SPANDIDOS
PUBLICATIONS

Amplification refractory mutation system polymerase chain reaction versus optimized polymerase chain reaction restriction-fragment length polymorphism for apolipoprotein E genotyping of majorly depressed patients

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Abstract. Major depressive disorder (MDD) is a prevalent, debilitating mood disorder that has been associated with several genetic polymorphisms. One such polymorphism, namely that of apolipoprotein E (APOE), has three allelic forms (ε2, ε3 and ε4) that encode for six unique isoforms of the APOE protein. A growing number of techniques have been developed for APOE genotyping; however, not all polymerase chain reaction (PCR)-based genotyping techniques are equally accurate or cost-effective. In order to find a more accurate and cost-effective APOE genotyping method for MDD screening in large populations, the present study comparatively evaluated two genotyping methods, amplification refractory mutation system PCR (ARMS-PCR) and optimized PCR restriction-fragment length polymorphism (PCR-RFLP), in blood samples taken from a population of 708 MDD patients. Although either of the two methods were able to detect all six unique APOE genotypes, comparisons of the two methods with Sanger sequencing demonstrated that ARMS-PCR (94%) was significantly more accurate than optimized PCR-RFLP (82%). ARMS-PCR should prove useful in quickly verifying ambiguous results obtained by other APOE genotyping methods and can be cost-effectively performed in the setting of a small laboratory or a population-based screening program.

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Introduction

Major depressive disorder (MDD) is a prevalent, debilitating mood disorder with a lifetime prevalence of 16% that contributes to increased rates of disability and suicide (1). The pathoetiology of MDD is complex and likely involves a combination of environmental and genetic factors. After several years of research, investigators have discovered several genetic polymorphisms associated with MDD; in particular, a comprehensive 2007 meta-analysis by López-León *et al* (2) provided statistically significant evidence for six MDD susceptibility genetic polymorphisms: APOE, DRD4, GNB3, MTHFR, SLC6A3 and SLC6A4.

One such polymorphism, namely that of apolipoprotein E (APOE), was initially discovered by Ramachandran *et al* (3) in 1996. APOE has a key role in transporting lipoproteins, fat-soluble vitamins and cholesterol through binding to low-density lipoprotein (LDL) and APOE receptors (4). The APOE gene is polymorphic and possesses three alleles: ε2, ε3 and ε4 (5). This polymorphism leads to six unique APOE protein isoforms (6,7). The APOE phenotyping method, an isoelectric focusing (IEF) technique that is based on simultaneously determining the charge differences (pI) between distinct APOE polypeptides, is a complex procedure requiring considerable expertise (8). By contrast, genotyping methods that detect sequence differences in the APOE alleles [single nucleotide polymorphisms (SNPs)] are simpler and more accurate than IEF; as a result, increasing numbers of PCR-based genotyping techniques have been applied to determine APOE genotypes (9-15).

Several common PCR-based genotyping techniques are currently in use, including amplification refractory mutation system PCR (ARMS-PCR), PCR restriction-fragment length polymorphism (PCR-RFLP), single-stranded conformational

Table I. Primers for amplification refractory mutation system polymerase chain reaction.

Primer name	Primer sequence	Product length (bp)
Arg112 (forward)	5'-CGCGGACATGGAGGACGTTC-3'	588
Arg158 (forward)	5'-ATGCCGATGACCTGCAGACGC-3'	451
Common primer (reverse)	5'-GTTCAGTGATTGTCGCTGGGCA-3'	
Cys112 (forward)	5'- CGCGGACATGGAGGACGTTT-3'	588
Cys158 (forward)	5'-ATGCCGATGACCTGCAGACGT-3'	451

Underlined letters denote allele-specific mismatches.

polymorphism (SSCP) and real-time PCR. However, not all PCR-based genotyping techniques are equally efficacious; for example, ARMS-PCR and PCR-RFLP have been found to be more accurate than SSCP (16,17). Since several different techniques have been employed to assess the mutation status of APOE with little evidence of their comparative accuracy, the present study comparatively evaluated two modern APOE genotyping methods: ARMS-PCR versus optimized PCR-RFLP (a modified PCR-RFLP method using the restriction enzymes *Afl*III and *Hae*II), in blood samples taken from 708 MDD patients in order to find a more accurate and cost-effective APOE genotyping method for MDD screening in large populations.

Materials and methods

Ethics statement. The present study was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (Chongqing, China). Written informed consent was obtained from all individuals prior to inclusion in this study.

Subject recruitment, blood sampling and genomic DNA extraction. In order to identify MDD candidates for recruitment, a structured clinical interview assessing the relevant Diagnostic and Statistical Manual of Mental Disorders (DSM-IV, 4th edition) (18) criteria was performed to diagnose candidates with a single depressive episode (19), and the 17-item version of the observer-rated Hamilton Depression Rating Scale (HDRS) was applied to define the severity of their depression (20). Only depressed candidates with HDRS scores >17 were recruited for the present study, while those with one or more confounding factors, including physical or mental disorders, were excluded.

A total of 708 peripheral blood samples (271 males, 437 females; age range, 15-79 years) were obtained from 708 MDD patients at the First Affiliated Hospital of Chongqing Medical University (Chongqing, China). Venous blood samples were collected in 10-ml Vacutainer tubes (BD Bioscience, Franklin Lakes, NJ, USA) containing the chelating agent EDTA and then separated into 200-μl blood samples that were stored individually at -80˚C. Genomic DNA was extracted from 0.2 ml of each blood sample using the QIAamp® DNA Blood Mini kit (Qiagen, Hilden, Germany). To remove RNA from the eluted DNA, 2 μl RNase (10 mg/ml) was added to the samples followed by incubation at 37˚C for 15 min.

APOE genotyping by ARMS-PCR. APOE genotyping by ARMS-PCR was performed with specific Cys primers (Cys112 and Cys158) as well as Arg primers (Arg112 and Arg158) (Table I). PCR was performed in a 20-μl reaction volume including 100 ng genomic DNA, 0.4 μl Cys primers (10 μM) or Arg primers (10 μM), 0.8 μl ARMS-reverse primer (common primer; 10 μM), 1.6 μl dimethylsulfoxide, 10 μl Green mix (GoTaq® Green Master Mix; M7122; Promega Corporation, Madison, WI, USA) and 4.8 μl nuclease-free water. PCR amplification was initiated by denaturation at 95˚C for 5 min, followed by amplification comprising 35 cycles of 95˚C for 30 sec, 63˚C for 30 sec and 72˚C for 30 sec, and final extension at 72˚C for 15 min. Amplified nucleotides were resolved by 2% agarose gel electrophoresis with a 2,000 + 1.5 Kbp ladder (Biomed, Beijing, China; DM0103) as a marker and stained with Gold View (1:20,000). Negative controls were used throughout the experiment as appropriate. The theoretical results determined using Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA) are shown in Table II. For this genotyping method, the detection of each sample was repeated in triplicate.

APOE genotyping by optimized PCR-RFLP. Optimized PCR-RFLP was performed in a 25-μl reaction mixture containing 100 ng purified genomic DNA, 0.2 μM apoE-forward and apoE-reverse primers (5'-ACAGAA TTCGCCCCGGCCTGGTACACTGCCA-3' and 5'-TCC AAGGAGCTGCAGGCGGCGCA-3', respectively; product length, 227 bp) 12.5 μl Green mix, and nuclease-free water. PCR amplification was initiated by denaturation at 95˚C for 5 min, followed by 36 cycles of 95˚C for 30 sec, 69˚C for 30 sec and 72˚C for 30 sec, and a final extension at 72˚C for 15 min. The *Afl*III digestion mixture contained 10 μl PCR products and five units of *Afl*III (R0541L; New England Biolabs, Ipswich, WI, USA) in the buffer supplied by the manufacturer (NEB 3 buffer). Similarly, the *Hae*II digestion mixture contained 10 μl PCR products mixed with 10 U *Hae*II (R0107L; New England Biolabs) in the buffer supplied by the manufacturer (NEB 4 buffer). The two reactions were allowed to proceed for at least three hours at 37˚C. The resulting fragments were separated on a 4% agarose gel with a 50-bp marker (Biomed; DM0903), and the bands were visualized by Gold View [3,6-Bis(dimethylamino) acridine zinc chloride hydrochloride] staining (1:20,000). The gel images were captured by a ChemiDoc XRS gel imaging system (1000 Alfred Nobel Driver, 94547; Bio-Rad

Table II. Theoretical results of amplification refractory mutation system polymerase chain reaction.

Genotype	Cys112 (bp)	Cys158 (bp)	Arg112 (bp)	Arg158 (bp)
ε2ε2	588	451	0	0
ε4ε4	0	0	588	451
ε3ε3	588	0	0	451
ε2ε3	588	451	0	451
ε3ε4	588	0	588	451
ε2ε4	588	451	588	451

Table III. Theoretical results of optimized polymerase chain reaction restriction-fragment length polymorphism.

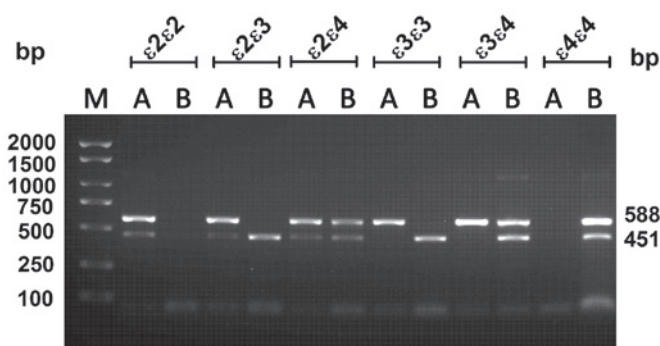| Genotype	*Afl*III digestion fragment (bp)	*Hae*II digestion fragment (bp)
ε2/ε2	177, 50	227
ε4/ε4	227	195, 32
ε3/ε3	177, 50	195, 32
ε2/ε3	177, 50	227, 195, 32
ε3/ε4	227, 177, 50	195, 32
ε2/ε4	227, 177, 50	227, 195, 32

Figure 1. Gel showing ARMS-PCR products. ARMS-PCR was performed with Cys primers (lane A) containing Cys112 (588 bp) and Cys158 (451 bp) primers or Arg primers (lane B) containing Arg112 (588 bp) and Arg158 (451 bp) primers. Every apolipoprotein E genotype was amplified with allele-specific primers, including ε2/ε2 (Cys112 and Cys158), ε3/ε3 (Cys112 and Arg158), ε4/ε4 (Arg112 and Arg158), ε2/ε3 (Cys112, Cys158, and Arg158), ε2/ε4 (Cys112, Cys158, Arg112 and Arg158), and ε3/ε4 (Cys112, Arg112 and Arg158). Lane M: 2,000 + 1.5 Kbp ladder. All products were separated on a 2% agarose gel. ARMS-PCR, amplification refractory mutation system polymerase chain reaction.
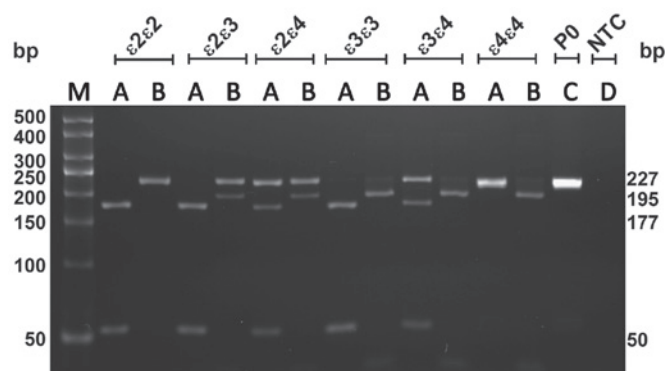

Figure 2. Gel showing optimized PCR restriction-fragment length polymorphism products. Lanes: A, PCR fragments digested by *Afl*III; B, PCR fragments digested by *Hae*II [namely, ε2/ε2: *Afl*III (177 and 50 bp) and *Hae*II (227 bp); ε3/ε3: *Afl*III (177 and 50 bp) and *Hae*II (195 and 32 bp); ε4/ε4 *Afl*III (227 bp) and *Hae*II (195 and 32 bp); ε2/ε3 (177 and 50 bp) and *Hae*II (227, 195 and 32 bp); ε2/ε4 *Afl*III (227, 177 and 50 bp) and *Hae*II (227, 195 and 32 bp); ε3/ε4 *Afl*III (227, 177 and 50 bp) and *Hae*II (195 and 32 bp)]; C, undigested PCR fragment (227 bp); D, template control; M, 50-bp ladder. All products were separated on a 4% agarose gel. PCR, polymerase chain reaction.

Laboratories Inc., Hercules, CA, USA). Negative controls were used as appropriate. The theoretical results which obtained by the Primer Premier 5.0 software are shown in Table III. Each of the 708 samples was analyzed three times using this genotyping method.

APOE genotyping by Sanger sequencing. APOE genotyping results from ARMS-PCR and optimized PCR-RFLP were confirmed against the gold standard for gene sequencing, Sanger sequencing (Invitrogen Life Technologies, Carlsbad, CA, USA). PCR was performed in a 25-μl reaction volume including 100 ng purified genomic DNA, 0.2 μM APOE-forward primer, 5'-TAAGCTTGGCACGGCTGTCCAAGGA-3', and APOE-reverse primer, 5'-ACAGAATTCGCCCCGGC CTGGTACAC-3' (9), 12.5 μl Green Mix (GoTaq® Green Master Mix; M7122; Promega Corporation) and nuclease-free water. PCR amplification was initiated by denaturation at 95°C for 5 min, followed by 36 cycles of 95°C for 30 sec, 65°C for 30 sec, and 72°C for 30 sec, and final extension at 72°C for 15 min. Negative controls were used as appropriate. The detection of each sample was repeated in triplicate.

Statistical analysis. Data were analyzed using SPSS 19.0 (International Business Machines, Armonk, NY, USA). Pearson's χ^2 test was applied to assess the diversity of the two genotyping methods. Comparing ARMS-PCR results with DNA sequencing results, the Pearson χ^2 value was 0.96, while comparing optimized PCR-RFLP results to DNA sequencing results, the Pearson χ^2 value was 0.001. This indicated that

ARMS-PCR results and PCR-RFLP results were not wholly consistent.

Results

APOE genotyping by ARMS-PCR. The results of the ARMS-PCR analysis showing the six unique APOE genotypes are displayed in Fig. 1. Amplification of the ε3/ε3 genotype, which contains Cys at codon 112 and Arg at codon 158, generated a 588-bp product when the Cys primers were used and a 451-bp product when the Arg primers were used. Amplification of the ε4/ε4 genotype, which carries Arg at codons 112 and 158, resulted in 588- and 451-bp products when the Arg primers were used, while no products were obtained when using the Cys primers. From the heterozygote ε2/ε3 genotype, which contains Cys at codon 112 as well as Cys and Arg at

Table IV. Apolipoprotein E genotype frequencies [n (%)] in the major depressive disorder study population (n=708).

Method	ε3/ε3	ε3/ε4	ε2/ε3	ε2/ε2	ε2/ε4	ε4/ε4
ARMS-PCR[a]	465 (65.7)	113 (16)	105 (14.8)	5 (0.7)	16 (2.3)	4 (0.6)
Optimized PCR-RFLP[b]	402 (56.8)	114 (16.1)	146 (20.6)	6 (0.8)	37 (5.2)	3 (0.4)
Sanger sequencing	460 (65)	111 (15.7)	115 (16.2)	4 (0.6)	13 (1.8)	5 (0.7)

[a]Pearson χ^2 test comparison with Sanger sequencing, P=0.96; [b]Pearson χ^2 test comparison with Sanger sequencing, P=0.001. ARMS-PCR, amplification refractory mutation system polymerase chain reaction; PCR-RFLP, polymerase chain reaction restriction-fragment length polymorphism.

Table V. Accuracy of ARMS-PCR versus optimized PCR-RFLP based on Sanger sequencing (n=708).

Method	True cases (n)	False cases[a] (n)	Accuracy (%)
ARMS-PCR	664	44	94
Optimized PCR-RFLP	581	127	82

[a]There were seven shared false cases between the two apolipoprotein E genotyping methods. ARMS-PCR, amplification refractory mutation system polymerase chain reaction; PCR-RFLP, polymerase chain reaction restriction-fragment length polymorphism.
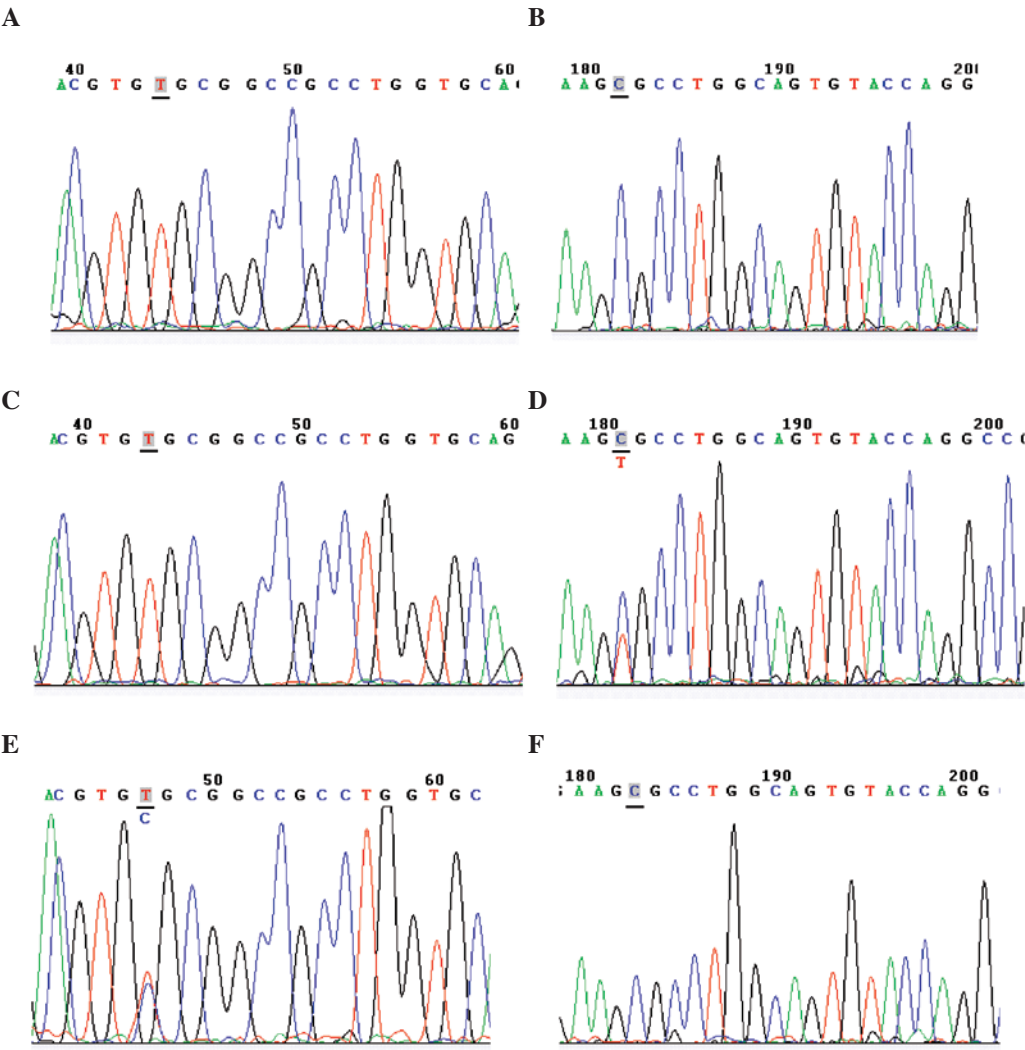

Figure 3. Representative findings from Sanger sequencing of apolipoprotein E isoforms. Isoform ε3ε3 gene sequences: (A) Position 112 in bold font (TGC) and (B) position 158 in bold font (CGC). Isoform ε2ε3 gene sequences: (C) Position 112 in bold font (TGC) and (D) position 158 in bold font (CGC, TGC). Isoform ε3ε4 gene sequences: (E) Position 112 in bold font (TGC, CGC) and (F) position 158 in bold font (CGC).

codon 158, 588- and 451-bp products were generated by using the Cys primers and a 451-bp product was obtained with the Arg primers. From the ε3/ε4 genotype, which contains Cys and Arg at codon 112 and Arg at codon 158, a 588-bp product was obtained with Cys primers, while 588- and 451-bp products were obtained with Arg primers. Amplification of the ε2/ε4 genotype, which possesses Cys and Arg at codons 112 and 158, generated 588- and 451-bp products when Cys or Arg primers were used. Our experimental results are consistent with the results reported by Kim *et al* (21).

APOE genotyping by optimized PCR-RFLP. Optimized PCR-RFLP is a modified method using two restriction enzymes, *Afl*III and *Hae*II, by which ε2, ε3 and ε4 alleles can be identified in a simple and unambiguous manner. As shown in Fig. 2, undigested 227-bp PCR fragments were separated from the 195- and 177-bp restriction products, with each genotype presenting a unique pattern following digestion with the two enzymes.

APOE genotyping by Sanger sequencing. As Sanger sequencing is considered the gold standard for genotyping, all results obtained from ARMS-PCR and optimized PCR-RFLP were compared against those from Sanger sequencing. Three representative APOE genotypes determined by Sanger sequencing are shown in Fig. 3. The APOE frequencies obtained by ARMS-PCR, optimized RFLP-PCR and Sanger sequencing are shown in Table IV.

APOE genotyping by ARMS-PCR is more accurate than optimized PCR-RFLP. Pearson's χ^2 test was applied to assess the diversity of the two genotyping methods, the results of which were not fully consistent (Table IV). After comparing the accuracy of the two genotyping methods against Sanger sequencing, ARMS-PCR (94%) was found to be more accurate than optimized PCR-RFLP (82%) in detecting APOE genotypes in these MDD patients (Table V).

Discussion

In order to find a more accurate and lower-cost APOE genotyping method for MDD screening in large populations, the present study comparatively evaluated two genotyping methods, ARMS-PCR and optimized PCR-RFLP, in blood samples from 708 MDD patients. Although the two APOE genotyping methods were able to detect all six APOE genotypes, Pearson's χ^2 test revealed that the APOE genotyping results of ARMS-PCR and optimized PCR-RFLP were not fully consistent. Comparison of the two methods with Sanger sequencing demonstrated that ARMS-PCR was significantly more accurate than optimized PCR-RFLP.

After years of research and development, several genotyping techniques for APOE have been introduced. The earliest methods for detecting APOE isoforms were based on protein isoelectric focusing electrophoresis (IEF) (22). Since IEF requires considerable expertise and expensive instrumentation, it was not particularly practical for small laboratories or population-based screening programs. Thereafter, molecular genetic techniques (23) based on PCR amplification and *Hha*I restriction enzyme digestion were introduced (9,15,24).

However, these *Hha*I-based assays were difficult to interpret as *Hha*I digestion yielded several small fragments; in addition, incomplete digestion by *Hha*I produced ambiguous results. Through utilizing two distinct restriction enzymes (*Afl*III and *Hae*II), the quality of the results was significantly improved but the cost of running the assay was also increased.

From these early methods, more advanced PCR-based APOE genotyping techniques have been recently developed, including allele-specific PCR (e.g. ARMS-PCR), single-stranded conformational polymorphism (SSCP) and real-time PCR. SSCP requires higher separation systems, and real-time PCR requires expensive reagents and instruments, and therefore, the cost of these methods is prohibitively high for small laboratories or population-based screening programs. Compared with these advanced genotyping methods, ARMS-PCR does not rely on restriction enzyme digestion, other treatment steps, or expensive reagents and instrumentation (25). In a BRAFV600E genotyping study, ARMS-PCR was found to be more sensitive and cost-effective than real-time PCR for BRAF mutational screening (26). Furthermore, ARMS-PCR was found to be more sensitive than automated dideoxy sequencing in detecting low BRAFV600E allele burdens in formalin-fixed and paraffin-embedded tumor specimens (27). The present study demonstrated that ARMS-PCR (94%) was significantly more accurate than optimized PCR-RFLP (82%) in detecting APOE genotypes in a population of 708 MDD patients. Furthermore, ARMS-PCR has distinct advantages over PCR-RFLP in terms of its cost of reagents and instrumentation, time consumption and simplicity of experimental processing, but faces a singular disadvantage to other genotyping methods in being unable to detect novel genetic mutations (25). Overall, this combination of factors make ARMS-PCR a superior APOE genotyping method for MDD screening in large populations.

It should be pointed out that the present study had several limitations: First, only blood samples from MDD patients were used; therefore the accuracy of APOE genotyping in healthy individuals or neuropsyciatric patients with clinical presentations similar to MDD, including bipolar disorder and schizophrenia, was not evaluated in the present study. Second, the entire population of 708 MDD patients in the present study was of Han Chinese ethnicity residing in the Chongqing metropolitan area; thus, future studies should use a more ethnically heterogeneous population sampled from multiple clinical sites in order to improve the validity of the conclusions. Third, the present study only compared ARMS-PCR to PCR-RFLP for genotyping of APOE polymorphisms in MDD patients; therefore, future studies should include other modern genotyping methods, including SSCP and real-time PCR, and assess other genetic polymorphisms associated with MDD, including DRD4, GNB3, MTHFR, SLC6A3 and SLC6A4.

In conclusion, the present study showed that ARMS-PCR was significantly more accurate than optimized PCR-RFLP in APOE genotyping of MDD patients. ARMS-PCR should prove useful in quickly verifying ambiguous results obtained by other APOE genotyping methods and can be cost-effectively performed in the setting of a small laboratory or a population-based screening program.

Acknowledgements

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