TR
İNÖNÜ UNIVERSITY
INSTITUE OF SCIENCE

GENETIC INVESTIGATION OF A HAPLOTYPE ON THE APH1B GENE IN SCHIZOPHRENIA PATIENTS AND THEIR FAMILIES

MASTER THESIS

Atife Nida KARAKAYA

Department of
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Thesis Advisor: Assoc. Prof. M.Mert SÖZEN

JANUARY 2023

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WORD OF HONOR

I declare that, this **"Genetic Investıgatıon of A Haplotype On The Aph1b Gene In Schizophrenia Patients and Their Families"** 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.

Atife Nida KARAKAYA

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ABBREVIATIONS

APH1B : Anterior Pharynx Defective

COMT : Catechol-O-methyl transferase

cSNP : Coding Single Nucleotide Polymorphism

dNTP : Deoxynucleotide Triphosphate

DSM-V : Diagnostic and Statical Manual of Mental Disorders-V

EGF : Epidermal Growth Factor

GABA : Gamma Aminobutyric

ICD-10 : International Classification of Disseases-10

iSNP : Intronic Single Nucleotide Polymorphism

mGluR : Metabotropic Glutamate Receptors

NCBI : National Cener for Biotechnology Information

NMDAR : NMDA Recptor

NRG1 : Neuregulin-1

nsSNP : Non-Synonymous Single Nucleotide Polymorphism

PCR : Polymerase Chain Reaction

rSNP : Regulatory Single Nucleotide Polymorphism

SNP : Single Nucleotide Polymorphism

sSNP : Single Nucleotide Polymorphism

SZ : Schizophrenia

ÖZET

Yüksek Lisans Tezi

ŞİZOFRENİ HASTALARI VE AİLELERİNDE APH1B GENİ ÜZERİNDEKİ BİR HAPLOTİPİN GENETİK ARAŞTIRILMASI

ATİFE NİDA KARAKAYA

İnönü Üniversitesi
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Şizofreni kompleks ve çok faktörlü olan ciddi bir psikotik bozukluktur. APH1B geni şizofreniyle ilişkilendirilme potansiyeline sahip genlerden biridir. Daha önce, APH1 genindeki iki tek nükleotid polimorfizminin (SNP) rs117618017 ve rs1047552'nin, yalnızca bir vaka çalışmasında hastalığın gelişimi üzerinde etkileri olduğu öne sürülmüştü. Bu çalışmada, Malatya-Türkiye'den bir grup aile üçlüsünde bu iki SNP'nin ilişkisini test ettik. Çalışmamızı aile temelli ilişkilendirme testi (FBAT) formatında tasarladık.. Yerel bir Türk popülasyonundan toplanan 25 şizofreni hastası ve etkilenmemiş ebeveynlerinden oluşan bir örneklemde 2 SNP'nin genotiplerini taradık. Bu varyantların hastalık ilişkilerini tek SNP, haplotip ve diplotip seviyelerinde test ettik. Çalışmamızın sonuçları, testlerimizde APH1B SNP rs117618017 ve rs1047552 ile şizofreni fenotipi arasında hiçbir genetik ilişki bulunmadığını gösterdi. Bu çalışmada kullandığımız örneklem nispeten küçük bir coğrafi bölgeden toplandığından ve aile üçlüsünün sayısı sınırlı olduğundan, taradığımız iki SNP ilişkisinin daha büyük hasta gruplarında test edilmesini gerektirmektedir.

Anahtar kelimeler: APH1B, Genetik İlişki, Şizofreni, Genetik, SNP, Haplotip, Diplotip, FBAT.

ABSTRACT

Master Thesis

GENETIC INVESTIGATION OF A HAPLOTYPE ON THE APH1B GENE IN SCHIZOPHRENIA PATIENTS AND THEIR FAMILIES

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Supervisor: Assoc.Prof.Mustafa Mert SOZEN

Schizophrenia is a severe psychotic complex-multifactorial disease. APH1B gene is one of the gene with a potential of association to schizophrenia. Previously, two single nucleotide polymorphisms (SNPs) rs117618017 and rs1047552 in the APH1 gene were suggested to have effects on the development of disease in a case only study. In this study, we tested the association of these two SNPs in a group of family trios from Malatya-Türkiye. We designed our study in family based association test (FBAT) format. We screened the genotypes of 2 SNPs in a sample consisted of 25 schizophrenia patients and their unaffected parents collected from a local Turkish population. We tested the disease associations of these variants at single SNP, haplotype and diplotype levels. The results of our study indicated there were no genetic associations were found in our tests between the APH1B SNPs rs117618017 and rs1047552 and the schizophrenia phenotype. Since the sample we have used in this study was collected from a relatively small geographic area, and the numbers of family trios were limited, the associations of two SNPs we have screened require to be tested in larger patient groups.

Key Words: APH1B, Association, Genetic Association, Schizophrenia, Genetics, SNP, Haplotype, Diplotype, FBAT.

1. INTRODUCTION

The name schizophrenia was derived by the first observer as disconnection or division of psychic functions. (Picchioni & Murray, 2007)

Awareness of psychiatric disorders and research on these psychiatric diseases are increasing day by day (Ayhan et al., 2016). Schizophrenia is a serious, lifelong disease with a rate of four percent worldwide (Winship et al., 2019). This ratio represents a large proportion that exceeds the disability burden of any infectious disease (Ayhan et al., 2016). Schizophrenia can have devastating effects on patients, their caretakers and their families, both material and spiritual effects. (Winship et al., 2019).

Schizophrenia is a debilitating illness, with positive symptoms, negative symptoms, and cognitive impairments. Delusions, hallucinations, and impaired reality test are three examples for positive symptoms. Negative symptoms include flat effects and social withdrawal. These symptoms and abnormalities are often a lifelong disability for the schizophrenic patient. The diagnosis of the disease is usually made in the twenties. In general, men are diagnosed around 5 years earlier than the women. The etiology of schizophrenia is still not fully understood since the cause of this disease is not only a single factor. There have been twin studies showing that schizophrenia is genetically based, and as a result of these studies, a genetic component of the risk has been well identified and it has been shown that it is inherited in the range of seventy-eighty percent (Ayhan et al., 2016). As a kind of positive symptoms, delusions can be seen in several types and can affect the functioning of the life of the affected person in different dimensions.

Delusions can be of varying degrees. Persecuting delusions and reference delusions are the most common in this disease. However, delusions of adding thoughts, withdrawing, and broadcasting are also associated with schizophrenia. Hallucinations may appear in any one of five senses despite most common ones are the auditory hallucinations. Hearing voices of interlocutors or the patient's self-commenting voices is considered characteristic, but threatening or accusing voices are more common (Tandon et al., 2009).

Negative symptoms include blunting or complete loss of a range of functions which can be conative or affective. Negative symptoms can be seen as; apathy (lack of interest), abulia (lost motivation), avolia (lack of initiative), anhedonia (inability to experience pleasure), alogia (poverty of speech), and diminished social drive. It is important to distinguish between primary and secondary negative symptoms in schizophrenia.

The fundamental symptoms to schizophrenia are primary negative symptoms, but extrinsic factors underlie the secondary negative symptoms for example, environmental deprivation and depression (Tandon et al., 2009).

1.1. Onset

Conceptual and attitude characteristics are present in infancy and beyond infancy. Common distinguishing features in schizophrenia are seen in the late adolescence and early twenties. Although schizophrenia is a cognitive disease, behavioral disorders exist from early childhood. The main features of the disease can generally begin in the late teens and early twenties. Of course, this situation places significant burdens on families and the social environment (Williams et al., 2009). The lifespan of individuals affected by schizophrenia is reduced by about 15 years. The main reasons for this are cardiovascular diseases and death situations resulting in suicide (Akbarian, 2014).

Selection of patients is based on a "The Diagnostic and Statistical Manual of Mental Disorders-V (DSM-V)".

1.2. Epidemiology and risk factors

Schizophrenia is seen universally and probably it has a lifetime risk of the disease of 1% across time, geography, and gender (Michael J. Owen et al., 2016). According to epidemiological data considering the isolation in diagnostic category of disease, the lifetime prevalence is between 0.30% and 0.66% while the incidence is 10.2-22.0 / 100,000 people per year. If other psychotic disorders like substance-induced psychotic disorder and bipolar disease are added to the lifetime schizophrenia rate, which includes brief psychotic disease or delusional disease, and psychotic disorder diagnostic categories not otherwise specified, this rate rises from 2.3% to 3.5% (van Os & Kapur, 2009). A later age of onset in women is likely to appear in comparison to the men and also, women show better social functioning and have better course of illness with fewer hospitalizations (Mueser &

McGurk, 2004). The environmental factors also play a key role dor development of schizophrenia and the neurodevelopmental hypothesis has been the dominant consideration to understand their contribution for more than three decades. This hypothesis draws attention to the identified risk factors affecting early neurodevelopment during pregnancy, such as infections and maternal stress, intrauterine growth retardation, nutritional deficiencies, pregnancy and delivery complications. Obstetric complications are particularly associated with bleeding during pregnancy, emergency cesarean section, preeclampsia, and low birth weight. Exposure to various infectious agents such as influenza, toxoplasma and herpes simplex virus type 2 are also reported to be associated risk factors with psychotic disorders. However, childhood difficulties such as parental death, neglect, socioeconomic factors, physical, sexual, and emotional abuse, and bullying have been associated with schizophrenia. Childhood compulsions have been found to be associated with the most severe positive symptoms in adulthood, particularly hallucinations and emotional symptoms. Childhood rural to urban migration doubles the risk of developing schizophrenia, and the increased years a child spends in the urban environment also increases the schizophrenia risk. The risk associated with urban migration continues in the second and third generations. It has been reported that socioeconomic status, usually determined by the father's occupation, is associated with an increased risk of psychosis. Living alone, being single or unemployed, living in a rented house in crowded conditions are also risk factors of schizophrenia. Additionally, in the people who were born in late winter or spring, the rates of schizophrenia have been reported higher. Also the individuals whose fathers are over 40 years of age, or younger parents under 20 years of age are reported to have a higher risk. Also, the use of cannabis is estimated to increase the risk two- to three-fold. There is solid evidence that psychostimulants such as amphetamine and cocaine can trigger psychosis (Michael J. Owen et al., 2016; Stilo & Murray, 2019; van Os & Kapur, 2009). However, the actual susceptibility for schizophrenia is determined by genetic factors. Twin studies show that the 80% of syndrome is inherited. Despite this genetic relationship, identifying the specific genetic variations at molecular level has been complicated, and not easy to understand. Schizophrenia has heritability higher than 80% and it is not due to only genetic factors, but also to the gene-environment interactions. Because of the gene-environment interactions, the effects of the environmental factors are connected with one or more genetic variants and vice versa. None of the above-discussed risk factors for schizophrenia are necessary or sufficient on their own, and this is becoming increasingly clear. Most of them makes

modest effects of doubling in risk, and none of them are specific factors for development of schizophrenia. Different factors making impact at various levels contribute to the progression and onset and disease. The developmental step in the direction of schizophrenia is about gene-environment interaction and the interactions between different environmental factors. More recently, attention has shifted to the possibility of gene-environment interactions (Michael J. Owen et al., 2016; Stilo & Murray, 2019; van Os & Kapur, 2009).

1.3. Diagnosis

The diagnosis of schizophrenia is generally determined by examining the patient's history and mental state. There is no biological marker or diagnostic test available. Schizophrenia generally manifests itself with psychosis (Michael J. Owen et al., 2016). The delusions and hallucinations in psychosis are generally not difficult to define. However, classification of these psychoses is difficult. Because psychoses are not specific to schizophrenia and occur in some of the other categories of psychotic diagnosis (van Os & Kapur, 2009). The systems based on criteria have been developed and have reduced complexity and simplified diagnosis. These systems include the International Classification of Diseases (ICD-10). Another one is the fourth edition of Manual of Mental Disorders (DSM-V). DSM-V describes the characteristic symptoms of schizophrenia. In ICD-10, severe symptoms must present for a month. However, a period of 6 months is required in DSM-V (DSM-V includes less severe and prodromal symptoms) (Schultz & Andreasen, 1999).

The criteria used to distinguish depend on duration, dysfunction and related substance use, strangeness of delusions, hallucinations, mania and depression (van Os & Kapur, 2009). Once the symptoms of schizophrenia become evident, social impairment persists and continues even after other severe symptoms have been alleviated with medications. Physchosocial impairment is becoming an increasing focus in treatment. Psychiatrists are increasingly recognizing the relationship between these negative symptoms and social impairments in patients with schizophrenia, and schizophrenia diagnosis is beginning to focus more on negative symptoms. Negative symptoms adversely affect and reduce the ability of patients to communicate meaningfully with their social environment. Many negative symptoms are cognitive, such as alogia, avolition, and attention deficit. These negative symptoms cause patients to be impaired in socio-economic terms, as they are unable to maintain productivity and are unable to communicate (Schultz & Andreasen, 1999).

1.4. Etiology of schizophrenia

Schizophrenia is a complex disease with many different manifestations. There is no single cause that contributes to the course of the disorder and its development. Schizophrenia is a disease that occurs with the interaction of many factors. While the etiology of schizophrenia is still largely unknown, it is thought that genetic and environmental risk factors as well as physiological processes together make individuals more susceptible to the development of this disorder (Cunningham & Peters, 2014).

1.4.1. Genetic factors

Schizophrenia is a multifactorial disease. A positive family history is a major risk factor. When the general population is examined, the lifetime risk remains below 1% while in first-degree relatives of patients that rate is 6.5%, and in the monozygotic twins of patients, it is even more than 40%. Extended studies concerning families, adoptions, and twins indicate that the risk is increased when the disease is seen in relatives (Picchioni & Murray, 2007). As with other common psychotic illnesses, the mode of transmission is complex and does not have Mendelian inheritance. For transmission of disease, the polygenic and oligogenic modes of transmission and threshold effect can be seen. A lot of researches have been carried out to identify the susceptibility genes using positional genetics (M. J. Owen et al., 2004). Also many attempts have been made for identifying the etiologically distinct subgroups because of the wide clinical heterogeneity. There are three basic approaches to identify disease genes in human populations; linkage studies, genetic association studies, and studies to identify chromosomal abnormalities in patients (M. J. Owen et al., 2004). As the subject of our research, genetics is discussed in more detail later.

1.4.2. Neurochemical factors

Dopamine and serotonin neurotransmitters have an important function in the pathogenesis and treatment of schizophrenia. However, current research also focuses other molecules including glutamine and g-amino-butyric acid (GABA) (Manford, 1998).

1.4.3. Dopamine assumption

Dopamine hypothesis states that the symptoms of schizophrenia are due to increased dopamine transmission, and the therapeutic effects of antipsychotic drugs are due to the

inhibitory effects on dopamine transmission. Important sources of this hypotheses are the studies which shows the administration of compounds including amphetamine which acts by increasing the concentration of extracellular dopamine can cause psychotic symptoms like the ones seen with schizophrenia. Supporting this, drugs that reduce dopamine levels (like reserpine and alpha methyl para-tyrosine) reduce psychotic symptoms (Howes et al., 2015; Schmidt & Wrieth, n.d.).

1.4.4. Serotonin assumption

The serotonin, also known as 5-hydroxytryptamine (5-HT) hypothesis is based on early studies on the interactions between 5-hydroxytryptamine and lysergic acid diethylamide (LSD) which is a hallucinogenic drug. Observation of the hallucinogenic effects of LSD with 5-HT blockade and the antipsychotic effects of antagonists of serotonin and dopamine like clozapine and risperidone have led to interest in the interaction between these two neurotransmitter systems as a possible pathophysiological target in development of schizophrenia (A. C. Yang & Tsai, 2017).

1.4.5. Glutamate assumption

In the brain, the main excitatory neurotransmitter is glutamate. The glutamatergic neurons use 60-80 percent of total metabolic activity of the brain (Howes et al., 2015). Glutamate plays a role in brain development, neuronal migration and differentiation, and axon formation. It is important in the nervous system, neuroplasticity, synaptic activity and changes in synapse structures (Özdemir & Güzel Özdemir, 2016).

It has long been known that the N-methyl-d aspartate (NMDA) antagonists phencyclidine (PCP) and ketamine at glutamate receptors induce schizophrenia-like positive, negative, and cognitive symptoms (Hu et al., 2015).

1.4.6. GABA assumption

The primary inhibitory neurotransmitter in the brain is Gamma aminobutyric acid (GABA) (Kern et al., 2014). There are three types of identified GABA receptors, called as ionotropic GABAA, metabotropic GABAB, and GABAC receptor (Wassef et al., 2003). It is not fully understood yet if the primary deficits in schizophrenia are depended on the abnormal functioning of the GABAergic system (Kern et al., 2014). The results of postmortem studies done with schizophrenia patients indicated the reduction in the GAD67 mRNA and protein as well as the GABA synthesizing enzyme. This suggests that the

decreased activity of GABA may have roles in schizophrenia pathophysiology (Egerton et al., 2017; Wassef et al., 2003). The synergistic effect of addition of a GABA agonist to an antipsychotic drug is suggested to improve the cognitive and negative symptoms of schizophrenia caused by prefrontal abnormalities (Wassef et al., 2003).

1.4.7. Neurodevelopmental model

The classical etiological hypothesis of schizophrenia asserts that, the disease begins during the early stages of neural development. Based on this, the central nervous system development can be affected by the genetic factors and the complications which can be faced before, during, or after birth resulting in permanent defects in the arrangement of axonal connections or other complex morphological features such as brain cell arrangement or brain asymmetry. In contrast, the observations from the studies on worsening of patients during the course of the disease and progressive exacerbation of negative and positive symptoms suggest that a degenerative process may be characteristic to schizophrenia. Evidence that cognitive impairment observed in schizophrenia patients is stable throughout the disease course and that gliosis, a marker of neuronal death, is absent in neurodegenerative diseases, is considered valid evidence of the neurodevelopmental origin of schizophrenia (Chiapponi et al., 2013).

1.5. Genetics of schizophrenia

Eighty percent of schizophrenia is inherited. While the probability of the disease is one percent in the general population, it can be six and a half percent in those with first-degree relatives of patients. People who had twins affected by schizophrenia are more than 40 percent likely to have schizophrenia. Extended family, twin, and adoption studies show a predisposition between the patient and the proband (Picchioni & Murray, 2007). In human populations, the genes related to schizophrenia are usually identified by one of three methods depending on the types of genetic variations. These are the cytogenetic methods, linkage studies and genetic association studies.

i. Cytogenetic Methods for Chromosomal abnormalities: One strategy for unraveling the genetic components of schizophrenia is the search for chromosomal abnormalities and familial syndromes seen with the in phenotypes of schizophrenia. There are many chromosomal abnormalities reported in families of patients affected psychiatric disorders including schizophrenia (Rujescu, 2019). This approach includes searching for

chromosomal abnormalities such as translocations, trisomies and deletions (Murphy, 2002).

ii. Linkage Studies: This approach is the analysis of pedigree samples of affected individuals to identify genetic regions associated with the disease (Arslan, 2018). Genetic linkage analysis examines the separation of traits in pedigrees, assesses whether certain phenotypes tend to be inherited together, and reveals the closeness of the genes responsible for these traits in the genome (Teare & Koref, 2014).

iii. Association studies: This approach focuses on the identification of allelic variations or single nucleotide polymorphisms (SNPs) that manifest themselves as variation in gene expression and/or disease-related functions (Arslan, 2018). Depending on the case, the association analyzes are more powerful than linkage analyzes. Furthermore, genome-wide association studies (GWAS) using single nucleotide polymorphism (SNP) marker loci through the whole genome increases the range of investigated genetic factors incredibely. Alsoi it is possible to get more comprehensive data using multiple family members, (Ott et al., 2015).

1.6. Candidate genes potentially acting in schizophrenia

The research methods mentioned above are suitable for the detection of the genes which has large impacts in the development of diseases caused by simpler genetic mechanisms. Whereas, their power is limited to detect genes with small or moderate effects which underlies complex disorders such as schizophrenia. This kind of studies requires a number of families. Therefore, researchers need to select specific candidate genes (M. J. Owen et al., 2004).

There are 3 very strong candidate genes that have proven to be effective in schizophrenia. Dysbindin (DTNBP1) located on the 6p chromosome, neurorogulin 1 (NRG1) located on the 8p chromosome, and G72 (DAAO) / G30, which is located on the 13q chromosome and is only expressed in humans (M. J. Owen et al., 2004).

1.6.1. Neuregulin-1 (NRG1)

Neuregulin-1 is a growth factor which belongs to the neuregulin family consisting of four types of proteins (neuregulin 1-4). Neuregulin-1 is encoded by the NRG1 gene. Neuregulins act by binding to the receptor tyrosines kineses of ErbB (epidermal growth

factor-like receptor) family. At least 31 isoforms of six proteins are produced from the NRG1 gene using the alternative splicing mechanisms and different 5' neighboring regulatory elements. Heregulin, Neu differentiation factor (NDF), glial growth factor (GGF), and acetylcholine receptor inducing activity (ARIA) are the examples for those isoforms. The isoforms of neuregulin have been classified according to their amino-terminal sequence, the presence of a transmembrane region, or the expression of α or β epidermal growth factor (EGF). The tpyes are named I-VI according to this classification (Kataria et al., 2019; O'Tuathaigh et al., 2007).

A transmembrane (TM) domain is present in the types I and II of neuregulin and in is called C-terminal transmembrane (TMc) domain. Whereas, a cysteine-rich domain (CRD) is present in the type III neuregulin in addition to the TMc domain. The type I, II, IV and V neuregulins which are also known as Ig-neuregulin1 carry an immunoglobulin [Ig] domain that is associated with the epidermal growth factor (EGF)-like domain (Mostaid et al., 2016; O'Tuathaigh et al., 2007). The effect of NRG1 gene on schizophrenia was first shown in the Icelandic population after LD mapping of chromosomal region8p21-22. Following this, a haplotype at the 5' end of NRG1 was found to be associated with schizophrenia. This haplotype association was also found in a larger sample from Scotland (Michael J. Owen, 2005).

Even though all types of neuregulin1 (I-VI) act by binding to the conjugated transmembrane receptor-tyrosine kinases of ErbB family, the main receptor for neuregulin1 is ErbB4. The putative imbalance of excitatory/inhibitory transmission in the cortex, hippocampus and striatum is possibly the neurobiological promoter for development of schizophrenia. The strong evidences indicate that the neuregulin-1 can disrupt normal excitatory/inhibitory neurotransmission via the ErbB4 receptor during preclinical period (Mostaid et al., 2016).

1.6.2. ERBB4

The ErbB4 is the unique autonomous ErbB receptor specific to neuregulin1. It can be activated as a tyrosine kinase when it interacts with the ligand. It is a 180kDa glycoprotein receptor tyrosine kinase. The ErbB4 receptor differs from the other ErbB receptors since it undergoes proteolytic processing after binding of the ligand. The active ErbB4 receptors form homodimer or heterodimer with another ErbB family member, causing the kinase activation and autophosphorylation of the receptors.

This phosphorylation triggers intracellular signal transduction pathways such as "phosphoinositide-3 kinase (PI3K) / Akt and Ras / mitogen-activated protein kinase (MAPK) cascades" (Mei & Xiong, 2008; Veikkolainen et al., 2011; Wang et al., 2019). It was also shown that the epidermal growth factor, heparin-binding epidermal growth factor-like growth factor, betacellulin and epiregulin can function as the ligaqnds of ErbB4. Activation of ErbB4 through its ligands leads to various cellular events such as cell proliferation, cell survival, chemotaxis and differentiation. While ErbB4 plays a role in the development of the mammary glands, heart, and central nervous system, it also has effects on the development of several disorders including cancer, cardiovascular diseases as well as psychiatric disorders (Kainulainen et al., 2000; Wang et al., 2019).

The ERBB4 gene is located on chromosome 2 and encodes for functionally different protein products or isoforms. (Figure 1.1) (Veikkolainen et al., 2011).

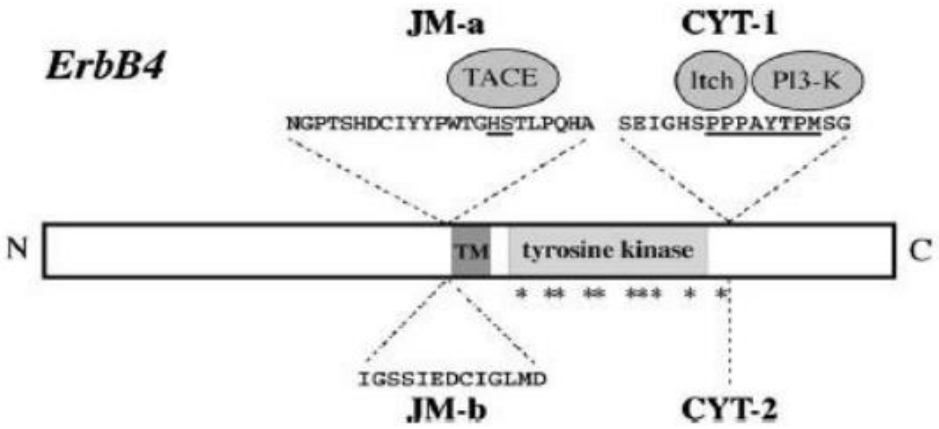


Figure 1.1: ERBB4 isoforms (Sundvall et al., 2008)

The downstream signaling cascades including "phosphoinositide 3 kinase (PI3-K)/Akt pathway" or "Ras/mitogen-activated protein kinase (MAPK) pathway" are activated after binding of ligand to ErbB4 receptor (Figure 1.2a). Apart from the activation of these signaling pathways, ErbB4 signals directly to the nucleus after proteolytic processes by the activities of two proteolytic enzymes, "tumor necrosis factor-α converting enzyme"" (TACE) and "γ-secretase" (Figure 1.2b). ErbB4 signaling is regulated by specific interactions of proteins such as Nedd4-like E3-ubiquitin ligase Itch and WW domain-

containing oxidoreductase (Wwox) which regulate the transcriptional activity, stability and intracellular targeting of ErbB4, (Figure 1.2c) (Sundvall et al., 2008).

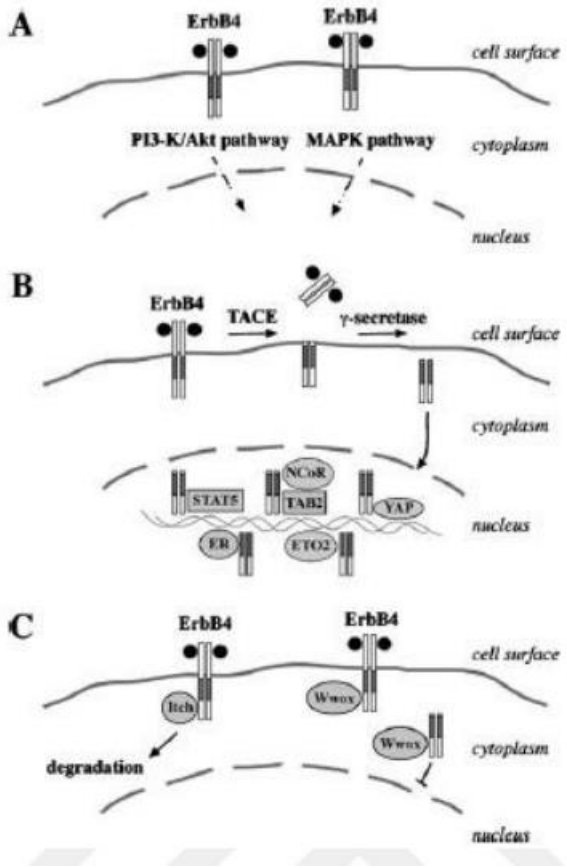


Figure 1.2: ERBB4 signals (Sundvall et al., 2008)

1.6.3. NRG1-ERBB4 signaling

Nrg-1 ligand acts by binding to three kinds of ErbB receptors tyrosine kinases (ErbB-2 to 4). The canonical Nrg-1/ErbB signaling pathway effects via forward and reverse signaling. Among ErbB receptors, ErbB-4 differs from the others in that it has both active kinase domains for ligand binding, which allows formation of functional dimers (either homodimers or heterodimers) upon the binding of Nrg-1. The functions of ErbB4 have been investigated and characterized extensively especially for their connections with the psychiatric disorders caused by neurodevelopmental abnormalities (Figure 1.3) (Kataria et al., 2019). A number of studies indicated that, the neuregulin-1 induced stimulation of the ErbB4 receptor has a lot of functions in neurodevelopment, including the expression of

several neurotransmitter receptors, development of glial cells, radial glia formation, neuronal migration, axon guidance, axon myelination, and dendritic development (Pan et al., 2011).

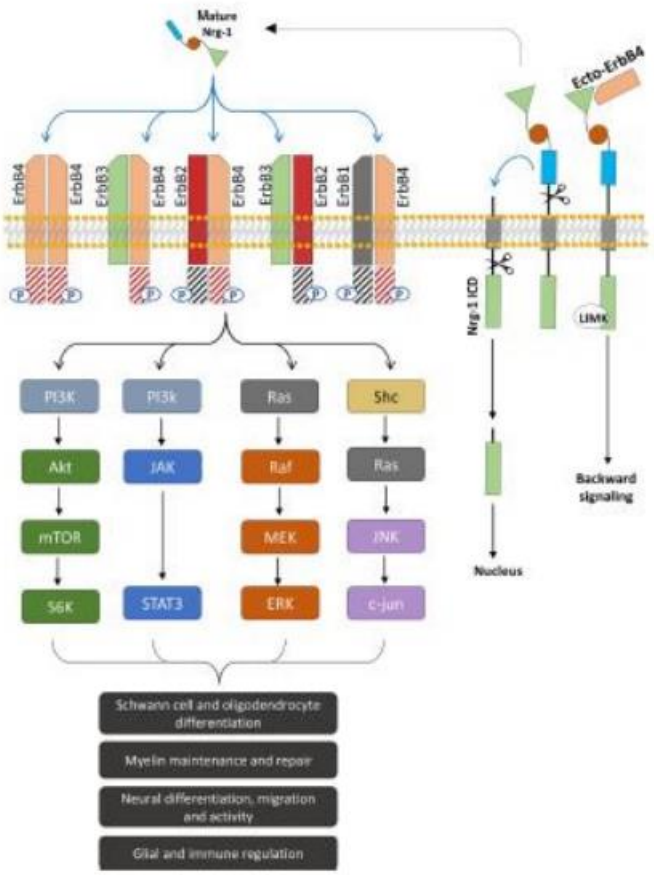


Figure 1.3: NRG1-ERBB4 signals (Kataria et al., 2019)

Also animal studies have indicated that the NRG1-ErbB4 signaling disorders in animals cause abnormal behaviors related to certain features characteristic to schizophrenia (Pan et al., 2011). In postmortem studies of the human brain, both decreased and increased levels and/or activities of NRG or ERBB were found to be associated with schizophrenia. In animal models, mice with the deletion of one copies of the ERBB2, ERBB3, ERBB4 or NRG, genes show reduced working memory, latent inhibition, impaired hyperactivity and inhibition in the open field, reduced fear conditioning, and abnormal social behavior. Significantly, many of deficits mentioned above were seen in mutant mice where Erbb4 is specifically reduced in interneurons, and it is clear that interneurons are targets for aberrant NRG1 signaling (Mei & Xiong, 2008). There is evidence that increased activation of

ERBB4 and NRG1 hyperphosphorylation in the prefrontal cortex in schizophrenia results in NMDA hypofunction, which results in loss of spikes and impairs synaptic transmission (Bennett, 2009). In a postmortem study in schizophrenic patients, a significant increase in NRG1-triggered ErbB4 activation and suppression of NMDA-type glutamate receptor activation was seen when compared with the normal controls (Tan et al., 2018). It is suggested that ERBB4 is required to maintain GABA activity and transmission (Tan et al., 2018).

1.6.4. APH1B

Aph-1b is part of γ-secretase, an enzyme complex that regulates type intramembrane proteolysis of type I proteins involved in multiple neurodevelopmental signaling pathways (Coolen et al., 2005).

γ-Secretase is an "aspartyl protease" that cleaves the I transmembrane proteins such as Notch or amyloid precursor protein from their intracellular domains when their larger extracellular domains are shed. The substrates of γ-Secretase has a variety of functions including cell adhesion, neurite outgrowth, cell fate and synapse formation indicating the importance of γ-secretase in neurogenesis and development. γ-secretase consists of four subunits called presenilin, nicastrin, presenilin enhancer 2 homolog (PEN2) and anterior pharynx defective 1 homolog (APH1). The active site of γ-secretase is in the presenilin subunit. The other three subunits functions to stabilize the complex and to recruit the substrates (Figure 1.4) (Osherovich, 2008). The catalytic prenicelin subunit has two conserved transmembrane aspartates constituting the active site. (Sato et al., 2007).

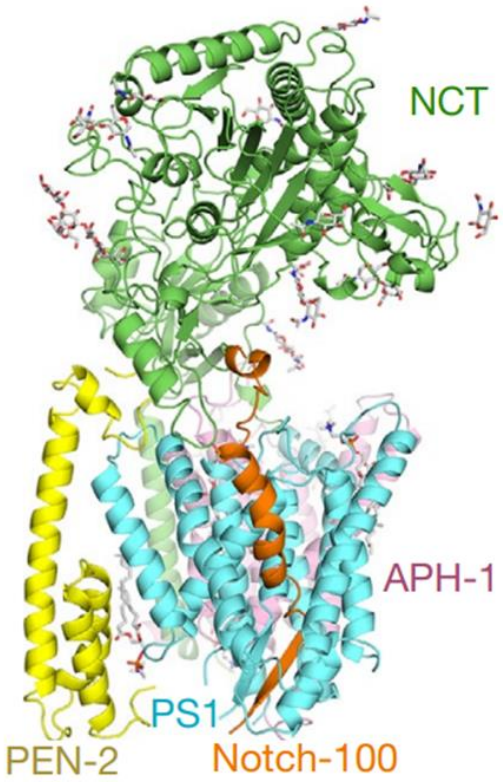


Figure 1.4: Structure of the γ-secretase-Notch-100 complex. Glycans and lipids are shown as bars (G. Yang et al., 2019).

As a component of neuregulin signaling pathway, γ-secretase is a new target in schizophrenia. γ-secretase was originally discovered as a genetic risk factor for Alzheimer's disease. When target proteins are digested by γ-secretase, activated intracellular and extracellular fragments are released which functions in transcriptional regulation and intercellular signaling acting in brain development and activity. Studies on the function of Aph1 revealed the presence of its variants one of which is called APH1A. This variant allows γ-secretase to recognize and cleave Notch which is a signaling protein. In addition, the other variant of APH1 which is called APH1B is known to function in neuregulin processing (Osherovich, 2008).

Two SNPs in the APH1B gene called rs117618017 and rs1047552 were seen to have a potential to be associated with schizophrenia after a study done by next generation sequencing (Hatzimanolis et al., 2013). Since there was not any other studies to approve this result, screening of this SNPs in more populations is required. In this study, we screened these SNPs in the families of a group of Schizophrenia patients from a Turkish population.

2. MATERIALS AND METHODS

2.1. Sample

Our study was carried out at İnönü University Turgut Ozal Medical Center Psychiatry Clinic between June 2019 and December 2019. 24 patients and their parents, aged 18-64 years, who were diagnosed with schizophrenia according to DSM-V diagnostic criteria, treated as an outpatient or an inpatient in the psychiatry outpatient clinic and service were included in the study. 100 healthy volunteers were taken as the control group. The procedures to be performed on all patients and control groups participating in the study were explained in detail and an informed consent form was obtained.

Patients with severe comorbidities, alcohol and substance addiction, psychotic disorder due to a general medical condition, psychotic disorder due to substance, psychotic disorders other than schizophrenia such as mood disorders with psychotic symptoms were not included in the study.

2.2. Application of the Research

Two cc blood samples were taken from each individual in the patient and control groups into 2 EDTA tubes. Sociodemography data form, Scale for the Assessment of Positive Symptoms (SAPS), Scale for the Assessment of Negative Symptoms (SANS), Brief psychiatric evaluation scale (CPS), General evaluation of functionality (IGD) scales were applied to 24 patients and their families whose blood was drawn, after a semi-structured interview by the physician who conducted the study.

2.2.1. Sociodemographic data form

This form was developed by the researcher in order to determine the socio-demographic characteristics of the participants. It includes sociodemographic characteristics such as gender, age, marital status, educational status of the participants in the study. In addition, data such as alcohol and substance use, whether they have a medical illness, duration of

illness, number of hospitalizations, and whether there is a psychiatric illness in the family were added to the form. The sociodemographic data form was applied to the participants by the interviewer (See ANNEX-1).

2.2.2. Scale for the Assessment of Positive Symptoms-SAPS

SAPS was developed by Andreasen in 1990 to measure the level, distribution and severity of the positive symptoms of schizophrenia, and its reliability and validity study was performed by Erkoç et al. It includes 4 subscales and 34 items: hallucinations, delusions, strange behavior, and formal thought disorder. Each item is scored between 0-5 and the total score varies between 0 to 170 (Erkoç Ş, 1991; H Soygür et al., 2007; Heidelberg et al., 1991) (See ANNEX-2).

2.2.3. Scale for the Assessment of Negative Symptoms-SANS

SANS was developed by Andreasen in 1990 to measure the level, distribution and severity of the negative symptoms of schizophrenia, and its reliability and validity study was performed by Erkoç et al. It includes 5 subscales and 25 items, including affective blunting, alogy, apathy, anhedonia, and attention deficit. Each item is scored from 0 to 5, and the total score ranges from 0 to 125 (Erkoç Ş, 1991; H Soygür et al., 2007; Heidelberg et al., 1991) (See ANNEX-3).

2.2.4. Brief psychiatric rating scale

It was developed to measure the severity of positive and some depressive symptoms in schizophrenia and other psychotic disorders. Safety and validity studies have not been conducted. Because it is sensitive to change, it can be used to measure change during treatment. While there were 16 items in the original version, it was later increased to 18 items and each item is evaluated with a score 0 to 6. Some items are filled in during the interview, while some items are evaluated within the last 72 hours (H Soygür et al., 2007) (See ANNEX-4).

2.2.5. Overall functionality assessment

It is a scale that helps to see the clinical course of people in its general framework by using a single measure. The overall functionality assessment covers the range from positive mental health to severe psychopathology, is a general measure of patients' psychological, social, and occupational functioning, and is intended to be a general method rather than a

diagnosis-specific scoring system. Functional impairments caused by physical or environmental limitations cannot be evaluated separately. The assessment made with the scale is scored by the clinician 1 to 100 for the current or past period, and the functionality of the person is graded. High scores on the scale mean high functionality (Aas, 2010; Fak et al., 2008) (See ANNEX-5).

2.3. Genetic Analysis

By comparing the genotype data to be obtained from the patient and control groups, it was determined whether there was a significant difference between the two groups as a result of the statistical tests. According to the results obtained from these tests, it was determined whether the SNP examined with schizophrenia disease is related in the sample population, whether there are risk alleles and genotypes that may predispose to schizophrenia in the examined SNP, or whether there are protective alleles and genotypes that are less common in patients.

2.3.1. DNA isolation

DNA samples of volunteers in the patient and control groups were obtained from blood samples collected in EDTA tubes, using ready-made commercial kits based on the colon, according to the method recommended by the manufacturer. In this step, first of all, column-based DNA isolation kits were used, thus minimizing the possible damages of the phenol compound. For the extraction of genomic DNA samples Purelink DNA Mini Kit by Invitrogen (California, USA) was used in this study according to instructions of manufacturer.

The reliability of the method was determined by using commercial kits in real-time PCR and conventional PCR experiments where the quality control of the isolated DNA samples was made.

2.3.2. DNA quality control and quantification and standardization

The isolated DNA samples were read with a spectrophotometer at 260 and 280 nm, and DNA concentrations and the amount of protein that could be contaminated were determined.

The main stocks were preserved by a little separation from all DNA samples, and the concentrations of the portions to be studied were adjusted to 20ng/uL. Standardized DNA

samples were stored in a fixed arrangement of 96 well plates, and the plate position of each sample was recorded. In the later stages, this order was preserved and all analyzes were carried out in this order.

DNA samples standardized to equal concentrations were PCR performed using previously validated PCR primers and PCR kits, and all DNA samples were tested for suitability for this process.

In case of protein contamination in DNA samples above the desired level, protein contamination was removed by passing the samples through the column again or by using the phenol-chloroform method. If the amount of DNA obtained was insufficient, DNA isolation of such samples from blood was repeated.

Inappropriate DNA samples were re-isolated, minimizing the problems that may be encountered during the PCR phase.

2.3.3. Determination of SNP Genotypes by Real-Time PCR

The SNP genotyping tests were done on a Step One Plus Real -Time PCR system from Applied Biosystems (California, USA). Applied Biosystems TaqMan ® Real-Time PCR Genotyping Master Mix (Catalog number: 4304437) and genotyping assay specific for SNP rs117618017 (C_163465265_10) and rs1047552 (C___7544089_10) were used. For genotyping, commercially available "end point fluorescence" based SNP genotyping kits, validated by the manufacturer, were used. For PCR, following conditions applied in each tube:

DNA (10 ng/µl)	2 µl
TaqMan Master Mix	5 µl
TaqMan SNP assay	0.19 µl
dH2O	4.81 µl

2.4. Statistical Analysis

2.4.1. Single SNP association analyses

This analysis was performed to understand if there is any difference between the patients and unaffected parents for the distributions of SNP genotypes and alleles. For this, the

groups of patients and the group of unaffected parents were compared for the screened SNPs, and whether if there is a statistically significant difference between two groups in terms of the frequencies of alleles or genotypes were determined by Pearson's chi-square test or Fisher's exact test. At this stage, H0 for this SNP is: "There is no difference between the patient and parents' groups in terms of the distribution of both alleles for the SNP examined." hypothesis was tested against the alternative hypothesis claiming the opposite, with 0.05 as the cutoff p-value. Then the same comparison was made for genotype frequencies. H0 for this SNP at this stage: "There is no difference between the patient and control groups in terms of the distribution of all three genotypes (homozygous allele 1, heterozygous and homozygous allele 2) for the SNP examined." hypothesis will be tested against the alternative hypothesis claiming the opposite, and the limit p value is 0.05.

2.4.2. Haplotype association analyzes

The haplotypes constructed by the screened SNPs were estimated by Haploview Software Version: 4.2 (Barret et al, 2005)

and confirmed by the manual methods using the patients and parents' genotypes in each family trio. In addition, diplotypes of each subject were determined according to genotypes of the members of family trios. Following these, the groups of probands and unaffected parents were compared to find if there is any significant difference in the distributions of haplotypes and genotypes.

2.4.3. Transmission disequilibrium tests

The family-based association tests were completed by transmission disequilibrium tests (TDT) of SNP alleles and haplotypes. This test was performed by using Haploview Software Version: 4.2.

3. RESULTS

In this study, we investigated the inheritance two SNPs (rs1047552 and rs117618017) located in human APH1B gene in the families of 28 schizophrenia patients (28 family trios) collected from Malatya-TÜRKİYE. General information about these SNPs are given in Table 3.1. We aimed to find the possible genetic associations between the alleles, genotypes and haplotypes of these polymorphisms and schizophrenia. We determined the genotypes of our subjects by a real-time PCR based method. After SNP genotyping, we estimated the haplotypes of individuals and proceeded for the association analyzes in family based association (FBAT) study format.

Table 3.1: General information about rs117618017 and rs1047552 SNPs

SNP	Position	Alleles	MAF (Global)	Amino acid Change	Blossom 62
rs117618017	Chr 15: 63277703	C/T	T: 0.0443	Thr 27 Ile	-2
rs1047552	Chr 15: 63305658	G/T	G: 0.0801	Phe 176 Leu	0*

3.1. Real-Time PCR Genotyping Results

The real time PCR results have been evaluated as multi component plots and allelic discrimination plots to confirm the reliability of Real-Time PCR assays.

The SNP rs1047552 has T and G alleles. T allele is the major allele in the general world population. In our sample, we detected all three possible genotypes (TT, TG, and GG). In Figure-3.1, multicomponent plots for three genotypes of rs1047552 are seen. The TaqMAN probe for T allele is labeled with the fluorescent dye FAM which gives blue illumination and the probe for G allele is labeled with HEX which illuminates green. In the panel of figure, the amplification in the tube of an individual who homozygote for T allele (TT genotype) is seen. B and C panels are the examples for GG and GT (heterozygote) genotypes.

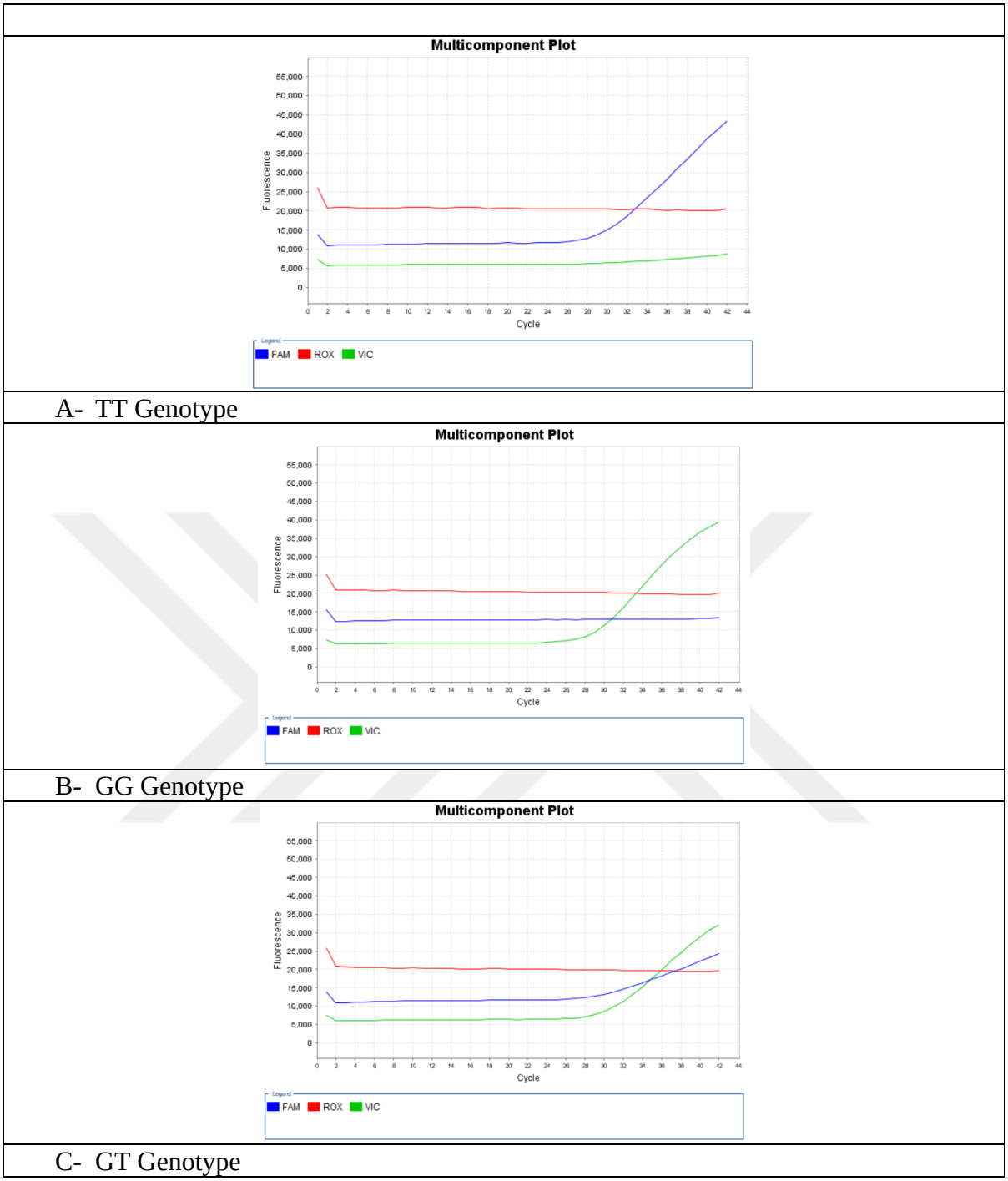


Figure 3.1: Multicomponent plots showing amplifications of rs1047552 alleles in various genotypes

In addition, we also confirmed the genotyping results by evaluating the allelic discrimination plots which shows al samples in the PCR plate together comparatively. Figure 3.2 is the allelic discrimination plot showing the result of rs1047552 SNP. All genotypes to be completely separated indicate our genotyping assays worked efficiently.

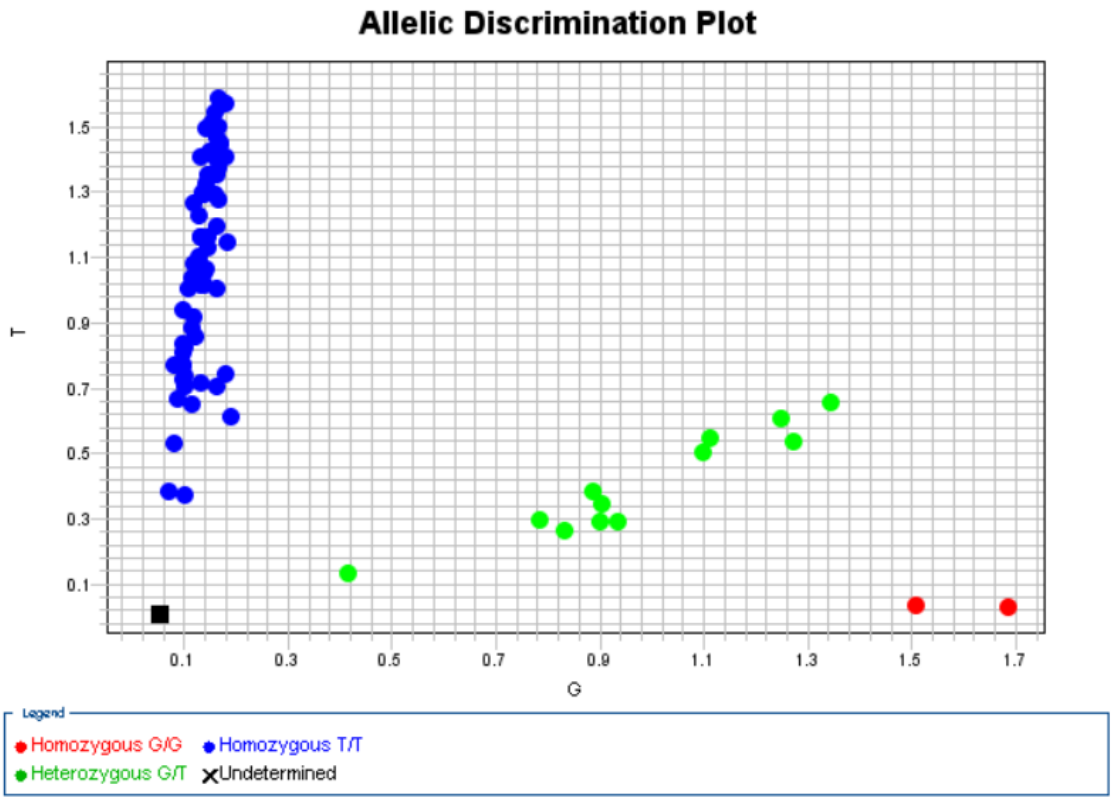
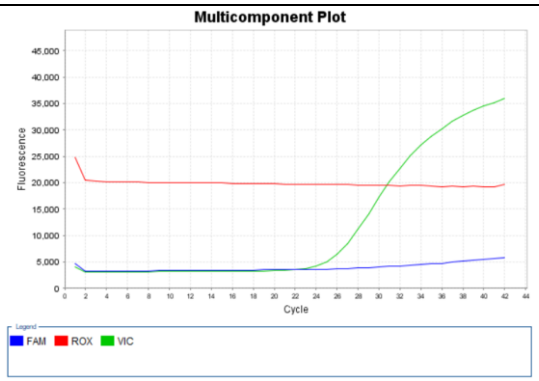
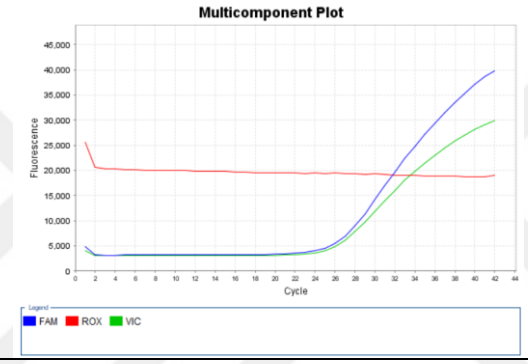


Figure 3.2: Allelic discrimination plot showing the separation of rs1047552 genotypes

The same evaluation steps were done for the second SNP rs117618017 which we have screened in this study. The multicomponent plots showing the amplifications of different genotypes are seen in Figure 3.3. At this SNP position, we had only two genotypes (CC and CT) in our sample. Since the frequency of T alleles low in the general world population, we did not catch any one with TT genotype which was not an unexpected situation.



Rs117 CC



Rs117 CT

Figure 3.3: Allelic Discrimination plots showing distributions of rs117618017 genotypes in the patient and control groups

Again, the signal strengths of both alleles in the amplification multicomponent plots and separation of genotypes in the allelic discrimination plot approves the reliability of our genotyping assay for this SNP.

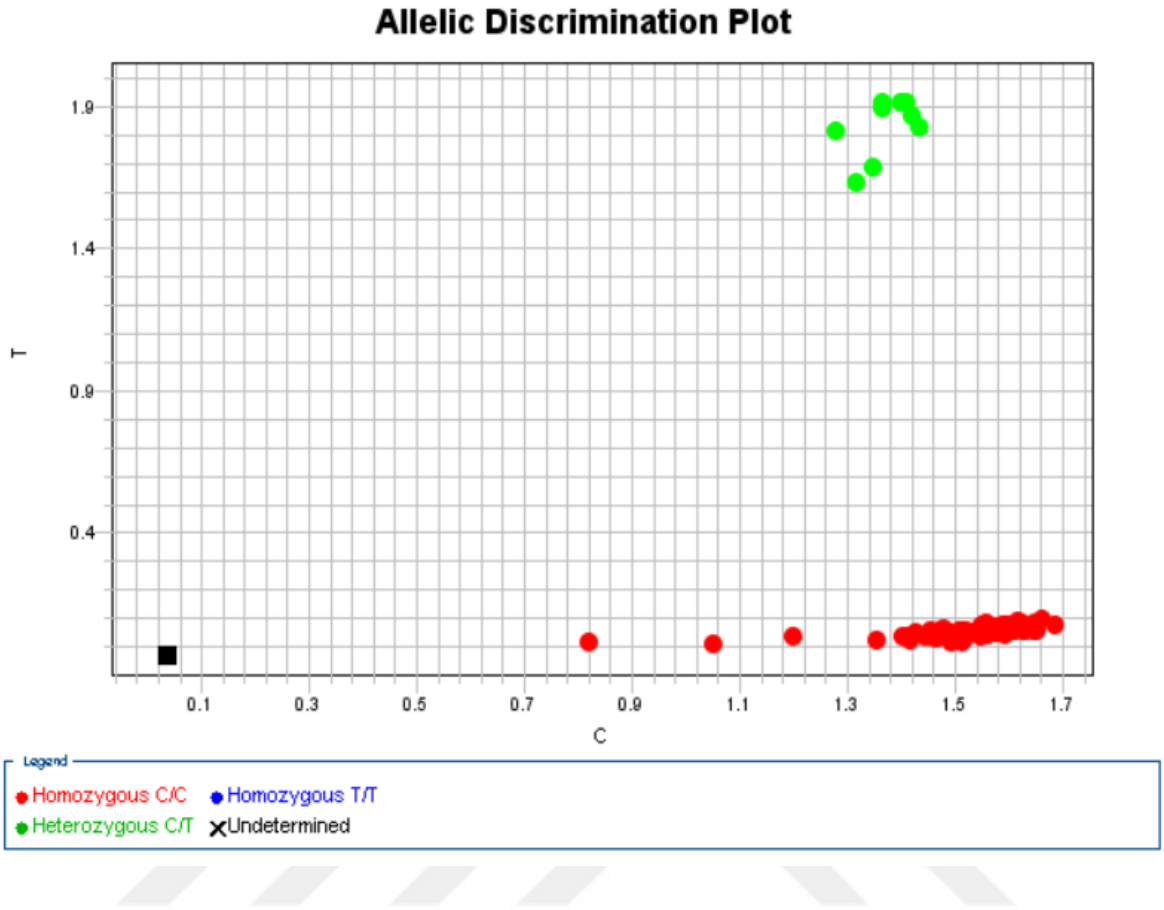


Figure 3.4: Allelic Discrimination plot showing distributions of rs117618017 genotypes

3.2. Analysis of SNP genotyping Results

The distribution of SNP genotypes and alleles in the probands and parents are presented separately as counts (n) and frequencies in Table 3.2. When the affected probands are compared with the parents for frequencies of the genotypes and alleles no significant differences were seen.

Table 3.2: Distribution of genotypes and alleles of SNPs in the probands and parents

	GENOTYPES				**ALLELES**		
rs117618017	**C C n (frequency)**	**C T n (frequency)**	**T T n (frequency)**	**Total**	**C n (frequency)**	**T n (frequency)**	**Total**
Probands	22 (0.88)	3 (0.12)	0 (0)	25	47 (0.94)	3 (0.06)	50
Parents	43 (0.86)	7 (0.14)	0 (0)	50	93 (0.93)	7 (0.07)	100
P-Value	0.81				0.82		
rs1047552	G G n (frequency)	G T n (frequency)	T T n (frequency)	Total	T n (frequency)	G n (frequency)	Total
Probands	0(0)	5 (0.20)	20 (0.78)	25	45 (0.90)	5 (0.10)	50
Parents	1 (0.02)	9 (0.18)	40 (0.80)	50	89 (0.89)	11 (0.11)	100
P-Value	0.86				0.85		

3.3. Haplotype Analysis

We determined the haplotypes manually by evaluating the genotypes of probands and their parents. Also, we used Haploview software for estimating the haplotypes in the group with their frequencies. Both methods produced the same results. The haplotypes we found in the affected probands and their parents are given and compared in Table 3.3. As seen in the table, The frequencies of each haplotype were similar when two groups are compared.

Table 3.3: Counts and frequencies of haplotypes in the pfrobands and parents.

Haplotype Frame: rs117618017- rs1047552				
Haplotypes	**T-C**	**T-T**	**G-C**	Total
Probands	42 (0.84)	3 (0.06)	5 (0.10)	50
Parents	82 (0.82)	7 (0.07)	11 (0.11)	100
p-value:	0.76	0.81	0.85	

3.4. Diplotype Analysis

We also determined the diplotypes (haploid pairs of haplotypes) of each individual and compared their distributions between the groups of probands and parents. The counts and frequencies of each diplotype we have detected in our sampe are given in the Table-3.4 as

counts and frequencies. The comparison of two groups showed the absence of any difference between the affected probands and unaffected parents.

Table 3.4: Counts and frequencies of diplotypes in the probands and parents.

Haplotype Frame: rs117618017- rs1047552						
Diplotype	**T-C-/T-C**	**T-C/T-T**	**G-C/T-C**	**G-C/G-C**	**G-C/T-T**	**Total**
Probands	17 (0.68)	3 (0.12)	5 (0.20)	0 (0)	0	25
Parents	34 (0.68)	6 (0.12)	8 (0.16)	1 (0.02)	1 (0.02)	50
p-value	1	1	0.67	NA	NA	

3.5. Family Based Association Test (FBAT)

At this step, we analyzed the transmission of particular SNP alleles and haplotypes with transmission disequilibrium test (TDT). For this test, we used Haploview software. As seen in the Table 3.5, none of the alleles showed a significant difference in terms of transmission.

Table 3.5: Single Locus TDT Results

SNP	**Over-transmitted**	**Transmitted**	**Untransmitted**	**Chi-Square**	**p-Value**
rs117618017	C	4	3	0.143	0.7
rs1047552	T	5	4	0.111	0.74

We have performed TDT for analyzing the transmission of haplotypes as well In Table 3.6, counts of families that transmitted and untransmitted particular haplotypes are seen and compared. The Chi-Squared test results indicate, there is no significant difference in the transmission of any haplotype to support an association with schizophrenia.

Table 3.6: Haplotype TDT

Haplotype	Transmitted	Untransmitted	Chi-square	p
T-C	8	6	0.286	0.593
G-C	4	5	0.111	0.739
T-T	3	4	0.143	0.705

4. DISCUSSION

In this study, we performed a family based genetic association study (FBAT – Family Based Association Test) in a group of Turkish schizophrenia patients and their parents to test the effect of two SNPs on the schizophrenia phenotype. The SNPs we have investigated were called rs117618017 and rs1047552 and located in the APH1B gene. To our knowledge, there is no previously published research to study the association of these two SNPs in the families of schizophrenia patients from Türkiye.

The Neuregulin pathway is known to affect brain development and its members has effects on schizophrenia. One of the members of this pathway, Aph1B was also reported to be effective in cleavage of neuregulin-1 protein. The deficiencies in the Aph1B protein cause pharmacological and behavioral abnormalities (Dejaegere et al, 2008). Following this, two SNPs in the gene coding for Aph1B gene rs117618017 and rs1047552 became prominent in a case-only study done by schizophrenia samples (Hatzimanolis et al, 2013). The minor alleles of these two SNPs were causing the amino acid changes which were predicted to damage protein structure. Because of that, these SNPs seemed as good candidates to be associated with schizophrenia. Nevertheless, this had to be confirmed by more association studies. To fill that gap in the literature, we tested the association of those two SNPs in a sample collected from Malatya – Türkiye.

We performed this study in family-based association test format (FBAT). For this, we collected the blood samples from the patients diagnosed for schizophrenia and their parents. After extracting their DNA samples, we determined the genotypes of rs117618017 and rs1047552.

We have evaluated the results of genotyping experiments with four methods:

1. The comparison of SNP genotypes and alleles between the groups of affected probands and unaffected parents.

2. The comparison of haplotypes constructed by these two SNPs between the groups of affected probands and unaffected parents.

3. The comparison of diplotypes constructed by these two SNPs between the groups of affected probands and unaffected parents.

4. Transmission Disequilibrium Tests.

As seen in the results, there was no significant difference between the group of patients and the group of unaffected parents for the distributions of genotypes and alleles of rs117618017 and rs1047552. The haplotypes constructed by these two SNPs and diplotypes of those haplotypes did not show any difference between two groups either. Moreover, transmission disequilibrium tests we have performed for the alleles and haplotypes did not indicate any over-transmitted allele or haplotype. All these results indicate, these SNPs were not associated with schizophrenia in our sample.

The association we expected not to be seen in our sample may be caused by several reasons. First of all, the numbers of subjects we used might be under what was necessary to show the difference between the probands and unaffected parents. On the other hand, since schizophrenia has heterogenous clinical manifestation, the molecular basis of our patients may be various genetic factors. Also, connected to this, the multifactorial characteristic of schizophrenia causes many different genes to affect the phenotypes of patients, and even in some of the patients the causative variant was APH1B gene, their number could be too low to show a difference in our sample.

As conclusion, even though we were not able to find an association between the SNPs rs117618017 and rs1047552 of APH1B gene and schizophrenia in our sample, screening of more family trios, or even the screening of the same SNPs in case-control groups may reveal their association with the disease. For the genetic basis of schizophrenia in the family trios studied in this project, screening of more SNPs or other genetic factors, or performing a more comprehensive screening covering the whole genome or at least whole exome should produce more roboust information.

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