Research Paper

Comparing PCR With High-resolution Melting Analysis for Apolipoprotein E Genotyping in Alzheimer's: A Case-control Study

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Citation Pourshaikhali,S., Saleh-Gohari, N., Saeidi, K., Fekri Soofiabadi, M. (2024). Comparing PCR With High-resolution Melting Analysis for Apolipoprotein E Genotyping in Alzheimer's: A Case-control Study. *Basic and Clinical Neuroscience, 15*(1), 37-48. http://dx.doi.org/10.32598/bcn.2022.206.2

http://dx.doi.org/10.32598/bcn.2022.206.2

Article info:
Received: 10 Jan 2021
First Revision: 18 Aug 2021
Accepted: 15 Dec 2021
Available Online: 01 Jan 2024

Keywords:
Alzheimer disease, *APOE* genotyping, HRM analysis, Multiplex T-ARMS PCR, *ε2*, *ε4*

ABSTRACT

Introduction: The apolipoprotein E (*APOE*) genotype has a heterogeneous distribution throughout the world. The present study aimed to characterize the *APOE* genotype (*rs429358*, *rs7412*) in healthy individuals compared with Alzheimer 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 tetra-primer amplification-refractory mutation system polymerase chain reaction (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 *ε2ε3* genotype (P=0.006, odd ratio [OR]=0.119) and *ε2* allele (P=0.004, OR=0.129) were more prevalent in the control group compared with the case ones, whereas *ε4* allele was associated with borderline risk of Alzheimer disease (P=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 *ε2 ε3* genotype and *ε2* allele are protective against Alzheimer whereas the *ε4* allele cannot be strongly considered as Alzheimer genetic risk factor in Kerman, Iran. The results may help to choose a better technique for *APOE* genotyping.

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Highlights

• In contrast to multiplex tetra-primer amplification-refractory mutation system polymerase chain reaction (T-ARMS PCR) test, high-resolution melting (HRM) analysis was not efficient in rs7412 genotyping.

• The *ε2ε3* genotype and *ε2* allele were more prevalent in healthy people compared to the patients with Alzheimer's disease.

• The *ε4* allele cannot be a genetic risk factor for Alzheimer's disease.

• Multiplex T-ARMS PCR test is a better option than HRM analysis for *APOE* genotyping due to having higher speed, accuracy, and simplicity.

Plain Language Summary

Alzheimer's is a major neurological disease leading to dementia. Contrary to widespread beliefs, this condition is not confined to old age, and its early onset has been documented before the age of 65. In this study, we delved into the principal genetic factor behind Alzheimer's disease, APOE genotyping, and compared it with a control group in Kerman, Iran. Our study employed two laboratory techniques intending to suggest an improved method for assessing the APOE gene genotype. The findings revealed that the *ε2* allele plays a protective role against the disease, and Multiplex-tetra ARMS PCR was deemed more suitable than a high-resolution melting analysis assay for studies involving larger populations.

1. Introduction

Every 3 seconds, there will be one new case of Alzheimer 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 60-70% of dementia cases have AD, and almost 60% of dementia cases are living in low and middle-income countries (WHO, 2023).

AD is a neurodegenerative disease with two important pathological hallmarks. The first one is the extraneuronal amyloid-beta (Aβ) deposition that develops toxic plaques, and the other one is the intracellular accumulation of hyperphosphorylated tau protein, which creates neurofibrillary tangles (NFTs) (Awasthi et al., 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 (Singh et al., 2016). There are two forms of AD based on the time of disease manifestation. Familial or early-onset AD (EOAD) accounts for nearly 5% of AD cases who develop the symptoms before 60. The other disease is sporadic or late-onset AD (LOAD), which accounts for about 95% of AD cases with over 60-65 years of onset. Overproduction or clearance impairment of Aβ is the basis of EOAD and LOAD, respectively. In EOAD, mutations in Amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*) genes cause the autosomal dominant form of AD (Bekris & 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 (Karch & Goate, 2015). *APOE* is an important lipoprotein and cholesterol transfer binding to the surface of different brain cells and rolling like high-density lipoprotein 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 (*ε2, ε3, ε4*) and six genotypes (*ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4*, and *ε4/ε4*).

It seems that the *APOE* genotype plays an essential role in the prognosis of AD since the *APOE ε4* allele is involved in AD pathogenesis. *APOE ε4* allele increases the disease risk with different mechanisms briefly including the Aβ-dependent pathway (reduce clearance and accelerate aggregation), Tau hyper-phosphorylation (Liu et al., 2008), neuro-inflammation exacerbate (Zhan et al., 2015), glucose cerebral metabolism, and lipid/cholesterol transport reduction (Wu et al., 2018; Safieh, 2019).

Over the years, in addition to the *APOE ε4* allele, genome-wide associated studies and sequence studies have identified >20 genes that have a bearing on LOAD genetics. Combining these genes created genetic risk scores (GRSs) as a better genetic prediction factor (Giri et al., 2016; Karch & Goate, 2015; Van Cauwenberghe et al., 2016). 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 which may have a minor effect on the probability of getting the disease (Chouraki et al., 2016) evaluated its association with incident AD and assessed its capacity to improve risk prediction over traditional models based on age, sex, education, and *APOE ε4*. In eight prospective cohorts included in the international genomics of Alzheimer's project (IGAP. Despite the importance of GRSs, *APOE ε4* 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 is the study of *APOE* genotyping.

Several methods have been used for APOE genotyping such as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (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 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 et al., 2019; Yang et al., 2019).

Here, these two techniques were chosen to be compared 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. Materials and Methods

Study design and sampling

This case-control study was done in southeastern (Kerman) Iran with more than 65-year-old participants. Peripheral blood samples of 90 AD and 90 control ones were collected into tubes containing EDTA. In addition to age, having National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA), Alzheimer criteria, and AD diagnosis by a neurologist were the other inclusion criteria for cases. Control subjects were recruited from healthy people who were sex and age-matched with AD samples.

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 the OD 260 nm to OD 280 nm 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.

Primer design

We designed primers using the online Primer 3, Primer designing tool and offline Oligo tools of Gene Runner software 3.05 (Hastings Software Inc. Hastings, NY, USA.

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

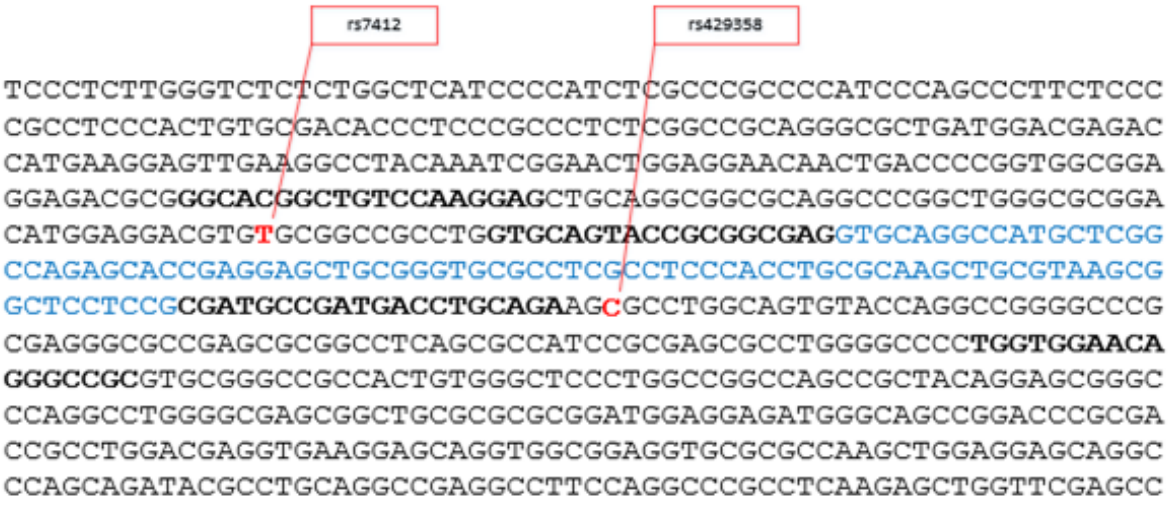

Figure 1. The high GC content of *APOE* gene

Note: For HRM analysis, primers of *rs7412* and *rs429358* were designed separately despite their 137bp distance (blue NTs).

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 the reaction was 10 µL, containing 5 µL of Type-it HRM PCR master mix (Qiagen, Germany), 0.3 µL forward and 0.7 µL reverse primer (10 Pmol/µl), 1 µL distilled water, and 3 µL DNA in a fix concentration of 40 ng per reaction.

PCR cycling program started at 95˚C for 5 minutes as a hold step to activate Hotstar Taq plus DNA polymerase of the master mix, followed by 40 times 2 step cycling consisting of 95˚C for 10 seconds as denaturation and 68˚C (for *rs429358*)/66˚C (for *rs7412*) for 30 seconds as annealing/extension step. At the end of each annealing/extension step, fluorescence data were acquired for cycle 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 seconds per step. All samples were performed in duplicate.

Multiplex T-ARMS PCR for *APOE* genotyping

PCR reaction performed with C1000 Touch thermal cycler (BioRad). Each microtube contained in a total volume of 31.45 µL with 40 ng DNA polymerase enzyme, 0.25 units of HotStarTaq DNA polymerase, 6% DMSO, 1Mm $MgCL_2$, 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 minutes, 25 cycles at 94˚C for 1 minute, 55˚C for 30 seconds, and 72˚C for 2 minutes followed by 72˚C for 5 minutes. PCR products were differentiated on 2% gel agarose and stained with Invitrogen dye (SYBR Safe DNA Gel Stain-Thermo Fisher).

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 Table 1). The thermal cycling program was 93˚C for 3 minutes, followed by 30 cycles repeated at 93˚C for 1 minute, 62˚C for 30 seconds, and 74˚C for 45 seconds. The final extension was 72˚C for 3 minutes. Sequence results were analyzed with Chromas software, version 2.6.

Statistical analysis

Statistical analysis was performed using SPSS software, 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. The chi-square test was used for qualitative data like sex. The chi-square test was fulfilled to evaluate both allele and genotype frequencies and also Hardy-Weinberg equilibrium (HWE) for all six geno-

Table 1. 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′

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Abbreviations: HRM: High-resolution melting; PCR-DNA: Polymerase chain reaction deoxyribonucleic; SNP: Single nucleotide polymorphism; T-ARMS PCR: Tetra-primer amplification-refractory mutation system polymerase chain reaction.

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

3. Results

Demographic findings

The average age of participants was 73.36±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=0.54). The average age and gender of participants are described in Table 2 separately for cases and controls. As shown, there was no significant difference in the sex and age of the participants in both groups.

The frequency of *ε3*, *ε2*, and *ε4* were 81.1%, 10% & 8.9% in control and 82.2%, 2.2%, and 15.6% in the case group, respectively (Table 3). Genotyping distribution showed that *ε2 ε3* genotype (P=0.006, OR=0.119) and *ε2* allele (P=0.004, OR=0.219) are protective against AD, and logistic regression displayed the dominant (P=0.00, OR=0.138) and codominant (P=0.00, OR=0.91) effects of *ε2* allele in inheritance models. The *ε4* allele was a risk factor with borderline significant trends (P=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=0.8).

Table 2. The mean age and gender of participants (n=90)

Variables		Case	Control	P
Age (y)		73.81±8.36	72.06±8.65	0.171
Sex	Male	57	46	0.097
	Female	33	44	

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Table 3. Frequencies of APOE alleles and genotypes and models of inheritance for *ε2* allele

Variables		No. (%)		OR (95% CI)	P
		Case (Alzheimer)	Control		
Allele	*ε3*	148(82.2)	146(81.1)	ref	
	ε2	4(2.2)	18(10)	0.219(0.072-0.66)	0.004
	ε4	28(15.6)	16(8.9)	1.72(0.89-3.32)	0.099
Genotype	*ε3ε3*	60(66.7)	73(70)	ref	
	ε2ε2	0	1(1.1)	1.53E9	1.000
	ε4ε4	2(2.2)	4(4.4)	1.90(0.33-10.78)	0.466
	ε2ε3	16(17.8)	2(2.2)	0.119(0.26-0.540)	0.006
	ε2ε4	2(2.2)	0	0	0.999
	ε3ε4	10(11.1)	20(22.2)	1.90(0.82-4.40)	0.132
Models of inheritance for *ε2* allele	Dominant	*(ε2ε2+ε2 ε3+ε2ε4)* 3(3.3) *(ε2ε2+ε2ε2+ε2ε2)* 87(96.7)	18(20) 72(80)	0.138(0.039-0.487)	0.00
	Recessive	*(ε2ε2)* 1(1.1) Other genotypes 89(89.9)	0 90(100)	0.989(0.967-1.011)	0.316
	Codominant	*(ε2ε3+ε2ε4)* 18(20) Other genotypes 72(80)	2(2.2) 88(97.8)	0.91(0.02-0.405)	0.00

OR: Odd ratio; CI: Confidence interval.

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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 TM distinguished homozygote samples. They were categorized into TT as wild-type homozygotes and CC as mutant homozygotes (Figure 2).

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

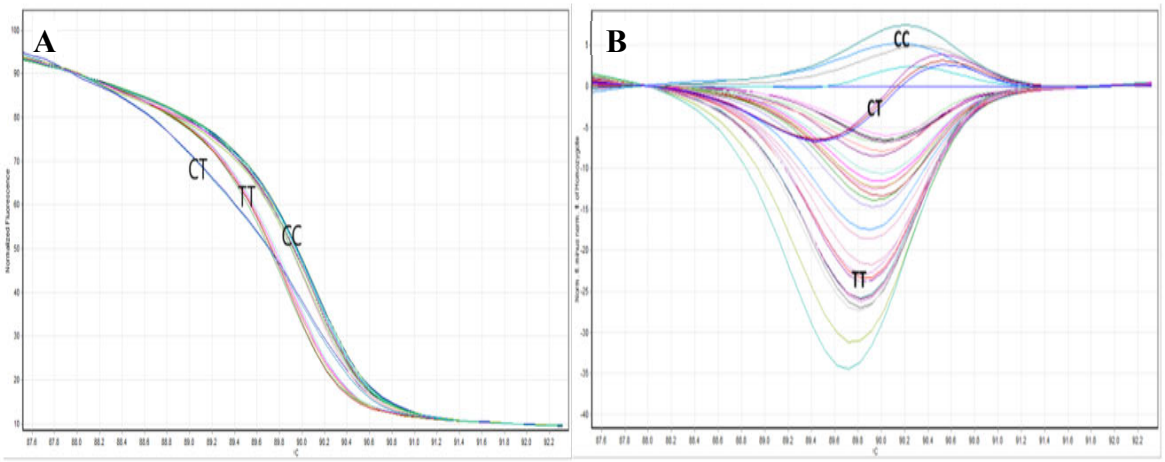

Figure 2. High-resolution melting analysis results of *rs429358*

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A) Normalized and shifted melting curves for the amplicon of *rs429358*, B) Normalized and temperature-shifted difference plot for the amplicon of *rs429358*

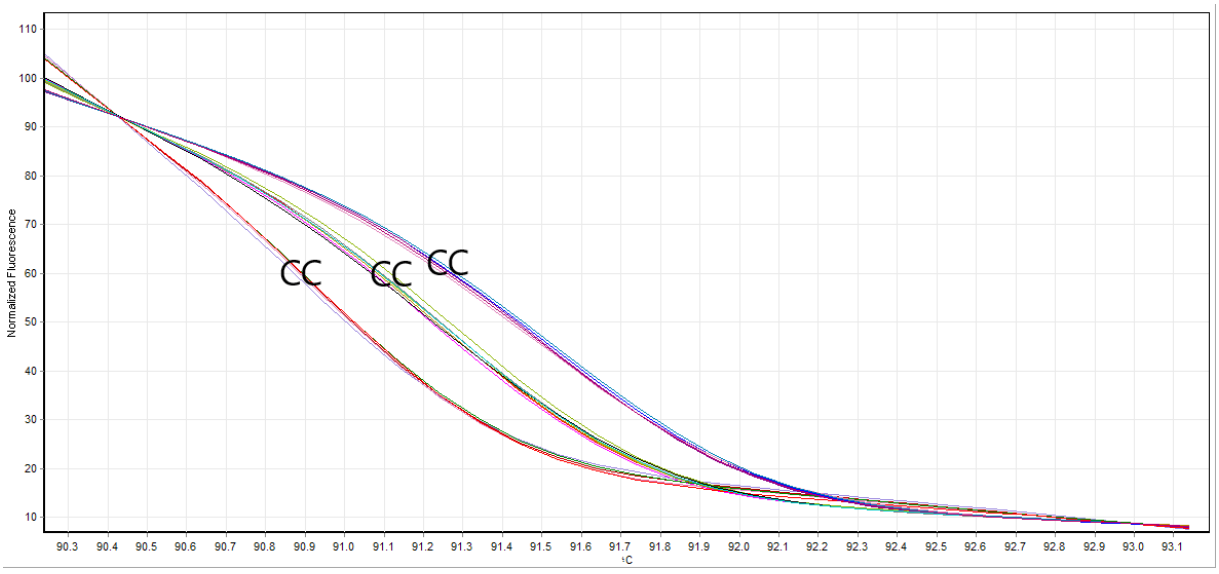

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Figure 3. Various normalized and shifted melting curves for the amplicon of *rs7412* with the same CC genotype

locus, wild-type, heterozygote, and homozygote classifications based on more than 0.5°C TM differences were inconsistent with sequencing results (Figure 3).

The results of multiplex T-ARMS PCR were interpreted based on the number and length of bands on the gel (Figure 4). The findings of HRM analysis and multiplex-T ARMS PCR showed the same genotype results of *rs429358*. Finally, PCR-DNA sequencing results were matched with the genotype classification of multiplex T-ARMS PCR (Figure 5).

4. Discussion

Although the frequency of *APOE* alleles is the most well-known genetic risk factor for AD, 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.

	115bp	253bp	307bp	444bp	514bp
ε3/ε3	+		+		+
ε2/ε2	+	+			+
ε4/ε4			+	+	+
ε3/ε4	+		+	+	+
ε2/ε4	+	+	+	+	+
ε2/ε3	+	+	+		+

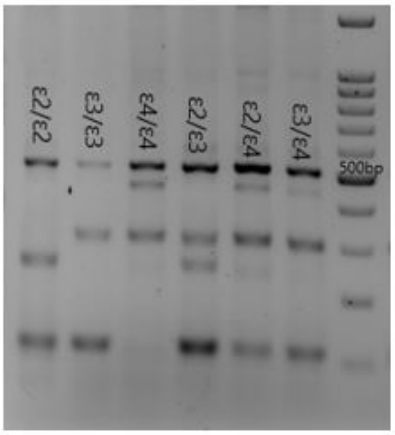

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Figure 4. Interpretation of the results of multiplex T-ARMS PCR on gel based on the number and length of bands

Note: *ε3ε3* genotype has 115, 307, and 514 bp bands

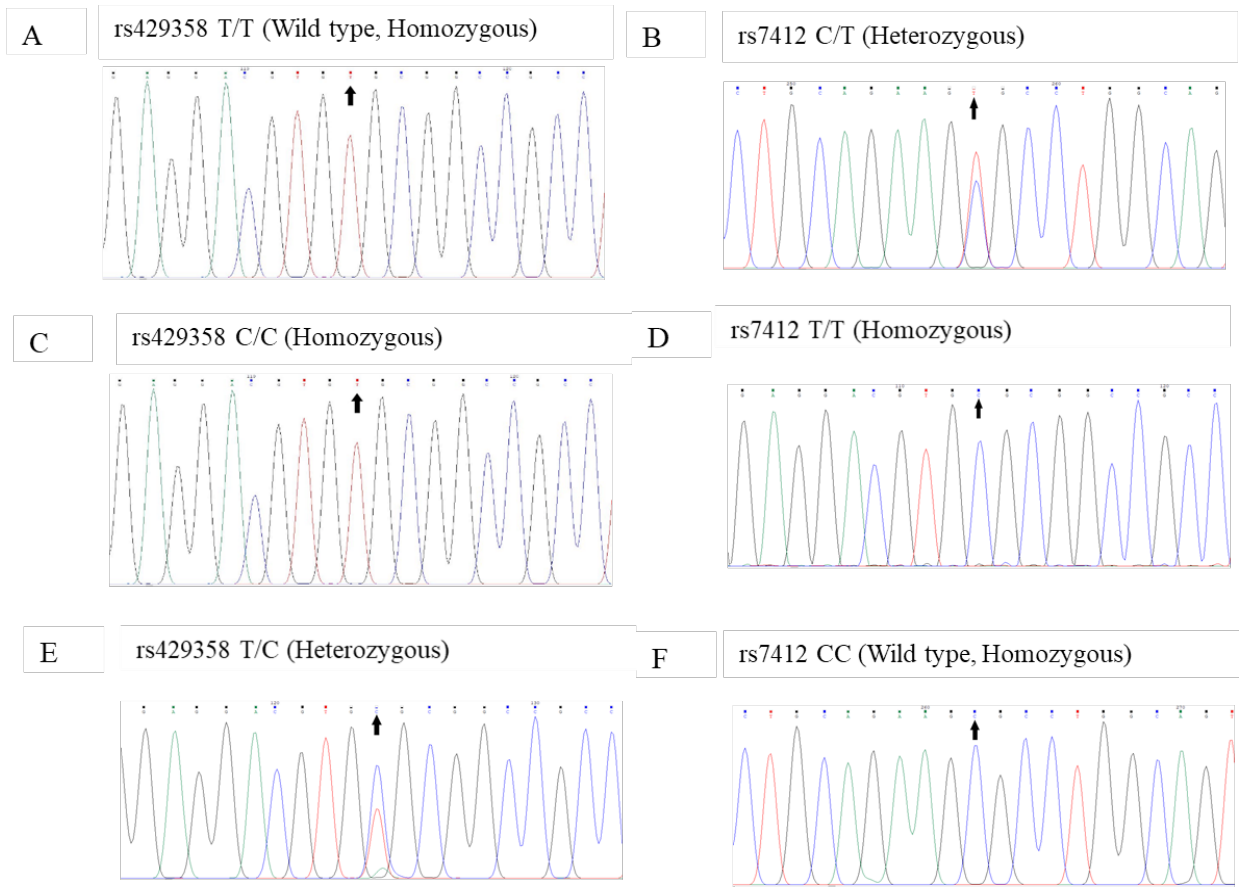

Figure 5. Results of PCR DNA-sequencing in *rs429358* and *rs7412* loci based on the multiplex T-ARMS PCR technique

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)

This study represented the protection role of *ε2* allele and *ε2ε3* genotype against AD that was in agreement with similar findings in Iran (Gozalpour et al., 2010), India (Agarwal & Tripathi, 2014), China (Zhan et al., 2015) and southern Italy (Panza et al., 2000). The protective role of the *ε2* allele is probably rooted in a more stable protein structure, reducing the cholesterol level, resistance against Aβ 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 *ε2* allele in controls (Ghayeghran et al., 2017). Unlike the *ε2* protective function, the *ε4* allele is generally considered a genetic risk factor for AD.

The prevalence of the *ε4* 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 *ε4* allele frequency globally to that date (Bazrgar et al., 2008). Further studies in Iran mostly accounted for the *ε4* allele as a genetic risk factor of AD (Ghayeghran et al., 2017; Gozalpour et al., 2010; Naji et al., 2018).

Our results showed a borderline statistical relationship of the higher *ε4* 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 *ε4* allele as a genetic risk factor for 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, a 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 *ε4* allele is significantly associated with dementia of the Alzheimer type (DAT) as the most common neuropathology of the disease. In contrast, the *ε4* allele has weak or no association with primary progressive aphasia (PPA) and frontotemporal dementia (FTD) forms of AD. This means that considering the neuropathology of AD samples as an included criterion may affect the results of *ε4* allele frequency in patients (Borges 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 save time and money.

Among techniques that have been set up for SNP genotyping, HRM analysis, and Multiple T-ARMS PCR are 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 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 the *ε2* allele and *ε2ε3* genotype are protective genetic factors against AD, and the *ε4* 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.

5. Conclusion

The study conducted in Kerman, southeastern Iran, compared *APOE* genotype characteristics in healthy individuals and Alzheimer's patients using HRM analysis and multiplex T-ARMS PCR techniques. The findings highlighted the superiority of Multiplex T-ARMS PCR over HRM analysis in terms of efficiency for *APOE* genotyping due to its speed, accuracy, simplicity, and cost-effectiveness, especially in large-scale applications. The results indicated that *ε2ε3* genotype and *ε2* allele appear to confer protection against Alzheimer's disease in the region, while the association of the *ε4* allele with Alzheimer's risk was observed to be borderline. These insights contribute to better understanding the genetic factors influencing Alzheimer's disease susceptibility in Kerman, Iran, and provide valuable guidance for selecting the most suitable genotyping technique for future research and clinical applications.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of Kerman University of Medical Sciences (Code: IR.KMU.REC, 12th May 2018) and conformed to the Declaration of Helsinki regarding research involving human subjects. Informed consent was also obtained from all participants or their families.

Funding

The paper was extracted from the master's thesis f Sara Pourshaikhali, approved by Department of Medical Genetics, Afzalipour Faculty of Medicine, Kerman University of Medical Sciences and was funded by Kerman University of Medical Sciences.

Authors' contributions

Conceptualization and supervision: Sara Pourshaikhali, Nasrollah Saleh-Gohari; Methodology: Sara Pourshaikhali, Kolsoum Saeidi and Mehrsa Fekri Soofiabadi; Investigation and writing: All authors; Funding acquisition and resources: Nasrollah Saleh-Gohari.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgments

The authors want to thank the Research Center for Hydatid Disease in Iran, Kerman University of Medical Sciences. The authors also appreciate Mohammad Ali Mohammadi and Ali Afkar for their helpful comments on the experimental design.

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