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Serum expression of EAAT2 and ADORA2A in patients with different degrees of Alzheimer's disease

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Abstract. – OBJECTIVE: This study aimed to explore the correlation between serum EAAT2 and ADORA2A levels and Alzheimer's disease (AD).

PATIENTS AND METHODS: A total of 68 patients with AD treated in our hospital from April 2017 to January 2019 were enrolled and assigned to group A, and 60 healthy individuals undergoing physical examinations in the same period were enrolled and assigned to group B. Enzyme-linked immunosorbent assay (ELISA) was used to measure the expression of serum EAAT2 and ADORA2A in the two groups, receiver operating characteristic (ROC) curve to assess the predictive value of diagnostic efficacy, Spearman correlation to perform correlation analysis, and multivariate logistic analysis to analyze risk factors of prognosis.

RESULTS: Patients from group A showed significantly higher serum ADORA2A level and lower serum EAAT2 level than individuals from group B (all $p<0.001$). The severity of AD was negatively correlated with the relative expression of serum EAAT2 (r=-0.7286, $p<0.001$), positively correlated with the relative expression of serum ADORA2A (r=0.7381, $p<0.001$). The sensitivity, specificity, and area under the curve (AUC) of EAAT2 alone for the diagnosis of AD were 85.00%, 82.35%, and 0.8853, respectively, and those of ADORA2A alone for the diagnosis of AD were 71.67%, 79.41.00%, and 0.8369, respectively. Univariate and multivariate Logistic regression analysis showed that disease severity, EAAT2, and ADORA2A were independent risk factors of the prognosis of AD.

CONCLUSIONS: Patients with AD have highly expressed ADORA2A and lowly expressed EAAT2 in the serum. EAAT2 and ADORA2A may play parts in the progression of AD, and they can act as potential serum biomarkers for the diagnosis and disease assessment of AD.

Key Words:
Hippocampus, EAAT2, ADORA2A, Alzheimer's disease.

Introduction

Alzheimer's disease (AD) is quite common in elderly patients with neurological diseases[1]. The aging population and changes of living habits give rise to an increase in the global incidence of AD, which seriously threatens the health of the elderly. Recently, AD has seen a spurt in its mortality to 55%, and it is among the list of diseases which cost the most for the treatment[2,3]. AD not only causes inconvenience to patients' lives, but also brings a heavy financial burden on patients and their families[4-6]. Patients with AD often miss timely treatment due to the absence of clear clinical symptoms of early AD and its complex pathogenesis, thereby facing a poor prognosis. Therefore, identifying the risk factors of AD is crucial for the prevention and treatment of AD[7-9]. Changes in neurotransmitters in the central nervous system of patients with AD can induce lesions of peripheral nerves and other complications[10]. Excitatory amino acid transporter 2 (EAAT2) can regulate peripheral nerve excitability[11,12]. In-depth studies of AD have found that the increase in the expression of EAAT2, a member of the glutamate family, can exacerbate related neurodegenerative diseases. ADORA2A, an important member of the adenosine signaling pathway that is closely related to the learning and memory function of the brain, can induce neurodegenerative diseases if its concentration is in disorder[13,14]. So far, little has been known about the relationship between EAAT2 and ADORA2A expression and the clinicopathological characteristics and disease severity of AD patients. This study aimed to investigate the expression and clinical significance of EAAT2 and ADORA2A in the serum of patients with AD.

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Patients and Methods

Basic Information

A total of 68 patients with AD treated in our hospital from April 2017 to January 2019 were enrolled and assigned to group A, and 60 healthy individuals undergoing physical examinations in the same period were enrolled and assigned to group B. The inclusion criteria of the study: patients diagnosed with AD according to the criteria for AD in the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria in the International Classification of Diseases of the World Health Organization (WHO)[15], patients with normal liver and kidney function, patients without other progressive memory and cognitive impairment, and patients without other malignant tumors. Exclusion criteria: patients previously treated by chemotherapy, immunotherapy, or radiation therapy before this study. This investigation was carried out with permission from the Ethics Committee of our Hospital and informed consent obtained from all participants and their families.

Reagents and Methods

Main Reagents and Instruments

EAAT2 and ADORA2A ELISA detection kits were purchased from Shanghai Hengfei Biotechnology Co., Ltd. The centrifuge machine was purchased from Hunan Pingfan Science and Technology Co., Ltd. Automatic Plate Washer was manufactured by Nanjing DeTie Laboratory Equipment Co., Ltd. Microplate analyzer was purchased from Shanghai LNB Instrument Co., Ltd.

Outcome Measures

The serum isolated from venous blood was stored in a refrigerator at -4°C. The expression of serum EAAT2 and ADORA2A was determined by enzyme-linked immunosorbent assay (ELISA).

Detection Method

The venous blood sample was centrifuged at 3500 r/min to isolate the serum. The concentrations of serum EAAT2 and ADORA2A were detected by ELISA in accordance with the instructions of kits. The kits and samples were transferred out from the refrigerator 30 minutes before the determination to make them return to the room temperature. We set a sample hole (100 µl of the sample), a standard hole (100 µl of the standard), and a blank hole (50 µl of the sample dilution solution). The microtiter plate was coated and incubated at 37°C for 2 hours. The solutions were removed and each well was dried. Working solution A (100 µl) was added to each well and the coated plate was incubated at 37°C for 1 hour. Then, the solutions were discarded, and the wells were dried, followed by 3 times of washing in the automatic plate washer. Working solution B (100 µl) was added to each well and the coated plate was incubated at 37°C for 1 hour. The solutions were removed and the wells were dried before being washed 3 times. Substrate solution (90 µl) was dispensed to each well and the coated plate was placed in the dark at room temperature for 20 minutes. After that, 50 µl stop solution was added to each well. Finally, the optical density at 450 nm of each well was detected by a microplate reader and the concentrations of EAAT2 and ADORA2A were calculated.

Statistical Analysis

Statistical analysis was carried out on SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The count data were compared between the two groups by the X^2 test. The measurement data were expressed as the ($x \pm s$), and compared between the two groups by the independent sample *t*-test. ROC curve was drawn to evaluate the value of serum EAAT2 and ADORA2A for the diagnosis of AD. A logistic regression model was established with EAAT2 and ADORA2A as independent variables. The probability value in the model was used to fit the area under the ROC curve. $p<0.05$ indicates a significant difference.

Results

Basic Information

The two groups of participants were not statistically different in age, sex, smoking, drinking, medical history, registered residence, and educational background (all $p>0.05$; Table I).

EAAT2 Expression in Groups A and B

According to ELISA results, the expression of serum EAAT2 in group A and group B was (3.20 ± 1.50) µg/ml and (5.40 ± 1.50) µg/ml, re-

Table I. Basic information of subjects [n (%)] ($\bar{x} \pm$ SD).

Group	N	EAAT2	*t/F*	*p*
Age (year)			0.443	0.506
≤ 67	30 (44.12)	30 (50.00)		
> 67	38 (55.88)	30 (50.00)		
Sex			0.142	0.707
Male	34 (50.00)	28 (46.67)		
Female	34 (50.00)	32 (53.33)		
BMI (kg/m^2)	19.23 ± 1.04	19.44 ± 1.00	1.161	0.248
Place of residence			0.280	0.596
Urban area	36 (52.94)	34 (57.63)		
Rural area	32 (47.06)	25 (42.37)		
Ethnicity			2.906	0.148
Han nationality	55 (80.88)	54 (90.00)		
Minority nationality	13 (19.12)	6 (10.00)		
Educational background			2.312	0.128
Bachelor degree or above	49 (72.06)	50 (83.33)		
Below bachelor degree	19 (27.94)	10 (16.67)		
Smoking			0.896	0.344
Yes	25 (36.76)	27 (45.00)		
No	43 (63.24)	33 (55.00)		
Drinking			0.630	0.427
Yes	27 (39.71)	28 (46.67)		
No	41 (60.29)	32 (53.33)		
Diabetes mellitus			1.062	0.303
Yes	16 (23.53)	19 (31.67)		
No	52 (76.47)	41 (68.33)		
Severity			-	-
Mild	20 (29.41)	-		
Moderate	20 (29.41)	-		
Severe	28 (41.18)	-		

spectively; the expression of serum ADORA2A in group A and group B was (10.20 ± 2.50) μg/ml and (5.80 ± 1.30) μg/ml, respectively. Patients from group A showed significantly higher serum ADORA2A level and lower serum EAAT2 level than participants from group B (all p<0.001). More details are shown in Figure 1 (A and B).

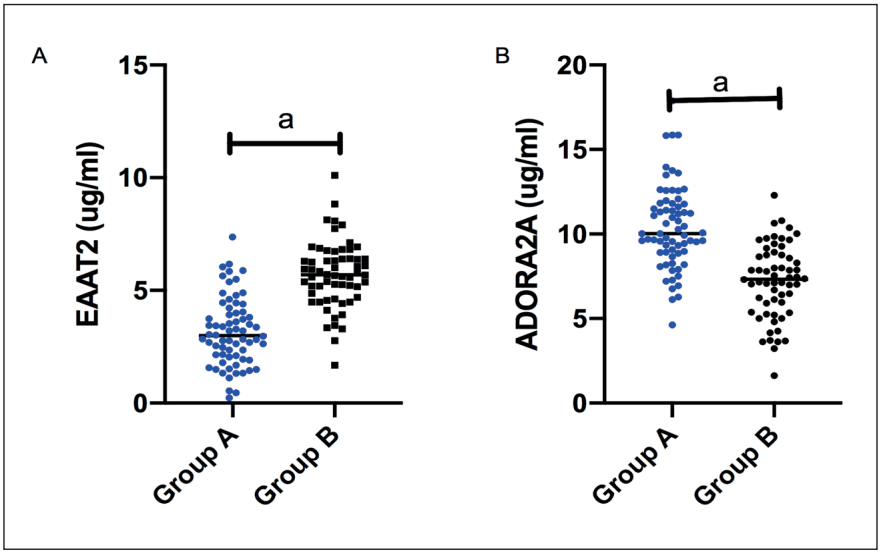

Figure 1. Comparison of the relative expression levels of serum EAAT2 and ADORA2A. **A,** Serum EAAT2 in group A was significantly lower than that in group B (p<0.001). **B,** Serum ADORA2A in group A was significantly higher than that in group B (p<0.001). Note: "a" indicates p<0.001.

Table II. The relationship between EAAT2 expression and the clinicopathological characteristics of AD.

Group	N	EAAT2	*t/F*	*p*
Age (year)			0.030	0.987
≤ 67	30	3.21 ± 1.40		
> 67	38	3.20 ± 1.35		
Sex			0.138	0.891
Male	34	3.15 ± 1.50		
Female	34	3.20 ± 1.50		
ADL (higher scores indicate better situation)			6.453	< 0.001
< 50	36	2.03 ± 1.10		
> 50	32	4.30 ± 1.76		
MMSE (higher scores indicate better situation)			7.134	< 0.001
< 20	35	2.00 ± 1.00		
> 20	33	4.50 ± 1.80		
ADAS-cog (lower scores indicate better situation)			7.391	< 0.001
< 12	34	4.60 ± 1.70		
> 12	34	2.10 ± 1.00		
Severity			16.310	< 0.001
Mild	20	4.00 ± 1.50		
Moderate	20	2.70 ± 1.50		
Severe	28	1.80 ± 1.00		

The Relationship Between the Expression Levels of EAAT2 and ADORA2A and the Clinicopathological Characteristics of AD

The Relationship Between EAAT2 Expression and the Clinicopathological Characteristics of AD

The expression of serum EAAT2 was related to the severity of the disease and scores of the activities of daily living (ADL), Mini-mental State Examination (MMSE), and Alzheimer's Disease Assessment Scale-Cognitive section (ADAS-cog), but was not correlated with the age and sex of patients. More details are shown in Table II and Figure 2.

The Relationship Between ADORA2A Expression and the Clinicopathological Characteristics of AD

The expression of serum ADORA2A was related to the severity of the disease and scores of ADL, MMSE, and ADAS-cog, but was not correlated with the age and sex of patients (Table III and Figure 3).

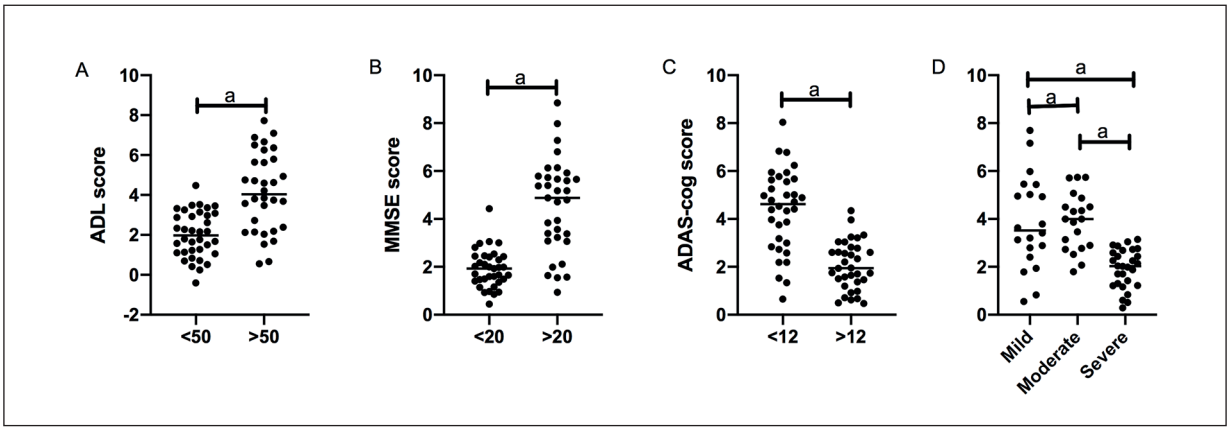

Figure 2. EAAT2 expression in patients with different clinicopathological characteristics. **A,** EAAT2 expression in patients with different ADL scores. **B,** EAAT2 expression in patients with different MMSE scores. **C,** EAAT2 expression in patients with different ADAS-cog scores. **D,** EAAT2 expression in patients with different AD severity. Note: "a" indicates $p<0.001$.

Table III. The relationship between ADORA2A expression and the clinicopathological characteristics of AD.

Group	N	ADORA2A	t/F	p
Age (year)			0.081	0.935
≤ 67	30	10.10 ± 2.40		
> 67	38	10.15 ± 2.60		
Sex			0.666	0.508
Male	34	9.60 ± 2.50		
Female	34	10.00 ± 2.45		
ADL			6.974	< 0.001
< 50	36	13.40 ± 2.46		
> 50	32	9.20 ± 2.50		
MMSE			7.235	< 0.001
< 20	35	12.80 ± 2.56		
> 20	33	8.44 ± 2.40		
ADAS-cog			6.866	< 0.001
< 12	34	9.00 ± 2.30		
> 12	34	13.00 ± 2.50		
Severity			26.370	< 0.001
Mild	20	8.20 ± 2.40		
Moderate	20	10.30 ± 2.50		
Severe	28	13.20 ± 2.30		

Correlation of EAAT2 and ADORA2A Expression with AD Severity

The expression of EAAT2 in patients with mild AD, those with moderate AD, and those with severe AD was (4.00 ± 1.50) μg/ml, (2.70 ± 1.50) μg/ml, and (1.80 ± 1.00) μg/ml, respectively. The expression of ADORA2A in patients with mild AD, those with moderate AD, and those with severe AD was (8.20 ± 2.40) μg/ml, (10.30 ± 2.50) μg/ml, and (13.20 ± 2.30) μg/ml, respectively. Patients with moderate and severe AD showed higher ADORA2A expression and lower EAAT2 expression than patients with mild AD (p<0.05). An increase in AD severity resulted in a rise in ADORA2A expression and a decrease in EAAT2 expression. Mild AD was labeled as "1", moderate AD as "2", and severe AD as "3". Then, we conducted Spearman correlation analysis on the relative expression of EAAT2 and ADORA2A in serum and the severity of AD. The severity of AD was negatively correlated with the relative expression of serum EAAT2 (r=-0.7286, p<0.001), positively correlated with the relative expression of serum ADORA2A (r=0.7381, p<0.001). More details are shown in Figure 4A-B.

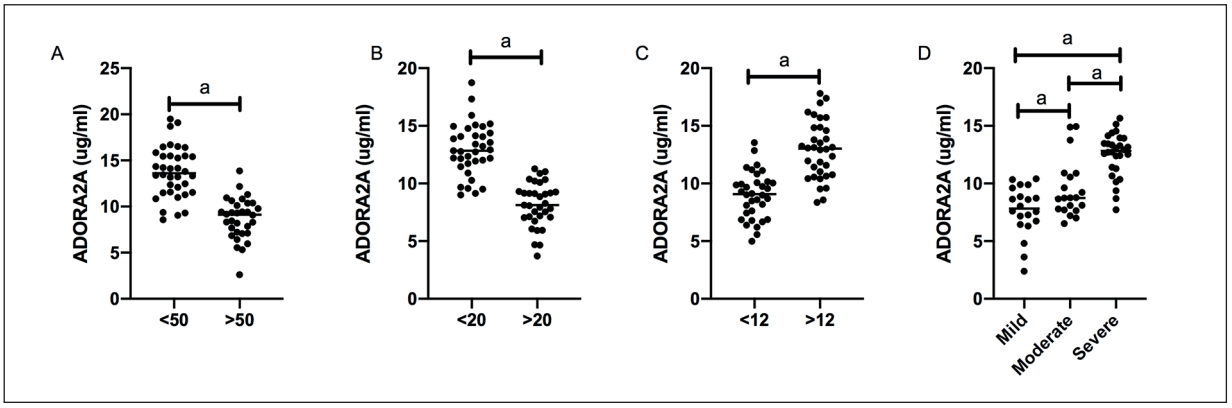

Figure 3. ADORA2A expression in patients with different clinicopathological characteristics.**A,** ADORA2A expression in patients with different ADL scores. **B,** ADORA2A expression in patients with different MMSE scores. **C,** ADORA2A expression in patients with different ADAS-cog scores. **D,** ADORA2A expression in patients with different AD severity. Note: "a" indicates p<0.001.

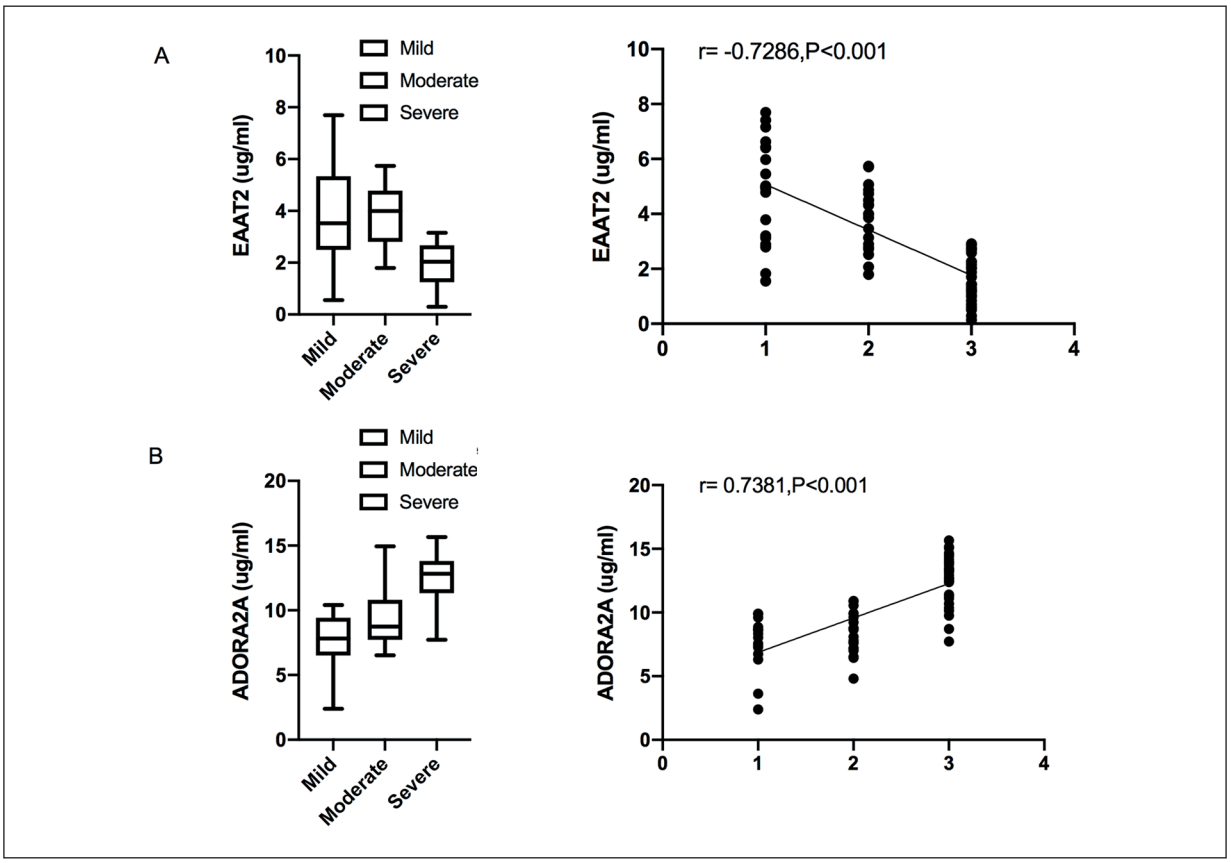

Figure 4. Correlation of EAAT2 and ADORA2A expression with AD severity. **A,** The relative expression of serum EAAT2 decreased with the increase of AD severity ($p<0.05$). The severity of AD was negatively correlated with the relative expression of serum EAAT2 (r=-0.7286, $p<0.001$). **B,** The relative expression of serum ADORA2A rose with the increase of AD severity ($p<0.05$). The severity of AD was positively correlated with the relative expression of serum ADORA2A (r=0.7381, $p<0.001$).

Diagnostic Value of EAAT2 and ADORA2A for AD

The sensitivity, specificity, and area under the curve (AUC) of EAAT2 alone for the diagnosis of AD were 85.00%, 82.35%, and 0.8853, respectively, and those of ADORA2A alone for the diagnosis were 71.67%, 79.41.00%, and 0.8369, respectively (Table IV and Figure 5).

Table IV. Diagnostic value of serum EAAT2 and ADORA2A for AD.

Factor	EAAT2	ADORA2A
AUC0.8853	0.8369	
95% CI	0.8273-0.9433	0.7689-0.9049
Std. Error	0.02958	0.03468
Cut-off value	4.466	8.610
Sensitivity (%)	85.00	71.67
Specificity (%)	82.35	79.41

Analysis of Risk Factors of AD Prognosis

Univariate Analysis of Risk Factors of AD Prognosis

According to the univariate analysis, AD patients with poor prognosis were significantly different from AD patients with good prognosis in the MMSE score, ADL score, severity of disease, EAAT2 expression, and ADORA2A expression (all $p<0.001$). The MMSE score, ADL score, severity of disease, EAAT2 expression, and ADORA2A expression were related risk factors for AD (Tables V and VI).

Multivariate Analysis of Risk Factors of AD

We assigned risk factors of AD to the multivariate conditional logistic regression analysis and found that MMSE score, severity of disease,

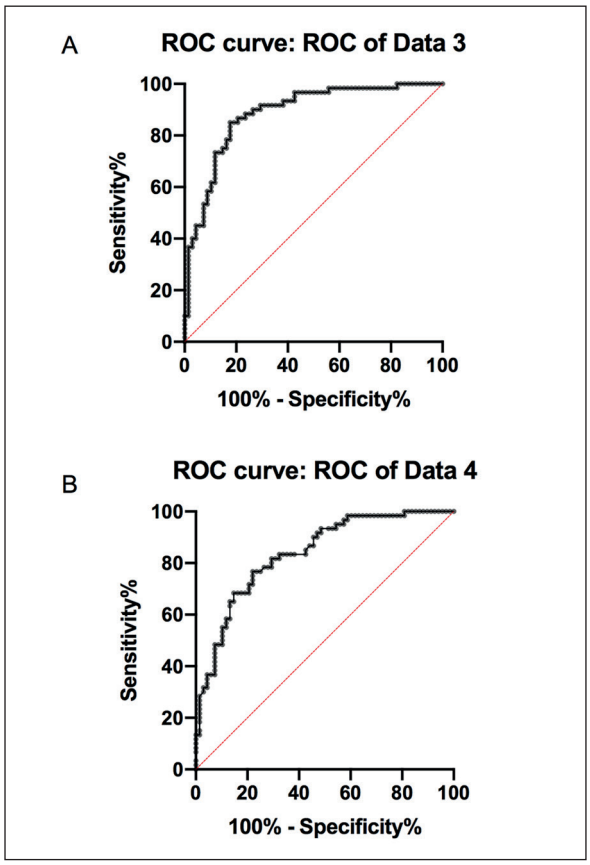

Figure 5. ROC curve of serum EAAT2 and ADORA2A for AD diagnosis. **A,** The sensitivity of serum EAAT2 for AD diagnosis was 85.00%, and the specificity was 82.35%. **B,** The sensitivity of serum ADORA2A for AD diagnosis was 71.67%, and the specificity was 79.41%.

Table V. Assignment of risk factors of AD.

Related factors	Assignment
Age (year)	< 67 = 0, ≥ 67 = 1
Sex	Male = 0, female = 1
ADL	< 50 = 0, >50=1
Severity	Mild = 0, moderate and severe = 1
MMSE	< 20 =0, >20 = 1
ADAS-cog	< 12 = 0, > 12 = 1
EAAT2 (μg/ml)	< 5.40 = 0, > 5.40 = 1
ADORA2A (μg/ml)	< 5.80 = 0, > 5.80 = 1
Smoking	No = 0, Yes = 1
Drinking	No = 0, Yes = 1

EAAT2 expression, and ADORA2A expression were independent risk factors for AD prognosis (Table VI).

Discussion

AD is a chronic neurodegenerative disease with complex pathogenesis and unspecified causes. EAAT2 and ADORA2A both have an essential effect on abnormal brain activity in patients with mental illness[16]. ADORA2A is differentially expressed in AD and controls the glial function and metabolic adaptation in the brain of patients with AD. The search for biological markers for the diagnosis and treatment of AD has notable clinical significance[17].

Here we quantified the expression of serum EAAT2 and ADORA2A in patients with AD and healthy individuals and found higher ADORA2A expression and lower EAAT2 expression in patients with AD than in healthy individuals. Horgusluoglu et al[18] on aging-caused cognitive impairment stated that ADORA2A antagonists could reverse synaptic dysfunction and alleviate AD. Glutamate accumulation is one of the main triggers for cerebral ischemic injury and other neurological diseases. EAAT2 can transport glutamate and protect hippocampal neurons[19,20]. Drug experiments on EAAT2 demonstrated that EAAT2 expression was reduced with the progression of AD. In previous researches[21,22], riluzole, a neuroprotective agent, stimulated the overexpression of EAAT2 in depression mice models to protect the nerve. As a potential target for the treatment of AD, EAAT2 is abnormally upregulated in a variety of immune disorders including AD. Sharma et al[23] suggested that the progression of AD could be controlled by adjusting the expression of EAAT2. Therefore, serum ADORA2A expression is upregulated in AD patients, while serum EAAT2 is downregulated. We, then, analyzed the relationship between the expression of EAAT2 and ADORA2A and the clinicopathological characteristics of AD, and found that

Table VI. Multivariate analysis of risk factors of AD.

Factors	β	SE	Wald	*p*	Exp (β)	95% CI
Severity	0.699	0.285	10.099	0.002	1.887	1.011-9.472
EAAT2	0.382	0.199	7.645	0.010	3.126	0.192-15.273
ADORA2A	0.473	0.190	9.165	0.024	2.036	1.175-6.200

the expression of EAAT2 and ADORA2A was related to the stage and severity of AD. So far, no studies have been made on the relationship between EAAT2 and the pathological characteristics of AD. Veitch et al[24] detected an increased ADORA2A expression in AD serum and found that serum ADORA2A expression in patients with more severe AD was higher, indicating a correlation of ADORA2A expression with the pathological stage of AD. The results of the above study are similar to what we found, which can well support our findings. Here we carried out a correlation analysis of the EAAT2 and ADORA2A expression and the severity of AD and draw a conclusion that an increase in AD severity led to a rise in ADORA2A expression and a decrease in EAAT2 expression. Spearman correlation analysis demonstrated that AD severity was positively correlated with the relative expression of serum ADORA2A and negatively correlated with the relative expression of serum EAAT2. ADORA2A has been verified to be closely related to disease conditions, and its expression is significantly increased or decreased with the progression of diseases[25]. We also analyzed the value of EAAT2 and ADORA2A in diagnosing AD and predicting the severity of AD. According to the ROC curve, both separate EAAT2 and ADORA2A showed good sensitivity and specificity in the diagnosis of AD. Imaging examinations, such as brain magnetic resonance and CT examinations are common in AD diagnosis, but they can cause misdiagnosis and missed diagnosis for the severity of AD. Routine imaging examinations combined with a specific marker can well improve the diagnostic efficiency[26,27]. We assigned risk factors of AD prognosis to the multivariate conditional logistic regression analysis and found that MMSE score, severity of disease, EAAT2 expression, and ADORA2A expression were independent risk factors of AD prognosis. No studies have been reported about the diagnostic efficacy and predictive value of serum EAAT2 expression for AD patients. In the present study, EAAT2 and ADORA2A showed a certain value in diagnosing AD and predicting the prognosis of AD.

This study indicated the value of EAAT2 and ADORA2A expression in AD patients, but it has some limitations. First, we failed to illustrate the regulation effect of EAAT2 and ADORA2A on AD and their biological functions. Second, we did not compare EAAT2 and ADORA2A with other routine AD markers, which make this study deficient. Therefore, we should make continuous efforts to address such problems and learn from the latest researches, aiming to perfect this study.

Conclusions

In summary, AD patients have highly expressed ADORA2A and lowly expressed EAAT2 in the serum. At present, the clinical biomarkers for the diagnosis of AD are incomplete. This study has found that EAAT2 and ADORA2A may be involved in the development of AD, and both can be adopted as potential new serum biomarkers for the diagnosis and assessment of AD.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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