

Original Article

Association of pro-inflammatory cytokines gene polymorphisms with Alzheimer's disease susceptibility in the Han Chinese population

Hongmei Yue*, Wei Han*, Li Sheng

*Department of Neurology, The First People's Hospital of Jining, Jining 272000, Shandong, China. *Equal contributors and co-first authors.*

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Abstract: Objective: The progressive neurodegenerative process in Alzheimer's disease (AD) is accompanied by chronic inflammation, including activation of microglia and astrocytes that express pro-inflammatory cytokines. Up to now, numerous studies of genetic epidemiology have assessed the association of pro-inflammation cytokines gene polymorphisms and risk of AD in different populations, but conflicting results were obtained due to the heterogeneity of the genetic background among populations. Methods: Here, we recruited 248 AD patients, and 226 matched healthy controls from Shandong Province to evaluate the influence of IL-6 rs1800795, IL-12 rs3212227 and TNF- α rs1800629 polymorphism patients with AD. Single nucleotide polymorphism locus was genotyped using PCR-RFLP. Results: The genotypic and allelic frequency of TNF- α , IL-6 did not show significant difference between AD and normal controls. However, the frequency of wild (AA) and homozygous mutant (CC) genotype IL-12 rs3212227 genotypes in cases and controls was found more in controls (43% and 5.7% respectively), but that of the heterozygous genotype was higher (60.15%) in cases with AD patients. Conclusion: Though no any relationship between IL-6, and TNF- α genotypes or alleles and AD susceptibility was revealed, we first identified IL-12 rs3212227 AC genotype confer genetically susceptibility to AD in Chinese population.

Keywords: Alzheimer's disease, IL-6, IL-12, TNF- α , polymorphism

Introduction

Alzheimer's disease (AD), accounting for 60% to 70% of cases of dementia, is a chronic neurodegenerative disease that usually starts slowly and gets worse over time [1-3]. As a person's condition declines, they often withdraw from family and society. Gradually, bodily functions are lost, ultimately leading to death. The cause of Alzheimer's disease is poorly understood [4]. About 70% of the risk is believed to be genetic with many genes usually involved. Other risk factors include a history of head injuries, depression, or hypertension. The disease process is associated with plaques and tangles in the brain. The main pathological feature of AD is the presence of abnormally accumulated proteins and loss of neurons in specific brain regions [5]. The progressive neurodegenerative process in AD is ac-

companied by chronic inflammation, including activation of microglia and astrocytes that express pro-inflammatory cytokines. It affects about 6% of people 65 years and older [6, 7]. In 2010, dementia resulted in about 486,000 deaths and in developed countries, AD is one of the most financially costly diseases [8-10].

Recently, numerous findings have provided evidence that the inflammatory process is an important pathological factor associated with AD. Nucleotide variations in genes encoding inflammatory molecules, such as interleukin (IL)-1 β , IL-6, IL-12 and tumor necrosis factor- α (TNF- α) may influence their biological activities and thus might influence the risk of AD. For example, Both experimental and clinical data indicate that brain expression, plasma and cerebrospinal fluid levels of IL-6 may affect plaque formation [11, 12], cognitive decline or

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Table 1. Demographic characteristic of study population

Characteristic	Case	Control	P-value
Male [n (%)]	158 (63.71)	142 (62.83)	0.342
Age (years)	79.94 ± 8.120	78.46 ± 6.257	0.765
Age at onset (years)	70.01 ± 7.80		
MMSE	7.56 ± 4.67	28.26 ± 1.08	0.0026

dementia both in cross-sectional and longitudinal follow-up studies [13, 14]. The IL-6 gene in humans is located on chromosome 7 (7p21). The (-174C/G) also known (rs1800795) polymorphism in the promoter region of the IL-6 gene was reported to affect the IL-6 gene transcription rates and IL-6 plasma levels in AD patients implicating its role in development of AD [15-17]. Meanwhile clinical research has suggested that there is a correlation between the IL-12 SNPs and levels of serum IL-12 with disease severity in AD patients. Many studies have focused mainly on 3'UTR, -1188A/C (rs3212227) in the IL-12 gene [18].

While, several polymorphisms in the promoter region of TNF- α have been associated with different TNF- α expression levels. Of these, the TNF- α -308G/A (also referred to as rs1800629) is the best studied. It involves the substitution of a guanine (G) by an adenine (A) and is associated with an increase in TNF- α expression levels [19, 20]. Up to now, numerous studies of genetic epidemiology have assessed the association of pro-inflammation cytokines gene polymorphisms and risk of cancer in different populations, but conflicting results were obtained due to the heterogeneity of the genetic background among populations. Furthermore, this support the need for replication studies among all ethnic groups.

Taken together the association of pro-inflammation cytokines and AD, the aim of the present study was to evaluate the influence gene polymorphisms of IL-6, IL-12 and TNF- α on the susceptibility to AD patients in Chinese population.

Patients and methods

Ethics statement

The Medical Ethics Committee of the First People's Hospital of Jining approved this study. Written informed consents conforming to the tenets of the Declaration of Helsinki were ob-

tained from each participant prior to the study.

Participants

A total of 248 sporadic AD cases, and 226 matched healthy controls were recruited from Department of Urology, the First People's Hospital of Jining, between January 2013 and January 2015. All subjects are Han Chinese. A clinical diagnosis of probable AD fulfilled the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the AD and Related Disorders Association. The evaluation included medical, neurological and neuropsychological examination, interview with a close informant, laboratory testing and computed tomography or magnetic resonance imaging. The details of patients' evaluation are described comprehensively elsewhere [21]. No AD patients had a family history of dementia. The control group was recruited from the Healthy Examination Center of the Jining Municipal Hospital and confirmed healthy and neurologically normal by medical history, general examinations, laboratory examination, and Mini Mental State Examination (MMSE) score ≥ 28 . Informed consent was obtained from each subject or from the guardian. **Table 1** summarized the baseline clinical characteristics of the patients and control groups.

Genotyping

Genome DNA from whole blood cells of each sample was extracted by using Blood Genomic DNA Miniprep Kit (Axygen, USA) according to the manufacturer's instructions. DNA samples were stored at -20°C until analysis. Genotyping for the IL-6 -174G/C, IL-12 -1188A/C, TNF- α -308G/A polymorphisms in genomic DNA ([Supplementary Figure 1](#)) were performed using the PCR and restriction fragment length polymorphism (RFLP). The genomic region encompassing polymorphism was amplified using the following primers: IL-6 F: 5'-AC-TTTCCCTAGTTGTGTCTTTC-3', R: 5'-AGAAT-GAGCCTCAGACATCTCCAGT-3', IL-12 F: 5'-GGC-ATTCTCTCCAGGTTCTG-3', R: 5'-CCATGGCAACTT-GAGAGCTG-3' and TNF- α F: 5'-AGGCAATAG-GTTTTGAGGGCCAT-3', R: 5'-TCCCTGCTCCGATT-CCG-3'. Polymerase chain reaction products were generated in a 10 μ L reaction volume containing 50 ng of genomic DNA, 1 \times PCR buffer, 2

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Table 2. Genotype and allele frequency of IL-6 rs1800795, IL-12 rs3212227 and TNF- α rs1800629 and Pearson's chi-square test in AD patients and normal controls

Genotype/ Allele	Patients (n=248)	Controls (n=226)	P-value	OR (95% CI)
IL-6 rs1800795	HWE* P=0.25 HWE P=0.21			
GG	147	146	0.620	0.905 (0.626-1.320)
GC	83	69	0.077	0.822 (0.681-1.484)
CC	18	11	0.122	4.560 (0.556-37.412)
G	377	361	0.314	0.841 (0.498-1.192)
C	119	91		
IL-12 rs3212227	HWE* P=0.28 HWE P=0.75			
AA	106	81	0.0521	0.805 (0.526-1.220)
AC	127	140	0.025	1.749 (1.104-2.417)
CC	15	5	0.0519	0.972 (0.653-1.521)
A	339	302	0.275	0.571 (0.328-0.981)
C	157	150		
TNF- α rs1800629	HWE* P=0.48 HWE P=0.56			
GG	172	169	0.559	0.869 (0.542-1.392)
GA	68	51	0.921	1.258 (0.787-2.010)
AA	8	6	0.350	3.358 (0.347-32.543)
G	412	389	0.356	0.813 (0.524-1.267)
A	84	63		

*Chi-square test for deviation from the Hardy-Weinberg equilibrium (a value of $P < 0.001$ was regarded as a deviation from the HWE).

mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP, 1 μ mol/L of each primer, and 0.25 U of Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA). Cycling conditions consisted of an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds and a final elongation step at 72°C for 1 minute. Polymerase chain reaction products were digested with 2 U of NcoI restriction enzyme at 37°C, according to the manufacturer's instructions (New England BioLabs, Ipswich, MA). IL-6 the amplified fragment of 204 bp was cleaved into two fragments of 24 and 180 bp. The uncut product of 204 bp was identified as CC genotype; the GC genotype was revealed by two fragments of 204 and 180 bp, and the GG by a fragment of 180 bp. And the IL-12 PCR products resulted in either two fragments of 173 and 70 bp (allele C) or a single fragment of 243 bp (allele A). Finally, the -308G allele contains an NcoI restriction site not present in the -308A allele; thus, in the presence of the -308G allele, the PCR product (107 bp) is cut into 2 fragments of 80 and 27 bp in length.

Statistic analysis

Data were statistically described in terms of mean \pm standard deviation (SD), or frequencies (number of cases) and percentages as required depending on their distribution. The Hardy-Weinberg equilibrium (HWE) was assessed for each variation to identify the deviation. The differences of the genotypes and alleles of detected genes between patients and normal controls were evaluated by using Pearson Chi-square test. Exact test was used instead when the expected frequency is less than 5. The odds ratio (OR) and 95% confidence intervals (95% CI) were calculated. Unpaired Student's t test or

Mann-Whitney tests were used for two-group comparisons. Statistical analysis of data was performed using the SPSS software package 18.0 (SPSS Inc. USA). P -value less than 0.05 was considered statistically significant.

Results

In this study, 248 AD patients (108 males and 140 females) and 226 controls (112 males and 114 females) were screened for IL-6 rs1800795, IL-12 rs3212227 and TNF- α rs1800629 polymorphisms using PCR-RFLP methods. No statistically significant differences were observed in age (age at AD patients compared with age at examination for control subjects) ($P=0.342$) and gender ($P=0.765$) between AD patients and control subjects. In addition, MMSE scores were significantly lower in AD patients than in control subjects ($P < 0.001$).

Firstly, the frequencies of genotypes and alleles of IL-6 rs1800795, IL-12 rs3212227 and TNF- α rs1800629 were detected in case and control groups. HWE of rs1800795, rs3212227 and rs1800629 in patients and controls

were listed in **Table 2**, and the results showed allelic distribution of detected SNP were not deviated from HWE in both case and control populations. IL-6 rs1800795 in the study population were as follows: 7.4% CC, 33.5% CG and 59.1% GG for the case study group and 4.9% CC, 30.4% GC, and 64.7% GG for the controls, indicating that the genotypes distributions were similar between the cases and the control groups. Also, genomic analysis did not reveal a difference between AD patients and healthy controls in allelic frequency at the -174 position for the IL-6 gene promoter. Similarly, the genotypic and allelic frequency of rs1800629 did not show significant difference between AD and normal controls. Then, Genotype and allele frequency of rs1800629 were detected in AD patients and normal control (**Table 2**). The genotypic and allelic frequency of rs1800629 between cases and health controls did not show significant difference.

Whereas, the frequency of wild (AA) and homozygous mutant (CC) genotype IL-12 rs321-2227 genotypes in cases and controls was found more in controls (43% and 5.7% respectively), but that of the heterozygous genotype was higher (60.15%) in cases with AD patients. Significant risk of AD was observed for AC (OR=1.74, 95% CI=1.10-2.41, $P=0.025$) genotype of IL-12. The genomic analysis did not reveal differences in allelic frequencies of the IL-12 (A/C) gene AD patients and healthy controls.

Discussion

The gene single nucleotide polymorphisms (SNP) have been thought to alter expressions or influence certain genes; thus, SNPs could be associated with an altered risk of multiple disease [22-28]. Up to now, the important role of pro-inflammatory cytokines during tumor development and prognosis are increasingly gaining interest. Several lines of evidence point to the involvement of interleukin-6 (IL-6) in pathogenesis of AD. Both experimental and clinical data indicate that brain expression [29], plasma [30-32] and cerebrospinal fluid levels of IL-6 may affect plaque formation, cognitive decline or dementia both in cross-sectional and longitudinal follow-up studies [33-35].

We did not find any evidence of an association between the IL-6 (-174C/G) polymorphism and

AD in the China population sample. The distribution of the studied polymorphism was similar to that observed in countries at the same geographic latitude, but different when geographical longitude was considered [36]. Previous studies (12 case-control and 2 prospective studies) assessing the connection between the IL-6 polymorphism and the risk of AD brought equivocal results. Faltraco et al. [37] reported risk reducing association of the IL-6 C allele in AD. Pola et al. [38] found that the G/G polymorphism was associated with increased risk of AD. In other studies on Italian populations the IL-6 C allele increased the risk of AD [39, 40], the C/C genotype increased the risk of AD in women [41], and the G/G genotype was lower in AD than in healthy controls [42]. Differences in study design and the geographical variations of IL-6 frequency may in part explain the different patterns of association between this polymorphism and AD in various studies. In most studies patients with AD had neuropsychological examination but in controls no test battery was used, apart from MMSE; therefore some cases with incipient AD might have been included. Moreover, all studies published to date analyzing the role of the IL-6 (-174C/G) polymorphism in AD have been underpowered. That is why we performed a meta-analysis assessing the significance of the studied polymorphism on all available data from 3107 AD patients and 10 014 controls. This meta-analysis, however, was not able to show the significance of the IL-6 (-174C/G) polymorphism for the risk of AD [31].

IL-12 is an important antitumor cytokine that plays important role in the development and progress of cancer. Variation in the DNA sequence lead to altered IL-12 production, and this can alter individual's susceptibility to cancer. The IL-12 3'UTR A>C polymorphism is a functionally important SNP that alters IL-12 production and it has been a reported potential biomarker for risks of numerous disease, such as hepatitis, psoriasis, Barrett's esophagus, asthma, and arteritis. More importantly, genetic variation in IL-12 was revealed to affect susceptibility to multiple sclerosis, another neurodegenerative disease with evident inflammatory responses. Considering the potential role of IL-12 in AD pathogenesis as well as the involvement of IL-12 polymorphisms in the predisposition to many inflammatory diseases. In our study, increased frequency of IL-12 rs3212227 AA

and CC homozygous genotype among controls but that of the heterozygous AC genotype was higher in cases with AD; thus, a significant risk of AD was observed for AC genotype of IL-12 rs3212227, which is consistent with the results of Zhu [43].

TNF- α gene is located in the class III region of the human major histocompatibility complex (MHC) on chromosome 6p21 [44, 45]. Among the several single nucleotide polymorphisms (SNPs) identified in TNF- α , TNF- α rs1800629 is the most extensively studied. The A allele of this polymorphism can lead to high binding affinity of nuclear factors to the TNF promoter, resulting in a high level of transcription activity and secretion levels of TNF- α . So, it was suggested to have a significant functional effect [46]. A variety of SNPs located in the promoter region of TNF- α gene has been investigated in AD patients by different groups with contrasting results. So, The aim of the present study was to better define the role of TNF- α polymorphisms in AD; In the present study, our results suggested that the genotypes distributions of TNF- α rs1800629 was almost the similar in the cases and the control groups. In previous study, the rs1800629 was mainly investigated in the Caucasian population and African American patients with a variation of A allele frequency from 13% to 29% with an OR = 1.05 from all Caucasian studies [47-49]. Recently, Wang and colleagues reported the single analysis of this SNP in Asian population with frequency of A allele (11%) slightly minor in respect to Caucasian sample, but with a positive result (OR=1.67) probably due to the very rare A allele frequency in controls (7%) [50].

Our results indicate a lack of association between pro-inflammatory cytokines SNP and AD in our China sample, suggesting that genetic, clinical and population heterogeneity are probably responsible for conflicting results in association studies.

Conclusion

In summary, though no any relationship between IL-6, and TNF- α genotypes or alleles and AD susceptibility was revealed, we first identified IL-12 rs3212227 AC genotype confer genetically susceptibility to AD in Chinese population.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Li Sheng, Department of Neurology, The First People's Hospital of Jining, 6th Health Road, Jining 272000, Shandong, China. Tel: 86-537-2253423; E-mail: lishengjin@sina.com

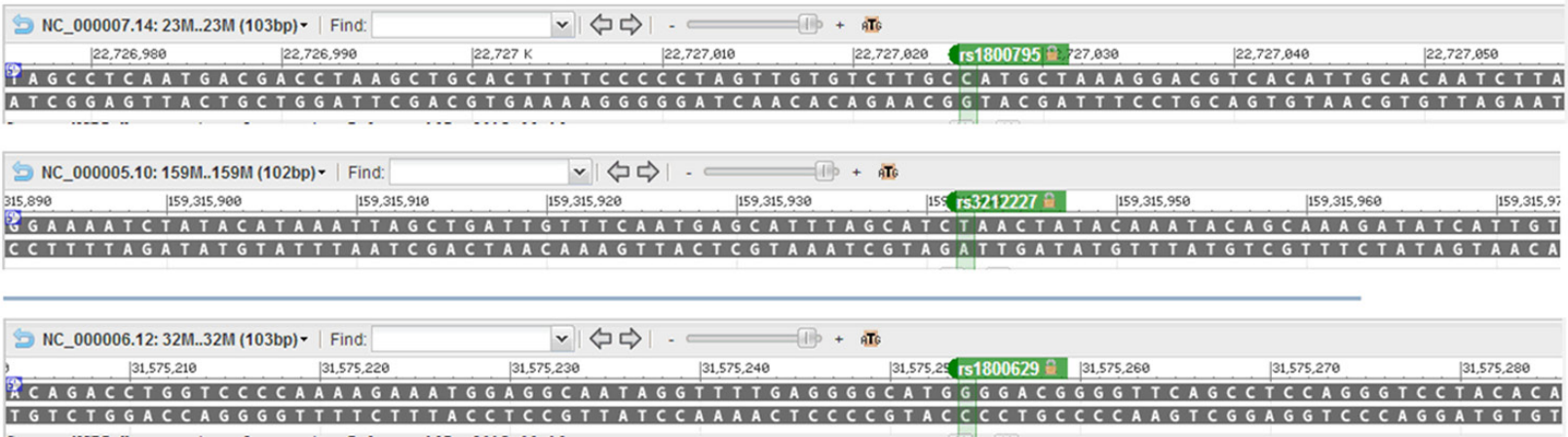
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Supplementary Figure 1. Genotype pictures for IL-6 rs1800795, IL-12 rs3212227 and TNF-α rs1800629 polymorphisms.