

## **An Association Analysis of Reelin Gene (RELN) Exon 22 (G/C), Rs.362691, Polymorphism with Autism Spectrum Disorder among Iranian-Azeri Population**

Leila Mehdizadeh Fanid <sup>1</sup>, Mohammad Ali Hosseinpour Feizi <sup>2</sup>, Mina Adampour Zare <sup>3</sup>,  
\*Hasan Shahrokhi <sup>4</sup>

<sup>1</sup>Assistant Professor of Cognitive Neuroscience. Department of Animal Biology Faculty of Natural Sciences. University of Tabriz, 29 Bahman Bolvard, Tabriz, Iran, IR. <sup>2</sup>Professor of Radiobiology, Department of Animal Biology Faculty of Natural Sciences. University of Tabriz, 29 Bahman Bolvard, Tabriz, Iran, IR. <sup>3</sup>Physiologist, Department of Animal Biology Faculty of Natural Sciences, University of Tabriz, 29 Bahman Bolvard, Tabriz, Iran, IR. <sup>4</sup>Child and Adolescent Psychiatrist MD, Research Centre of Psychiatry and Behavioral Science, Tabriz University of Medical Science, Tabriz, Iran, IR.

### **Abstract**

#### **Background**

Autism spectrum disorder (ASD) is a intricate childhood neuropsychiatric disorder that is described by deficits in communication of verbal and non-verbal, reciprocal social interactions, stereotypic behaviors, interests, and activities. The studies of post-mortem neuro-anatomical anomalies have indicated that migration alterations could occur early during development (first trimester) in autistic brain. Since the Reelin gene, plays a crucial role in these migratory processes, it is subsequently considered as a potential candidate gene for autism.

#### **Materials and Methods**

In this case-control study, we recruited 74 patients with ASD and 88 healthy controls from Iranian-Azeri Population. Genomic DNA isolated from blood leukocytes of cases and control individuals by the proteinase K and using salt-out method. Single nucleotide polymorphisms (SNP) genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique.

#### **Results**

The allele and genotype frequencies did not show significant difference between autistic and control groups ( $P>0.05$ ). No significant relationship was observed between the genders and genotypes in autism group ( $P>0.05$ ).

#### **Conclusion**

The current study showed that the SNPs rs362691 could not be used as a useful molecular biomarker to predict genetic susceptibility for ASD among Iranian-Azeri patients.

**Key Words:** Autism, Children, Molecular marker, Polymorphism, Reelin gene.

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#### **\*Corresponding Author:**

Hasan Shahrokhi, Child and Adolescent Psychiatrist MD, Research Centre of Psychiatry and Behavioral Science, Tabriz University of Medical Science, Tabriz, Iran, IR.

E- Email: shaahrokhi.hasan@yahoo.com

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## 1- INTRODUCTION

Reelin is a large secreted signaling protein that plays an important role in the development of nervous system. During neuronal development, it is mainly secreted by Cajal-Retzius cells at the surface of the neocortex and is responsible for the proper positioning of newly generated post mitotic neurons from the ventricular zone to form distinct brain areas and in regulation of synaptic plasticity (1-3). Reelin binds to the very low density lipoprotein (VLDL) receptors and to the apolipoprotein E receptor 2 or (ApoER2) on migrating neurons and induces tyrosine phosphorylation of Disabled-1 (Dab1) gene, an adaptor protein that interacts with the cytoplasmic domains of both receptors (4). Therefore, altered Reelin level may lead to deficits in neuronal development and function. Biochemical analyses of postmortem autistic brain have showed that Reelin involved in the pathology of autism (5).

Autism spectrum disorder (ASD) is an intrinsic childhood neuropsychiatric disorder that is described by considerable perturbations in social, communicative, and behavioral functioning. The autism is a heterogeneous disturbance and influences by both environmental (6, 7) and genetic factors (8-10). Autism has onset in early childhood and usually diagnosed before 3 years of age. The median of worldwide prevalence estimates of autism is 62/10,000 with nearly four times higher in males than in females (11).

Various studies have shown that the risk of autism is higher among siblings of affected individuals than in the general population, indicating that autism is one of the most strongly genetic childhood-onset psychiatric disorders with high heritability. Therefore, this hypothesis is accepted that several susceptibility genes are interacting together with a complex manner of heredity leading to the typical phenotypes of the autism (12). At least two to four

genes and perhaps more genes involved in the disorder (12-14). As respects, Reelin gene plays an important role in the proper positioning of newly generated post mitotic neurons and the development of nervous system. It seems to be a candidate for autism disorder. Therefore, in the current study, the association a single nucleotide polymorphism (SNPs), a G/C SNP in exon 22 (dbSNP: rs.362691, V997L), including one coding region SNPs in the Reelin gene with autism was investigated. The polymorphism in exon 22 (L997V) alters amino acid composition of the Reelin protein.

## 2- MATERIALS AND METHODS

### 2-1. Selection of Samples

The patient group included of both male and female autistic subject from North West of Iran (n=74; mean age=8.57± 0.07 and range aged 3-24 years at the time of sampling, 53 males and 18 females). All subjects were diagnosed with ASD following a thorough psychiatric assessment, developmental history, and a review of the data provided by their teachers and parents. Oral and written informed consents were obtained from at least one parent (responsible parent) of all participants, and the research protocol was approved by the ethics committee of Tabriz University of Medical sciences (ID number: 5/4/12152).

For control group, 88 volunteers were recruited from local children's Hospital in same age range. They were also examined to rule out any neurological, psychiatric, or learning problems. Control group were from those patients that were admitted to Children's Hospital for other reasons. As a result volunteers went under careful examination to rule out any neurological disorder before taking part in this study. Written consent was also obtained from control participants. Furthermore, none of these children was on medication, and this information was gathered from one of their

parents. The control group consisted of 88 children.

## 2-2. Polymerase chain reaction (PCR) and Restriction fragment length polymorphism (RFLP)

Peripheral blood samples (2cc) were taken from all the participants by someone who skilled in bloodletting and was moved in the laboratory in flask containing liquid nitrogen and delivered to the laboratory for molecular genetics study. After red blood cell lysis, genomic DNA was extracted from white blood cells using the proteinase K and salting out method, and was stored at  $-20^{\circ}\text{C}$ . The target sequence containing the polymorphic site was amplified by forward

5'-ACAGTGGAGGAGAGTCATACTG-3' and reverse

5'-ACAGTGGAGGAGAGTCATA\*CTG-3' site-specific primers.

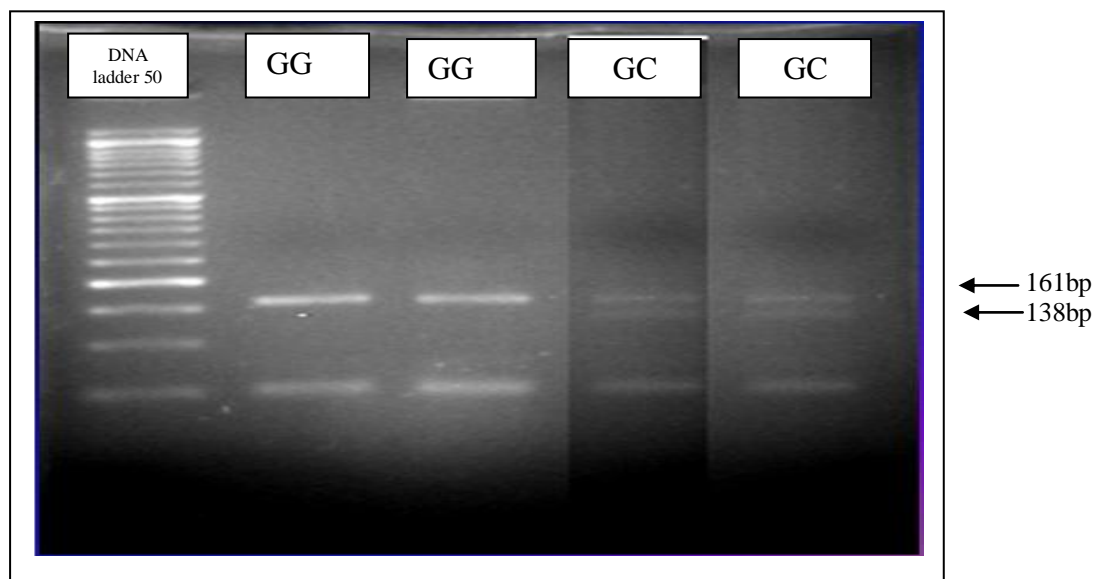
\*The 21<sup>st</sup> base of reverse primer was mutated to create a BseI RFLP site. PCR was performed in a total volume of 25  $\mu\text{l}$  reaction mixture containing 2.5  $\mu\text{l}$  10X reaction buffer, 2 mM  $\text{MgCl}_2$ , 0.2 mM dNTPs, 0.4  $\mu\text{M}$  of each primer, 0.1-0.5 $\mu\text{g}$  DNA and 1.0 U of Taq polymerase (Cinagen, Iran). The PCR was performed by thermal cycler (Sensoquest, GmbH, Germany) at 35 cycles including of steps: denaturation at  $95^{\circ}\text{C}$  for 5 min, annealing for 30 s at  $62^{\circ}\text{C}$  and extension for 30s at  $72^{\circ}\text{C}$  and final extension for 5 min at  $72^{\circ}\text{C}$ . The amplification of rs.362691 SNPs results in the product of 161bp. The PCR products digested with 4U of BseI restriction enzyme (Thermo science) for SNPs rs.362691 in a final volume of 10 $\mu\text{l}$  at  $65^{\circ}\text{C}$  for 7h and finally fragments are separated on 4 percent agarose gel containing ethidium bromide and visualized under UV-light. On the 161bp fragment, wild-type allele resulted in two fragments of 138 and 23bp. Also, variant alleles were present as an undigested 161-bp fragment.

## 3- RESULTS

In order to investigate the association of rs.362691 (G/C) with increased risk of autism spectrum disorder, the total of 74 autism patients ( $8.57 \pm 0.07$ ) and 88 healthy participants (mean age  $7.79 \pm 0.14$ ) were genotyped. Seventy one (62%) of cases was boy and 24.32% was girl. Among 88 controls, 75.0% were female and 25.0% were male. The image of the gel is illustrated in (**Figure.1**). Pearson's Chi-square test was used to compare observed genotype and allele frequencies with those that are expected in a population with Hardy-Weinberg equilibrium. The frequencies of genotype and allele did not show aberration from Hardy-Weinberg equilibrium in both patient and control groups. The frequencies of CC, CG and GG genotypes were 0, 36.4, and 63.6 % in patients and 0, 49.2 and 50.8% in controls, respectively. No significant association was observed between rs.362691 (G/C) polymorphism and increased risk of autism spectrum disorder (OR = 1.692, 95% CI: 0.830-3.449; P-value: 0.146).

According to the results of the statistical analysis, no significant difference in the frequency of two variant alleles between cases and controls was observed; although, the rs.362691 -C allele had lower frequency compared with the G allele in both groups, in fact, the G is the major allele while the C is the alternative allele in the studied population. Consistently, no statistically considerable difference detected in the overall distribution of genotype frequencies between patients and healthy individuals ( $P > 0.05$ ). However, GG homozygous dominants were more frequent than CG heterozygous and CC homozygous recessives, indicating that GG homozygous Reelin rs.362691 (G/C) was overrepresented and, conversely, CC homozygosis was remarkably underrepresented in the general Azeri population (**Table.1**).

GG: homozygous recessive; GC: heterozygous.



**Fig.1:** Banding pattern of homozygous and heterozygous genotypes is clearly distinguishable on 4 percent agarose gel.

**Table1:** Genotype and allele frequencies in autism and control groups.

Rs.362691	Case (%) n=74	Control (%) n=88	OR (95% CI)	P-Value
<b>Genotypes</b>				
G/G	58(63.6)	60(50.8)		
G/C	16(36.4)	28(49.2)	1.692(0.830-3.449)	0.146
C/C	0	0		
<b>Alleles</b>				
C	16(10.81)	28(15.90)	0.641(0.259-1.573)	0.290
G	132(89.19)	148(84.09)	1.561(0.636-3.867)	0.289

Estimated relative risks with odds ratios (OR) and 95 % confidence intervals (95 % CI) and P- values for association between rs.362691 and ASD risk.

**Table 2:** The relationship between genotype and gender in autism group

Genotype	Gender		P-value	95% Confidence intervals
	Male	Female		
GG	12(75)	4(25)		
GC	43(74.1)	15(25.9)	0.944	0.267-3.420
CC	0	0		

GG= homozygous recessive; GC= heterozygous; CC= homozygous dominant.

#### 4- DISCUSSION

In 1951, spontaneous appearance of an autosomal recessive mutation was presented by Falconer the in a colony of inbred mice at the Institute of Animal Genetics, Edinburgh (15). In about 18

days, a mouse homozygous for the mutation difficulty could hold its hind quarters upright, and when the mouse tried to walk, it fell onto its side. The mutation was named "Reeler" (rl). In the Edinburgh strain of reeler mouse the mutant allele at

the reeler locus is signified *rl*. The pathological changes underlying the symptoms in reeler mouse are most obvious in the cerebral cortex and cerebellum (16). Post-mitotic neurons migrate from the ventricular zone at the normal developmental stage and proceed as far as the intermediate zone of the cortex (16). However, early-born neurons can not advance into the cortical plate region. The majority of neurons that arrive later are unable to bypass their predecessors, and result in the formation of a misplaced cortical plate in which the oldest neurons are on the outside (outside-in pattern). In reeler mouse the cerebellum is greatly reduced in size and the Purkinje cells, normally arranged as a single layer between the molecular and granular layers, can not migrate from the inner layer of the cerebellar cortex.

Other behavioral and biochemical data also showed that reductions in levels of Reelin in brain or blood, following postnatal hypoxia (17), prenatal viral infection in mid-gestation (18, 19) and in heterozygous reeler mutants (20) cause abnormalities in behavior such as decrease in Prepulse Inhibition (PPI), increase in anxiety and decrease in memory formation. Additionally, mutation in Reelin gene have been associated with significant learning disability, Cerebellar hypoplasia, ataxia and cognitive decline in human and mouse (21). Based on this information, we hypothesized that the gene that encodes reelin protein might act as a potential RELN candidate gene for autism. To date, a number of studies have investigated association of the SNPs of the gene with autism in various populations (22-28).

The contradictory results obtained through these studies demand replication of Reelin gene correlation studies in larger groups from different populations worldwide. Therefore, to assess the possibility of a correlation between a single nucleotide

polymorphism of Reelin gene, rs.362691, and autism risk a case-control study was performed with samples from Iranian-Azeri patients. In the current study, no significant association was observed between gender (and genotypes in case group (**Table.2**).

The findings of this research work did not detect any significant association between SNPs rs.362691 with autism in the studied population OR (95%CI): 1.692(0.830-3.449), P=0.146) (**Table.1**). Similarly, Dutta et al. (2008) studied six SNPs, including rs.362691 in an Indian population and concluded that there is no association of the Reelin gene polymorphism with ASDs (29). Also, no evidence of association of rs.362691 with autism found among International Molecular Genetic Study of Autism Consortium (IMGSAC) samples (25). In contrast, a significant association of SNP rs362691 with epilepsy was showed in an Indian population.

These apparent controversies association studies of Reelin gene markers with autism might be explained on the basis of genetic complexity of the disorder. It has also been hypothesized that autism is a disturbance of polygenic heredity so the effect of a single SNP could be subtle. Therefore, more studies, including more SNPs of Reelin, should be investigated (30).

Also, in the current study, we found that the G allele was more prevalent in both Azeri patients and controls compared with the C allele. In fact, the G is the major allele while the C is the minor allele in the studied population. In this study, significant difference in the allele frequency between cases and controls was not detected. In contrast, among autistic patients selected from the European autistic population by Bonora et al. (2003) (25), the G allele was identified as the minor allele while in the South African population studied by Sharma et al. (2013), the G allele was observed to be the major

allele (31). To some extent, variation in allelic frequencies reflects racial differences, which might be responsible for the inconsistent results.

#### 4-1. Limitations of the study

Working with autism spectrum disordered children can be challenging. Not having enough volunteers is one of the impediments in this project.

### 5. CONCLUSION

In conclusion, it should be pointed out that rs.362691 was not associated with autism spectrum disorder in Iranian-Azeri patients based on our results. So, this polymorphism (rs.362691), could not be applied as a functional molecular biomarker to predict genetic susceptibility for autism spectrum disturbance in Iranian-Azeri patients.

**6- CONFLICT OF INTEREST:** None.

### 7- ACKNOWLEDGMENTS

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