte genes (Additional file 3). A total of SMC-derived cell, Macrophage, CD8+T cell, Smooth muscle cell (SMC), NK cell, Lymphatic Endothelial cell were obtained in normal group (Fig. 4A). A total of SMC, SMC-derived cell, Macrophage, common myeloid Progenitor, Lymphatic Endothelial cell were obtained in the disease group

cell, CD4+T cell (Fig. 4B). Among them, we projected FBLN5 onto the cell distribution and found that in normal group FBLN5 high specificity expressed on SMC-derived cell and Smooth muscle cell (Fig. 4C-D). However, in disease group, in addition to the above two types of cells, FBLN5 was also highly expressed in endothelial cells (Fig. 4E-F).

Discussion

Stroke is a deadly disease, and its mortality have been rising worldwide in recent years. Carotid atherosclerotic is one of important reasons. Although fat, smoking and chronic diseases are considered risk factors, the etiology of unstable plaque in carotid atherosclerotic is unclear due to conflicting evidence⁽¹¹⁾. Understanding the molecular mechanism of unstable plaque is of great importance for diagnostic and treatment. Numerous studies have defined the gene signatures that distinguish stable plaques samples with unstable ones, and also many studies confirmed that ncRNA fulfill their role in unstable plaque in carotid atherosclerotic by binding and down-regulating mRNAs⁽¹²⁻¹⁴⁾. The present study distinguishing from previous studies is the identification of FBLN5 to be a prediction diagnostic biomarker and miRNA-532-3p and miRNA-128 as its upstream regulator. More importantly, through single-cell sequencing analysis we identified that the potential mechanism of FBLN5 and upstream ncRNA may influence atherosclerotic endothelial cells and smooth muscle cells.

A total of 497 differentially expressed genes (DEGs) were identified. The expression of 221 genes was increased, while the expression of 276 genes was decreased. According to the module recognition heatmap and scatter diagrams, the blue and turquoise modules were related to the instability of plaques ($p < 0.05$) (Fig. 2C and 2D).

GO and KEGG analyses identified the common genes between DEGs and hub modules as immune-related and inflammation-related modules (Fig. 2F).

We selected the eight best candidate genes using a mix of Cox regression and LASSO regression methods (FBLN5, FMOD, GAL, GEM, SLC14A1, STBN1, TMEM119, and GREM1). These genes were utilized to build a Cox regression risk model that could predict whether or not IPH was present.

Through searching for upstream microRNAs of hub genes and identifying differentially expressed ncRNAs, microRNA-mRNA interactions of 13 microRNAs and 5 hub genes were constructed. In particular, mir-532-3p was linked to four hub genes. Among its downstream genes, FBLN5 was the most significantly differentially expressed gene. We speculated that mir-532-3p-FBLN5 might play a significant impact on the stability of carotid plaque.

By the observation of atherosclerotic cell level, it was found that FBLN5 was highly expressed on smooth muscle cells and SMC-derived cells in the normal samples. The expression of FBLN5 in the same cells of the disease group was also highly expressed and more importantly, endothelial cells as a new type of

cells with high specific expression appeared in the disease group. We identified that FBLN5 is primarily expressed in SMC, SMC-derived cells, and endothelial cells, according to our findings, and may be controlled by mir-128, mir-532-3p to affect the development of these cells in atherosclerosis.

A secreted extracellular matrix protein with an Arg-Gly-Asp (RGD) motif and calcium-binding EGF-like domains is encoded by FBLN5. Through the interaction of integrins and the RGD motif, it enhances endothelial cell adhesion. It is highly expressed in arteries that are still growing. In balloon-injured arteries and atherosclerotic lesions, however, its expression is reinduced, particularly in intimal vascular smooth muscle cells and endothelial cells. Numerous studies have confirmed that FBLN5 is one of factors in the modules that predict the outcome of coronary calcification^(15, 16). Tomoyuki Nakamura found that FBLN5 directly interacts with elastic fibres through its amino-terminal and provide anchorage of elastic fibres to cells to stabilize and organize vasculature⁽¹⁷⁾. As a result, this gene's protein may play a function in vascular development and remodeling. After ligation of the carotid artery, FBLN5(-/-) animals showed severe carotid intima hyperplasia, smooth muscle cell proliferation, and migration.⁽¹⁶⁾ Our study coincidentally demonstrates that in the patient with carotid atherosclerosis, the expression of FBLN5 and anchoring protein in both smooth muscle cells and endothelial cells are reduced, resulting in weakness of inter-cell connections and increased cell proliferation and migration. As the ncRNA of FBLN5, miR-128 affects VSMC proliferation, migration, differentiation, and contractility by targeting Kruppel-like factor 4 and modulating the methylation status of the pivotal VSMC gene myosin heavy chain 11 (Myh11)⁽¹⁸⁾. Elizabeth J Hennessy observed that CHROME lncRNA regulates cellular and systemic cholesterol homeostasis in the atherosclerotic plaques by inhibiting miRNA-128-3p expression⁽¹⁹⁾. miRNA-532 is associated with human vulnerable plaques and modified low-density lipoprotein or tumor necrosis factor α exposure. Chenjing Wang found that lncRNA CASC2 suppressed cell reproduction and promoted cell apoptosis by regulating the miR-532-3p/PAPD5 axis in ox-LDL-mediated VSMCs⁽²⁰⁾.

It has been reported that miR-133b are related to subclinical atherosclerosis⁽²¹⁾. MiR-330-3p was characterized by Wen-Hsien Lee as a potential new diagnostic or therapeutic method for targeting cardiomyocytes under acute hypoxic stress in conditions such as acute myocardial infarction⁽²²⁾. Downregulation of HOXA-AS3 dramatically slowed the progression of atherosclerosis by modulating the miR-455-5p/p27 Kip1 axis, suggesting that it could be a potential target for atherosclerosis treatment⁽²³⁾.

Our results provide novel molecular targets predicting advanced plaque progression. However, more factors need to be combined to predict adverse clinical outcomes. And the functional pathways of ncRNA and FBLN5 deserve further discussion so that cardiovascular events can be more effectively avoided.

Conclusion

FBLN5, miRNA-532-3P, and miRNA-128 could be used as biomarkers for diagnosis and prognosis, as well as possible targets for medicinal therapy. The biological functions of the axis in the etiology and progression of carotid atherosclerosis require more research into the underlying mechanisms.

Methods

Datasets

Gene expression profiles of GSE163154 were selected from the Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>), which was utilized to get carotid atherosclerosis gene expression profiles. The samples are carotid atherosclerotic plaques deriving from carotid endarterectomy surgery and include 16 stable atherosclerotic lesion segments and 27 unstable ones, based on whether there is intraplaque bleeding. Series matrix file and platform data tables (GPL6104) were downloaded..

DEG analysis

The probe names in the matrix files were replaced with gene symbols, and GSE163154 was annotated with the GPL6104 platform data tables. Our study comprised a total of 43 samples, with 27 demonstrating the presence of IPH and 16 demonstrating the lack of IPH. The presence of IPH plaque in the sample was used to estimate plaque stability. The limma R program was used to find differentially expressed genes (DEGs).⁽⁷⁾ $|\log_2(\text{fold change})| > 2$ and adjusted $p < 0.05$ were set as the threshold for DEG screening. Advanced volcano plot was performed using the OmicStudio tools at <https://www.omicstudio.cn/tool>. Clustering DEGs was performed by using heatmap tools in Hiplot (<https://hiplot.com.cn>), a comprehensive web platform for scientific data visualization⁽⁸⁾.

Construction of co-expression network

Based on the expression data profile of GSE163154, the WGCNA package in R was used to design a co-expression network. The impute package in R was used to inspect the microarray quality, detecting whether any genes had missing data and ensuring that the samples were good. WGCNA analysis should be performed on the top 20% of genes in the GSE163154 dataset. To visualize the sample tree and identify outliers, we used sample clustering. The soft thresholding power (β) value was calculated using WGCNA's pickSoftTreshold function after we computed Pearson's correlation matrices for paired genes.

Gene Ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) analysis

GO enrichment and KEGG pathway analyses of the intersection between genes in the hub modules and DEGs were performed using the clusterProfiler package (for enrichment analysis) and org.hs.eg.db package (for ID conversion) in R 3.6.3, a search tool for the retrieval of interacting genes/proteins⁽⁹⁾.

Identification of hub genes

To identify novel genes related to intracellular signaling and alterations in transcription, a Cox regression analysis was carried out jointly. First, we combined the levels of expression of screened genes with the presence or absence of IPH in patients. The DEGs with a p.05. were then identified using a univariate Cox proportional hazard regression analysis. The glmnet package in R software was then used to do the least absolute shrinkage and selection operator (LASSO) Cox regression analysis to find the genetic model with the best prognostic value. Finally, a prognostic signature for predicting the prognosis of IPH was created using multivariate Cox regression analysis. The expression of differentially expressed inflammatory response-related genes (DEIRGs) and the regression coefficients obtained in the regression model were used to compute the risk score for each patient. To calculate the risk score for each patient, the regression coefficient of the gene was multiplied by the gene's expression and then totalled. The calculation formula is as follows:

$$\text{Risk score (patients)} = \sum_{i=1}^n \text{coefficient (gene}_i\text{)} \times \text{expression value of (gene}_i\text{)}$$

Gene_i is the ith chosen gene, and coefficient (gene_i) is the gene_i's estimated regression coefficient from the Cox proportional hazards regression analysis. The accuracy of the prognostic prediction models was evaluated using time-dependent (ROC curves).⁽¹⁰⁾ We downloaded GSE100927 from GEO, which contains the expression profile data of the carotid atherosclerotic group and the normal group, and used R for analysis and visualization. An AUC of >0.60 was deemed a good forecast, while an AUC of >0.75 was acknowledged as having high predictive value. Thus, ROC curve analysis was performed and AUC values were calculated to determine the prognostic accuracy of the expression of these hub genes as parameters.

Identification of key microRNAs

starBase V3.0 is a database that predicts the function of ncRNA and protein-coding gene. We imported diagnostic genes predicted by LASSO into starBase V3.0 database to retrieve their upstream microRNAs. And GSE11794 was downloaded from GEO database, which describes the microRNA expression profile of symptomatic and asymptomatic patients with carotid atherosclerosis. All the patients presented with significant carotid stenosis (60%-100%). But only symptomatic ones suffered a transient ischemic attack, transient monocular blindness ipsilateral to the study artery, or minor or non-disabling ipsilateral stroke. Using GEO2R, we obtained the data of differential analysis, and utilized ggplot2 package, OmicStudioclassic package and OMicStudioKits package in R version 3.6.1 to screen the differentially expressed genes. The parameter was set to P . Advanced volcano plot was performed using the OmicStudio tools at <https://www.omicstudio.cn/tool>. The upstream miRNAs predicted by LASSO were intersected with DEM to obtain the key miRNAs that might affect the stability of carotid plaque. Cytoscape V3.7.2 was used to map the network interaction between genes and key miRNAs. The

differently expressed matrix of hub genes in GSE163154 was performed using the OmicShare tools, a free online platform for data analysis (<https://www.omicshare.com/tools>).

Performing scRNA-seq (10X Genomics) to study the transcriptomic profiles of atherosclerosis

In order to understand the mechanism and localization of core genes in carotid atherosclerosis more accurately, our study downloaded single-cell data of normal arteries (GSM4704932 in GSE155468) and single-cell data of atherosclerosis (GSM4705589 in GSE155512) from GEO database. 10x genomics analysis was performed on each of the two samples, and the selected genes were highlighted in the distribution of the main aggregating cells.

Abbreviations

WGCNA: Weighted gene co-expression network analysis; ROC: Receiver operating characteristic; DGEs: Differentially expressed genes; IPH: Intraplaque hemorrhage; GEO: Gene Expression Omnibus; KEGG: Kyoto encyclopedia of genes and genomes; LASSO: Least absolute shrinkage and selection operator; DEIRGs: Differentially expressed inflammatory response-related genes; DEMs: Differentially expressed miRNAs; BP: Biological processes; SMC: Smooth muscle cell.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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Authors' contributions

Conceptualization, LZ and JH; methodology, JH; software, LZ; validation, XYZ and RHC; formal analysis, LZ and JH.; resources, LZ and JH; data curation, LZ and JH; writing—original draft preparation, LZ and JH; writing—review and editing, XYZ and RHC; visualization, LZ and JH; supervision, HLD; project administration, HLD; All authors read and approved the final manuscript.

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Supplementary Materials

The Supplementary Material can be found online at the end of the article.

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Figures

Figure 1

Flowchart for the study

Figure 2

Identification of genes associated with carotid atherosclerosis. (A) Volcano plot of the DEGs. Red, upregulation; blue, downregulation. (B) The heatmap of DEGs. GO annotation and KEGG pathway enrichment analysis of DEGs in blue and turquoise modules. (C) A dendrogram based on a dissimilarity metric of all differentially expressed genes (1-TOM). (D) Relationship between module eigengenes and

intraplaque bleeding as a heatmap. (E) Intersection of DEGs and clinical-module genes. (F) The top 10 pathways of enrichment. FDR q value < 0.05. GO BP is the blue module. GO CC is the red module. GO MF is the green module. KEGG is turquoise module. KEGG of the turquoise module.

Figure 3

(A) Ridge trace. (B) Cross-validation score. (C) The ROC analysis for target gene co-expression in training. (D) Volcano plot of the DEM. Red, upregulation; blue, downregulation. Thirteen genes related to plaque

rupture were identified in the volcanic plot. Except for hsa-miR-4739 downregulation, other miRNAs were significantly up-regulated. (E) Intersection of DEMs and upstream microRNAs of hub genes. There are 13 overlapping miRNAs (F) Network of intersection microRNAs and hub genes. hsa-miR-532-3p is the most core miRNA, which has 4 lines with hub genes. (I) (H) The box plot of GSE100927 after data standardization, blue represents different disease sets, and red represents normal sets.

Figure 4

(A) tSNE projection of 1132 cells in the normal artery. Each dot represents a single cell, which is colored differently depending on the type of cell. (B) Atherosclerosis tissue tSNE projection of 1132 cells (C) Violin plots depicting FBLN5 expression in the 7 normal sample clusters. (D) As seen in the tSNE graphic, FBLN5 was utilized to designate clusters based on cell identity. The legend is labeled in log scale, and the data are colored according to expression levels. (E) Violin plots depicting FBLN5 expression in the 7 atherosclerosis sample clusters. (F) Violin plots depicting FBLN5 expression in the 7 atherosclerotic clusters.

Supplementary Files

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