Original Article
MicroRNA-146a expression in the peripheral blood of Alzheimer's disease patients and its correlation with disease severity

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Abstract: Objective: The study aimed to explore the expression of microRNA-146a (miRNA-146a) in the peripheral blood of patients with Alzheimer's disease (AD) and its correlation with disease severity. Methods: We recruited 122 AD patients (AD group) who were further divided into mild, moderate, and severe subgroups according to the Mini-Mental State Examination (MMSE) score and 122 healthy people (control group) during the period of January 2017 to January 2019. The levels of miRNA-146a from peripheral blood, Toll-like receptor 4 (TLR4) on peripheral blood monocytes, and C-reactive protein (CRP) and procalcitonin (PCT) in serum were compared between the groups and among the subgroups. The correlations among miRNA-146a, TLR4, CRP, and PCT levels and MMSE scores were analyzed among the three subgroups. Moreover, receiver operating characteristic curve analysis was used to assess the diagnostic value of the above indicators in AD. Results: Compared with the control group, miRNA-146a, TLR4, CRP and PCT levels increased significantly in the AD group (P<0.05), and also rose markedly as the disease progressed (P<0.05). According to the correlation analysis, significant negative correlations existed between miRNA-146a, TLR4, CRP and PCT levels and MMSE scores (P<0.05), while a positive correlation existed between levels of miRNA-146a and TLR4 (P<0.05). Furthermore, the receiver operating characteristic curve analysis demonstrated that miRNA-146a and TLR4 are of great clinical value (AUC>0.90), and CRP and PCT are of certain clinical value for screening of AD (AUC>0.70). Conclusion: MiRNA-146a, which may participate in the pathogenesis of AD via TLR signaling pathway, is closely correlated with TLR4. In the diagnosis of AD, miRNA-146a and TLR4 are diagnostically significant.

Keywords: Alzheimer's disease, microRNA-146a, Toll-like receptor 4

Introduction

Alzheimer's disease (AD) is a common irreversible neurodegenerative disorder, mainly characterized by progressive decline in global mental and intellectual function, associated with personality changes as well as behavioral abnormalities [1]. Approximately 47 million people are reported to suffer from AD worldwide. In 2050, the number is expected to reach 130 million. Due to the large population base and accelerated aging process in China, the number of AD patients in this country ranks first, globally [2, 3]. Clinically, there are many ways to diagnose AD. However, the primary one is mainly based on clinical manifestations, psychological evaluation and medical history. The reason

for this is that positron emission tomography (PET) is an expensive technique and biomarkers such as Tau and extracellular amyloid-β (Aβ) have not been widely used for early detection. As a result, some AD patients do not receive an accurate diagnosis and proper treatment in the early stages. In the traditional views, the main pathological features of AD are the deposition of Aβ, loss of neurons and synapses, intracellular neurofibrillary tangles and abnormal neuronal cell death [4, 5]. However, recent studies have unveiled that the inflammatory response of the central nervous system plays a critical role in the pathogenesis of AD, which provides new insights for researchers to explore more effective therapeutic measures for the pathogenesis of AD.

Toll-like receptors (TLRs), a class of transmembrane proteins, can recognize several pathogen-associated molecular patterns and induce inflammatory immune responses in the body [6]. A previous study showed that TLR was significantly increased in the brain tissue and peripheral blood of AD patients, suggesting that TLR may be involved in the pathogenesis of AD [7]. Besides, microRNAs (miRNAs) are a class of highly conserved, non-coding single-stranded small RNAs made up of 18 to 25 nucleotides in length, which are involved in various physiological processes of the body. It has been reported that MiRNA-146a is abundantly expressed in various tissues and organs of the nervous system, can cross the blood-brain barrier in an exocrine manner so as to be released into the peripheral blood and it can be stabilized in the peripheral blood by combining with proteins [8, 9]. Additionally, miRNA-146 may be involved in the immune-inflammatory mechanism of AD through the TLR signaling pathway [10]. Based on these research findings, we herein investigated the expression levels of miRNA-146a and TLR4 in the peripheral blood of AD patients, and their correlations with disease severity to provide a relevant basis for the clinical diagnosis and treatment of AD.

Materials and methods

General data

One hundred and twenty-two AD patients admitted to Shaoxing 7th People's Hospital from January 2017 to January 2019 were selected as the AD group, and 122 healthy people matched in age and sex were enrolled as the control group during the same time period. There were 69 males and 53 females with a mean age of 73.5±5.8 years in the AD group, and 65 males and 57 females with a mean age of 73.8±5.1 years in the control group. No statistically significant differences were found in age and sex ratio between the two groups (P>0.05). Written informed consent was obtained from all patients and their families and ethical approval for the study was given by the Ethics Committee of Shaoxing 7th People's Hospital.

Inclusion and exclusion criteria

The included patients were diagnosed with AD according to the criteria issued by the National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) [11]. All patients presented with the manifestations of dementia with insidious onset and clear history of cognitive impairment that was evaluated by self-report or direct observation, and their initial and most prominent cognitive dysfunctions were evident on medical history and physical examination in both amnestic and non-amnestic presentations.

In addition, patients with other types of dementia or cognitive impairment, liver and kidney diseases, anemia, infectious diseases, and hyperthyroidism were all excluded. Those with the long-term use of addictive or analgesic drugs and diseases that may cause inflammatory reactions (e.g., colds, pneumonia, arthritis) were also excluded.

Methods

All subjects were evaluated using the Mini-Mental State Examination (MMSE) before enrollment under the guidance of physicians who had unified and formal training in this neuropsychological scale [12]. The scale measured cognitive abilities such as memory, recall, orientation, language, attention and calculation, with a total score of 30 points. A low score indicates a high level of mental impairment. The score of ≥27 indicated normal cognitive functions.

According to the MMSE score, the patients in the AD group were further divided into three subgroups, namely, the mild group (20-26 points) with 42 cases, the moderate group (10-19 points) with 49 cases, and the severe group (0-9 points) with 31 cases.

Outcome measures

Fasting venous blood samples were drawn from each subject into three 5 mL tubes in the next morning after enrollment. A tube of blood was used to isolate monocytes and detect the expression of TLR4 on their surface using flow cytometry (CytoFLEX FCM flow cytometer, Beckman Coulter, Inc., USA). Another sample of blood was centrifuged at 3000 r/min for 5 min to separate serum, followed by measurement of PCT levels using an automated electrochemiluminescence immunoassay (Roche COBAS

Table 1. Primer sequences

Primer	Sequence (5'-3')
MiR-146a (Upstream)	GTCGTATCCAGTGCAGGGTCCGAGGT
MiR-146a (Downstream)	GCACTGGATACGACAACCCA
β-actin (Upstream)	ACTCTTCCAGCCTTCCTTCC
β-actin (Downstream)	CGTACAGGTCTTTGCGGATG

E601 analyzer, Roche Diagnostics International Ltd., Switzerland) and determination of CRP levels using an immunoturbidimetric assay (Roche/Hitachi cobasc501 analyzer, Roche Diagnostics International Ltd., Switzerland). The last tube of blood was used to extract RNA from the peripheral blood using an RNA extraction kit. Then, reverse transcription reaction and reverse transcription polymerase chain reaction (RT-PCR) were performed according to the instructions of the reverse transcription kit and SYBR RT-PCR kit. The initial denaturation was carried out at 95°C for 30 s, followed by 35 cycles of 95°C for 5 s and 60°C for 30 s. Subsequently, the relative expression of miR-146a in each group was determined by the $2^{-\Delta\Delta Ct}$ method, with β-actin as the internal reference. Each measurement was repeated three times and then the results were averaged. Primer sequences are shown in **Table 1**.

Statistical analysis

Data analyses were performed with the SPSS 22.0 software. The measurement data were expressed as mean ± standard deviation ($\overline{x}$ ± sd). One-way analysis of variance was used for multigroup comparison, and the Bonferroni method was adopted for inter-group comparison. Chi-square test (χ^2 test) was adopted for the comparison of enumeration data expressed as the percentage or case (n). Moreover, the Pearson correlation was applied to analyze the correlations among miRNA-146a, TLR4, CRP and PCT levels and MMSE scores in the AD group. P<0.05 was considered statistically different. Receiver operating characteristic (ROC) curve analysis was used to assess the clinical value of miRNA-146a, TLR4, CRP and PCT in diagnosing AD.

Results

Comparison of miRNA-146a, TLR4, CRP, and PCT levels and MMSE scores between the two groups

In the AD group, the levels of miRNA-146a, TLR4, CRP, and PCT increased markedly (P<

0.001), while the MMSE score decreased significantly (P<0.001), as compared with the control group. See **Table 2**.

Comparison of miRNA-146a, TLR4, CRP, and PCT levels and MMSE scores among the three subgroups

As AD aggravated, the levels of miRNA-146a, TLR4, CRP and PCT increased markedly (P<0.05), while the MMSE score decreased significantly (P<0.05) in the AD group, as compared with the control group. See **Table 3**.

Correlations among miRNA-146a, TLR4, CRP, and PCT levels and MMSE scores in AD patients

The correlation analysis demonstrated that there was a significant negative correlation between miRNA-146a, TLR4, CRP and PCT levels and MMSE scores (P<0.05), while a positive correlation between levels of miRNA-146a and TLR4 (P<0.05). See **Table 4**.

ROC curves for miRNA-146a, TLR4, CRP, and PCT levels

According to ROC curve analysis, miRNA-146a and TLR4 are of great diagnostic value (AUC>0.90), and CRP and PCT of certain value in diagnosing AD (AUC>0.70). See **Table 5** and **Figure 1**.

Discussion

Currently in China, early screening for AD is based mainly on the MMSE scale. However, patients screened by the MMSE scale often have already developed moderate-to-severe AD with cognitive decline [13]. Besides, considering the 50% incidence of AD type pathology in patients with mild cognitive impairment, exploring biomarkers is essential in the diagnosis of AD.

MiRNA-146a, a member of the miR-146 family, is abundantly expressed in the brain. Studies have unveiled that miRNA146a expression is increased significantly in the human neocortex and limbic system and positively associated with disease severity [14, 15]. The *in vitro* experiments reported by Zhang et al. further revealed that low-density lipoprotein receptor-related protein-2 (LRP2) expression was

Table 2. Comparison of miRNA-146a, TLR4, CRP, and PCT levels and MMSE scores

Group	MiRNA-146a	TLR4 (%)	CRP (mg/L)	PCT (µg/L)	MMSE score (point)
Control Group (n=122)	18.02±3.79	56.15±8.33	5.12±2.62	0.45±0.35	27.44±0.91
AD Group (n=122)	27.33±4.05	75.48±11.17	10.74±4.29	1.01±0.59	15.51±8.05
t	18.554	15.319	12.33	8.98	16.256
P	<0.001	<0.001	<0.001	<0.001	<0.001

Note: AD: Alzheimer's disease; TLR4: Toll-like receptor 4; CRP: C-reactive protein; PCT: procalcitonin; MMSE: Mini-Mental State Examination.

Table 3. Comparison of miRNA-146a, TLR4, CRP, and PCT levels and MMSE scores among the three subgroups

Subgroup	MiRNA-146a	TLR4 (%)	CRP (mg/L)	PCT (µg/L)	MMSE score (point)
Mild group (n=42)	22.48±3.31	69.95±7.98	8.67±3.12	0.62±0.24	22.42±2.27
Moderate group (n=49)	27.98±3.89[a]	76.32±8.19[a]	10.78±4.31[a]	1.07±0.44[a]	15.17±3.32[a]
Severe group (n=31)	31.19±4.42[a,b]	84.13±8.78[a,b]	13.47±4.55[a,b]	1.49±0.58[a,b]	7.84±1.07[a,b]
F	48.914	26.23	12.794	37.457	293.723
P	<0.001	<0.001	<0.001	<0.001	<0.001

Note: Compared with the mild group, [a]P<0.05; Compared with the moderate group, [b]P<0.05. MiRNA-146a: microRNA-146a; TLR4: Toll-like receptor 4; CRP: C-reactive protein; PCT: procalcitonin; MMSE: Mini-Mental State Examination.

Table 4. Correlations among miRNA-146a, TLR4, CRP, and PCT levels and MMSE scores in AD patients

Variables	MiRNA-146a	TLR4 (%)	CRP (mg/L)	PCT (µg/L)	MMSE score (point)
MiRNA-146a		0.344[*]	0.187	0.122	-0.498[*]
TLR4	0.344[*]		0.105	0.114	-0.513[*]
CRP (mg/L)	0.187	0.105		0.123	-0.336[*]
PCT (µg/L)	0.122	0.114	0.123		-0.219[*]
MMSE score (point)	-0.498[*]	-0.513[*]	-0.336[*]	-0.219[*]	

Note: Compared with the control group, [*]P<0.05. MiRNA-146a: microRNA-146a; TLR4: Toll-like receptor 4; CRP: C-reactive protein; PCT: procalcitonin; MMSE: Mini-Mental State Examination.

Table 5. ROC curves for miRNA-146a, TLR4, CRP, and PCT levels

Variables	Cut off value	AUC	95% CI	Sensitivity	Specificity
MiRNA-146a	22.721	0.946	0.919, 0973	0.885	0.893
TLR4 (%)	66.906	0.902	0.858, 0.947	0.861	0.893
CRP (mg/L)	5.919	0.882	0.838, 0.926	0.803	0.820
PCT (µg/L)	0.491	0.829	0.775, 0.882	0.795	0.811

Note: MiRNA-146a: microRNA-146a; ROC: receiver operating characteristic; AUC: area under curve; CI: confidence interval; TLR4: Toll-like receptor 4; CRP: C-reactive protein; PCT: procalcitonin.

decreased in AD cell models. The suppression of LRP2 can enhance Aβ1-42-induced toxicity in cells and cause apoptosis, while overexpression of LRP2 attenuates the toxicity in cells and reduces apoptosis. By inhibiting LRP2 expression, overexpression of miRNA-146a failed to stimulate Akt phosphorylation, induced caspase-3 activity, and thus resulted in apoptosis, suggesting that miRNA-146a may be involved in the pathogenesis of AD via the LRP2/Akt signaling pathway [9]. Furthermore, the Rho-associated, coiled-coil containing protein kinase 1 (ROCK1) is a target gene of microRNA-146a. Wang et al. identified that overexpression of miRNA-146a could inhibit ROCK1 protein levels and induce abnormal Tau hyperphosphorylation. In a mouse model, a specific inhibitor of miRNA-146a was used for intervention, and the results showed that ROCK1 protein levels significantly increased while Tau phosphorylation reduced in the hippocampus. According to the mouse behavior, there was also an improvement in the recovery
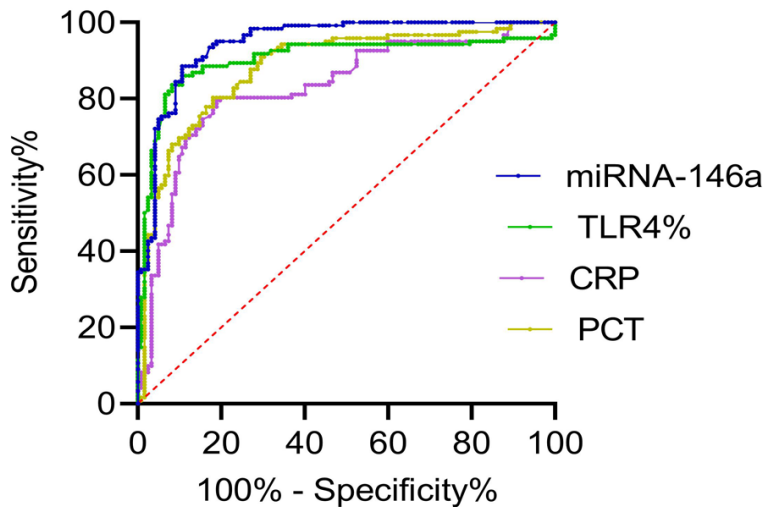
Figure 1. ROC curves for miRNA-146a, TLR4, CRP, and PCT levels. MiRNA-146a: microRNA-146a; ROC: receiver operating characteristic; TLR4: Toll-like receptor 4; CRP: C-reactive protein; PCT: procalcitonin.

ease severity. In addition, we found that TLR4 had great clinical value in diagnosing AD if the cut off value was 66.906% (AUC=0.902). Recently, evidence supporting miRNAs as key regulators in TLRs signaling pathway has emerged [20-22]. Taganov et al. first reported that miRNA-146a was closely linked to innate immunity as miRNA-146a was overexpressed in the human monocytic THP-1 cell line after lipopolysaccharide-induced activation of the TLR4 signaling pathway [10]. Our study showed that miRNA-146a expression levels in the peripheral blood of AD patients were positively correlated to TLR4 expression levels, suggesting that miRNA-146a may be involved in the development and progression of AD via the TLR signaling pathway.

of memory function [16]. In this study, we found that miRNA-146a expression in the peripheral blood of AD patients was significantly increased compared with the normal population and it was positively associated with disease severity, indicating that miRNA-146a plays a critical role in the development and progression of AD. Moreover, the ROC curve results demonstrated that the AUC of miRNA-146a for AD was 0.946 if the cut off value was 22.721, confirming that miRNA-146a has great diagnostic value.

Toll-like receptors (TLRs), type I transmembrane receptors, are widely distributed in nerve cells, and are considered as the only class of transmembrane proteins in mammals that can transmit extracellular information to cells and induce an inflammatory response [17]. As the first and most widely studied class of receptors in the Toll-like receptor family, TLR4 can respond to a variety of exogenous or endogenous ligands, inducing innate immune and inflammatory responses [18]. It was identified that TLR4 was closely related to the clinical course of AD [19]. The TLR4-dependent signaling pathway can activate microglia, regulate the expression of inflammatory factors, cause Aβ deposition and neuronal degeneration, and thus participate in the pathogenesis of AD. Our study revealed that the TLR4 expression levels on monocytes from peripheral blood in AD patients were significantly increased compared with the control group and positively correlated with dis-

In clinical practice, PCT and CRP are both important indicators of common infectious diseases or inflammatory reactions, which can provide a valid basis for the diagnosis and treatment of inflammatory or infectious diseases [23, 24]. In this study, the CRP and PCT levels used as evaluation indicators in AD patients were increased compared with the normal population. As the disease progressed, the levels were also increased. The results of ROC curve illustrated that CRP and PCT levels have certain clinical value in the diagnosis of AD, while the cutoff values were almost identical with the healthy people's, so further studies are desirable.

In summary, miRNA-146a, which may be involved in the development and progression of AD through the TLR signaling pathway, is closely associated with TLR4. In the diagnosis of AD, miRNA-146a and TLR4 are of great clinical value. However, the sample size in our study is small, therefore, we will use larger sample sizes to get a more precise conclusion in the future.

Disclosure of conflict of interest

None.

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