GENETIC POLYMORPHISM OF SERUM EXOSOME-ASSOCIATED MICRORNAS IN PATIENTS WITH ALZHEIMER'S DISEASE

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ABSTRACT

Objective*: This research aims to study the genetic polymorphism of serum exosome-associated MicroRNAs (MicroRNAs, miRNA) in patients with Alzheimer's disease (AD).*

Methods: *From June 2018 to June 2020, 120 AD patients were treated in the Affiliated Hospital of Medical University, including 63 males and 57 females, aged 50 to 85 years, with an average age of 69.22±5.76 years. 120 healthy subjects served as the control group, including 62 males and 58 females, aged 48 to 86 years, with an average age of 68.53±6.59 years. After fasting for 12 hours, peripheral blood was collected from each patient. Total Exosome Isolation Kit was used to isolate exosomes. Genotyping was carried out by PCR. The expression levels of miR-23a-3p and miR-197-5p genes were detected by real-time PCR. Cognitive function was assessed through MMSE. The level of IL-6 was detected by Western blot.*

Results: *There were no statistically significant differences between the control group and the AD group in age, gender, body mass index, systolic blood pressure (SBP), diastolic blood pressure (DBP), fasting blood glucose (FBG), total cholesterol (total cholesterol, TC), low density lipoprotein (LDL), high density lipoprotein (HDL) and triglycerides (TG) (P>0.05). Compared with the control group, the frequency of AA genotype and A allele decreased in the AD group (P<0.05), and the frequency of AG genotype, GG genotype and G allele increased (P<0.05). Compared with the control group, the frequency of TT genotype and T allele decreased in the AD group (P<0.05), and the frequency of TC genotype, TT genotype and T allele increased (P<0.05). Compared with the control group, the expression levels of miR-23a-3p genotypes AA and AG+GG and miR-197-5p genotypes TT and TC+CC increased. The MMSE scores decreased, and the level of IL-6 in the AD group All increased (P<0.05). In the control group, miR-23a-3p genotypes AA and AG+GG, miR-197-5p genotypes TT and TC+CC gene expression levels, MMSE scores and IL-6 levels did not differ (P>0.05). In the AD group, the expression level of miR-23a-3p genotype AG+GG was higher than that of AA and the expression level of miR-197-5p genotype TC+CC was higher than that of TT gene. Both of their MMSE scores were lower, and IL-6 levels were higher (P<0.05).*

Conclusion: *The polymorphism of miR-23a-3p and miR-197-5p changed the expression levels of miR-23a-3p and miR-197-5p. The change may regulate the inflammatory response in the brain of AD patient and further aggravate the susceptibility to AD. MiR-23a-3p genotype AG+GG and miR-197-5p genotype TC+CC were associated with an increased risk of cognitive decline in AD patients.*

Keywords: *Alzheimer's disease, serum exosome, microrna, polymorphism.*

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Introduction

AD is prevalent in the elderly. It is a slowly developing neurodegenerative disease, mainly manifested as memory loss[(1)]. AD has become the biggest public health challenge in the world. It is estimated that the AD patients will increase year by year[(2)]. AD brings huge economic burden to patients' families and the society, and has become a major disease endangering global public health[(3)].

The formation of AD is a complex process with multiple stages and factors. Environmental and genetic factors are likely to interact and promote each other, eventually leading to AD. MiRNA is a short non-coding RNA (containing about 20-24 nucleotides), which is involved in mRNA silencing

and post-transcriptional regulation of gene expression through its ability to bind 3' untranslated regions[4]. Therefore, miRNA is a key participant in the normal function of cells. Exosomes are membrane vesicles with a size of 40-100 nm released from various cell types[5]. Recent studies have shown that exosomes carry mRNA and miRNA in addition to functional proteins[6]. Functionally, exosomes are considered to represent a new intercellular communication mechanism[7].

In addition, they are actively secreted from cells, which can help eliminate interference from passively secreted miR[8]. The abnormal expression of miR has been detected in AD[9]. There are also studies to screen the differential exosome miRNA biomarkers and neuron-derived exosome proteins in serum between healthy people and AD patients[10].

However, the polymorphism of serum exomemeir-23a-3p and miR-197-5p in AD patients is still unclear. Our aim is to explore the genetic polymorphism of serum exosome related miRNAs in AD patients.

Data and methods

General data

From June 2018 to June 2020, 120 AD patients were treated in the Affiliated Hospital of Medical University, including 63 males and 57 females, aged 50 to 85 years, with an average age of 69.22±5.76 years.

All patients were diagnosed according to the standards of National Institute of Neurological and Communicative Disorders and Stroke and AD and Related Diseases Association (NINCDS-ADRDA). Another group of 120 healthy subjects was recruited as control group, including 62 males and 58 females aged between 48 and 86 years, with an average age of 68.53±6.59 years.

Inclusion criteria:

• Mini mental state examination score ≤24, clinical dementia rating scale (CDR) >0.5;

• AD diagnosis based entirely on NINCDS-ADRDA standard under joint diagnosis by psychiatrists and neurologists;

• Behavioral and psychological symptoms of dementia.

Exclusion criteria:

• Patients with other organic diseases;

• Patients with malignant tumors;

• Patients with frontotemporal dementia;

• Pseudodementia in patients with depression.

Medical ethics

This study was approved by the hospital ethics committee, and all subjects signed the informed consent form.

Methods

Sample collection

After fasting for 12 hours, peripheral blood was collected from each patient. Serum was separated by centrifugation at 3,000xg for 10 minutes at room temperature and 12,000xg for 5 minutes at 4°C. Samples were stored at -80°C until needed.

Separation of exosomes

Exosomes were isolated using the total exosome isolation kit (Invitrogen Life Technologies). Serum was centrifuged at 2,000×g for 30 minutes to remove cells and debris. Thereafter, 400μL of clarified serum was transferred to a new test tube, and 0.2 volume of total exosome separation reagent was added. Turbine mixing was carried out to mix the serum/reagent mixture thoroughly until a homogeneous solution is formed. The samples were incubated at 4°C for 30 minutes. After incubation, the samples were centrifuged at 10,000×g for 10 minutes at room temperature. The supernatant was discarded, and the sediment containing foreign bodies was resuspended in 200μL phosphate buffer (PBS) at the bottom of the test tube. The size distribution was confirmed by nanoparticle tracking analysis (Zetaview, Germany).

Genotyping

Genomic DNA was extracted from serum exosome of subjects using QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany). PCR primers were synthesized by Sangon Biotech (Shanghai, China). The extracted genomic DNA was used as a template for PCR, and the reaction conditions were: at 95°C for 5 minutes; at 94°C for 20 s; at 58°C for 30 s; at 72°C for 30 s, repeated for 35 cycles; 72°C for 5 minutes; saved at 4°C. PCR products were analyzed by Sanger sequencing of Sangon Biotech (Shanghai, China).

Real-time PCR(qRT-PCR)

According to the manufacturer's instructions, total RNA was extracted from the serum exosome of the subjects using TRIzol reagent (Invitrogen). CDNA was synthesized by reverse transcription PCR using PrimeScript RT kit (Baojiu City, Shiga Prefecture, Japan). The real-time quantitative PCR(qRT-PCR)

was performed on ABI Prism 7500 system (Applied Biosystems, Foster City, CA, USA) using OneStep SYBR RT-PCR kit (Quantace, Valencia, CA, USA). The relative expression levels of SIRT2 mRNA and miRNA were calculated by $2^{-\Delta\Delta Ct}$ method with GAPDH as internal control, and the samples of each group were measured in triplicate. Primers were synthesized by Sangon Biotech (Shanghai, China).

MMSE score

MMSE is used to evaluate cognitive function. The score scale has a total score of 30 points. MMSE contains 11 questions, and is divided into 2 parts. It takes about 5 to 7 minutes to complete the scale. In the first part, participants are asked to give oral answers to questions focusing on memory, attention and direction. The second part evaluates the patient's ability to follow oral and written commands, including writing sentences and drawing polygonal maps, with a total score of 9 points. Compared with individuals with at least 8 points, those with scores between 24 points and 30 points are considered to be under normal cognitive education for many years; the score between 19 and 23 indicates borderline cognitive impairment, and the score below 19 points indicates cognitive impairment.

Western blot

Proteins were collected by lysis with radioimmunoassay (RIPA) buffer supplemented with 1:100 protease inhibitor (Life Technologies) and phosphatase inhibitor cocktail I and II(Sigma-Aldrich). They were quantified using the Bicinchonic Acid Protein Assay Kit (Life Technologies). The samples were separated on SDS polyacrylamide gel for western blot analysis. Separated proteins were transferred to PVDF membrane (Bio-Rad, Hercules, CA, USA). After sealing the membrane with 5% skimmed milk, the blot was incubated with the appropriate primary antibody at 4° overnight.

After washing, the blot was incubated with HRP-coupled anti-rabbit anti-secondary antibody for 1 hour at room temperature. After washing, Immobilon Western HRP (Millipore; The United States) was used to observe the immune response band and FluorChem HD2(proteinsimple; United States) was used for the test.

Statistical analysis

Chi-square test of goodness of fit was used to test whether genotype frequency conformed to Hardy-Weinberg equilibrium (HWE). According to allele frequency and distribution of genetic models (additive model, dominant model and recessive model), the correlation between SNP and AD risk was determined, and OR and 95% confidence interval (CI) were used in the comparison.

Chi-square test was used for statistical analysis of classification variables. One-way ANOVA or independent sample T-test was used for statistical analysis of continuous variables. The statistical analysis used in this study was conducted by GraphPad PRISM (Version 7.0, La Jolla, California, USA) and SPSS20.0 software (IBM, Chicago, Illinois). All the tests are double-tailed. $P<0.05$ indicated statistically significant differences.

Results

General characteristics of control group and AD group

This study included 120 AD patients and 120 controls. Comparing the general characteristics of the two groups, the results showed that there was no statistical difference in age, sex, body mass index, SBP, DBP, FBG, TC, LDL, HDL and TG between the two groups ($P>0.05$), so that the above factors could be excluded to make the data more comparable. (Table 1).

Item	Control group	AD Group	χ^2 value/*t* value	*P* value
n	120	120	-	-
Gender (male: female)	62:58	63:57	8.364	0.238
Age (year)	68.53±6.59	69.22±5.76	6.521	0.634
Body mass index (kg/m^2)	25.34±2.47	25.68±2.21	3.226	0.852
SBP (mmHg)	125.89±10.56	125.37±1.53	8.624	0.763
DBP (mmHg)	77.43±3.55	77.26±3.17	9.564	0.596
FBG (mmol/L)	4.78±0.56	4.72±0.48	7.231	0.743
TC (mmol/L)	4.86±0.52	4.82±0.47	11.237	0.582
LDL (mmol/L)	2.65±0.31	2.61±0.28	10.231	0.463
HDL (mmol/L)	1.33±0.27	1.43±0.29	12.005	0.751
TG (mmol/L)	1.29±0.26	1.25±0.23	8.063	0.634

Table 1: General situation of control group and AD group.

Genotype and allele frequency distribution of mir-23a-3p

In the control group of this study, the frequency distribution of different genotypes of miR-23a-3p (A>G) is in Hardy-Weinberg equilibrium.

Compared with control group, the frequency of AA genotype and A allele in the AD group decreased

(P<0.05), while the frequency of AG genotype, GG genotype and G allele increased (P<0.05). (Table 2).

miR-23a-3p (A>G)		Control group (%)	AD Group (%)	OR (95%CI)	*P* value
Gene type	AA	66 (55.00%)	24 (20.00%)	1.00	-
	AG	25 (20.83%)	45 (37.50%)	1.38 (1.14-1.65)	0.011
	GG	29 (24.17%)	51 (42.50%)	1.44 (1.06-1.73)	0.013
	AG+GG	54 (45.00%)	96 (80.00%)	1.87 (1.55-1.97)	<0.001
Allelic genes	A	157 (65.42%)	93 (38.75%)	1.00	-
	G	83 (34.58%)	147 (61.25%)	1.89 (1.53-2.21)	<0.001

Table 2: Genotype and allele frequency distribution of mir-23a-3p (A >G).

Genotype and allele frequency distribution of mir-197-5p

In the control group of this study, the frequency distribution of different genotypes of miR-197-5p (T>C) is in Hardy-Weinberg equilibrium. Compared with control group, TT genotype and T allele frequency in the AD group decreased (P<0.05), while TC genotype, TT genotype and t allele frequency increased (P<0.05). (Table 3).

miR-197-5p (T>C)		Control group (%)	AD Group (%)	OR (95%CI)	*P* value
Gene type	TT	58 (48.33%)	18 (15.00%)	1.00	-
	TC	35 (29.17%)	53 (44.17%)	1.83 (1.56-2.11)	0.013
	CC	27 (22.50%)	49 (40.83%)	1.75 (1.47-1.96)	0.014
	TC+CC	62 (51.67%)	102 (85.00%)	2.23 (1.85-2.45)	<0.001
Allelic genes	T	151 (62.92%)	89 (37.08%)	1.00	-
	C	89 (37.08%)	151 (62.92%)	1.89 (1.37-2.32)	<0.001

Table 3: Genotype and allele frequency distribution of mir-197-5p (T> C).

The relationship between polymorphism of mir-23a-3p and miR-197-5p and gene expression level

The expression levels of miR-23a-3p and miR-197-5p were detected by real-time PCR.

Compared with control group, the gene expression levels of miR-23a-3p genotype AA and AG+GG and miR-197-5p genotype TT and TC+CC in the AD group increased (P<0.05). In the control group, there was no difference in gene expression level between AA and AG+GG of miR-23a-3p genotype and TT and TC+CC of miR-197-5p genotype (p>0.05); in the AD group, the expression level of miR-23a-3p genotype AG+GG was higher than that of AA and the expression level of miR-197-5p genotype TC+CC was higher than that of TT gene (P<0.05). (Figure 1, Table 4).

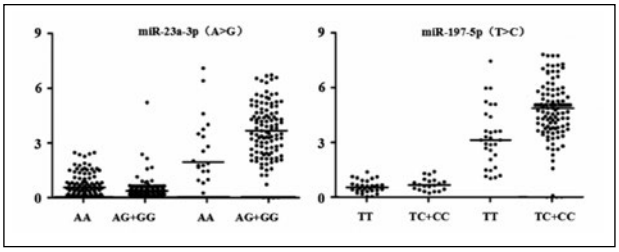

Figure 1: Correlation between miRNA polymorphism and expression level.

Gene type		Control group (%)	AD group (%)	*t* value	*P* value
miR-23a-3p (A>G)	AA	0.87±0.23	2.22±0.16	6.597	0.258
	AG+GG	0.82±0.15	3.74±0.32	8.634	0.024
t value		3.264	9.135	-	-
P value		0.398	0.012	-	-
miR-197-5p (T>C)	TT	0.95±0.13	3.16±0.34	5.627	0.367
	TC+CC	0.93±0.25	4.86±0.18	15.214	0.011
t value		2.063	11.325	-	-
P value		0.458	0.006	-	-

Table 4: Correlation between miRNA polymorphism and expression level.

The relationship between the polymorphism of miR-23a-3p and miR-197-5p and cognitive decline

Cognitive ability was evaluated by MMSE score. Compared with control group, MMSE scores of both miR-23a-3p genotype AA and AG+GG and miR-197-5p genotype TT and TC+CC in the AD group decreased (P<0.05). In the control group, MMSE scores of miR-23a-3p genotype AA, AG+GG and MMSE scores of miR-197-5p genotype TT, and TC+CC had no difference (p>0.05); in the AD group, the MMSE score of miR-23a-3p genotype AG+GG was lower than that of AA and the MMSE score of miR-197-5p genotype TC+CC was lower than that of TT (P<0.05). (Table 5).

Gene type		Control group (%)	AD group (%)	*t* value	*P* value
miR-23a-3p (A>G)	AA	28.69±1.14	12.46±1.38	13.568	0.018
	AG+GG	28.55±1.06	5.23±0.24	12.654	<0.001
t value		2.056	16.325	-	-
P value		0.856	0.006	-	-
miR-197-5p (T>C)	TT	28.37±1.12	14.18±1.39	11.152	0.006
	TC+CC	28.26±1.25	5.15±0.25	16.524	<0.001
t value		1.236	12.325	-	-
P value		0.452	0.013	-	-

Table 5: Correlation between miRNA polymorphism and MMSE score.

The relationship between the polymorphism of miR-23a-3p and miR-197-5p and IL-6 inflammation level

The level of IL-6 was detected by western blot. Compared with control group, the IL-6 levels of miR-23a-3p genotype AA and AG+GG and miR-197-5p genotype TT and TC+CC in the AD group increased (P<0.05). In the control group, there was no difference in the IL-6 level between miR-23a-3p genotype AA and AG+GG and miR-197-5p genotype TT and TC+CC (p > 0.05); in the AD group, the IL-6 level of miR-23a-3p genotype AG+GG was higher than that of AA and the IL-6 level of miR-197-5p genotype TC+CC was higher than that of TT (P<0.05). (Figure 2, Table 6).

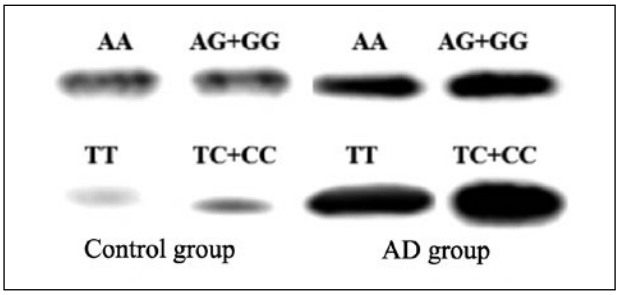

Figure 2: Correlation between miRNA polymorphism and IL-6.

Gene type		Control group (%)	AD group (%)	*t* value	*P* value
miR-23a-3p (A>G)	AA	0.98±0.11	1.89±0.13	13.26	0.015
	AG+GG	1.06±0.14	2.78±0.23	15.326	<0.001
t value		3.026	13.284	-	-
P value		0.746	0.015	-	-
t value	TT	0.72±0.13	2.36±0.11	8.264	0.012
P value	TC+CC	0.79±0.18	3.25±0.13	16.241	<0.001
		2.527	13.629	-	-
		0.629	0.013	-	-

Table 6: Correlation between miRNA polymorphism and IL-6.

Discussion

Searching for non-invasive AD biomarkers is one of the fastest developing fields in AD research. Many researchers have previously evaluated the potential benefits of diagnosing and treating various diseases (including cancer, infection and neurodegenerative diseases) in miRNA analysis[11]. In this study, the serum exosome miRNA is the measured object. It has been shown that many cells of nervous system release exosomes in the form of extracellular vesicles, indicating that they play an active role in the function, development and pathology of the system. More importantly, other recent studies have shown that exosomes play a role in the degradation of toxic Aβ and the accumulation of toxic peptides when there are too many scavenging pathways[12]. In this study, the miRNA levels of two serum exosomes (miR-23a-3p and miR-197-5p) in AD patients were significantly higher than those in the control group. Exocrine miRNA in serum may represent the research direction of the occurrence and treatment of AD. SNP on precursor and mature miRNA has been shown to affect the level of mature miRNA, and has been shown to be associated with many diseases[13]. In this study, SNPs with functional importance, namely miR-23a-3p (A>G) and miR-197-5p (T>C), were selected to evaluate their association with AD. The A>G polymorphism of miR-23a-3p is located in the promoter region of miR-23a-3p, which has the binding site of V-Ets oncogene homolog 1 (Ets-1). G allele of miR-23a-3p interferes with Ets-1 binding and leads to higher expression level of mature miR-23a-3p. A large number of reports have associated neuroinflammation with the decline of AD cognitive function[14]. A recent clinical study reported that the MMSE score of AD patients with high inflammation score is easier to accelerate within 3 years than that of AD patients with low inflammation score[15]. As expected, our results confirmed that both SNPs affected the levels of mature miR-23a-3p and miR-197-5p. In addition, our study found that miR-23a-3p genotype AG+GG had more relation with cognitive decline in AD patients than AA and miR-197-5p genotype TC+CC had more relation with cognitive decline in AD patients than TT.

Elevated concentrations of pro-inflammatory cytokines (such as IL-1β and IL-6) are also related to cognitive impairment[16]. More and more evidence shows that miR-23a-3p plays a role in stimulating inflammatory response in the brain of AD patients[17]. As positive regulators of AD inflammation, the increased levels of miR-23a-3p and miR-197-5p may increase the expression of pro-inflammatory cytokines in AD patients, and the functional polymorphism of miR-23a-3p and miR-197-5p may lead to cognitive degradation in AD patients by affecting the inflammatory process of the brain[18-20]. Our data showed that there was a positive correlation between risk-related AG+GG and TC+CC genotypes and cognitive decline of AD patients. Our results also confirmed that AG+GG and TC+CC genotypes could increase the expression level of proinflammatory cytokine IL-6. This is consistent with the following phenomenon:

individuals with AG+GG and TC+CC genotypes have higher levels of IL-6 stimulation. Although the potential mechanism of this association cannot be inferred directly from this study, we believe that AD patients with AG+GG and TC+CC genotypes may have higher levels of miR-23a-3p and miR-197-5p, thus enhancing the inflammatory response and leading to the development of AD. However, due to limited AD samples in our study, this association should be verified further in a larger queue.

To sum up, the polymorphism of miR-23a-3p and miR-197-5p changed the expression levels of miR-23a-3p and miR-197-5p. The change may regulate the inflammatory reaction in the brain of AD patients and further aggravate the susceptibility to AD. The miR-23a-3p genotype AG+GG and miR-197-5p genotype TC+CC were associated with the increased risk of cognitive decline in AD patients.

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