

Investigation of Gene Polymorphisms Leading to Formation of Tau and Beta-amyloid in Alzheimer's disease

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Abstract
Alzheimer's disease, a chronic neurodegenerative disease, is the most common cause of dementia. Clusterin (CLU) is a protein that is thought to regulate pathways such as apoptosis and the inflammation associated with Alzheimer's disease. Sortilin-related Receptor 1 (SORL1) proteins bind to amyloid precursor proteins and effectively decrease the amount of toxic Aβ proteins. The Complement receptor 1 (CR1) gene encodes a protein considered to play crucial roles in development and progression of various autoimmune and infectious diseases. The study group consists of 68 patients with Alzheimer's disease and 75 healthy individuals without any neurological disease were included to the control group. CLU and CR1 genotypes were determined with PCR-RFLP while SORL1 genotypes were determined using

capillary sequencing. SPSS 17 program was used for statistical analyses and $p<0.05$ was considered as statistically significant. The allelic association test showed that allelic distributions of rs202081077 on the Clusterin gene, rs641120 on SORL1 gene and rs202213311 on CR1 gene were significantly different in Alzheimer's patients. Clusterin (rs202081077) TT genotype and T allele frequency ($p=0.044856$, $p=0.003871$); SORL1 (rs641120) TT genotype and T allele frequency ($p=0.000001$, $p=0.00000$); CR1 (rs202213311) GG genotype and G allele frequency were higher in the Alzheimer group than in the control group ($p<0.05$). These polymorphisms may be effective in Alzheimer's disease.

Keywords: Alzheimer's disease, polymorphism, SORL1, CLU, CR1

1. Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disease, which is the most frequent cause of dementia in patients over the age of 65 years and displays exponential increase with age (Katzman and Saitoh, 1991). There were 24 million reported cases of Alzheimer's patients worldwide in 2010 (Kukull et al., 2002). It is estimated that 4.6 million new cases of AD develop worldwide every year (Ferri et al., 2005). It is predicted that by 2050, more than 25% of the world's population will be over the age of 65 (Olhansky et al., 1993). These figures imply that AD will be one of the most important health problems of the future (Taneli et al., 1999). Objectives of genetic studies on Alzheimer's disease include understanding the pathophysiology of the disease and the ability of the molecules encoded by genetic risk factors to target new drugs. As well as the ability of the identified genetic variants and molecules to be used as biomarkers for early diagnosis (Ertekin, 2007). Some studies which aim to explain the pathophysiology of Alzheimer's disease, have reported that increased Ca+2 concentration in brain leads to the formation of amyloid plaques and neurofibrillary tangle (NFT), which promote the activation of neutral proteinases (Small, 2008). The morphology and molecular composition of the neurofibrillary tangles depends on both the cell type and the location in brain. Cortical and subcortical NFT is morphologically similar but chemically different. It was thought that NFT formation might be related to the degeneration of specific types of neurons (Tabaton et al., 1988).

One of the most important molecular markers of NFT is Tau protein accumulating intracellularly with twisted ribbon-like assemblies (Reitz and Mayeux, 2014). Tau is a protein from the family of microtubule-associated proteins (MAP) encoded by the 17th chromosome. In Alzheimer's disease, Tau protein is abnormally phosphorylated and aggregated in NFT. Thus, the differences in protein composition of NFTs might depend on the antigens expressed by the cells in several specific pathways. Most cases of AD are sporadic, with less than 1% of all Alzheimer cases being inherited (Goedert and Spillantini, 2006). E4 allele of apolipoprotein E2 is the only genetic risk factor introduced in sporadic AD (Strittmatter et al, 1993). Mutations identified up to date constitute of about 50% of familial AD. Genes which have not been found yet and autosomal non-dominant transitional forms are proposed for the remaining 50% (Gürvit, 2004). Many other polymorphisms can also affect the risk of AD development. Differences in a new gene, the Sortilin-related Receptor 1 (SORL1) have been associated with AD. SORL1 is a glycoprotein that is mainly found in cortical neurons, hippocampus, cerebellum and spinal cord regions as well as being expressed in the kidney and other organs in very small amounts (Fiete et al., 2007). This protein may play a role in amyloid precursor protein metabolism. It has been suggested that the reduction in the expression of SORL1 dislocates APP to β-secretase complex, thus increases amyloid β production (Rogaeva et al., 2007).

Clusterin (CLU) which is also known as Apolipoprotein J, is located at position p21.1 on chromosome 8. It is thought to be involved in many diseases from Alzheimer's to cancer. Although the role of the Clusterin in Alzheimer is unclear, there is much evidence to support its association with AD. It has been shown that Clusterin mRNA and protein levels increase in AD (Kyriazis et al., 2008). Clusterin, a component of amyloid plaques, regulates Alzheimer-related pathways such as apoptosis and inflammation (Sala et al, 2009; Zhang et al, 2005; Falgarone et al., 2009). As well as acting as amyloid-beta chaperone to alter amyloid-beta aggregation and/or excretion (Yerbury et al., 2007; Bell et al., 2007).

Complement receptor 1 (CR1 or CD35) gene located on chromosome 1q32, has been thought as responsible for the late-onset of AD (Fonseca et al., 2016). CR1 is a membrane receptor for C3b and C4b complement fragments and expresses in different cell types, especially in the red blood cells and leukocytes of the circulatory system (Crehan et al., 2012). CR1 plays an important role in the pathway of immune complexes covered by C3b and C4b. CR1 expression levels in erythrocytes vary and CR1 is a polymorphic molecule which has different molecular weights. It also plays an important role in the

pathogenesis and development of various autoimmune and infectious diseases. Therapeutic potential of soluble CR1 (sCR1) is currently the subject of many studies (Rochowiak and Niemir, 2010). CLU, SORL1 and CR1 genes are identified common variants associated with late-onset AD (Rosenberg et al., 2016). Despite the several encouraging findings on these genes, to our knowledge, this analysis is the first to investigate the polymorphisms of related gene variants together as early-stage biomarkers for AD. The aim of this study was to determine the variations of rs641120, rs202081077 and rs202213311 in CLU, SORL1 and CR1 genes involved in Alzheimer's pathogenesis. As well as the identification of individual and combined effects, as a risk factor in development of Alzheimer's disease.

2. Materials and Methods

Following the Ethics Committee approval (2012/436-994) and informed patient consents, two sample groups were created. Healthy individuals without any neurological disease and patients with Alzheimer's disease were included in the study. The study group was consist of 68 patients and 75 subjects were included to the control group. We examined CLU (rs202081077), SORL1 (rs641120), CR1 (rs202213311) gene polymorphisms. In control group; the individuals had no medical history of Alzheimer's disease, any neurological disease, hypertension, and lipid abnormality, metabolic disorders including Diabetes Mellitus, kidney failure or hepatic insufficiency. In the study group; Alzheimer's disease was diagnosed using the criteria of the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) (McKhann et al., 2011). Morover, all patients completed a Mini Mental State Examination test (Keskinoğlu et al., 2009). 5 ml of peripheral blood sample was taken and sent to our laboratory with cold chain. DNA isolation was performed according to the protocol of the kit (Invitrogen, Purelink Genomic DNA Kit K182001). The concentration of the obtained DNA samples was measured with a NanoDrop II spectrophotometer. The Touch Down PCR method was also used for genotyping (Don et al, 1991). Primer sequences were given in Table 1. The denaturation temperature was 95 ºC, the bonding temperatures are between 67-55 ºC (Table 2) and the elongation temperature was 72 ºC.

Table 1. Primer sequences used in genotyping

Gene	**CLU**
Rs No	rs202081077
Forward Primer	5'-CGACTCACCCACAGACAAGA -3'
Reverse Primer	5'-CTGGGCAACAGAGTGAGACC -3'
Gene	**SORL1**
Rs No	rs641120
Forward Primer	5'-TTTCTAGTCTATTACCAGCAACCTAA -3'
Reverse Primer	5'-GCTGTTAAGTGGAAAGATGGTTAAGAT -3'
Gene	**CR1**
Rs No	rs202213311
Forward Primer	5'-TCCTAATGGGAGACACACAGG -3'
Reverse Primer	5'-TCCCCTTGAGGGTCACTTGT -3'

Table 2. Touch down PCR conditions

Step	Tempature	Time
Initial denaturation	95 ºC	5 min
1. bonding cycle temperature	67 ºC	4 cycle
2. bonding cycle temperature	65 ºC	3 cycle
3. bonding cycle temperature	63 ºC	2 cycle
4. bonding cycle temperature	61 ºC	2 cycle
5. bonding cycle temperature	59 ºC	2 cycle
6. bonding cycle temperature	57 ºC	2 cycle
7. bonding cycle temperature	55 ºC	23 cycle

PCR products were run on a 3% agarose gel and 236 bp, 150 bp and 194 bp and they were detected by photographing the gel with a polaroid camera under UV light (Figure 1 and 2).

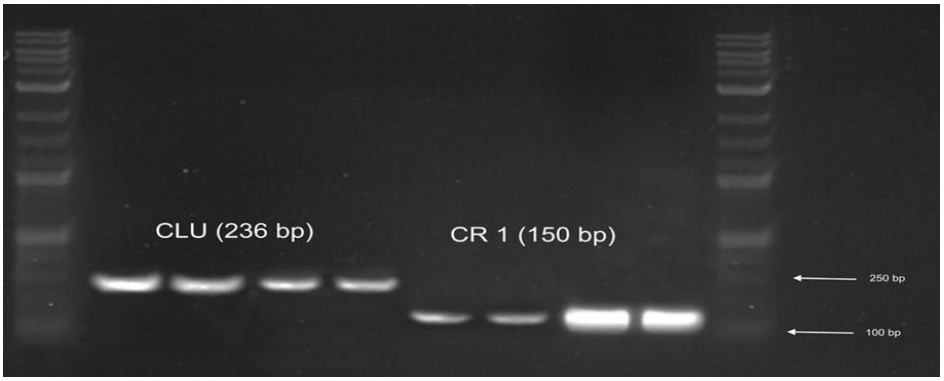

Figure 1. 3% agarose gel image of CLU and CR1 PCR products (Marker: 50 bp DNA marker).

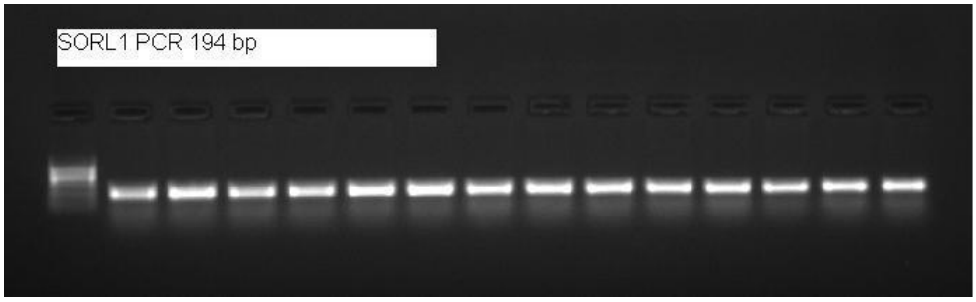

Figure 2. 3% agarose gel image of SORL1 PCR products (Marker: 50 bp DNA marker).

The resulting PCR products were cut using appropriate restriction enzymes. The characteristics of the cutting enzymes are shown in Table 3, and the results of the restriction endonuclease enzymes are shown in Figure 3. Besides, SORL1 genotypes were determined using capillary sequencing.

Table 3. Restriction endonuclease enzyme digestion properties

Gene name	Pcr product size	Restriction site	Restriction enzyme	Restriction product
CLU	236 bp	5'…CGA(T)CG↓…3' 3'…↑GCA(T)GC…5'	Hyp99I	123 bp 113 bp
SORL1	194 bp	5'..CAC(T)NN↓NNA(G)TG..3' 3'…GTA(G)NN↑NNC(T)AC…5'	Ms1I	140 bp 54 bp
CR1	150 bp	5'…A↓AGCTT…3' 3'…TTCGA↑A …5'	HindIII	94 bp 56 bp

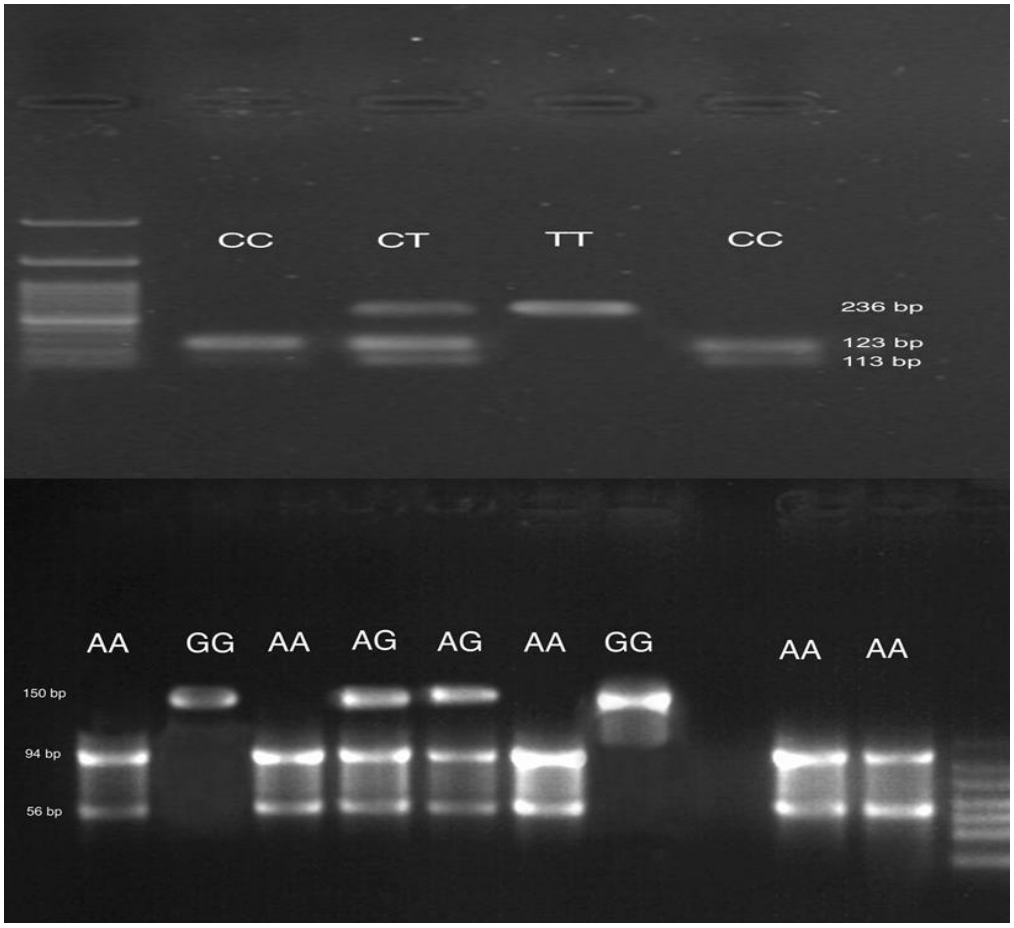

Figure 3. Restriction endonuclease enzyme digestion products for CLU and CR1

2.1 Statistical Analysis

SPSS Software version 17.0 (SPSS Inc., Chicago, Illinois, USA) program was used for statistical analyses. The frequency, rate, average, and standard deviation values were used for the descriptive statistics of the data. The Chi square test ($\chi 2$) was used to compare categorical data, and Fisher's Exact test was used to compare numerical data. Allele frequencies were calculated using the gene counting method. $P<0.05$ was considered as statistically significant.

3. Results

The genotype and allele distributions of the study and control groups are identified in Table 4. There was a significant difference in CLU (rs202081077) genotypes and alleles between the study and control group. Patients with homozygous TT genotype had a significantly higher frequency of individuals (16.1%) than the control group (5.3%, p=0.045). The frequency of individuals with T allele was significantly higher in study group (28.7%) than the control group (14.7%, p=0.0039). There were significant differences between both genotypes and alleles of study and control groups ($p <0.001$) for SORL1 (rs641120) gene. The frequency of homozygous TT genotype (17.6%) was higher in study group than in control group (2.6%, p=0.000001). The frequency of individuals with T alleles was found to be higher in the study group (40.4%) than the control group (12.0%, p=0.000). The difference between study and control group genotypes was statistically significant ($p<0.005$) for CR1 gene. There was a statistically significant difference between alleles ($p<0.001$). The frequency of patients with homozygous GG genotype (14.7%) was significantly higher than the control group (2.7%, p=0.002). The frequency of individuals with the G allele was significantly higher in the study group than the control (27.2% and 8.7% respectively, p=0.000037). Genotype and allele distributions of Alzheimer and control groups are summarized in Table 4.

4. Discussion

In this prospective randomised controlled trial, we investigated the possible effect of the CLU (rs202081077), SORL1 (rs641120) and CR1 (rs202213311) polymorphisms in the pathogenesis of Alzheimer's Disease and evaluated the ability of the identified genetic variants as biomarkers for early diagnosis of AD. Our results demonstrated that genotypes and allelic distribution of the CLU (rs202081077), SORL1 (rs641120) and CR1 (rs202213311) polymorphisms were statistically significant in Alzheimer's Disease. Several recent studies concluded the association between autosomal dominant AD and APP, PSEN1, PSEN2 and APOE4 genes (Karch and Goate, 2015) as a genetic risk factor. Studies in genetically engineered mice suggest that the primary neurotoxic formation is a mutant Tau protein rather than NFT (Tanzi, 2005; Bondareff et al., 1990). For this reason, both NFTs and amyloid plaques may be the end products, rather than the mediators of the pathogenesis of AD. However, mutations in familial AD increase the production of amyloid β but not Tau. We examined rs202081077 polymorphism in the CLU gene to determine the Alzheimer's risk. The distribution of allelic frequency between study and control groups indicated that the C allele has a significant protective effect. A meta-analysis concluded that some polymorphisms in the CLU and CR1 genes are associated with AD in white individuals, but not in other ethnic groups. However the association of APOE4 allele with AD was found in both white and Arabic patients (Jun et al., 2010). In our study, when rs202213311 polymorphism in CR1 gene was examined in terms of Alzheimer's risk, distribution of allelic frequency between study and control groups indicated that allele A has a protective effect. Rogaeva et al. (Rogaeva et al., 2007) concluded that genetic and specific environmental factors, as well as changes in SORL1 expression or function have been linked to the pathogenesis of AD and may be a modest effect on risk for the disease. Definitive identification of genetic effects in SORL1 has not yet been constituted. There are many different allelic variants in different genomic regions in SORL1 gene for different populations. Besides these variants are found in the possible intronic regulatory sequences which determine the cell type or the tissue specific expression of SOLR1. These variants act as risk factors for the disease by altering the physiological role of SORL1 in the production of APP holoprotein (Kok et al., 2011).

Table 4. Genotype and allele distributions of Alzheimer and control groups

a) CLU, b) SORL1 c) CR1

		Alzheimer group		Control group		
a	**CLU**	**n**	**%**	**n**	**%**	**p value**
	Genotype					
	CC	40	58.9	57	76	
	CT	17	25	14	18.7	
	TT	11	16.1	4	5.3	**0.044856**
	Allele					
	C	97	71.3	128	85.3	
	T	39	28.7	22	14.7	**0.003871**
b	**SORL1**	**n**	**%**	**n**	**%**	**p value**
	Genotype					
	CC	25	36.8	59	78.7	
	CT	31	45.6	14	18.7	
	TT	12	17.6	2	2.6	**0.00001**
	Allele					
	C	81	59.6	132	88	
	T	55	40.4	18	12	**0.0000004**
c	**CR1**	**n**	**%**	**n**	**%**	**p value**
	Genotype					
	AA	41	60.3	64	85.3	
	AG	17	25	9	12	
	GG	10	14.7	2	2.7	**0.001911**
	Allele					
	A	99	72.8	137	91.3	
	G	37	27.2	13	8.7	**0.000037**

In the presence of SORL1, APP is covered by holoprotein retromer. In the absence of SORL1, APP retromer leaves the sorting pathway and is released into the late endosomal pathway which will be exposed to β- and γ-secretase cleavage, forming the Aβ protein (Rogaeva et al., 2007). It has been suggested that genetic variants in SOLR1 may be a risk factor for late-onset Alzheimer's disease (Bettens et al., 2008), based on a study of 550 individuals with late-onset Alzheimer's disease and 637 healthy subjects in Belgian population. In another Italian study, 13 polymorphisms in the SORL1 gene were investigated in 708 patients and 358 healthy voluntees. (Cellini et al., 2009) As a result of this study, it has been shown that 4 polymorphisms may contribute to the late onset of AD pathogenesis. One of these polymorphisms is rs641120, which is also found to be significant in our study. In addition, our results are compatible with the study conducted by Cellini et al. (Cellini et al., 2009). In a recent meta-analysis, the rs641120 polymorphism of SORL1 gene has been shown to reduce the risk for Alzheimer's disease by displaying a protective effect (Wang et al., 2016). In our study, when rs641120 polymorphism in SORL1 gene was examined in terms of Alzheimer's risk, the distribution of allelic frequency between study and control groups; allele C indicated a significant protective effect. This study was the first polymorphism research in our population on CLU, SORL1 and CR1 genes in Alzheimer's patients. Although there is a published data on neurological diseases relating to these genes in Alzheimer's disease (Narayan and Dragunow, 2017), none of these polymorphisms have been studied in the same study before. On the other hand we are in the opinion that the results obtained from this study will also be valuable as being the first data created for our population.

5. Conclusion

The polymorphism of Clusterin rs202081077, SORL1 rs641120 and CR1 rs202213311 genes likely to play a role in the pathogenesis of the disease. In conclusion, investigation of the polimorphisms of related gene variants together may have diagnostic value as early stage biomarkers in Alzheimer’s disease.

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