

# A non-invasive, rapid method to genotype late-onset Alzheimer's disease-related apolipoprotein E gene polymorphisms

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## Abstract

The apolipoprotein E gene  $\epsilon 4$  allele is considered a negative factor for neural regeneration in late-onset Alzheimer's disease cases. The aim of this study was to establish a non-invasive, rapid method to genotype apolipoprotein E gene polymorphisms. Genomic DNA from mouth swab specimens was extracted using magnetic nanoparticles, and genotyping was performed by real-time PCR using TaqMan-BHQ probes. Genotyping accuracy was validated by DNA sequencing. Our results demonstrate 100% correlation to DNA sequencing, indicating reliability of our protocol. Thus, the method we have developed for apolipoprotein E genotyping is accurate and reliable, and also suitable for genotyping large samples, which may help determine the role of the apolipoprotein E  $\epsilon 4$  allele in neural regeneration in late-onset Alzheimer's disease cases.

**Key Words:** nerve regeneration; neurodegeneration; late-onset Alzheimer's disease; apolipoprotein E gene; real-time PCR; DNA sequencing; risk factor; allele; neural regeneration

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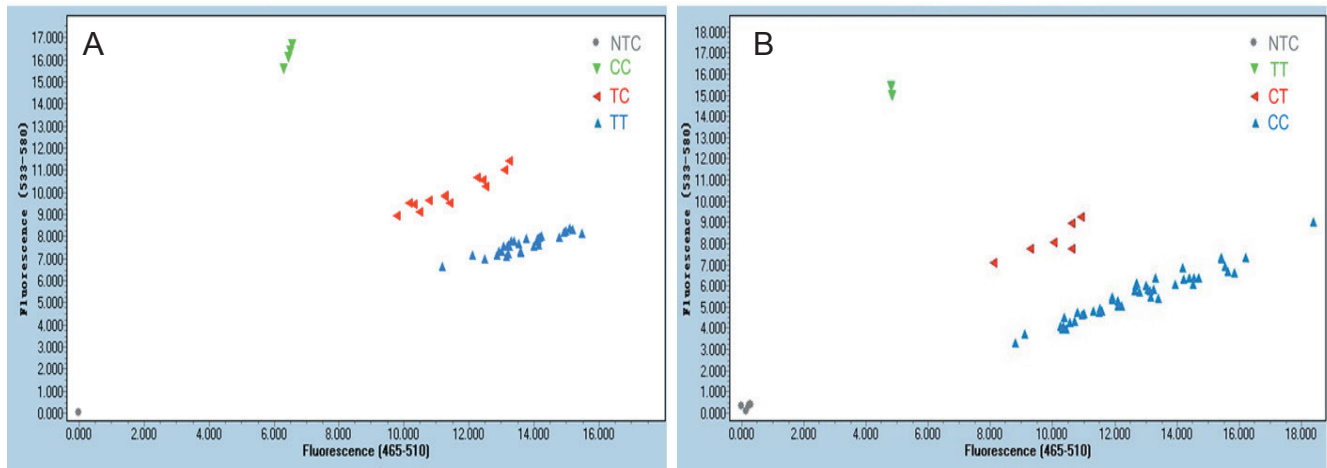
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## Introduction

Alzheimer's disease is the most common form of dementia related to aging, and is a progressive neurodegenerative disease affecting millions of people worldwide<sup>[1-2]</sup>. Clinically, Alzheimer's disease presents with anterograde episodic memory impairments, intellectual disturbances, language problems, and decline in other cognitive domains<sup>[3-4]</sup>. Neuropathologically, Alzheimer's disease is characterized by extracellular senile plaques (formed by aggregates of cleaved  $\beta$ -amyloid (A $\beta$ ) protein), intracellular neurofibrillary tangles (mainly composed of hyperphosphorylated tau protein), and neuronal loss<sup>[5-8]</sup>. Alzheimer's disease is typically divided into early-onset and late-onset forms. Early-onset Alzheimer's disease is rare, whereas late-onset Alzheimer's disease (with an onset age of over 65) accounts for approximately 95% of all Alzheimer's disease cases<sup>[9-11]</sup>. Early-onset Alzheimer's disease can be identified by genetic mutations in amyloid precursor protein, presenilin-1, and presenilin-2. Late-onset Alzheimer's disease lacks obvious genetic mutations<sup>[12-14]</sup>, however many studies have shown that apolipoprotein E (APOE)  $\epsilon 4$  gene is the most important known genetic risk factor<sup>[15-16]</sup>. Thus, is APOE  $\epsilon 4$  a genetic maker of late-onset Alzheimer's disease?

APOE is polymorphic, and encodes a 34.2 kDa glyco-

sylated protein, APOE<sup>[17]</sup>. APOE is located on chromosome 19q13.2, and comprised of four exons and three introns, covering 3,597 bp<sup>[18-19]</sup>. The two APOE single nucleotide polymorphisms associated with Alzheimer's disease are rs429358 and rs7412. The three common variants resulting from these polymorphisms are  $\epsilon 2$  (rs429358, T; rs7412, T),  $\epsilon 3$  (rs429358, T; rs7412, C) and  $\epsilon 4$  (rs429358, C; rs7412, C). In humans, these variants result in three homozygous ( $\epsilon 2/\epsilon 2$ ,  $\epsilon 3/\epsilon 3$ , and  $\epsilon 4/\epsilon 4$ ), and three heterozygous ( $\epsilon 2/\epsilon 3$ ,  $\epsilon 2/\epsilon 4$ , and  $\epsilon 3/\epsilon 4$ ) phenotypes. APOE  $\epsilon 2$ ,  $\epsilon 3$ , and  $\epsilon 4$  determine the three major protein isoforms of APOE, specifically, APOE2, E3, and E4<sup>[20-22]</sup>. On the mature APOE polypeptide chain, APOE2 has cysteine residues at positions 112 and 158, APOE3 has a cysteine and arginine residue at positions 112 and 158, respectively, and APOE4 has arginine residues at both sites. Despite differing by only one or two amino acids at residues 112 and/or 158, the structure and function of APOE protein isoforms are profoundly altered<sup>[23-25]</sup>. Carriers of one or two APOE  $\epsilon 4$  alleles have a three- or eleven-fold, respectively, increased risk for late-onset Alzheimer's disease<sup>[26]</sup>. As neuronal loss is one of the pathological characteristics of late-onset Alzheimer's disease, in recent years emphasis has been placed on neural regeneration research. The APOE  $\epsilon 4$  allele is considered a negative factor for neural



**Figure 1** Results of rs429358 (A) and rs7412 (B) genotypings using the Roche LightCycler 480II system. NTC: Negative template control.

regeneration<sup>[27-28]</sup>. APOE4 exhibits greater neurotoxicity than APOE2 and APOE3, and affects neural regeneration mainly by generating neurotoxic fragments that lead to pathological mitochondrial dysfunction and cytoskeletal collapse<sup>[29]</sup>. However, the exact mechanism whereby the APOE  $\epsilon$ 4 allele inhibits neural regeneration in late-onset Alzheimer's disease is not completely understood<sup>[30-31]</sup>.

APOE genotyping is crucial to APOE polymorphism analysis. Peripheral venous blood is the conventional tissue source for APOE polymorphism analysis<sup>[32-33]</sup>. Blood yields high-quality genomic DNA and can meet various research purposes. However, because of invasiveness, taking blood samples decreases compliance among the elderly, especially neuropsychiatric patients<sup>[34-36]</sup>. Moreover, blood specimens often need cold storage, thereby increasing the cost. In contrast, buccal mucosa sampling through mouth swabs is non-invasive and generates high-quality genomic DNA for single nucleotide polymorphism genotyping. Specimens can be stored at room temperature<sup>[37-40]</sup>. Ilveskoski et al.<sup>[41]</sup> reported successful APOE genotyping from buccal swabs using the restriction enzyme *HhaI*, although the process was labor- and time-consuming (taking a total of ten hours). Magnetic nanoparticles are novel materials for genomic DNA isolation in molecular biology. Wang and Su<sup>[42]</sup> have shown magnetic nanoparticles effectively and rapidly enrich trace amounts of DNA. Real-time PCR is a high-throughput method for single nucleotide polymorphism analysis<sup>[33]</sup>. Thus, we aimed to establish a method for genomic DNA extraction from mouth swab specimens using magnetic nanoparticles coupled with APOE genotyping by real-time PCR using TaqMan-BHQ probes.

## Results

### Rapid APOE genotyping by real-time PCR

Real-time PCR amplification took 50 minutes. The whole procedure was operated in closed tubes. After amplification, genotypes were automatically read by the Endpoint Genotyping module. Representative rs429358 and rs7412 genotyping results are shown (Figure 1).

### APOE genotyping by DNA sequencing

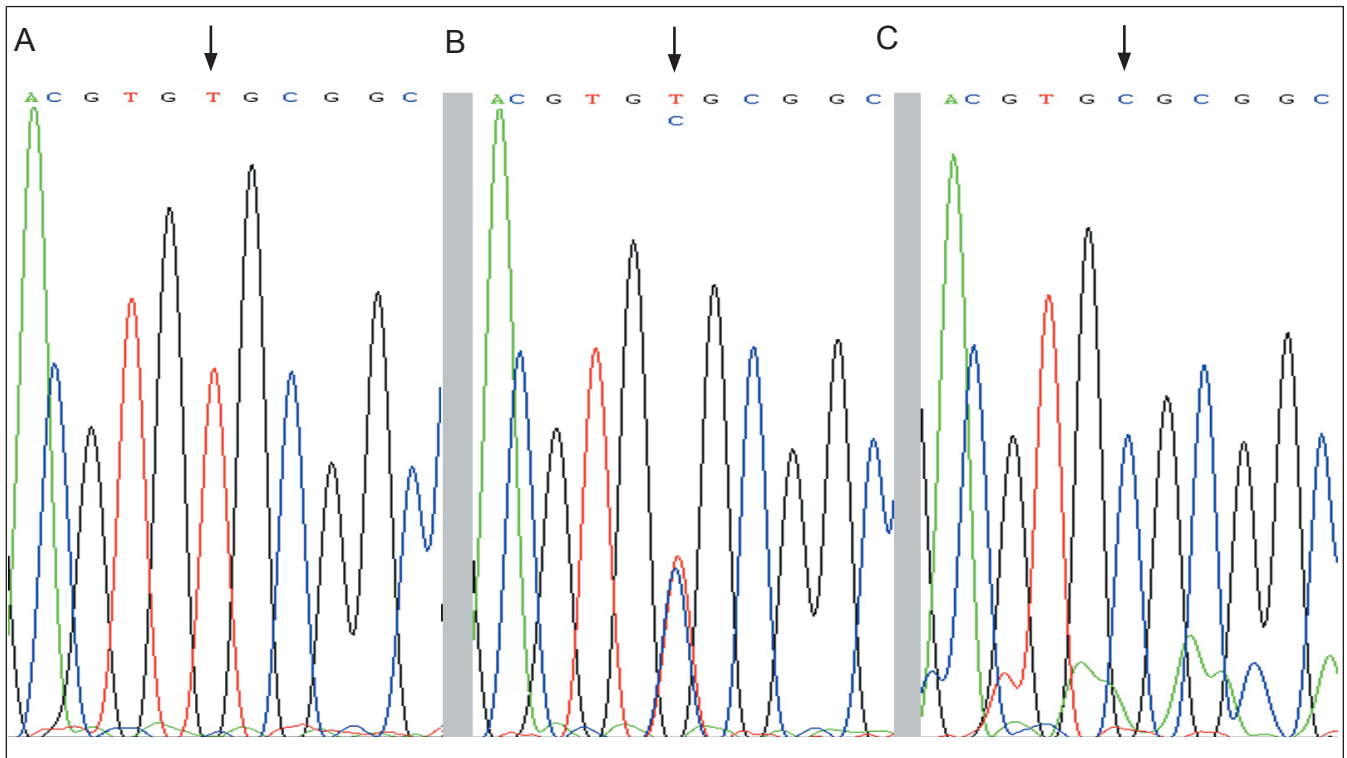
DNA sequencing was performed by the Beijing Genomics Institute in China, using the ABI3730 DNA Sequencer (Applied Biosystems). Typical results are presented (Figures 2, 3).

### Genotype analysis of the APOE $\epsilon$ 4 allele, a risk factor for late-onset Alzheimer's disease

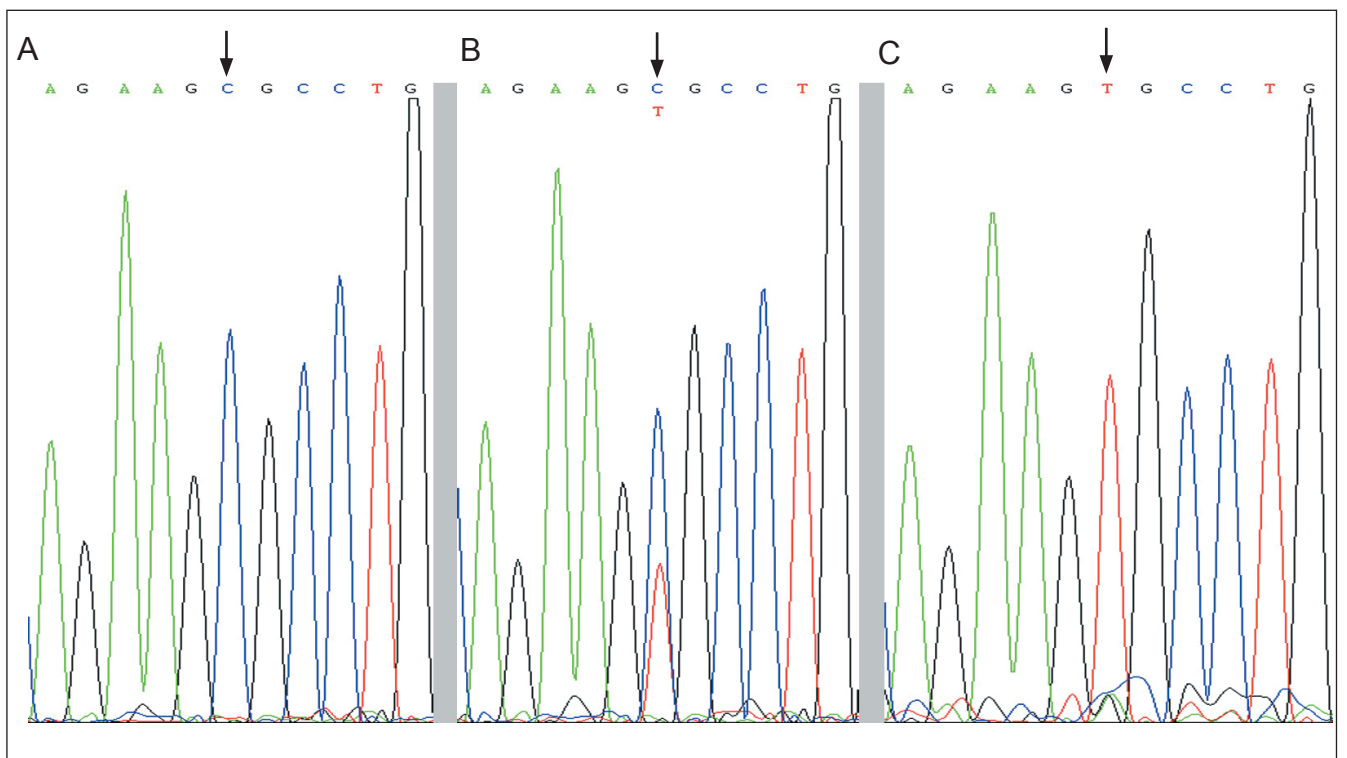
APOE genotyping by real-time PCR using TaqMan-BHQ probes, showed 100% correlation with DNA sequencing results, demonstrating reliability of our protocol. Genotype and allele frequencies, and Hardy-Weinberg equilibrium tests are summarized (Table 1). Both cases and controls were in Hardy-Weinberg equilibrium ( $P > 0.05$ ), indicating our subjects were genetically randomly selected. APOE genotype distributions between cases and controls were significantly different ( $P = 0.004$ ). The APOE  $\epsilon$ 4 allele was associated with high risk ( $P = 0.001$ ; odds ratio (OR) 3.958, 95% confidence interval (CI) 1.681–9.319) for developing late-onset Alzheimer's disease, supporting the APOE  $\epsilon$ 4 allele as a risk factor for late-onset Alzheimer's disease<sup>[16]</sup> (Tables 2, 3).

## Discussion

The APOE  $\epsilon$ 4 allele is well known to play an important role in the pathological processes of late-onset Alzheimer's disease, in particular, A $\beta$  generation or deposition, neurofibrillary tangle formation, lipid homeostasis, neuronal survival, and intracellular signal transduction<sup>[43-45]</sup>. In the central nervous system, APOE is considered important for repairing and maintaining myelin and neuronal membranes during growth and injury<sup>[46-47]</sup>. Of the three isoforms, APOE4 is the least stable, and less effective at accomplishing these functions. The APOE  $\epsilon$ 4 isoform shows higher affinity for binding A $\beta$  and stabilizing amyloid fibrils, thereby enhancing A $\beta$  accumulation in plaques. Late-onset Alzheimer's disease patients with one or two APOE  $\epsilon$ 4 alleles have more senile plaques and A $\beta$  deposition<sup>[48-51]</sup>. APOE plays a fundamental role in regulating transport of cholesterol and phospholipids among cells<sup>[52]</sup>. APOE  $\epsilon$ 4 genotypes are associated with less APOE protein in plasma. Plasma low density lipoprotein levels are higher in APOE  $\epsilon$ 4 allele carriers<sup>[53-56]</sup>. Cholinergic



**Figure 2** Screenshots of rs429358 genotyping obtained by DNA sequencing. (A) rs429358 T/T; (B) rs429358 C/T; (C) rs429358 C/C. Arrows show polymorphic sites.



**Figure 3** Screenshots of rs7412 genotyping obtained by DNA sequencing. (A) rs7412 C/C; (B) rs7412 C/T; (C) rs7412 T/T. Arrows show polymorphic sites.

signal transduction is impaired in the majority of late-onset Alzheimer's disease patients. Among these patients, *APOE*  $\epsilon 4$  allele carriers have greater deficits in cholinergic activity in the hippocampus and cortex, and fewer cholinergic neurons<sup>[43-44]</sup>.

*APOE*  $\epsilon 4$  allele effects on neural regeneration in late-onset Alzheimer's disease cases are complex and can be summarized as follows: (1) *APOE*  $\epsilon 4$  inhibits neurite outgrowth; (2) *APOE*  $\epsilon 4$  leads to mitochondrial dysfunction including

**Table 1 Genotype and allele frequencies of rs429358 and rs7412 and Hardy-Weinberg equilibrium (HWE) tests in cases and controls**

Subjects	Genotype frequency [n(%)]			HWE (P)	Allele frequency [n(%)]	
	C/C	C/T	T/T		C	T
<b>Cases</b>						
rs429358	2(4)	19(38)	29(58)	1.00	23(23)	77(77)
rs7412	45(90)	4(8)	1(2)	0.13	94(94)	6(6)
<b>Controls</b>						
rs429358	49(86)	8(14)	0(0)	1.00	106(93)	8(7)
rs7412	0	9(16)	48(84)	1.00	9(8)	105(92)

An intergroup comparison was performed using the chi-square test.

**Table 2 APOE phenotype distribution in cases and controls**

APOE phenotypes	rs429358/rs7412 and rs429358/rs7412 nucleotide combination	Frequency [n(%)]	
		Cases (n = 50)	Controls (n = 57)
ε2ε2	T/T and T/T	1(2)	0(0)
ε2ε3	T/T and T/C	4(8)	8(14)
ε2ε4	T/T and C/C	0(0)	1(2)
ε3ε3	T/C and T/C	24(48)	41(72)
ε3ε4	T/C and C/C	19(38)	7(12)
ε4ε4	C/C and C/C	2(4)	0(0)

An intergroup comparison was performed using the chi-square test. APOE: Apolipoprotein E.

**Table 3 APOE genotype distribution in cases and controls**

APOE genotypes	Nucleotide combination	Frequency [n(%)]	
		Cases (n = 50)	Controls (n = 57)
ε2	rs429358 T and rs7412 T	6(6)	9(8)
ε3	rs429358 T and rs7412 C	71(71)	97(85)
ε4	rs429358 C and rs7412 C	23(23)	8(7)
P	0.004		

An intergroup comparison was performed using the chi-square test. APOE: Apolipoprotein E.

membrane potential alterations, mitochondrial motility reductions, and mitochondrial respiratory enzymes dysfunction; (3) APOE4 impairs the cytoskeleton and causes tau hyperphosphorylation; (4) APOE4 inhibits synaptogenesis, increases Aβ and lysosomal leakage, and causes neuronal apoptosis; and (5) animal experiments show APOE4 memory and learning impairments in mice<sup>[29, 57]</sup>.

Much remains to be determined to explain APOE ε4 allele effects on neural regeneration in late-onset Alzheimer's disease cases. Thus, APOE genotyping of large samples is indispensable. Besides compliance, efficiency and cost of sample collection need to be considered when screening large samples. Thomson and colleagues<sup>[58]</sup> recommended buccal cells as the first choice for PCR DNA analysis.

In our study, 107 mouth swab specimens were collected. The process of specimen collection was non-invasive and no discomfort was reported. The procedure of collecting mouth swab specimens was easy to manage, with no specialized facilities or even skilled collection staff required. It took only several seconds to collect one mouth swab specimen. Compared with blood specimens, the cost of mouth swab specimen collection was reduced. According to the manufacturer, one sterile mouth swab costs < 0.1 dollar. No anticoagulant was needed and specimens could be stored at room temperature for more than two weeks.

We used magnetic nanoparticles to extract genomic DNA from the mouth swab samples. Compared with traditional human genomic DNA extraction kits, magnetic nanoparticles have several advantages. They are highly efficient at enriching genomic DNA because of their large specific surface area, which can notably enlarge the reaction interface. Due to their superparamagnetic effects, magnetic nanoparticles are easy to use, enabling them to be moved and isolated with external magnetic fields supplied by magnetic stands or common magnetic irons<sup>[59]</sup>. Furthermore, magnetic nanoparticles degenerate easily, supporting environmental protection. In our assay, the time spent in extracting genomic DNA from one mouth swab specimen was just one hour.

PCR-restriction fragment length polymorphism analysis is the most common method applied to genotype APOE polymorphisms<sup>[60-61]</sup>. However, PCR-restriction fragment length polymorphism analysis is not an effective method, as it requires a number of reaction steps (*i.e.*, PCR, overnight enzyme digestion, electrophoresis, and ultraviolet irradiation). Moreover, it is prone to insufficient restriction enzyme digestion resulting in inaccurate results. DNA sequencing is the most accurate method for APOE genotyping, but it is labor-consuming and requires expensive detection equipment that small laboratories can often not afford. Real-time PCR is a time- and labor-saving, high-throughput protocol

for single nucleotide polymorphism analysis. To date, SYBR Green, hybridization probes, and TaqMan-MGB probes have been used in real-time PCR for *APOE* genotyping<sup>[32-33]</sup>. SYBR Green, a double stranded DNA-fluorescent dye, is inexpensive, however, it is not specific enough and tends to be affected by primer-dimer formation<sup>[61]</sup>. Hybridization and TaqMan-MGB probes are highly specific, although high price restricts their application. TaqMan-BHQ is a specific fluorescence probe that is cheaper than hybridization or TaqMan-MGB probes. TaqMan-BHQ probe application shows an increasing trend in recent years, especially in small laboratories. Our assay demonstrates that TaqMan-BHQ probes are accurate and reliable for *APOE* genotyping, with confirmation of our results by PCR with DNA sequencing. Genotyping with TaqMan-BHQ probes requires no post-PCR sample handling, reducing the chance of sample contamination and mix-up. We used negative template controls for real-time PCR amplification, and observed no contamination. Samples were rechecked on different days, with comparable results, showing good assay reproducibility. A single real-time PCR reaction took approximately 50 minutes, and the Roche LightCycler 480 II system has the potential to run 384 reactions at one time.

In conclusion, our method for *APOE* genotyping is non-invasive, fast, and economical, with the potential for high-throughput application. It is suitable for screening *APOE*  $\epsilon 4$  allele carriers among large samples, which may help elucidate the influence of the *APOE*  $\epsilon 4$  allele in late-onset Alzheimer's disease cases.

## Subjects and Methods

### Design

A novel test method for clinical research.

### Time and setting

Experiments were performed in the Central Laboratory of Peking University Shenzhen Hospital, China from July 2011 to April 2012.

### Subjects

A total of 50 late-onset Alzheimer's disease cases (mean age  $74.2 \pm 5.8$  years, 24 males and 26 females), diagnosed according to criteria of the National Institute of Neurological and Communicative Disorders and Stroke, and the Alzheimer's Diseases and Relative Disorders Associations<sup>[62]</sup>, and 57 age-matched healthy controls (mean age  $72.98 \pm 4.76$  years, 29 males and 28 females), were recruited from the in- and out-patient departments of the Center of Health Examination, Peking University Shenzhen Hospital. All participants were from the Han Chinese population. Written informed consent was obtained from subjects or surrogates. The study was performed in accordance with the *Declaration of Helsinki* and approved by the Ethics Committee of Peking University Shenzhen Hospital, China.

### Methods

#### *Preparation of mouth swab specimens*

Sterile mouth swabs (Jiangsu KangJian Medical Apparatus,

Jiangyan, Jiangsu Province, China) were used to collect cheek epithelial cells from subjects. The inside of both cheeks was rubbed and swabs were swirled five times. Tips of mouth swab specimens were cut into 1.5 mL microcentrifuge tubes and stored at room temperature until DNA extraction.

#### *Human genomic DNA extraction*

The Magnetic Nanoparticles DNA Extraction Kit (Wawasye Nanotech, Wuhan, Hubei Province, China), including magnetic nanoparticles, and lysis, binding, washing, and elution buffers, was used. Lysis buffer (200  $\mu$ L) was added to mouth swab specimens and vortexed for seconds. Tubes were centrifuged briefly at  $12,000 \times g$  at room temperature, and then incubated at  $80^\circ\text{C}$  for 30 minutes. Tubes were again briefly centrifuged at  $12,000 \times g$  and supernatants transferred to clean microcentrifuge tubes. Magnetic nanoparticles (20  $\mu$ L) were added and mixed by inversion 20 times. Mixtures were then incubated at room temperature for 10 minutes. Tubes were again briefly centrifuged and supernatants discarded. Tubes were placed into a magnetic stand to preserve magnetic nanoparticles when discarding supernatants. Washing buffer (100  $\mu$ L) was added and mixed by inversion 20 times. Supernatants were discarded using the magnetic stand. The washing procedure was repeated, and magnetic nanoparticles permitted to air dry for five minutes. Following this, elution buffer (50  $\mu$ L) was added and incubated at  $70^\circ\text{C}$  for 10 minutes. After brief centrifugation, supernatants (including genomic DNA) were transferred to clean microcentrifuge tubes and stored at  $-20^\circ\text{C}$  until use.

#### *APOE genotyping by real-time PCR*

Primer and probe sequences for rs429358 genotyping were designed by Shanghai Genecore Biotechnologies (Genecore, Shanghai, China), and were: forward primer 5'-ACC TCG CCG CGG TAC TG-3', reverse primer 5'-GGG CAC GGC TGT CCA A-3'; TaqMan probe pair 5'-FAM-CGG CCG CAC ACG TCC TCC-BHQ-3', 5'-HEX-CGG CCG CGC ACG TCC T-BHQ-3'. For rs7412 genotyping, sequences according to Koch et al.<sup>[33]</sup> were used: forward primer 5'-CGC GGC CCT GTT CCA-3', reverse primer 5'-CTC CGC GAT GCC GAT G-3'; TaqMan probe pair 5'-FAM-ACT GCC AGG CGC TTC TGC AGG-BHQ-3', 5'-HEX-CAC TGC CAG GCA CTT CTG CAG GT-BHQ-3'. All primers and probes were synthesized and purified by Genecore.

Real-time PCR amplification reactions for rs429358 and rs7412 genotyping were performed using the Roche LightCycler 480II system (Roche Diagnostics, Basel, Switzerland). Reactions contained 5  $\mu$ L of  $2 \times$  premix Ex Taq (TaKaRa, Dalian, Liaoning Province, China), 2.5  $\mu$ mol/L of each primer, 1.25  $\mu$ mol/L of each TaqMan probe, and 2  $\mu$ L of genomic DNA. Sterile water was added to a final volume of 10  $\mu$ L. Negative template controls, using sterile water instead of genomic DNA, were included in every amplification. Cycling parameters were: an initial denaturation at  $95^\circ\text{C}$  for 30 seconds, followed by 40 cycles of denaturation at  $95^\circ\text{C}$  for 5 seconds, annealing and extension at  $60^\circ\text{C}$  for 31 seconds, and finally cooling to  $4^\circ\text{C}$ . After amplification, genotypes were automatically determined by the Roche LightCycler 480

service software, equipped with the Endpoint Genotyping module.

#### DNA sequencing

DNA sequencing was performed to validate *APOE* genotyping obtained by real-time PCR. The primer pair sequences (5'-GGG CAC GGC TGT CCA A-3' and 5'-CGC GGC CCT GTT CCA-3') used, produced a 300 bp fragment encompassing rs429358 and rs7412, and were designed and synthesized by Genecore. PCR amplification reactions were performed using the ABI 2720 Thermal Cycler (Applied Biosystems, CA, USA). PCR reaction volumes were 25  $\mu$ L, and included 12.5  $\mu$ L of 2  $\times$  premix Ex Taq (TaKaRa), 5  $\mu$ mol/L of each primer, 2  $\mu$ L of genomic DNA, and 9.5  $\mu$ L of sterile water. Appropriate negative controls were included in each run. Amplification conditions were: an initial denaturation at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 18 seconds.

Southern blots were performed to verify amplification of the expected 300 bp *APOE* products. PCR products (5  $\mu$ L) were mixed with 6  $\times$  loading buffer (1  $\mu$ L) (TaKaRa) and electrophoresed on 2% agarose gels. Amplicons were measured using the DL1000 molecular mass marker (TaKaRa). PCR products of 300 bp were sent to Beijing Genomics Institute (Shenzhen, Guangdong Province, China) for DNA sequencing.

#### Statistical analysis

Genotype distributions and allele frequencies of rs429358 and rs7412 were obtained by direct counting. To determine if genotype distributions were in Hardy-Weinberg equilibrium, tests were performed using  $R^{[63]}$ . Other analyses were performed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Genotype frequencies between two groups, were compared using chi-square tests and assessment of *APOE*  $\epsilon$ 4 allele risk for late-onset Alzheimer's disease determined.  $P \leq 0.05$  was considered statistically significant.

**Author contributions:** Yi L was responsible for the funding and the whole research including experiment design, data analysis and manuscript preparation. Wu T designed and performed the experiment, collected and analyzed data and wrote the manuscript. Luo WY was responsible for collecting subjects and revised the manuscript. Wu J and Zhou W were responsible for the theoretical and experimental instructions. All authors approved the final version of the paper.

**Conflicts of interest:** None declared.

**Peer review:** A high-sensitivity, non-invasive, fast and economical method to genotype *APOE* gene polymorphisms by collecting buccal mucosa epithelial cells from cases with late-onset Alzheimer's disease has been established, which is suitable for clinical research.

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