TR
INONU UNIVERSITY
INSTITUTE OF SCIENCE

EFFECT OF GENE METHYLATION STATES ON SCHIZOPHRENIA

MASTER THESIS

Ezgi KARAASLAN

Department of Molecular Biology and Genetics

Thesis Advisor: Assoc. Prof. Dr. Ceren ACAR

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WORD OF HONOR

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

Ezgi Karaaslan

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ABBREVIATIONS

5mC	5-methylcytosine
CGI	CpG island
CpG	Sitozin-fosfat-guanin
DNMT	DNA methyltransferase
ECM	Extracellular matrix
MMP	Matrix metalloproteinas
MMP-9	Matrix metalloproteinase-9
ncRNA	Noncoding RNA
SZ	Schizophrenia
T.gondii	Toxoplasma gondii
TSS	Transcriptional start sites
WHO	World Health Organization

ABSTRACT

Master Thesis

EFFECT OF GENE METHYLATION STATUS ON SCHIZOPHRENIA

Ezgi KARAASLAN

Inonu University
Graduate School of Science
Department of Molecular Biology and Genetics

51+ix Pages

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Supervisor: Assoc. Prof. Dr. Ceren ACAR

Schizophrenia is a neuropsychiatric disorder caused by disease in brain development in which genetic and environmental factors play a role. Despite neurobiological, clinical and genetic studies, the interactions and pathogenic mechanisms of the two main etiological factors are not fully understood. Although genetic factors alone have an important effect on the development of the disease, the presence of environmental factors on the genes that play a role in schizophrenia may explain the risk factors of the disease.

Therefore, studies have focused on the effects of genetic and environmental factors on the development of schizophrenia. As environmental factors can cause changes in gene expression through epigenetic mechanisms, it is thought to alter gene expression networks involved in neuronal regulation. Genes are epigenetically activated or silenced in response to environmental factors during or after gametogenesis and embryogenesis. In general, methylation of 5'cytosine in the DNA sequence on gene promoters prevents binding of transcription factors and reduces gene expression. Studies conducted in this direction confirm the results of both postmortem human brain and peripheral blood samples examining changes in DNA methylation in candidate gene promoters.

For this purpose, differences in DNA methylation pattern of Matrix metalloproteinase-9 (MMP-9), which has recently been associated with glutamate regulation and contribute to pathological synaptic plasticity in schizophrenia, were investigated. Pyro-sequencing method was used to determine DNA methylation of MMP-9 at positions CpG7-1, CpG7-2, CpG7-3, CpG7-4, CpG7-5 in peripheral blood cells from 40 schizophrenic patients and 32 healthy samples. MMP-9 methylation changes were compared with the mean MMP-9 methylation of healthy subjects. Significant differences were found in the position of CpG7-3, CpG7-4, CpG7-5 and the mean methylation pattern.

Keywords: DNA methylation, epigenetics, MMP-9, schizophrenia

ÖZET

Yüksek Lisans Tezi

GEN METİLASYON DURUMLARININ ŞİZOFRENİYE ETKİSİ

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Şizofreni, genetik ve çevresel faktörlerin rol oynadığı beyin gelişimindeki bozuklukların neden olduğu nöropsikiyatrik bir hastalıktır. Nörobiyolojik, klinik ve genetik çalışmalara rağmen iki ana etiyolojik faktörün etkileşimleri ve patojenik mekanizmaları tam olarak anlaşılamamıştır. Hastalığın gelişiminde genetik faktörlerin tek başına önemli bir etkisi olmasına rağmen, şizofrenide rol oynayan genler üzerinde çevresel faktörlerin etkisinin bulunması hastalığın risk faktörlerini açıklayabilir. Bu nedenle çalışmalar genetik faktörlerin ve çevresel faktörlerin şizofreni gelişimindeki etkilerine odaklanmıştır.

Çevresel faktörlerin epigenetik mekanizmalar yoluyla gen ekspresyonunda değişikliklere neden olabileceğinden, nöronal regülasyonda yer alan gen ekspresyon ağlarını değiştirdiği düşünülmektedir. Genler, gametogenez ve embriyogenez sırasında veya sonrasında çevresel faktörlere yanıt olarak epigenetik olarak etkinleştirilir veya susturulur. Genel olarak, gen promotörleri üzerindeki DNA dizisindeki 5'sitosinin metilasyonu, transkripsiyon faktörlerinin bağlanmasını önler ve gen ekspresyonunu azaltır.

Bu doğrultuda yürütülen çalışmalar, aday gen promotörlerinde DNA metilasyonundaki değişiklikleri inceleyen hem ölüm sonrası insan beyni hem de periferik kan örneklerinin sonuçlarını doğrulamaktadır. Bu amaçla, son zamanlarda glutamat regülasyonu ile ilişkilendirilen ve şizofrenide patolojik sinaptik plastisiteye katkıda bulunan Matrix metalloproteinaz-9'un (MMP-9) DNA metilasyon paternindeki farklılıklar araştırıldı. 40 şizofreni hastasından ve 32 sağlıklı numuneden alınan periferik kan hücrelerinde CpG7-1, CpG7-2, CpG7-3, CpG7-4, CpG7-5 pozisyonlarında MMP-9'un DNA metilasyonunu belirlemek için pyrosekanslama yöntemi kullanıldı. MMP-9 metilasyon değişiklikleri, sağlıklı kontrollerin ortalama MMP-9 metilasyonu ile karşılaştırıldı. CpG7-3, CpG7-4, CpG7-5 pozisyonunda ve ortalama metilasyon modelinde önemli farklılıklar bulundu.

Anahtar kelimeler: DNA metilasyonu, epigenetik, MMP-9, şizofreni

1. INTRODUCTION

1.1. Schizophrenia

Schizophrenia (SZ) is defined as a complex behavioral and cognitive neuropsychiatric disorder caused by disorders related with genetic and environmental factors in development of brain [1]. Early neurodevelopmental factors, imbalances in neurotransmitter signaling, obstetric complications, infections, stress and exposure to any trauma are among the main causes of the pathology of the disease [2]. Among the neuropsychiatric disorders, SZ is a mysterious subject for researchers, as it is the disease that most negatively affects human life [3]. SZ affects approximately 1% of the world's population [4]. Most of the individuals with SZ are isolated from the social environment over time and cannot continue their work and school life [5].

Similar behaviors that define SZ are found in written documents from ancient Mesopotamia, ancient Greece and Rome, the Middle Ages, the time of the ancient Egyptian Pharaoh and Europe from the fifteenth to the seventeenth centuries [3, 6] . In the early 19th century, knowledge of the mind increased and psychiatry took its place in the medical world [6]. In the 1890s, German psychiatrist Emil Kraepelin divided patients with severe mental disorders into two categories: manic-depressive illness (bipolar) and dementia praecox (SZ) [7].

The term SZ was first used by Eugen Bleuler (1857–1939) at the beginning of the twentieth century. SZ consists of two words of Greek origin. It is formed by combining the words "schizo" meaning split and "phren" meaning mind. The word SZ means mental and emotional splitting of the person [3]. According to Bleuler, SZ has four symptoms. These include; Assocation disorder (thought flow disorders), Affect Disorder (affective disorders), Autism (autism), Ambivalence [8].

With the 20th century, the use of the criteria in the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders and the World Health Organization (WHO) International Classification of Diseases provided a reliable approach to psychiatric diagnoses [1, 8].

1.1.1. Epidemiology

Describing the epidemiology of a disease can help us understand the causes of disease development [9]. The spread of SZ differs in each society. According to researches, it affects approximately 1% of the world's population and causes a lifelong disability [4]. The

prevalence of SZ, which is considered to be the most detailed study conducted in Finland, was 0.87. Prevalence rates vary geographically and range up to five times [10]. According to the data of the WHO, there are approximately 20 million SZ patients in the world [7].

The prevalence of SZ was 1.4–4.6 per 1000 and the incidence 0.16–0.42 per 1000. In a systematic review conducted in Turkey, the lifetime prevalence of SZ was found to be 8.9 per 1000 people [11]. The life expectancy of patients with SZ is 15-20 years shorter than the general population. Deaths due to cardiovascular diseases were recorded more in female patients and deaths due to suicide in male patients. The main causes of high mortality in patients SZ are the prevalence of comorbid medical disorders and low overall healthcare quality [12].

1.1.2. Symptoms of schizophrenia

In SZ, the first episode of psychosis is usually seen in the early twenties, but is possible to be seen in the early adolescence or early forties. SZ has been characterized by various symptoms. The severity and frequency of symptoms may vary from patient to patient, depending on the course of the disease. We can divide these symptoms into four groups as positive, negative, cognitive, and mood symptoms [1, 12].

Positive symptoms consist of hallucinations, delusions, and speech disorders and behavioral disorders. Negative symptoms refer to the decrease or loss of mental functions [5]. These are weakened emotions, poverty of speech, and loss of interest and motivation. Negative symptoms tend to become more chronic. Cognitive symptoms consist of disruptions and deficiencies in working memory, attention, and executive functions such as organization and abstraction [1, 13]. The main clinical features are shown in **Table 1.1.**

Table 1. 1: Clinical features of SZ [1].

Main features	Delusions, hallucinations, impaired motivation, decreased speech
Additional features	Cognitive impairment, episodes of elated and/or depressed mood
Heritability estimate	Roughly 80%
Average age of onset	16–30 years
Drug treatment	Antipsychotics

1.1.3. Schizophrenia risk factors

Season of birth (seasonality of birth)

According to studies conducted for many years, patients with SZ are more likely to be born in late winter and early spring. This is because the second trimester (14.-27. weeks) of pregnancy coincides with the period when influenza is at its highest. It is explained by the increased risk of SZ in offspring due to maternal infections during this period [14].

Birth complications

According to the studies, it divides birth complications into three categories. These are pregnancy complications, abnormal fetal growth and development, and delivery complications [15]. Complications during and after delivery have been reported as a risk factor for schizophrenia. Causes such as emergency cesarean section, bleeding during pregnancy, pregnancy poisoning and low birth weight have been associated with SZ. In addition, the use of forceps and low birth weight suggest that psychosis is associated with an earlier age of onset [16].

Sex differences

Sex difference plays an important role in SZ [17]. According to the researches, it has been concluded that the incidence of SZ is higher in men than women [18]. According to psychiatrists, the age of development of SZ in women is later than men, and the course of the disease is less severe than in men. In the early stages of the disease, negative symptoms are more common in male patients. In some studies, positive symptoms such as

hallucinations were observed with a higher rate in female patients. In studies of structural brain abnormalities in female and male SZ patients, greater abnormalities were found among males [17].

Parental age

Recent studies have shown that advanced parental age is a high risk for SZ [14]. In particular, paternal age is a risk factor. Epidemiological studies have revealed that children of fathers aged 35 and over are 3 fold more likely to develop SZ than fathers at a younger age. Because spermatogonia reproduce much more than oocytes throughout life, and fathers' age is an important risk factor for some autosomal dominant diseases due to new mutations [19, 20]. At the same time, advanced paternal age is probably related to the accumulation of de novo mutations or the bridging of impaired epigenetic regulation with genetic factors [21].

Infections and the immune system

In recent years, the number of studies on infection as a risk factor for SZ has increased. Although SZ is a neurodevelopmental disorder, infection is a possible candidate risk factor since it has been known for a long time that microbial pathogens cause brain abnormalities during pregnancy [22].

It shows that mothers exposed to an influenza epidemic in the second trimester of pregnancy are in a higher risk group for the development of SZ [14]. Rubella in the prenatal period is the cause of some childhood psychiatric disorders. A study was conducted with the assumption that rubella passed during pregnancy may also increase the risk of SZ during adulthood. In the study conducted by Brown in New York, the risk of SZ in children born to pregnant mothers who were diagnosed with rubella was examined. And as a result, it was found that 20% of individuals with prenatal measles were diagnosed with adult SZ [23].

Toxoplasma gondii (T.gondii), an intracellular parasite, is a major cause of miscarriage and stillbirth after primary infection in pregnant women [24]. In the Child Health and Development Study cohort, maternal serum testing showed a two-fold higher risk of SZ among individuals exposed to high maternal T.gondii immunoglobin G (IgG) antibody in utero [25]. Even though these findings provide the strongest evidence to date that prenatal infection contributes to the risk of SZ, the mechanisms by which infections may cause SZ are not adequately explained [23].

Ethnicity

Ethnicity defines shared history such as religion, race and country. Ethnic origin, which is affected by biological, social and cultural factors, is not an indicator of genetic differences alone [14, 26]. In fact, people from ethnically disadvantaged groups are more likely to be exposed to different environmental toxins, such as heavy metals or dangerous chemicals. Similarly, disadvantaged ethnic groups have life in lower socioeconomic groups, conditions more conducive to the spread of infections, and difficulty accessing medical systems. Still, the data linking these factors to schizophrenia are unclear at best [27].

Cannabis

Cannabis is the most widely used illegal drug [28]. Studies show that SZ patients are more likely to use cannabis. Lifetime cannabis use was observed in 64.4% of SZ patients. People who use cannabis are 2 to 25 times more likely to develop SZ than non-users [14, 29]. According to Arsenault et al., people who used cannabis during adolescence were found to have twice the risk of schizophrenic disorder later on [30].

Cannabis use is a risk factor for SZ as it will affect brain function through biochemical changes. Therefore, it may also have an effect on cognitive functioning in patients with SZ. Cannabis use can precipitate or even cause an episode of SZ [14, 29]. According to some studies, there is evidence that SZ symptoms will decrease when cannabis use decreases [28].

Urban and rural life

There is evidence that urban life is associated with SZ compared to rural life [18]. According to research conducted in Chicago in the 1930s, applications for SZ came mostly from downtown. These and similar results have shown that people with SZ tend to migrate to the city. Individuals born in urban centers have a 2 to 4 times higher risk of developing SZ than in rural areas [14]. In a Danish study, there is evidence that the number of years spent in childhood in an urban area increases the risk of developing SZ [18].

Migration

Studies on immigrant populations have found high incidence rates of SZ. Examples include people of African Caribbean descent in the UK, Surinams in the Netherlands, people of Dutch Antilles and Moroccan descent, and several immigrant groups in Denmark. This hypothesis, proposed by Odegaard in 1932, showed that the admission of Norwegian

immigrants to the United States for initial SZ was twice that of Native Americans and Norwegians living in Norway [9].

1.1.4. Role of genetic factors in schizophrenia

The diagnosis of SZ is not made by any laboratory test but by clinical observation [19]. Once diagnosed with SZ, medical follow-up is important to understand any problems that may develop with the disease. Since there are many factors affecting the etiology of SZ, it is difficult to attribute a single cause of the disease [31]. It is not surprising to us that SZ has a mixed foundation. Because the brain is the most complex of all organs and our knowledge about brain functions is limited [32].

SZ has long been known to have a genetic component with non-Mendelian inheritance [33]. The inheritance rate of SZ is estimated to be approximately 80%. When we examine the family histories of the patients, the risk of SZ is higher than the general population. Studies have shown that biological relatives of these patients have a risk of SZ. Especially in monozygotic twins and first degree relatives, this rate was seen as 50%. When we look at these rates, the importance of inheritance is high in SZ. This strong and consistent evidence leads researchers to investigate the genetic basis of SZ [31, 34].

Genetic risk factors are affected by external factors and create genetic susceptibility that will trigger the symptoms of the disease [35]. However, the pathological architecture of SZ is not explained by genetic abnormality. Understanding how genetic factors influence the development of SZ is important to better understand the role of environmental factors in disease [36].

The effect of genetic and environmental risk factors on the etiology of SZ can also be examined using adoption studies [37]. In all adoption studies, the risk of SZ has increased in biological relatives of patients with SZ. For example, in 361 families in Finland, 4.9% of the adopted children of schizophrenic mothers were found to have SZ [38].

There are genetic risks in the genetic makeup of SZ caused by different DNA sequence variations. The most studied and known are single nucleotide polymorphism and copy number variants [33]. Findings from recent studies have shown that differences in DNA methylation patterns may be the cause of monozygotic twin mismatch. It has been thought that there may be a mechanism for various environmental risk factors for SZ [19].

It is unlikely that the disease is associated with a single gene. It is believed that there is more than one gene, and the effect of each gene is low. Interactions between genes and between gene products as well as interactions with environmental risk factors are likely to contribute to disease risk [32, 39].

1.2. Epigenetic

The term epigenetics was first used in 1942 by Conrad Waddington, an embryologist and developmental biologist. Epigenetics is a combination of the words genetics and epigenesis and means on top of the genes [40, 41]. Holiday and Pugh first described DNA methylation, one of the epigenetic modifications, in the 1970s [42]. With the discovery of the regulation of X inactivation and genomic imprinting in mammals by epigenetic mechanisms some time after these definitions, the hereditary structure of epigenetic gene editing mechanisms has been emphasized. Thus, in the 1990s, epigenetics was defined as the study of inherited changes in gene expression that occur without a change in DNA sequence, including modification of DNA methylation and remodeling of chromatin [43, 44].

Epigenetic mechanisms are an inherited way of regulating a variety of genomic functions, including gene expression, by covalent modifications of DNA, histones, and non-coding RNAs (ncRNAs). [45]. Several different epigenetic regulation mechanisms are known. The nature of these different mechanisms is not fully understood. Each of the mechanisms interacts with each other. These are:

(a) DNA methylation;

(b) modification of histone molecules by a series of chemicals (eg methylation, acetylation, ubiquitination);

(c) exchange of histone molecules with the corresponding isoforms;

(d) regulation of access to DNA by manipulation of chromatin by nucleosome reshapers [46].

The best known modifications of these epigenetic mechanisms are DNA methylation, histone modification, and non-coding RNAs. One feature of epigenetic modifications is that they can be inherited. Nutritional style and environmental factors may cause cells with the same DNA to differ phenotypically [47]. Understanding how epigenetic mechanisms are initiated, maintained, and inherited is an important issue in biological research [48]

Although all cells in an organism contain the same genetic information, not all genes are expressed by all cell types at the same time and at the same rate. Epigenetic mechanisms mediate diversified gene expressions in various cells and tissues in multicellular organisms [43]. Among the epigenetic mechanisms, DNA methylation and histone modifications play an important role in gene regulation and carcinogenesis [49].

Epigenetic mechanisms tell the cell to correctly interpret genes and signals from the environment. During cell division epigenetic modifications are chemically stable and preserved, thus the phenotypic compatibility with the daughter cell is maintained. This modifications take part in chromatin packaging and depending on the modification they either alow or inhibit inhibit the accessibility of genes to regulatory proteins [41].

Researching the effects of environmental factors on human health is one of the important research topics of biological sciences [50]. According to the evidence obtained from many studies, it is accepted that both genetic and environmental factors play an important role in the pathogenesis of SZ [51].

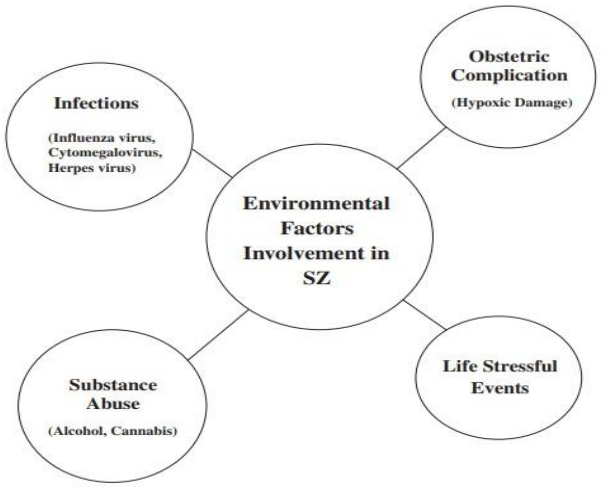

Figure 1. 1: Environmental factors involved in pathogenesis of SZ [52].

Environmental risk factors are the mechanism that affects cells during the formation and development of the disease in individuals at risk of SZ [53]. Regulation of gene expression in the nervous system is not only controlled by transcriptional mechanisms. Many epigenetic mechanisms play a role in the regulation of gene expression [54]. Epigenetic mechanisms are involved in regulating important functions related to the brain, such as neurogenesis, neurodegeneration, neuronal activity, and cognition. Epigenetics is an

important factor in understanding the genesis and development of SZ and other psychotic disorders. Environmental risk factors for SZ are active in critical periods for early neurodevelopment, where significant neurogenic reprogramming processes lead to cell replication and tissue differentiation. Environmental factors may continue to shape epigenetics throughout adulthood and lifetime [55].

Epigenetic processes are dynamic and regulate cell type-specific gene programs, enabling cells to respond to environmental factors. Epigenetic changes over time indicate familial clustering. This may explain the clustering of some common diseases in families, so the epigenetic model may play a critical role in transmitting 'susceptibility' across generations [2].

Most environmental factors appear to affect brain development in general without specificity to SZ [56]. Increasing genetic studies and a large number of epidemiological data in recent years show that environmental factors are important in causal investigation of major psychological disorders [20].

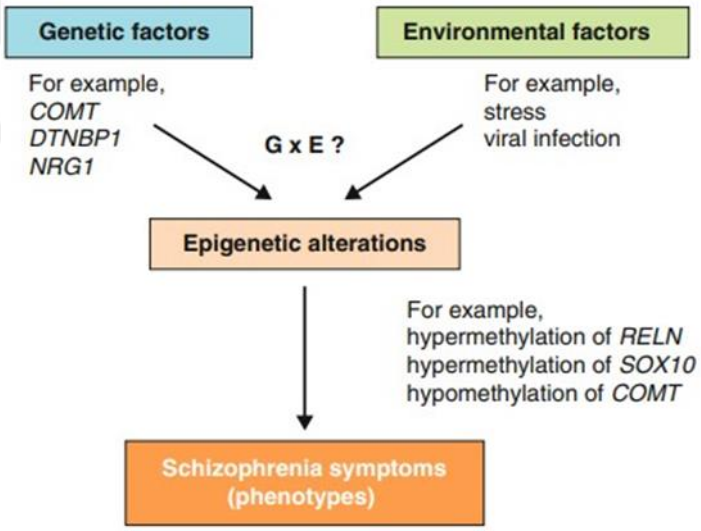

Figure 1. 2: A schematic view of the possible relationship between SZ symptoms, epigenetic modifications and other factors. Genetic and environmental factors may influence epigenetic status independently or through interactions with each other, and in this case some epigenetic changes may lead to SZ symptoms. Epigenetic modifications may also be the way to partially explain gene-environment interactions (G × E). [57].

1.2.1. Psychiatry and epigenetics

The importance of epigenetics in psychiatric diseases increased after the discovery of mutations in genes involved in the epigenetic mechanism in neurodevelopmental disorders such as Rett and fragile X syndromes [41, 57].

Aside from changes in DNA sequence, there is growing evidence that epigenetic modifications of DNA and histone proteins can contribute to complex phenotypes. Epigenetic factors are stable and have regulatory roles in a large number of genomic activities. Therefore, epigenetic psychiatric disease makes it a promising way of research in etiological studies [45].

SZ is generally thought to result from the coexistence of genetic risk polymorphisms or mutations that interact with dangerous environmental factors. In order to understand the etiology of SZ, genetic relationship and linkage studies have been carried out in recent years. However, these studies resulted in poor replication and risk alleles with small effect sizes. The association of psychiatric diseases with epigenetics is based on two main findings. First, the evidence we have obtained from research shows that epigenetic regulation is below normal cognition and cognitive dysfunction occurs as a result of misregulation of the epigenetic mechanism. Second, epigenetic research provides new insights into the inherited and non-inherited factors of psychiatric disorders [45].

Considering that epigenetic modifications are sensitive to environmental factors and reversible, epigenetic research gain importance in the area of psychiatry [41]. Recent studies have revealed that epigenetic mechanisms, especially DNA methylation, generate and sustain behavioral changes in animal models. Therefore, it is very important to characterize basic signaling and neuronal circuit processes. Experimental and clinical studies are needed to understand the molecular mechanisms of gene-environment interaction that increase the risk of SZ [58].

We know that epigenetic modifications can be involved in some of the molecular changes associated with SZ, but there are some difficulties in this area. These are: there is no clear link between SZ and epigenetic changes, lack of cell-specific chromatin analysis that prevents identification of epigenetic changes in specific cell populations and insufficient knowledge of the stability or rotation of epigenetic markers on certain chromatin loci [59].

1.2.2. DNA methylation

We know that DNA methylation occurs in mammals ever since DNA was identified as genetic material. In 1948, Rollin Hotchkiss discovered the modified cytosine using paper chromatography and suggested that the modified cytosine naturally existed in DNA. Until the 1980s, there were not many studies showing that DNA methylation plays a role in gene regulation and cell differentiation. However, we now know that DNA methylation is one of the most important epigenetic mechanisms regulating gene expresion [43]. Especially, DNA methylation ensures that the life-long and normal expression pattern in multicellular organisms is maintained [60].

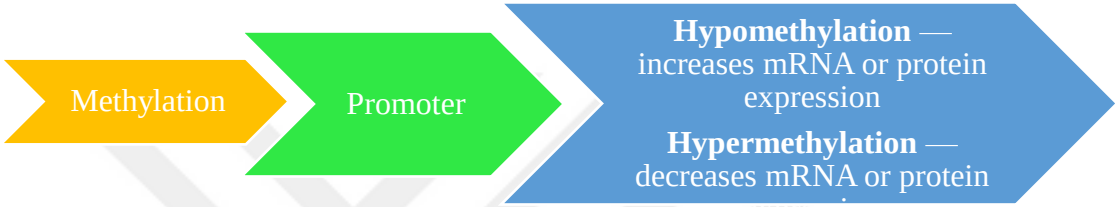

Figure 1. 3: Effect of DNA methylation on promoter regions.

DNA methylation is the best known epigenetic mechanism. It is the addition or removal of a methyl group to a cytosine nucleotide in a DNA sequence by covalent bonds [47]. Although different species have been found in prokaryotes and some eukaryotes, the DNA methylation process usually takes place in specific dinucleotide regions called CpG (cytosine- phosphate-guanine) islands (CpGs) [61].

Some methylation patterns are inherited and are dynamically reshaped at different reprogramming stages throughout an organism's life cycle. DNA methylation acts as a suppressor signal in gene transcription. Methylated DNA regions inhibit the binding of transcription factors and initiate chromatin compression leading to gene silencing [62]. DNA methylation is a more stable epigenetic modification compared to histone tail modifications, which are generally readily reversible [63]. Like DNA sequences, DNA methylation patterns are transferred from maternal chromatids to daughter chromatids during mitosis, and this is called the epigenetic inheritance system [64].

Changes in the amount and distribution of 5-methylcytosine (5mC) in human DNA are of great importance for development and diseases [65].

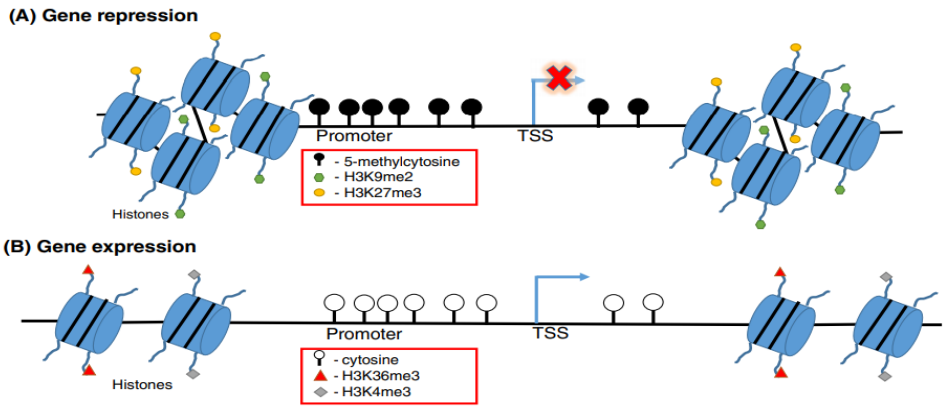

Figure 1. 4: Epigenetic regulation of A) gene repression and B) gene expression [66].

1.2.3. Enzymes involved in DNA methylation mechanism

DNA methylation is catalyzed by a family of enzymes called DNA methyltransferases (DNMTs). The DNMT family includes DNMT1, DNMT3 (3a and 3b) and the newly recognized DNMT 3L. DNMT1 is considered to be the main enzyme that catalyzes methylation. It is involved in the maintenance of DNA methylation during cell division. DNMT1 is ubiquitously expressed in somatic tissues [60, 61]. DNMT1 is approximately 1620 amino acids long [67]. DNMT2 shows weak DNA methyltransferase activity in vitro [68]. DNMT3A/3B performs *de novo* DNA methylation during embryonic development and determination of cell fate [60, 61]. DNMT3a and DNMT3b have substantial primary structural homology. However, they are encoded by different genes that are mapped to different chromosomes. DNMT3 enzymes have architectural features similar to DNMT1, with a large N-terminal regulatory region attached to a C-terminal catalytic domain. DNMT3a has a lower level of methyltransferase activity than DNMT1. DNMT3b and DNMT3a, which have different expression patterns, do not perform de novo methylation at the same degree [67].

1.2.4. CpG islands and patterns of DNA methylation across the genome

More than half of the genes in vertebrate genomes contain regions rich in short CpG known as CpG islands (CGI). Most of the recent studies on DNA methylation has been done on CGIs at transcriptional start sites (TSSs) and attempts to unravel the general function of DNA methylation [69]. 60% of human genes have CGI promoters and 70% of these promoters are methylated. Genes with tissue-specific transcribed CGI promoters are expressed in early embryos and later in somatic cells [69, 70].

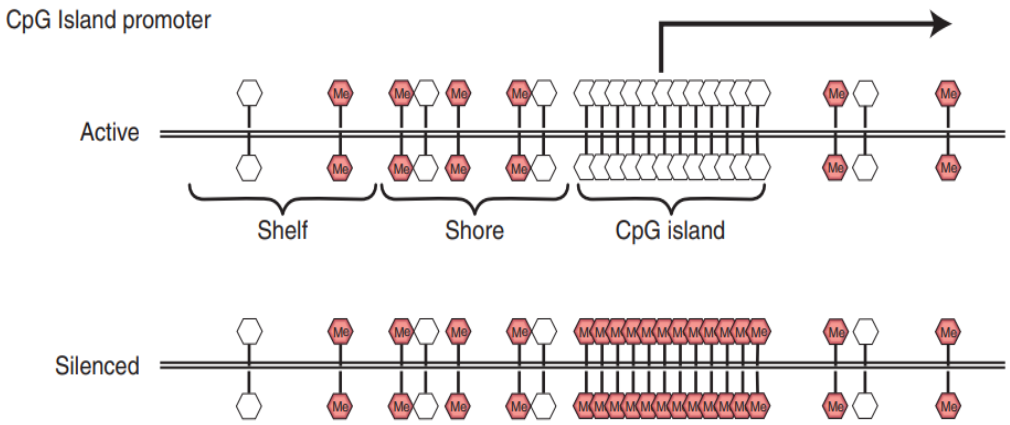

Figure 1. 5: CGIs are regions of high CpG density [71].

To briefly summarize what we know about DNA methylation in mammals: Most CGI are not methylated when found at TSSs. The methylation that takes place on the CGI of TSSs is associated with silencing in long term. Although non-CGI methylation is more dynamic and more tissue-specific than CGI methylation, its function in mammals is currently unknown [69].

1.2.5. DNA methylation in schizophrenia

Epigenetic mechanisms have an important place in neurodevelopment. Neuroscience research has shown its important role in brain development, synaptic flexibility, learning, and memory. For these reasons, epigenetic research within psychiatry has recently gained traction [72]. DNA methylation has important functions in the brain. There are two important processes in neurogenesis that occur during development and in adults. These are: forming the fates of cells in the central nervous system and regulating gene expression programs depending on the signals received by the cell [73].

The fact that the SZ genetic risk locus is located in non-coding regions such as introns and promoter regions suggests that gene regulation plays a critical role in the development of the disease [74]. However, most of the SZ risk loci are associated with gene expression and these epigenetic modifications have shown that genetic risk is important in the pathogenesis of SZ [75]. Many genetic susceptibility loci associated with SZ have been identified, but these alone are insufficient to explain the high heritability estimates of schizophrenia. SZ is characterized by gene expression levels in the cerebral cortex and other brain regions. Genes with irregular expression in the brain are expected to show epigenetic changes in their regulatory regions [2]. To date, it is supported by a postmortem human brain

study focusing mainly on changes in DNA methylation in candidate gene promoters [63]. Among these studies, RELN, GAD1, COMT and BDNF were the most investigated genes [75].

Matrix metalloproteinase 9 (MMP-9) is another gene that plays an important role in schizophrenia, and DNA methylation studies on this gene are limited. In this study, we aimed to examine the DNA methylation model of the MMP9 gene and bring it to the literature.

1.3. Matrix Metalloproteinase-9 (MMP-9)

Proteases are one of the five main catalytic classes (metalloproteinases and serine, cysteine, threonine, and aspartic proteinases), and metalloproteinases are the largest class [76]. Matrix metalloproteinases (MMPs) are a large group of zinc-dependent proteases tasked with separating and rebuilding connective tissue components such as collagen, elastin, gelatin, and casein. The extracellular matrix plays a role in the organization and differentiation of cells, and acts as a barrier against microorganisms and information exchange between cells. MMPs play an important role in the degradation of the extracellular matrix (ECM) during developmental steps such as growth and morphogenesis [76, 77]. MMPs also play a role in processing bioactive molecules such as cell surface receptors, neurotrophic factors, chemokines, and cytokines. Consequently, MMPs participate in cell signaling and remodeling of the pericular microenvironment through the release or processing of cell surface receptor ligands [78]. MMPs are a family of zinc-dependent enzymes that are active at pH 5, primarily secreted as proenzymes (inactive zymogens) or pro-MMPs in their latent form, and require proteolytic activation [79].

MMP-9 is found in cells in vesicles dispersed throughout microtubules and microflaments, and is secreted in a Golgi-dependent pathway in vesicles [80].

MMPs are mosaic proteins containing an N-terminal prodomain, C-terminal hemopexin-like domain, and catalytic Zn^{2+} protease domain [81]. ECM remodeling plays a role in many physiological (eg, embryogenesis, tissue remodeling, wound healing, angiogenesis) and pathophysiological (eg, atheroma, arthritis, metastasis, fibrosis, heart disease) processes [80, 82]. MMPs do not only degrade extracellular matrix (ECM) proteins related to nervous system physiology. It is known to also activate growth factors and their receptors, cytokines and ECM receptors [83].

MMP-9 is encoded by a gene located on chromosome 20 (20q13.12) in the human genome and also encodes a signal peptide. It is a type IV collagenase and 92-kDa protein belonging to the family of zinc and calcium-dependent endopeptidases. It is also known as gelatinase B as it can cleave down gelatin [76, 83].

MMP-9 plays a crucial role in synaptic plasticity underlying brain pathologies [84]. Investigation of MMP-9's roles in physiology and pathology in the brain is critical for new therapeutic and diagnostic methods [76].

MMP-9 mRNA and protein expression was detected at low levels in various regions of the brain, mainly in neurons, such as the hippocampus, cerebellum, and cerebral cortex. Levels of MMP-9 mRNA and protein increase as a result of various stimuli. These increase may result from brain infiltration of both MMP-9 originating from brain cells and leukocytes if the blood-brain barrier is compromised [76]. MMP-9 activity and mRNA is present near synapses [80].

MMP-9 is the most well characterized matrix metalloproteinase in the central nervous system. It is expressed by neurons in adult brain and glial cells. It is released in response to increased neuronal activity under physiological and pathological conditions [78].

1.3.1. MMP-9 in schizophrenia

Evidence from studies on the genetic basis of SZ targets an important role region that was not suspected until recently: the extracellular matrix (ECM). Evidence of genetic susceptibility to abnormalities affecting ECM and ECM-related factors further supports ECM abnormalities in SZ [85]. Adult brain ECM consists of hyaluronic acid, glycoproteins and chondroitin sulfate proteoglycans (CSPGs) predominantly belonging to the lectic family. Some components of the ECM can express a variety of basic biological functions on proteolysis. Degradation of ECM components by MMPs can alter cellular behavior and phenotypes [86].

SZ is thought to be associated with changes in neuronal circuits, disruption of synaptic connectivity, and changes in dendritic spines [78]. In recent studies, we know of MMP-9 as a molecule that contributes to pathological synaptic plasticity in SZ, but the mechanisms affecting the underlying SZ pathology still remain unclear [84]. MMP-9 plays an important role in brain disorders with its ability to process and activate various cytokines and chemokines involved in immune and inflammatory responses [87].

MMP-9 has been seen as a potential risk factor in recent studies examining the pathology of SZ. MMP-9 is widely found in neurons, astrocytes and glia, albeit at low levels in the brain under normal physiological conditions. MMP-9 plays an important role in the development and maturation of neural circuits and homeostatic regulation of their integrity in the normal brain. Therefore, the dysregulation of MMP-9 expression may contribute to the onset of SZ by disrupting neurodevelopmental processes. Deficiencies of synaptic connections between neural circuits are thought to be an essential pathophysiological feature of SZ. It has been suggested that these deficiencies are the result of abnormal developmental synaptic pruning. In this case, it causes the onset of the disease in late adolescence and early adulthood. Increasing evidence suggests that MMP-9 is involved in postnatal fine tuning of brain circuit maturation by regulating synaptic pruning [88].

Mental health disorders occur when homeostasis is lost or impaired and with abnormalities in thoughts, perceptions, feelings, behaviors, and relationships with others. The increase in the number of individuals with mental disorders in populations has become an important and increasing research interest [80].

MMP-9 concentrations in patients diagnosed with SZ are higher than healthy controls in most published studies [88-90]. MMP-9blevels were found to be higher in patients with SZ not taking medication than controls, and previous use of antipsychotics was associated with higher MMP-9 concentrations [91]. MMP-9 levels were higher in resistant SZ groups who were on clozapine treatment compared to healthy controls [92].

The study conducted by Gao et al. in 2018 is the first DNA methylation analysis performed in the MMP-9 gene in patients with SZ. This study showed that the epigenetic pattern and gene expression of MMP-9 of peripheral blood mononuclear cells were different between SZ and its groups [93]. More studies are needed on the specific, mechanical role of MMP-9 in neuropsychiatric disorders that may help develop new groups of psychiatric drugs [94].

1.4. Analysis of DNA Methylation by Pyrosequencing

DNA sequencing is one of the most important platforms for molecular biology studies. As a result of increasing biological studies, the need for alternative DNA sequencing methods has increased and studies have been carried out by different research groups to develop new methods [95].

Many DNA sequencing methods have been found from the past to the present. However, Sanger and pyrosequencing are more preferred among these methods today. In the Sanger method, DNA fragments of different lengths are synthesized by including both nucleotides and dideoxy terminators. Approximately 800 bases can be read by the Sanger sequencing method [96]. In addition to its many advantages, the Sanger sequencing method has some limitations. As a result of increasing biological studies, the need for alternative DNA sequencing methods has increased and studies have been carried out by different research groups to develop new methods. The Pyrosequencing method is a new DNA sequencing technology first developed at the Royal Institute of Technology (KTH) and is the first alternative to the Sanger method for de novo DNA sequencing [95].

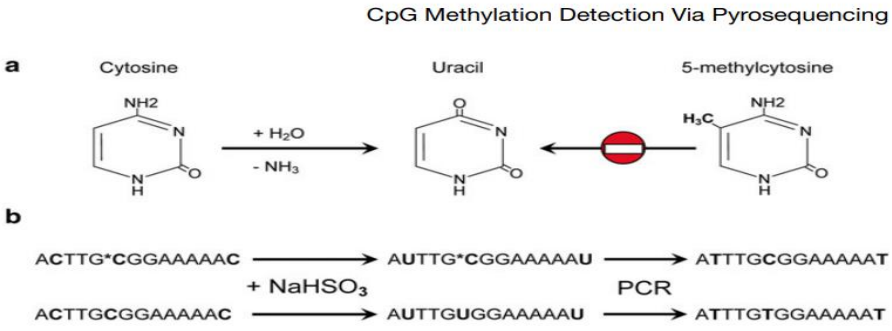

Figure 1. 6: Deamination of cytosine via sodium bisulfide conversion.

2. MATERIALS AND METHODS

2.1. Collection of Samples

We investigated the methylation status of the MMP-9 gene in a group of SZ patients and individuals without SZ symptoms (healthy controls) in the population of Malatya-Turkey. The case group consisted of 40 individuals diagnosed with SZ, and the control group consisted of 32 individuals without SZ symptoms. Patient samples were provided by Malatya İnönü University Faculty of Medicine Department of Psychiatry faculty member Prof. Dr. Şükrü Kartalcı. Relatives of the patients were not included in this study. The samples in the control group samples were selected from individuals who did not have a history of psychiatric illness in themselves or in their families. Blood samples were collected after informed consent was signed by the participants. Our study was approved by the Inönü University Malatya Clinical Research Ethics Committee (research protocol code: 2020-96). The patient samples were given a code number.

2.2. DNA Isolation

Blood samples were isolated with a commercial DNA isolation kit according to the manufacturer's protocol. This protocol consists of four basic steps; blood lysate, DNA binding, DNA washing and DNA elution.

The protocol steps are as follows:

1. 20 µl of proteinase K was pipetted to the bottom of a sterile 1.5 ml microcentrifuge tube and 200 µl of whole blood sample was added to the microcentrifuge tube.

2. 200 µl Buffer AL was added to sample and vortexed for 15 seconds to obtain a homogeneous solution.

3. Sample was incubated at 56 ° C for 10 minutes.

4. After incubation, the tubes were centrifuged to remove drops from the inside of the lid of the microcentrifuge tube.

5. 200 µl of ethanol (96-100%) was added to the sample and mixed by pulsing vortexing for another 15 seconds.

6. After mixing, the tubes were centrifuged to remove the drops inside the cap.

7. The mixture from the blood lysate was transferred to the QIAamp Mini Spin column without wetting the rim.

8. The samples were centrifuged at 6000 x g (8000 rpm) for 1 minute.

9. The spin columns were placed in a clean collection tube and the tube containing the precipitate discarded.

10. 500 µl of Buffer AW1 was added and the tube was centrifuged at 6000 x g (8000 rpm) for 1 minute.

11. The spin column was placed in a clean collection tube and the collection tube.

12. 500 µl of Buffer AW2 was added without wetting the rim.

13. The samples were centrifuged at full speed for 3 minutes.

14. The spin column was placed in a clean 1.5 ml microcentrifuge.

15. 150 µl of Buffer AE or distilled water was added and incubated at room temperature for 1 minute and then centrifuged at 6000 x g (8000 rpm) for 1 minute.

16. In a second elution step, 50 µl of Buffer AE or distilled water was added and incubated at room temperature for 1 minute, and then centrifuged at 6000 x g (8000 rpm) for 1 minute.

17. The obtained DNA was stored at +4 ° C.

2.3. DNA Quantification

The concentrations of the DNA samples were measured by reading the absorbances below 260 nm and 280 nm. The concentrations of all DNA samples were adjusted to 10 ng / µL. The DNA was stored at 4 ° C for methylation analysis.

2.4. DNA Methylation Analysis

EpiTect Bisulfite procedure basic steps steps:

- bisulfite-mediated conversion of unmethylated cytosines,
- binding the transformed ssDNA to the membrane of an EpiTect spin column,
- washing,
- desulfonation of membrane-bound DNA,
- washing the membrane-bound DNA to remove the desulfonation agent,

- and elution of pure, transformed DNA from the spin column.

2.4.1. Sodium bisulfite conversion of unmethylated cytosines in DNA

Bisulfite DNA Conversion

1. DNAs to be used in bisulfite reactions were solved. By adding 800 µl of RNase-free Water to each part, required number of Bisulfite Mixture was dissolved. The Bisulfite Mixture was vortexed until completely dissolved.

2. Bisulfite reactions were prepared in 200 µl PCR tubes. Order of the components added is listed in the Table 2.1 bellow.

Table 2. 1: Bisulfite reaction components

Component	**Volume per reaction (µl)**
DNA solution (1 ng – 2 µg)	Variable* (maximum 20 µl)
RNase-free water	Variable*
Bisulfite Mix (dissolved), see step 1	85
DNA Protect Buffer	35
Total volume	140

3. PCR tubes were capped and the bisulfite reactions mixed well. Tubes were stored at room temperature (15-25 ° C).

4. Bisulfite DNA conversion was performed in a thermal cycler. The program used thermal cycler is given in **Table 2.2.**

Table 2. 2: Bisulfite conversion thermal cycler conditions

Step	**Time**	**Temperature**
Denaturation	5 min	95°C
Incubation	25 min	60°C
Denaturation	5 min	95°C
Incubation	85 min	60°C
Denaturation	5 min	95°C
Incubation	175 min	60°C
Hold	Indefinite*	20°C

5. PCR tubes containing the bisulfite reactions were placed in the thermal cycler and the thermal cycle incubation was initiated.

2.4.2. Cleanup of Bisulfite Converted DNA

1. 560 µl of freshly prepared Buffer BL containing 10 µg / ml carrier RNA was added to each sample. The solutions were vortexed and then centrifuged briefly.

Buffer BL is prepared according to the **Table 2.3.**

Table 2. 3: Carrier RNA and Buffer BL volumes

Number of samples	**1**	**4**	**8**	**16**	**24**	**48**
Volume of Buffer BL*	620 µl	2.5 ml	5 ml	10 ml	15 ml	31 ml
Volume of carrier RNA solution**	6.2 µl	25 µl	50 µl	100 µl	150	310

2. The required number of EpiTect spin columns and collection tubes were placed in a rack. The entire mixture from each tube in step 1 was transferred to the corresponding EpiTect spin column.

3. The spin columns were centrifuged for 1 minute at maximum speed. The flow was discarded and the spin columns were placed back in the collection tubes.

4. 500 µl of Buffer BW was added to each spin column and centrifuged for 1 minute at maximum speed. The flow was discarded and the spin columns were placed back in the collection tubes.

5. 500 µl of Buffer BD was added to each spin column and incubated for 15 minutes at room temperature.

6. The spin columns were centrifuged for 1 minute at maximum speed. The flow was discarded and the spin columns were placed back in the collection tubes.

7. 500 µl of Buffer BW was added to each spin column and centrifuged for 1 minute at maximum speed. The flow was discarded and the spin columns were placed back in the collection tubes.

8. Step 7 was repeated once.

9. The spin columns were placed in the new 2 ml collection tubes and the spin columns were centrifuged for 1 minute at maximum speed to remove residual liquid.The spin columns were placed in clean 1.5ml microcentrifuge tubes with open caps and the spin columns were incubated for 5 minutes at 56 ° C in a heating block.

10. Spin columns were placed in clean 1.5 ml microcentrifuge tubes. 20 µl of EB Buffer was dispensed into the center of each membrane. The purified DNA was eluted by centrifugation at approximately 15,000 xg (12,000 rpm) for 1 minute.

2.4.3. Polymerase chain reaction

1. A master mix was prepared by adding 12.5 µl PyroMark PCR Master Mix, 2.5 µl CoralLoad Concentrate, 2.5 µl PyroMark test primers A and B and 5.5 µl Molecular Biology Grade Water per reaction. Each volume delivered was multiplied by the number of reactions determined in step 2 to determine the volumes to collect.
2. 23 µl of the prepared mixture was divided into the appropriate number of 0.2 ml (PCR) tubes.

3. 2 µl of Molecular Biology Grade Water was added to NTC tubes and 2 µl of bilsulfite converted DNA to the remaining tubes.

4. Tubes are centrifuged and load into a thermal cycler. Programmed the thermal cycler according to **Table 2.4.**

Table 2. 4: PCR cycles

Step	**Time**		**Temperature**
Initial	15 min		95 ºC
Denaturation		30 s	94 ºC
Annealing	45 cycles	30s	55 ºC
Extension		30s	72 ºC
Final Extension	10 min		72 ºC
Hold	∞		4 ºC

5. When PCR is complete, the tubes were removed and stored at –20ºC.

2.4.4. Pyrosequencing

1. The bead mix was prepared by adding 40 µl of binding buffer, 29 µl of Molecular Biology Grade Water and 1 µl of beads per reaction.

2. 70 µl of this mixture was added to 22 wells of a 24-well PCR plate. The last two wells were left empty for negative controls and no beads mixtures were added into these wells.

3. 10 µl of PCR products was added into the corresponding well of the 24-well PCR plate.

4. A strip was used to close the wells and mixed up at 1400 rpm for at least 15 min.

5. 20 µl of 1x sequencing primer in annealing buffer was added to 22 wells on the PyroMark Q24 plate.

6. The last two wells were reserved for the primer controls. The sequencing primer was added into one well, and a mixture of biotinylated PCR primer (2 µl) and sequencing primer (20 µl) was added into the other well.

7. PyroMark Q24 plate was placed on the vacuum preparation workstation and filled all the trays of the station with the corresponding solutions.

8. Template for the plate was prepared on the PyroMark Q24 Advanced software.

9. The volumes checked to add to the cartridge for each nucleotide, enzyme mix and substrate mix and added the volumes to the cartridge and place it on the PyroMark Q24 Advanced instrument.

10. Switch on the vacuum was switched in the suction probe, and place it on the prime tray with distilled water, aspirating approximately 70 ml.

11. The 24-well PCR plate removed from agitation and the lids off and placed in the vacuum preparation workstation.

12. The suction probe was placed into the PCR plate wells with the vacuum switched on, captured all the solution from the wells for about 15 s.

13. The beads/samples were washed with 70% ethanol for 5 s.

14. Continued to the tray with Denaturing solution, flushed for 5 s.

15. Aspirated for 10 s on the Washing Buffer tray and tilt the suction probe to beyond vertical 90º for a few s.

16. The vacuum suction was placed probe on top of the PyroMark Q24 plate without touching the liquid.

17. The vacuum was switched off and the handle was lowered on the PyroMark Q24 plate.

18. The suction probes were shaken gently from side to side for 30 s to 1 min, to release the beads on the PyroMark Q24 plate containing sequencing primers.

19. The vacuum suction probe was rinsed on the rinse tray containing distilled water, by agitating for 10 s.

20. 70 ml of distilled water was aspirated in the preparation tray

21. The suction probe was moved vertically beyond 90º for a few seconds and its vacuum was removed.

22. The plate was placed on the metal tray and heated at 80 ºC for 5 minutes.

23. Within a 30 second interval, the sequencing plate was placed in the PyroMark Q24 Advanced, the lid was closed, and the run was started.

24. When run was complete, the PyroMark Q24 plate and unused contents of the cartridge were discarded by inversion and the cartridge was rinsed with deionized water.

25. Each well of the cartridge was filled with water and cleaned by pressing the top with your fingers, forcing the water to pass through the small needles at the bottom.

26. The previous step was repeated at least 3 times for each well.

27. It was left to dry at room temperature.

28. All trays were removed from the vacuum preparation workstation and rinsed with deionized water. It was dried at room temperature.

2.5. Data analysis

PyroMark Q24 Advanced software performed percent methylation analysis at each variable CpG site and data displayed as a Pyrogram®. Percent methylation value was taken for each variable position and an Excel® spreadsheet containing values for all tissues tested for each primer set was made.

2.6. Statistical analysis

The conformity of the data to the normal distribution was examined using the Shapiro-Wilk test. Numerical data were summarized with median, minimum and maximum values. In group comparisons, Mann Whitney U test was used for two independent groups and Kruskal-Wallis test was used for three independent groups. Relationships between numerical variables were analyzed using the Spearman rank correlation coefficient. The significance level was accepted as 0.05 in all tests.

3. RESULTS

Region containing exon 7 using Hs_MMP9_05_PM PyroMark CpG assay (PM00194768). Analyzes the 5'-T**CG**A**CG**GTGATGGGGGGCAACT**CG**G**CG**GGGGAGCTGTG**CG** -3' sequence. The mean methylation values of the CpG-containing sequence of exon 7 were calculated. In total, 5 CpG sites were included, designated as CpG7-1, CpG7-2, CpG7-3, CpG7-4, CpG7-5. MMP-9 methylation changes of schizophrenia patients were compared with the mean MMP-9 methylation of healthy controls.

We screened the methylation patterns of the CpG7 (CpG7-1, CpG7-2, CpG7-3, CpG7-4, CpG7-5) locus in the MMP-9 gene in a SZ patient group and a control group selected from the Malatya-Turkey population. As a result of pyrosequencing, a significant difference was found in the mean methylation status and methylation position at position 3, position 4 and position 5. Statistical analysis of MMP-9 methylation in SZ and healthy control group is shown in **Table 3.1.**

Table 3. 1: Methylation of MMP-9 in SZ and healhty control group**.**

	Control (n=32)	Schizophrenia (n=40)	*p*
Position 1 Meth (%)	46.5(19-89)	54.5(5-65)	0.147
Position 2 Meth (%)	100(44-100)	100(10-100)	0.256
Position 3 Meth (%)	61.5(13-100)	73(7-95)	**0.042**
Position 4 Meth (%)	100(58-100)	100(100-100)	**0.049**
Position 5 Meth (%)	45.5(6-92)	72(17-96)	**0.002**
Average	67.7(51-87)	80.7(32.4-90.8)	**0.002**

An example of the pyrosequencing result of the SZ patient and the HC group is shown in **Figure 3.1** and **Figure 3.2.** The methylation percentages of all SZ patients and the HC group are presented in **Table 3.2** and **Table 3.3**.

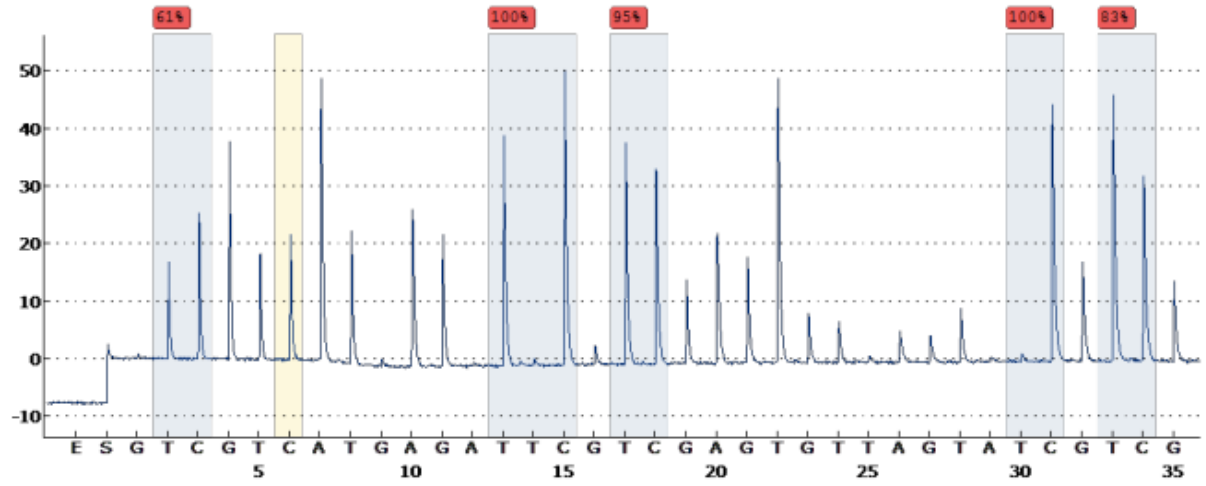

Figure 3. 1. Pyrosequencing result of E1 coded schizophrenia patient

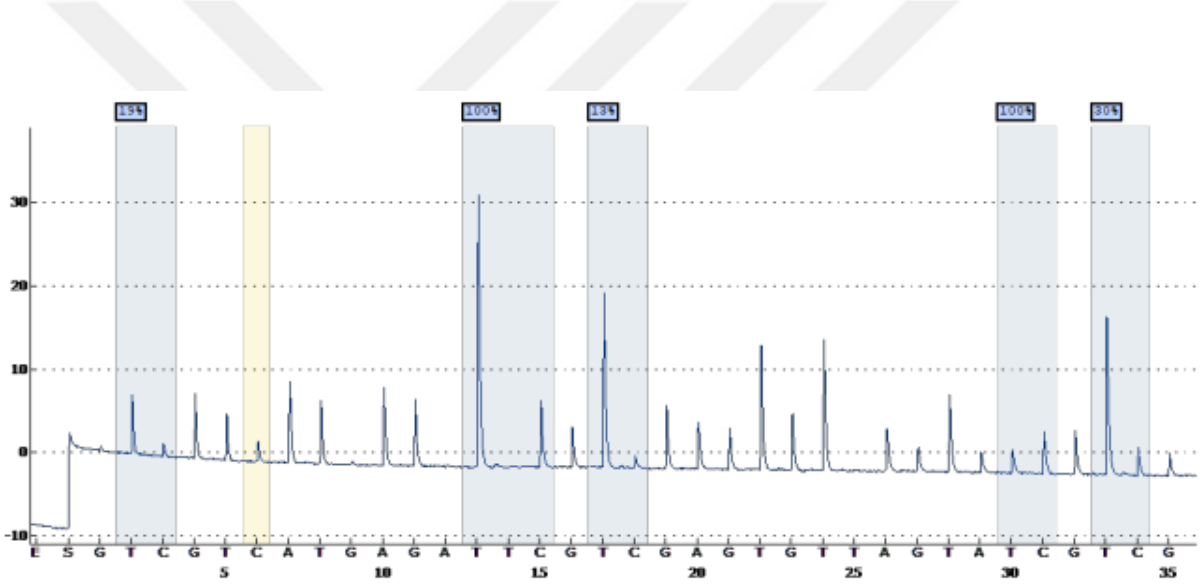

Figure 3. 2: Pyrosequencing result of A83 coded healthy control

Table 3. 2 : Pyrosequencing results of patients with schizophrenia

Sample ID	Position 1 Meth (%)	Position 2 Meth (%)	Position 3 Meth (%)	Position 4 Meth (%)	Position 5 Meth (%)
2	54	100	69	100	53
6	39	100	21	100	51
12	49	100	57	100	77
17	64	100	71	100	72
22	49	100	53	100	33
27	56	100	49	100	31
56	39	100	45	100	35
58	45	100	46	100	20
74	45	91	40	100	17
3	5	100	10	100	36
15	6	10	7	100	39
48	47	100	57	100	58
5	40	100	70	100	70
24	44	100	73	100	69
57	28	100	66	100	58
28	37	100	68	100	85
34	47	100	62	100	61
E1	61	100	95	100	83
E2	59	100	84	100	79
E3	57	100	81	100	86
E4	64	100	94	100	96

E5	60	100	90	100	77
E6	64	100	94	100	76
E7	63	100	87	100	82
E8	62	100	73	100	82
E9	60	100	83	100	83
E10	63	100	77	100	72
E11	50	100	86	100	81
E12	60	100	75	100	78
E13	57	100	73	100	70
E14	63	100	92	100	71
E15	56	100	77	100	79
E16	55	100	73	100	80
E17	53	100	90	100	78
E18	59	100	69	100	62
E19	57	100	78	100	77
E21	45	100	83	100	84
E22	50	100	78	100	64
E23	46	100	58	100	51
E25	65	100	83	100	81

Table 3. 3 : Pyrosequencing results of patients with schizophrenia

Sample ID	Position 1 Meth (%)	Position 2 Meth (%)	Position 3 Meth (%)	Position 4 Meth (%)	Position 5 Meth (%)
A22	59	57	67	58	14
A79	47	100	48	100	36
A80	33	100	42	100	32
A81	46	100	40	100	27
A82	36	100	40	100	27
A83	19	100	13	100	30
A88	40	100	42	100	25
A93	45	100	42	100	32
A95	69	64	66	63	15
A23	55	100	62	100	56
A98	41	100	39	100	39
A24	56	100	58	100	50
A29	71	85	53	94	92
A20	40	100	46	100	49
A78	45	100	70	100	25
A40	37	100	36	100	26
A99	43	100	47	100	42
T3	25	100	41	100	35
T11	46	100	86	100	56
T12	44	100	91	100	74
A37	89	100	86	100	6

T9	62	100	64	100	60
A97	51	100	33	100	38
EK1	47	100	68	100	63
EK2	59	100	68	100	75
EK3	59	100	92	100	84
EK4	23	44	100	100	70
EK5	53	100	79	100	78
EK6	43	100	83	100	81
EK7	57	100	83	100	79
EK8	55	100	77	100	76
EK9	60	100	61	100	66

The relationship between SANS, SAPS and methylations was analyzed with Spearman's rho correlation coefficient and no significant difference was found. The results are shown in **Table 3.4.**

Table 3. 4: The relationship between SANS, SAPS and methylations

Correlations

			SAPS	SANS
Spearman's rho	Position 1 Meth (%)	Correlation Coefficient	-,155	,063
		Sig. (2-tailed)	,340	,699
		N	40	40
	Position 2 Meth (%)	Correlation Coefficient	-,033	-,256
		Sig. (2-tailed)	,841	,111
		N	40	40
	Position 3 Meth (%)	Correlation Coefficient	-,163	,083
		Sig. (2-tailed)	,315	,611
		N	40	40
	Position 4 Meth (%)	Correlation Coefficient	.	.
		Sig. (2-tailed)	.	.
		N	40	40
	Position 5 Meth (%)	Correlation Coefficient	-,198	,038
		Sig. (2-tailed)	,220	,817
		N	40	40

The relationship between age and age of onset of psychosis and methylations was analyzed with Spearman's rho correlation coefficient and no significant difference was found. The results are shown in **Table 3.5.**

Table 3. 5: Relationship between age, age of onset of psychosis and methylations

Correlations

			Age	Age of onset of psychosis
Spearman's rho	Position 1 Meth (%)	Correlation Coefficient	,042	-,134
		Sig. (2-tailed)	,797	,411
		N	40	40
	Position 2 Meth (%)	Correlation Coefficient	-,088	-,195
		Sig. (2-tailed)	,588	,228
		N	40	40
	Position 3 Meth (%)	Correlation Coefficient	-,171	-,231
		Sig. (2-tailed)	,291	,151
		N	40	40
	Position 4 Meth (%)	Correlation Coefficient	.	.
		Sig. (2-tailed)	.	.
		N	40	40
	Position 5 Meth (%)	Correlation Coefficient	-,272	-,241
		Sig. (2-tailed)	,090	,134
		N	40	40

The relationship between age and age of onset of psychosis and SANS SAPS values was analyzed with Spearman's rho correlation coefficient and no significant difference was found. The results are shown in **Table 3.6.**

Table 3. 6: The relationship between age, age of onset of psychosis and SANS, SAPS

Correlations

			Age	Age of onset of psychosis
Spearman's rho	SAPS	Correlation Coefficient	,055	-,011
		Sig. (2-tailed)	,735	,949
		N	40	40
	SANS	Correlation Coefficient	-,068	,209
		Sig. (2-tailed)	,677	,196
		N	40	40

The relationship between antipsychotic drug type and methylation values was summarized with median, minimum and maximum values. The results are shown in **Table 3.7.**

Table 3. 7: The relationship between antipsychotic drug types and methylation

Case Summaries

	Antipsychotic Drug Types								
	Atypical (n=29 +1 typical)				Typical+Atypical				
	N	Median	Minimum	Maximum	N	Median	Minimum	Maximum	*p*
Position 1 Meth (%)	30	50	5	64	10	56	40	65	0,315
Position 2 Meth (%)	30	100	10	100	10	100	91	100	0,770
Position 3 Meth (%)	30	74	7	95	10	72	40	92	1,000
Position 4 Meth (%)	30	100	100	100	10	100	100	100	1,000
Position 5 Meth (%)	30	77	20	96	10	71	17	81	0,379

The relationship between antipsychotic drug type and SANS SAPS values was summarized with median, minimum and maximum values, and significant results were found. The results are shown in **Table 3.7.**

Table 3. 8: Relationship between antipsychotic drug type and SANS SAPS

Case Summaries

	Antipsychotic Drug Types and SANS SAPS								
	Atypical				Typical+Atypical				
	N	Median	Minimum	Maximum	N	Median	Minimum	Maximum	*p*
SAPS	30	43	16	90	10	55	40	76	0,083
SANS	30	56	16	84	10	76	42	86	**0,015**

The relationship between gender and SANS SAPS values was summarized with median, minimum, and maximum values. The results are shown in **Table 3.9.**

Table 3. 9: Relationship between gender and SANS SAPS

Case Summaries

	Gender						
	Male			Female			
	N	Mean	Std. Deviation	N	Mean	Std. Deviation	*p*
SAPS	26	48,42	17,03	14	50,79	18,23	0,685
SANS	26	60,81	19,07	14	56,07	21,80	0,480

The relationship between marital status and SANS SAPS values was summarized with median, minimum, and maximum values. The results are shown in **Table 3.10.**

Table 3. 10: Relationship between marital status and SANS SAPS

Case Summaries

	Marital Status												
	Single				Married				Divorced				
	N	Median	Minimum	Maximum	N	Median	Minimum	Maximum	N	Median	Minimum	Maximum	*p*
SAPS	26	46	25	90	10	42,5	16	78	4	45	29	64	0,507
SANS	26	56	20	86	10	60,5	16	76	4	77	64	86	0,159

The relationship between urban/rural life and SANS SAPS values is summarized with median, minimum and maximum values. The results are shown in **Table 3.11.**

Table 3. 11: Relationship between urban/rural life and SANS SAPS

Case Summaries

	Urban Rural								
	Urban				Rural				
	N	Median	Minimum	Maximum	N	Median	Minimum	Maximum	*p*
SAPS	25	45	25	90	15	46	16	78	0,890
SANS	25	65	16	86	15	56	22	86	0,956

The relationship between antipsychotic drug type and methylation values is summarized with median, minimum and maximum values. The results are shown in **Table 3.7.**

Table 3. 12: Relationship between antipsychotic drug types and methylation

Case Summaries

	Antipsychotic Drug Types								
	Atypical (+1 typical)				Typical+Atypical				
	N	Median	Minimum	Maximum	N	Median	Minimum	Maximum	*p*
Position 1 Meth (%)	30	50,0	5	64	10	55,5	40	65	0,315
Position 2 Meth (%)	30	100,0	10	100	10	100,0	91	100	0,770
Position 3 Meth (%)	30	74,0	7	95	10	72,0	40	92	1,000
Position 4 Meth (%)	30	100,0	100	100	10	100,0	100	100	1,000
Position 5 Meth (%)	30	76,5	20	96	10	70,5	17	81	0,379

4.DISCUSSION

SZ is a common and debilitating illness characterized by chronic psychotic symptoms and psychosocial deterioration [97]**.** SZ is a multifactorial disease and the biggest risk factor is the individual's family history. While the risk of disease is about 1% in the general population, it is 6.5% in first-degree relatives of patients and over 40% in monozygotic twins of affected individuals [39]. Although genetic factors are important risk factors for schizophrenia, they alone have failed to explain the high heritability estimates of schizophrenia. Some environmental factors are thought to affect the onset of the disease and have been proposed as a mechanism to explain the risk of SZ [51].

Recent work in epigenomics has increased the understanding of gene-environment interaction by identifying molecular mechanisms that mediate environmental effects on gene expression and activity. These epigenetic findings are important for understanding the causes of not only SZ but also complex multifactorial psychiatric disorders such as autism [51].

To date, DNA methylation patterns have been the most studied epigenetic mechanism in SZ. Recent studies report differences in DNA methylation patterns between schizophrenia patients and control subjects in both peripheral blood and postmortem brain tissue. The first studies investigating the relationship between DNA methylation changes and SZ started in the early 2000s with the examination of COMT, RELN, GAD1 and SOX10 genes. Among these genes, RELN and GAD1 most showed aberrant methylation in SZ patients [98]. In another study conducted in 2010, it was observed that the FOXP2 methylation level in the left parahippocampal area was higher in the postmortem brain tissues of SZ patients than in the right parahippocampal area [99]. A study of the FAM63B gene in SZ samples revealed that it was significantly hypomethylated [100]. In a study on synaptic plasticity, it was shown that GRIN-2B is hypomethylated in SZ patients [101]. In another study examining EGR-1 methylation patterns, there was no significant difference in methylation patterns between SZ patients and controls [102]. BDNF, which is the main regulator of the function and plasticity of neurons, is one of the genes that play an important role in the pathology of schizophrenia. Results from postmortem brain samples from SZ patients who committed suicide showed greater methylation at the BDNF promoter IV[103].

Also, the first epigenome-wide study to examine DNA methylation in major psychosis investigated 12,000 GC-rich regions, including CpG islands, in the brain's prefrontal cortex. Evidence for psychosis-related DNA-methylation differences has been found at multiple loci

in the frontal cortex, including glutamatergic and GABAergic neurotransmission, brain development, and other processes functionally linked to disease etiology [104].

Recent research indicates changes in glutamate signaling in schizophrenia that lead to abnormal synaptic plasticity. MMP-9 has been shown to regulate glutamate receptors and modulate physiological and morphological synaptic plasticity [78]. Accordingly, in a study conducted in the Chinese population, 51 patients with deficit schizophrenia (DS), 53 patients with non-deficit schizophrenia (NDC), and 50 healthy controls (HC).When comparing NDS patients with DS patients, significantly lower DNA methylation was found in the CpG regions in exon 4 and exon 5 of MMP-9. A positive correlation was found between MMP9 expression and negative symptoms in SZ patients [93].

In this study, we examined the DNA methylation pattern at 5 positions in the CpG7 region of the MMP-9 gene in 40 SZ patients and 32 HC samples selected from Malatya Turkey population. The mean methylation levels of the 5 regions in MMP-9 were significantly different between the SZ and HC groups. No significant difference was found at positions CpG7-1 and CpG7-2, but significant difference was found at positions CpG7-3, CpG7-4 and CpG7-5.

In addition, different statistical analyzes were made for criteria such as SANS SAPS values, methylation percentages, age of onset of psychosis, type of antipsychotic drug, urban rural life, gender, marital status, but a significant difference was found only between SANS SAPS values and antipsychotic drug type.

This study provided evidence for DNA methylation differences of MMP-9 in peripheral blood samples from SZ patients in the Turkish population, and results were obtained that supported previous studies. In the continuation of the study, gene expression should be examined and evaluated together with methylation patterns. Also this study, which we have done using peripheral blood samples, can be supported by post mortem brain samples.

Understanding the interactions of DNA-epigenetic modifications and investigating gene expression together can provide a better understanding of the mechanism of gene regulation in psychiatric disorders and is a hot area for gaining a different perspective in elucidating disease pathology. Additionally, "Is DNA methylation a cause or consequence of schizophrenia?" The question still remains a mystery. Large-scale studies involving family members are needed to answer this question.

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