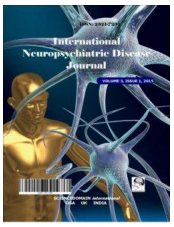

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Investigating the Frequency and Association of BACE1-AS rs147542312C/T with Alzheimer's Disease in Northwest of Iran

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Authors' contributions

This work was carried out in collaboration between all authors. Author MKK designed the study, wrote the protocol and edited the manuscript. Author MV performed the experiments and the statistical analysis and wrote the first draft of the manuscript. Author MAHF managed the analyses of the study and literature searches. Authors MT and MF evaluated the clinical values and approved the AD cases and controls. All authors read and approved the final manuscript.

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ABSTRACT

Background and Aims: Alzheimer's disease is the most common neurodegenerative disorder worldwide. The long noncoding RNA, BACE1-AS, acts as a regulator of BACE1 transcript which its product involves in Alzheimer's disease pathobiology. We investigated the frequency and association of rs147542312C/T of *BACE1-AS* gene with Alzheimer's disease in northwest of Iran.
Place and Duration of Study: Department of Genetics and Animal Biology (Faculty of Natural Science, University of Tabriz) and Neurosciences Research Center, Tabriz University of Medical Sciences, between February 2016 and May 2017.
Methodology: Genomic DNA was extracted from peripheral blood cells of 180 people, including 87 cases and 93 healthy controls by standard salting out method. The samples were genotyped using tetra-primer as well as three-primer ARMS-PCR methods. To further verify the results, some

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samples were sequenced randomly. Tetra-primer and three-primer ARMS-PCR methods revealed no T allele either in cases or in controls and the results were verified with sequencing.
Results: The results obtained showed that all cases and controls were genotypically CC homozygotes. This study did not reveal any association between the rs147542312C/T polymorphism of *BACE1-AS* with Alzheimer's disease. So the rs147542312C/T polymorphism of *BACE1-AS* do not present or may occur in a very low frequency in northwest of Iran.
Conclusion: The results imply that the rs147542312C/T polymorphism of *BACE1-AS* can't be considered as an informative genetic change for prevalence of Alzheimer's disease in northwest of Iran.

Keywords: Alzheimer's disease; polymorphism; BACE1; BACE1-AS; association study.

1. INTRODUCTION

Alzheimer's disease (AD) is characterized by deposition of extraneuronal amyloid-β (Aβ) plaques and intraneuronal neurofibrillary tangles (NFTs) in the brain which are accompanied by synaptic dysfunction and neurodegeneration [1]. It is the most common neurodegenerative disorder which occurs in familial (early age-of-onset) and nonfamilial (late age-of-onset) forms. Common form of the disease is nonfamilial which happens at people mostly over 65 years old [2]. Late age-of-onset or sporadic AD is a multifactorial condition that might be affected by individual's genetic background as well as some environmental factors including diabetes mellitus, hypertension, obesity, lack of physical activity, depression [3] and oxidative stress sources [4,5]. However, about 70% of its risk factors are genetical [6] involving different genes such as *APOE, CD33*, *PICALM*, *TREM2*, *CR1* and many others [7]. In general, genes that involve in the pathways leading to Aβ peptides production and/or clearance might be considered as critical genetic factors in AD pathobiology. Based on the amyloid hypothesis, rises in production or defects in clearance of Aβ peptides from the brain are the leading causes of AD [8].

The amyloid-β peptides are produced in amyloidogenic pathway via processing of the APP (amyloid-precursor protein) by β and γ-secretases [9,10]. The main enzyme in production of Aβ peptides, β-secretase, is encoded by the *BACE1* gene on the chromosome 11q23.2-11q23.3. The gene has 9 exons and 8 introns and is regulated by a long non-coding antisense RNA (lncRNA) known as BACE1-AS [11]. BACE1-AS lncRNA is transcribed from the opposite strand of *BACE1* gene. Its transcription results in transcripts with ~2 Kb lengths. It is partially complement with the exon 6 encoding region of the BACE1 mRNA, where their pairing leads to elevation of the BACE1 mRNA stability and translation. Elevation of β-secretase then boosts Aβ production and plaque formation [11].

It was recently reported that single nucleotide polymorphisms (SNPs) of lncRNAs can play important roles in their function though in some cases associate with pathologic conditions [12,13]. Due to critical regulatory role of BACE1-AS in β-secretase level, we proposed that its SNPs might contribute to BACE1-AS dysfunction and thereby to misregulation of BACE1 mRNA translation and Alzheimer's disease pathobiology. So, this study was intended to evaluate the association of *BACE1-AS* variations with Alzheimer's disease. For achieving this, we chose rs147542312C/T of the *BACE1-AS* gene which is a functional splice-site variation [14], and evaluated its frequency and association with Alzheimer's disease in northwest of Iran.

2. MATERIALS AND METHODS

2.1 Patients

In this case-control study 87 patients (50 female and 37 male) with AD symptoms and 93 healthy people (58 female and 35 male) without any familial AD history in their first-degree relatives were enrolled. AD subjects were recruited from the Neurology Department at the Emam Reza Hospital of Tabriz where they were diagnosed by neuroscience specialist according to the Alzheimer's disease and Related Disorders Association (ADRDA) clinical diagnostic criteria. All cases and controls were examined with mini–mental state examination (MMSE) test [15]. Subjects with MMSE scores of lower than 20, were considered as AD but those with upper than 28, as healthy controls. The mean ages of AD and control subjects were 75.05 and 72.63 years respectively. The AD subjects were sporadic with age-of-onset of above 65 years old. All cases and controls were residences of northwest of Iran with Turkish ethnicity. All enrollments or their next of kin provided informed consent. The study

was approved by the ethics committee of Tabriz University of medical sciences.

2.2 Genomic DNA Extraction and Analyzing Its Quality and Quantity

Four ml peripheral blood was taken from all the case and control individuals and collected in EDTA tubes. Genomic DNA was extracted from blood leucocytes by standard salting out method [16]. The quality and quantity of the genomic DNA samples were checked by 1% electrophoresis and spectrophotometry at 260 and 280 nm.

2.3 Tetra-primer ARMS-PCR

Tetra-primer ARMS-PCR is a practical approach that was recruited by researchers for genotyping of single nucleotide polymorphisms (SNPs) [17-19]. This method was used for genotyping the case and control samples. The primers used were: forward outer (FO) primer 5'-TCCACCGTCCTGAGTTAAAGTG-3', reverse outer (RO) primer 5'-ACACTTAGGAGGTGCCAAAGC-3', forward inner (FI) primer 5'-CCCCATCCTTAGTCCACTCAC-3' and reverse inner (RI) primer 5'-ATCAAGGCAGCCTCCTCCA-3'. PCR reactions were done at 25 µl final volume including 2.5 µl PCR Buffer 10X, 0.8 µl MgCl2 50 mM, 0.5 µl dNTP 10 mM, 0.5 µl each primer (10 pmol), 0.2 µl Taq DNA pol., 100 ng DNA and ddH_20 up to final volume. The reactions were followed as: primary denaturation at 95°C for 5 min., amplification of DNA amplicons for 35 cycles at 95°C 30 sec., 63°C 30 sec. and 72°C 45 sec., and the final amplification step at 72°C for 5 min. The PCR products were run on 2% agarose gel electrophoresis. The amplicon sizes for FO and RO, FO and RI, FI and RO primers were 577, 368 and 246 bp long respectively. The 368 and 246 bp bands on the gel represent the presence of T and C alleles in the samples, respectively.

2.4 Three-primer ARMS-PCR

To verify the results of tetra-primer ARMS-PCR and functionality of the T allele-specific primer, we studied all the samples by three-primer ARMS-PCR, as well. So, we used the same FO, RO and FI primers together with another T allele-specific primer 5'-CCCCATCCTTAGTCCACTCAT-3' for genotyping. The optimum conditions for these PCR reactions were the same as the previous ones and the only difference was that the T and C alleles-specific reactions were amplified in separate reactions. So, on the gel electrophoresis the size of amplicons was 246 bp for both T and C alleles. In this case, presence of electrophoretic bands in both T and C allele-specific reactions represents heterozygote sample.

2.5 Sequencing

To further verify the results of the PCR reactions, the products of some PCR samples were sent for sequencing by Macrogen, Inc. (South Korea), in random. The sequences were analyzed with ChromasPro version 2.0.1.

2.6 Data Analysis

Because we just observed the C allele in all samples, there was no need for further statistical analysis.

3. RESULTS

3.1 Genotyping with Tetra-primer ARMS-PCR Revealed No T Allele

Genomic DNA was extracted by salting out method from blood samples. Quality and quantity of the DNA was then checked by electrophoresis and spectrophotometry. Tetra-primer ARMS-PCR was used for identifying the genotype of rs147542312C/T in subjects with AD and healthy controls. The PCR products were run on agarose gel electrophoresis. Fig. 1 shows the electrophoresis of 5 samples. In this protocol, amplification by outer primers produces a 577 bp long amplicon as an internal control while amplification by C or T allele-specific primers produces 246 bp or 368 bp long amplicons respectively. Gel electrophoresis of both case and control samples revealed no T allele, so then all subjects were CC homozygotes.

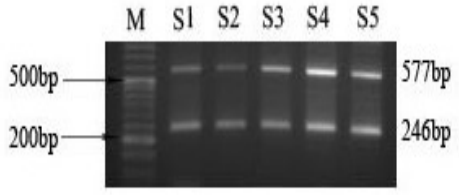

Fig. 1. Gel electrophoresis showing the results of tetra-primer ARMS-PCR reactions. M; DNA size marker, S; sample. 577bp amplicon is as an internal control and 246bp amplicon shows the C allele for 5 samples

3.2 Genotyping with Three-primer ARMS-PCR Also Revealed No T Allele

We observed no T allele in the tetra-primer ARMS-PCR tests. Therefore, for verifying the results obtained, we conducted another experiments with three-primer ARMS-PCR strategy. In these experiments, the T or C allele-specific primers were identical except for the 3'-end nucleotides which were T or C, respectively. All the control and case samples were then genotyped with three-primer ARMS-PCR. Again, the results obtained (Fig. 2) revealed no T allele in all the samples, which further confirmed the results of tetra-primer ARMS-PCR method.

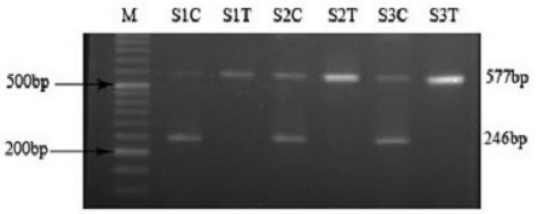

Fig. 2. Agarose gel electrophoresis representing the results of three-primer ARMS-PCR. Each lane represents the PCR products obtained from a combination of FO, RO and C or T allele specific primers. The 577 bp band is product of FO and RO primers as an internal control, while the 246 bp band is product of RO and C or T allele specific primers. M; DNA size marker, S; sample

3.3 Sequencing Verified Absence of T Allele in the Samples

To further verify the results of ARMS-PCR genotyping experiments, we sequenced 10 percent of samples in random. Again, sequencing showed no T allele in the samples. All results validated that there was no T allele in the studied population and the genotypes of both cases and controls were CC homozygote. The Table 1 outlines genotype and allele frequencies of the rs147542312C/T from the *BACE1-AS* gene in patients, controls and total population.

4. DISCUSSION

According to the 2013 Alzheimer's Association report, AD is the most common neuro-degenerative disorder [20]. So, considering the factors affecting the severity of the disease is necessary and of great importance. The effect of β-secretase enzyme and its product, Aβ peptides, on the pathology of AD has greatly been studied [21-25]. These studies have revealed that up-regulation of *BACE1*, the gene encoding β-secretase, might lead to the Aβ peptides overload, and hence the progression of the AD [11,22,25]. Moreover, the antisense transcript of β-secretase (BACE1-AS) increases the stability of the BACE1 transcript leading to its elevated activity [11]. On the other hand, it was reported that polymorphisms can affect the function of a gene product (RNA or protein) [26,27]. With this in mind, one may propose that polymorphisms of BACE1-AS possibly would influence its structural and/or functional behavior. One class of polymorphisms having such behavioral effects is splice-site variations or splice variants [28,29].

In the present study, we evaluated the frequency and probable association of rs147542312C/T, a splice-site variation (14), from *BACE1-AS* gene with the Alzheimer's disease by tetra-primer ARMS-PCR. We observed no T allele at rs147542312C/T site of the *BACE1-AS* gene and all samples showed the CC homozygote genotype. We further verified the observed results by repeating the genotyping tests with three-primer ARMS-PCR method as well as sequencing of some PCR samples in random. These results imply absence or very low frequency of the T allele in northwest of Iran. Moreover, according to the results obtained, presence of any association between the *BACE1-AS* rs147542312C/T with the Alzheimer's disease might be excluded.

Table 1. Genotyping results; genotype and allele frequencies

		Case		Control		Number of samples studied	Frequency in the population
		Number	Frequency	Number	Frequency		
Allele	C	174	0.48	186	0.52	360	1
	T	0	0	0	0	0	0
Genotype	CC	87	0.48	93	0.52	180	1
	CT	0	0	0	0	0	0
	TT	0	0	0	0	0	0

Absence of a polymorphism in a population might occur due to some reasons: it may occur due to small sample size [30], which causes the studied sample not to be an appropriate representation for the main population. The number of samples enrolled in this study seems low, however, considering the high prevalence of AD (one out of nine) at people over 65 years old in the US [31], the number of samples studied seems adequate, though the AD prevalence is almost the same worldwide [31,32]. There is no strict data about the AD prevalence in Iran, however, implementing the study with more samples would be helpful to get more exact results. One another reason might be the genetic background of the population [33]. If a polymorphism is not present in the genetic background of a population or it has been created recently, then the frequency of the polymorphism would be very low in that population. The other important factor might be the effect of the polymorphism on the fitness [31]. The more the polymorphism affects negatively the fitness, the more the frequency of that polymorphism decreases in the population; as such it may even be eliminated from the population.

5. CONCLUSION

In conclusion, genotyping 87 AD cases and 93 healthy controls from northwest of Iran in present study, showed no T allele at rs147542312C/T site of the *BACE1-AS* gene and all samples were CC homozygotes. This observation implies absence or very low frequency of it in the northwest of Iran. All together, the results imply no association of the rs147542312C/T site of the *BACE1-AS* gene with Alzheimer's disease.

CONSENT

All authors declare that written informed consent was obtained from the patients or their next of kin for taking part in the research project.

ETHICAL APPROVAL

The study was approved by the ethics committee of Tabriz University of medical sciences.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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