Title: Multiplex-Tetra ARMS PCR Versus High-Resolution Melting Analysis Assay Method for Characterization of Apolipoprotein E Genotype in Alzheimer Patients: A Case-Control Study

Running title: Completing the Puzzle of Apolipoprotein E Genotyping Geographical Distribution

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Abstract

Introduction: Apolipoprotein E (APOE) genotype has heterogeneous distribution throughout the world. The present study was aimed to characterize the APOE genotype (rs429358, rs7412) in healthy individuals compared with Alzheimer's cases in Kerman, southeastern Iran, by two standard mutation scanning methods.

Methods: In this case-control study, 90 Alzheimer patients as a case group and 90 healthy individuals as a control group were examined. APOE genotyping was carried out using high resolution melting (HRM) analysis assay and Multiplex T-ARMS PCR techniques.

Results: In contrast to Multiplex T-ARMS PCR, HRM analysis was not efficient in rs7412 genotyping. The results of Multiplex T-ARMS showed that $\epsilon_2\epsilon_3$ genotype (P-value=0.006, OR=0.119) and ϵ_2 allele (P-value=0.004, OR=0.129) were more prevalent in the control group compared with the case ones, whereas ϵ_4 allele was associated with borderline risk of Alzheimer disease (P-value =0.099, OR=1.76).

Conclusion: We concluded that Multiplex T-ARMS PCR could be considered as a better option than HRM analysis for APOE genotyping in terms of speed, accuracy, simplicity, and cheapness in large-scale use. Also, the present study revealed that $\epsilon_2\epsilon_3$ genotype and ϵ_2 allele are protective against Alzheimer whereas cannot strongly consider ϵ_4 allele as Alzheimer genetic risk factor in Kerman, Iran. The results may help to choose a better technique for APOE genotyping.

Keywords: Alzheimer disease, APOE genotyping, HRM analysis, Multiplex T-ARMS PCR, ϵ_2 , ϵ_4

1. Introduction

Every 3 seconds, there will be one new case of Alzheimer's disease (AD) worldwide. There will be more than 50 million AD cases worldwide in 2019, and with the aging population, it will be more than 152 million by 2050 (Bhatt et al., 2019). The disease is the most common cause of dementia, so that (60-70) % of dementia cases have AD, and almost 60% of dementia cases are living in low- and middle-income countries (LMICs)(*Fact Sheet of Dementia*, 2021).

AD is a neurodegenerative disease with two important pathological hallmarks. The first one is the extraneuronal amyloid-beta ($A\beta$) deposition that develops toxic plaques, and the other one is the intracellular accumulation of hyperphosphorylated tau protein, which creates neurofibrillary tangles (NFTs) (Manika Awasthi, Swati Singh, Veda P. Pandey, 2016). These two hallmarks lead to abnormal cytoskeletal formation, synaptic dysfunction, and neuronal cell death, mainly in the hippocampus and cerebral cortex, associated with remembering and performing cognitive actions(Sandeep Kumar Singh, Saurabh Srivastav, Amarish Kumar Yadav Saripella Srikrishna, 2016). There are two forms of AD based on the time of disease manifestation. Familial or Early-Onset Alzheimer disease (EOAD) accounts for nearly 5% of AD cases who develop the symptoms before 60. The other disease is Sporadic or Late-Onset Alzheimer's disease (LOAD), which accounts for about 95% of AD cases with over 60-65 years old age of onset. Overproduction or clearance impairment of $A\beta$ is the basis of EOAD and LOAD, respectively. In EOAD, mutations in APP (Amyloid precursor protein), PSEN1 (presenilin 1), and PSEN2 (presenilin 2) genes cause the autosomal dominant form of AD(Lynn M. Bekris, Chang-En Yu & Tsuang, 2011).

Unlike EOAD with a Mendelian pattern of inheritance, LOAD has several environmental and genetic risk factors. AD's most substantial genetic risk factor is the Apolipoprotein E (APOE) gene(Goate & Celeste, 2015). APOE is an important lipoprotein and cholesterol transfer binding to the surface of different brain cells and rolling like HDL in the peripheral. This gene is polymorphic in two loci (rs429358, rs7412), causing alternations in structure and function of APOE protein and leading three alleles ($\epsilon 2$, $\epsilon 3$, $\epsilon 4$) and six genotypes ($\epsilon 2/\epsilon 2$, $\epsilon 2/\epsilon 3$, $\epsilon 2/\epsilon 4$, $\epsilon 3/\epsilon 3$, $\epsilon 3/\epsilon 4$, and $\epsilon 4/\epsilon 4$).

It seems that the APOE genotype plays an essential role in the prognosis of AD since the APOE $\epsilon 4$ allele involves in AD pathogenesis. APOE $\epsilon 4$ allele increases the disease risk with different mechanisms briefly including the $A\beta$ -dependent pathway (reduce clearance and accelerate

aggregation), Tau hyper-phosphorylation (Chia-Chen Liu¹, Takahisa Kanekiyo², Huaxi Xu¹, 2008), neuro-inflammation exacerbate (Yin-Ying Fan et al., 2017), glucose cerebral metabolism, and lipid/cholesterol transport reduction (Long Wu, 1 Xin Zhang, 2018; Mirna Safieh¹, 2019).

Over the years, in addition to the APOE ϵ 4 allele, Genome-wide associated studies (GWAS) and sequence studies have identified more than 20 genes that have a bearing on LOAD genetics. Combining these genes created Genetic risk scores (GRSs) as a better genetic prediction factor (Giri, Lü, & Zhang, 2016; Goate & Celeste, 2015; Caroline Van Cauwenberghe, Christine Van Broeckhoven, & Kristel Sleegers, 2016). In fact, GRSs involve common genetic variants associated with a disease. It has been described for different diseases such as coronary artery diseases, diabetes, or neurodegenerative diseases like AD. GRSs disclose the capacity of all variants together that may have a minor effect on the probability of getting the disease (Chouraki et al., 2016). Despite the importance of GRSs, APOE ϵ 4 is much more predictive than GRSs alone; combining them with some extra environmental data like lifestyle can be a more favorable AD prediction (Stocker et al., 2018). The first step in assessing AD genetic predisposition in an area where no genetic studies have been performed appears to be the study of APOE genotyping.

Several methods have been used for APOE genotyping such as PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) (Hixson & Vernier, 1990), TaqMan probe (Zhong et al., 2016), PCR-DNA sequencing (Johansson et al., 2013), and microarrays (Calabretta et al., 2009). Although they are all effective, they are not affordable for large-scale population screening. High-resolution melting (HRM) analysis is an accurate, rapid, and cost-effective closed-tube method for detecting mutations based on the curves (Wittwer, 2009). Multiplex tetra-primer amplification refractory mutation system (T-ARMS) PCR is also an improved form of ARMS PCR that can detect homozygote, heterozygote, and wild type just in one reaction (Heidari, Hadadzadeh & Fallahzadeh, 2019; Yang et al., 2019).

Here, these two techniques were chosen to compare in terms of sensitivity, specificity, and cost in APOE genotyping. Characterization of APOE genotype (rs429358, rs7412) in healthy individuals compared with AD cases was also determined by HRM analysis and Multiplex T-ARMS PCR methods.

2. Methods

2.1. Study design and sampling

This case-control study was done in southeastern (Kerman) of Iran with more than 65 years old participants. This study conformed to the Declaration of Helsinki regarding research involving human subjects and was accepted by the Ethical Committee of the Kerman University of Medical Science, Kerman, Iran (IR.KMU.REC.12th May 2018). Informed consent was also obtained from all participants or their families. Peripheral blood samples of 90 AD and 90 control ones were collected into tubes containing EDTA. In addition to age, having NINCDS-ADRDA (National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association), Alzheimer's Criteria, and AD diagnosis by a neurologist were the other inclusion criteria for cases. Control subjects were recruited from healthy people that were sex and age-matched with AD samples.

2.2. Extraction of genomic DNA

According to the manufacturers' instructions, genetic DNA was extracted from peripheral blood leukocytes using the Genomic DNA Isolation Kit (Norgen biotech, Canada). DNA concentrations were determined by Nanodrop ND-1000 spectrophotometer (Thermo-fisher Scientific, Rockford, IL, USA). The measurement of OD260nm to OD280nm ratio showed the purity of the nucleic acid within the scope of 1.8–2. DNA concentrations were adjusted to 40 ng/mL and stored at -20°C for further analysis.

2.3. Primer design

We designed primers using the online Primer3(<http://bioinfo.ut.ee/primer3-0.4.0/>), Primer designing tool(<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and offline Oligo tools of Gene Runner software.

The appropriate amplicon length of HRM single nucleotide polymorphism (SNP) genotyping primers is nearly 100 bp (Reil & Bux 2015). Therefore, primers for two rs429358 and Rs7412 locus were separately designed, albeit 137bp apart(Figure 2).

Figure1. The high GC content of APOE gene. For HRM analysis, primers of rs7412 and rs429358 were designed separately despite their 137bp distance (blue NTs).

The primers were amplified 118 and 94 bp to detect Rs7412 and rs429358 alleles, respectively. Multiplex T-ARMS PCR primers were chosen from the previous study(Ward et al., 2012). PCR DNA-sequencing primers were designed to amplify 719 bp of the APOE gene encompassed both SNPs (Table 1). The specificity of primers and the absence of secondary structures were checked by blasting in the National Center for Biotechnology Information (NCBI).

Table1. Primers used for HRM analysis, Multiplex T-ARMS PCR, and PCR-DNA sequencing

Use for	application	primers	sequence
HRM	rs7412	F	5'-CGATGCCGATGACCTGCAGA-3'
		R	5'- GCGGCCCTGTTCCACCA-3'
	rs429358	F	5'- GGCACGGCTGTCCAAGGAG-3'
		R	5'- CTCGCCGCGGTACTGCAC-3'
Multiplex T-ARMS PCR	Common outer primers	FO	5'-ACTGACCCCGGTGGCGGAGGA-3'
		RO	5'-CAGGCGTATCTGCTGGGCCTGCTC-3'
	Inner primers at rs429358	FI-1	5'GGCGCGGACATGGAGGACGgGC-3'
		RI-1	5'-GCGGTACTGCACCAGGCGGCctCA-3'
	Inner primers at rs7412	FI-2	5'-CGATGCCGATGACCTGCAGAcGC-3'
		RI-2	5'-CCCGGCCTGGTACACTGCCAGtCA-3'
PCR-DNA sequencing	Both SNPs	F	5'-GGACGAGACCATGAAGGAGTT-3'
		R	5'-GCTTCGGCGTTCAGTGATTGT-3'

2.4. HRM analysis for APOE genotyping

For APOE genotyping, real-time amplification and HRM analysis were carried out with the Rotor gene 6000. The final volume of reaction was 10 μ L, containing 5 μ L of Type-it HRM PCR master mix (Qiagen, Germany), 0.3 μ L forward and 0.7 μ L reverse primer (10 Pmol/ μ l), 1 μ L distilled water and 3 μ L DNA in a fix concentration of 40ng per reaction.

PCR cycling program started with 95°C for 5 min as hold step to activate Hotstar Taq plus DNA polymerase of the master mix, followed by 40 times 2-step cycling consist of 95°C for 10 secs as denaturation and 68°C (for rs429358) / 66°C (for rs7412) for 30 secs as annealing/extension step. At the end of each annealing/extension step, fluorescence data were acquired to cycling A on the

green channel. After the last PCR cycle, the melting program immediately started with a ramp from 70°C to 95°C, raising by 0.1 degrees each step and pausing for 2 secs per step. All samples were performed in duplicate.

2.5. Multiplex T-ARMS PCR for APOE genotyping

PCR reaction performed with C1000 Touch thermal cycler (BioRad). Each microtube contained in the total volume of 31.45 µl with 40ng DNA polymerase enzyme, 0.25 units of HotStarTaq DNA polymerase, 6% DMSO, 1Mm mgcl₂, 15 pmol of any primers (FO, RO, FI-1, RI-1, FI-2, RI-2), 2.5 µl AMS buffer, 0.5 µl DNTP, 0.5 µl BSA and adequate water. Multiplex T-ARMS PCR program consisted of an initial denaturing step at 94°C for 3 min, 25 cycles at 94°C for 1 min, 55°C for 30 secs, and 72°C for 2 min followed by 72°C for 5 min. PCR products differentiated on 2% gel agarose and stained with Invitrogen dye(SYBR Safe DNA Gel Stain - Thermo Fisher).

2.6. Polymerase chain reaction-DNA sequencing

Several samples were amplified and sequenced by the Macrogen company in South Korea to confirm the genotyping results. Amplification was carried out in 27.7 µl with the same components of Multiplex T-ARMS PCR except for primers (specific primers for the sequence are shown in table1). The thermal cycling program was 93°C for 3 min, followed by 30 cycles repeated in 93°C for 1min, 62°C for 30 secs, and 74°C for 45 secs. The Final extension was 72°C for 3 min. Sequence results were analyzed with Chromas version 2.6.

2.7. Statistical analysis

Statistical analysis was performed using SPSS version 16.0. The Kolmogorov-Smirnov test was used to check out the normality of age data. Since it does not have a normal distribution, the Mann-Whitney trial investigated the significant difference between the two sample groups. Chi-square test was used for qualitative data like sex. Chi-square test was fulfilled to evaluate both allele and genotype frequencies and also Hardy-Weinberg equilibrium (HWE) for all six

genotypes. Logistic regression was conducted to determine the independent roles of the alleles and genotypes against AD and also models of inheritance.

3. Results

3.1. Demographic findings

The average age of participants was $73/36 \pm 7/80$. Mann-Whitney test showed no significant difference in age between case and control groups ($p=0.70$). The gender distribution of participants consists of 56.7% men and 43.3% women, with no significant differences among them ($P\text{-value}=0.54$). The average age and gender of participants are described in Table 2 separately for cases and controls. As you see, there was no significant difference in the sex and age of the participants in both groups.

Table2. The average age and gender of participants

	Case(n=90)	Control(n=90)	p-value
Age, year	73.81 $\pm$ 8.36	72.06 $\pm$ 8.65	0.171
Sex (Male. Female)	M=57. F=33	M=46. F=44	0.097

The frequency of $\epsilon 3$, $\epsilon 2$, and $\epsilon 4$ were 81.1, 10, and 8.9 percent in control and 82.2, 2.2, and 15.6 percent in the case group, respectively (Table 3). Genotyping distribution showed that $\epsilon 2$ $\epsilon 3$ genotype ($P\text{-value}=0.006$, $OR=0.119$) and $\epsilon 2$ allele ($P\text{-value}=0.004$, $OR=0.219$) are protective against AD and logistic regression displayed the dominant ($P\text{-value}=0.00$, $OR=0.138$) and codominant ($P\text{-value}=0.00$, $OR=0.91$) effects of $\epsilon 2$ allele in inheritance models. The $\epsilon 4$ allele seemed to be a risk factor with borderline significant trends ($P\text{-value}=0.099$, $OR=1.76$) (Table 2). The chi-square test showed no significant difference between the observed genotypes and the common genotypes in the population, and therefore the population was in equilibrium ($P\text{-value}=0.8$).

Table3. Frequencies of APOE Alleles and genotypes and models of inheritance for $\epsilon 2$ allele

	Case (Alzheimer)		control	OR (95% CI)	P-value
Allele					
$\epsilon 3$	148(82.2%)		146(81.1%)	ref	
$\epsilon 2$	4(2.2%)		18(10%)	0.219(0.072-0.66)	0.004
$\epsilon 4$	28(15.6%)		16(8.9%)	1.72(0.89-3.32)	0.099
Genotype					
$\epsilon 3 \epsilon 3$	60(66.7%)		73(70%)	ref	
$\epsilon 2 \epsilon 2$	0(0%)		1(1.1%)	1.53E9	1.000
$\epsilon 4 \epsilon 4$	2(2.2%)		4(4.4%)	1.90(0.33-10.78)	0.466
$\epsilon 2 \epsilon 3$	16(17.8%)		2(2.2%)	0.119(0.26-0.540)	0.006
$\epsilon 2 \epsilon 4$	2(2.2%)		0(0%)	0.000	0.999
$\epsilon 3 \epsilon 4$	10(11.1%)		20(22.2%)	1.90(0.82-4.40)	0.132
Models of inheritance for $\epsilon 2$ allele					
Dominant	($\epsilon 2 \epsilon 2 + \epsilon 2 \epsilon 3 + \epsilon 2 \epsilon 4$)	3(3.3%)	18(20%)	0.138(0.039-0.487)	0.00
	($\epsilon 2 \epsilon 2 + \epsilon 2 \epsilon 2 + \epsilon 2 \epsilon 2$)	87(96.7%)	72(80%)		
Recessive	($\epsilon 2 \epsilon 2$)	1(1.1%)	0(0%)	0.989(0.967-1.011)	0.316
	Other genotypes	89(89.9%)	90(100%)		
Codominant	($\epsilon 2 \epsilon 3 + \epsilon 2 \epsilon 4$)	18(20%)	2(2.2%)	0.91(0.02-0.405)	0.00
	Other genotypes	72(80%)	88(97.8%)		

3.2. Molecular analyses

Homozygotes, heterozygotes, and wild type of rs429358 variant were determined by HRM analysis. Heterozygotes have a specific HRM melting profile with a smaller melt curve (Wittwer, 2009). Differences in T_M distinguished homozygote samples. They were categorized into TT as wild-type homozygotes and CC as mutant homozygotes (Figure 2).

Figure 2. High-resolution melting analysis results of rs429358. (A) Normalized and shifted melting curves for the amplicon of rs429358 (B) Normalized and temperature-shifted difference plot for the amplicon of rs429358.

Although rs429358 was detected conveniently by HRM analysis, the method was not efficient for rs7412. The results of rs7412 genotyping by HRM analysis did not match with what sequence results showed. In this locus, wild-type, heterozygote, and homozygote classifications based on more than 0.5°C T_M differences were inconsistent with sequencing results (Figure 3).

Figure3. Various normalized and shifted melting curves for the amplicon of rs7412 with the same CC genotype

The results of Multiplex T-ARMS PCR were interpreted based on the number and length of bands on the gel (figure4). The findings of HRM analysis and Multiplex-T ARMS PCR showed the same genotype results of rs429358. And finally, PCR-DNA sequencing results were matched with genotype classification of Multiplex T-ARMS PCR (figure5)

	115bp	253bp	307bp	444bp	514bp
$\epsilon 3/\epsilon 3$	+		+		+
$\epsilon 2/\epsilon 2$	+	+			+
$\epsilon 4/\epsilon 4$			+	+	+
$\epsilon 3/\epsilon 4$	+		+	+	+
$\epsilon 2/\epsilon 4$	+	+	+	+	+
$\epsilon 2/\epsilon 3$	+	+	+		+

Figure4. Interpretation the results of Multiplex T-ARMS PCR on gel base on the number and length of bands. e.g., $\epsilon 3\epsilon 3$ genotype has 115, 307, and 514bp bands

Figure 5. Result of polymerase chain reaction DNA-sequencing in rs429358 and rs7412 loci base on the Multiplex T-ARMS PCR technique. (A) rs429358 T/T (Wild type, Homozygous); (B) rs7412 C/T (Heterozygous); (C) rs429358 C/C (Homozygous); (D) rs7412 T/T (Homozygous); (E) rs429358 T/C (Heterozygous); (F) rs7412 CC (Wild type, Homozygous)

4. Discussion

Although the frequency of APOE alleles is the most well-known genetic risk factor of Alzheimer's disease, allele frequency and distribution of this gene vary in regions and countries (Singh et al., 2006; Ward et al., 2012). Our study had two parallel aims to investigate. First, it tried to determine the frequency of APOE alleles in AD and control groups to suggest if

it can be a helpful test in predicting AD in our population. The second was to propose an efficient and affordable technique for APOE genotyping in large-scale populations.

This study represented the protection role of $\epsilon 2$ allele and $\epsilon 2\epsilon 3$ genotype against AD that was in agreement with the similar findings in Iran (Gozalpour, KKamali, KMohammd, HR Khorram Khorshid, MOhadi, MKarimloo, A Mirabzadeh, 2010), Indian (Agarwal & Tripathi, 2014), China (Zhan et al., 2015), and southern Italy (Panza et al., 2000). The protective role of the $\epsilon 2$ allele probably roots in a more stable protein structure, reducing the cholesterol level, resistance against A β deposition, lower densities of neurotic plaque, etc. (Grothe et al., 2020; Suri et al., 2013). Despite all, some evidence showed no protective role of the $\epsilon 2$ allele in controls (Ghayeghran, Akbarshahi1, & Zivar Salehi2, 2017). Unlike the $\epsilon 2$ protective function, the $\epsilon 4$ allele is generally considered a genetic risk factor for AD.

The prevalence of the $\epsilon 4$ allele in AD patients varies worldwide. The highest prevalence is estimated in northern Europe and the least in Asia and southern Europe (Ward et al., 2012). In a study done in south Iran, Barzegar et al. reported the frequency of APOE alleles similar to those found in Taiwan, Oman, Lebanon, India, Turkey, Greece, Spain, Sardinia Islands of Italy and two other Iranian populations (conducted in Tehran and Kermanshah). Their study reported the lowest $\epsilon 4$ allele frequency globally to that date (Saadat, 2008). Further studies in Iran mostly accounted for the $\epsilon 4$ allele as a genetic risk factor of AD (Ghayeghran, Akbarshahi, & Zivar Salehi2, 2017; Gozalpour et al., 2010; Naji T1, Ebrahimi A2, & Anari M3, 2018).

Our results showed a borderline statistical relationship of the higher $\epsilon 4$ allele frequency in patients than controls. Statistically, borderline relationship ranges are neither completely acceptable nor completely unrelated. It means that we cannot strongly consider the $\epsilon 4$ allele as a genetic risk factor of AD in this area. Considering some test circumstances may justify the antithesis results reported in various studies of Iran or other countries.

Geography, isolation by distance, genetic drift, and less likely pre-historical selection can result in a different distribution of APOE genotyping in other regions and even within countries (Singh et al., 2006; Ward et al., 2012). In addition to the mentioned factors, stricter choice of inclusion criteria may lead to more realistic results. For example, considering the two following criteria can influence the better results deduction. Firstly, the $\epsilon 4$ allele is significantly associated with Dementia of the Alzheimer Type (DAT) as the most common neuropathology of the disease. In contrast, the $\epsilon 4$ allele has weak or no association with Primary Progressive Aphasia (PPA) and

Frontotemporal Dementia (FTD) forms of Alzheimer's disease. It means that considering the neuropathology of AD samples as an included criteria may affect the results of $\epsilon 4$ allele frequency in patients (D et al., 2017; Rogalski et al., 2011). Secondly, we need to consider that a large sample size at the national scale could provide more reliable APOE allele frequencies to present to health policymakers. To reach this goal, proper and optimal techniques can help time and money-saving.

Among techniques that have been set up for SNP genotyping, HRM analysis and Multiplex T-ARMS PCR appear to be appropriately effective and inexpensive. Few studies have been done to determine the APOE genotype by HRM analysis. A study in China in 2015 displayed the HRM analysis as a suitable method for APOE genotyping (Zhan et al., 2015). Still, the strategy of this study showed that four examiners were needed to determine the APOE genotype for each sample which seemed expensive and time-consuming. In our survey, HRM analysis distinguished rs429358 heterozygotes and homozygotes variants effectively in just one reaction. However, it has poor performance in detecting the rs7412 variant, so that no concordance was observed between the results of the HRM analysis and Sanger sequencing results in rs7412 loci.

Although HRM analysis is a closed-tube, sensitive and low-cost method, it is a sensitive technique to salt concentration, pipetting, primer design, amplicon length, GC content, and the need to standardize DNA concentration, fresh samples, etc. (Słomka et al., 2017; Tindall et al., 2009).

Regarding the APOE gene region with high guanine-cytosine (GC) repetitive content (Figure 1), there was not much choice in designing, redesigning, and choosing primers with enough resolution in rs7412 loci. It seems that the HRM analysis setup is complicated and time-consuming for APOE genotyping.

In contrast to HRM analysis, Multiplex T-ARMS PCR could genotype the APOE gene quickly and effectively. Although it needed post-PCR preparation, it could determine two variants by just one PCR reaction. This technique was not sensitive to DNA quality and concentration, pipetting, and required no DNA concentration adjusting or specific DNA extraction kit to arrange salt density. Moreover, the cost per reaction of Multiplex T-ARMS PCR became far less than the HRM analysis technique.

The study demonstrated that $\epsilon 2$ allele and $\epsilon 2 \epsilon 3$ genotype are protective genetic factors against AD, and $\epsilon 4$ allele might be a factor predisposing to AD in a southeastern Iranian population. A

large sample size and more carefully selected inclusion criteria may lead to better validity of the results.

Moreover, the present study highlighted the accuracy, simplicity, and cost-effectiveness of Multiplex T-ARMS PCR in APOE genotyping compared with the HRM analysis technique.

Suggestion

For better inference about genetic criteria of Alzheimer's disease, investigating other rare variants and GRSs on a large scale is suggested.

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Ethical considerations

The Ethical Committee approved this study of the Kerman University of Medical Science, Kerman, Iran (IR.KMU.REC.12th May 2018)

Conflict of interest

The authors declare no conflict of interest.

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