TR
INONU UNIVERSITY
INSTITUTE OF SCIENCE

INVESTIGATION OF GENETIC ASSOCIATION BETWEEN SCHIZOPHRENIA AND A TWO-SNP HAPLOTYPE ON NRG1 GENE

MASTER THESIS

Ayşenur SAYGILI

Department of Molecular Biology and Genetics

Thesis Advisor: Assoc. Prof. Mustafa Mert Sözen

JANUARY 2023

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WORD OF HONOR

I declare that this “Investigation of Genetic Association Between Schizophrenia and A Two-SNP Haplotype on NRG1 Gene” 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 isconsisted of that are displayed in reference appropriate to the method and I confirm this with my honor.

Ayşenur SAYGILI

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ABBREVIATIONS

COMT	: Catechol-O-Methyl Transferase
cSNP	: Coding Single Nucleotide Polymorphism
dNTP	: Deoxynucleotide Triphosphate
DSM-V	: Diagnostic and Statistical Manual of Mental Disorders-V
EGF	: Epidermal Growth Factor
GABA	: Gamma Aminobutyric
ICD-10	: International Classification of Diseases 10
iSNP	: Intronic Single Nucleotide Polymorphism
mGluR	: Metabotropic Glutamate Receptors
NCBI	: National Center for Biotechnology Information
NMDA	: N-Methyl-D-Aspartate
NMDAR	: NMDA Receptor
NRG1	: Neuregulin-1
nsSNP	: Non-Synonymous Single Nucleotide Polymorphism
PCR	: Polymerase Chain Reaction
rSNP	: Regulatory Single Nucleotide Polymorphism
SNP	: Single Nucleotide Polymorphism
sSNP	: Synonymous Single Nucleotide Polymorphism
SZ	: Schizophrenia

ABSTRACT

Master Thesis

INVESTIGATION OF GENETIC ASSOCIATION
BETWEEN SCHIZOPHRENIA AND A
TWO-SNP HAPLOTYPE ON NRG1 GENE

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Inonu University
Institute of Science
Department of Molecular Biology and Genetics

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Supervisor: Asst. Prof. Mustafa Mert SÖZEN

The earliest gene reported to be linked with schizophrenia is NRG1 which has a number of susceptibility alleles at many SNP (single nucleotide polymorphism) loci. In this study, the genetic association of schizophrenia with two SNPs located in the NRG1 gene -rs4560751 and rs3802160- has been investigated.

The study has been planned in case-control format. The case group was consisted of 96 schizophrenia patients and the control group was 100 healthy controls living in Malatya-Turkiye. Two SNPs mentioned above have been genotyped in these groups and the distributions of their alleles and genotypes were compared between two groups. The statistical significances of differences were tested with Pearson's Chi-Squared method.

The statistical tests showed there were no significant difference between the case and control groups for the distributions of the alleles or genotypes of two NRG1 SNPs.

As conclusion, our study revealed that the SNPs rs4560751 and rs3802160 in the NRG1 gene were not associated to schizophrenia in our sample.

Keywords: Schizophrenia; Genetics; Association; Neuregulin 1; Polymorphism; Turkey

ÖZET

Yüksek Lisans Tezi

NRG1 GENİNDEKİ İKİ SNP'Lİ BİR
HAPLOTİP İLE ŞİZOFRENİ HASTALIĞININ GENETİK
İLİNTİSİNİN İNCELENMESİ

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Şizofreni ile bağlantılı olduğu bildirilen, birçok SNP (tek nükleotid polimorfizmi) lokusunda bir dizi duyarlılık aleline sahip olan en eski gen NRG1'dir. Bu çalışmada şizofreninin NRG1 geninde yer alan iki SNP -rs4560751 ve rs3802160- ile genetik ilişkisi araştırılmıştır.

Çalışma vaka kontrol formatında planlanmıştır. Olgu grubunu Malatya-Türkiye'de yaşayan 96 şizofreni hastası, kontrol grubunu ise 100 sağlıklı kontrol oluşturdu. Yukarıda belirtilen iki SNP, bu gruplarda genotiplendi. Alellerinin ve genotiplerinin dağılımları iki grup arasında karşılaştırıldı. Farklılıkların istatistiksel önemi Pearson'ın Ki-Kare yöntemi ile test edildi.

İstatistiksel testler, iki NRG1 SNP'nin alellerinin veya genotiplerinin dağılımları için vaka ve kontrol grupları arasında anlamlı bir fark olmadığını gösterdi.

Sonuç olarak, çalışmamız, NRG1 genindeki SNP'ler rs4560751 ve rs3802160'ın örneklemimizde şizofreni ile ilişkili olmadığını ortaya koydu.

Anahtar Kelimeler: Şizofreni; Genetik; Bağlantı; Neuregulin 1; Polimorfizm; Türkiye

1. INTRODUCTION

1.1. Description and History

Schizophrenia (SZ) is a multifactorial psychiatric chronic disease (MIM181500) and in the human general population, it has a prevalence between 0.5% to 1% [1]. Positive symptoms, negative symptoms, and cognitive deficits are clinical features of the disease. Positive symptoms are delusions and hallucinations. and negative symptoms are withdrawal from social life, flat mood affect, and anhedonia. Synaptic connection problem is one of the most common pathologies of schizophrenia. This synaptic connectivity problem has been demonstrated in functional and structural neuroimaging studies, as well as causing numerous human deaths [2]. SZ is considered a complex and multifactorial disease. Multiple susceptibility genes, epigenetic, stochastic factors and environmental factors, contribute to the development of schizophrenia [3]. Schizophrenia is a heterogeneous complex disorder. The cognitive and behavioral symptoms are caused by the impaired brain development due to the genetic, environmental factors, or both [4].

Three basic principles are considered in defining this disease; a) For Kraepelin's symptoms of schizophrenia; emphasis on avolition, chronicity and poor outcome; b) the inclusion of the Bleuler view pointing to negative symptoms as the primary basis of pathology; and c) Schneiderian's emphasis on distortions in reality assessment or positive symptoms [5].

Kraepelin (1919) grouped manic-depressive insanity (known as bipolar disorder in present-day) and early dementia into a single group, before defining them as two separate conditions with the present-day equivalent of schizophrenia. The symptoms mentioned in schizophrenia are the symptoms that include abnormalities in cognition and emotion such as inability to speak (alogia), avolition, lack of pleasure (anhedonia), suppression of emotion (affective) and attention deficit, which we call negative symptoms today [6].

Bleuler (1950) tried to define schizophrenia and divided the symptoms of schizophrenia into two broad categories as basic and accessory symptoms.He defined the continuity of associations, loss of emotional response, attention and willpower, the simultaneous occurrence of opposing feelings/ideas and wishes regarding a situation (ambivalence), and autism as the main symptoms. He emphasized cleavage in associations as the most basic symptom of the disorder, and named the disease 'schizophrenia', which means 'cleavage of the mind' for this reason. Accessory symptoms are delusions and hallucinations [7].

Kurt Schneider (1930), wanted to describe the basic symptoms like Bleuler. For this reason, he focused on various 'first-order' symptoms characterized by the lack of boundaries between the self and the extra-self and a loss of sense of personal autonomy such as the insertion of thought, broadcasting, delusions of being controlled, making vocal comments and talking among themselves [8].

In the 1950s, with the International Classification of Diseases (ICD-10) from the publications of the World Health Organization as well as DSM-4 and 5 (Diagnostic and Statistical Classification of Mental Disorders) guidelines by the APA (American Psychological Association), schizophrenia was evaluated as a syndrome and defined as a schizophrenic spectrum.

1.1.1. Epidemiology and Risk Factors

SZ is a serious chronic psychological condition that causes 80–90% loss of function and reduces life expectancy by 10–20 years, which has a heavy impact on both the individuals living with this disease and towards the society [4]. SZ affects approximately 0.5% to 1% of world population [9]. Schizophrenia is affected by many factors including susceptibility of genes, epigenetic, stochastic, and environmental factors [3]. There are many genes associated with schizophrenia [10]. Epidemiological data show that the lifetime prevalence is between 0.30% and 0.66%.

The incidence of disease is between 10.2 to 22.0 in 100,000 people per year when isolation is taken in the account in the category of schizophrenia diagnosis. The rate of diagnosis of schizophrenia increases from 2.3% to 3.5% when categories like brief psychotic disorder, delusional disorder, unspecified psychotic disorder are included, including other psychotic disorders such as substance-induced psychotic disorder and bipolar disorder [11].

While it was thought that there was no difference between the genders in the incidence of schizophrenia for many years, studies have reported that the ratio in males is higher than in females, and the male/female ratio varies between 0.9 and 2.4 (1.4 as average) [12]. The age of onset of the disease is later in women than in men [13]. According to Aleman et al., the difference in incidence between men and women has been more apparent in the last 30-40 years [14]. While the age of onset is between 15 to 25 years of age in males, and 25 to 35 years in females [15]. The onset of the disease in individuals before the age of 10 and after the age of 60 is very rare [16].

The reasons for the prevalence of schizophrenia, which have been researched for many years, are that it is higher in individuals with low socioeconomic status, lower in married individuals than singles, and higher in people with some childhood difficulties (neglect, bullying, parental death, physical or sexual abuse and psychological / emotional abuse) when evaluating schizophrenia [17]. In addition, among the triggering factors, viral infections, migration, the season of birth (born in late winter or in spring), stressful life events have also been observed to affect the disease. In viral infection case, having the flu after the 6^{th} month of pregnancy can be considered as another risk factor [18]. The association of the increased risk of psychotic diseases to the exposure of the other infectious agents, such as toxoplasma and type-2 herpes simplex virus was also shown [19].

Substance abuse, suicide attempt and harmful behaviors are among the leading causes of death in schizophrenia patients. An estimated of two- to three-fold increased risk has been reported between cannabis use and schizophrenia. Metabolic syndrome caused by antipsychotics used in the treatment of schizophrenia is another risk factor shortening the life expectancy [20].

Genetic epidemiological results from families, twins, and adoption studies have indicated the presence of polygenic inheritance in schizophrenia, along with schizophrenia being a multifactorial disease that develops due to genetic and environmental factors [21,22]. It is known that the biggest risk factor for people diagnosed with schizophrenia is a positive family history. Susceptibility to the disease is seen at a higher rate in families with a schizophrenic individual than in

the normal population. According to family studies, the schizophrenia risk in relatives is 10% in first-degree relatives and 5% in second-degree relatives [23].

In cases where only one parent has schizophrenia, the risk of developing schizophrenia in children is 12.5%-13.8%, while studies have reported that this risk increases to 35-46% when both parents have schizophrenia [24]. In a study that will provide evidence for the effect of heredity, the rate of comorbidity in 12 pairs of monozygotic twins raised separately was found to be 58% [25]. Twin studies have estimated that approximately 80% inherited susceptibility to schizophrenia [26]. Despite the genetic relationship in schizophrenia, identifying the relevant molecular genetic variation has not been easy.

The high heritability of schizophrenia being 80% is due not only to genetic influences, but also to environmental factors in which genes interact. The concept of interactions between the gene and the environment means that the influence of the environmental factor causes at least more than one genetic variant. All these risk factors are neither necessary nor sufficient on their own. Different factors effecting the progression and onset of the disease at various levels. Although two different considerations are thought of as gene-environment or environment-environment interaction, there has been more recent interest in the possibility of gene × environment interaction [27, 28].

1.1.2. Diagnostic Criteria

Currently, since there is no objective test for SZ, “The International Statistical Classification of Diseases and Related Health Problems 10th Edition (ICD-10)”, DSM-5 and DSM-4 diagnostic systems are frequently used to diagnose schizophrenia. Following the publication of the “Diagnostic and Statistical Manual of Mental Disorders (DSM-I)” by the American Psychiatric Association in 1958, updated editions were published in 1968 (DSM-II), 1980 (DSM-III), and 1994 (DSM-IV), respectively. Finally, the revised DSM-IV was published in 2007. DSM-V was published in 2013 with new information [29,30].

1.1.2.1. Diagnostic criteria for schizophrenia in ICD-10

1. Echoing, insertion, withdrawal, transmission of thoughts.

2. Delusions of control, being affected and passiveness (affecting body movements, private thoughts, actions or senses), delusional perceptions.

3. By interpreting the patient's behaviors, hearing hallucinations and hallucinatory sounds coming from certain parts of the body are felt in the patient.

4. Completely improbable, uncultured, persistent delusions. For example, having political or religious identity or super-faith powers and abilities (controlling the weather, communicating with strangers from another world, etc.).

5. Persistent hallucinations that may be different but long-lasting. These hallucinations must be inconsistent with the affect, transient, or present every day for weeks or months without interruption.

6. Disruption in the current of thought and other thought insertions; resulting confusion (incoherence), inappropriate speech, or neologism.

7. Catatonic behavior, excitation, holding the body in a certain position, negativism mutism (selective mutism), stupor (stunned state) and waxy flexibility.

8. Negative Symptoms include evident apathy, slurred speech, blunted emotional responses, and inappropriateness. These symptoms often lead to withdrawal from social life and decreased social performance. These symptoms are not related to depression and antipsychotic drug treatment.

9. Change in all behavior. Evident and persistent quality changes associated with some aspects of personality; these can manifest as loss of interest, purposelessness, laziness, self [31].

1.1.2.2 Diagnostic criteria in DSM-5 for schizophrenia

A. Characteristic symptoms: At least two criteria from the following list during one-month period (or less if successfully treated) for a significant portion of that time.

1. Delusions

2. Hallucinations

3. Disorganized speech

4. Highly catatonic or disorganized behavior

5. Negative symptoms, as affective dullness, alogia (inability to speak) or avolition (loss of will).

If the bizarre (out of ordinary) delusions are present or if the hallucinations consist of voices constantly commenting on the person's behavior or thoughts (more than two voices speaking to one another), only one symptom from Criterion A is sufficient.

B. Social or occupational dysfunction: Significant impairment in work life, interpersonal relationships, and self-care.

C. Duration: The symptoms must be persistent minimum for 6 months.

D. The schizoaffective disorder and mood disorder with psychotic features are excluded. In this case, manic, major depressive, or mixed episodes are not seen at the same time as the symptoms of active phase.

E. Exclusion of substance use/general medical condition

F. Association with a pervasive developmental disorder [32].

1.1.3. Etiology

SZ is a very different and complex disease, and it is not caused by a single cause that contributes to the treatment response, the course of the disease, and the development of this disease, but the interaction of many factors. Investigations into the etiology of schizophrenia continues. However, the combination of genetic and physiological processes, as well as the environmental risk factors are thought to act in the development of schizophrenia [33].

1.1.4. Genetics of Schizophrenia

Schizophrenia is a multifactorial brain disorder. A positive family history is the biggest risk factor for schizophrenia. In general human population, the risk through the lifetime is between 0.5% and 1%. Whereas this risk is 6.5% in first-degree relatives of the people affected by schizophrenia. Moreover, in monozygotic twins of schizophrenia patients, it is greater than 40%. The heritability of susceptibility to schizophrenia has been calculated to be approximately 80% in twin studies [26].

Large family, adoption, and twin studies indicate that this risk reflects genetic closeness between the relative and the index case. However, examining the genes that cause schizophrenia is a challenging process due to heterogeneous subtypes of the disease and not following Mendelian inheritance. After developing the idea that genetics is an important risk factor in catching the disease and showing a multifactorial transition gained importance, new findings were obtained in the field of molecular genetics with segregation, linkage and association studies, and many chromosome regions and candidate genes were revealed. [34,35]. The factors are mentioned below in order.

Segregation studies: Determines the accuracy of the disease frequency in the pedigree of a family by comparing it with the genetic transmission method. Due to the multifactorial nature of the disease, the diagnostic modality has not been fully determined. This reduces the reliability of this method [36].

Linkage studies: Linkage studies directly examine the genetic markers and disease phenotype and the transmission of alleles across generations in the pedigree of a family to be investigated. Marker alleles look for functions that indicate they are associated with a locus. In parametric linkage analysis, the extended pedigrees are used. A disease-marker model is specified, and its transmission is evaluated. In nonparametric linkage analysis, allelic similarity is sought in relatives who are affected. This eliminates the need to create a disease model. With linkage studies, defect in protein structure or the presence of a different nucleic acid sequence can be detected [37].

In a publication by Lewis et al., on 1208 loci with schizophrenic family, a connection was found between chromosomal regions 1p, 2q, 3p, 5q, 6p, 8p, 11q, 13q, 14p, 20p and 22p and schizophrenia. In the meta-analysis study by Badner and Gerson, it was determined that 8p, 13q, 22q chromosome regions are associated with schizophrenia.

Segurado et al.'s study with 347 families revealed the importance of chromosome regions 9p, 10q, 14q and 18q in schizophrenia. With a linkage study with 236 Japanese families showed that the 1p, 14q, 20p chromosome regions are associated with schizophrenia, the study by DeLisi et al. also showed the importance of the 2q and 8p chromosome regions [38]. With the innovations in the field of molecular genetics, studies supporting each other have increased and

many schizophrenia susceptibility candidate genes such as NRG1 (Neuregulin 1), NRG3 (Neuregulin 3), ErbB4, COMT (Catechol-O-methyl transferase) have been revealed [9, 39-40].

Association studies: The new techniques of genotyping increased the power of genetic association studies [41]. For population-based association study; a potential problem is sufficient such as the presence of undetected problems that can lead to false positives or overlooked real effects.

If the prevalence of disease also differs significantly between subpopulations, it will tend to be different, as will the proportions of patients and healthy controls have sampled from each subpopulation, and the frequencies of alleles or genotypes between cases and controls at any locus [42].

SNP markers used in association studies are the alternatives of one of four types of nucleotide alleles. Due to their low mutation rates, most of them are in a biallelic structure. In general, association studies can only yield results for variables of common cause that are strong enough. Common causes depend on the effect sizes and marker frequencies of the sample at hand. However, the frequency of minor allele may need to be larger than 5% [43]. The various types of association studies on the population are as follows:

i. Candidate Polymorphism

The focus of these studies are on te individual polymorphisms suspected to cause disease [44].

ii. Candidate Gene

These studies may involve differential coding (including flanking regions of the coding sequence, insertion and regulatory regions) of 5-50 SNPs within a gene [44].

iii. Fine Mapping

These are studies that may contain approximately several hundred SNPs, usually conducted in a 1-10 Mb candidate region. The candidate region may be identified by linkage studies and may cover 5-50 genes [44].

iv. Genome Wide Association Study (GWAS)

The majority of common genetic variations can be represented by genotyping a selected tagging SNP set. This kind of genome-wide association studies makes it possible to discover the new genes that affect complex disorders including SZ. It is much more powerful than linkage studies to detect small effects in the gene [44]. To date, five genome-wide association studies (GWAS) have been conducted for schizophrenia. The first of these is the study of people recruited in the USA who identify as Caucasian. Of this case group, which consisted of 322 people in total, 178 were found to be in the patient group [45].

The second study which consisted of 600 cases and 2,771 controls from pooled DNA samples who were Ashkenazi Jewish were used. Despite the strong effect of RELN SNP seen only in females, no significant genome-wide association was found [46].

In the third study, no significant genome-wide association was found after using 574 triads of schizophrenia patients and 605 controls collected from Bulgaria [47]. Research done with 738 patients and 733 control samples did not find any evidence for the effect of common SNPs in schizophrenia [48]. In another study, 479 patients and 2,937 controls were compared for the WTCCC SNP. This study was replicated in 7,897 controls, 1,664 patients and 3,541 controls and 6,666 cases and two other datasets [49].

By the advances in methods for high-throughput genotyping and the help of International HapMap Project, many markers have been identified. However, it is not possible to find all variants with cost leading to limitations on computational and statistical efficiency [50]. In order to avoid the cost of multiple tests, haploid SNP tests and joint tests of SNP groups are used. The genome-wide association studies (GWAS) using SNP marker loci are the associative mapping tool of choice. For all individuals in the history table of family members, SNP genotypes can be determined and analyzed using genome-wide association analysis [51].

By introducing a new approach to disease gene mapping by analyzing SNP haplotypes from population-based, large-scale association studies. Haplotype groups are tested for disease association [52].

1.2. SNP (Single Nucleotide Polymorphism)

There are genetic and environmental components that affect the common diseases. The probability of identifying risk alleles for these factors are low.
The presence of these low penetrating alleles in the population has a high incidence rate (>1%) [53]. Through the genome, the most common form of variations are SNPs. The SNPs are used widely for studying the genetic differences that can be seen between the populations or individuals [54].

This kind of variations sometimes affect protein structure. Many proteins have several common, inherited isoforms similar to blood group antigens [55]. These genetic variants, including SNPs, also affect susceptibility to the common disorders. SNPs are changes on a base at a specific location in the genome, usually accompanied by two alleles [56].

A SNP is a variation of a single genetic code. SNPs are usually biallelic, although multiallelic SNPs exist. SNPs can contribute to changes in the exon and intron region of the genomic sequence [57]. Typically, these changes are found in most genes approximately every 300-1000 base pairs (bp). This means that there is an average of 3,000,000 differences in the two human genes [58]. Today, the total number of variable positions among the human population is thought to be much larger, at more than 10,000,000 [59]. Moreover, SNPs can have a major impact on the immune response against pathogens and the consequences of diseases [60]. The SNP may have a protective role, affecting the disease progression rate and even the pathogen induced cellular immune response [61].

1.2.1. Application

SNPs are usually accepted as useful biomarkers which can be used in the diagnosis or prognosis of diseases since they are common, easy to analyze, can be genotyped with low cost [62]. Therefore, SNPs have recently gained importance in disease association studies. In human studies during the past few decades, progress have been made in identification hundreds of a number of SNPs associated with complex clinical conditions of many common diseases [63–64].

1.2.1.2. Types

Depending on where a SNP occurs, it can have different consequences at the phenotypic level:

A. Silent SNPs (non-coding region SNPs) (intronic SNPs, iSNPs; regulatory SNPs, rSNPs):

SNPs are found heterogeneously among the genomes. There are many more SNPs in non-coding regions than in coding regions. However, SNPs have no direct accepted effect on an individual's phenotype. They are being used as indicators in evolutionary studies or population genetics.

B. SNPs in the coding region (coding SNPs, cSNPs):

SNPs in coding sequences do not significantly affect protein amino acid sequence due to genetic code degeneration. However, SNPs with altered protein function and structure are an important and sufficient cause of many distinct Mendelian monogenic diseases. These SNPs are analyzed for diagnostic reasons. SNPs that change protein structure are important for drug metabolism. Therefore, these types of SNPs have been investigated for pharmacogenetics. There are two subtypes of this type of SNP:

1. Synonymous SNPs (sSNP): These types of SNPs do not affect protein sequence but can alter protein activity.

2. Non-synonymous SNPs (nsSNPS): These SNPs can change the protein amino acid sequence and have two subtypes according to the mutation type:

a) Missense mutation SNPs: It is the result of a base change in the sequence by causing a protein change or disorder as a result of mutation. The missense mutation causing the SNP can be identified with the aim of calculating the risk of a particular disease.

b) Nonsense mutation causing SNPs: Such SNPs often result in inactive protein yield due to either a missing protein as a result of an immature stop codon or a point mutation and a nonsense codon [65-67].

1.3. Neurochemical Factors

Important neurotransmitters involved in the pathogenesis and treatment of schizophrenia are dopamine and serotonin. Recent research has revealed that glutamine and g-amino-butyric acid (GABA) act on this disease.

1.3.1. Dopamine Hypothesis

Increased concentration of dopamine secretion in individuals with schizophrenia is excessive. The Dopamine hypothesis prevailed 10 years ago. With the research done, it has been proven that administering amphetamine and a group of other substances that increase extracellular concentrations of dopamine can cause psychotic symptoms.

The similar symptoms also seen with schizophrenia. The drugs reducing the dopamine levels such as alpha methyl-para-tyrosine and reserpine also reduced the psychotic symptoms [68,69]. The weakness of the initial dopamine patterns was their inability to explain cognitive deficiencies and negative symptoms in schizophrenia. Only the positive symptoms are tend to be induced by dopamimetics, while antipsychotics' limited effect against negative symptoms. Because of this the negative symptoms are thought to be related to the reduction of dopamine [70]. The Dopaminergic system regulates the additional neurotransmitter receptor (D2) systems in the brain [71].

1.3.2. Serotonin Hypothesis

In SZ, serotonin hypothesis is based on the research on the interactions of hallucinogenic drug lysergic acid diethylamide (LSD) and 5-HT (serotonin is also known as 5-hydroxyriptamin) [72].

Serotonin is a biogenic monoamine, which intermediates the effects of the 5-HT receptor. The serotonin has very high functioning functions in the central nervous system. The serotonin is linked to the effect of the system on the front computer, the brain stem, and the cerebellum [73].

The serotonin warehouses are corrected by negative symptoms in phenphuramine schizophrenia, a venting agent. The results of research suggest that

selective 5-HT2 antagonists can be useful in negative symptoms of schizophrenia. This idea is also supported by the indication that serotonin antagonists, such as ritanserin, are particularly useful in negative symptom schizophrenia [74]. The serotonin hypothesis should be considered complementary to dopamine hypothesis [75].

1.3.3. Glutamate Hypothesis

The main stimulant in the brain is the glutamate, which contains glutamatergic neurons, which use approximately 60 to 80% of all brain metabolic activity. G protein-linked metabotropic receptors (mGluR1-8) and ionotropic receptors AMPA (alpha-amino-3-hydroxia-5-methyl-isocsazol-propionic acid) and NMDA (N-methyl-D-aspartate) [76].

Glutamate is involved in the formation of an axon in neuronal migration and differentiation for brain development. It also takes important roles in the nervous system, neuroplasticity, synaptic activity and synaptic changes. It is known that N-methyl-d-aspartate (NMDA) antagonists from glutamate receptors who have been researched and thought for years reduce positive, negative and cognitive symptoms, such as schizophrenia, such as phenylene (PCP) and ketamine. 6,5% of schizophrenia patients with emotional and neural fluctuations have been reported to be positive for NMDAR-antibody by meeting diagnostic criteria. Cortical NMDAR exposure studies using brain tissue showed changes in transcript and protein expression depending on the brain region being examined and the receptor sub-unit [77]. Consistent evidence has been reported that glutamatergic neurons and dendrites in the cerebral cortex of schizophrenia patients have morphological changes and decreased marker levels of the axon terminals [78].

1.3.4. GABA Hypothesis

Gamma aminobutyric acid (GABA) is investigated as the primary inhibitor neurotransmitter in the brain in schizophrenia pathophysiology. The GABA receptors in three different types have been defined as Ionotropic GABAA and GABAC receptors and the metabotropic GABAB receptor. The post-mortem studies in individuals with schizophrenia revealed the reduction of GABA synthesizer enzyme, GAD67 mRNA and protein. Therefore, it suggests that the

GABA activity of schizophrenia, which may be found in pathophysiology, has decreased.

Deficiencies in GAD-67 and decreased exposure of parvalboom, synaptic changes in the dorsolateral prefrontal cortex can disrupt the inhibitor balance by GABA intermediate neurons, which can cause shortcomings in memory [79].

1.3.5. Dopamine, Glutamate and GABA Hypothesis

The presinaptic dopamine disfunction is linked to psychotic symptoms and the relationship of dopamine with negative and cognitive symptoms are still not understood enough. In this context, glutamate models containing NMDA receptor blockage can better explain the causes of schizophrenia.The combination of NMDA and dopamine functions can best describe all clinical aspects of schizophrenia synaptically [80]. Glutamate can affect all other neurotransmitter systems, causing an inhibit effect through other mechanisms of glutamate. Dopamine disfunction seen in schizophrenia can be interpreted as being secondary to a modified glutamatergic function. Many transmitters, including GABA, regulate the dopaminergic mesoortical system; their disorders can increasingly intensify this disease by causing dopamine disorder in schizophrenia [81].

NMDA hypophonia causes schizophrenia if it is present in some GABA intermediate neurons in the cerebral cortex. Due to the incorrect genetic programming of the positive GABA intermediary neurons of parvalbumin, the dysfunction results in the inability to inhibit glutamatergic neurons in the prefrontal cortex, which results in the excessive activation of glutamate neurons. Glutamate is responsible for organizing the signal path of mesolimbic and meso cortical dopamine via GABA. Glutamate over release causes dopamine increase in the mesocortical pathway and dopamine reduction in the mesolimbic pathway, affecting positive and negative symptoms [82].

1.4. Neuregulin (NRG) 1

As a family of growth factors, four neuregulins (NRG1-4) make effect by binding to the receptor tyrosine kinases from epidermal growth factor-like receptor (ErbB) family. The Nrg-1 gene spans 2.4 mbp (megabase pairs) in mice and 2.6 mbp in humans and rats [83].

The NRG1 gene is located on the human chromosome 8; NRG1 gene is necessary for the development of the nervous system and heart [84]. It turned out to be important in brain development during the embryo period and after birth. NRG1 is a large gene (1.4 MB) and encodes about 15 different peptides. NRG1 is also divided into six classes within itself; The six classes are derived from different amino terminal sequences. There are the NRG1 gene, six types of Neuragulin-1 proteins known in humans (type I-VI), and about 31 different isoforms [85]. These isoforms include NDF (Neu differentiation factor Heregulin), GFF (glial growth factor) and ARIA (acetylcholine receptor inducing activity). The isoforms of neuregulin-1 have been classified as type I-VI, depending on whether they express the α or β epidermal growth factor (EGF) of isoforms and contain a transmembrane region, depending on the N-terminal sequence [86-89].

Type I and II Neuregulin-1 isoforms contain a transmembrane region called a C-terminal transmembrane region (TMc), while NRG type III contains a cysteine-rich (CRD) N-terminal transmembrane region (TMn) in addition to TMC. Type I-II-IV-V neuregulin-1 also contains an immunoglobulin (Ig) like domain and is called Ig-NRG1.

Ig domain is associated with the epidermal growth factor (EGF) -like region [84]. NRG1 type III contains no Ig-like space [90]. The neuregulin-1 protein is functionally activated by one of three types of transmembrane field proteas (i.e., pro-NRG1, BACE1 and ADAM17), known as ADAM19, formed by proteolytic digestion of the membrane-type precursor. A bioactive piece of extracellular NRG1 is released, except for NRG1 type III [91, 92].

The interaction of NRG1 gene with schizophrenia was first shown in in the Icelandic population after LD mapping from 8p21-22. A haplotype associated to the disease was found at the gene's 5'end. Later, the same haplotype was shown in a study associated with an abundant sample from Scotland but samples from the UK did not yield the same result [93]. These factors were the alternative splicing products of a single gene (NRG1) [94].

NRG1 gene has been revealed that, with its properties in neurodevelopment, also plays a role in the pathogenesis of psychiatric disorders. NRG1 gene plays a role in tissue formation differentiation and systems of organs.

All NRG1 types bind to the receptor-tyrosine kinases of ErbB family (ErbB3 and ErbB4). However, the main receptor for neuregulin-1 is the ErbB4 receptor. The default imbalance of stimulant/inhibitory delivery in the cortex, striatum and hippocampus is a possible neurobiological supporter for schizophrenia. In the preclinical period, NRG1 can disrupt normal stimulant/inhibitory neurotransmission through the ErbB4 receptor [95]. The Nrg-1 / ErbB network regulates the myelin lifespan, quality, and remyelination [96]. The mutations in the NRG1 gene were found to be associated with susceptibility to schizophrenia and bipolar disorders [97].

The NRG2 gene has two isoforms of NRG2α and NRG2β, which differ from EGF-like regions. NRG2 is produced in the developing nervous system. During the embryonic period, acts in the heart, lung, and bladder development. Expression of NRG2 in adult brain is highest in the cerebellum, hippocampal dentate gyrus (granule cells) and olfactory bulbs, whereas it was weaker in the striatum, hippocampal CA1 - CA3 neurons and neocortex [98]. NRG1 Type III interacts with ErbB receptors located on neurons or glia. Although there are N- and C-terminal regions in the cytoplasm; most isoforms of neuregulin1 are synthesized as a precursor transmembrane polypeptide (pro-NRG1s) including an EGF domain. Type III neuregulin1 is thought to act in the cell surface mechanism. The mechanism of Type IV, Type V, and Type VI pro-NRG1s is not fully understood, but the process is assumed to be as in Type I and Type II [99].

Several SNPs in the NRG1 gene were screened for testing their associations to schizophrenia. In a study, in which 49 markers were screened in a case-control group, interactions between schizophrenia and the SNPs rs4560751 and rs3802160 in the NRG1 gene have been reported [100]. In this study, we tested the associations of those two SNPs in a case-control group consisting of schizophrenia patients and controls from Malatya-Turkey. The first SNP we have screened was rs4560751 which is a G to T change in the genomic position “chr8: 31672512”. The second one, rs3802160 is an A to G alteration located in the genomic position “chr8:32547111” (GRC h38.p13).

Our scientific literature database search showed that, as a case-control study, the present work is the first one to be done to investigate the potential associations of NRG1 SNPs “rs4560751 and rs3802160” with schizophrenia in the cases and control samples collected from a Turkish Population.

2. MATERIAL AND METHODS

2.1. Collection of Samples

All procedures and applications in this study have been ethically approved according to Declaration of Helsinki by the local ethical committee (Protocol No: 2021/08). All the subjects volunteered in the groups of cases and normal controls were provided written informed consents forms. Every subject in both groups have been clinical evaluated and diagnosed by a senior psychiatrist in the Psychiatry Department at Medical School of Inonu University.

In the patient group there are 96 people from Malatya (Turkey), who have nothing to do with schizophrenia (age average: 36.9 years, min: 18, max: 64. SD=11.6). All diagnoses have been done according to The Structured Clinical Interview for the DSM-V (SCID-5) [101]. The average age at which patients are diagnosed is 24.4 (SD: 8.6). All patients declared to have Turkish ethnicity.

In this study, we kept patients with schizophrenia form disorder, schizoid disorder, mood disorder showing psychotic traits, substance-induced psychotic disorder, paranoid personality disorder schizoaffective disorder, schizotypal disorder and psychotic disorder separate from this research. The group of controls included 100 healthy subjects of Turkish ethnicity, according to their self-identification. None of the samples in the control group are linked.

2.2. Isolation of DNA

Peripheral blood samples of the subjects collected in the EDTA coated tubes were used for DNA extraction. To protect the confidentiality of the volunteers, a coding system was applied to all blood and DNA samples. “Invitrogen Purelink DNA mini kit (California, USA)” has been used for extraction of DNA.

DNA extraction method:

1. In a sterile microfuge tube, 200µl of blood sample was mixed with 20 µl of Proteinase K.

2. 20 µl RNase A added to the sample. Mixed by brief vortexing and incubated at

room temperature for 2 minutes.

3. 200 µl PureLink™ Genomic Lysis/Binding Buffer was added and mixed by vortexing to homogenize the solution.

4. The mix was incubated at 55°C for 10 minutes to promote digestion of proteins.

5. 200 µl 96-100% ethanol was added to the lysate and mixed.

6. Proceed immediately to Purification Protocol, next page.

7. A PureLink™ Spin Column was placed in a Collection Tube.

8. The lysate (~640 µl) was added into the prepared Genomic Lysis/Binding Buffer and ethanol and into the spin column.

9. The columns were centrifuged at 10,000 × g for 1 minute at room temperature.

10. The collection tube was discarded, and the spin column placed into a clean PureLink ™ Collection Tube supplied with the kit.

11. 500 µl Wash Buffer 1 prepared with ethanol (page 2) was added to the column.

12. The column was centrifuged at 10,000 × g for 1 minute at room temperature.

13. The collection tube was discarded, and the spin column was place into a clean PureLink™ collection tube supplied with the kit.

14. 500 µl Wash Buffer 2 prepared with ethanol was added to the column.

15. The column was centrifuged at maximum speed for 3 minutes at room temperature and the collection tube was discarded.

16. The spin column was placed in a sterile 1.5-ml microcentrifuge tube.

17. 100 µl of PureLink™ Genomic Elution Buffer was added to the column.

18. The column was incubated at room temperature for 1 minute and centrifuged at maximum speed for 1 minute at room temperature to elute the DNA.

2.3. DNA Quality Control and Quantity Determination and Standardization

Isolated DNA samples were read by spectrophotometer at 260 and 280 nm to determine DNA concentrations and the amounts of protein that could be contaminated. It was wanted to reduce contamination by keeping the main stocks, with some separation from all DNA samples. The concentrations of the portions to be studied are set to be 20ng/uL. Standardized.

DNA samples were stored in DNA storage plates (plate) with 96 wells by placing them according to a fixed order, and the plate position of each specimen was recorded. In later stages, this order was maintained, and all analysis was

carried out in this order. DNA samples whose concentrations were standardized to be equal were previously PCR made using validated PCR primers and PCR kits, and all DNA samples were tested whether they were suitable for this process.

2.4. Identifying SNP of Genotyping with Real-Time PCR

The genotyping of the SNP rs3802160 and rs4560751 to be screened was done with real-time PCR. For genotyping, it has been used with SNP genotyping kits based on "end point fluorescence" sold commercially, validated by the manufacturer firm. For this, a real-time PCR reaction would be performed using allele-specific fluorescent-marked probes, and allelic discrimination was made using the manufacturer's proposed computer program based on the color of the resulting glow.

We determined the genotypes of each sample using the genotyping assays designed specifically by “Applied Biosystems” for the SNPs rs4560751(“TaqMan ® Catalog number: C__28957721_10”) and rs3802160 (“TaqMan ® Catalog number: C__27503359_10”) with the TaqMan ® PCR Master Mix (“Catalog number: 4304437”) on “Step One Plus real-time PCR system of Applied Biosystems (California, USA)”. The reactions have been done with the conditions suggested by the producer (the total volume: 10 µL, template genomic DNA: 2 µL in concentrations of: 20 ng/ µL), TaqMan ® Universal PCR Master Mix: 5 µL, TaqMan ® genotyping assay: 0.5 µL of and water: 2.5 µL). A hot start step (10 minutes at 95 ºC) was applied before 40 PCR cycles of: 15 seconds at 95ºC and 1 minute at 60ºC. SNPs were detected at the end of PCR with endpoint plate reads. We analyzed the SNP associations by comparing the genotypes of the patients and controls.

2.4.1. Statistical Analysis

The counts and frequencies of SNP genotypes and alleles of cases and controls were determined using genotype data from real-time PCR. The Hardy–Weinberg equilibrium was tested in both groups with Pearson’s Chi-Square method. In the case of a difference between two groups for the counts of alleles or genotypes, again the Chi-Squared test was used to determine the statistical significances of that differences. H0 for this SNP at this stage: "There is no

difference between patient and control groups in terms of distribution of both alleles for the SNP studied." The hypothesis was tested against the alternative hypothesis claiming the opposite, with 0.05 taken as the limit p value. The same comparison was then made for genotype frequencies. H0 for this SNP at this stage: "There is no difference between patient and control group in terms of distribution of all three genotypes (homozygous allel-1, heterozygote and homozygous allel-2) for the SNP studied." The hypothesis will be tested against the alternative hypothesis claiming the opposite, with 0.05 taken as the limit p value. The G*Power software was used to determine the statistical power [102].

3. RESULTS

In this study, we have screened two SNPs (rs456051 and rs3802160) located in human NRG1 gene in a group patients (96 samples) diagnosed for schizophrenia and a group of 100 controls (100 samples) collected from Malatya-TURKEY to uncover a potential association between these two SNPs and development of Schizophrenia. We have used TaqMan Real-Time PCR method for genotyping and analyzed the genotyping results for single SNP association of each screened SNP by comparing two groups for the distributions of genotypes and alleles.

3.1. Genotyping Results for rs3802160 Polymorphism:

We evaluated real time PCR results in multi component plots and allelic discrimination plots and used the SNP data for statistical analysis.

3.1.1. Real-Time PCR Genotyping Results as Multi-component Plots for rs3802160

The SNP rs3802160 has G and A alleles. We were able to detect all three genotypes constituted by these two alleles (GG, AG, and AA) in our case and control groups. To prove reliability of genotyping assays, first we evaluated real-time PCR results as multi-component plots which show the amplification in each sample tube separately.

Examples for real time PCR primary results showing amplifications of DNA fragments from different alleles are given in Figure-3. In the Figure 3.1, an individual who is homozygote for G allele is seen with increase of only FAM signal by the PCR cycles. Figure 3.2 shows an A homozygote in which only VIC

signal is increased significantly. The 3.3 Figure shows increase of signals from both fluorescent labels and indicates presence of both alleles A and G because of heterozygote genotype of the individual. These results show both genotyping assays which include PCR primers and allele specific probes work efficiently and able to distinguish all possible genotypes.

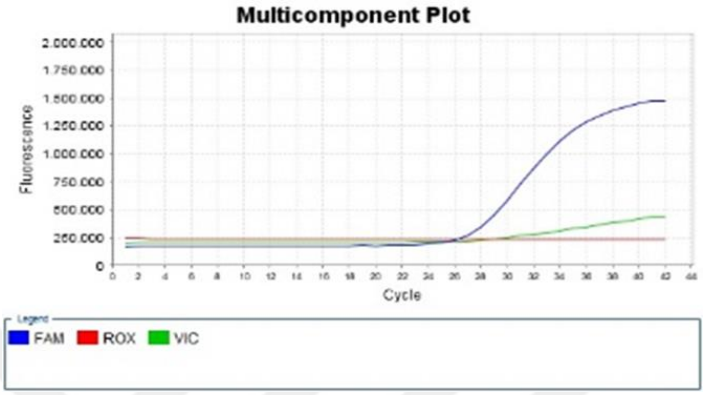


Figure 3.1: rs3802160, G/G Genotyping Results

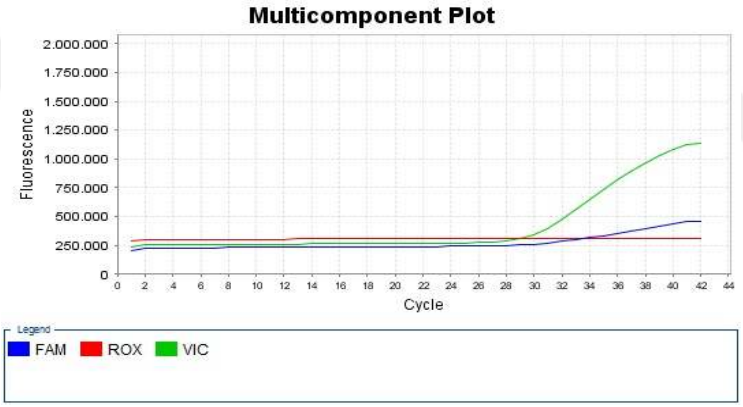


Figure 3.2: rs3802160, A/A Genotyping Results

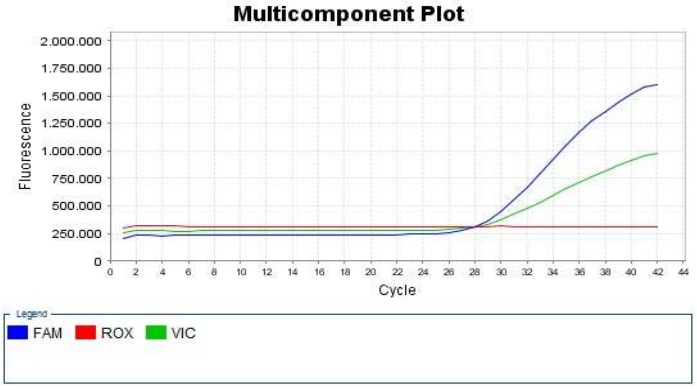


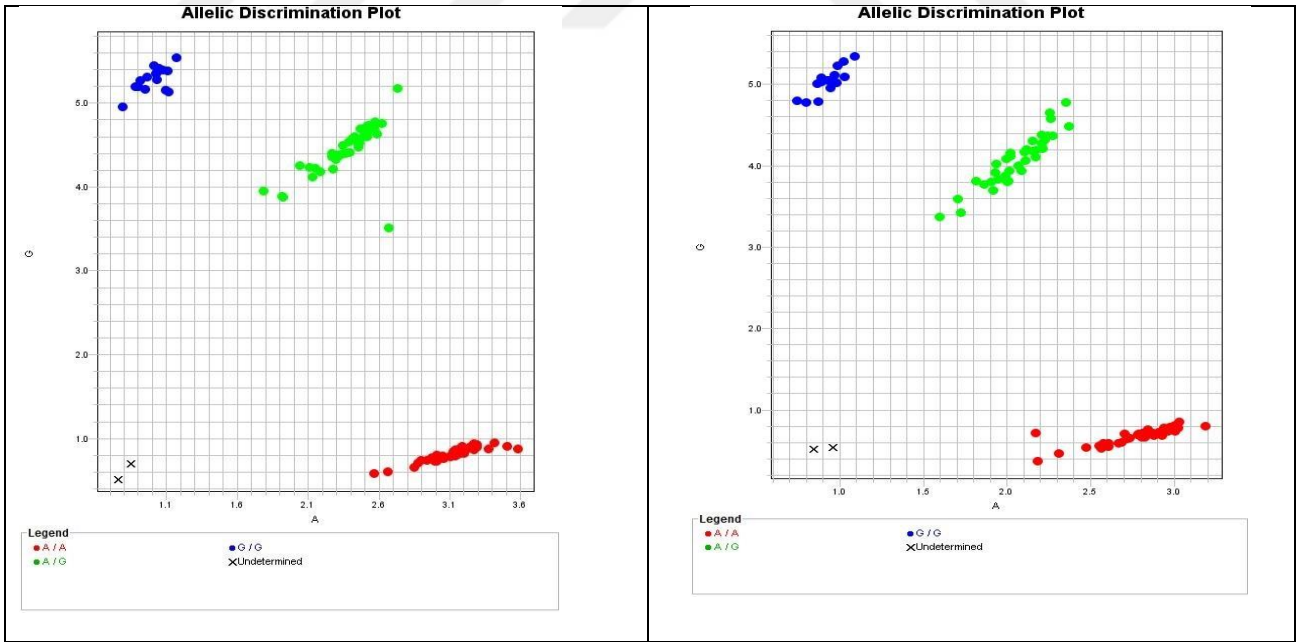
Figure 3.3: rs3802160, A/G Genotyping Results

Figure 3: Multicomponent plots showing amplification of rs3802160 alleles in samples with different genotypes.

3.1.2. Real-Time PCR Genotyping Results as Allelic Discrimination Plots for rs3802160

The real-time PCR results were also expressed as cartesian plots showing allelic discrimination in the whole plate carrying 94 samples and two negative (no-template) controls. The PCR plates were arranged as a patients plate and a controls plate. Excess of samples were collected in separate plates and genotyped with repeat samples. Allelic discrimination plots also visualize the separation between different genotypes in the whole plate and show the quality of template DNA, reliability and efficiency of PCR reactions and allele specific probes in a different way.

Figure-3.4 shows cartesian plots produced by real-time PCR software. In panel-the patient group, in panel-b control group can be seen. In these pictures, we can see all genotypes are separated from the others by the sufficient signal strengths successfully.



a. Case (Patients) Group

b. Control Group

Figure 3.4: Allelic Discrimination plots showing distributions of rs3802160 genotypes in the groups of patients and controls. (Two samples shown as "x" -Undetermined- are no-templete -negative- controls).

1.3. Analysis of Single-SNP Genotyping Results of rs3802160 Polymorphism:

The results of real-time PCR tests were also analyzed and exported as genotype list showing every single individual's genotype by the software. All further association analyses were done on these genotyping results. Numbers of individuals carrying each genotype in case and control groups were determined and these two groups were compared for the distributions of genotypes. We have also calculated counts and frequencies of both alleles and compared the groups of patients and controls for the allelic distributions. The genotype and allelic distributions were expressed as numbers and frequencies. For the SNP rs3802160, Distributions of genotypes are given in Table-3.1. In addition, Table-3.2 shows the distributions of the alleles of SNP rs3802160. The counts of genotypes and alleles were compared with Pearson's Chi-Squared test between case and control groups to evaluate if there is a significant difference.

The p-values are given at the bottom lines of the tables 3.1 and 3.2. The p-values calculated above 0.05 indicated that there was no significant difference between two groups (cases and controls) in terms of distributions of alleles and genotypes of rs3802160.

Table 3.1: Comparison of the patients and controls for distributions of genotypes of rs3802160.

rs3802160	A A	A G	G G	Total
Case Count *(Frequency)*	40 *(0,42)*	40 *(0,42)*	16 *(0,17)*	96
Control Count *Frequency*	43 *(0,43)*	41 *(0,41)*	16 *(0,16)*	100
p	0,98			

Table 3.2: Comparison of the patients and controls for distributions of alleles of rs3802160.

rs3802160	A	G	Total
Case Count *(Frequency)*	120 *(0,625)*	72 *(0,375)*	192 *(1)*
Control Count *Frequency*	127 *(0,635)*	73 *(0,365)*	200 *(1)*
p	0,84		

The expected numbers of three genotypes can be seen in case and control groups were calculated according to Hardy-Weinberg Low. The actual numbers from genotyping results were compared with the expected numbers by Chi-Squared tests. The expected and observed numbers of each genotype and Chi-square test results as p-values are given in the Table 3.3. The 3.3 panel shows the Hardy-Weinberg test results for the case group. The results of the same test applied to controls group were shown in the panel of Table 3.4. The genotype distributions in patient and control groups fit Hardy-Weinberg Equilibrium as seen in the p-values greater than 0.05.

Table 3.3: Hardy-Weinberg Equilibrium Test for Case Group:

rs3802160	A A	A G	G G	Total
Observed	40	40	16	96
Expected	*37,5*	*45*	*13,5*	*96*
p-Value	0,28			

Table 3.4: Hardy-Weinberg Equilibrium Test for Control Group:

rs3802160	A A	A G	G G	Total
Observed	43	41	16	100
Expected	*40,3*	*46,4*	*13,3*	*100*
p-Value	0,25			

1.4. Dominant and Recessive Models:

We have also tested disease association under dominant and recessive models for both alleles. For this test, we combined homozygotes of subjected alleles with heterozygotes, and compared the numbers of carriers (Allele-Present) with the number of homozygotes of alternative allele (Allele-Absent). We compared the case and control groups and tested the statistical significance of difference between two groups with chi-square test. For the alleles of rs3802160, we were not able to detect any significant difference between case and control groups (Table 3.5).

Table 3.5: Comparison of the patients and controls for dominant models of A and G alleles of rs3802160

rs3802160	A-Present	A-Absent	G-Present	G-Absent
Case				
Count	80	16	56	40
(Frequency)	*(0,83)*	*(0,17)*	*(0,58)*	*(0,42)*
Control				
Count	84	16	57	43
(Frequency)	*(0,84)*	*(0,16)*	*(0,57)*	*(0,43)*
p	0,9		0,85	

3.2. rs4560751 Genotyping Results:

The results produced in the same way as the previous SNP were analyzed for the second SNP rs4560751.

3.2.1. Real-Time PCR Genotyping Results as Multicomponent Plots for rs4560751

The second SNP we have screened was rs4560751. Two alleles seen at this SNP locus are G and T. All three possible genotypes (GG, TT and GT) at this position were detected in our study. Multicomponent plots showing examples for three genotypes are given in Figure-3.5-3.6-3.7. In the TaqMan kit which we have used for real time PCR, the probe specific for G allele was labelled with VIC and the other probe which targets T allele was labelled with FAM fluorescent dye. The multicomponent plots indicate all primers and allele specific probes in the SNP assay we have used for rs4560751 worked properly and we were able to detect both alleles and to distinguish between all three possible genotypes.

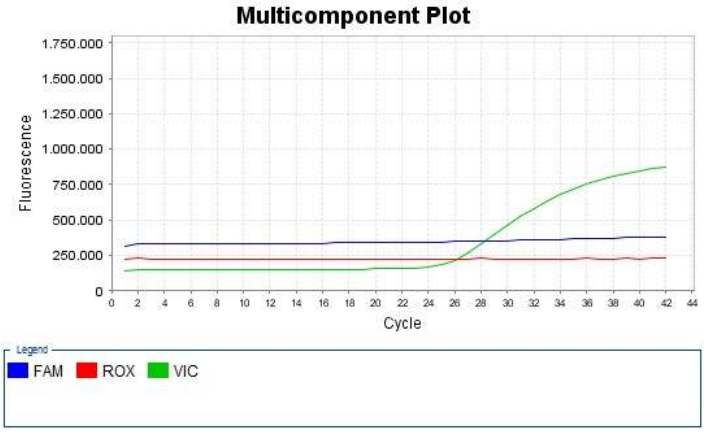


Figure 3.5. rs4560751**,** G/G Genotyping Results

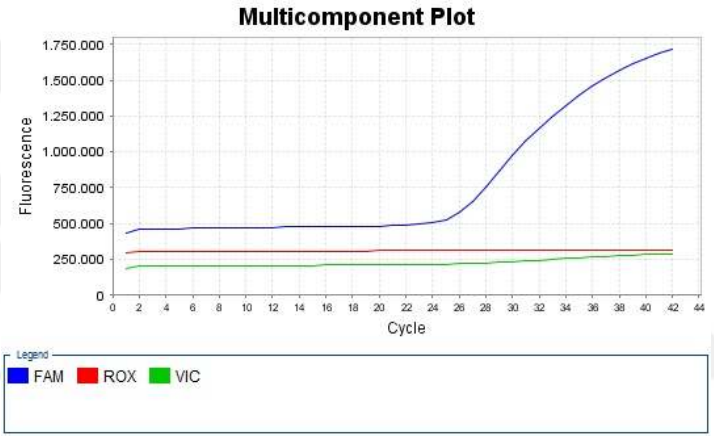


Figure 3.6. Rs4560751**,** T/T Genotyping Results

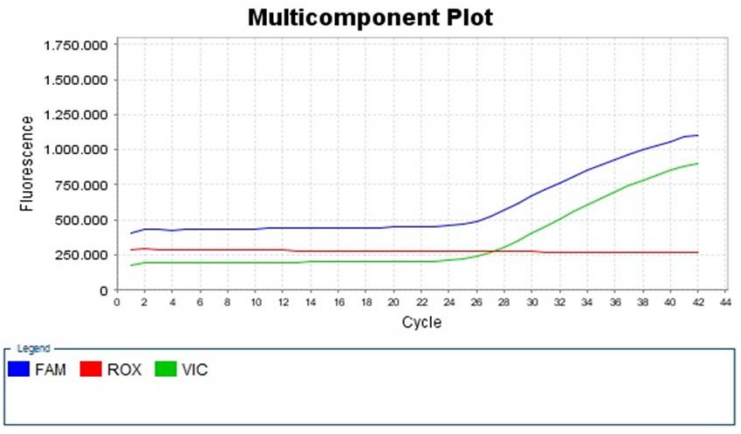
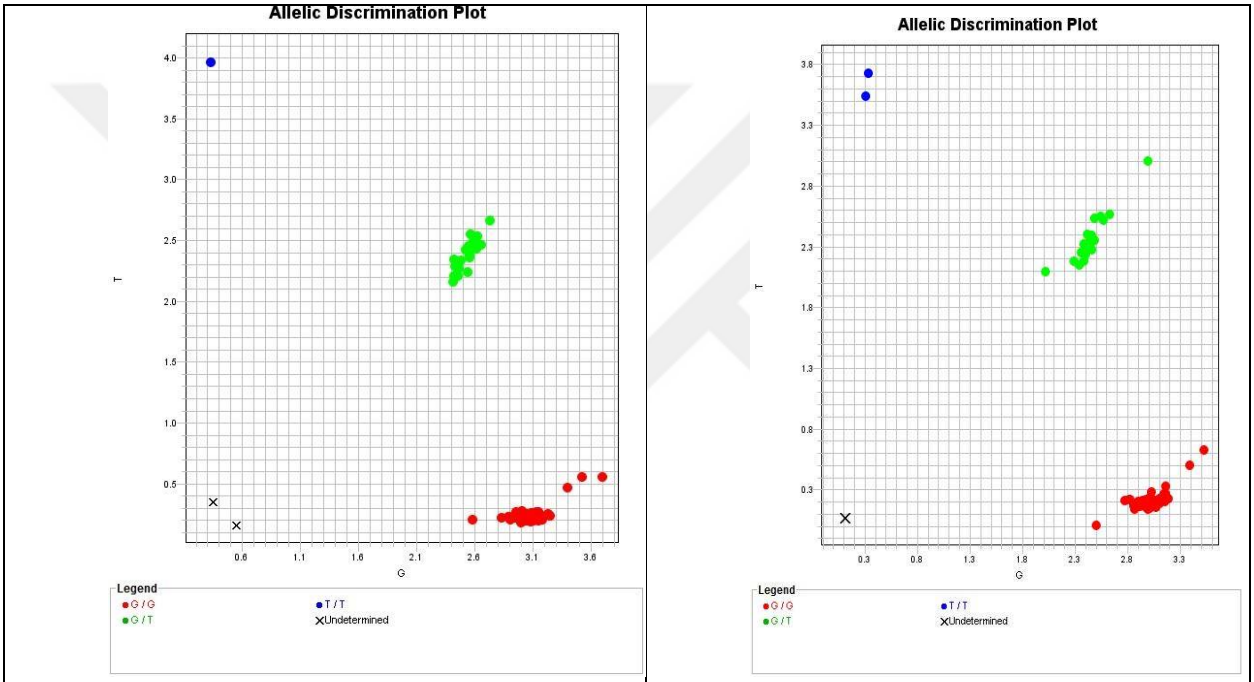


Figure 3.7. Rs4560751**,** G/T Genotyping Results

3.2.2. Real-Time PCR Genotyping Results as Allelic Discrimination Plots for rs4560751

Figure-3.8 presents cartesian plots showing allelic discrimination of case and control plates produced by real-time PCR software. As in the previous SNP screen, one plate was patients, and the other plate was controls' plate. We arranged each plate to house 94 samples and 2 negative (no template) controls. The excess samples were collected in a separate plate and genotyped in following PCR reactions. The cartesian plot of patients' plate is given in panel A and that of control plate is seen in the panel B of Figure-3.8.



A. Case (Patients) Group	B. Control Group

Figure 3.8: Allelic Discrimination plots showing distributions of rs4560751 genotypes in the patient and control groups

3.2.2.1 Analysis of Single-SNP Genotyping Results of rs4560751 Polymorphism:

The distribution of genotypes of rs4560751 as counts and frequencies are given in Table-3.6 and Table-3.7 shows the distribution of alleles of the same SNP. Chi-Square tests comparing case and control groups for these counts indicates there is no significant difference between our case and control groups for rs4560751 (p-values given in the bottom line of the table).

Table 3.6: Comparison of the patients and controls for distributions of genotypes of rs4560751.

rs4560751	G G	G T	T T	HWE-p	Total
Case					
Count	75	20	1	0,79	96
(Frequency)	*(0,78)*	*(0,2)*	*(0,01)*		
Control					
Count	73	25	2	0,93	100
(Frequency)	*(0,73)*	*(0,25)*	*(0,02)*		
p	0,66				

Table 3.7: Comparison of the patients and controls groups for distributions of alleles of rs4560751.

rs4560751	G	T	Total
Case			
Count	170	22	192
(Frequency)	*(0,9)*	*(0,1)*	*1*
Control			
Count	171	29	200
(Frequency)	*(0,85)*	*(0,15)*	*1*
p	0,37		

Table 3.8: Hardy-Weinberg Equilibrium Test for Case Group:

rs4560751	G G	G T	T T	Total
Observed	75	20	1	96
Expected	75,26	19,48	1,26	96
p-Value	0,8			

Table 3.9: Hardy-Weinberg Equilibrium Test for Control Group:

rs4560751	G G	G T	T T	Total
Observed	73	25	2	100
Expected	73,1	24,8	2,1	100
p-Value	0,93			

Both case and control groups were in Hardy- Weinberg Equilibrium for the genotypes of rs4560751 (Table 3.8-3.9).

3.2.2.2. Dominant and Recessive Models for Alleles of rs4560751

As seen in the Table 3.10, there was no significant difference between case and control groups when we group the samples according to presence or absence of a particular allele for testing the dominant and recessive models.

Table 3.10: Comparison of the patients and controls for dominant models of A and G alleles of rs4560751

rs4560751	G-Present	G-Absent	Total	T-Present	T-Absent	Total
Case						
Count	95	1	96	21	75	96
(Frequency)	*(0,99)*	*(0,01)*	*1*	*(0,22)*	*(0,78)*	
Control						
Count	98	2	100	27	73	100
(Frequency)	*(0,98)*	*(0,02)*	*1*	*(0,27)*	*(0,73)*	
Chi-Square	-			0,7		
p	-			0,4		

All our results indicate that alleles and genotypes of the SNPs rs3802160 and rs4560751 has no association with the development of schizophrenia in our patients group.

4. DISCUSSION

In this study, we carried out a genetic association study in case-control format to understand if the NRG1 SNPs rs3802160 and rs4560751 are associated with schizophrenia in a group of Turkish patients. To our knowledge, there is no other published studies to investigate the association of these two SNPs in a sample from Turkiye.

The NRG1 gene is the first one to be found genetically linked to schizophrenia in two earlier studies [100 ,101]. After this linkage was found, the screening of this gene in different populations revealed more polymorphisms associated with the disease [102,103]. The association between the schizophrenia and NRG1 gene have been supported by the meta-analysis studies [104]. One of the case-control studies done in a German sample indicated the interaction between two SNPs rs3802160

and rs4560751 located in the NRG1 gene [105], but the disease associations of these SNPs were not studied and approved in any other populations.

The situation in which the genetic association between a genetic factor like a SNP and a multifactorial disease to be present in one population, but not to in another one is notably common. Because of that, the associations of genetic factors with the diseases like schizophrenia has to be supported by similar studies done in the samples from different populations [3,106] Since association of rs3802160 and rs4560751 with schizophrenia was shown only in one population, we attempted to find more evidence to support this case. We collected our schizophrenia patients and control samples from Malatya (a city located in the Eastern Anatolian Georgaphic Region of Turkiye). Since we have collected our samples only from a limited region, our results may not be expected to reflect the entire population living in Turkiye. We used the DNA samples of subjects in the case and control groups to determine their genotypes at rs4560751 and rs3802160 SNP loci and compared two groups for the frequencies and counts of alleles and genotypes of these SNPs. The statistical tests we have done to evaluate the significance of differences between two groups indicated the differences were not significant.

Based on this, there were no sufficient evidence supporting the associations of genotypes or alleles of rs4560751 and rs3802160 SNPs with schizophrenia. Following these comparisons, we tested the disease associations of alleles at each SNP loci in dominant models, but these tests did not show any associations either.

There are several reasons that may cause the associations not to appear in our sample. The first two possible reason are the sample size we used might not be sufficient to distinguish the differences of allele and genotype frequencies between the cases and controls and the differences seen in the risk alleles between different populations. Also, the heterogeneity that can be seen in the clinical presentation of schizophrenia which can affect the diagnosis might be another reason. In addition, the regional limitations because of our samples were collected from a relatively small geographic area could be preventing the appearance of differences of allelic and genotypic distributions between the case and control groups. According to this, there is still a possibility to find associations of these SNPs with schizophrenia if a case-control group in a larger size from a wider geographic area can be screened. Lastly, because schizophrenia is a multifactorial – polygenic disease whose

phenotype is affected by a number of genes and various other factors, instead of these two polymorphisms, a lot of other variations in the NRG1 gene as well as the other genes may be the reason underlying the disease in most of our patients.

The associations found between schizophrenia and a number of other genes including NRG3, ERBB4, DAOA, COMT, DRD2, DRD3, DRD4, TLR2 and BDFN supports this idea [107-111]. According to this, we can assume that the subjects in the group of patients who were not carrying the risk alleles at two SNP loci we have screened may be carrying the risk alleles of other SNPs in the NRG1 gene, or the ones in the other associated genes. Also, the disease risk factor may be one of haplotypes in the NRG1 gene as well as the epistatic interactions between different genes [112].

The absence of association between a gene and a disease in some populations does not eliminate the possibility of that gene's contribution to the disease phenotype. For the NRG1 gene, its involvement has been approved by recent functional studies revealing the behavioral phenotypes similar to schizophrenia deficits were seen in the haploin sufficient mouse models [113]. When this is considered, we can assume the NRG1 polymorphisms or variants could have effects on the development of schizophrenia phenotype. Nevertheless, to uncover this involvement probably requires the screening of more variants and analyzing the associations at haplotype and epistatic levels.

As conclusion, during this study, we did not find sufficient evidence supporting the presence of any associations between development of schizophrenia phenotype and two SNPs in the NRG1 gene (rs4560751 and rs3802160) in the patient group we have collected from a Turkish population living in Malatya-Turkiye. More studies screening the SNPs in the NRG1 gene as well as other schizophrenia associated genes in case-control format or more extensive studies to be done by better developed methods like next generation sequencing techniques probably should make understanding the genetic basis of schizophrenia in our patients possible.

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