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MiR-4299 suppresses non-small cell lung cancer cell proliferation, migration and invasion through modulating PTEN/AKT/PI3K pathway

W.-B. YANG, W.-P. ZHANG, J.-L. SHI, J.-W. WANG

Department of Cardiothoracic Surgery, Heping Hospital affiliated to Changzhi Medical College, Changzhi, Shanxi, China

Abstract. – OBJECTIVE: The aim of the present study was to investigate clinical significances and biological roles of miR-4299 in non-small cell lung cancer (NSCLC)

PATIENTS AND METHODS: Expression of miR-4299 in NSCLC tissues and matched non-tumor tissues was determined by quantitative real-time PCR (qRT-PCR). The correlations between miR-4299 expression and clinicopathological characteristics and prognosis were also analyzed. MTT assay and Transwell assay were performed to determine the proliferation, migration and invasion. Western blotting was used to examine the expressing patterns of PTEN/AKT/PI3K signaling pathway-related proteins.

RESULTS: We found that the expression level of miR-4299 was downregulated in NSCLC tissues and cell lines. Low miR-4299 expression was positively correlated with TNM stage (p=0.002), histological grade (p=0.002) and lymph node metastasis (p=0.028). Moreover, Kaplan-Meier survival analysis showed that the patients with low miR-4299 expression had shorter survival time than those with high miR-4299 expression (p=0.0011). More importantly, multivariate analysis suggested that decreased miR-4299 expression was a poor independent prognostic predictor for NSCLC patients (p=0.009). Functionally, overexpression of miR-4299 inhibited the proliferation, migration and invasion in A549 cells. Mechanistically, the results of Western blot showed that miR-4299 exhibited its tumor-suppressive role by modulating PTEN/AKT/PI3K signaling pathway.

CONCLUSIONS: We firstly indicated that miR-4299 may be a candidate independent marker for NSCLC prognosis and suppressed the progression of NSCLC by modulating the activation of PTEN/AKT/PI3K signaling pathway, suggesting that miR-4299 could be a potential target for developing therapies in treating NSCLC.

Key Words

miR-4299, NSCLC, Prognosis, PTEN/AKT/PI3K pathway, Proliferation, Migration, Invasion.

Introduction

Lung cancer remains the most prevalent and lethal cancer in both men and women worldwide[1]. Non-small cell lung cancer (NSCLC) accounted for approximately 80-85% of all lung cancers[2]. Although several diagnostic techniques and treatments for NSCLC have been developed, the 5-year survival rate of patients with NSCLC remains < 15%[3]. Tumor recurrence and metastasis are frequent and great challenges for the effective treatment of NSCLC patients[4]. Therefore, it is very important to understand the molecular events underlying NSCLC progression and to identify novel therapeutic targets to improve NSCLC prognosis.

MicroRNA (miRNA), a kind of non-coding RNA molecule with a length of about 21-23 nucleotides, regulate gene expression by binding to 3'-untranslated regions of target mRNA[5]. New evidence indicated that miRNAs play crucial roles in many biological processes such as differentiation, cell proliferation, migration, invasion and apoptosis[6,7]. Deregulated miRNAs and their roles in tumor progression have also attracted much attention[8]. In NSCLC, growing studies reported that miRNAs served as tumor suppressor or tumor promoters by targeting certain mRNAs or modulating several tumor-related signal pathways[9,10]. For instance, Chi et al[11] reported that miR-203 suppressed cell proliferation, invasion, and migration of NSCLC by targeting RGS17. Wang et al[12] found that miR-224 promoted NSCLC cell proliferation by directly targeting RASSF8. In addition, several miRNAs were reported to be involved in the regulation of several tumor-related signal pathways, such as Wnt/β-catenin pathway, Notch-1 signaling pathway and NF-κB pathway[13-15]. Because of the important role of miRNAs in tumor behavior, the potential of miRNAs as biomarkers for diagnosis and prognosis of NSCLC also attracted much attention.

Corresponding Author: Wei-Bin Yang, MM; e-mail: czqq61@163.com

MiR-4299, located on chromosome 11, has been shown to be dysregulated in follicular thyroid carcinoma and colon adenocarcinoma[16,17]. However, the expression, function and potential molecular mechanism of miR-4299 in other tumors remain largely unclear. Recently, a miRNA microarray showed that miR-4299 expression was significantly down-regulated in NSCLC tissues[18]. However, to our best knowledge, the biological function and clinical significance of miR-4299 has not been investigated. Our present study may provide a better understanding of the pathogenesis and development of NSCLC.

Patients and Methods

Patients and Tissue Samples

Paired NSCLC and adjacent non-cancerous lung tissues were obtained from 168 patients who received curative resection of NSCLC in Heping Hospital affiliated to Changzhi Medical College between January 2007 and December 2012. The diagnosis and histological grade of all tissues were independently confirmed by two pathologists based on WHO classification. All patients did not process chemotherapy or radiotherapy prior to surgery. Follow-up information was collected every 3 months via telephone or by mail. Related important clinical information was collected from each patient's medical records. This study was performed with the approval of the Research Ethics Committee of Heping Hospital affiliated to Changzhi Medical College. The written consent was obtained from each enrolled patient.

Cell Culture and Transfection

Four NSCLC cell lines (A549, SK-MES-1, H1299, 95-D) and normal lung cell lines (HELF) were purchased from the Cell Bank of the Institute of Biochemistry and Cell Biology, China Academy of Sciences (Xuhui, Shanghai, China). All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA). Mature miR-4299 mimics and negative control (NC) were synthesized by GenePharma (Jinan, Shandong, China). For RNA transfection, the cells were seeded into each well of 24-well plates, incubated overnight. Transfection was performed when A549 cells were grown to 80% confluence, using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNAs from NSCLC tissues and cell lines were extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the first strand cDNA was generated using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Otsu, Shiga, Japan) or miRNA cDNA Kit (CWBio, Guangzhou, Guangdong, China). qRT-PCR was performed on Applied Biosystems 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR® Premix Ex Taq™ II (TaKaRa, Otsu, Shiga, Japan). The GADPH gene was used as a reference control for miR-4299. PCR products were verified by melting curve analysis. Data analysis was performed using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in the present study were as the followings: miR-4299, 5'-GGCGCUGGUGACAUG-3' (forward), 5'-GTGCAGGGTCCGAGG-3' (reverse); GAPDH, 5'-AACTTTGGCATTGTGGAAGG-3' (forward), 5'-ACACATTGGGGGTAGGAACA-3' (reverse).

Cell Proliferation Assay

For cell proliferation analysis, 4×10^3 of the A549 cells after transfection were plated into 96-well plates. Cells were then cultured for 1, 2, 3 and 4 days, respectively. A 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) solution of 20 µl was added into 100 µl culture media and cells were incubated for a further 4 h at 37°C. The cell growth curve was constructed using the values at OD490 nm as ordinate axis.

Migration and Invasion Assays

For the transwell migration assay, 3×10^4 transfected cells were placed in the upper chamber of each insert (Corning, Corning, NY, USA). For the invasion assay, the upper chamber was coated with 150 mg Matrigel (Biosystems, Foster City, CA, USA). 500 ul of high-glucose Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10 % fetal bovine serum (FBS) were added to the lower chamber wells. The incubation time in the migration or invasion assays was 24 hours or 48 hours, respectively. After 48 h, the non-migrated cells were removed from the insert with a cotton swab. The cells migrated or invaded through the membrane were stained with 0.1% crystal violet and counted under microscope.

Western Blot Analysis

Cell lysates were prepared using RIPA buffer according to the standard method. Protein concentrations were determined by the Bradford assay. Equal amounts of total protein were loaded for electrophoresis in sodium dodecyl sulfate-polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) microporous membranes. The membranes were blocked with 5% non-fat milk for 1 h at room temperature, and were then incubated at room temperature with primary antibodies against. β-actin was used as endogenous controls. The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit IgG. Antibody against β-actin and all the secondary antibodies were obtained from Professor Zhang (Fudan University, Shanghai, China). Signals were visualized using ECL Substrates.

Statistical Analysis

All data are presented as mean values ± SD and analyzed by SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Statistical significance between groups was analyzed by Student's *t*-test or x^2-test, as appropriate. Survival curves were constructed using the Kaplan-Meier method and compared using the log-rank test. A Cox regression model was applied for the univariate analysis and multivariate analysis of prognostic factors. $p<0.05$ was considered statistically significant.

Results

MiR-4299 was Downregulated in NSCLC Tissues and Cell Lines

To evaluate the expression of miR-4299 in NSCLC, qRT-PCR was performed. As shown in Figure 1A, we found that miR-4299 expression was significantly down-regulated in NSCLC tissues compared to matched normal lung tissues ($p < 0.01$). Besides, we also detect the expression of miR-4299 in NSCLC cell lines and normal lung cell lines. As shown in Figure 1B, the results showed that miR-4299 expression in the 4 NSCLC cell lines was also markedly downregulated, compared with that of the HELF. Among the four NSCLC cell lines, miR-4299 decreased the most in A549 cell lines, thus, we chose A549 for *in vitro* experiment.

Downregulation of miR-4299 was Associated with Advanced Clinicopathological Features of NSCLC

To determine the clinical significance of miR-4299 in NSCLC, clinical formation was collected and analyzed. According to the median value of relative miR-4299 expression, the patients were categorized into low and high expression groups. As shown in Table I, we found that miR-4299 expression was positively correlated with TNM stage (p=0.002), histological grade (p=0.002) and lymph node metastasis (p=0.028). However, there were no significant correlations between miR-4299 expression and other clinicopathological features, such as age, sex, history of smoking and tumor size (p>0.05).

miR-4299 Expression was Associated with the Poor Survival of NSCLC Patients

We used Kaplan-Meier analysis and log-rank test to investigate the effects of miR-4299 expression on overall survival. As shown in Figure 1C, we observed that the patients in the low miR-4299 group had a shorter overall survival than those in the high miR-4299 group (p=0.0011).

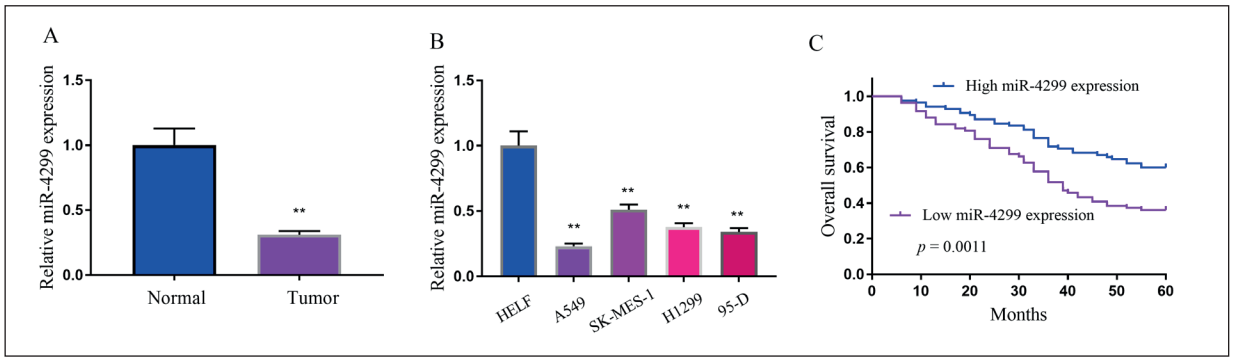

Figure 1. Down-regulation of miR-4299 expression in NSCLC samples and cell lines. **A**, RT-PCR was used to detect miR-4299 expression in human NSCLC tissues and pair-matched adjacent normal tissues. **B**, RT-PCR was used to detect miR-4299 expression in four NSCLC cell lines and normal HELF cells. **C**, Kaplan-Meier plots showed overall survival according to the level of miR-4299 in NSCLC patients. *p<0.05, **p<0.01.

Table I. Correlation between miR-4299 expression and clinicopathological characteristics of NSCLC patients.

Clinicopathological features	No. of cases	miR-4299 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.544
< 55	77	40 (51.9)	37 (48.1)	
≥ 55	91	43 (47.3)	48 (52.7)	
Sex				0.550
Male	105	50 (47.6)	55 (52.4)	
Female	63	33 (52.4)	30 (47.6)	
History of smoking				0.258
Ever	100	54 (54)	47 (47)	
Never	68	30 (44.1)	38 (55.9)	
Tumor size				0.122
≤ 3 cm	103	46 (44.7)	57 (55.3)	
> 3 cm	65	37 (56.9)	28 (43.1)	
TNM stage				0.002
I/II	107	43 (40.2)	64 (59.8)	
III/IV	61	40 (65.6)	21 (34.4)	
Histological grade				0.002
Well and moderately	110	45 (40.9)	65 (59.1)	
Poorly	58	38 (65.5)	20 (34.5)	
Lymph node metastasis				0.028
Negative	105	45 (42.9)	60 (57.1)	
Positive	63	38 (60.3)	25 (39.7)	

In order to further determine the prognostic value of miR-4299 in NSCLC patients, the Cox proportional hazard regression model was performed. As shown in Table II, the results of univariate analysis showed that TNM stage, histological grade, lymph node metastasis, and miR-4299 expression were statistically significant risk factors affecting the overall survival of patients with NSCLC. More importantly, multivariate analysis confirmed that miR-4299 expression was an independent prognostic factor for overall survival of NSCLC (p=0.008).

Ectopic Expression of miR-4299 Inhibited the Proliferation, Migration and Invasion of NSCLC cells

To explore the potential role of miR-4299 expression in NSCLC tumorigenesis, we transfected A549 cells with miR-4299 mimics, and the expression of miR-4299 was significantly up-regulated after transfection, as suggested by the qRT-PCR ($p < 0.01$, Figure 2A). The MTT assay was used to assess short-term cell viability. As shown in Figure 2B, we found that miR-4299 ectopic expression significantly inhibited the cell growth

Table II. Univariate and multivariate analyses for overall survival by Cox regression model.

Parameters	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p*	HR	95% CI	*p*
Age	1.332	0.452-1.933	0.377	—	—	—
Sex	1.569	0.633-2.328	0.292	—	—	—
History of smoking	1.783	0.894-2.558	0.167	—	—	—
Tumor size	2.132	0.694-3.453	0.093	—	—	—
TNM stage	4.563	1.478-8.669	0.001	3.232	1.184-6.673	0.005
Histological grade	3.784	1.589-6.784	0.003	3.017	1.218-5.337	0.008
Lymph node metastasis	3.329	1.273-5.673	0.016	2.673	1.026-4.778	0.033
miR-4299 expression	4.673	1.675-7.663	0.004	3.163	1.218-5.667	0.009

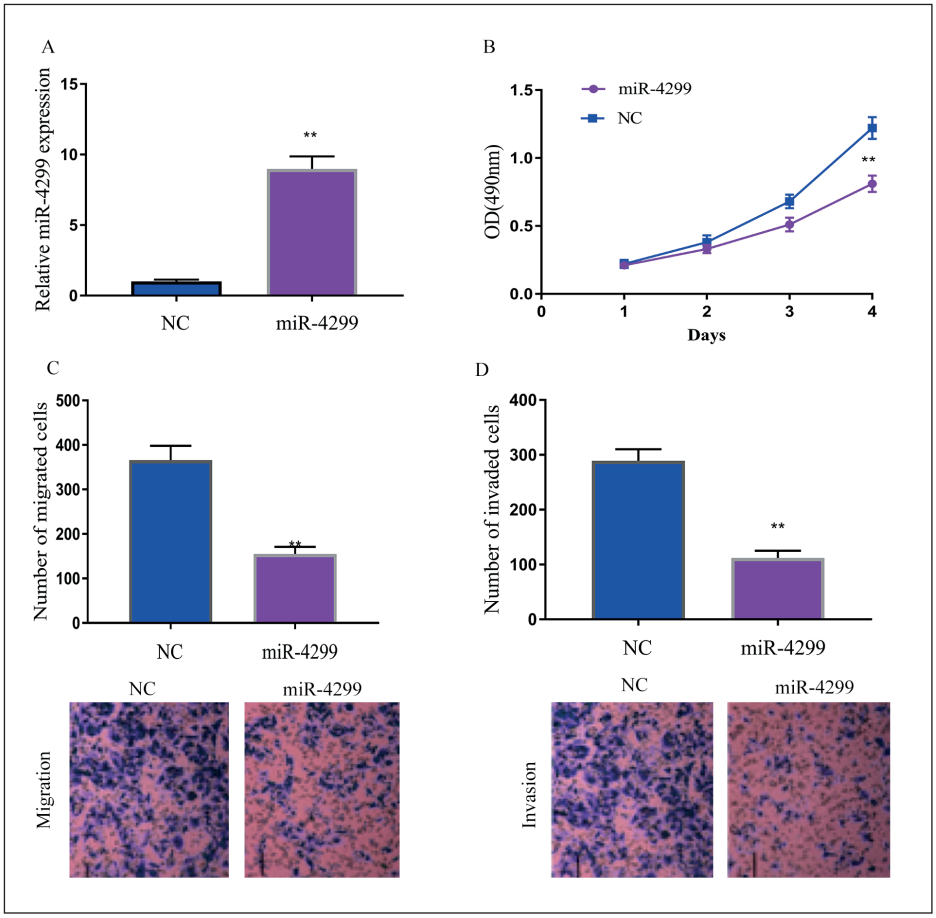

Figure 2. MiR-4299 mimics inhibited A549 cell proliferation, migration and invasion. (**A**, Expression levels of miR-4299 were determined by Real-time PCR in A549 cells transfected with miR-4299 mimics or NC. **B**, MTT assay was performed to detect the role of miR-4299 on A549 cells proliferation. **C**, Transwell analysis was used to determine effect of miR-4299 on migration in A549. **D**, Transwell analysis was used to determine effect of miR-4299 on invasion in A549. *p<0.05, **p<0.01.

rates after 3 and 4 days of transfection. Then, we analyzed the migratory and invasive capabilities of 549 cells overexpressing miR-4299 by using transwell chambers. As shown in Figure 2C, the migratory ability of A549 cells was reduced following transfection with miR-4299 mimics. Besides, we also found that overexpression of miR-4299 significantly promoted the invasion of A549 cell lines (Figure 2D). Overall, our findings revealed that miR-4299 served as a tumor suppressor in NSCLC.

MiR-4299 regulated PTEN/AKT/PI3K Signaling in NSCLC cells

In order to explore the potential mechanism by which miR-4299 modulated the proliferation, migration and invasion of NSCLC cells, our attention focused on PTEN/AKT/PI3K signaling, which has been confirmed to play an important role in tumor behavior. Then, we up-regulated miR-4299 levels to explore its role on PTEN/AKT/PI3K related proteins. We found that phosphorylated AKT and phosphorylated BSK3β were reduced and PTEN protein was increases in A549 cells transfected with miR-4299 mimics (Figure 3). Taken together, our results suggested that miR-4299 showed its tumor-suppressive role by regulating PTEN/AKT/PI3K signaling.

Discussion

Currently, the majority of NSCLC patients present advanced stages of the disease[19]. Prediction of NSCLC patients is very important for individual treatment. The currently used system to predict prognosis is based on clinicopathological parameters, which do not accurately stratify patients[20]. Thus, there is an increasing interest in the discovery of biomarkers for NSCLC, particularly those that can predict overall survival. In the present study, we firstly explored the potential of miR-4299 as a biomarker for prediction of NSCLC patients. Our results of RT-PCR indicated that miR-4299 expression was significantly down-regulated in NSCLC tissues and cell

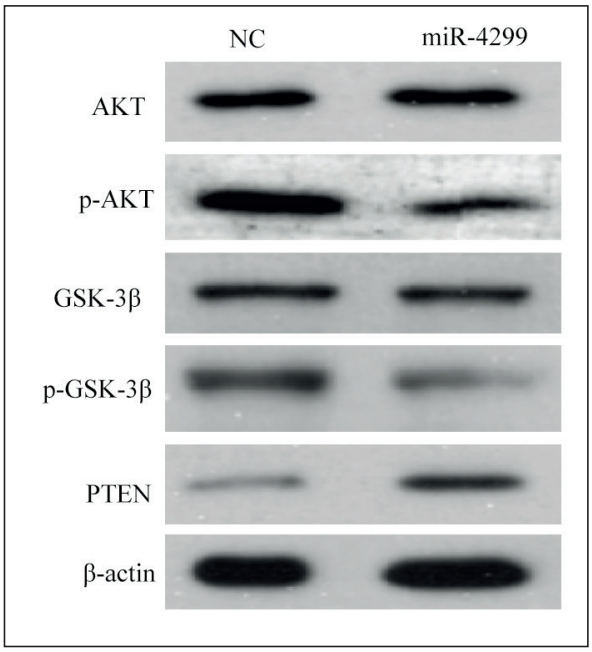

Figure 3. MiR-4299 overexpression regulated PTEN/AKT/PI3K pathway in NSCLC. Western blotting analysis was used to examine PTEN/AKT/PI3K signaling pathway related proteins.

lines. Subsequent clinical assay revealed that low miR-4299 was significantly associated with TNM stage, histological grade and lymph node metastasis, suggesting that miR-4299 may suppress clinical progression of NSCLC. Furthermore, the Kaplan-Meier survival analysis indicated that low expression of miR-4299 was significantly associated with worse prognosis. Importantly, the univariate and multivariate survival analyses showed that down-regulation of miR-4299 was an independent factor for predicting overall survival in NSCLC patients. Taken together, our findings suggested miR-4299 as a potential prognostic biomarker.

In order to explore the biological function of miR-4299 in NSCLC progression, we used miR-4299 mimics to up-regulate the expression of miR-4299 in A549 cells. Subsequently, a series of *in vitro* experiments were performed; we found that overexpression of miR-4299 could significantly suppress the proliferation, migration and invasion of A549 cells. Interestingly, the function of miR-4299 has also been reported in other tumors. Miao et al[16] reported that miR-4299 expression was lowly expressed in human follicular thyroid carcinoma and its knockdown modulated the invasive ability of human thyroid carcinoma cells by targeting ST6GALNAC4. Hu et al[17] showed that the levels of miR-4299 were associated with chemotherapy resistance in colon cancer patients, and high miR-4299 expression was significantly correlated with better survival of colon cancer patients. Both two studies indicated that miR-4299 play a negative regulator in development and progression of tumors. Our results further confirmed miR-4299 as a tumor suppressor in NSCLC. This trend was in line with previous studies.

The PI3K/AKT pathway is a signal transduction pathway involved in the regulation of several cellular functions including cell proliferation, metastasis and apoptosis[21,22]. AKT can promote cell survival through the direct phosphorylation of protein BAD[23]. A number of potential therapeutics targeting the PI3K/AKT signaling cascade have been developed[24,25]. Recently, a tumor suppressor called PTEN can dephosphorylate the phospholipid products of PI3K[26,27]. In addition, several miRNAs have been reported to be involved in the regulation of PTEN/PI3K/AKT signaling in several tumors[28-30]. In order to explore the potential mechanism by which miR-4299 exhibited its anti-cancer role in NSCLC, our attention focused on the effect of miR-4299 on PTEN/AKT/PI3K pathway. The results of Western blot showed that the up-regulation of miR-4299 modulated the activity of PTEN/ PI3K/AKT pathway. However, the detailed mechanism remained to be illustrated in further study.

Conclusions

To the best of our knowledge, we have first demonstrated that overexpression of miR-4299 acts as an unfavorable prognostic biomarker in NSCLC patients. Furthermore, miR-4299 served as an anti-oncogene by modulating PTEN/AKT/PI3K pathway.

Conflict of interest

The authors declare no conflicts of interest.

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