**TR**
**INONU UNIVERSITY**
**INSTITUTE OF SCIENCE**

# AN INVESTIGATION ON THE ASSOCIATION OF CERTAIN POLYMORPHISMS IN NEUROGULIN 1 GENE WITH SCHIZOPHRENIA

**AYŞE ASİYE CULUM**

**MASTER THESIS**

**DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS**

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Title of Thesis: **An Investigation on the Association of Certain Polymorphisms in Neuroglin 1 Gene with Schizophrenia**

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The thesis mentioned above is evaluated by our committee and accepted as the master thesis in Molecular Biology and Genetic Department.

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**WORD OF HONOR**

I declare that, this “An Investigation on the Association of Certain Polymorphisms in Neurogulin 1 Gene with Schizophrenia” titled study that I submit as a master thesis is written on my own without consulting an assist that contrary to academic ethics and tradition, and all literature, both in text and reference, I utilize is consisted that are displayed in reference appropriate to the method and I confirm this with my honor.

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## ABSTRACT

Master Thesis

AN INVESTIGATION ON THE ASSOCIATION OF CERTAIN POLYMORPHISMS IN NEUROGULIN 1 GENE WITH SCHIZOPHRENIA

Ayşe Asiye CULUM

Inonu University
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Schizophrenia is a multifactorial disorder. Many characteristic single nucleotide polymorphisms (SNPs), accompanying their narrow impact, collectively can describe around 30% of the essential risk of schizophrenia. Finding susceptibility genes that play role in development of schizophrenia can explain the risk factors of the disease. Neuregulin 1 or NRG1 is crucial for the ordinary development of the nervous system. *NRG1* was initially involved in schizophrenia in the Icelandic community after linkage disequilibrium aligning over 8p21–22 displayed association between schizophrenia. We investigated two *NRG1* SNPs (rs3924999 and rs2954041) to find association with schizophrenia.

We included 178 patients with schizophrenia and 180 healthy individuals as controls from a Turkish population living in Malatya. SNP genotyping was carried out with PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) method and case and control groups were compared for a potential difference between distributions of genotypes and alleles.

The case-control study we have done showed there was no significant difference in terms of genotype and allelic distributions of rs3924999 and rs2954041 SNPs between case and control groups we have screened. We were not able to find enough evidence to support presence of a single SNP association between schizophrenia and two SNPs concerned in this study.

# ÖZET


Yüksek Lisans Tezi

NEUROGULİN 1 GENİNDEKİ BAZI POLİMORFİZMLERİN ŞİZOFRENİ İLE İLİŞKİSİ ÜZERİNE BİR ARAŞTIRILMA

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Şizofreni multifaktöriyel bir hastalıktır. Küçük etkileriyle beraber birçok özgün tek nükleotit polimorfizmi (SNP) beraberce şizofreninin esas riskinin yaklaşık %30'unu açıklayabilir ve şizofrenide rol oynayan yatkınlık genlerini bulmak hastalığın risk faktörlerini açıklayabilir. Neuregulin 1 veya NRG1 sinir sistemi normal gelişimi için çok önemlidir. *NRG1*, ilk olarak İzlanda toplumunda şizofreni ile arasında ilişkisi olduğu ortaya çıkan 8p21-22 üzerindeki bağlantı eşitsizlik sıralamasından sonra şizofreniye dahil edildi. Şizofreniyle ilişki bulmak üzere iki *NRG1* SNP'ini (rs3924999 ve rs2954041) araştırdık.

Çalışmaya Malatya'da yaşayan Türk popülasyonundan 178 şizofreni hastası ve kontrol olarak 180 sağlıklı bireyi dahil ettik. SNP genotiplemesi, PZR-RFLP (polimeraz zincir reaksiyonu - restriksiyon fragment uzunluk polimorfizmi) yöntemi ile gerçekleştirildi ve hasta ve kontrol grupları, genotip ve alel dağılımları arasındaki muhtemel farlılık için karşılaştırıldı.

Yaptığımız hasta-kontrol çalışması, rs3924999 ve rs2954041 SNPlerinin genotipik ve alelik dağılımları açısından taradığımız hasta ve kontrol grupları arasında önemli bir farklılık olmadığını gösterdi. Bu çalışmada şizofreni ve ilgili iki SNP arasında tek SNP ilişkisinin varlığını destekleyen yeterli kanıt bulamadık.

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And my grandfather….

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**ABBREVIATIONS**

| | |
|---|---|
| AKT1 | protein kinase B |
| APS | ammonium persulfate |
| BDNF | brain-derived neutrophic factor |
| bp | base pair |
| COMT | catechol-O-methyl transferase |
| CNS | central nervous system |
| CRD | cysteine-rich domain |
| cSNP | coding single nucleotide polymorphism |
| DISC1 | disrupted-in-schizophrenia 1 |
| DISC2 | disrupted-in-schizophrenia 2 |
| dNTP | deoxynucleotide triphosphate |
| DTNBP1 | dysbindin |
| DSM-IV | Diagnostic and Statistical Manual of Mental Disorders-IV |
| EGF | epidermal growth factor |
| EGFR | epidermal growth factor receptor |
| EST | expressed sequence tag |
| GABAergic | gamma aminobutyric acidergic |
| HGMD | Human Gene Mutation Database |
| HGVBase | Human Genome Variation Database |
| ICD-10 | International Classification of Diseases-10 |
| Ig | immunoglobulin |
| iSNP | intronic single nucleotide polymorphism |
| mGluR | metabotropic glutamate receptors |
| NCBI | National Center for Biotechnology Information |
| NMDA | N-Methyl-D-aspartate |
| NMDAR | NMDA receptor |
| NMJ | neuromuscular junction |
| NRG1 | neuregulin-1 |
| nsSNP | non-synonymous single nucleotide polymorphism |
| OMIM | Online Mendelian Inheritance in Man |
| PAGE | polyacryamide gel electrophoresis |
| PCP | phencyclidine |
| PCR | polymerase chain reaction |
| PRODH | proline dehydrogenase |
| RFLP | restriction fragment length polymorphism |
| rSNP | regulatory single nucleotide polymorphism |
| S | spacer region |
| SNP | single nucleotide polymorphism |
| sSNP | synonymous single nucleotide polymorphism |
| TEMED | *N',N',N',N'*-tetramethylethylenediamine |
| TMc | C-terminal transmembrane domain |
| TMn | transmembrane domain |
| ZDHHC8 | zinc finger and DHHC domain containing protein 8 |

# 1 INTRODUCTION

All common diseases, and also schizophrenia are strongly influenced by genetic factors. Studies have surely displayed that inheritance has the most powerful factor over schizophrenia. It is estimated that the heritability of schizophrenia is about 80% in twin studies after variance analysis. However, examining the genetic ground is disappointing, because there isn't any classifying pathology or testing standard. Describing the alterations of genes is hard to separate physiological or biochemical differences combined with the disorder. This disease does not show Mendelian inheritance; instead it is multifactorial. Diagnosing of collective genes accompanying their narrow impact, and further effect of environmental cause could more describe the complicated model of inherited accumulation. Numerous typical single nucleotide polymorphisms (SNPs), each with narrow impact, accumulatively could describe around 30% of the essential risk of schizophrenia, and discovering the susceptibility genes that play the major role in schizophrenia can explain the risk factors of the disease (Maier, Zobel, & Kühn, 2006; Singh, Kumar, Agarwal, Phadke, & Jaiswal, 2014).

## 1.1 Schizophrenia

Schizophrenia is a mental disease described through positive indicia like deceptions, illusions and confused thinking, and negative indicia like depression, public recession, and lack of interest (OMIM 181500; http://www.ncbi.nlm.gov/Omim/) (H. J. Williams, Owen, & O'Donovan, 2009).

The basis of the word is Greek; consist of *skhizein* ("to cleave") and *phrēn* ("intelligence"). But schizophrenia isn't a "cleave of individuality", or "collective individuality" disease. Conversely, it expresses a "cleaving of psychiatric activities", reflecting the presentation of the illness. Genetics and intrauterine life, mental and public courses are crucial contributing causes (Picchioni & Murray, 2007).

### 1.1.1 Onset

Quite conceptual and attitude marks are generally exist in infancy and beyond. The common hallmarks are mainly seen in the tardy teenagers and the early 20s. Schizophrenia characteristically exists in early majority or tardy

maturity. Results differ, however the characteristic progress is fractional forgiveness after getting worse, and a distinctive decrease in public and professional activity (H. J. Williams et al., 2009). Schizophrenia decreases the lifetime of an influenced person approximately 15 years, accompanying cardiovascular disease and autocide amongst the main factors for raised annihilation (Akbarian, 2014).

### 1.1.2 Subtypes

The variety of schizophrenia is identified well and there are a few endeavors to describe its importance while explaining its assorted definition. The Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV) studying team suggested lowering the five sub-categorizations of schizophrenia involved in DSMI-IV-TR:

i. **Catatonic type** is associated with obvious psychomotor discomfort including indolence, negativism, solidity, enthusiasm, and posing;
ii. **Disorganized type** is characterized by obvious disconnecting of affiliations, thinking failure, and smooth influence;
iii. **Paranoid type** is associated with anxiety accompanying one or more systematic misbelieves or existence of repeated illusions connected with one subject;
iv. **Schizoaffective type** is characterized by a mixture of significant mental state discomfort in combination with mental symptomatology;
v. **Undifferentiated type** is taken into account when a sufferer exists with mental indicia that fit standards for schizophrenia but not for any certain group;
vi. **Residual type** is analyzed through the presence of positive symptoms at a low intensity only.

The International Classification of Diseases-10 (ICD-10) explains two extra groups:

vii. **Simple schizophrenia** is analyzed through progressive beginning of an ambition in the lack of significant misbelieves;
viii. **Latent schizophrenia** is associated with obvious distance and weird attitudes (Tandon, Nasrallah, & Keshavan, 2009).

### 1.1.3 Diagnosis and Treatment

Diagnosis is depended on monitored attitude and the individual's informed involvements (Picchioni & Murray, 2007). The treatment is antipsychotic medication targeting dopaminergic, seratonergic, and monoaminergic receptor systems, mostly by suppressing dopamine receptor activity, however the most of sufferers still encounter a poor reaction to medication (Akbarian, 2014; Becker & Kilian, 2006).

### 1.1.4 Neurology

Morphologically, the brain size and its molecular structure involving particular glial, synaptic, and neuronal populations in the dorsolateral prefrontal cortex, dorsal thalamus, and hippocampus are reduced in schizophrenic patients (P. J. Harrison & Weinberger, 2005). Hyperstimulation of mesolimbic pathway and hypostimulation of the mesocortical pathway brings about by dopaminergic and glutamatergic dysfunction is found in schizophrenia. Possibly the outcome of deterioration or damaged neuronal growth, grey matter is reduced, and white matter anomalisms could be determined (Michael J. Owen, 2005).

### 1.1.5 Causes

A compound of environmental and genetic factors acts in the development of schizophrenia (van Os & Kapur, 2009). Schizophrenics possibly have additional natal obstacles, especially immature born, low born weight, birth in winter or early spring, prenatal hypoxia and old paternal age. It is clear that fetal and prenatal environmental risks have a deep impact on brain development (Singh et al., 2014). Distinct environmental risk factors have a role in adulthood, such as urban life, migrant status, drug use, and social isolation (Picchioni & Murray, 2007; van Os & Kapur, 2009).

Prenatal exposure to famine alters DNA methylation. Adverse childhood experiences are combined with brain function and structure in patients with schizophrenia. Cannabis use in adolescents influences catechol-O-methyl transferase (*COMT*), which encodes COMT glutamatergic co-activator that degrades dopamine, gene methylation, and generally recognized as a factor increasing schizophrenia risk (Bosia, Pigoni, & Cavallaro, 2015).

### 1.1.6 Prevalence and Heritability

Schizophrenia is a typical disease with a lifetime prevalence of about 1%. It is a multifactorial disorder, and the most contributory factor is a positive ancestry (Maier et al., 2006; Picchioni & Murray, 2007). The heritability is high, but the causative genes have kept indefinable. The inheritability of susceptibility to the disease is about 80% (Maier et al., 2006; Riley & Kendler, 2006). The percentages are variable, because the contribution of environmental factors is not constant. Schizophrenic's first degree relatives are under the risk of 6.5% (Picchioni & Murray, 2007). Monozygotic concordances are calculated at 41–65% checked against dizygotic's of 0–28% (Craddock, O'Donovan, & Owen, 2005). Determination of the liability genes is crucial for comprehending the pathophysiology and pathogenesis of schizophrenia, and giving the expectancy of additional efficient medication. Nevertheless, researching chromosomes and liability genes is not fast and results are disappointing, possibly the reason that the presence of more than one liability gene, accompanying their narrow impact, and also the combination of epigenetic factors and environmental agents (P. Harrison, 2003).

The inheritance type is complex and not Mendelian. Probably polygenic, oligogenic or an admixture of the two with a threshold effect is the most noticed transmission modes. There isn't any predicted and easy correlation between genotype and phenotype, so identifying the liability genes is hard. Thousands of individuals are needed for basic discovery and much more for duplication (M J Owen, Williams, & O'Donovan, 2004)**.**

### 1.1.7 The Identification of Genetic Risk Factors

The genetic risk identifying duty for schizophrenia is not so easy, for the reason of ambiguity and complication at diverse issues. Heterogeneity at loci and alleles, phenotype heterogeneity, the presence of environmental agents, sequence and constitutional variants, the possible addition of an admixture of ordinary and infrequent variants, interaction of many genes, and narrow effects of personal risky genotypes make the picture complex. The determination and analysis methods are not fully developed to solve this complexity (Alaerts & Del-Favero, 2009).

Identification of schizophrenic genes is determined in three principal ways in human populations. The determination of chromosomal abnormalities and genetic polymorphisms in sufferers are found after linkage and association studies:

**Chromosomal abnormalities:** Chromosomal translocations and deletions may be associated with schizophrenia, and pass through families. These possible markers can be utilized for finding the location of the susceptibility genes. Two chromosomal abnormalities provide assuring clue for locating of a liability gene. 22q11 deletion syndrome (Velo-cardio-facial syndrome) is caused increased risk of schizophrenia in adults. Susceptibility genes in 22q11 include zinc finger DHHC domain containing protein 8 (*ZDHHC8*), catechol-O-methyltransferase (*COMT*), and and proline dehydrogenase (*PRODH*) genes (Craddock et al., 2005). Another chromosomal abnormality is found in a huge Scottish family and it is an equal reciprocal translocation between (1q42; 11q14.3). This abnormality cosegregates with other psychiatric disorders and schizophrenia. The translocation in *DISC1* and *DISC2* on chromosome 1 disturbs these two genes. There isn't any open reading frame on *DISC2*. *DISC1* expression is controlled by anti-sense RNA expressed by *DISC2*. DISC1 regulates cytoskeleton, so *DISC2* or *DISC1* distribution may affect neurite structure and/or intracellular transport, neuronal migration (Bray & Owen, 2001; M J Owen et al., 2004).

**Genetic linkage:** Genetic data is collected from families which have many influenced individuals, then, these data is used to determine the separating genome regions related with the disease. Genetic markers are used for investigating the susceptibility gene. Much more families are need to find the location of genes for complex diseases like schizophrenia (Owen et al., 2004).

The linkage study consequences are frustrating in schizophrenia for now. The importance in genome wide grade couldn't be succeeded, and the findings couldn't be duplicated. It is mostly due to small sample sizes. After meta-analyses of schizophrenia linkage, liability genes on chromosomes 8p, 13q, and 22q were supported. Also chromosome regions on 5q, 3p, 11q, 6p, 1q, 22q, 20q, and 14p are found to be related to the disease (Craddock et al., 2005)

**Association studies:** More (or less) characteristic alleles in patients distinguish from population are identified in association studies. Genetic indicators are need to be found in the region itself or closer regions and sequences

related to liability. Because of this, prior knowledge of the possible location and the action of definite gene is important ahead of the study. Certain candidate genes must be elected by investigators via association (Owen et al., 2004; Owen, 2005). Statistically significant alterations are investigated in allele frequencies in case group and correlate with healthy group in association analysis (Alaerts & Del-Favero, 2009).

Most of the certain candidate genes have functions on the neuropharmacological pathways. In this manner, serotonergic and dopaminergic neurotransmission genes are on the top of the interest. The attention quickly enlarges to genes have a role in neurodevelopment and neuromodulation, and gamma aminobutyric acidergic (GABAergic), glutamatergic systems (Owen et al., 2004).

### 1.1.8 Hypothesis about Pathophysiology

The role of dopamine in the mesolimbic pathway of the brain plays role in schizophrenia (Laruelle et al., 1996). There are two main hypotheses about the pathophysiology of schizophrenia:

**The dopamine hypothesis of schizophrenia:** The hypothesis is depend on the observations that dopamine-releasing stimulants cause psychosis. Traditional antipsychotic drugs restrict dopamine neurotransmitter receptors ($D_2$ receptors) (Coyle, Tsai, & Goff, 2003). Such schizophrenia indicia appear because of extreme dopamine delivery, and excessive stimulation of $D_2$ receptors give rise to (the positive symptoms of) schizophrenia (Jones & Pilowsky, 2002).

The dopamine hypothesis was dominant 10 years ago. But the restriction of the theory is low response of cortical atrophy, cognitive impairments, and negative symptoms to classic antipsychotics (Coyle et al., 2003). Additionally, novel irregular antipsychotic drugs have attitude profits alike to traditional medicines with decreased affection for $D_2$ receptor. Thus, dopaminergic system might be one of the factors included in schizophrenia. Dopaminergic system regulates additional neurotransmitter systems in the brain. Therefore, other systems influenced by dopaminergic system requires to be cautiously tested (Konradi & Heckers, 2003).

**The glutamate hypothesis of schizophrenia**: Mainly the glutamatergic system collaborates very near with the dopaminergic process on the intracellular

grade and neuronal-circuitry. Traditional antipsychotic medicines act on the glutamate system. It is obvious that the glutamate system has been entirely involved in schizophrenia. For that reason, N-Methyl-D-aspartate (NMDA) receptor antagonists increase mental indicia in schizophrenia (Konradi & Heckers, 2003). An alternative hypothesis is arisen from the clinical observations that dissociative anesthetics, including ketamine and phencyclidine (PCP), develop a affection that is clinically identical to schizophrenia. It was firstly thought that this is connected to the defection of glutamatergic neurotransmission by blocking NMDA receptors (NMDAR). Resolute anesthetics are tied to sites among the channel of NMDAR (Coyle et al., 2003).

Glutamate also can affect dopamine function (Coyle et al., 2003). The atypically decreased grades of glutamate receptors discovered in the postmortem schizophrenic brains (Konradi & Heckers, 2003). Also a strong evidence supports that hypofunction of a subset of NMDAR might be responsible for the symptomatic characteristics of schizophrenia.

The dopamine theory in schizophrenia anticipates atypically raised function among the dopamine neurotransmitter system as the first step of deficiency. The glutamate theory in schizophrenia explains atypically declined function among the glutamate neurotransmitter system, especially of NMDA receptors. Patients could experience failures in one or two systems. Additionally, these two systems are treated by the inhibition of dopamine $D_2$ receptor, thus, modulation of abnormally low glutamate system. Therefore, NMDA receptor function could be affected by overstimulation of $D_2$ receptors (Konradi & Heckers, 2003).

### 1.1.9 Mechanism

Entire susceptibility genes involved in schizophrenia have a role in glutamergic transportation or in growth of neurons and neurodevelopment. Numerous liability genes seem to be correlated to the regulation of glutamate transmission, and synaptic plasticity, especially NMDA receptor activation. L-glutamate and glycine are needed for effective NMDA receptor channel motivation. Glia and the activity of neurons are regulated by glutamate via the activation of diverse glutamate receptors. Ion channels (ionotropic glutamate receptors) and G-protein-coupled receptors [metabotropic glutamate receptors (mGluRs)] are found in glutamate receptors. NMDA receptors are one of the

ionotropic glutamate receptors. The flow of $Ca^{2+}$ by ionotropic glutamate receptors is very important; as a result that $Ca^{2+}$ stimulates intraneuronal signal transduction cascades which regulate cell death and cellular plasticity. Kinases and phosphatases are activated by $Ca^{2+}$, which stimulates signal transduction cascades, and $Ca^{2+}$ acts on the signaling proteins, phosphorylation state of receptors, ion channels, and transcription factors. The phosphorylation results as a changed neuronal excitement, and the expression of additional genes. Creating novel synapses and encouragement of present synapses and the regulated dissociation in apoptosis are achieved by synthesized proteins according to the signal transduction pathway. So glutamate is an important substance in neuronal death and neurodevelopment, neurotoxicity, and neuroplasticity. Permeability of $Ca^{2+}$ and physiological features of it interpret the activity of NMDA receptor, and NMDA receptor is especially critical for the induction of cellular programs (Konradi & Heckers, 2003).

### 1.1.10 Candidate Genes

There are three very strong candidates that proved likely to have role in schizophrenia: dysbindin (*DTNBP1*) gene on chromosome 6p, neuregulin 1 (*NRG1*) gene on chromosome 8p, and a fewer understood gene locus, *G72 (DAAO)/G30* on chromosome 13q, which is expressed only in humans. Dysbindin acts on synaptic function and structure in the brain, has a role in both pre- and postsynaptically. NRG1 is the regulator of glial cells, myelination, NMDA activation and effects expression of NMDA and GABA receptors. *G72* interplays with D-amino acid oxidase (DAAO) coding gene. It is engaged in splitting of D-serine that regulates NMDARs. Other susceptibility genes might include the gene codes protein kinase B (AKT1) and the disrupted-in-schizophrenia 1 (DISC1) (Figure 1.1). *DISC1* translocation was detected to cosegregate with the schizophrenic families, and typical mutations of the gene have associations with schizophrenia in outbred communities. Synaptic and postsynaptic transmissions are very important for schizophrenia because of the phosphorylation. Thus, phosphokinases are potent candidate proteins, and polymorphism in these genes effect intercellular signal transmission. The gene expresses AKT1 was found to be associated with schizophrenia. Also many noncoding variants were found to be associated with these genes. Not only the genetic alterations changing the protein

structure, but also alterations in the regulatory and splice regions can influence the expression. These may be cause narrow or drastically complex results (Maier et al., 2006; Michael J. Owen, 2005; Singh et al., 2014).

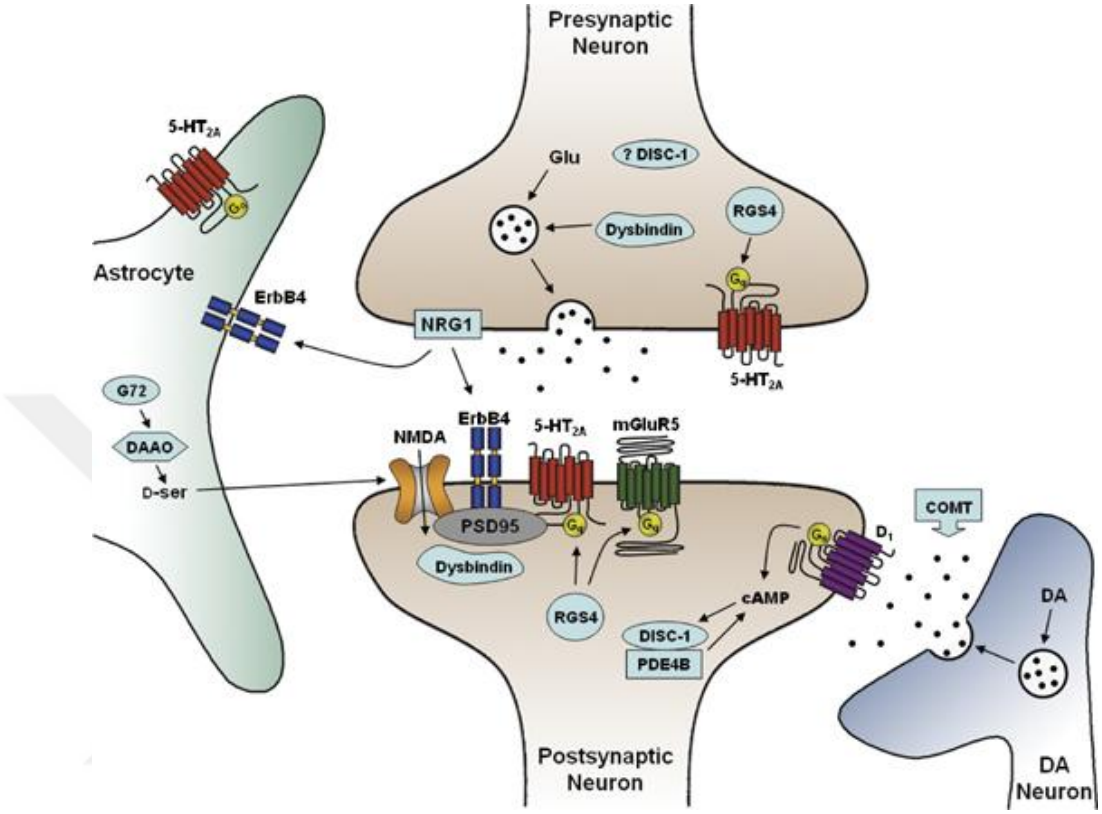


**Figure 1.1.** Assumptive acts at a glutamatergic synapse of schizophrenia genes: NRG1 (neuregulin-1), ErbB4 (ErbB-type tyrosine kinase receptor B4), DISC-1 (disrupted in schizophrenia-1), G72, Dysbindin, RGS4 (regulator of G protein signaling 4), COMT (catechol-O-methyltransferase), PDE4B (phosphodiesterase 4B), and DAAO (D-amino acid oxidase), NMDA (N-methyl-D-aspartate glutamate receptor), 5-$HT_{2A}$ (serotonin receptor 2A), mGluR5 (metabotropic glutamate receptor 5), $D_1$ (dopamine receptor 1), Glu (glutamate), DA (dopamine), D-ser (D-serine), cAMP (cyclic adenosine monophosphate), PSD95 (postsynaptic density protein 95), $G_q/G_s$ (G proteins) (Gray & Roth, 2007)

## 1.2 Neuregulin 1

The neuregulin family has four members, and Neuregulin 1 or NRG1 is one of these. Neurogulin family has their actions on the epidermal growth factor receptor (EGFR) family. It is essential for the ordinary development of the nervous system, heart (Britsch, 2007). *NRG1* is an extremely big gene (1.4 MB) and encodes around 15 distinct peptides. It includes many regulatory elements and

promoter sequences. It is yielded in several isoforms by alternative splicing (P. J. Harrison & Weinberger, 2005).

Separate amino-terminal sequences of NRG1 are the determinants for the classification of six NRG1 isoforms. The type III isoforms include a transmembrane domain (TMn) in a cysteine-rich domain (CRD). Epidermal growth factor (EGF)-like domain is contained in all six isoforms. An immunoglobulin (Ig)-like domain is obtained in Types I, II, IV and V, between the N-terminal sequence and the EGF domain, with or without the spacer region (S). At types III and VI, the N-terminal-specific region is linked immediate to the EGF domain. Splicing in the C-terminal regions and linker regions additionally produces variants. C-terminal transmembrane domain (TMc) exists among the C-terminal regions and linker regions (Figure 1.2 a) (Mei & Xiong, 2008).

Although Type III NRG1 has cytoplasmic N- and the C-terminal regions, many NRG1 isoforms are synthesized as transmembrane precursor polypeptides (pro-NRG1s) with EGF domain located in the extracellular region. The β-site of amyloid precursor protein cleaving enzyme, tumor necrosis factor-α converting enzyme or meltrin β (displayed by the lightning arrow, Figure 1.2) splits the extracellular region. After the cleavage, soluble mature NRG1s are created. But this cleavage hasn't taken place in Type III NRG1, as a result, Type III NRG1 is considered to have a role in cell contact. Type IV, Type V and Type VI pro-NRG1s mechanism is not totally understood however it is assumed that the process is like in Type I and Type II (Figure 1.2 b) (Mei & Xiong, 2008).

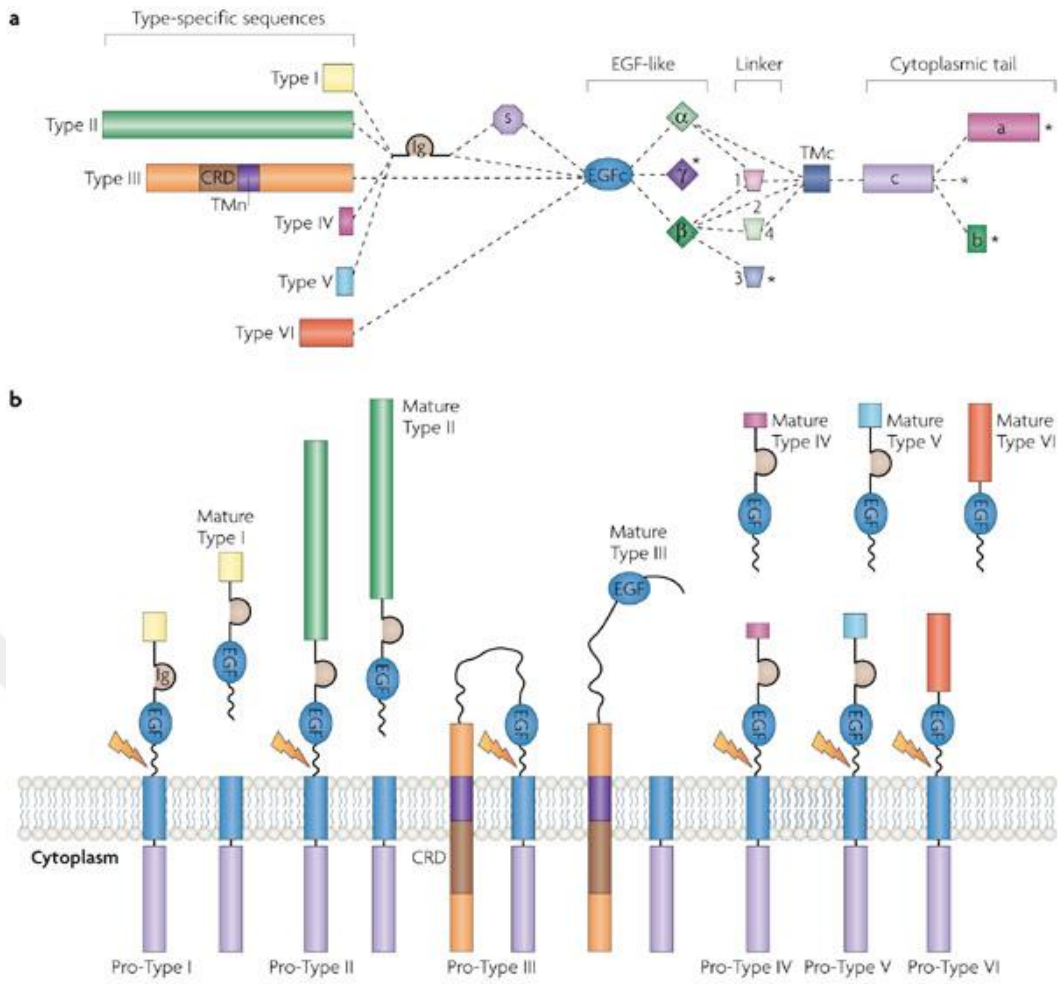


**Figure 1.2.** Different types of NRG1 (Mei & Xiong, 2008)

NRG1 was firstly associated in schizophrenia in the Icelandic community after linkage disequilibrium aligning over 8p21–22 demonstrated association between schizophrenia and a multi-marker haplotype at the 5' end of *NRG1*. Then, identical haplotype was found associated in an abundant sample from Scotland, and not strong evidence in UK sample (Craddock et al., 2005).

There are meaningful associations in a 300 kb region near the *NRG1*exon 1. Furthermore in Asian population, original haplotypes are associated with a very low frequency, so the association do not occur, but the association is found in many ordinary variants at the same loci with schizophrenia (Maier et al., 2006).

### 1.2.1 Roles

In the prevalent pathogenic model, schizophrenia is explained as a neurodevelopmental disease that causes abnormal synaptic connectivity.

Neuregulin is essential for neurodevelopment, cellular differentiation and neuronal migration. NRG1 also controls synaptic plasticity by interacting with the postsynaptic density proteins. Ionotropic glutamate receptors are collaborated with these proteins (P. Harrison, 2003). Roles of NRG1 in neural development include:

**a)** NRG1 delivered from neurons advances the establishment and sustenance of radial glial cells. Glial cells are crucial for the radial migration of neurons from ventricular zones to the pial surface.

**b)** NRG1 is needed for the tangential migration of GABAergic interneurons in the cortical region. Corridor cells express NRG1, and NRG1 provides thalamocortical axon navigation among the diencephalon.

**c)** The quantity of NRG1 released from substrate axons regulates peripheral nerve myelination and ensheathment.

**d)** Myelination of axons and oligodendrocyte development in the CNS may be controlled via NRG1 released from axons.

**e)** Neuromuscular junction (NMJ) establishment requires NRG1, possibly NRG1 may have influences on terminal Schwann cell durability and differentiation.

**f)** Establishment of synapse in central nervous system (CNS) is activated by NRG1. Characteristic excitatory synapses, consisted among glutamatergic terminals and spines are illustrated in Figure 1.3 (Figure 1.3.) (Mei & Xiong, 2008).

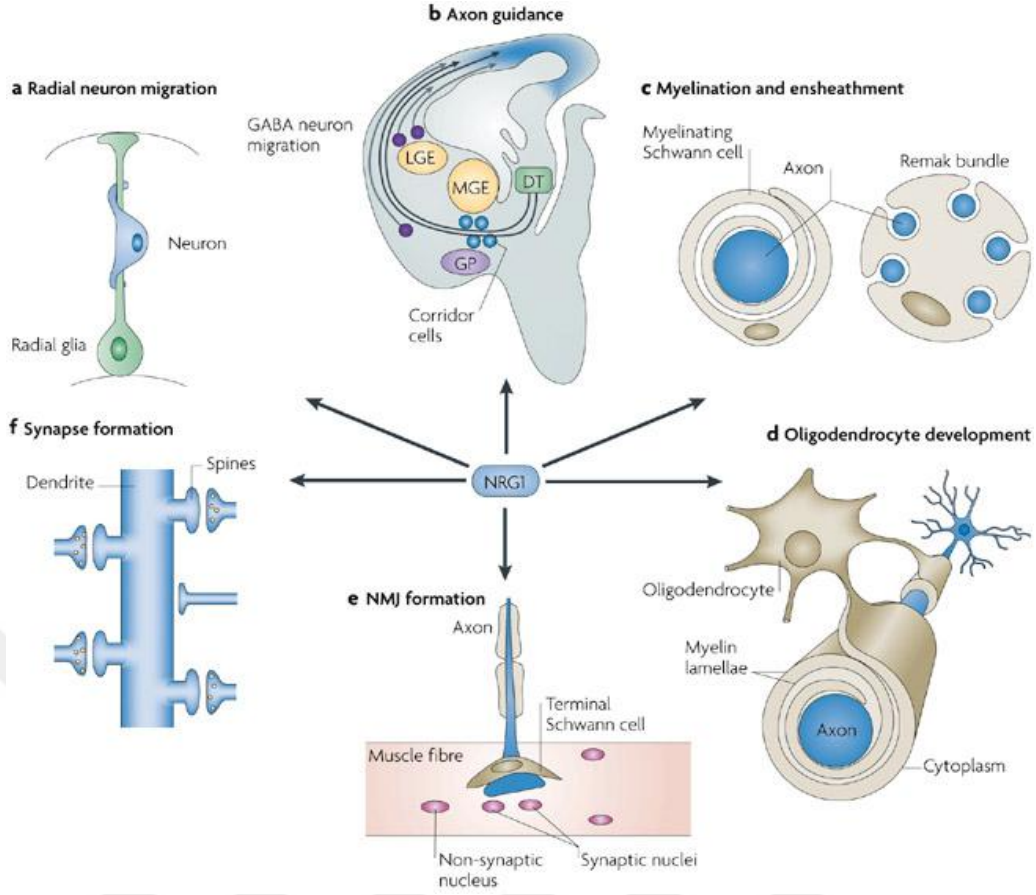


**Figure 1.3.** Roles of NRG1 (indicated by blue shading) in neural development: LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; GP, globus pallidus; DT, dorsal thalamus (Mei & Xiong, 2008)

Impaired synaptic plasticity may contribute to schizophrenia. Interplays between Neuregulin 1-ErbB4 may have an importance in the pathological mechanism of schizophrenia (D. Li, Collier, & He, 2006). Changes in expression or probably mRNA splicing may act on schizophrenia (Craddock et al., 2005). It has been proposeded that NRG1 polymorphisms associated with schizophrenia may affect gene expression levels (O'Tuathaigh et al., 2007). A broad series of neuroreceptors, especially NMDA glutamatergic receptors might be affected by NRG1 due to its effects on changing their expression and functions which can cause disease liability. There are white matter abnormalities in schizophrenia, and altered NRG1 function results in abnormal myelination. Myelination is found decreased in schizophrenic patients. Changes in NRG1 cause an activity defect of glia, and this leads to synaptic damage (O'Donovan, Williams, & Owen, 2003).

Current antipsychotics have their therapeutic efficiencies about 75% of sufferers, however conceptual deterioration is frequently the additional disenabling and enduring specialty of schizophrenia. It is a challenging task to advance realistic remedy improvement in schizophrenia, chiefly the reason of the absence of a combining heterogeneous genetic risk structure and neuropathology (Akbarian, 2014).

## 1.3 Single Nucleotide Polymorphism

A lot of common diseases have complex genetic characteristics, including multiple genetic and environmental factors providing to liability. Determination and probability of risk alleles are few. The presence of these low penetrant alleles in the population is at a huge frequency (>1%). Some of these variations affect protein structure. Most of the proteins have various characteristic, heritable isoforms such as blood type antigens. Characteristic genetic variants, involving single nucleotide polymorphisms (SNPs), affect liability to common disorder. Single nucleotide polymorphisms are changes in one base at a particular location in the genome, mostly accompanying two alleles. Typically, these alterations are placed in every 300-1000 base pairs (bp) in several genomes. This means there is an average of 3,000,000 differences between any two human genomes. Today the total number of variant positions among all humans is far greater and estimated to be more than 10,000,000 (Aitken, Smith, Schwarz, & Morin, 2004; Garvin, Saitoh, & Gharrett, 2010; Gut, 2001; Kwok & Gu, 1999; Mooney, 2005; Syvanen, 2001).

### 1.3.1 Use

SNPs are easy to count, cheap for genotyping, introduce co-dominant markers with an easy, obvious mutation design and delivered within the genome. These features make SNPs utilize to a broad range of studies. They can be used as markers for building genetic maps or for conducting searches for mutations that correlate with specific diseases or predispositions to disease. Useful respond or side effect to drug is a complicated issue controlled with many genes. Some variations also affect the response and adverse side effects to a drug and the approach specific medication to every single genetic portrait is the new field of

pharmacogenomics (Aitken et al., 2004; Garvin et al., 2010; Gut, 2001; Kwok & Gu, 1999; Mooney, 2005; Syvanen, 2001).

### 1.3.2 Types

It might have individual results at the phenotypic level according to the location of the SNP:

**A. SNPs in non-coding region (intronic SNPs, iSNPs; regulatory SNPs, rSNPs):** SNPs disperse through the genome heterogenously. Non-coding regions contain much more SNPs than coding regions. However, SNPs haven't got any straight accepted effect on the phenotype of a person. They are used in evolutionary studies and population genetics as indicators. SNPs in not protein coding regions may influence degradation of messenger RNA, splicing, the non-coding RNA sequences, or binding of transcription factor.

**B. SNPs in coding region (coding SNPs, cSNPs):** SNPs in coding sequences don't significantly alter the protein amino acid sequence because of the genetic code degeneracy. Protein function or structure changing SNPs is important and adequate reason of many obvious Mendelian inherited monogenic diseases. These SNPs are analyzed for testing reasons. Protein structure changing SNPs are included in medication metabolism. Therefore, these kinds of SNPs are studied for pharmacogenetics. These kind of SNPs have two subtypes:

**1. Synonymous SNPs (sSNP):** These kinds of SNPs do not affect the protein sequence, however could even influence protein activity.

**2. Nonsynonymous SNPs (nsSNPs):** These SNPs alter the protein amino acid sequence. nsSNPS have two subtypes according to the type of mutation:

**a) Missense mutation causing SNPs:** Alteration in protein amino acid and its defect causing disorder is the result of one base alteration in the sequence. Missense mutation causing SNP could be diagnosed to calculate the risk for a specific disorder.

**b) Nonsense mutation causing SNPs:** These type of SNPs cause generally inactive protein yield due to truncated, incomplete protein as a result of immature stop codon, or nonsense codon by a point mutation (Aitken et al., 2004; Garvin et al., 2010; Gut, 2001; Kwok & Gu, 1999; Mooney, 2005; Syvanen, 2001).

### 1.3.3 Importance

The single nucleotide mutation proportions are comparatively low (~$10^{-8}$) over similar sites, therefore, this represents an infinite sites model. In association studies, genotype of variants in sufferers and healthy individuals are determined, and the susceptibility to a variety of diseases is tested. The genotype or allele frequencies at researched region are correlated in community of patients and healthy individuals. Detecting the association with susceptibility or increased risk of disorder is assessed by the high frequency in cases. Ethnic mixture and stratification of the population are the main problems of these studies, due to the separation of cases and controls into at least two ethnic groups or subgroups without a purpose. Stratification appears when one of these subgroups has elevated frequency of disease. Thus, any polymorphism genetically marks in the high-risk subgroup will probably result in an inaccurate association. Family-based studies or collecting samples from dissimilar ethnic groups can be used to overcome this problem. Gene-gene or gene-environment interactions are another problem that generates unstable results. Efficiency of a variant generally depends on the presence of specific genetic or environmental factors. If so, there will be an association when these factors exist together in communities or subgroups. Consequently, when genetic risk factors are not so strong, there can be association, however, the associations couldn't be duplicative. Small amounts of sample sizes wouldn't reflect the accurate statistical significance due to the weak effects (Aitken et al., 2004; Garvin et al., 2010; Gut, 2001; Kwok & Gu, 1999; Mooney, 2005; Syvanen, 2001).

### 1.3.4 Databases

The primary database of polymorphisms is dbSNP, and involves more than 5,000,000 validated human SNPs. Online Mendelian Inheritance in Man (OMIM), Human Genome Variation Database (HGVBase), the Human Gene Mutation Database (HGMD) and Swiss-Prot contain disease-associated polymorphisms (Mooney, 2005).

Finding the liability genes illuminates the pathophysiology and the aetiology of schizophrenia. Uncovering the underlying processes in schizophrenia, signal transduction, and brain maturation with candidate genes have great interest for last

2-3 years, and comprehensive studies are performed in animals, *in vitro*, and postmortem samples. *NRG1* gene is among the promising candidate genes for schizophrenia. In this study, we researched two *NRG1* SNPs (rs3924999 and rs2954041) to find association with schizophrenia. Second exon contains rs3924999, and conserved in all isoforms. In this SNP, a G base in 12nd position alters to A base that leads to amino acid alteration from arginine (Arg), a basic amino acid to glutamine (Gln), an acidic amino acid (Arg 38 Gln) (http://www.ncbi.nlm.nih.gov/SNP/). rs2954041 is located in fifth intron and may be associated with schizophrenia. It is suggested that the fifth intron could be associated with functional loci; such as rs392499 or other loci (J. Z. Yang et al., 2003).

Finding candidate genes and their roles in schizophrenia will open a road to discover novel targets for novel therapeutics.

# 2 LITERATURE REVIEW

Chromosome 8p may harbor a candidate gene for schizophrenia. Stefansson and colleagues declared that *NRG1* might have a role in the aetiology of schizophrenia. After association and linkage studies, they discovered an association in a risk haplotype about 290 kb at the 5' end of *NRG1* in Icelandic population. Stefansson *et. al* showed five SNPs [SNP8NRG221132 (A/G) (rs73235619); SNP8NRG221533 (A/G) (rs35753505); SNP8NRG241930 (C/A) (rs62519682); SNP8NRG243177 (A/G) (rs6994992), SNP8NRG433E1006 (not annotated in dbSNP130)], and two microsatellites (478B14-848, 420M91395) associated with schizophrenia, and these seven markers called Icelandic haplotype ($Hap_{ICE}$) (Stefansson et al., 2002).

The association between $Hap_{ICE}$ and Northern European population with schizophrenia was investigated. Risk haplotype was found to be further frequent in sufferers than healthy individuals (N. M. Williams et al., 2003).

A linkage disequilibrium study was performed to conclude the association among rs3924999, rs2954041, SNP8NRG221533 (rs35753505) on *NRG1* gene and in Chinese Han trios with schizophrenia with PCR-RFLP method and denaturing high performance liquid chromatography. After the transmission disequilibrium test, two transmitted alleles (rs3924999, rs2954041) are found to be associated with the disease (J. Z. Yang et al., 2003).

A Chinese family study (J. Z. Yang et al., 2003) showed an association among a *NRG1* polymorphism (Arg38Gln, rs3924999) with schizophrenia; but results have not replicated yet. For validating the results, the beginning age of the disease in a Chinese population has taken into account, but there wasn't an important association between the *NRG1* Arg38Gln polymorphism and schizophrenia (C. J. Hong et al., 2004).

Results of the studies about *NRG1* are inconsistent and they must be replicated. Three SNPs [SNP8NRG221533 (rs35753505), SNP8NRG241930 (rs62519682), SNP8NRG243177 (rs6994992)] and two microsatellites (478B14-848, 420M91395) from $Hap_{ICE}$ were genotyped in Han Chinese. The marker SNP8NRG221132 (rs73235619) was abandoned because of heterozygosity. The association was unable to be found between five markers, but strong linkage

disequilibrium was observed in all SNPs and microsatellite pairs (Zhao et al., 2004).

Three *NRG1* SNPs and its seventeen microsatellite markers were screened in European and Irish samples. Probable liability regions that cover about 911 kb which recognized by Stefansson et al. were investigated in the study (Stefansson et al., 2002). The Icelandic core haplotype exhibited strong intermarker linkage disequilibrium. Haplotype configuration might be different in population to population; therefore a distinct shape of association existed in Irish population (Corvin et al., 2004).

Han Chinese community has investigated for the association of *NRG1* with schizophrenia by Stefansson et al. with case control and family trio studies. 1.1 Mb *NRG1* including twenty five microsatellite markers, seven marker haplotype at the 5'end of the gene and SNPs were screened. There wasn't a significant association in the seven marker haplotype in Chinese population. But a novel haplotype ($Hap_{China\ 1}$) was found immediately upstream of the of the Icelandic haplotype (T. Li et al., 2004).

NRG1 has roles in neurotransmitter systems and neurodevelopment. The association between *NRG1* genotype and schizophrenia was genotyped in Finnish population with SNP8NRG221533 (rs35753505). There wasn't significant difference between sufferers and healthy individuals in *NRG1* genotype or allele frequencies (Kampman et al., 2004).

The association between *NRG1* gene and schizophrenia has been found in Scottish, Icelandic, Irish and mixed UK populations [SNP8NRG221132 (rs73235619); SNP8NRG221533 (rs35753505); SNP8NRG241930 (rs62519682); SNP8NRG243177 (rs6994992), SNP8NRG433E1006 (not annotated in dbSNP130), 478B14-848, 420M91395]. These populations derived from the same origin, thus, this enables researching for association and/or linkage of *NRG1* polymorphisms and haplotypes with schizophrenia. The seven marker core Icelandic/Scottish *NRG1* haplotype, SNP8NRG255133, SNP8NRG249130 and SNP8NRG243177 (rs6994992) (telomeric) and microsatellites 478B14-428, 420M9-1395, D8S1810 and 420M9-116I12 (centromeric) were not related with schizophrenia in Irish study of high-density schizophrenia study as a result of the linkage disequilibrium study (Thiselton et al., 2004).

*NRG1* markers and haplotypes were tested in schizophrenia patients from Portuguese descent. A haplotype covers $Hap_{ICE}$ and two haplotypes in the 3' end of *NRG1* were associated with schizophrenia, but, there wasn't association with $Hap_{ICE}$ itself (Petryshen et al., 2005).

Lin *et al.* investigated the association of schizotypal personality to *NRG1* variants (rs3924999, rs2954041, rs35753505). A allele of rs3924999 was found to be associated with schizotypal personality (Lin, Liu, Liu, & Hung, 2005).

SNP8NRG221533 (rs35753505), SNP8NRG241930 (rs62519682), and SNP8NRG243177 (rs6994992) from Stefansson et al. and rs1081062 in intron 1 of *NRG1* were genotyped in a Japanese sample. Association wasn't found in allele frequencies of SNPs; but, there were associations in minor allele homozygotes of SNP8NRG241930 (rs62519682), SNP8NRG243177 (rs6994992), and rs1081062 with schizophrenia (Fukui et al., 2006).

Seven microsatellite markers in 8p21-12 were screened for linkage analysis, and the association study was performed for three SNPs, two microsatellite markers and their haplotypes in Korean population with schizophrenia. There was a meaningful linkage in 352 kb upstream of the 5'- end of the first exon of *NRG1*, and an association in the G allele of SNP8NRG241930 (rs62519682) in patients with auditory hallucination (Kim et al., 2006).

NRG1 acts on the tyrosine kinase receptor ErbB4 for a number of its reactions. NRG1 and ErbB4 interactions analyzed in gene knock-out animals, and found that these interactions regulate attitudes. Therefore, these animals can be a schizophrenia model. Fourteen DSM-IV schizophrenic patients are genotyped for ErbB4 mutation, and discovered 15 SNPs. Also a significant interaction between the NRG1 and ErbB4 is found (Norton et al., 2006).

The association with the 5' end of the *NRG1* was investigated in Danish schizophrenics. This haplotype first noted in the Icelandic population with a significant association (Ingason et al., 2006).

ATTT motifs and AT-rich regions of the *NRG1* gene were detected for various polymorphisms. Caucasian and African American schizophrenics and healthy individuals were genotyped for allele frequencies. Association was found in genotype distribution and haplotype blocks placed in the 5' and 3' of *NRG1* in the African Americans. Results from rs6150532 were striking, because rs6150532 is an insertion/deletion variant located in conserved region of an intron. It is

between the two narrow, alternatively spliced exons. However, association couldn't be detected in Caucasians. In spite of the small sample sizes, the relation between *NRG1* and schizophrenia is found in African American samples (Lachman et al., 2006).

Population of Central Valley of Costa Rica is used to investigate the association of NRG1 with schizophrenia. Six $Hap_{ICE}$ were genotyped, but there wasn't association with $Hap_{ICE}$ in the Central Valley Costa Rica population (Walss-Bass et al., 2006).

A case-control association study is performed for schizophrenia and bipolar disorder together. Entire *NRG1*, with the linkage disequilibrium blocks and up- and downstream of the coding region were screened. Thirty six haplotype tagging SNPs were chosen for association of *NRG1* from the International HapMap project Utah residents of northern and western European ancestry data. A new *NRG1* region involved in schizophrenia and bipolar disorder was identified in the Scottish population (Thomson et al., 2007).

The association of $Hap_{ICE}$ and additional markers with schizophrenia trios was tested in Bulgarian population. Only SNP8NRG221533 (rs35753505) yielded significant evidence for association (Georgieva et al., 2008).

Results of the studies about *NRG1* are inconsistent and they must be replicated. Ethnic mixture and stratification of the population are the main problems of these studies. Also the genes that interplay with *NRG1* might contribute to the incoherence. A significant interaction between the NRG1 and its receptor ErbB4 was found after studies. Eighteen polymorphisms in *NRG1* and *ErbB4* were screened for interaction in Japanese individuals. There wasn't significant difference between sufferers and healthy individuals in allele frequencies including rs3924999 and rs2954041. In the interaction analysis, significant interaction was observed between rs2919381 in *NRG1* and rs7560730 in *ErbB4*. Thus, interaction between variants in *NRG1* and *ErbB4* might contribute to susceptibility for schizophrenia in a Japanese population (Shiota et al., 2008).

Hong *et al.* conducted a study to find association between prepulse inhibition and two SNPs of *NRG1* (rs10503929 and rs3924999) in schizophrenia patients. rs3924999 was found to be associated with prepulse inhibition (Hong et al., 2008)

*NRG1* is one of the most researched genes associated with schizophrenia. Results of the studies about *NRG1* are inconsistent. According to a meta-analysis, associations in SNP8NRG221132 (rs73235619), and two microsatellites 420M9-1395(0) and 478B14-848(0) (members of $Hap_{ICE}$ block) were detected in schizophrenia; however the sample sizes were almost narrow compared to other markers. Otherwise, other markers had big sample sizes, but had no association (Gong et al., 2009).

Okochi *et al.* investigated methamphetamine-*NRG1* SNPs (rs3924999, rs35753505, rs62519682, rs6994992) associations. There was no significant association between *NRG1* and methamphetamine induced psychosis (Okochi et al., 2009).

Qednow *et al.* analyzed *COMT* (rs4680), serotonin-2A receptor (rs6311/rs6313) and *NRG1* (rs3924999) polymorphisms to find association with acoustic startle response and prepulse inhibition in schizophrenia. AA allele of rs6311/rs6313 showed increased, but male individuals with rs4680 showed decreased prepulse inhibition (Quednow et al., 2009).

Associations between five SNPs of $Hap_{ICE}$ placed in the region including the *NRG1* [SNP8NRG221132 (A/G) (rs73235619); SNP8NRG221533 (A/G) (rs35753505); SNP8NRG241930 (C/A) (rs62519682); SNP8NRG243177 (A/G) (rs6994992), SNP8NRG433E1006] and three SNPs within the brain-derived neutrophic factor (BDNF) gene [rs6265 (A/G); rs4923463 (A/G); rs11030104 (A/G)] were tested in Italian population. There wasn't significant difference between sufferers and healthy individuals in allele frequencies (Squassina et al., 2010).

Schmechting *et al.* researched the association of two endophenotypes of schizophrenia; smooth pursuit and antisaccade eye movement with rs3924999 in healthy individuals. There was no significant association with these two endophenotypes and SNP, but there was association with gender and increased hypermetric performance (Schmechtig et al., 2010).

In a family study, the risk of schizophrenia was found in a missense polymorphism in the *NRG1* gene Val>Leu in exon 11, in the Central Valley region of Costa Rica. rs6994992, rs3924999, and Val>Leu missense polymorphism in exon 11 were researched for interrelation from an insulated community from the Central Valley region of Costa Rica. Insulated communities

could carry narrow genetic heterogeneity and raise power to discover risk variants in liability genes. Schizophrenia, bipolar disorder patients and unrelated controls were genotyped for three *NRG1* variants. There wasn't significant difference with schizophrenia or bipolar disorder and healthy individuals. The dorsolateral prefrontal cortex was diagnosed for the type IV *NRG1* in a postmortem with schizophrenia, bipolar and major depressive disease sufferers, and healthy individuals. It was discovered that rs6994992 genotype has an impact on expression. There wasn't association with schizophrenia in the missense polymorphism Val>Leu in exon 11 (Moon et al., 2011).

The association of SNP8NRG241930 (rs62519682) from the *NRG1* gene with schizophrenia is found in Iranian population (Shariati, Behmanesh, & Galehdari, 2011).

*NRG1* variants (rs73235619, rs62519682, rs6994992, rs3924999, rs2439272, rs10503929) were investigated for finding the association with prepulse inhibition. rs62519682, T allele of rs6994992 and C allele of rs2439272 was found related to prepulse inhibition. Prepulse inhibition was also related to rs3924999 or rs10503929 if combined with rs2439272 (Roussos, Giakoumaki, Adamaki, & Bitsios, 2011).

Schizophrenic or bipolar I disorder sufferers accompanying psychotic symptoms, their healthy family members, and unrelated healthy controls from Caucasians were included to a family study. The association hasn't found in COMT Val158Met and BDNF Val66Met polymorphisms in every phenotype. However, there was an association in rs221533 of the *NRG1* gene. TT homozygous individuals had a low achievement compared with C allele carriers with premorbid adaptation in adolescence (Walshe et al., 2012).

Yang investigated the association between schizophrenia and the SNPs of *NRG1*; rs3924999, rs7014762, rs11998176. rs3624999 was found to be associated with schizophrenia (Yang, 2012).

Event-related potentials indicate the capability to fulfill incoming data. P300 is a part of it and a common measurement of cognitive efficiency. Kang *et al*. researched the association between P300 and rs2954041, SNP8NRG221533 (rs35753505), rs392499. They found a significant association with AT haplotype. There was no differences between control and schizophrenia group in allele frequencies and genotype distributions (Kang et al., 2012).

6-kb block in *ErbB4* was identified in European subjects. This block was between chromosome locations 212,156,823 and 212,162,848, and found associated with schizophrenia. Also there was an association in 25-kb block in *NRG1* between 32,291,552 and 32,317,192. These locations can be the binding sites for various transcription factors (Agim et al., 2013).

Schizophrenia shows substantial genetic and pathway heterogeneity. Hatzimanolis *et al.* showed that two or further destruction variants in the neuregulin signaling pathway genes coding NRG1, NRG3, ErbB4, β- and γ-secretase complex are necessary to cause disease. They also found after sequencing analysis that rs3924999 is a tolerable possibly damaging variant (Hatzimanolis et al., 2013).

Kukshal *et al.* researched two microsatellites and 35 SNPs of *NRG1* in Indian population. Haplotype 221121 (rs35753505, rs6994992, rs1354336, rs10093107, rs3924999,mrs11780123) particularly one microsatellite (420m9-1395), three SNPs (rs4733263, rs35753505, rs6994992) are found associated with schizophrenia (Kukshal et al., 2013).

According to a meta-analysis, rs3924999, rs2954041 and rs764059 was investigated for association with schizophrenia with Chinese, Malay and Indian groups. But no significant association was found (Chern, Yee, Foon, Jen, & Yen, 2013).

Tosato *et al.* researched several SNPs of *NRG1* and *ErbB4* in Scottish and German population. They found that few regions of *ErbB4* and *NRG1* have a function in biologic mechanism underlying in phenotype of schizophrenia (Tosato, Zanoni, Bonetto, & Tozzi, 2014).

Diez *et al.* investigated rs392499 and rs6994992 (*NRG1*); rs10748842 (*NRG3*) to find association with P300. They found no association between *NRG* variants and cognitive processes in schizophrenia (Díez et al., 2014).

Variants of *NRG1* (rs35753505, rs3924999, rs2054041) were analyzed for finding the association with schizophrenia in Pakistani population. There was significant association with rs35753505 (Nawaz et al., 2014).

*DISC1* (rs821626, rs6675281), *NRG1* (rs3924999, rs10503929, rs3735782, rs3735781), *BDNF* (rs6265) and *NOTCH4* (rs367398) polymorphisms were researcher for the association with clinical symptoms. Only *NOTCH4* variant

showed association with clinical symptoms of schizophrenia (Terzi, Kastelic, & Dol, 2015).

He *et al.* conducted a research to find association between *NRG1* and *DISC1* with schizophrenia. They suggested that *NRG1* rs3924999 and *DISC* rs821616 interactions or synergistic effects have a role on the development of schizophrenia. They also found that *DISC1* GA or GA/AA genotypes are associated with the increased risk of schizophrenia (He et al., 2016).

# 3 MATERIALS AND METHODS

## 3.1 Patients and Controls

We have screened rs3924999 (G/A) and rs2954041 (G/T) single nucleotide polymorphisms (SNP) located in *NRG1* gene in a group of schizophrenia patients (case group) and a control group from Malatya – Turkey for a potential single locus association with schizophrenia. The case group included 178 patients diagnosed for schizophrenia (121 men and 57 women; between 18 and 64 years of age (average: 36.9, SD: 11.6) and the control group included 180 individuals who did not have any symptoms of schizophrenia (90 men and 90 women who were older than 50 years of age). The samples were selected from the patients followed in the Psychiatry Clinic at Inonu University, Turgut Ozal Medical Center, Malatya. The participants were interviewed and diagnosed according to the Diagnostic and Statistical Manual for Mental Disorder IV (DSM-IV) criteria by Dr. Şükrü Kartalcı who is an experienced physician and faculty member of Inonu University School of Medicine, Department of Psychiatry. The relatives of the patients were not included in this study. Samples in the control group were chosen from the individuals who had no history for psychiatric illnesses in their families. Both control and case subjects were collected from Malatya, Turkey. Participants were informed about the study, then; after having their consent, blood samples were collected. Our study was confirmed by the Inonu University Malatya Ethics Committee of Clinical Researches (research protocol code: 2013/154). Code numbers were assigned to the patients for ethic reasons.

## 3.2 DNA Extraction and Quality Control

Venous blood samples were collected in EDTA covered tubes. DNA was extracted from 200 µL of whole blood using Qiagen QIAamp DNA Mini Kit according to the instructions of the producer:

1. 20 µL proteinase K was pipeted into the buttom of a 1.5 mL microcentrifuge tube.
2. 200 µL uncoagulated whole blood sample was added to the microscentrifuge tube.
3. 200 µL Buffer AL was added to the sample, and mixed pulse-vortexing for 15 seconds.

4. The tube was incubated at 56℃ for 10 minutes.
5. 1.5 mL microcentrifuge tube was centrifuged briefly to remove drops from inside of the lid.
6. 200 µL 100% ethanol was added to the sample, mixed again by pulse-vortexing for 15 seconds. After mixing, 1.5 mL microcentifuge tube was briefly centrifuged to remove drops from inside of the lid.
7. The mixture was applied carefully to the spin column and centrifuged at 8000 rpm for 1 minute. The spin column was placed in a clean 2 mL collection tube, and discarded the tube containing the filtrate.
8. The spin column was opened carefully and 500 µL Buffer AW1 was added and centrifuged at 8000 rpm for 1 minute. Spin column was placed in a clean 2 mL collection tube, and the collection tube containing the filtrate was discarded.
9. The spin column was opened carefully and 500 µL Buffer AW2 and centrifuged at 14,000 rpm for 3 minutes.
10. The spin column was placed in a new 2 mL collection tube and the old collection tube with filtrate was discarded, centrifuged at 14,000 rpm for 3 minutes.
11. The spin column was placed in a clean 1.5 mL microcentrifuge tube and the collection tube containing the filtrate was discarded. 200 µL Buffer AE was added to the spin column. The tube was incubated at room temperature for 5 minutes, and then centrifuged at 8000 rpm for 1 minute.

DNA samples were qualified by electrophoresis on 0.8 % agarose gels.

### 3.3 Quantification of DNA samples

Concentrations of DNA samples were measured reading the absorbances under 260 nm and 280 nm. A Bio-Tek Epoch Microplate Reader Take3 Plate was used for quantification. The concentration was automatically calculated by instrument's data analysis software Gen5.

The concentrations of DNA samples were adjusted to 10 ng/µL. Standardized DNA was stored in +4℃ for polymerase chain reaction step.

## 3.4 SNP Genotyping with PCR-RFLP (Polymerase Chain Reaction – Restriction Fragment Length Polymorphism) Method

### 3.4.1 PCR Amplification of Specific DNA Fragments Covering Targeted Polymorphisms

Fragments of *NRG1* gene covering each SNP concerned in this study were amplified separately as two different amplicons. Amplicon-1 covering rs3924999 was a 193 bp fragment, and Amplicon-2 covering rs2954041 was a 203 bp fragment (Table 4.1). PCR primers were designed using Primer3 software (Untergasser et al., 2012). Sequences of PCR primers and PCR amplicons indicating polymorphic sites are given in Table 3.2.

**Table 3.1.** An overview to SNPs and RFLP fragments

| SNP | Alteration | Region | Amplicon | Restriction Enzyme | Size of Fragments bp |
|---|---|---|---|---|---|
| **rs3924999** | G/A | 2nd exon | 193 bp | *Mun*I (37°C) | CC: 193<br>CT: 193-102-91<br>TT: 102-91 |
| **rs2954041** | G/T | 5th intron | 203 bp | *Tru1*I (65°C) | GG: 130-103<br>GT: 130-103-74-29<br>TT: 130-74-29 |

**Table 3.2.** Sequences of amplicons and PCR primers used for amplification of *NRG1* fragments including targeted SNPs. Primer sequences and restriction enzyme recognition sites are underlined and alternative alleles of targeted SNPs are italicized

| | |
|---|---|
| **SNP: rs3924999** | |
| DNA sequence of amplicon-1 PCR product | GAACCA**CTTGAATCTGAGAGAGGAGTA**TTCAGAACTGGTTTCACACCGAA GGACTAGTTTGGAACCTGCAGCCGATTCCTGGCTTTTCATCTCTTT**CAAT *T/C*G**GGGAGGCAAGGCTAAAAGAAGAAAAGAGAATGAAAAACAACTCTGA TCACCAGGCAATCTTTCAAAGAGAAA**CATTTAATCTGGAGTTAGCCTGAT CA** |
| Forward primer for amplicon-1 | 5′-CTTGAATCTGAGAGAGGA**GTA**-3′ |
| Reverse primer for amplicon-1 | 5′-GATCAGGCTAACTCCAGATTAAATG-3′ |
| **SNP: rs2954041** | |
| DNA sequence of amplicon-2 PCR product | AGCGGATAACAATTTCACACAGGA**ACATTTTTACTTTACTTTTTGAC**ATT ATTCATTGTTTGTTGCTGATGAAT***G/T*****AA**ACCTCTTTTCGAGTGTGTGTT CCCT*TTAA*TAGGCACTGGTAATTTATGTACTAAGATTTATTTCTGTATGG AAATTACAAGTGAAATTTCATATTGAATTTATATTTAGCCTGTTCTAGTA CATAGTCTCT**GCATAGTATATCCATGGCATCCTAC** |
| Forward primer with 5’ M13 tail for amplicon-2. M13 sequence is shown in italic characters. | 5′-*AGCGGATAACAATTTCACACAGGA*ACATTTTTACTTTACTTTTTGAC-3′ |
| Reverse primer for amplicon-2 | 5′-GTAGGATGCCATGGATATAC-3′ |

PCR was carried out using ThermoFisher Scientific Taq Polymerase. Content of PCR mixture is given in Table 3.3.

**Table 3.3.** Master mix of PCR

| **Master Mix** | **Stock Concentration** | **1 tube (µL)** | **Final Concentration** |
|---|---|---|---|
| $dH_2O$ | | 12.9 | |
| Buffer | 10X | 2.5 | 1X |
| dNTP | 10 mM | 0.5 | 0.125 mM |
| $MgCl_2$ | 25 mM | 1.5 | 0.9375 mM |
| Forward Primer | 10 µM | 1.25 | 12.5 µ |
| Reverse Primer | 10 µM | 1.25 | 12.5 µ |
| Taq Polymerase | 5 U/µL | 0.1 | 0.5 U |
| DNA | 10 ng/µL | 5 | 50 ng |
| **Total Volume** | | **25** | |

PCR reactions were performed in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, CA, USA). The instrument was heated to 94ºC for 5 minutes for initial denaturing step. Steps of PCR reactions include:

**Denature DNA:** 94ºC for 45 seconds;

**Primer annealing:** 57ºC for 45 seconds;

**Extension:** 72ºC for 44 seconds.

These steps were repeated for 30 cycles. After 72ºC for 7 minutes final extension, amplicons were cooled to 20ºC end of the reaction. Amplicon quality was examined visually after electrophoresis on 2% agarose gel.

### 3.4.2 Genotyping SNPs with RFLP Method

**Genotyping of rs3924999 with MunI Restriction Endonuclease:**

***Mun*I (*Mfe*I):** *Mun*I recognizes 5'…C↓AATTG…3', and accesses the DNA from the major groove and distorts the DNA. The source of the enzyme is *Mycoplasma fermentans*. *Mun*I belongs to *Eco*RI branch (Pingoud et al., 2005).

rs3924999 is a single nucleotide polymorphism located at the 32595840th position of chromosome 8 (GRCh38 38.1/141). The alternative alleles for this position are T and C. While T allele forms the recognition site for restriction enzyme *Mun*I which is "CAATTG" C allele modifies this sequence to "CAATCG" which is not recognized by the enzyme. In our study, the amplicon-1 which is a fragment of the second exon of *NRG1* gene covering SNP rs3924999 was digested with the enzyme *Mun*I (Thermo Scientific) at 37ºC for 3 hours. Reaction conditions for digestion are given in Table 3.4. The digestion products were analyzed with gel electrophoresis.

**Table 3.4.** Master mix of *Mun*I

| Master Mix | Stock Concentration | 1 tube (µL) | Final Concentration |
|---|---|---|---|
| PCR Product | | 10 | |
| $dH_2O$ | | 18 | |
| Buffer G | 10X | 3.2 | 1X |
| *Mun*I | 1500 U | 0.2 | 300 U |
| **Total Volume** | | **31.4** | |

The full length of amplicon-1 covering the first SNP rs3924999 was 193 bp. The amplicon has no digestion site for *Mun*I if the sample is homozygous for the

C allele. T allele introduces a recognition site for *Mun*I and causes the amplicon-1 to split into two fragments (102 bp and 91 bp). Thus, the heterozygote samples were seen with three fragments in lengths of 193 bp, 102 bp and 91 bp (Figure 3.1).

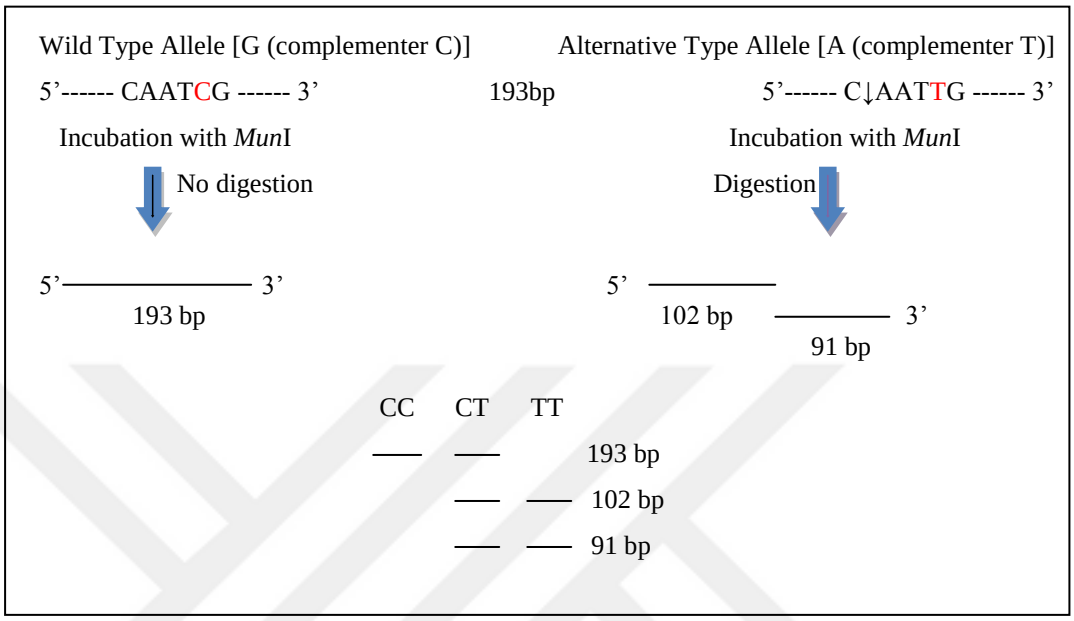


**Figure 3.1.** The diagram for restriction sites and fragments for *Mun*I

We analyzed restriction endonuclease treated PCR products with electrophoretic methods. *Mun*I digestion products of amplicon-1 were separated with 1.5% agarose gel electrophoresis and visualized under UV transluminator after staining with ethidium bromide. The results were also confirmed on polyacrylamide gel electrophoresis.

**Genotyping of rs2954041 with Tru1I Restriction Endonuclease:**

***Tru*1I (*Mse*I):** *Tru*1I recognizes 5'…T↓TAA…3'. The source of the enzyme is *Thermus ruber* RFL1.

rs2954041 is located on the 32665107$^{th}$ position of chromosome 8 (GRCh38 38.1/141) with T and G alternative alleles. While T allele is recognized by the enzyme *Tru*1I whose recognition site is "TTAA", G allele is not recognized. In our study the amplicon-2 which is a fragment of the fifth intron of *NRG1* gene covering SNP rs2954041 was digested with the enzyme *Tru*1I (Thermo Scientific) at 65ºC for 3 hours. Reaction conditions for digestion are given in Table 3.5.

**Table 3.5.** Master mix of *Tru*1I

| Master Mix | Stock Concentration | 1 tube (µL) | Final Concentration |
|---|---|---|---|
| PCR Product | | 10 | |
| $dH_2O$ | | 20 | |
| Buffer R | 10X | 3.1 | 1X |
| *Tru*1I | 300 U | 0.1 | 30 U |
| **Total Volume** | | **33.2** | |

The amplicon-2 covering SNP rs2954041 was a 209 bp PCR fragment. A 5' non-human tail was introduced to the PCR product with the forward primer to adjust the lengths of restriction fragments for enabling the separation with gel electrophoresis. The digestion of the fragment with *Tru1*I is expected to produce 130 bp, 74 bp and 29 bp restriction fragments for TT homozygotes and 130 bp and 103 bp fragments for GG genotypes. The expected fragments for heterozygote genotype were 130, 103, 74 and 29 bp (Figure 3.2). The resulting products were analyzed by electrophoresis on 10 % polyacrylamide gels and stained with silver nitrate.

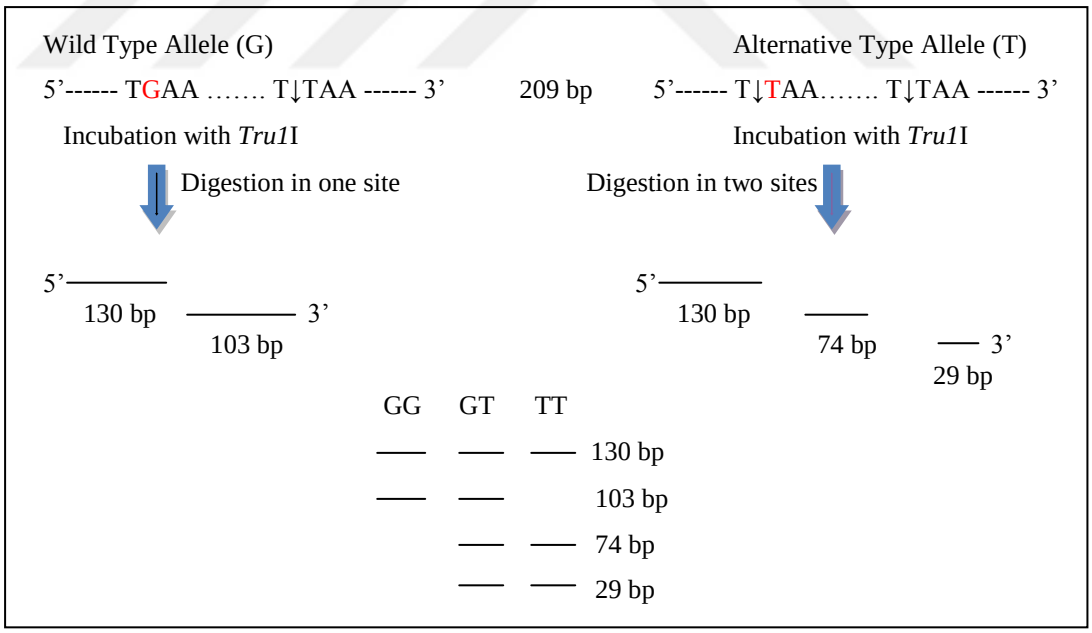


**Figure 3.2.** The diagram for restriction sites and fragments for *Tru1*I

### 3.5 Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel with 10 % concentration was used to separate fragments with different lengths after restriction for detecting each genotype. Polyacrylamide gel with 10 % concentration was prepared as follows:

| | |
|---|---|
| Acrylamide/Bis-acrlamide (19:1) | 5 gr |
| 10X TEA Buffer | 5 ml |

The solution volume was adjusted to 50 mL with distillated water and filtrated with 0.22 µm pore sized Sartorius syringe filter. Then, 7.5 µL *N',N',N',N'*-tetramethylethylenediamine (TEMED) and 250 µL of 10% ammonium persulfate (APS) were added to 25 mL acylamide/bis-acrylamide solution. The content was mixed well with gently shaking. It was quickly poured between PAGE glasses for preventing early polymerization. The comb was replaced and left about two hours for polymerization. After polymerization, gels were placed on BIO-RAD Mini-PROTEAN Tetra Cell PAGE System filled with 1X TAE buffer. A pre-run step was performed with bromophenol blue containing loading buffer. 100 volts of current was applied for 10 minutes, and then 10 µL of restriction endonuclease reaction product sample was loaded on each well. 100 volts of current was applied for 90 minutes, and the bands were visualized after staining with silver nitrate.

### 3.6 Silver Nitrate ($AgNO_3$) Staining of Polyacrylamide Gels

1. The gels were kept for 10 % acetic acid solution at least for 10 minutes.
2. The gel was washed by shaking with distillated water until the greasy layer was completely removed.
3. The gel was placed in the solution containing 0.1 % $AgNO_3$ and 0.2 % formaldehyde onto a shaker for 60 minutes.
4. The $AgNO_3$ solution was poured out and gel was washed with distillated water twice briefly.
5. The gel was developed in the third solution containing 3.4 % sodium carbonate ($Na_2CO_3$) and 0.2 % formaldehyde.

6. As soon as the bands appeared, the reaction was stopped by addition of 10 % acetic acid.

The images of gels were taken with Syngene G:BOX gel imagining system.

### 3.7 Statistical Analysis

The distributions of alleles and genotypes were represented as count and percentage. The Hardy-Weinberg equilibrium was tested with chi-square method. Genotype and allele frequencies between case and control groups were compared with chi-square test for determining a possible single SNP association.

# 4 RESULTS

We have screened rs3924999 (G/A) and rs2954041 (G/T) single nucleotide polymorphisms (SNP) located in *NRG1* gene in a group of schizophrenia patients (case group) and a control group from Malatya – Turkey for a potential single locus association with schizophrenia. The case group included 178 patients diagnosed for schizophrenia (121 men and 57 women; between 18 and 64 years of age (average: 36.9, SD: 11.6) and the control group included 180 individuals who did not have any symptoms of schizophrenia (90 men and 90 women who were older than 50 years of age) (Table 4.1). All genotypes were separated with 10 % polyacrylamide gel electrophoresis, and dyed with ethidium bromide diluted in distillated water and silver nitrate staining for validation.

**Table 4.1**. Demographic features of participants

| | **Men** | **Women** | **Age** | **Total** |
|---|---|---|---|---|
| **Control** | 90 | 90 | 36.9 ± 11.6 | 180 |
| **Case** | 121 | 57 | 36.9 ± 11.6 | 178 |
| **Total** | 211 | 147 | | 358 |
| **%** | 59 | 41 | | 100 |

## 4.1 Genotyping of rs3924999

Restriction digestion of amplicon-1 with *Mun*I enzyme produced two fragments (102 and 91 bp) if the sample was homozygous TT for rs3924999. C homozygotes were not digested and heterozygosity was seen as three fragments (193, 102 and 91 bp). A silver stained polyacrylamide gel is seen on Figure 4.1. Figure 4.2 shows a gel stained with ethidium bromide.

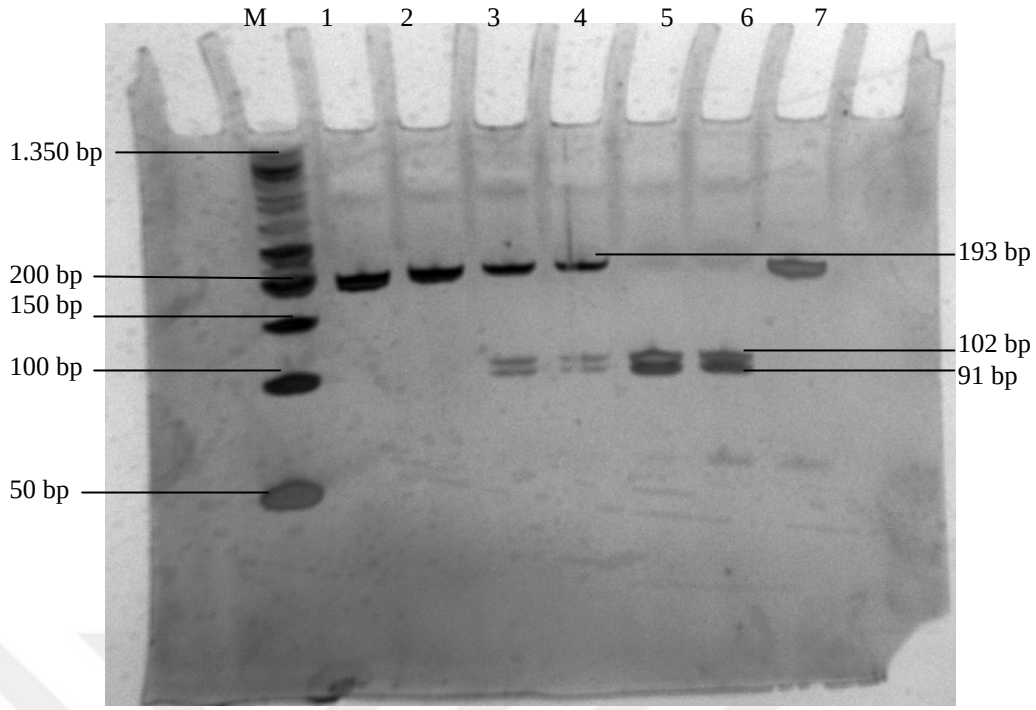


**Figure 4.1.** Genotypes of *Mun*I restriction with silver nitrate stained 10 % polyacrylamide gel. M: 50 bp marker; 1, 2: CC; 3,4: CT; 5,6: TT; 7: unrestricted amplicon

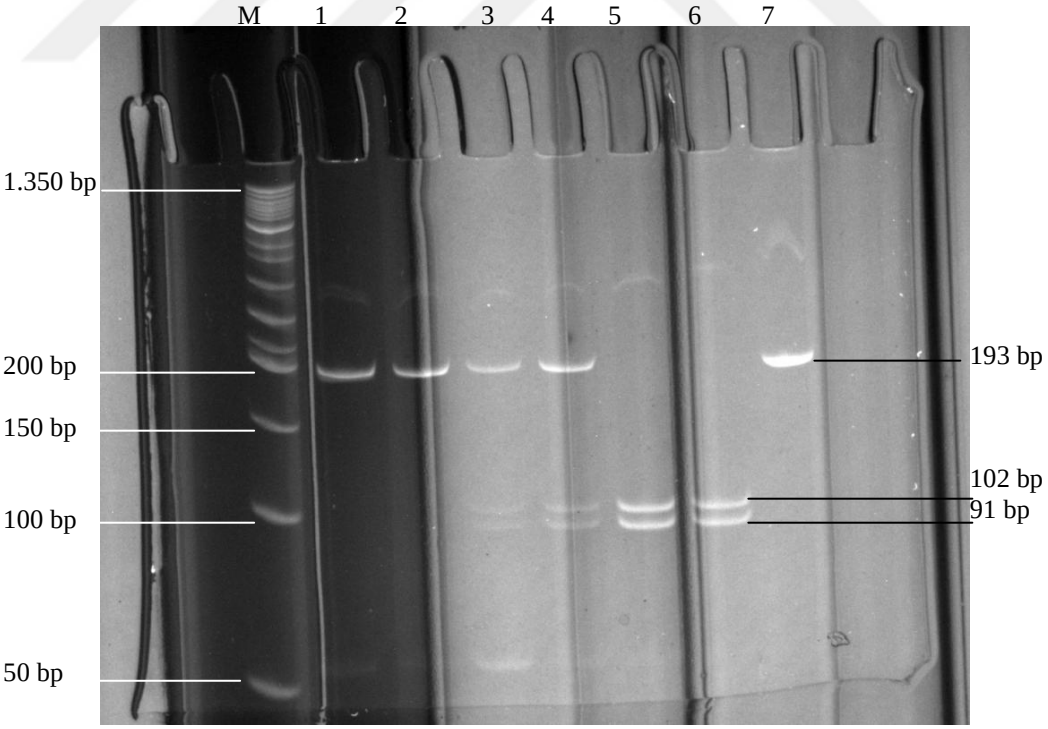


**Figure 4.2.** Genotypes of *Mun*I restriction with 10 % polyacrylamide gel with ethidium bromide staining. M: 50 bp marker; 1, 2: CC; 3,4: CT; 5,6: TT; 7: unrestricted amplicon

## 4.2 Genotyping of rs2954041

Restriction digestion of amplicon-2 with *Tru*1I enzyme was expected to produced two fragments (130 and 103 bp) if the sample was homozygous GG for rs2954041. None of our samples were homozygote for G allele. Homozygotes for T allele were digested at two points and produced three fragments (130, 74 and 29 bp). And heterozygotes were seen with four restriction fragments as expected (130, 103, 74 and 29 bp). A polyacrylamide gel stained with ethidium bromide and visualized under UV is seen on Figure 4.3.

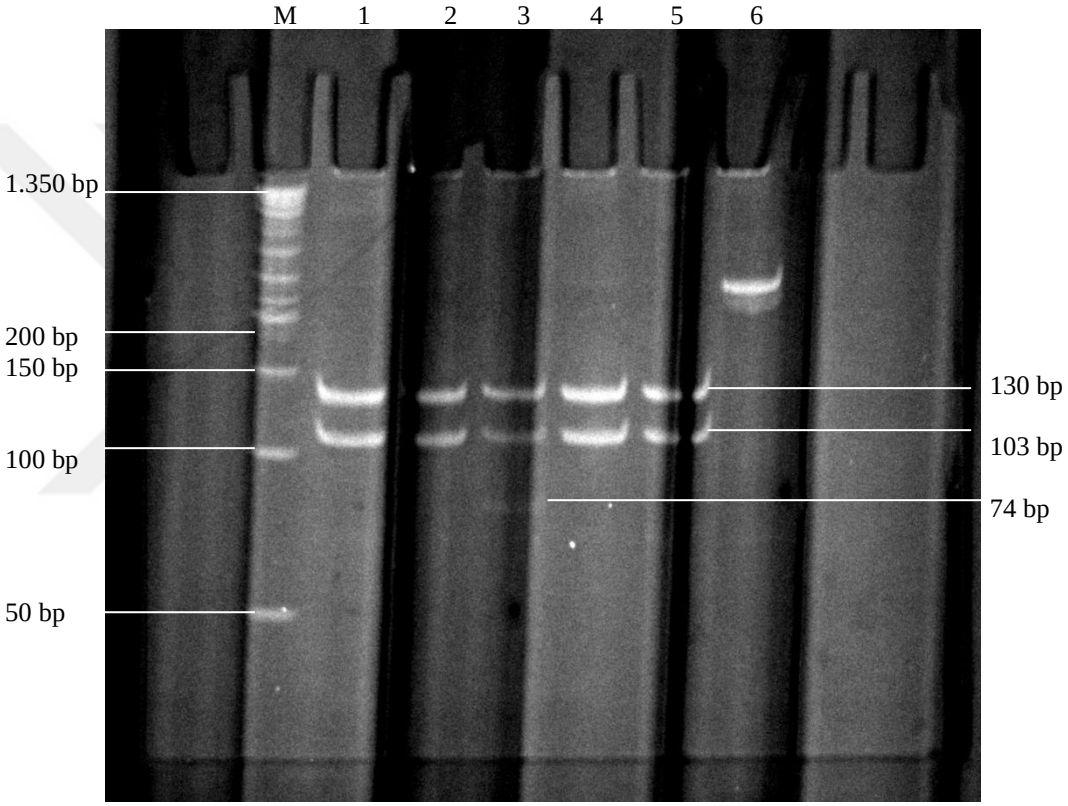


**Figure 4.3.** Genotypes of *Tru*1I restriction with 10 % polyacrylamide gel with ethidium bromide staining. M: 50 bp marker; 1, 2, 4, 5: GG ; 3: GT ; 6: unrestricted amplicon

## 4.3 Statistical Analysis

The distributions of alleles and genotypes were represented as count and percentage. The Hardy-Weinberg equilibrium was tested with chi-square method for both SNPs in patient and control groups separately. Genotype and allele frequencies between case and control groups were compared with chi-square test for determining possible single SNP associations.

The numbers of individuals in case and control groups carrying each genotype of each SNP (genotype counts) are given in Table 4.2. Genotype frequencies are presented in Table 4.3. Both case and control groups were found in Hardy-Weinberg equilibrium. Comparison of case and control groups for distribution of genotypes showed there was no statistically significant difference between two groups since p-values calculated with chi-square tests were all above 0.05.

There were results from 165 controls and 170 schizophrenic patients for rs3924999. 65 of controls had CC, 79 were CT, and 21 were TT genotype. 68 of patients had CC, 73 were CT, and 29 were TT genotype. There were results from 169 controls and 159 schizophrenic patients for rs2954041. There was not TT genotype both in controls and patients. 166 of controls were GG, 3 were GT genotype. 158 of patients were GG, 1 was GT genotype (Table 4.2).

**Table 4.2.** Distributions of genotypes found in the patient and control groups for screened SNPs

**Genotype Counts**

| SNP | HW-p * | Genotypes | | | p |
|---|---|---|---|---|---|
| **rs3924999** | | **CC** | **CT** | **TT** | |
| Controls (n=165) | 0.40 | 65 | 79 | 21 | 0.4699 |
| Patients (n=170) | 0.39 | 68 | 73 | 29 | |
| **rs2954041** | | **GG** | **GT** | **TT** | |
| Controls (n=169) | 0.9822 | 166 | 3 | - | 0.34 |
| Patients (n=159) | 0.9937 | 158 | 1 | - | |

* HW-p: Hardy-Weinberg p-value

The distribution of genotypes for rs3924999 in controls was 39 % for CC, 48 % for CT, and 13 % for TT genotypes. Distribution in patients as follows: 40 % for CC, 48 % for CT, and 13 % for TT genotypes. The distribution of genotypes for rs2954041 in controls was 98.22 % for GG, and 1.78 % for GT genotypes. Distribution in patients as follows: 99.37 % for GG, and 0.63 % for GT genotypes (Table 4.3).

**Table 4.3.** Genotype frequencies found in the patient and control groups for screened SNPs

**Distribution of Genotypes (Frequencies)**

| **SNP** | **Genotypes** | | |
|---|---|---|---|
| **rs3924999** | **CC** | **CT** | **TT** |
| Controls (n=165) | 0.39 | 0.48 | 0.13 |
| Patients (n=170) | 0.40 | 0.43 | 0.17 |
| **rs2954041** | **GG** | **GT** | **TT** |
| Controls (n=169) | 0.9822 | 0.0178 | - |
| Patients (n=159) | 0.9937 | 0.063 | - |

The allele frequencies were calculated for each SNP. Distribution of each allele was compared with chi-square test between case and control groups for determining a potential allelic association with the disease. Table 4.4 shows the distribution of alleles in case and control groups, and Table 4.5 shows frequencies of alleles in case and control groups. Chi-square tests were performed to compare case and control groups for distribution of alleles at both polymorphic points. The p-values obtained for both SNPs were higher than 0.05 and no significant difference was found between case and control groups.

The allele count for rs3924999 in controls was 209 for C and 121 for T alleles; in patients as follows: 209 for C and 131 for T alleles. The allele count for rs2954041 in controls was 3 for G and 335 for T alleles; in patients as follows: 1 for G, and 317 for T genotypes (Table 4.4).

**Table 4.4.** Distribution of alleles in the patient and control groups for screened SNPs

**Allele Counts**

| SNP | Alleles | | p |
|---|---|---|---|
| **rs3924999** | **C** | **T** | |
| Controls (n=165) | 209 | 121 | 0.6119 |
| Patients (n=170) | 209 | 131 | |
| **rs2954041** | **G** | **T** | |
| Controls (n=169) | 3 | 335 | 0.346 |
| Patients (n=159) | 1 | 317 | |

The allele frequencies for rs3924999 in controls were 0.633 for C and 0.367 for T alleles; in patients as follows: 0.615 for C and 0.385 for T alleles. The allele frequencies for rs2954041 in controls were 0.9911 for G and 0.089 for T alleles; in patients as follows: 0.9968 for G, and 0.0032 for T genotypes (Table 4.5).

**Table 4.5.** Allele frequencies found in the patient and control groups for screened SNPs

**Allele Frequencies**

| SNP | Alleles | | p |
|---|---|---|---|
| **rs3924999** | **C** | **T** | |
| Controls (n=165) | 0.633 | 0.367 | 0.6119 |
| Patients (n=170) | 0.615 | 0.385 | |
| **rs2954041** | **G** | **T** | |
| Controls (n=169) | 0.9911 | 0.0089 | 0.346 |
| Patients (n=159) | 0.9968 | 0.0032 | |

As seen in the tables and p-values from chi-square tests comparing our case and control groups for genotype and allelic distributions, we did not find any evidence for association of SNPs rs3924999 and rs2954041 with schizophrenia in our population.

# 5 DISCUSSION AND CONCLUSION

Schizophrenia is a mental disease described through positive indicia like deceptions, illusions and confused thinking, and negative indicia like depression, public recession, and lack of interest (H. J. Williams et al., 2009). *NRG1* is a huge gene (1.4 MB), and has many roles in CVS including neurodevelopment and transmission. Because of its importance, any abnormality of the gene disrupts normal development of CVS. Thousands of SNPs are exist among the gene. It has been suggested that *NRG1* polymorphisms associated with schizophrenia may affect gene expression levels (O'Tuathaigh et al., 2007). A vast series of neuroreceptors, especially NMDA glutamatergic receptors might be affected by NRG1 due to its effects on changing their expression and functions which can cause disease liability. There are white matter abnormalities in schizophrenia, and altered NRG1 function results in abnormal myelination. Myelination is found decreased in schizophrenic patients. Changes in NRG1 cause an activity defect of glia, and this leads to synaptic damage (O'Donovan et al., 2003). Uncovering the underlying processes in schizophrenia, signal transduction, and brain maturation with candidate genes have great interest for last 2-3 years, and comprehensive studies are performed in animals, *in vitro*, and postmortem samples.

NRG1 was initially involved in schizophrenia in the Icelandic community after linkage disequilibrium (Stefansson et al., 2002), then, identical haplotype was found associated in an abundant sample from Scotland, and not strong evidence in UK sample. These populations derived from the same origin, thus, this enables researching for association and/or linkage of *NRG1* polymorphisms and haplotypes with schizophrenia. Additionally, the association between $Hap_{ICE}$ and Northern European population with schizophrenia was investigated. Risk haplotype was found to be further frequent in sufferers than healthy individuals (N. M. Williams et al., 2003). But haplotype markes varies among other populations.

*NRG1* gene is one of the hopeful candidate genes for schizophrenia. In this study, we researched two *NRG1* SNPs (rs3924999 and rs2954041) to find association with schizophrenia. Second exon contains rs3924999, and conserved in all isoforms. So a default in this exon will affect all isoforms. In this SNP, a G base in 12nd position alters to A base that leads to a missense polymorphism;

amino acid alteration from arginine (Arg) to glutamine (Gln) (Arg 38 Gln) (http://www.ncbi.nlm.nih.gov/SNP/). This change makes the SNP very important. Arg is an aliphatic, basic and essential amino acid; Gln is a neutral, asidic and polar amino acid that contains amide in its side chain. This alteration may affect the conformation, configuration and activation of the protein. rs2954041 is located in fifth intron and may be associated with schizophrenia. The SNPs in introns may have great importance, because introns contain splices signals, and sometimes regulator sequences. Alteration in introns could cause degradation of messenger RNA, defaults in splicing and the non-coding RNA sequences, or binding of transcription factor, high or low transcription, and alterations in protein activity. An interesting study was arise from Roussos *et al*. If rs3924999 or rs10503929 combines with rs2439272, they have an effect on prepulse inhibition (Roussos et al., 2011). This interaction gives an idea that rs3924999 can influence the regulatory regions of the network. Hatzimanolis *et al.* found after sequencing analysis that rs3924999 is a tolerable possibly damaging variant (Hatzimanolis et al., 2013). He *et al.* suggested that *NRG1* rs3924999 and *DISC* rs821616 interactions or synergistic effects have a role on the development of schizophrenia (He et al., 2016).

rs3924999 and rs2954041 were firstly found to be associated with schizophrenia in Chinese Han trios after a linkage disequilibrium study by Yang *et al.* They calculated the association with transmission disequilibrium test, but association could not able to be replicated in other studies, as our study (J. Z. Yang et al., 2003). For validating the results, the beginning age of the disease in a Chinese population has taken into account, but there was not an important association between the *NRG1* Arg38Gln polymorphism and schizophrenia. Genotype distributions of rs392499 in this study for cases were, GG: 0.061; GA: 0.320; AA: 0.618 in Chinese population, and no significant difference was found between case and control group (C. J. Hong et al., 2004). The distribution of genotypes in other Chinese population (Kang et al., 2012) is correlated in Yang's study (J. Z. Yang et al., 2003) in control group but not in case group. Yang investigated the association between schizophrenia and the SNPs of *NRG1*; rs3924999, rs7014762, rs11998176. rs3624999 was found to be associated with schizophrenia. The same situation is seen in genetically related Korean

population. This observation is same in rs2954041 genotype distributions (Yang, 2012) (Tables 5.1, 5.2, 5.3, 5.4).

The association with schizophrenia was found in Korean and Chinese Han populations (Yang, 2012; Yang et al., 2003) although it was failed in same Chinese population (C. J. Hong et al., 2004; Kang et al., 2012). It may due to the stratification, sample sizes or the method of study and calculation. Kukshal *et al*. and Yang *et al.* studies were linkage disequilibrium studies; additionally Kukshal *et al.* had a very large sample size and used Bonferroni correction by assessing the asociation (Kukshal et al., 2013; J. Z. Yang et al., 2003)

rs3924999 and rs2954041 were generally found to be associated after linkage disequilibrium and haplotype studies. Also Yang *et al.* firstly found association with schizophrenia after a linkage study with transmission disequilibrium test. Kukshal *et al.* researched haplotype 221121 (rs35753505, rs6994992, rs1354336, rs10093107, rs3924999,mrs11780123) particularly one microsatellite (420m9-1395), three SNPs (rs4733263, rs35753505, rs6994992) are found associated with schizophrenia (Kukshal et al., 2013). Furthermore, the method being used and the test for assessing the association is a very important issue. Kukshal *et al.* used linkage disequilibrium and found association. It is clearly seen that allele frequencies of rs3924999 in Kukshal's study in North Indian population are correlated to our results. Our study is a case-control study, an association may be found after a linkage disequilibrium study in Turkish population.

In many populations, no significant association was found with rs3924999 and rs2954041 in schizophrenia like our study. Eighteen polymorphisms in *NRG1* and *ErbB4* were screened for interaction in Japanese individuals. There wasn't significant difference between sufferers and healthy individuals in allele frequencies including rs3924999 and rs2954041 (Shiota et al., 2008). The genotype distribution of rs392499 Shiota's study in Japanese population is different from other populations, but similar to other Japanese, Slovenian and Turkish populations. In allele frequency, the results are correlated with Japanese (Okochi et al., 2009) and Turkish populations. In rs2954041 genotype distribution and allele frequency, there is a correlation with Chinese Han population (Kang et al., 2012; J. Z. Yang et al., 2003) (Tables 5.1, 5.2, 5.3, 5.4).

In a family study, rs6994992, rs3924999, and Val>Leu missense polymorphism in exon 11 were researched for interrelation from an insulated community from the Central Valley region of Costa Rica. There wasn't significant difference with schizophrenia or bipolar disorder and healthy individuals (Moon et al., 2011). The study performed with an insulated community from the Central Valley region of Costa Rica, so this study is different for its population from other studies. Insulated communities could carry narrow genetic heterogeneity and raise power to discover risk variants in liability genes. When we compare the genotype distribution to other populations, a similarity can be seen with Slovenian and Turkish population (Costa Rica GG: 0.419; GA: 0.485; AA: 0.099 in cases). It is striking to see a similarity in populations with different origins and environmental factors. It may because of the similarity of the pathways that cause schizophrenia (Table 5.1).

According to a meta-analysis, rs3924999, rs2954041 and rs764059 was investigated for association with schizophrenia with Chinese, Malay and Indian groups. But no significant association was found (Chern et al., 2013). Variants of *NRG1* (rs35753505, rs3924999, rs2054041) were analyzed for finding the association with schizophrenia in Pakistani population. There was significant association with rs35753505 (Nawaz et al., 2014). The abundance of GG genotype is seen in this population, and the frequency of A allele is too low in rs3924999, and the same situation is seen in rs2954041 genotype distributions and allele frequencies (GG: 0.9286; GT: 0.0714; TT: 0 in control). The results are correlated with our study in rs2954041 genotype distribution (GG: 0.9822; GT: 0.0178; TT: 0 in controls). There may be no evolutionary factors to change allele frequencies in both populations (Tables 5.1, 5.3).

Most of rs3924999, rs2054041 studies are about interactions with other genes and the mechanisms of schizophrenia. These studies showed that these two SNPs have roles in pathophysiology of schizophrenia. These studies also involve genotype distributions and allele frequencies, so these studies' results can be compared with others. Lin *et al.* investigated the association of schizotypal personality to *NRG1* variants (rs3924999, rs2954041, rs35753505). A allele of rs3924999 was found to be associated with schizotypal personality (Lin et al., 2005). Hong *et al.* conducted a study to find association between prepulse inhibition and two SNPs of *NRG1* (rs10503929 and rs3924999) in schizophrenia

patients. rs3924999 was found to be associated with prepulse inhibition (Hong et al., 2008). Hong studied in Chinese population, and the genotype distributions are correlated with Chinese Han and Korean populations (Tables 5.1, 5.2).

*NRG1* variants (rs73235619, rs62519682, rs6994992, rs3924999, rs2439272, rs10503929) were investigated for finding the association with prepulse inhibition. Prepulse inhibition was related to rs3924999 or rs10503929 if combined with rs2439272 (Roussos et al., 2011). Kang *et al*. researched the association between P300 and rs2954041, SNP8NRG221533 (rs35753505), rs392499. They found a significant association with AT haplotype. There was no differences between control and schizophrenia group in allele frequencies and genotype distributions (Kang et al., 2012). Kang's study population was Chinese Han, and the genotype distributions and allele frequencies of rs3924999 are correlated with Chinese, Chinese Han and Korean populations; in rs2954041, they are correlated with Chinese and Chinese Han populations (Tables 5.1, 5.2, 5.3, 5.4).

He *et al.* suggested that *NRG1* rs3924999 and *DISC* rs821616 interactions or synergistic effects have a role on the development of schizophrenia (He et al., 2016).

Okochi *et al.* investigated methamphetamine-*NRG1* SNPs (rs3924999, rs35753505, rs62519682, rs6994992) associations. Unfortunately, there was no significant association between *NRG1* and methamphetamine induced psychosis in Japanese population (Okochi et al., 2009). The genotype distributions and allele frequencies are correlated with Shiota's Japanese population (Tables 5.1, 5.2).

Qednow *et al.* analyzed *COMT* (rs4680), serotonin-2A receptor (rs6311/rs6313) and *NRG1* (rs3924999) polymorphisms to find association with acoustic startle response and prepulse inhibition in schizophrenia. No significant association was found with rs392499 (Quednow et al., 2009). Schmechting *et al.* researched the association of two endophenotypes of schizophrenia; smooth pursuit and antisaccade eye movement with rs3924999 in healthy individuals. There was no significant association with these two endophenotypes and SNP (Schmechtig et al., 2010). *DISC1* (rs821626, rs6675281), *NRG1* (rs3924999, rs10503929, rs3735782, rs3735781), *BDNF* (rs6265) and *NOTCH4* (rs367398) polymorphisms were researcher for the association with clinical symptoms. Only *NOTCH4* variant showed association with clinical symptoms of schizophrenia

(Terzi et al., 2015). Our genotype distributions are correlated with Slovenian distributions; this may be the result of the genetic relatedness (Table 5.1).

Diez *et al.* investigated rs392499 and rs6994992 (*NRG1*); rs10748842 (*NRG3*) to find association with P300. They found no association between *NRG* variants and cognitive processes in schizophrenia (Díez et al., 2014). Hatzimanolis *et al.* found rs3924999 is a tolerable possibly damaging variant (Hatzimanolis et al., 2013). Hatzimanolis *et al.* sequenced the region covers rs392499 in cases in European Caucasians. The allele frequencies are similar in North Indian and Turkish populations which shares the same ancestry (Table 5.2).

**Table 5.1.**Comparison of rs3924999 genotype distributions/counts in previous studies

| **Population** | **Sample size** | **Genotype Distributions/Counts (rs3924999)** | | | **P** | **Reference** |
|---|---|---|---|---|---|---|
| | | **GG** | **GA** | **AA** | | |
| Chinese Han | 492 controls | 33 | 243 | 216 | | Yang *et al.*, 2003 |
| | 246 cases | 26 | 126 | 94 | | |
| Chinese | 269 controls | 0.071 | 0.331 | 0.599 | 0.869 | Hong *et al.*,2004 |
| | 228 cases | 0.061 | 0.320 | 0.618 | | |
| Japanese | 514 controls | 0.602 | 0.344 | 0.054 | 0.23 | Shiota *et al.*, 2008 |
| | 416 cases | 0.611 | 0.358 | 0.031 | | |
| Japanese | 534 controls | 339 | 171 | 24 | 0.652 | Okochi *et al.*, 2009 |
| | 171 cases | 103 | 58 | 10 | | |
| Costa Rica | 479 controls | 208 | 209 | 62 | | Moon *et al.*, 2011 |
| | 272 cases | 114 | 132 | 27 | | |
| Chinese Han | 120 controls | 0.083 | 0.500 | 0.417 | 0.129 | Kang *et al.*, 2012 |
| | 287 cases | 0.132 | 0.544 | 0.324 | | |
| Korean | 198 controls | 0.061 | 0.387 | 0.552 | 0.022 | Yang *et al.*, 2012 |
| | 143 cases | 0.032 | 0.321 | 0.647 | | |
| North Indian | 1019controls | | | | 0.02 | Kukshal *et al.*,2013 |
| | 1007 cases | | | | | |
| Pakistani | 60 controls | 0.833 | 0.166 | 0 | 0.17 | Nawaz *et al.*, 2014 |
| | 100 cases | 0.75 | 0.23 | 0 | | |
| Slovenian | 94 controls | 0.500 | 0.500 | | 0.914 | Terzi *et al.*, 2015 |
| | 138 cases | 0.493 | 0.507 | | | |
| **Turkish** | **165 controls** | **0.39** | **0.48** | **0.13** | **0.4699** | **Culum, 2016** |
| **(this study)** | **170 cases** | **0.40** | **0.43** | **0.17** | | |

**Table 5.2.** Comparison of rs3924999 allele frequencies in previous studies

| Population | Sample size | Allele Frequency (rs3924999) | | P | Reference |
|---|---|---|---|---|---|
| | | G (wild) | A (variant) | | |
| Chinese Han | 492 controls | 0.31 | 0.69 | 0.007752 | Yang *et al.*,2003 |
| | 246 cases | 0.36 | 0.64 | | |
| Chinese | 269 controls | 0.236 | 0.764 | 0.597 | Hong *et al.*, 2004 |
| | 228 cases | 0.221 | 0.779 | | |
| Japanese | 520 controls | 0.773 | 0.227 | 0.40 | Shiota *et al.*,2008 |
| | 416 cases | 0.790 | 0.210 | | |
| Japanese | 534 controls | 0.795 | 0.205 | 0.363 | Okochi *et al.*, 2009 |
| | 171 cases | 0.772 | 0.228 | | |
| Korean | 198 controls | 0.255 | 0.745 | 0.014 | Yang *et al.*, 2012 |
| | 143 cases | 0.192 | 0.808 | | |
| Chinese Han | 120 controls | 0.333 | 0.667 | 0.058 | Kang *et al.*, 2012 |
| | 287 cases | 0.404 | 0.596 | | |
| North Indian | 1019 controls | 0.60 | 0.40 | 0.19 | Kukshal *et al.*,2013 |
| | 1007 cases | 0.57 | 0.43 | | |
| European Caucasian | | | | | Hatzimanolis *et al.*, 2013 |
| | 48 cases | 0.52 | 0.48 | | |
| **Turkish (this study)** | **165 controls** | **0.633** | **0.367** | **0.6119** | **Culum, 2016** |
| | **170 cases** | **0.615** | **0.385** | | |

**Table 5.3.** Comparison of rs2954041 genotype distributions/counts in previous studies

| Population | Sample size | Genotype Distributions/Counts (rs2954041) | | | P | Reference |
|---|---|---|---|---|---|---|
| | | GG | GT | TT | | |
| Chinese Han | 492 controls | 166 | 262 | 64 | | Yang *et al.*, 2003 |
| | 246 cases | 64 | 131 | 51 | | |
| Japanese | 491 controls | 0.487 | 0.395 | 0.118 | 0.11 | Shiota *et al.*, 2008 |
| | 396 cases | 0.416 | 0.450 | 0.134 | | |
| Chinese Han | 120 controls | 0.333 | 0.525 | 0.142 | 0.81 | Kang *et al.*, 2012 |
| | 287 cases | 0.310 | 0.526 | 0.164 | | |
| Pakistani | 70 controls | 0.9286 | 0.0714 | 0 | 0.15 | Nawaz *et al.*, 2014 |
| | 100 cases | 0.75 | 0.25 | 0 | | |
| **Turkish (this study)** | **169 controls** | **0.9822** | **0.0178** | **0** | **0.34** | **Culum, 2016** |
| | **159 cases** | **0.9937** | **0.063** | **0** | | |

**Table 5.4.**Comparison of rs29544041 allele frequencies in previous studies

| Population | Sample size | Allele Frequency (rs2954041) | | P | Reference |
|---|---|---|---|---|---|
| | | G (wild) | T (variant) | | |
| Chinese Han | 492 controls | 0.60 | 0.40 | 0.0009309 | Yang *et al.*, 2003 |
| | 246 cases | 0.53 | 0.47 | | |
| Japanese | 520 controls | 0.684 | 0.316 | 0.06 | Shiota *et al.*,2008 |
| | 416 cases | 0.641 | 0.359 | | |
| Chinese Han | 120 controls | 0.596 | 0.404 | 0.55 | Kang *et al.*, 2012 |
| | 287 cases | 0.573 | 0.427 | | |
| **Turkish (this study)** | **169 controls** | **0.9911** | **0.0089** | **0.346** | **Culum, 2016** |
| | **159 cases** | **0.9968** | **0.0032** | | |

This is the first study that screens rs3924999 and rs2954041 in Turkish population. As conclusion we were not able to find an evidence for presence of an association between schizophrenia and two SNPs (rs3924999 and rs2954041) located in the *NRG1* gene in the case-control groups we have collected from Malatya-Turkey. Results of the studies about *NRG1* are inconsistent and they must be replicated. Ethnic mixture and stratification of the population are the main problems of these studies. Also the genes that interplay with *NRG1* might contribute to the incoherence. This may be because of limited number of individuals included in the study. Screening of larger groups from a wider geographic region may produce data to approve the association found in the previously reported populations.

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Biologist, Ozel Malatya Hospital, Malatya (15.09.2004-28.06.2006)

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Biologist, Malatya Pathology and Cytology Laboratory, Malatya (02.12.2000-30.09.2001)

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**Publications:**

Yurekli, M., Culum, A., "The effects and angiogenic features of adrenomedullin in obese rats", *The FASEB Journal*, 2015, 29:1, Supp. 884.4.

Yurekli, M., Culum, A.A., "The investigation of relationship between adrenomedullin vascular growth endothelial factor in obese and calorie restricted rats", *Medical Science and Discovery*, 2016, 3:3, 124-9.