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Original Article

Correlation analysis between APOE gene polymorphism and Alzheimer's disease

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Abstract: Alzheimer's disease (AD) is a condition of neurodevelopmental degeneration with an occult onset. It was found that apolipoprotein E (APOE) is associated with a variety of neurological disorders. This study was performed to investigate the association between APOE gene polymorphism (SNP) and AD. Leukocyte DNA of AD patients was extracted and the APOE gene polymorphism was analyzed by restriction fragment length polymorphism polymerase chain reaction (PCR-RFLP) and Sanger sequencing. The distribution of different alleles in different populations was analyzed to explore the association between different APOE gene polymorphisms and AD. APOE expression in different APOE homozygous genotype mice was detected by Western blot. The concentration of Aβ in cerebrospinal fluid was analyzed by enzyme-linked immunosorbent assay (ELISA). For APOE polymorphism distribution, the E4/E4 ratio was significantly higher in the AD group than in the healthy control group ($P < 0.05$). The frequency of E4 allele was obviously higher, while E3 allele was markedly lower than that of the control group ($P < 0.05$). The expression level of APOE in E4/E4 genotype mice was lower than that in E3/E3 and E2/E2 mice ($P < 0.05$), while the concentration of Aβ in cerebrospinal fluid was significantly higher than that the latter ($P < 0.05$). The E4 genotype of APOE gene was correlated with AD. E4/E4 homozygous genotype may downregulate APOE expression level, but it may increase the expression and accumulation of Aβ to participate in the occurrence and development of AD.

Keywords: Alzheimer's disease, APOE, gene polymorphism, Aβ

Introduction

Alzheimer's disease (AD) is a neurodevelopmental degeneration with occult onset [1]. The incidence of AD is gradually elevated with the increase of age. It was found that the incidence of AD is about 5% in the 65-year-old population, while it is about 20% in the population over 85 years old [2]. AD mainly presents in the clinic with memory impairment, aphasia, apraxia, visuospatial dysfunction, and executive dysfunction [3]. With the aging process of society as a whole, the incidence of AD has gradually increased, seriously affecting the health and quality of life of the elderly and bringing a heavy burden to the family and society [4]. In-depth study of the pathological mechanisms closely related to AD is of great significance for prevention or attempts to delay occurrence.

The specific etiology of AD is unclear. The current epidemiological study reports a relationship to genetic factors, physiological diseases, head injury, lifestyle, and state [5]. A genetic study of AD found that multiple genes were associated with the development and progression of human AD. APP, PSEN1 and PSEN2 gene mutations often existed in the early-onset family AD patients [6-8]. Recent research has demonstrated that the PSEN1 gene mutation may be one of the major causes of familial AD in China. PSEN1 gene mutation can be found prior to detection in the clinical diagnosis of familial AD [9]. The study of late-onset familial AD suggests that APOE is associated with this type of AD [10].

APOE is a 299 amino acid protein encoded by the APOE gene. APOE is a type of cholesterol transporter widely distributed in the liver, brain, and blood that is extremely important for the maintenance of blood lipid balance [11]. APOE has three different subtypes, APOE2, APOE3 and APOE4. The different subtypes are due to polymorphisms of the APOE gene [11], with the expression of different subtypes of APOE being

Table 1. The primer sequence of PCR-RFLP

Primer	Sequence
APOE-F	5'-GATCAAGCTTCCAATCACAGGCAGGAAG-3'
APOE-R	5'-GATCCG-GCCGCACACGTCCTCCATG-3'

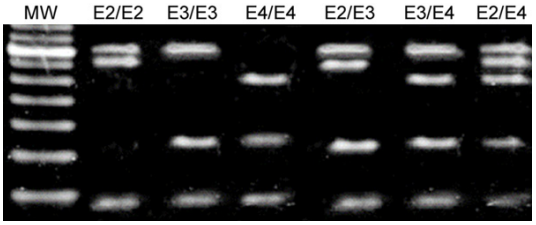

Figure 1. APOE gene polymorphism detected by PCR-RFLP.

associated with cardiovascular disease, atherosclerosis, and AD [12]. This study was done to investigate the association between the APOE gene polymorphism and AD to explore their mechanistic relationship.

Objects and methods

Main reagents and instruments

Blood DNA kit (Qiagen), tissue DNA extraction kit (QIAgen), ultramicro spectrophotometer (Thermo), *Hha*I restriction endonuclease (NEB), agarose (Thermo), shrimp alkaline phosphatase (SAP) (TAKARA), exonuclease (TAKARA), Big Dye II (ABI), ABI 3500 gene analyzer (ABI). H1850R centrifuge (Hunan Xiangyi Laboratory Instrument Development Co., Ltd.), primary and secondary antibodies (Biodesign), mouse Aβ detection ELISA kit (Abcam), other reagents (Sinopharm).

Objects

A total of 91 AD patients admitted to Yan'an University Xianyang hospital (Shaanxi, China) from June 2014 to December 2016 aged (71 ± 6.2) years were enrolled, including 52 males and 39 females. All patients were diagnosed according to AD diagnostic criteria and the third edition of the "Chinese Schizophrenia Program and Diagnostic Criteria", based on occult onset, progressive aponea, memory impairment, cognitive impairment and psychiatric symptoms, and neurological deficits. At the same time, 60 healthy volunteers were selected as the control group with average age at 69 ± 7.3 years old. There was no significant difference on age and gender between the control group and the experimental group. This study was approved by the Ethics Committee of Yan'an university Xianyang hospital (Shaanxi, China). All subjects had signed informed consent.

Blood sample collection and DNA extraction

A total of 10 mL peripheral blood was extracted from the subjects in the morning using EDTA blood-collecting tube. After centrifugation at 2000 g for 10 min, the white blood cell layer between the plasma and blood cells were isolated for leukocyte DNA extraction. Leukocyte DNA samples were extracted using the blood DNA Kit (Qiagen). Microscopic spectrophotometer was used to detect DNA content.

APOE gene polymorphism analysis

APOE gene polymorphism was analyzed by PCR-RFLP. Specific primers were designed according to the sequence of the APOE gene (GeneBank accession number: NM_0013026-89) (**Table 1**). PCR amplification was performed using extracted DNA. PCR amplification procedure: 95°C pre-denaturation for 10 min, followed by 35 cycles of 95°C for 60 s, 63°C for 60 s and 72°C for 60 s. The PCR product was purified by ethanolic sodium acetate to remove the excess primers and ions. Next, the PCR product was redissolved and then digested by *Hha*I restriction endonuclease at 37°C for 6 h. The 5 μL digested product was electrophoresed on 1.5% agarose gel. The E2/E3 type was 91 bp + 83 bp; E3/E3 type was 91 bp + 48 bp; E4/E4 was 72 bp + 48 bp; the heterozygous E2/E3 type was 91 bp + 83 bp + 48 bp; E2/E4 was 91 bp + 83 bp + 72 bp + 48 bp; and E3/E4 was 91 bp + 72 bp + 48 bp. Genotype was determined according to the results of agarose gel electrophoresis.

Sanger sequencing

The PCR product was treated with 0.1 U of SAP and 0.1 U of EXO to remove the single nucleotide that did not participate in the reaction and non-specific amplification products. PCR products were diluted at 1:5 after digestion. A total of 1 μl sample was used as template for sequencing PCR using Big Dye II and Taq enzyme. The reaction conditions: 95°C pre-denaturation for 3 min, followed by 32 cycles of 95°C for 30 s, 50°C for 30 s, and 60°C for 3 min, and 72°C

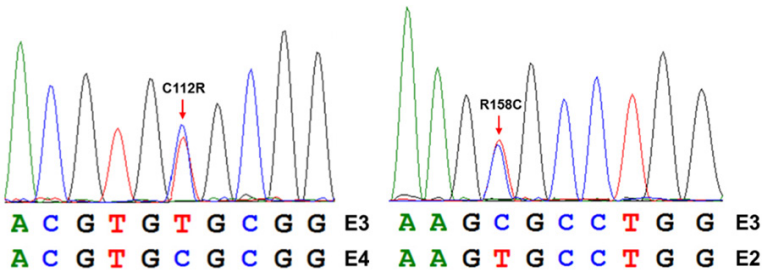

Figure 2. Sanger sequencing verification of APOE gene polymorphism.

extension for 10 min. The product of sequencing PCR was precipitated by ethanol and centrifuged to remove ethanol. Finally, it was dissolved in formamide and then tested directly on an ABI 3500 gene analyzer to detect APOE gene mutation [10].

The impact of different APOE genotypes on gene expression

To investigate the effect of different APOE genotypes on gene expression, we selected 6-week old C57BL6 mice (purchased from Peking University Experimental Animal Center) with E2/E2, E3/E3 and E4/E4 genotype APOE genes, respectively. There were three male and three female mice in each genotype group, with average body weights of 31.6 ± 2.5 g. The mice were raised on normal food, with free drinking and eating, and maintained at a temperature of 25°C with relative humidity held at 60 ± 10%. All treatments were performed in accordance with the animal disposal guidance of the National Institutes of Health (NIH) Animal Protection Committee.

After the mice were sacrificed, the cerebrospinal fluid (CSF) was extracted and the brain tissue was collected. The cells were digested by cell lysates after homogenization and the protein was extracted to test APOE expression using Western blot. The primary antibody was rabbit anti-mouse APOE monoclonal antibody (1:1000) and rabbit anti-mouse β-actin monoclonal antibody (1:1000), while the secondary antibody was goat anti-rabbit IgG antibody (1:200). The membrane was then developed by DAB and analyzed on gel imaging system.

ELISA

The CSF sample was detected by an ELISA kit (Abcam) to determine the content of β-amyloid protein (Aβ). 100 μL CSF was added to each well of a 96-well ELISA plate and incubated with 100 μL PBS (pH 7.4) overnight at 4°C. After washing with PBS, the plate was treated by 2% H_2O_2-ethanol solution to block endogenous peroxidase. The non-specific antibody binding site was blocked by 1% BSA solution. After washing with PBS, the plate was added with 1:1000 diluted rabbit anti-mouse APOE specific antibody at 37°C for 2 h. After washing off the excess antibody, the plate was added with 1:200 diluted biotin-labeled goat anti-rabbit secondary antibody at 37°C for 1.5 h. Finally, the OPD was used for development for 6 min and the reaction was stopped by 0.2 mM H_2SO_4. The content of APOE was measured using a microplate reader (Biotek).

Statistical analysis

All statistical analyses were performed using SPSS20.0. The difference between the groups was analyzed by ANOVA or t-test. The population representation of the samples was detected using the Hardy-Weinberg equilibrium method. The correlation between APOE gene polymorphism and prognosis of breast cancer patients was analyzed by multivariate logistic regression. The results were presented as an odds ratio (ORs) and 95% confidence interval (CIs). $P < 0.05$ was defined as statistical difference.

Results

APOE gene polymorphism in AD patients

The polymorphism of APOE gene in all the subjects was analyzed by PCR-RFLP (**Figure 1**). The APOE genotype was determined by electrophoresis and then verified by Sanger sequencing. The differences of the three alleles E2, E3 and E4 of APOE were the amino acids at 112 and 158 (**Figure 2**). Cys changes were at the 112 and 15 sites of E2, which corresponded to C-C, respectively. The loci of E4 were Arg corresponding to the base of T-T. The locus 112 and 158 sites of E3 were Cys and Arg, corresponding to C-T, respectively. The APOE genotype of all the subjects was calculated (**Table 2**). The

Table 2. APOE gene mutation in AD patients

Group	Cases (n)	E2/E2	E3/E3	E4/E4	E2/E3	E2/E4	E3/E4
AD	91 (100%)	0 (0.00%)	35 (38.46%)*	32 (35.16%)#	5 (5.49%)	2 (2.20%)	17 (18.68%)*
Control	60 (100%)	1 (1.67%)	48 (80.00%)	1 (1.67%)	3 (5.00%)	1 (1.67%)	6 (10.00%)

*P < 0.05, compared with control; #P < 0.01, compared with control.

Table 3. APOE allele distribution in AD patients

Group	E2	E3	E4
AD	3.85%	50.55%*	45.60%*
Control	3.33%	87.5%	7.5%

*P < 0.05, compared with control.

proportion of E3/E4 was significantly lower, while the ratio of E3/E4 and E4/E4 genotypes was obviously higher than that of the control group (P < 0.05).

APOE allele distribution

The allele distribution of the subjects in different groups was calculated (**Table 3**). It was found that the allele frequency of E4 in the AD group was significantly higher than that in the control group (P < 0.05). In addition, it was revealed that the E3 allele frequency in the control group was significantly higher than that in the AD group (P < 0.05). There was no significant difference in E2 allele frequency between the two groups (P > 0.05).

The impact of different APOE genotype on gene expression

The effects of different APOE genotypes on APOE gene expression were analyzed by animal experiments and Western blot analysis (**Figure 3**). These data show that the expression level of APOE gene in brain tissue with APOE genotype E2/E2 is significantly higher than that of E3/E3 and E4/E4. The expression of APOE in three homozygous genotypes was E2/E2 > E3/E3 > E4/E4.

The influence of different APOE genotype on Aβ expression

After the mice were sacrificed, the levels of Aβ in CSF of three different homozygous APOE genotypes were analyzed by ELISA (**Figure 4**). The concentration of Aβ in CSF of mice with APOE genotype E4/E4 was markedly higher than that in E2/E2 and E3/E3 mice, while the concentration of Aβ in CSF of E3/E3 mice exhibited no statistical difference compared with E2/E2 group.

Discussion

AD is a neurodevelopmental degeneration with occult onset. At present, numerous studies have considered that a variety of genes are involved in the occurrence and development of AD [1, 8]. APP, PSEN1 and PSEN2 gene mutations often exist in the early-onset family AD patients [8] and various genes, including APOE, SORL1 and MAPT, are closely associated with the development of late-onset AD [10, 13]. In this study, we examined the APOE genotype of AD patients and analyzed the relationship between different APOE genotypes and AD. We further analyzed the concentration of Aβ in the CSF of different APOE genotype mice so as to explore the mechanism of different APOE genotypes in the pathogenesis of AD.

In this study it was found that the proportion of E4/E4 in the APOE genotype was significantly higher in AD patients than in the healthy control group, while the ratio of E3/E3 genotype was significantly lower than that of the healthy group. By comparing the proportion of different alleles, it was also observed that the E4 allele in AD patients was markedly higher than the proportion of healthy control group. A large number of studies have proven that APOE genes are associated with a variety of human neurological disorders, including brain amyloidosis (CAA) and AD. It has been suggested that APOE gene polymorphisms are closely related to the susceptibility of the above two diseases [14]. Mormino *et al.* proposed that APOE4 genotype is associated with age-related memory loss [15]. Altmann *et al.* found that the E4 homozygosity of APOE gene was associated with the susceptibility to AD, which is consistent with our results [16].

APOE is an important lipoprotein that is involved in the process of lipid metabolism by regulating

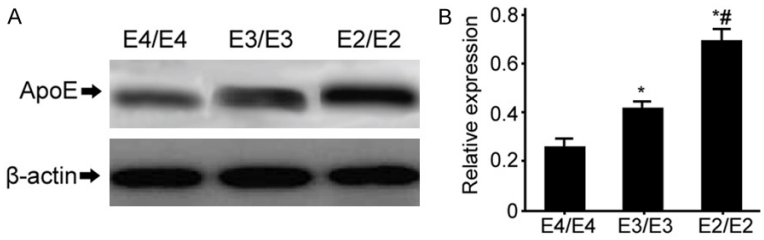

Figure 3. Western blot detection of APOE expression. A. Western blot result. B. Relative expression level of APOE in different APOE genotypes mouse brain tissue. *P < 0.05, compared with E4/E4; #P < 0.05, compared with E3/E3.

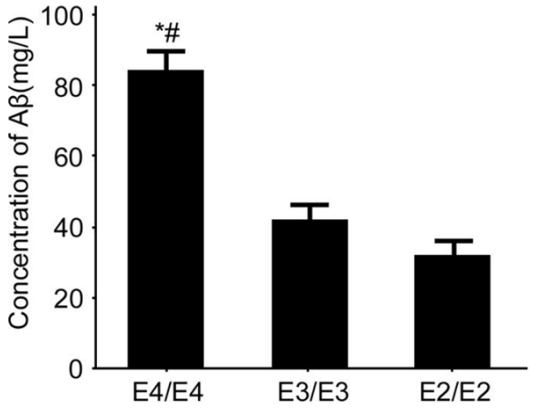

Figure 4. The influence of different APOE genotype on Aβ expression. *P < 0.05, compared with E2/E2; #P < 0.05, compared with E3/E3.

lipid transport [10]. APOE binding with LDL receptor in plasma can regulate lipid levels in peripheral blood. In the brain, APOE is mainly secreted by astrocytes and regulates the enrichment of cholesterol and phospholipids in CSF [11]. APOE has three different alleles. Different alleles appeared in the pathogenesis of AD [11]. Kim *et al.* found that different APOE genotype-expressing proteins produce different effects on lipid metabolism [17]. Different genotypes have different binding capacities with different lipoproteins, such as lower APOE2 and LDL receptors. The stability of the three alleles was also different. APOE4 often presents as the shape of a melting ball, which often has a highly pathogenic activity and can damage the membrane structure and improve the sensitivity of the protease [11].

There is still controversy about the mechanism of APOE4 in the pathogenesis of AD. A study observed that Aβ play a key role in the pathogenesis of AD and accelerate development of the disease [18]. Dardy's experiment exhibited that overexpression of Aβ leads to abnormal phosphorylation of Tau protein, thus damaging calcium ion homeostasis in neuronal cells and inducing neuronal apoptosis [19]. In addition, excess Aβ may form Aβ aggregates, while the formation of Aβ aggregates is an important phenomenon in the pathogenesis of AD [20]. Kuszczyk *et al.* observed direct binding of Aβ aggregates and APOE by electron microscopy [21]. It was found that the affinity of APOE4 and Aβ aggregates was the highest. Aβ aggregates in blood vessels and senile plaques were directly related to APOE4 expression [22]. In this study, we also found that in different genotype mice CSF, Aβ expression was highest in APOE4 genotype mice, which was consistent with other studies.

In this study, we analyzed the APOE genotype of AD patients and healthy controls. We found that E4 may be associated with susceptibility to AD and that the E4 genotype of APOE could be involved in the pathogenesis of AD through Aβ. However, this research did not analyze the expression of heterozygous APOE genotype and its effect on AD. Future studies may be required to investigate the expression of different APOEs and their relationship with Aβ accumulation and neuronal cell apoptosis by culturing hybrid mice.

Conclusion

The E4 genotype of APOE gene was correlated with AD. E4/E4 homozygous genotype may downregulate APOE expression level, but it may increase the expression and accumulation of Aβ to participate in the occurrence and development of AD.

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Disclosure of conflict of interest

None.

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