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Original Article

Analysis of circulating lncRNA expression serve as a fingerprint for the progression of acute coronary syndromes

Hao Chi, Xinye Liu, Jin Wang, Qian Zheng, Jingye Li, Yong li, Bin Zhang, Dongwei Yang

The Fourth Department of Cardiology, Zhengzhou Central Hospital Affiliated to Zhengzhou University, Zhengzhou 450000, Henan, China

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Abstract: Circulating long non-coding RNAs (lncRNA) can function as biomarkers for diagnosis, treatment, and prevention of diseases. However, little systematic study regarding whether lncRNAs can be used as biomarkers for the occurrence or progression of acute coronary syndrome (ACS) was reported. In this study, we aimed to screen the potential fingerprint for the occurrence or progression of ACS. In this study, we applied lncRNA microarray technology to analyze lncRNA expression in healthy controls, healthy control with three or more risk factors (hypertension, dyslipidemia, active smoker), patients with stable angina (SA), non-ST elevation ACS (NSTE-ACS), and ST-segment elevation myocardial infarction (STEMI). The candidate lncRNAs were validated by individual reverse transcription quantitative real-time PCR (RT-qPCR) arranged in the training and validation set. Three lncRNAs entitled PAX8-AS1, LINC01254 and ENSG00000254528.3 were significantly increased in patients with NSTE-ACS and STEMI compared with the other groups after the multiple stages. The areas under the receiver operating characteristic (AUC) curves of the validated three plasma lncRNAs signature in NSTE-ACS comparing with non-ACS groups were 0.869, 0.728 and 0.903 while the merged was 0.977. For the STEMI group compared with non-ACS groups, the AUC was 0.836, 0.844, 0.751 and the merged was 0.962. In conclusion, these data provide evidence that plasma lncRNAs have the potential to be sensitive, minimally invasive biomarkers for the detection of ACS, especially for NSTE-ACS and STEMI.

Keywords: Acute coronary syndromes, fingerprint, circulating, lncRNA, miRNA

Introduction

Coronary artery disease (CAD) is a leading cause of death worldwide [1, 2]. Clinical presentations of CAD include silent ischemia, stable angina pectoris, unstable angina, myocardial infarction, heart failure, and sudden death. Acute coronary syndrome [3, 4] (ACS) is not only one of the severest diseases but also an economic burden to society, costing Americans more than 150 billion dollars annually. The two subtypes of ACS are unstable angina (UA) (38%) and acute myocardial infarction (AMI), including ST-elevation myocardial infarction (30%) and non-ST-elevation myocardial infarction (25%) [5, 6]. Thus, the early diagnosis of non-ST elevation ACS (NSTE-ACS) and ST-segment elevation myocardial infarction (STEMI) is essential for improved prognoses [7].

Currently, the clinical diagnosis of ACS relies on assessment of symptoms. Additional tests have been added to these assessments including electrocardiogram, coronary computed tomographic angiography, muscle and brain fraction of creatine kinase, or blood tests such as troponin I or T. However, the current methods are insufficient for a highly sensitive and specific diagnosis, especially in distinguishing AMI from UA. It is therefore urgent to discover more effective biomarkers to precisely diagnosis the subtypes of ACS [8, 9].

Long non-coding RNAs (lncRNAs) are a group of non-coding RNAs with > 200 nucleotides [10]. Despite having no coding ability, lncRNAs are involved in several processes, including gene imprinting, chromatin remodeling, cell cycle regulation, splicing regulation, mRNA degrada-

tion and translation regulation [11]. Importantly, plasma lncRNAs directly released from primary tumors or the circulating cancer cells might provide biomarkers for human malignancies [12, 13]. LncRNAs are known to play important roles during cellular development and differentiation, and a large range of biological processes, such as modulation of tumor proliferation and invasiveness, and reprogramming of induced pluripotent stem cells [14, 15]. However, to our knowledge to date, little study has been performed regarding the circulating lncRNAs for dynamic monitoring of ACS patients.

The circulating lncRNA expression was screened through microarry in 9 patients diagnosed with NSTE-ACS, 9 patients with STEMI, 9 patients with SA, 9 healthy control with three or more risk factors (hypertension, dyslipidemia, active smoker) and 9 healthy volunteers. A risk score analysis was performed to determine the potential ability of candidate lncRNA in predicting NSTE-ACS and STEMI from SA, healthy controls with three or more risk factors, or healthy controls.

Materials and methods

Patients and study design

This study was approved by the Institutional Ethics Committee of Zhengzhou Central Hospital Affiliated to Zhengzhou University, and written informed consent was also obtained from each participant. The ACS patients' information was described below:

Patients with coronary heart disease were classified as having SA or ACS. For the ACS we enrolled NSTE-ACS and STEMI. Diagnostic criteria was referred to the ACC/AHA/AATS/PCNA/SCAI/STS Focused Update of the Guideline for the Diagnosis and Management of Patients With Stable Ischemic Heart Disease, AHA/ACC Guideline for the Management of Patients with Non-ST-Elevation Acute Coronary Syndromes, and ACCF/AHA Guideline for the Management of ST-elevation Myocardial Infarction. In addition, all patients presented stenosis (≥ 50% in at least one main coronary artery) as confirmed by coronary angiography. The healthy controls were healthy volunteers without human malignant tumor or genetic disease.

A total of 900 patients with chest pain who underwent diagnostic coronary angiography in Zhengzhou Central Hospital Affiliated to Zhengzhou University were enrolled from March 2011 to July 2017. The patients were divided into five groups as follows: 300 patients with STEMI, 300 patients NSTE-ACS, 300 patients with SA, 300 patients without CAD but with health factors and 300 healthy controls. Patients without CAD but with factors are defined as patients that did not have coronary stenosis as confirmed by coronary angiography but had three or more risk factors for coronary heart disease (hypertension, dyslipidemia, active smoker). Nine cases were randomly selected from each group for analysis of gene expression profiles. The differential expressions of lncRNAs between groups were analyzed according to the following criteria: fold-change (FC) ≥ 2 and $P < 0.05$. The study was performed according to the guidelines of the Declaration of Helsinki.

After the screening phase, we performed individual RT-qPCR in the training phase to further filter signals of the screened lncRNAs. Subsequently, we detected the number of plasmatic lncRNAs included as the NSTE-ACS and STEMI signature in a validation set including 200 samples in each group. The detailed information of patients and controls was listed in **Table 1**.

RNA isolation and RT-qPCR assay

Circulating cell-free total RNA was isolated from frozen serum samples using Trizol reagent (Invitrogen, CA, USA) and purified by the miRNeasy kit (Qiagen, CA, USA) as described before. Briefly, 3 volumes of Trizol were mixed with 1 volume of serum samples and incubated for 5 min at room temperature. Chloroform was added, and after 5 minutes at room temperature the mixture was centrifuged at 14,000 g and 4°C for 15 min. The upper aqueous phase was transferred to a fresh reagent tube and 1.5 volumes ethanol were added. The total RNA quality and concentration was determined by UV spectrophotometry. Double-stranded complementary DNA was synthesized by reverse transcription in accordance with the cDNA synthesis kit; qPCR was performed using the Script SYBR Green PCR kit (both Toyobo, Osaka, Japan). The data obtained were calculated by the $2^{-\Delta\Delta CT}$ method as described before. For the analysis of the expression levels of lncRNAs, both the internal reference (U6) and external

Table 1. Clinical information of patients enrolled in each group

Feature	STEMI	NEST-ACS	SA	NC-R	NC	*P* value
All cases	300	300	300	300	300	
Age						0.221
< 60	100	107	82	102	103	
≥ 60	200	193	218	198	197	
Gender						0.872
Male	177	180	171	169	178	
Female	123	120	129	131	122	
Risk factors						
Hypertension						< 0.001
Negative	78	82	114	80	288	
Positive	222	218	186	220	12	
Dyslipidemia						< 0.001
Negative	111	102	120	109	279	
Positive	189	198	180	191	21	
Active smoker						< 0.001
Negative	69	89	82	83	280	
Positive	231	211	218	217	20	
Medications						< 0.001
Antiplatelet agents	207	216	194	22	/	
β-Blockers	78	97	91	12	/	
CCB	48	44	39	78	/	
ACEI/ARB	91	87	98	12	/	
Statins	194	199	187	9	/	

normalization cel-miR-39 was applied for normalization.

Risk score analysis

Risk score analysis was performed to evaluate the associations between the concentrations of the lncRNA expression levels. The upper 95% reference interval of each lncRNA value in controls was set as the threshold to code the expression level of the corresponding lncRNA for each sample as 0 and 1 in the training set. A risk score function (RSF) to predict DA group was defined according to a linear combination of the expression level for each lncRNA. For example, the RSF for sample i using information from three lncRNAs was: RSFi = ∑3j-1Wj. sij. In the above equation, sij is the risk score for lncRNAj on sample i, and Wj is the weight of the risk score of lncRNAj. The risk score of three lncRNAs was calculated using the weight by the regression coefficient that was derived from the univariate logistic regression analysis of each lncRNA. Frequency tables and ROC curves were then used to evaluate the diagnostic effects of the profiling and to find the appropriate cutoff point, and to validate the procedure and cutoffs in the next validation sample set.

Statistical analysis

The lncRNA data was expressed as the mean (interquartile interval), and other variables were expressed as the mean (SD). Chi-square test analysis of variance was used to evaluate statistical differences in demographic and clinical characteristics. Statistical analysis was performed using STATA 10.0, and presented with GraphPad Prism 5.0 software. Results were considered statistically significant at $P < 0.05$.

Results

PAX8-AS1, LINC01254 and ENSG00000254528.3 were highly expressed in the plasma samples of patients with NSTE-ACS or STEMI

The differentially expressed lncRNAs were screened by microarray technology in five groups including healthy controls (NC), healthy control with three or more risk factors (hypertension, dyslipidemia, active smoker) entitled with (NC-R), patients with stable angina (SA), non-ST elevation ACS (NSTE-ACS), and STsegment elevation myocardial infarction (STEMI). As presented in **Figure 1A**, the abnormal expression landscape of lncRNAs in the five groups were observed. Significant aberrant expressed lncRNAs were filtered according to the following criteria: (i): fold-change (FC) ≥ 2 and P < 0.05; (ii): the expression density > 50% in all samples. For the fingerprint exploration for ACS, the NSTE-ACS and STEMI were labelled as ACS positive group, while the SA, NC-R and NC group were labelled as control. The Venny analysis was employed to further screen the special lncRNAs dysregulated in NSTE-ACS and STEMI group comparing with SA, NC-R and NC group. We first compared the expression of NSTE-ACS, STEMI, SA or NC-R with NC group. Four candidate lncRNAs were obtained (**Figure 1B**). The four candidate lncRNAs were further detected by RT-qPCR in a training sample set and validation set (40 samples each group and 201 samples each group). As presented in **Figure 2**, we

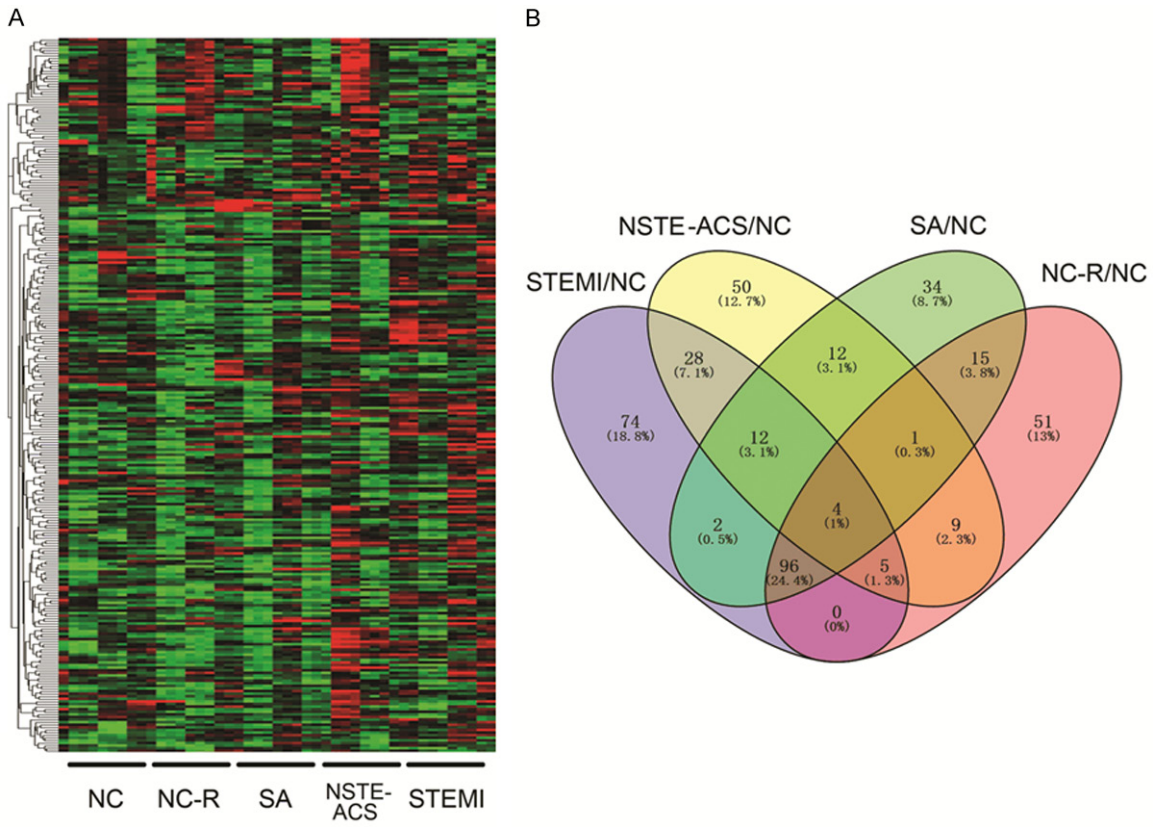

Figure 1. The microarray-based screening of lncRNAs in patients with ACS and control groups. A. Cluster analysis of the different expression of the lncRNAs. B. Venny analysis of different expressed lncRNA in NC, NC-R, SA, NSTE-ACS and STEMI. NC: healthy control; NC-R: control patients with risk factors. SA: stable angina; NSTE-ACS: non-ST elevation ACS; STEMI: ST-segment elevation myocardial infarction.

obtained that the three lncRNAs including PAX8-AS1, LINC01254 and ENSG000002545-28.3 were highly expressed in the plasma samples of patients with NSTE-ACS or STEMI comparing with either SA, NC-R or healthy controls. The other lncRNA XLOC_004924, as presented in **Figure 2**, although it might be differentially expressed in STEMI comparing with control group, or patients with SA or other risk factors; however, no difference was obtained for NSTE-ACS group. The result indicated that they might not distinguish main subgroup from ACS, thus we could not consider this lncRNA as potential fingerprint.

The three lncRNA panel might predict NSTE-ACS and STEMI from patients with SA or other risk factors

To assess the diagnostic value of the three plasma lncRNA profiling system, we used a risk score formula to calculate the risk score function for ACS samples by comparing with SA, NC-R and healthy controls, respectively. ROC curves analyses were conducted to assess the diagnostic sensitivity and specificity of the three lncRNA signatures for NSTE-ACS or STEMI groups comparing with the control groups by using risk score functions (RSFs), The areas under the curve (AUC) for NSTE-ACS were 0.869, 0.728 and 0.903 while the merged was 0.977 for the plasma samples in validation sets (**Figure 3A**). In addition, then we compared the STEMI group with the rest of the groups, we found a significant AUC of 0.836, 0.844, 0.751 and the merged was 0.962, indicating that the three lncRNAs might predict the NSTE-ACS and STEMI gropus from patients with SA or other risk factors in an early stage (**Figure 3B**).

Double-blind test for the three panel lncRNAs as fingerprints

Another 50 plasma samples were tested in a double-blind fashion to validate the predictive ability of the three lncRNA-based signatures

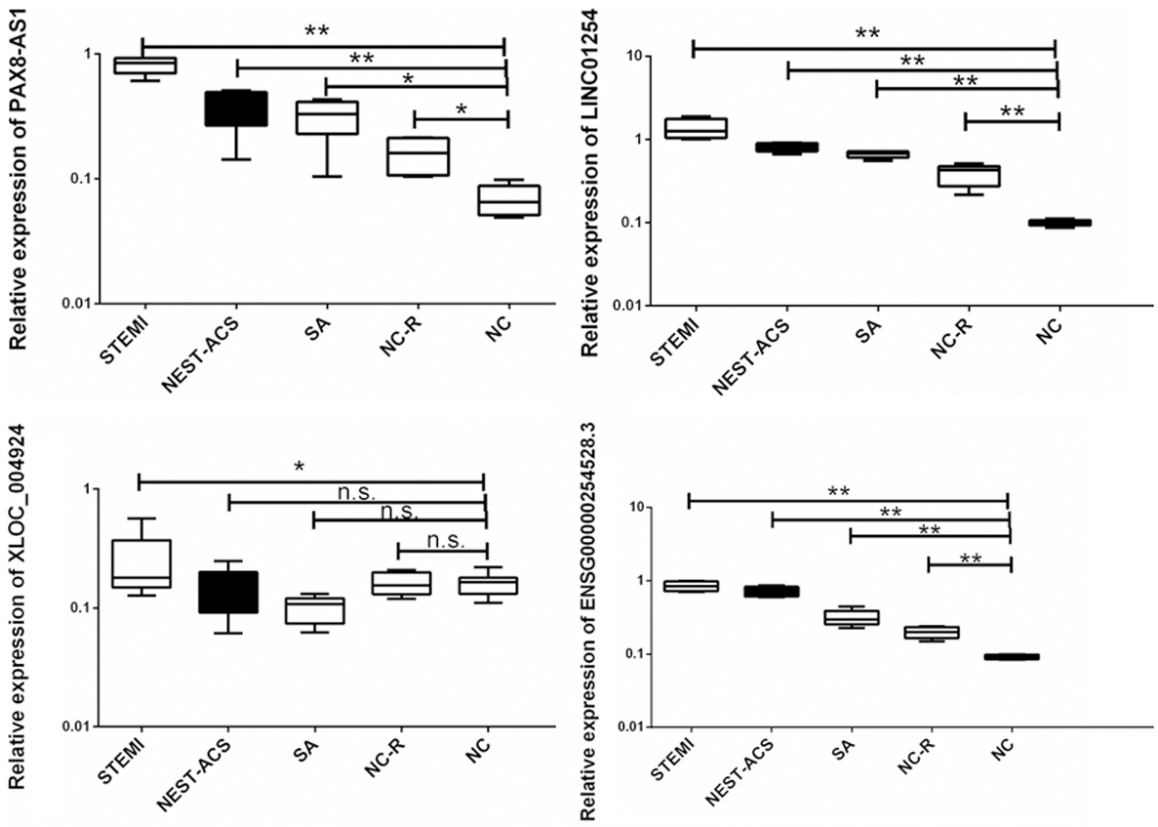

Figure 2. Different expression of PAX8-AS1, LINC01254, ENSG00000254528.3 and XLOC_004924 in each group. Increased level of PAX8-AS1, LINC01254, ENSG00000254528.3 and XLOC_004924 was confirmed by RT-PCR in groups. Data were presented as box plot of the mean with SD. *indicated $P < 0.05$, **indicated $P < 0.01$.

for ACS diagnosis. We used the same risk score formula to analyze the expression of the three lncRNAs in those plasma samples and classified them into a high-risk group and a low-risk group. On the basis of the pathological diagnosis, the accuracy rate of the three-lncRNA profile as NSTE-ACS and STEMI signature was 88.4%.

Discussion

Circulating cell-free lncRNAs biomarkers show promise as biomarkers for cancer diagnosis. However, unlike other ncRNA (e.g., microRNAs), lncRNAs with their tissue or organ specific expression and low stability in human fluid, present a major challenge in the development of cell-free lncRNAs as cancer biomarkers [16, 17]. It has been proven that lncRNAs serve a function in a number of biological processes by functioning as important regulators of gene regulation at transcriptional, posttranscriptional and epigenetic levels. With the rapid development of genome microarrays and genome sequencing technology, a type of transcription without protein encoding, originally considered to be non-RNA genome encoding transcription 'noise', has become a research focus in recent years [18, 19]. Considering the abundance, variability, function and mechanisms of lncRNAs, they may be the regulatory core of RNA, and an increasing number of studies have demonstrated that the differential expression of lncRNAs is closely associated with tumorigenesis and tumor development, which provides a new basis for understanding the mechanism underlying these processes [20, 21].

Between the years 2011 and 2013, approximately 15,000 ACS cases were reported in Malaysia, with more than 75% afflicting patients older than 50 years of age [22]. To date, the rates of myocardial infarction, readmission of patients with ACS and death remain high, underscoring the need for more aggressive health awareness, education, and non-pharmacological approaches to counter this rising prevalence of ACS as well as innovative early

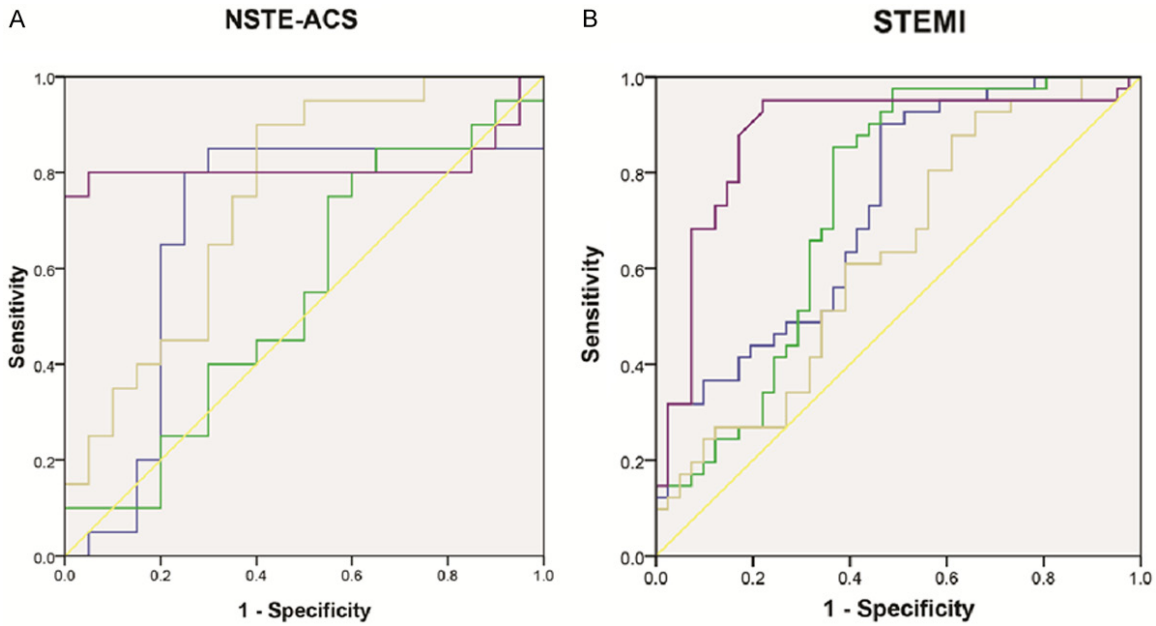

Figure 3. ROC analysis of the three potential biomarkers for NSTE-ACS and STEMI comparing with control groups.

and reliable biomarkers to facilitate rapid and more accurate diagnosis and subsequent treatment [23]. In the diagnosis of ACS, the identification and evaluation of the use of circulating lncRNAs have thus far focused on patients of higher age groups, as older people are more at risk for ACS where its prevalence is indeed higher in the elderly. Apart from age, other risk factors for ACS include smoking, diabetes, hypertension, obesity, and hypercholesterolemia. In Malaysia, approximately 23% of ACS cases involved individuals below 50 years of age and the majority of these patients were male (~80%) [24]. Here in this study, we identified the cell-free circulating lncRNA in patients with ACS and different control groups based on the high throughput technology. Through the risk score analysis, we revealed three lncRNA, PAX8-AS1, LINC01254 and ENSG00000254528.3, in both training set and validation set, might act as potential fingerprint for NSTE-ACS and STEMI.

In conclusion, we have identified unique lncRNA biomarkers for early screening of ACS which may serve as a novel non-invasive approach for diagnosis and dynamic monitoring of ACS.

Disclosure of conflict of interest

None.

Address correspondence to: Dongwei Yang, The Fourth Department of Cardiology, Zhengzhou Central Hospital Affiliated to Zhengzhou University, North Tongbai Road 16#, Zhengzhou 450000, Henan, China. Tel: 86-371-67690965; Fax: 86-371-67690965; E-mail: dongweiyang1@yeah.net

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