Relationship between Alzheimer's disease and mitochondria coenzyme II Gene

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Abstract: We aimed to investigate the relevance between Alzheimer's disease (AD) and gene mutations of mitochondrial cytochrome oxidase subunit III (COX3) and coenzyme II (ND2), and to provide genetic markers for the diagnosis of Alzheimer's disease (AD) and further provide some feasible basis for preventive treatment. Polymerase chain reaction-restriction fragment length polymorphism technique was used, and genotypes and gene frequencies were detected in 60 patients with Alzheimer's disease (AD), who meet the ICD-10 diagnostic criteria (AD group), 10 AD families and 60 normal old people (control group). (1) Gene variation on nt5460 gene locus of mitochondria ND2 of the patient group is G→A, and the variation rate is 13.3%, P=0.006 < 0.05. Gene variation G→A of the patient group perfomred statistical significance. (2) In the families, it is also found that in the gene variation of G→A, the variation rate is 33.3%, P>0.05. There is obvious gene variation in the families, but this variation does not perform statistical significance. (3) There is no gene variation on nt9861 gene locus of mitochondria COX3 of the patient group. Gene variation of T→C is not found both in the patient group and the control group. There is possible a gene mutation of G→A on nt5460 gene locus of mitochondria ND2 of the AD patients. Although gene mutation of G→A is found in the families, it performed no statistical significance. At the same time, it is found that there is no relation between AD patients and Gene variation of T→C on nt9861 gene locus of mitochondria COX3.

Keywords: AD, mitochondrial gene, gene mutation, DNA sequencing.

INTRODUCTION

Previous studies [Cavelier, et al., 1995] detected that the activity of cytochrome oxidase in the brain of patients with Alzheimer's disease was about 30% lower than that of normal value and COX subunit I and III (COX1, COX3) were encoded by mitochondria DNA (mtDNA). A researcher [Wallale, 1992] found that there were locus mutations on gene COX1 and COX3 through enzyme digestion method and amplification method, and the mutated genes were C→T, A→G, T→C, G→A, etc.. Research reports at home and abroad indicated that gene mutation of G→A or T existed on nt5460 gene locus of mitochondria II (ND2) and gene mutation of T→C existed on nt9861 gene locus of mitochondria COX3. This study mainly uses case-control study and method of relevance of patient family and selects nt9861 gene locus of mitochondria COX3 and nt5460 gene locus of mitochondria ND2 to explore the relevance between Alzheimer's disease (AD) and the change of mitochondria DNA (mtDNA), with the purpose of providing biochemical markers for the diagnosis of Alzheimer's disease (AD) and that Alzheimer's disease (AD) can be found early and given immediate prevention and control.

MATERIALS AND METHODS

Materials

Patient group: AD patients who had been hospitalized and accepted outpatient treatment in Xi'an Mental Health Center during the year 2010 to 2011. There were 60 patients meeting with the AD diagnostic criteria in ICD-10, including 32 male patients and 28 female patients. The age range of the patients was from 61 to 83 years old. Patients with pseudo dementia, dementia caused by other reasons and mental retardation were excluded. Five cases with positive family history were selected from the remaining patients. 18 cases from the selected five patients' family were collected (including family members with AD symptoms or with no AD symptoms). Before entering the study, all selected patients and family members were asked for agreement. Ethical approval was given by the Medical Ethics Committee of Shaanxi Xi'an Mental Health Center.

Control group: 60 normal people were selected as controls from the national key gene laboratory resource center from forensic department of Xi'an Jiaotong University College of Medicine. The ages of 60 patients were from 60 to 80 years old and there were 30 males and 30 females among the control group. There were no other major body diseases, genetic diseases and history of mental illness among people in control group who had normal intelligence quotient (ID).

Methods

Tools: ICD-10 dialogistic criteria and scales such as SDH (Hascgawa dementia scale), MMSE (Mini mental status examination) and CCSE (Cognitive capability screening

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examination), etc. were employed for selection of cases; HIS (Hachinski Inchemic Score) was employed for screening of cases.

DNA extraction: each person was drawn with 3 ml of elbow venous blood and the blood was kept at -20°C, then chelex100 method was employed for DNA extraction: 100ul venous blood was taken from the centrifuge tube. 1ml deionized double-distilled water was added into the blood and it was shaken for 5-10 seconds at high speed. It was kept for 10 minutes at room temperature. After 2 minutes of centrifugation under 15000rpm, the supernatant was discarded. The above three steps were repeated once again. 100µl suspended 5% Chelex100 solution was added and it was shaken for 5-10 seconds at high speed. It was kept in 56°C water for 20 minutes, then it was shaken for 5-10 seconds. After 8 minutes of 100°C warm bath, it was shaken for 5-10 seconds. After 2 minutes of centrifugation under 15000rpm, it was kept under 4°C in reserve.

Primer design: the primers were designed voluntarily according to the standard sequence. When the primer sequence was nt5460, the upstream primer was 5'-CTAACCGGCTTTTTGCCC-3' and the downstream primer was 5'-ACCTAGAAGGTTGCCTGGCT-3'; the length of PCR was 84 bp; the upstream primer of nt9861 was 5'-CCCACCAATCACATGCCTAT-3' while the downstream primer was 5'-TGTAGCCGTTGAGTTGTGGT -3' and the length of PCR was 940bp.

Mitochondria DNA amplification: reaction system of PCR was 30µl and the reaction mixture included following components: 6pmol of 3' end and 5' end primers respectively, 2 X pfu PCR MIX 15µl, 10ng DNA template.

Cycle parameter of PCR for nt5460 was: it was pre-denatured for 5 minutes at 95°C; it was denatured for 30 seconds at 94°C; it was annealed for 30 seconds at 55°C; it was extended for 1 minute and 20 seconds at 72°C; there were 35 cycles in all. After fully extension for 7 minutes at 72°C, it was kept at 4°C. Cycle parameter of PCR for nt9861 was: it was pre-denatured for 5 minutes at 95°C; it was denatured for 30 seconds at 94°C; it was annealed for 30 seconds at 56°C; it was extended for 1 minute and 20 seconds at 72°C. There were 35 cycles in all. After fully extension for 7 minutes at 72°C, it was kept at 4°C.

mtDNA PCR amplification and the purified product were conducted for agarose gel electrophoresis, in which the gel concentration was 2% agarose gel (including 05µg/ml EB), the interelectrode distance was 15cm and 4µl sample was added (3µl sample and 1µl bromphenol blue). The deposition condition was 20 minutes under 120V and the electrode buffer was 1XTAE, the result was recorded by gel instant imaging system.

Purification of PCR amplified product used millionpore 96 PCR purification plate: TE buffer was added into PCR product and the total volume was adjusted to 100ul. The solution was added into MILLIPORE Multiscreen (R) PCR96 PLATE. PCR96 PLATE was put on the vacuum pump, and the negative pressure value was adjusted to -20inches Hg until the solution in the hole was sucked out to dry. 50ul TE buffer was added into each holes to clear and then it was extracted to dry. 30ul deionized water was added to dissolve and it was kept for 30 minutes after shaking, then the solution was suck out. The purified DNA was kept at 4°C or -20°C in reserve. Purified PCR product was kept for more than 12 hours at -20°C and for less than 12 hours at 4°C. DNA in agarose gel electrophoresis product was detected in accordance with criteria and DNA content in samples was calculated and recorded accurately.

Sequencing for mitochondria DNA: the reaction system was 10ul and the reaction mixture contains Big-Dye Terminator Ready Reaction mix 3.1 (fluorescence labeling ddNTP, dNTP, TaqDNA polymerase, MgC12, Tris-HCL buffer; PH 9.0) 0.7µl, 5' or 3' extremity primer was 0.3µM, the PCR amplified product was 10ng. The reaction condition was 10 seconds at 96°C, 5 seconds at 50°C, 4 minutes at 60°C, and there are 25 cycles in total.

The purification of product after the sequencing reaction: 12.5µl (100% alcohol: 3M sodium ethoxide = 37.5ml: 2.5 ml) mixed liquid was added to the pipe bottom of each tube, it was shoken and blended. It was centrifuged for 30 minutes at 4°C with 4000 rpm. The 96 pore plate was inverted immediately, and it was centrifuged to 500 rpm and then it was stopped. 25µl of 70% alcohol was added to each pore and it was centrifuged for 15minutes at 4°C with 4000rpm. The 96 pore plate was inverted immediately, and it was centrifuged to 500rpm and then it was stopped. The above two steps were repeated once again. The alcohol was vaporizeed clean at room temperature and it was dried in reserve.

mtDNA sequencing analysis: 10µl Hi-Di Formamide which had been centrifuged and dried after purification was added into centrifuge tube. After denaturation for 10 minutes at 95°C, it was placed on the ice immediately. It was moved into 96 pore upper sample plate after 5 minutes. Electrophoresis determination: the collection software Data Collection 2.0 of the 3730 type DNA sequencer was opened and the sample form was compiled, and the sample information was typed in, then it was saved. The equipment was set as standard sequencing program (default). The 96 pore upper sample plate was put into the 3730 type DNA sequencer and the sample was placed for electrophoresis. After the

Table 1: The gene variation comparison between patient group and control group

	Variation cases (A)	No variation cases (G)	Aberration rate (%)
Patient group	8	52	13.3
Control group	0	60	0.00
Total	8	112	6.67
X^2 value	8.57		
P value	0.006<0.05		

electrophoresis was finished, the files were saved for next analysis.

Data processing and sequence analysis

The Sequence Analysis 5.1 software was opened and the files to be analyzed were loaded.

The software was commanded for automatic typing.

After generating the corresponding sequence analysis file, it was saved for further analysis.

Sequence alignment

The blast alignment software was used to compare the sequence information of samples obtained after sequence analysis and the Anderson sequence (the determination of base sequence in the total length of mtDNA had been completed for the first time in 1981, thus the sequence reported by Anderson was regarded as standard sequence) in order to show the variant base.

The SPSS 13.0 statistical software was used for data processing. The Hardy-Weinberg heritage equilibrium test and $\chi 2$ test were used to test the genetype of the patient group and the control group, and the difference of each allele gene frequency was compared, the $P<0.05$ was regarded as the statistical difference.

RESULTS

1. Conduct Hardy-Weinberg genetic equilibrium test for the genetype in family and allele gene, the genetype A/A, A/G, G/G and T/T, T/C, C/C was in good equilibrium coincidence.

2. The variation of the nt5460 genetic locus of the mitochondria ND2.

The experimental result showed that the base of 60 cases of control group was G, and the G base took 100%; in the 60 cases, base of 8 cases was A, taking 13.3%; the base of other 52 cases was G, taking 86.7%. The gene variation was G→A and the aberration rate was 13.3%. Upon test, $\chi 2=8.57$, $P=0.006<0.05$, and the gene variation G→A of patient group has statistical significance.

Among all these six patient families, two of them had gene variation G→A, and the other four families had no relationship to this condition, therefore the variation rate of which was 33.3%, the x2 examination result was 2.4 and $P>0.05$. Although an obvious gene variation was found in the families, it had no statistical significance.

3. Variation of the nt9861 gene locus on mitochondria COX3

The experimental result showed that the base of all the 60 cases of control group was T which took 100%; the base of all the 60 cases of patient group was T which took 100%. T→C variation of gene had not been found.

DISCUSSION

AD (Zhao and Chen, 2004) is a progressive, degenerative encephalopathy syndrome and the clinical symptoms mainly are cognitive decline and non-cognitive mental symptom. previous studies are mainly in the field of neuroscience, our study objects are Alzheimer's disease patients who were cognitive decline and psychiatric symptom in psychiatry. The pathogenesis of Alzheimer's disease is mainly considered as genetic and environmental factors. A lot of studies have indicated a close relationship between energy metabolism and decay in [Li et al., 2007], and the mitochondria is the main device to get energy in the living body, the important process of the material energy conversion such as the tricarboxylic acid cycle and oxidative phosphorylation are all carried out in mitochondria, in the processing of this kind of energy conversion, the cytochrome oxidase (COX) and oxidation-reduced coenzyme II (ND2) all play a very important role. In addition, damage and mutation of DNA will increase with rising age. The mitochondria DNA is lack of histone protection and the repair system of nucleic acid, and more sensitive to environment and the damage of free radicals, so its mutation is higher than the nuclear DNA mutation incidence rate. The mitochondrial diseases caused by the mitochondria DNA gene mutation include the oxidation respiratory chain disease, fatty acid metabolism disease and glycogenosis. The symptoms of the nervous system and the involvement of the muscle system are the most common; central nervous system involvement can lead to encephalopathy, dementia, parapoplexy and etc. (Zhao, 2007).

Our study result shows that the nt5460 of DN2 in mitochondria DNA of AD patient has the G→A gene mutation, and its mutation has statistical significance, and it is basically comply with the ne5460 exist G→A or T gene mutation ND2 of previous study report (3), which indicates that the main mutation is G→A rather than the mutation of G→T, and it is also pointed out that maybe AD patients have the G→A gene mutation of nt5460

locus in ND2, according to this mutation, the diagnosis of AD can be used as a basis or not.

Although the G→A mutation is also found in this family study, it has no statistical significance. It is likely because that the AD is a multiple-factor inheritance disease, and its morbidity is caused by various factors. Because there are too few studies on the families, it is necessary to expand the number of family for further study and genetic analysis. There is no gene T→C mutation of the nt9861 gene locus in COX3 during this study, which indicates that the AD patients have no relationship to T→C mutation of the nt9861 locus in COX3, but it is not comply with the previous report that T→C gene mutation exists on nt9861 of COX3 (2). It is likely because that the objects we select are AD patients with psychogeny and those objects are different from previous study objects. In addition, the experimental method in this study is method of designing T→C primer and direct sequencing, which is different from nt5460 method; DNA extracted in this experiment is DNA fragment but not the complete DNA. The study needs to be continued in the future.

In summary, there is possible a gene mutation of G→A on nt5460 gene locus of mitochondria ND2 of the AD patients. Although gene mutation of G→A is found in the families, it has no statistical significance. At the same time, it is found that there is no relation between AD patients and Gene variation of T→C on nt9861 gene locus of mitochondria COX3.

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