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Study of apolipoprotein E genetic polymorphism in patients with atherosclerotic cerebral infarction*

LUO Ben-yan(罗本燕)†[1], CHEN Zhi(陈智)[2], CHEN Feng(陈峰)[2], LI Xia(李霞)[1], PAN Xiao-ping(潘小平)[2]

([1] *Department of Neurology*; [2] *Institute of Infectious Diseases, The First Affiliated Hospital, Medical College, Zhejiang University, Hangzhou 310003, China*)

†E-mail: lobenyan@263.net

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Abstract: Objective: To explore the frequency and significance of ApoE gene polymorphisms in Chinese patients with atherosclerotic cerebral infarction (ACI). Methods: Polymerase chain reaction and gene sequencing, single nucleotide polymorphisms of ApoE gene were used to analyze 33 cases of patients with ACI and 35 controls. Results: The frequencies of ApoE gene single nucleotide polymorphisms 465C/G, 462C/G and 451delC in the ACI group were significantly higher than those in the control group ($P < 0.05$). The prevalence of polymorphism 486G/T in the control group was significantly higher than that in the ACI group ($P = 0.011$). Conclusions: 465C/G, 462C/G and 451delC polymorphisms might be associated with ACI. 486GT allele might have protective effect on the pathogenesis of ACI.

Key words: Cerebral infarction, Atherosclerotic, Genetics, ApoE allele, Single nucleotide polymorphism
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INTRODUCTION

Atherosclerotic Cerebral Infarction (ACI) is one of the most common cerebral vascular diseasse. Its pathogenesis has not yet been completely expounded, although more and more studies showing that genetic factors may play an important role, especially genetic mutations. Located at No.19 chromosome long arm, Apolipoprotein E (ApoE) gene is an important gene involved in lipid metabolism. ApoE, a product of its coding, participates in the transport and metabolism of cholesterol in the body and plays a key role in the formation and repair of the synapses. This study is an attempt to analyze the single nucleotide polymorphism (SNP) by examining the ApoE gene sequence of patients with ACI in order to find the SNP loci associated with the pathogenesis of atherosclerotic cerebral disease.

SUBJECTS AND METHODS

Subjects

ACI group: A total of 33 cases with atherosclerotic cerebral infarction were chosen from patients who came to the hospital from Nov.2001 to Mar. 2002, included 19 males and 14 females, with average age of 68.31 ± 10.07. All the cases were firmly diagnosed by cranial CT or MRI and other examinations according to the diagnostic criteria formulated by the 4th National Academic Conference on Cerebral Vascular Diseases, excepting tumor, hemorrhagic cerebral vascular diseases and ischemic stroke cases resulting from other causes.

Control group: 35 healthy volunteers (averaging 65.37 ± 10.94 in age) selected for the study, 17 were males and 18 were females. None of them had a history of cerebral vascular disease or endocrine diseases.

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There was no significant difference in age and sex between the two groups($P > 0.05$).

Exclusion criteria: Subjects who had tumor, dementia, diabetes, familial hypercholesterolemia, dysfunction of heart, liver and kidney were excluded from the study.

Methods

Extraction of DNA

Procure: Three ml of blood sample (Conc, 1mg/ml) was into an EDTA anticoagulant-containing tube for 1 hr and then centrifuged; after which the plasma was removed to leave blood cells for extraction of whole blood genomic DNA according to the procedure described in the instructions sheet (in the whole blood genomic DNA extraction Kit purchased from the Shanghai Bo Cai Bio-technology Co., Ltd). The obtained genomic DNA was then dissolved in a TE buffer solution and preserved at -20℃ for future use.

PCR reaction's conditions

Designing the primers for synthesis of ApoE gene:

A1: 5'-ACAGAATT-CGCCCCGGCCTGG-TACAC-3',

A2: 5'-TAAGCTTGG-CACGGCTGTC-CAAGGA-3'.

PCR reaction's total volume: 50 μl, containing 50 – 100 ng template DNA, 2 mmol/L dNTP, each of up-and-down-stream primers: 20 pmol, 50 mmol/L KC1, 1.5 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl, pH8.3, 1.5u Taq DNA polymerase.

95℃ pre-degeneration for 3 min, 35 cycles (94℃, 45 s, 62℃, 45 s, 72℃, 30 s), 72℃ extension 8 min, 4℃ holding. The PCR amplification product was identified by agarose gel electrophoresis, its length was 244 bp.

Sequencing of the ApoE gene PCR amplification product

The above PCR amplification product was purified with a DNA extraction kit according to the procedure described in the instruction sheet. After being quantitated, the product was used for sequencing by an automatic sequencer (MegaBase 1000, Pharmacia, USA) with a sequencing reagent kit (DYENAMIC™ ET dye terminator Kit, MegaBase USA), the sequencing primer being primer A.

The normal sequence of ApoE gene is reported in McLean *et al*.(1984).

Data processing

X^2 test was used to compare the distribution frequencies of each SNP locus between the two groups. The level of significance was set at $P < 0.05$.

RESULTS

ApoE gene PCR amplification product: see Fig.1.

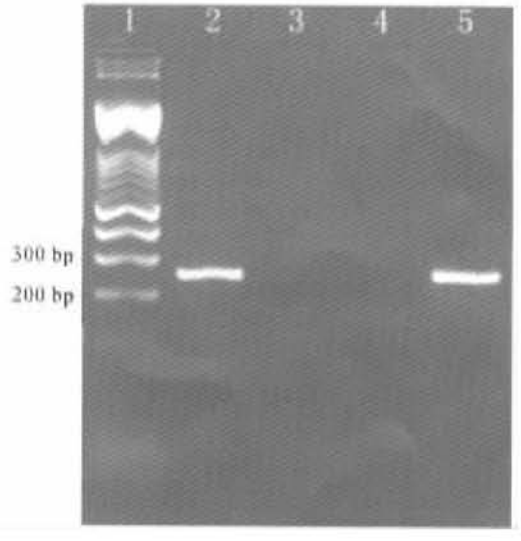

Fig.1 ApoE gene PCR amplification product

ApoE gene sequencing spectrum: see Fig.2.

ApoE genetic single nucleotide polymorphism analysis:

The results of ApoE genetic sequencing and analysis of its single nucleotide polymorphism were used to compare the distribution frequencies of each SNP locus between the AC1 group and control group. Statistical results showed that there was significant difference in the frequencies of the 4 SNP Loci (i.e. 486 G/T, 465 C/G, 462 C/G, and 451 delC) occurrence between the two groups.

DISCUSSIONS

ApoE is an important serum lipoprotein participating in the transport of cholesterol and in the metabolism of low-density lipoprotein (LDL) in the body. The genetic mutation of ApoE gene results in ApoE functional changes which leads

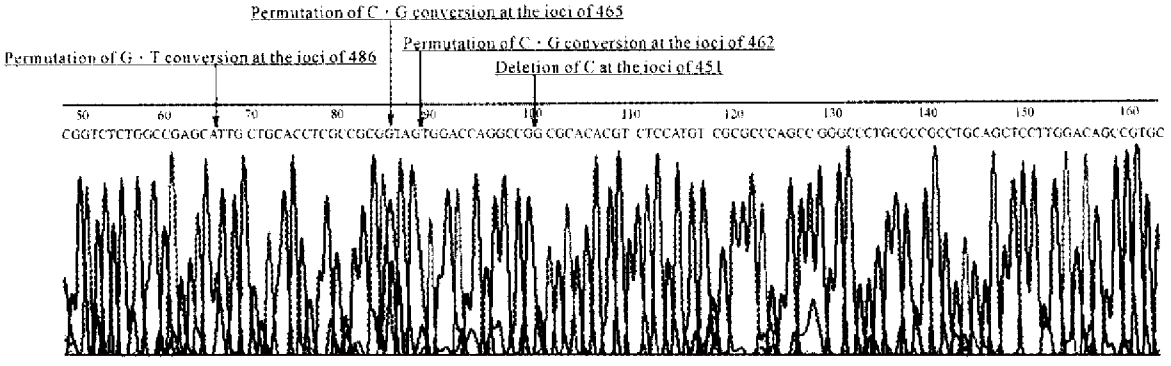

Fig.2 ApoE gene sequencing spectrum

Table 1 Frequencies and distribution of apoE genetic single nucleotide polymorphism (SNP) loci in ACT group and control group

	Single nucleotide polymorphism loci (%)									
Group	530del G	517del C	507del C	486G/T	472C/T	465C/G	462C/G	451del C	441del C	432del C
ACI	69.56	30.77	27.59	40.00	45.16	28.12	28.12	33.33	42.42	39.39
Control	58.04	22.58	34.37	71.87	36.36	6.06	3.03	11.43	60.00	45.71
X^2	0.749	0.489	0.327	6.399	0.513	5.626	6.050	4.740	2.101	0.277
P value	0.387	0.484	0.567	0.011	0.474	0.018	0.014	0.029	0.147	0.598

to the elevation of serum total cholesterol and low density lipoprotein cholesterol (LDC-C) levels and accelerates the development of arteriosclerosis. ApoE in the brain is mainly synthesized by star-shaped cells and is associated with the metabolism and redistribution of cholesterol and phospholipid in the process of the formation of synapses, and participates in the repair and reformation of neurons and directly influences the growth of synapses. Therefore, ApoE genetic polymorphism confers risk of atherosclerosis (Siest *et al.*, 1995) and influences the clearance and repair of injured tissues following an ACI (Kitagawa *et al.*, 2001).

This study involved analysis of the ApoE genetic sequences in patients with ACI and in normal persons; and revealed four significant SNP loci, i.e. 486 G/T, 465 C/G, 462 C/G, and 451 del C. The results of the study revealed that over 28% of the patients with ACI had a genetic mutation of C→G conversion at the loci of 465 and 462 nucleotide of ApoE gene, while among the 35 cases in the control group no such mutation occurred at the corresponding loci ($P < 0.05$). The results suggested that the 465 C/G, 462 C/G polymorphism loci were closely associated with the pathogenesis of ACI. It is worth noting that among approximately 40% of the patients with ACI, the nucleotide at locus 486 was a GT heterozygote, while the 486 GT heterozygote carriers accounted for about 71.87% of the healthy controls. The distribution frequency of 486 GT in the control group was significantly higher than that of the ACI group ($P = 0.011$). This result suggested that 486 GT allele might have a protective effect to some extent on the pathogenesis of ACI. In addition, we have not found 486 TT homozygote in the ApoE genetic sequence determined in this study. However, from the results of the study, we could infer that the possibility that ACI in 486 TT homozygote carriers may probably be lower. Furthermore, statistical analysis showed that the incidence of ApoE genetic mutation at locus 451 nucleotide C deletion in patients with ACI was higher than that of the control group, and that there was significant difference between the two groups ($P = 0.029$); which suggested that 451 del C polymorphism may be associated with the pathogenesis of ACI to some extent.

In recent years, there have been several published reports of studies of ApoE genetic polymor-

phism and its relation with ACI. However, these studies mainly dealt with ApoE genotype analysis based on restriction fragment length polymorphism (RFLP), i.e. according to the difference of the residues at loci 112 and 158 of ApoE amino acid sequence which could be divided into three kinds of alleles, i.e. Apo ε_2, ε_3, ε_4; and with analysis of the distribution frequencies of these genotypes in the normal population and in patients with ACI. McCarron (McCarron *et al*., 1999) summarized 9 papers about cases controlled studies reported on Medline before 1999. He considered Apo ε_4 allele is a strong risk factor in the pathogenesis of ACI. However, MacLeod *et al*. (2001) considered that there is lack of association between ApoE genotypes and ACI. So the relationship between ApoE genotypes and ACI is still controversial. This may be related to the difference of geographical area of the subjects as well as racial difference.

Actually the manifestation of ApoE genetic polymorphism was not only limited to the amino acid variation at loci 112 and 158 but also to the mutations such as the conversion, insertion, and deletion of single base, which is widely distributed in the genetic sequence, could not be neglected also. SNP, as a kind of genetic polymorphism, is characterized by its wide distribution, large number and being easy-to-examine in batches (Wang *et al*., 2001). Disease-association analysis based on SNP has been widely used in different fields of life science. The search of SNP locus associated with ACI will lay a foundation for the research and development of ACI genetic chips in future.

Because ApoE genetic polymorphism may be associated with a variety of diseases such as Alzheimer's disease (AD) (Martin *et al*., 2000) and cardio and cerebral vascular diseases, its genetic SNP analysis has drawn more and more attention. Presently, the ApoE genetic SNP databank has gradually been strengthened and a few SNP loci have been reported, including SNP 517, SNP 507, SNP 465 and SNP 462 determined in this study. However, all the loci reported above originated from the study of Alzheimer's disease (AD); the analysis and research on ApoE genetic SNP remain a blank.

In recent years, the morbidity of ACI and other cerebral vascular diseases among the middle aged and elderly population has increasingly risen year by year. Early diagnosis, and prevention and treatment of cerebral vascular diseases have become a problem calling for urgent solution. This study has filled in the blank in this field. We discovered three ApoE genetic SNP loci i.e. 465 C/G, 462 C/G and 451 del C, which may be associated with the pathogenesis of ACI and one SNP locus (486 GT) which might have a protective effect on the pathogenesis of ACI. This provided valuable data for the study of genetic diagnosis and gene therapy for the future. We convince that in the near future the presence of ACI genetic chips based on ApoE genetic SNP loci will bring good news to many patients with ACI for their disease can be diagnosed and treated at an early stage.

References

Kitagawa, K., Matsumoto, M., Kuwabara, K., Ohtsuki, T. and Hori, M., 2001. Delayed, but marked, expression of apolipoprotein E is involved in tissue clearance after cerebral infarction. *J Cereb Blood Flow Metab*, **21** (10): 1199 – 1207.

MacLeod, M. J., De Lange, R. P., Breen, G., Meiklejohn, D., Lemmon, H. and Clair, D. S., 2001. Lack of association between apolipoprotein E genoype and ischaemic stroke in a Scottish population. *Eur J Clin Invest*, **31** (7): 570 – 573.

Martin, E. R., Lai, E. H., Gilbert, J. R., Rogala, A. R., Afshari, A. J., Riley, J., Finch, K. L., Stevens, J. F., Livak, K. J., Slotterbeck, B. D., Slifer, S. H., Warren, L. L., Conneally, P. M., Schmechel, D. E., Purvis, I., Pericak-Vance, M. A., Roses, A. D. and Vance, J. M., 2000. SNPing away at complex disease: analysis of single-nucleotide polymorphisms around ApoE in Alzheimer disease. *Am J Hum Genet*, **67**(2): 383 – 394.

McCarron, M. O., Delong, D. and Alberts, M. J., 1999. ApoE genotype as a risk factor for ischemic cerebrovascular disease: a meta-analysis. *Neurology*, **53**(6): 1308 – 1311.

McLean, J. W., Elshourbagy, N. A., Chang, D. J., Mahley, R. W. and Taylor, J. M., 1984. Human apolipoprotein E mRNA. cDNA cloning and nucleotide sequencing of a new variant. *J Biol Chem*, **259**(10): 6498 – 6504.

Siest, G., Pillot, T., Regis-Bailly, A., Leininger-Muller, B., Steinmetz, J., Calteau, M. M. and Visvikis, S., 1995. Apolipoprotein E: an important gene and protein to follow in laboratory medicine. *Clin Chem*, **418**: 1068 – 1086.

Wang, D. G., Fan, J. B., Siao, C. J., Berno, A., Young, P., Sapolsky, R., Ghandour, G., Perkins, N., Winchester, E., Spencer, J., Kruglyak, L., Stein, L., Hsie, L., Topaloglou, T., Hubbell, E., Robinson, E., Mittmann, M., Morris, M. S., Shen, N., Kilburn, D., Rioux, J., Nusbaum, C., Rozen, S., Hudson, T. J. and Lander, E. S., 1998. Large-Scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome. *Science*, **280**(5366): 1077 – 1082.