Original Research Article

Proteasome activator 28A: A clinical biomarker and pharmaceutical target in acute cerebral infarction therapy

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Abstract

Purpose: To determine the dynamic changes in serum levels of PA28α in patients with acute cerebral infarction (ACI), and to investigate its correlation with infarct size and neurological deficit of the disease.
Methods: A total of 100 ACI patients and 100 healthy volunteers were recruited from The First Affiliated Hospital of Xinxiang Medical University as case and control groups, respectively. Their serum levels of PA28α were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The potential of PA28α in predicting the incidence of ACI was assessed by plotting ROC curves. Multivariate logistic regression analysis was conducted to investigate the risk factors of ACI. In addition, an ACI model in rats was established, and ACI rats were classified into 1, 3, 5, 7 and 14 day subgroups based on the duration post-ACI. Rats in the sham group served as control.
Results: Serum level of PA28α was significantly higher in ACI patients than in controls. Moreover, the serum level of PA28α at admission was positively correlated to the NIHSS score and infarct volume of ACI patients. The level of PA28α in ACI rats gradually increased post-ACI, reaching a peak on day 7. The number of apoptotic brain cells in ACI rats gradually decreased after ACI. In addition, PA28α level was negatively correlated to the number of apoptotic brain cells in ACI rats ($R^2 = 0.5148$, $p < 0.001$).
Conclusion: The serum level of PA28α is elevated in ACI patients, and is positively correlated to infarct volume and neurological deficit of the disease. The dynamic change in brain cell apoptosis post-ACI is negatively correlated to the serum level of PA28α. These findings may provide theoretical basis for the diagnosis and treatment of ACI.

Keywords: Acute cerebral infarction, Proteasome activator 28A, Biomarker, Pharmaceutical target, Apoptotic brain cells

INTRODUCTION

Acute cerebral infarction (ACI) is characterized by high morbidity, disability and recurrence, which seriously threatens human health [1]. At present, there are more than 7 million stroke patients in China, involving 65 % of ischemic stroke cases [2]. Its pathogenesis has become the focus of medical research. So far, the pathogenesis of ACI has not been fully elucidated, and atherosclerosis (AS), a long-term

chronic inflammatory pathological process is generally considered as the main pathogenic factor of ACI [3].

Changes in vascular endothelial cell function, coagulation function, inflammatory factors, and some biomarkers in cerebrospinal fluid (CSF) may be attributed to the development of cerebral small vessel disease (CSVD). A high level of homocysteine in AS patients is closely related to CSVD, and causes a decline in the volume of cerebral white matter lesions, luminal infarction, and cognition [4]. The blood level of dimethylarginine is increased in CSVD patients, which correlates with the degree of white matter lesions and the occurrence of silent infarction [5].

The PA28 (proteasome activator 28) family, also known as 11S or REG, is composed of three members: PA28α, PA28β and PA28γ [6]. When observed under an electron microscope, PA28α is a cyclic particle. It binds to 20S without the assistance of ATP to form a PA28α-20S complex, which increases the binding ability of 20S core particles with various substrate peptides, and enhances the maximum response speed of protein degradation [7]. A growing number of studies have demonstrated abnormally expressed PA28 in malignant tumors [8]. Using proteomic methods, PA28α is differentially located in the nuclei of normal ovarian epithelial cells and cytoplasm of cancer cells. Therefore, PA28α may serve as a potential biological marker for ovarian cancer. The potential function of PA28α in ACI remains unclear. The aim of his study was to investigate the dynamic change of PA28α post-ACI, and its clinical significance. The findings may provide clinical references for guiding the treatment of ACI.

METHODS

Participants

A total of 100 patients with ACI who were admitted to The First Affiliated Hospital of Xinxiang Medical University were recruited for this study.

Inclusion criteria

ACI was diagnosed based on the guidelines of the *Report of the WHO Task Force on Stroke and other Cerebrovascular Disorders* [9], and the findings of the head MRI and diffusion weighted imaging. The patients were admitted within 24 h of the onset of ACI, and clinical data were completely recorded.

Exclusion criteria

(1) History of stroke; (2) Combined with liver or kidney diseases; (3) Combined with severe cardiopulmonary diseases; (4) Combined with systemic infectious diseases, malignant tumors or metabolic immune diseases, or patients with long-term use of hormonal drugs, and non-steroidal anti-inflammatory drugs or immunosuppressive drugs. During the same period, 100 healthy volunteers receiving physical examinations in this hospital were recruited to constitute the control group. They were confirmed not to have had stroke by the examination of head MRI. Written informed consent was obtained from each participant. This study was approved by the Ethics Committee of The First Affiliated Hospital of Xinxiang Medical University (18-XXMU-no.03). The study was conducted in line with the Declaration of Helsinki [10].

Subgrouping

Based on the neurological deficit classification of the National Institute of Health Stroke Scale (NIHSS) [11], ACI patients were classified into mild group (NIHSS ≤ 5, n=28), moderate group (5 < NIHSS ≤ 15, n = 40) and severe group (NIHSS > 15, n = 32). In addition, they were classified based on infarct volume: small volume group (< 5 cm^3, n=35), moderate volume group (5 cm^3 - 10 cm^3, n = 40) and large volume group (> 10 cm^3, n = 25) [12].

Serum sample collection

Peripheral blood samples (5 ml) were collected from ACI patients at admission and from healthy volunteers during physical examinations. The samples were placed in EDTA anticoagulant tubes for 2 h, and centrifuged at 3,000 rpm for 20 min (centrifugal radius = 12 cm). The supernatant was transferred to EP tubes and stored at -80 °C pending its use.

Establishment of ACI rats and subgrouping

A total of 30 male Sprague-Dawley (SD) rats, with 4 - 5 months and weighing 240 - 280 g) were provided by Experimental Animal Center of Xinxiang Medical University. This study was approved by the Animal Ethics Committee of Xinxiang Medical University Animal Center (no. 18-AN-021). All procedures were conducted in accordance with the 'Animal Research: Reporting *in vivo* Experiments guidelines 2.0' [13]. Rats were fast for 12 h, but they had a free access to water. According to previously reported method [14], the middle cerebral artery occlusion

(MCAO) was performed for establishing an ACI model in rats. In brief, rats were anesthetized by intraperitoneal administration of pentobarbital sodium (150 mg/kg). A midline incision was cut in the rat neck to isolate the right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA). A nylon thread with a diameter of < 0.25 mm at the terminal end was inserted from ECA, which passed through the middle cerebral artery (MCA) alongside ICA. The nylon thread was finally inserted in the MCA, where it was 19.0 ± 1.0 mm away from the bifurcation of CCA. The thread was fixed until there was a mild resistance to the insertion. Anal temperature of rats maintained at 37 °C using a constant temperature water bag during the procedure. Rats had free access to food and water after MCAO.

Neurological deficits of rats were assessed at the indicated time points and graded using the following criteria [15]. (1) 0 score: Both forelimbs were extended outwards, and there were no symptoms of neurological deficits; (2) 1 score: Left forelimb flexion; (3) 2 scores: The ability to resist lateral push was weakened, and forelimb flexion; (4) 3 scores: The ability to resist lateral push was weakened, forelimb flexion and circling; (5) 4 scores: Inability to walk, and loss of consciousness. Rats graded 1 - 3 scores were included and classified into 1, 3, 5, 7 and 14 d group, with 6 rats in each group. They were sacrificed 1, 3, 5, 7 and 14 days after ACI via cervical dislocation (after they were anesthetized using peritoneal administration of pentobarbital sodium at a dose of 40 mg/kg), respectively. Six rats in the sham operation group were taken as control. They were similarly operated, but the nylon thread was only inserted in CCA without the involvement of ICA. Rat brains were collected and paraffin- embedded.

Reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNAs were extracted using TRIzol, and the concentrations were measured using NanoDrop 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Using the PrimeScript™RT Master

Mix (TaKaRa, Tokyo, Japan), reversely transcribed cDNAs and further subjected to qPCR using TBGreen®Premix Ex Taq™II (TaKaRa, Tokyo, Japan) on the CFX-96 RT-qPCR system (Bio-Rad Technologies, Inc., Hercules, CA, USA). Thermal cycling conditions were 30 s at 95 °C, followed by 40 cycles for 5 s at 95 °C and 30 s at 60 °C. with GAPDH with serving as the internal reference of PA28α. The sequences of primers are shown in Table 1.

TUNEL assay

Paraffin-embedded sections of rat brains were dewaxed and dehydrated for TUNEL staining. Apoptotic cells were stained TUNEL-positive, and they were examined using a microscope for computation (20×).

Statistical analysis

SPSS statistical analysis software (version 26.0) was used for statistical processing. Normally distributed measurement data were expressed as mean ± standard deviation (SD), while enumeration data were expressed as percentage. Differences between groups were compared by independent *t*-test, while differences between groups were compared by one-way ANOVA. The ROC curves were depicted to assess the potential of PA28α in predicting the incidence of ACI. Multivariate logistic regression analysis was conducted to investigate risk factors of ACI. Pearson's correlation test was conducted to assess the correlation between PA28α level and the number of apoptotic brain cells in ACI rats. Statistical significance was set at $p < 0.05$.

RESULTS

Baseline characteristics and clinical profile of participants

Baseline characteristics and clinical data of recruited ACI patients and healthy volunteers were compared.

Table 1: Primer sequences used in PCR

Gene		Primer sequence
GAPDH (Homo)	Forward	5'-GGGAGCCAAAAGGGTCAT-3'
	Reverse	5'-GAGTCCTTCCACGATACCAA-3'
PA28α (homo)	Forward	5'-CTGATGACCAGCCTCCAC-3'
	Reverse	5'-ATTCCCTTTGTTTCTCCC-3'
GAPDH (Rattus)	Forward	5'-TCACTGGCATGGCCTTCC-3'
	Reverse	5'-GGCGGCACGTCAGATCC-3'
PA28α (Rattus)	Forward	5'-GGCCACACTGAGGGTCCAT-3'
	Reverse	5'-CACAGGTCTTCACGGAACACA-3'

There were no significant differences in the age, sex, total cholesterol, high density lipoprotein cholesterol, smoking, diabetes and hypertension history between ACI patients and controls. However, the low-density lipoprotein cholesterol level (LDL-C) was markedly higher in ACI patients than in controls ($p < 0.05$) (Table 2). It is indicated that LDL-C may influence the development of ACI.

Serum level of PA28α in ACI patients

Compared with controls, serum level of PA28α was markedly higher in ACI patients ($p < 0.05$; Figure 1 A), suggesting the potential involvement of PA28α in the development of ACI. As in depicted ROC curves, AUC of PA28α in predicting ACI was 0.9747 (sensitivity = 93 %, specificity = 91 %, cut-off value > 1.555, Youden index = 0.84) (Figure 1 B).
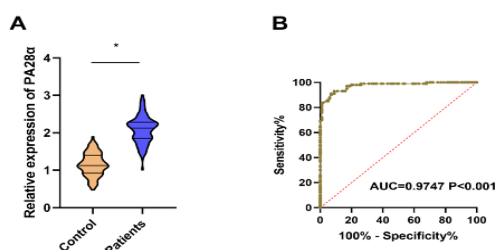

Figure 1: Serum level of PA28α in ACI patients and its clinical significance. (A) Serum level of PA28α was significantly higher in ACI patients than healthy volunteers; (B) ROC curves for identifying the prognostic potential of PA28α in ACI

PA28α is risk factor for ACI

To further investigate the potential risk factors for ACI, multivariable logistic regression model was introduced with ACI as the dependent variable, and LDL-C and PA28α level as independent variables. It is shown that LDL-C and PA28α were significantly correlated to the incidence of ACI (Table 3).

Serum level of PA28α, neurological deficit and infarct volume

Based on classification of NIHSS scores, the serum level of PA28α was higher in ACI patients with moderate and severe neurological deficits than those in the mild group, but was more pronounced in the severe group compared with that of moderate group (Figure 2 A). Consistently, serum level of PA28α was higher in ACI patients with a larger infarct volume (Figure 2 B). Thus, the serum level of PA28α was positively correlated to neurological deficits and infarct volume of ACI patients.

Dynamic change for PA28α level post-ACI

An ACI model in rats was established to further examine the function of PA28α in the development of ACI. Compared with the sham rats, the relative level of PA28α in ACI rats gradually increased a post-ACI, reaching a peak on day 7. It was gradually reduced from day 14, which was still higher than the baseline (Figure 3 A). On the other hand, the number of apoptotic brain cells in ACI rats gradually decreased after ACI, which achieved the bottom at day 7, but increased from day 14, although it was still lower than the normal level (Figure 3 B). Thus, PA28α influenced cell apoptosis after the incidence of ACI.
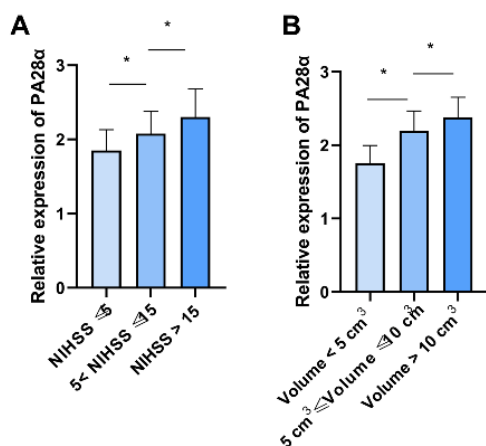
Table 2: Compare of baseline clinical data between the two groups (n = 100)

Variable	Control	Patients	t/χ²	P-value
Sex (male/female)	50/50	50/50	-	-
Age (years)	65.14±5.59	65.81±5.70	0.836	0.404
TC（mmol/L）	5.87±1.09	5.70±1.03	1.136	0.257
LDL-C（mmol/L）	3.04±0.92	3.35±0.35	3.125	0.002
HDL-C（mmol/L）	1.18±0.32	1.20±0.29	0.400	0.690
Smoking (Yes/No)	33/67	40/60	1.057	0.304
Diabetes (Yes/No)	25/75	21/79	0.452	0.502
Hypertension (Yes/No)	37/63	43/57	0.750	0.386

Note: TC: Total cholesterol; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High density lipoprotein cholesterol

Table 3: Multivariate logistic regression analysis on risk factors of acute cerebral infarction

Variable	OR	95%CI	p-value
LDL-C (mmol/L)	2.954	1.597-4.684	<0.001
PA28α	4.024	1.869-5.491	<0.001

Note: LDL-C: Low-density lipoprotein cholesterol

Figure 2: Correlation between serum level of PA28α, neurological deficit and infarct volume of ACI patients (A) Serum level of PA28α was higher in ACI patients with higher NIHSS scores (B) Serum level of PA28α was higher in ACI patients with a larger infarct volume
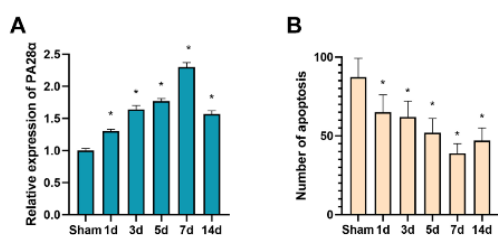

Figure 3: Dynamic change in PA28α level at post-ACI, (A) Dynamic changes in serum level of PA28α in rats at post-ACI, (B) Dynamic changes of apoptotic cell number in rats at post-ACI

Correlation between PA28α and number of apoptotic cells

Pearson's correlation test results indicate that PA28α level was negatively correlated with the number of apoptotic cells in ACI rats (R^2 = 0.5148, p < 0.001), further confirming the role of PA28α in promoting the development of ACI by influencing cell apoptosis (Figure 4).
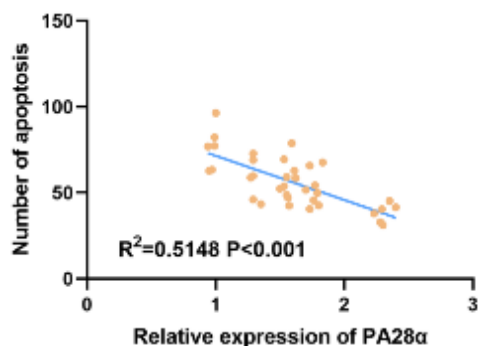

Figure 4: Correlation between PA28α and the number of apoptotic cells in ACI rats. Serum level of PA28α was negatively correlated to the number of apoptotic cells in ACI rats

DISCUSSION

Epidemiological investigations have shown that there are more than 2.5 million new onsets of stroke in China, and stroke deaths are as high as 1 million every year [16]. It has become a serious public health problem. The ACI accounts for 60-80 % of stroke cases, serving as the main subtype [17]. The ACI has a rapid onset, which triggers a series of waterfall pathophysiological cascades like inflammation, free radical damage, oxidative stress and neuronal apoptosis in a short period. It rapidly progresses, and the expanded necrotic lesions immediately result in neurological, motor and language disorders [18]. Hence, an effective assessment of the infarct volume and neurological deficits of ACI patients at admission using non-invasive clinical indices is urgently needed.

There are two main directions in the research of applying blood biomarkers in diagnosing ACI. Group biomarkers are the first research focus, which make up for the shortcomings of a single biomarker [19]. However, regression analysis, effectiveness validation in a wide range of diseases, and serological diagnosis model of group biomarkers that can be applied in clinical practice are lacking [20]. Current studies focus on the search of novel blood biomarkers for diagnoses of ACI in the early stage [21]. Some plasma biomarkers are closely related to the prognosis of ACI, including brain tissue injury, inflammation, endothelial dysfunction, coagulation and thrombosis-associated biomarkers [22]. The PA28α is abnormally expressed in multiple types of human malignant tumors, and it has been reported that PA28α is significantly upregulated in prostate cancer, which enhances the migratory capacity of cancer cells, and is considered as a potential diagnostic marker [23,24]. These results have consistently showed that the serum level of PA28α was significantly elevated in ACI patients, and was a risk factor of the incidence of ACI.

Using ROC curves in the present work, it was demonstrated that the sensitivity and specificity of PA28α in diagnosing ACI were 93 and 91 %, respectively (cut-off value > 1.555, Youden index = 0.84). Moreover, a high level of PA28α indicated larger infarct volume and higher NIHSS scores, suggesting that PA28α was positively correlated to the severity of ACI. To further investigate the role of PA28α in influencing the development of ACI, an ACI model in rats was successfully established. With the prolongation of ACI, PA28α was gradually upregulated and achieved peak on day 7, but declined from day 14. Besides, the number of apoptotic brain cells

of ACI rats showed a declining trend and reached the lowest level on day 7, but was elevated from day 14. Thus, PA28α inhibited cell apoptosis in ACI rats, thereby mediating the development of ACI.

Overall, the serum level of PA28α increases in ACI patients, which is a risk factor of ACI, by regulating cell apoptosis. thereforePA28α may serve as an appliable biomarker for predicting the incidence of ACI.

CONCLUSION

The serum level of PA28α significantly increases in ACI patients, and is positively correlated to infarct volume and severity of the disease. The dynamic change of brain cell apoptosis at post-ACI is negatively correlated to serum level of PA28α. The results of this study may provide new strategies for the diagnosis and treatment of ACI.

DECLARATIONS

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None provided.

Ethical approval

This study was approved by the Ethics Committee of The First Affiliated Hospital of Xinxiang Medical University (18-XXMU-no.03).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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