ASSOCIATION STUDY AND META ANALYSIS OF *NRG 1* GENES (rs2954041) WITH SCHIZOPHRENIA

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A project report submitted in partial fulfilment of the requirements for the award of Bachelor of Chemical Engineering with Honours

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May 2023

DECLARATION

I hereby declare that this project report is based on my original work except for citations and quotations which have been duly acknowledged. I also declare that it has not been previously and concurrently submitted for any other degree or award at UTAR or other institutions.

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APPROVAL FOR SUBMISSION

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ABSTRACT

Schizophrenia is the most persistent mental illness. The etiology of schizophrenia is still unknown however, various studies have shown that there are genetic factors in the pathogenesis of schizophrenia. The *neuregulin-1* (*NRG1*) gene is one of the most potent schizophrenia risk genes. Neurotransmitter activity is correlated with each of the four *NRG1* genes. The objective of this study is to examine the association between rs2954041 of *NRG1* with schizophrenia in Malaysian populations (Malay, Chinese and India). Meanwhile, gender for the pathogenesis of schizophrenia was also observed in this review. Meta-analysis was used to combine the findings for this study with several studies addressing a range of related research hypotheses. There was a total of 44 samples for both healthy controls and patients in this study. DNA sequencing was used to confirm the genotyping of *NRG1* SNP rs2954041 using the polymerase chain reaction-restriction fragment length polymorphism technique (PCR-RFLP). Statistical analysis of the observatory data shows that there was an association ($p < 0.005$) of rs 2954041 of *NRG 1* with schizophrenia by ethnic. Meanwhile, there was no association ($p > 0.005$) of rs2954041 of *NRG 1* with schizophrenia by gender and the total pooled samples. The significant association of rs2954041 of NRG1 with schizophrenia by ethnic might be a false association which was affected by the small sample sizes. The homozygous genotype, G/G (guanine/guanine) appeared the most in this study, the risk allele is the thymine(T) which the low frequency of 'T' resulted in an insignificant result. The results suggest that the rs2954041 of *NRG1* do not play an important role in development of schizophrenia, however, further study is necessary to further confirm the result.

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LIST OF SYMBOLS / ABBREVIATIONS

DSM-5	Diagnostic and Statistical Manual of Mental Disorder
CBT	Cognitive Behavioural Therapy
TMAP	Texas Medication Algorithm Project (TMAP)
FGA	First Generation Antipsychotics
SGA	Second Generation Antipsychotics
GABA	Gamma-Amino Butyric Acid
CNS	Central Nervous System
PNS	Peripheral Nervous System
L_DOPA	L-3,4-dihydroxyphenylalanine
MAO	Monoamine Oxidase
COMT	Catechol-O-methyltransferase
AADC	Amino Acid Decarboxylase
HVA	Homovanillic Acid
TCA	Citric Acid Cycle
NGR 1	*Neuregulin 1*
DAAO	D-Amino Acid Oxidase
NMDARs	N-methyl-d-aspartate receptors
PSD	Postsynaptic Density
EFG	Epidermal Growth Factor
CRD	Cysteine Rich Domain
GGF	Glial Growth Factor
SMDF	Sensory Motor Neuron-Derived Factor
AAO	Age on Set
PCR-RFLP	Polymerase Chain Reaction Restriction-Fragment Length Polymorphism
MINI	Mini International Neuropsychiatric Interview
DSM III	Third Edition of Diagnostic and Statistical Manual of Mental Disorders
ICD-10	International Classification of Diseases
ORs	Odd Ratios

I^2	I-squared value

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

1.1.1 Introduction

A psychotic mental disorder is a serious mental disorder that causes abnormal thinking and perception. When a psychotic episode occurs, a person is not able to identify what is real and what is not, usually with delusions, bizarre behavior, and confused language hallucinations. There are two major symptoms of psychotic mental disorders: hallucinations and delusions.

Hallucinations are the symptoms that the patient will hear or see things that are not real, while delusions are the symptoms that the patient will believe something that is not true. Generally, most of the patients will not realize that the hallucination is unreal, and they will believe what they see and hear are real. These symptoms will not only exist in people with psychotic mental disorders but will also exist in people with other health problems, such as bipolar disorder.

Schizophrenia is the most common type of psychotic disorder. Schizophrenia patients will experience changes in perception and unprovoked misconceptions that persist for more than six months. Often, most of the patients are found to have impaired social functioning, which means they cannot live without the help of others. In Malaysia, the number of newly diagnosed schizophrenia patients is increasing, and the prevalence of schizophrenia in Malaysia is ranked 4th worldwide. According to the Clinical Practice Guideline (CPG), more than 60% of schizophrenia patients found in Malaysia were male. There were 54% Malays, 28% Chinese, 9% Indians, and 9% of others.

The risk factors causing schizophrenia might be due to brain and body factors, genetic factors, and environmental factors. Although there are several factors that contribute to schizophrenia, genetic factors play a significant role in the risk factors that cause schizophrenia. Hence, the association between genetics and schizophrenia is important as it could facilitate the development of a treatment.

1.1.2 Introduction to Meta-Analysis

The word "meta" comes from Greek, and its meaning is "after or beyond. The whole term "meta-analysis" means an analysis of analyses, and this term was first mentioned and explicitly used in the 1970s.

Meta-analysis is a quantitative technique and a research process that is used to summarize the findings from single and multiple studies. In other words, meta-analysis using mathematical and statistical methods to summarize the findings of the systematic review Meta-analysis is able to provide a more precise estimation by combining all the information and results from all the relevant studies. Typically, meta-analyses are mostly conducted on randomized controlled trials (RCT), which consist of higher-level evidence to obtain more accurate and reliable results. Meta-analysis is required to follow some principles, such as that it must be done systematically and there must be many outcomes in order to perform meta-analysis.

By performing meta-analysis, there are several advantages. Firstly, applying the meta-analysis approach is able to reduce the effort, time, and costs of researchers as it combines and summarizes all the information. We all know that each study will have some inconclusive and conflicting information, despite having the same research topic. With the use of meta-analysis, is able to solve this problem. Moreover, the generalizability of the individual findings can be improved by summarizing all the preliminary studies with different populations of patients and sample size. This action allows the meta-analysis result to be generalized to a wider population. Not only that, but the result from meta-analysis is also considered to provide the evidence with the highest accuracy, as the bias of the narrative reviews can be solved by performing meta-analysis.

On the contrary, there are also several weaknesses or limitations when using meta-analysis. The first weakness will be the publication bias. The meta-analysis will ignore the unpublished data, as research with a positive impact will be published frequently while research with no significant result often remains unpublished. Due to ignoring the unpublished data, it might cause the overestimation of the actual size of an effect. Thus, the result of the meta-analysis cannot truly be representative of the data. Pooling data through meta-analysis may lead to problems, for example, coverage limitation, nonlinear

correlations, and non-homogeneous data that are irrelevant to the hypothesis.

1.2 Importance of the Study

Schizophrenia is a type of psychotic mental disorder. Schizophrenia affects around 24 million people, or one in three hundred people worldwide, which is approximately 0.32%. According to the World Health Organization (2022), the rate of schizophrenia is one in two hundred twenty-two people (0.45%) among adults. Besides, schizophrenia patients 2 to 3 times tend to die early if compared to normal people due to the physical diseases for example cardiovascular disease, metabolic disorder, and infectious disease.

Therefore, it is important for humans to know the etiology of schizophrenia to prevent the schizophrenia disease. However, the main cause of schizophrenia is still unknown. According to most studies, genetics is one of the most attractive factors that lead to schizophrenia. Thus, it is important to carry out a meta-analysis on the association of *NRG1* gene with schizophrenia. Meanwhile, analysis of the relationship between schizophrenia between gender and ethnicity is also required, as some studies show the effect of these factors on schizophrenia. By understanding and gaining more information, the higher the chance of curing or preventing schizophrenia, as the remedy can suit the case.

1.3 Problem Statement

According to the research, the schizophrenia prevalence in Malaysia was reported at around 7.7 to 43 per 100,000 people between 2000 and 2013 (Chee and Aziz, 2014). Schizophrenia appears suddenly without any warning, so it is necessary to understand the etiology of schizophrenia. However, based on the current studies, the exact cause of schizophrenia remains unknown. Although the exact cause is unknown, it is certain that schizophrenia is a type of disease with a biological basis that is similar to diabetes or cancer. Hence, genetics will be the most potent etiology of schizophrenia.

Studies of genetic association studies for schizophrenia have been conducted repeatedly over the past two decades, leading to a widely held view of inconsistent results. To change this held view, meta-analysis is applied in

the study to identify the difference between healthy controls and schizophrenia patients.

1.4 Aim and Objectives

The aim of this study is to investigate the association and meta-analysis of *NRG1* genes (rs2954041) with schizophrenia.

(i) To identify the association of *NRG1* gene with schizophrenia in Malaysia by using meta-analysis.

(ii) To analyse the relationship of schizophrenia between gender and ethnic in Malaysia populations.

1.5 Scope and Limitation of the Study

The scope of study for this project is to investigate the association and meta-analysis of genes with schizophrenia. Besides, the relationship between gender, ethnicity, and schizophrenia is also included in this project by using the technique of meta-analysis.

The limitation of this study is the small sample size of schizophrenia patients involved, which might decrease the precision of the result. Not only that, but publication bias also exists, where the result obtained from meta-analysis only depends on the published article, which ignores the unpublished articles, and this might cause the inaccuracy of the result.

CHAPTER 2

LITERATURE REVIEW

2.1 Schizophrenia

2.1.1 Introduction of Schizophrenia

The term "schizophrenia" was created by a Swiss psychiatrist called Paul Eugen Bleuler during the year 1900 (Ryder and Craft, 2022). This term is derived from Greek roots, as schizo means split and phrene means mind.

Schizophrenia is not a disorder that involves a split personality, and it is different from multiple personality disorder. Schizophrenia is a type of mental illness that is severely disabling and affects up to 1% of the global population. Schizophrenia will affect the thinking, action, and expression of emotion in humans and others. The public will usually misunderstand that schizophrenia means a split personality. However, schizophrenia occurs when a person cannot differentiate between imagination and reality. People with schizophrenia will seem to have lost control of reality as they interpret it abnormally. Not only this, but people with schizophrenia will normally also have problems at work, in relationships, and in society. This is because they might feel scared and withdraw as they are disconnected from reality. This lifelong disease cannot be cured; however, with the proper treatment, it can be managed or controlled.

The basic characteristic that defines schizophrenia still remains elusive. Emil Kraepelin is the one who described the condition comprehensively for the first time, and he has named it "dementia praecox," which means an onset in young life followed by a deterioration in mental functioning (Liddle, 2000). However, based on the long-term studies that show the term dementia that was used by Emil Kraepelin, which implied a relentless worsening trend, this is not confirmed, as many cases showed at least partial recovery over decades. Meanwhile, psychiatrist Eugen Bleuler, who coined the term "schizophrenia," presents the characteristic of this condition as fragmentation of mental activity, which is one of the central features of the concept of schizophrenia.

Schizophrenia consists of several different types, which are classified based on the patient's symptoms. Several types of schizophrenia exist: paranoid schizophrenia (the most common type), catatonic schizophrenia, disorganized schizophrenia, simple schizophrenia, residual schizophrenia, and undifferentiated schizophrenia.

The severity of schizophrenia varies from person to person; some people will have multiple psychotic episodes in their lives, but they live normally in between, whereas some people will just have one psychotic episode throughout their whole lives. However, the patient's life will certainly be greatly affected over time without proper treatment.

2.1.2 Symptoms of Schizophrenia

The first sign of schizophrenia appears differently in men and women. For men, they usually show the first sign of schizophrenia in their mid-teens or early 20s at the same time; for women, it is usually shown in their early 20s and 30s (Bhandari, 2022). The prodromal period is the period between the onset of symptoms and full psychosis. It can last for days, weeks, or years, and as there is usually no specific trigger, it is hard to discover.

Schizophrenia consists of three stages and has its own distinct signs and symptoms (Eske, 2021). The first stage is called prodromal, as it happens before the overt psychotic symptoms appear. At this stage, a person can experience behavioral and cognitive changes that are able to develop into psychosis when time passes. The psychotic symptoms, such as delusions and hallucinations, will manifest during the active stages. Next, the final stage is called residual," and at the same time, this stage is also named "recovery stage," as in this stage the psychotic symptoms still appear but are not as severe as in the active stage.

The symptoms of schizophrenia can be categorized as positive or negative. Positive symptoms mean "add on," which refers to additional thoughts or actions that are not based on reality. There are several symptoms categorized as positive, which include delusions, hallucinations, and catatonia. Firstly, delusion is a disorder of the mind's content, as people believe something that is not true or not supported by any evidence. They will not be

persuaded by anyone that the belief is wrong, even given the evidence. Hallucinations are false sensations, or, in simple words, the things that people see, hear, taste, and feel that seem real but only exist in their minds. Meanwhile, catatonia is a type of psychomotor disorder that reflects the connection between mental function and the movement of the body. It is a behavior that reduces responsiveness to immediate surroundings. For example, people with catatonia symptoms might stop speaking, and their bodies will be fixed in a certain position for a long period of time.

Besides, the negative symptoms of schizophrenia are those that lead to a loss of normal functioning. The negative symptoms often persist in the life of the person with schizophrenia even when the positive symptoms are no longer present. Some of the negative symptoms that commonly appear are apathy, alogia, active flattening, lack of pleasure, and social isolation. When it’s hard to get anything completed or unable to initiate and persist in an activity due to a lack of motivation, it's called apathy. Next, alogia is a lack of spontaneity and conversational flow. People with alogia will respond to the questions with very short answers, or they will answer slowly. Active flattening causes people to only show little emotion, such as when their voice sounds flat when they are talking, or they might not have any facial emotion about the conversations that are going on around them (Jennifer, 2020).

In fact, many people with schizophrenia are unaware of their physical discomfort. The symptoms of hallucinations and delusions make it difficult to convince the patient to take their medication, or they might even fear the side effects as they think the medicine will harm them.

2.1.3 Etiology of Schizophrenia

The etiology of schizophrenia is still unknown. Schizophrenia has a very strong genetic component, but a single gene is not able to develop schizophrenia in a person (Clarke, 2022). However, based on the current study, the etiology of schizophrenia is multifactorial.

The factors can be the complex interactions of environmental and genetic factors. Schizophrenia is known to be a highly heritable disease based on the strong evidence provided by family, twin, and adoption studies. By

identifying its common and unique genetic risk factors, considerable progress has been made. Table 2.1 shows the risk details for relatives with schizophrenia. Next, by referring to genome-wide association studies, studies show that schizophrenia's genetic architecture may include multiple common variants; each of them has a little effect, but when all of them work together, they are able to increase the risk of developing schizophrenia. According to the National Institute of Mental Health (2016), many different types of genes will cause schizophrenia; however, there is no single gene that can cause the disorder itself.

Table 2.1: Risk Details for Relatives with Schizophrenia (Salleh, 2004).

Relationship	**Percentage of Risk (%)**
Identical Twins	57.70
First-degree relatives	
Parents	4.40
Sisters and Brothers	8.50
Children	8.20
Second-degree relatives	
Aunts and Uncles	2.00
Nephew and Nieces	2.20
Grandchildren	2.80
Half-sisters and Half-brother	3.20
Third-degree relatives	2.90
Risk of offspring of 0-2 schizophrenic parents	
Neither parents' schizophrenic	8.20
One parent schizophrenic	13.80
Both parents' schizophrenic	36.60
General population	0.86

Meanwhile, due to the concordance rate for identical twins being only around 55%, individual genetic makeup is not sufficient to develop schizophrenia. Thus, a non-hereditary form of the disorder must exist (Michel, 2016). However, the nearly 15% to 40% risk that is from environmental sources is less clear (Robinson and Sarah, 2021). Much research has been done on the environmental risk factors for schizophrenia. The environmental factors that are associated with schizophrenia include obstetric complications, cannabis use, urban living, winter or spring birth (season of birth), childhood

adversity, and infections.

Besides, brain structure and function are also one of the etiology of schizophrenia that are still under investigation. It is found that a number of key brain systems are changing, such as the prefrontal and medial temporal lobe regions, which are involved in working and declarative memory (Karlsgodt, et al., 2010). Schizophrenia patients may have differences in the volume of certain brain regions, including the thalamus, hippocampus, and nucleus accumbent, and in the connections between brain regions.

In fact, there are several risk factors or etiology that may increase the risk of a person having schizophrenia, including genetic factors, environmental factors, and abnormalities in brain structure and function.

2.1.4 Diagnosis

The diagnosis of schizophrenia is established through a series of clinical interviews, such as observation of the patient's behaviour and the observer's mental state, while excluding other similar disorders with known causes (Frohlich, 2016). With the help of physical exams, tests, and screenings, it is possible to exclude other possible diseases that may cause similar symptoms as schizophrenia and also check for any associated complications.

The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) is a manual to assess and diagnose mental disorders, and DSM-5 outlines the specific criteria for schizophrenia (Frances, 2020). According to the DSM-5, the diagnosis of schizophrenia requires the presence of the symptoms in the patient. The symptoms mentioned will be hallucinations, delusions, disorganized speech, catatonic behaviours, negative symptoms, and dysfunction in areas such as work, relationships, and self-care. Two or more of the symptoms (at least one must be either hallucination, delusion, or disorganized speech) must be present for 6 months while active for only one month to be diagnosed as schizophrenia.

2.1.5 Treatment

Treatment of schizophrenia includes psychosocial and pharmacologic therapy (Shenai, et al., 2022), and both therapies must be used to optimize long-term

results. Firstly, psychosocial therapy can be divided into different categories which are individual, family, and cognitive behavioral (Patel, et al., 2014).

Individual psychosocial therapy is involved with social skills training as it aims to teach independent living and social skills to people with schizophrenia (Kern et al., 2009). Next, family psychoeducation is said to be the most important development in psychosocial therapy, as family involvement in the treatment process will have a positive impact (Bellack and Mueser, 2001). The purpose of family psychoeducation is to build a cooperative relationship with the families to provide useful information related to schizophrenia and its treatment, while also teaching family members strategies for reducing stress, communication, and problem solving. It is well known that people with schizophrenia will have hallucinations and delusions, so cognitive behavioral therapy (CBT) is required. The objective of this therapy is to help people think and behave (Jay, 2021). In other words, CBT is used to explore and address the integration of feelings, behaviors, and thoughts that lead to the current problems and induce psychological distress.

Without the use of antipsychotic medication, it is difficult to implement an effective recovery program for most people with schizophrenia. The purpose of pharmacologic therapy is to control the signs and symptoms of schizophrenia effectively by using the lowest possible dose. According to the Texas Medication Algorithm Project (TMAP), there are a total of six algorithm stages for schizophrenia treatment (Tami, et al., 2008).

Stage 1: Treatment of patients, including monotherapy with the use of second-generation antipsychotics (SGA): Clozapine, Aripiprazole, Olanzapine, Risperidone, Quetiapine, and Ziprasidone SGA is preferred compared to first-generation antipsychotics (FGA) as it has a lower risk of serious side effects. When the patient has received an adequate dose and treatment duration, it is able to be based on the response of the patient to determine the further treatment. If the patient shows a response, then the stage one treatment will continue; if there is little or no response from the patient, they will move to stage two treatment; and if the patient has a background history of substance abuse suicide, they will be considered for stage three.

Stage 2: Stage two is similar to stage one in that stage two treatment will use the SGA that wasn't used in stage one treatment or even use FGA. Similarly, if the patient shows a response, the treatment will continue; if the patient shows no or little response, they will proceed to stage three treatment.

Stage 3: Clozapine monotherapy is used. In stage three, observation of the count of white blood cells is important, as clozapine monotherapy has to stop immediately if agranulocytosis occurs. The patient has to proceed to stage four treatment if there is no response to stage three treatment.

Stage 4: Stage 4 treatment includes the use of clozapine or either one (FGA, SGA, or electroconvulsive therapy). Similarly, the patient with no or little response has to proceed to stage five treatment.

Stage 5: Stage five is monotherapy with a FGA or SGA that has not been tried in stage one or stage two. Proceed to stage six treatment if there is no or little response.

Stage 6: Stage six treatment is a combination therapy with FGA, SCG, and electroconvulsive therapy. However, stage six treatment is only recommended at the last stage of TMAP, as the usage of two or more antipsychotics is not suggested as it may increase the risk of drug interactions and medication errors.

The main purpose of both psychosocial and pharmacologic therapy is to help the patient manage the condition by targeting symptoms and preventing the chance of relapse, so the patient is able to integrate into the community and live the same as normal people.

2.2 Neurotransmitters in the pathogenesis of schizophrenia

Neurotransmitters are chemicals that transmit electrical signals between brain cells. The molecule must meet several criteria to be considered a neurotransmitter: it has to be synthesised and stored in the presynaptic neuron, released from the presynaptic terminal, and produce a response in the postsynaptic cell. Based on their molecular and chemical properties, neurotransmitters can be classified into different classes, which are amino acids, peptides, and monoamines. Gamma-aminobutyric acid (GABA) and glutamate are amino acid neurotransmitters; dopamine (DA) and serotonin (5-

hydroxytryptamine) are monoamine neurotransmitters; and somatostatin and norepinephrine are peptide neurotransmitters (Jana, 2022).

GABA and glutamate are the major neurotransmitters in the brain. Inhibitory GABA and excitatory glutamate will cooperate in order to control processes, which include the overall excitability level of the brain (Hampe et al., 2017). Glutamate plays a main role in shaping learning and memory, while GABA controls the hyperactivity of nerve cells associated with stress, fear, and anxiety.

Dopamine, serotonin, and glutamate are important neurotransmitter pathways in the human nervous system. These neurotransmitter pathways control many important emotional and behavioral characteristics. Therefore, the genes from these neurotransmitters will be the focus of investigations into the susceptibility of individuals to psychiatric and behavioral disease.

i) Dopamine Neurotransmitters

Dopamine is a type of monoamine neurotransmitter called catecholamines, and it is found in the medulla of the adrenal gland and in peripheral sympathetic nerves. Dopamine plays an important role in many brain functions, including learning, motor control, emotional function, and executive function (Ko and Strafella, 2012). Meanwhile, dopamine is a therapeutic target for many disorders of the central nervous system (CNS), such as Parkinson's disease and schizophrenia.

At the presynaptic terminal, dopamine is synthesized from tyrosine, and most of the tyrosine is obtained from the diet. The first step will be the hydroxylation of the amino acid tyrosine to L-DOPA (L-3,4-dihydroxyphenylalanine) with the help of the tyrosine hydroxylase (TH) enzyme. The following steps in the synthesis of dopamine will be the decarboxylation of L-DOPA to dopamine by means of the aromatic amino acid decarboxylase (AADC) enzyme. The dopamine will then be taken up and transported into the secretory vesicles for storage and then released into the synaptic cleft in the presynaptic from the secretory vesicle.

Dopamine that is taken up by the presynaptic cells can either be recycled into vesicles for further usage or it can be degraded. The degradation process required two enzymes, which are monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT). MAO is an enzyme that has the function of terminating catecholamine action in the brain and periphery. MAOs exist in two isoforms: (1) MAO-A, expressed both in the brain and in the periphery. (2) MAO-B, which focuses on the CNS. Both MAO isoforms are able to degrade dopamine and other monoamine compounds. In general, MAO-B is used for catabolizing dopamine in the CNS.

In the presence of Mg2+, COMT catalyses the methylation of hydroxyl groups on catechol nuclei to produce the dopamine metabolite 3-methoxy tyramine (Mannisto and Kaakkola, 1999). In other words, COMT adds a methyl group to the hydroxyl group on the 3-position of the benzene ring in order to inactivate the catecholamines. In fact, the pathway to synthesise dopamine and the series action of MAO and COMT degrade the dopamine to the stable metabolite, which is homovanillic acid (HVA), are shown in figure 2.1.

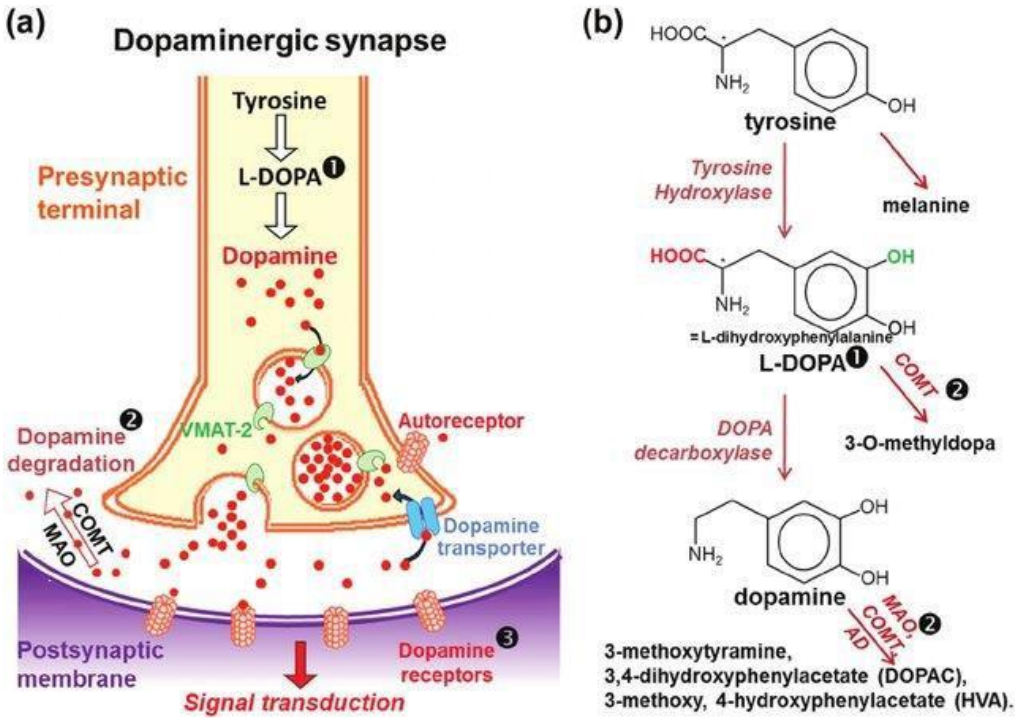


Figure 2.1: Dopaminergic Synapse and Metabolism of Dopamine (Jones, et al., 2014).

By referring to the studies that show that the gene level of COMT in glial cells that are located in the frontal cortex is increasing in schizophrenia patients (Lakhan and Vueira, 2009). Disruption of the dopamine-glutamine interaction and abnormalities of the glia in schizophrenia may be due to the increased COMT level. COMT polymorphisms appear to disrupt the function of the neurocognitive system, which increases the susceptibility to schizophrenia (Liao, et al., 2009).

According to the study, the development of schizophrenia might be due to the abnormal function of the dopamine neurotransmitter in the brain. This is because, the decrease in dopamine activity might lead to the development of the negative symptoms of schizophrenia.

ii) Serotonin Neurotransmitters

Serotonin (5-hydroxytryptamine, 5HT) is synthesized in the neurons originating from the raphe nucleus in the midline of the brainstem in the central nervous system (CNS). Serotonin plays a key role in human behavior such as memory, sleep, personality, appetite, sexuality, mood control, emotions, and neuroendocrine function. Meanwhile, serotonin regulates smooth muscle tone, especially in the vascular gastrointestinal tract in the periphery (Boyer and Shannon, 2005).

Various studies have shown that the dopamine system and the serotonin system interact at a fundamental neurophysiological level. The function of dopamine in the midbrain is inhibited by the serotonin system, while serotonin also inhibits synaptic release and possible dopamine synthesis in the striatum.

As shown in Figure 2.2, serotonin is synthesized in the presynaptic neurons by hydroxylation and decarboxylation of tryptophan. Once the serotonin is produced, it is then merged into the vesicle, where it will reside until it is needed for neurotransmission. Serotonin will be released into the intrasynaptic space after the stimulation of the axon. Next, serotonin will bind to the postsynaptic receptors in order to affect neurotransmission.

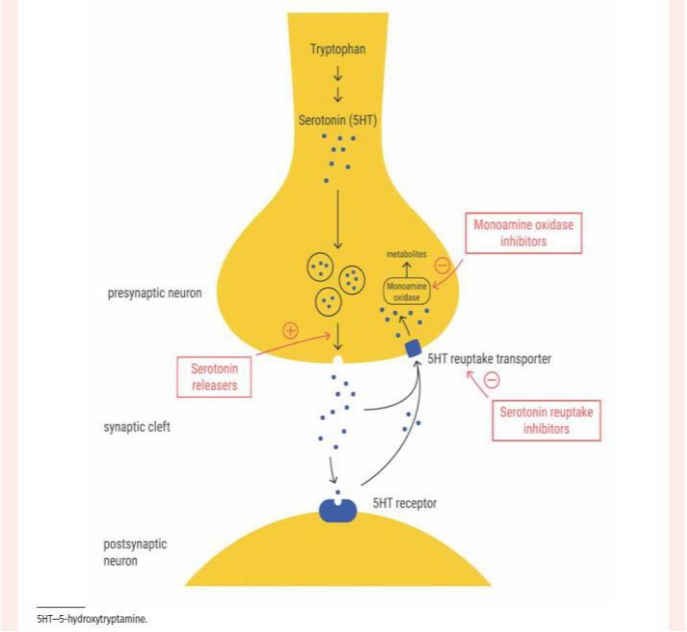


Figure 2.2: Synthesis of Serotonin and Serotonin Synapse (Foong et al, 2018).

i) Glutamate Neurotransmitters

The central nervous system (CNS) has several receptor types for glutamate, an excitatory neurotransmitter. The metabolism of glutamate is important for maintaining optimal levels in the extracellular space. Therefore, glutamate plays a key role in the regulation of cognition, emotion, and memory.

Glutamatergic synapses are used to regulate the building of neural network connections in the development of the spinal cord and brain. Meanwhile, it also regulates the cellular processes that are critical to plasticity and synaptic transmission.

Glutamine can be constructed from α-ketoglutarate (α-KG) which is an intermediate of the citric acid cycle (TCA cycle). In the presynaptic terminal, the glial cell will release glutamine, which will then be metabolized by the mitochondrial enzyme glutaminase to form glutamate. After packaging into synaptic vesicles via an ATP-dependent transport process, the glutamate-filled vesicles are docked and released from the presynaptic. High-affinity glutamate transporters that are present in the presynaptic terminal and the glial cells are used to remove the glutamate from the synaptic cleft. The release of glutamate into the synaptic cleft is terminated by its intake into the neuron and

the glial cells surrounding it through the specific transporters. Figure 2.3 shows the synthesis of glutamate and the circulation between neurons and glial cells.

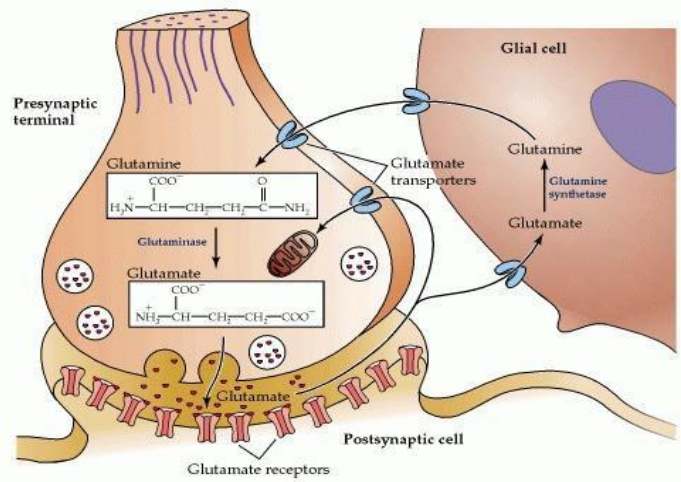


Figure 2.3: The Synthesis of Glutamate and the Circulation between Neurons and Glial Cells (Purves, et al., 2001).

Furthermore, several genes, including *neuregulin 1* (*NRG1*), G72, dysbindin-1, and D-amino acid oxidase (DAAO), are linked to susceptibility to schizophrenia and might affect glutamate synaptic function. In glutamate synaptic vesicles, the *NRG1* gene is present to regulate the N-methyl-d-aspartate receptors (NMDARs) expression, interact with postsynaptic density (PSD), and activate the *ErbB4* receptor that colocalizes with NMDARs. Meanwhile, G72 will interact with the DAAO to oxidize D-serine (a modulator of NMDARs). Figure 2.4 is the glutamatergic synapse.

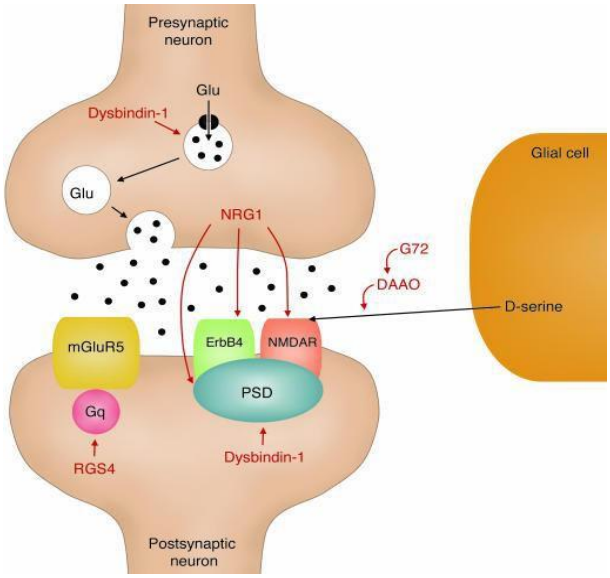


Figure 2.4: Glutamatergic Synapse (Owen et al, 2004).

In fact, recent studies have shown that the pathological changes in schizophrenia involve an imbalance or change in gamma-aminobutyric acid (GABA), serotonin, glutamate, and acetylcholine (Brisch, at el., 2014).

2.3 *NRG1* Gene

2.3.1 Introduction on *NRG1* Gene

The *NRG1* gene, also known as "*Neuregulin 1*," is a protein-coding gene. The protein coded by the *NRG1* gene is a membrane glycoprotein that intervenes in intercellular signaling and also plays an important role in the growth and development of multiple organ systems (Tan, et al., 2007). *NRG1* is not a single protein entity; it consists of multiple families (>16) of transmembrane and secretory isoforms that are encoded by one of the largest human genes, which is around 1.6 MB (Schwab, et al., 2013).

NRG1 is the most well-characterized of a four-member gene family (*NRG1-NRG4*), all of which share an epidermal growth factor (EFG) (Shi and Bergson, 2020). *NRGs* include a family of growth factors that are able to stimulate the *ErbB* receptor tyrosine kinases (*ErbB2, ErbB3, and ErbB4*). According to the research, *NRGs* and their receptor, ErbBs, are identified as the susceptibility genes for diseases including schizophrenia.

The *NRG1* gene works by stimulating the *ErbB* protein (Bublil and Yarden, 2007). ErbB1 receptor cannot bind to the *NRG1* gene but is able to form a heterodimer with *ErbB4* protein, and it can also bind to EGF. *ErbB2* will work as the coreceptor by attaching to the ligand with *ErbBs* to form heterodimers (Tzahar, et al., 1996). Next, the homodimer is catalytically inactive when ErbB3 is bound to the *NRG1* gene, and this causes the kinase to not function. Lastly, *ErbB 4* is the only *ErbB4* family member that can bind to the *NRG1* gene (Savci, et al., 2022). Figure 2.5 shows the four members of epidermal growth.

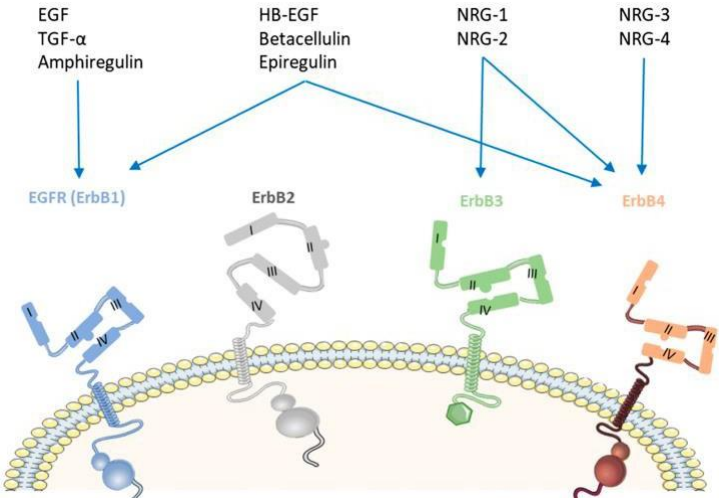


Figure 2.5: The Four Members of the Epidermal Growth Factor (El-Gamal, et al, 2021).

As shown in Figure 2.6, *NRG1* produces six subtypes of proteins (I-VI) and at least thirty-one types of isoforms (Ou, et al., 2021) by alternative splicing. These six subtypes of proteins (I–VI) are characterized by distinct extracellular domains, and all subtypes share an EGF-like core domain. Types I, II, IV, and V contain the immunoglobulin (ig)-like domain that is between the EGF domain and the N-terminal sequence. Type II contains a sole cysteine rich domain (CRD), and type II has a Glial Growth Factor (GGF)-specific domain.

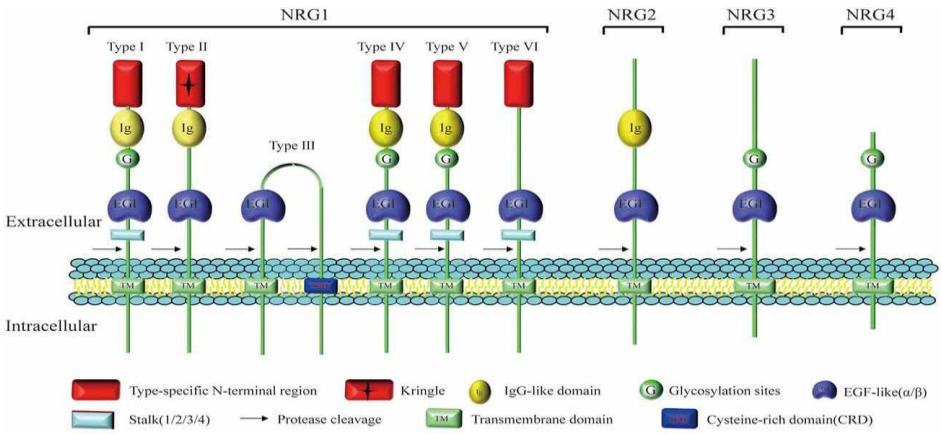


Figure 2.6: *Neuregulin* (*NRGs*) Structure and Homology (Ou et al, 2021).

Type I proteins are called neuregulin, acetylcholine receptor inducing activity (ARIA), and neu differentiation factor (NDF) (Ledonne and Mercuri, 2020). This protein lacks a signal peptide, but it is produced as a transmembrane protein that is proteolytically cleaved in order to release the growth factors. *NRG1* type II is known as Glial Growth Factor (GGF), and it is a secreted protein. Next, type III is called sensory motor neuron-derived factor (SMDF), and it requires cell- to-cell contact for trophic activity as the type III isoform is tethered to the membrane (Friedman, 2012). All six subtypes of *NRG1* are able to be detected in the brain; however, the abundance of each form varies widely and is related to developmental stage as well as neuronal activity.

Most of the *NRG1* isoforms are generated as membrane-anchored precursors, which are termed pro-NRG1s, with the EGF domain located extracellularly. Pro-*NRG1s* go through cleavage of the proteolytic in a membrane-juxtaposed region which is located at the carboxy-terminus of the EGF-like domain (Lin and Xiong, 2008). This resulted in the release of disseminated mature *NRGthrough proteolyticpt* for type III). Besides, some of the *NRG1* isoforms do not have a transmembrane and are released directly outside of the cell (Douglas, 2003).

In fact, *NRG1* isoforms that differ in their N-terminal region or EGF-like domain function differently in vivo. These functional differences may be due to differences in the pattern's expression or to differences that reflect intrinsic biological characteristics (Falls, 2003)

2.3.2 Role of *NRG1* gene in human

Neuregulin 1 is a multifunctional protein that promotes proliferation, divergence, and survival of multiple cell types such as cardiomyocytes, epithelial cells, and neurons by enhancing phosphorylation of *ErbB2*, *ErbB3,* and *ErbB4* (Lemmens, et al., 2007). *NRG1* also plays an important role in activity-dependent maturation and excitatory synapse structure and function plasticity (Li, et al., 2007). Synaptic activity causes the activation and recruitment of *ErbB4* at synapses.

Both the *NRG1* and *ErbB4* signaling pathways play an important role in the development of neural networks, including radial neuronal migration, myelination, axon guidance, synapse formation, and the development of oligodendrocytes. *NRG1* and *ErbB4* are critically involved in synaptic plasticity, and they also enhance and inhibit long-term potentiation (LTP) in a complex manner with activity in different regions of the brain. In addition, the *NRG1* and *ErbB* signaling pathways are able to affect the glutamatergic system directly by regulating the expression and function of N-methyl-D-aspartate (NMBA) receptors (Mei and Nave, 2014). The signaling of *NRG1* is complex and bidirectional. In canonical forward signaling of *NRG-ErbB* can stimulate the Raf-MEK-ERK and PI3K-Akt-S6K pathways, which are used to regulate the expression of genes and signal transduction, as shown in Figure 2.7.

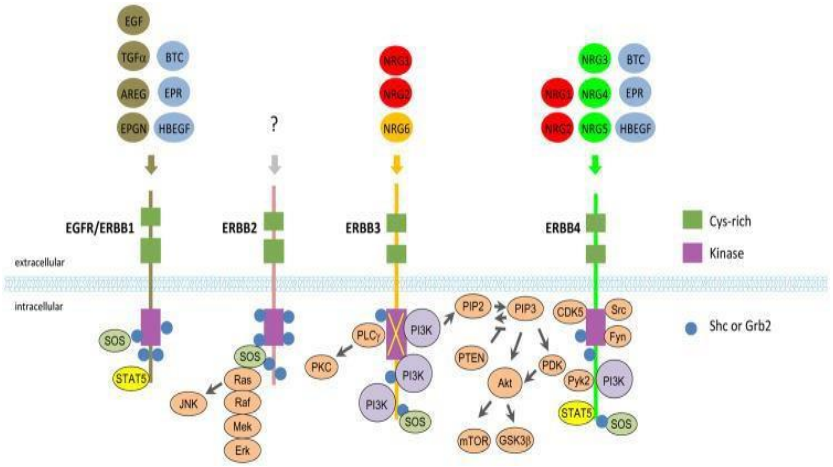


Figure 2.7: Canonical Signalling of *NRG-ErbB* (Mei and Nave, 2014).

Besides, the *NRG1* gene can affect several aspects of the development of neurons in the brain, such as migration of neurons and modulation of neurotransmitters for glutamate, acetylcholine, and gamma-aminobutyric acid (GABA) (Friedman, 2012). By doing studies and research in conditional *NRG1/ErbB*-deficient mice, it is suggested that the *NRG1* gene and *ErbB* are also important in cardiac development and functionality. Administration of *NRG1* induces cell cycle re-entry in differentiated cardiomyocytes after myocardial infarction (MI), reduces infarct scar size, improves the function of the myocardium, and also alleviates cardiac hypertrophy.

Moreover, *NRG1* is important in peripheral nervous system (PNS) and central nervous system (CNS) development. According to Carrol et al (1997), it emphasizes the role of *NRG1* in PNS repair by suggesting that *NRG1* signaling is regulated during the injury of peripheral nerves. Once the peripheral nerve is injured, a series of repair events called Wallerian degeneration will occur, as shown in Figure 2.8.

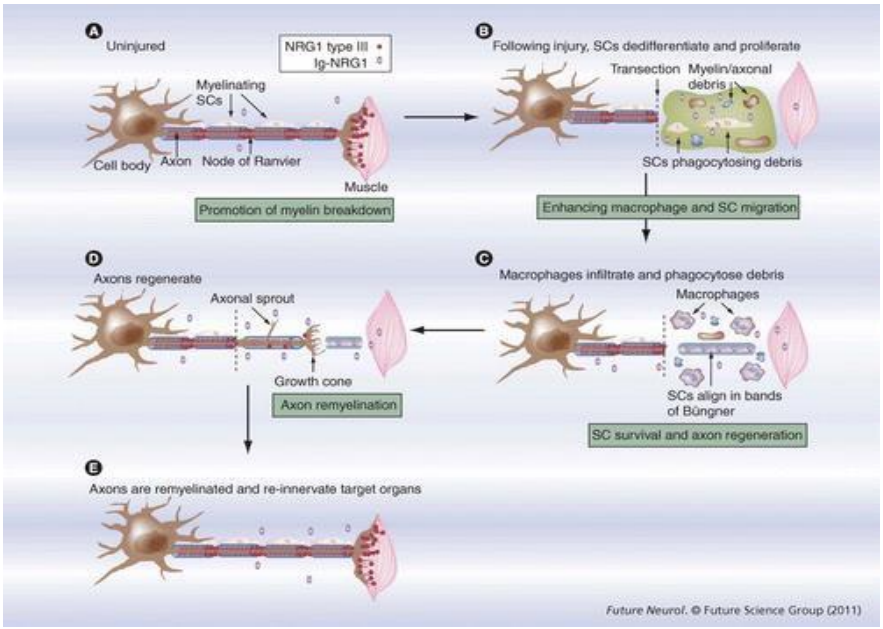


Figure 2.8: Involvement of *NRG1* in Multiple Stages of the Process of Nerve Repair (Fricker and Bennett, 2011).

Firstly, fragments of distal stamps and Schwann cells will start to clear the myelin debris and axonal debris within 2 days after injury, while the

Schwann cells will also proliferate and dedifferentiate (Li, et al., 1997). *NRG 1* is involved in promoting the initial clearance of myelin. The expression levels of *NRG1*, *ErbB 2,* and *ErbB 3* were elevated in Schwann cells after injury and persisted long after demyelination. *NRG 1* type III neuronal expression is reduced and does not return to normal levels until axons re-dominate their target organs. Next, the distal stump is infiltrated by macrophages, and macrophages and Schwann cells will complete the debris clearance within two weeks' time, while the dedifferentiated Schwann cells will be lined up in Bungner bands. Although it has not been shown in vivo, *NRG1* is important in macrophage recruitment after nerve injury, as *NRG1* is known to increase the motility of macrophages (Calvo, et al., 2010). After that, axons are regenerated with the help of *NRG 1* from the proximal stump after the Schwann cell cannal and re-engage with Schwann cells. Lastly, axons will re-dominate their target organs and be re-myelinated by Schwann cells with the help of *NRG1* to restore full function (Fricker and Bennett, 2011). During the neural repair process in the PNS and CNS, treatment with *NRG1* is suggested, which may improve the functional outcome after injury.

In conclusion, *NRG1* genes are essential for the development of cardiac function and the maintenance of the structure and functional integrity of the human heart.

2.3.3 Association of *NRG1* gene with Schizophrenia

The interaction of *neuregulin* (*NRG1*) and family members of *ErbB* to regulate the signaling pathway is important for the formation and proper functioning of all organ systems. As the signaling pathway is essential to organ functioning, dysregulation of *NRG1/ErbB* will have a negative impact on humans, including leading to heart failure, impaired reproductive performance, cancer, neuropsychiatric disorders, and neurodegenerative diseases.

The neuropsychiatric disorder will be schizophrenia, as, by genome-wide studies, the *neuregulin 1* (*NRG1*) gene that is located at chromosome 8p, specifically a 30cm region around 8p22q11.2, has been identified as a schizophrenia susceptibility gene (Stefansson, et al., 2003). According to recent studies, variations in genetic and structural microdeletions of the

tyrosine kinase receptor for the *NRG1* gene have also been implicated in schizophrenia.

NRG1 and its receptor-*ErbB4* have been implicated in schizophrenia, and this attracts most of the attention of scientists due to their involvement. The involvement of *NRG1* and *ErbB4* includes neural development, glutamate regulation, and synaptic plasticity. An earlier study suggests that ErbB4 heterozygous mice are associated with behavioral phenotypes of schizophrenia (Agim, et al., 2013). Meanwhile, some of the studies also showed that abnormalities on the *NRG1/ErbB4* signaling pathways will lead to neuronal migration dysfunction.

Any defect in the *NGR1/ErbB4* signaling pathways will contribute to schizophrenia. This is especially true when the loss of *ErB4* reduces the density of interneuron in the postnatal cortex. As the interneuron density decreases and the degree of differentiation deteriorates, there is a variable number of GABAergic neurons. Migration regulation of GABAergic interneurons is lost due to their expression in *NRG1,* and *NRG1's* isoform is altered (Savci, et al., 2022). This condition is associated with the GABAergic interneuron neurodevelopmental pathology that was observed in schizophrenia.

Moreover, the function of N-methyl-D-aspartate (NMDA) receptors in the brain is regulated by the *NRG1* gene. Due to its interaction and functional impact with NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, postsynaptic density protein-95 (PSD-95) has been implicated in the synaptic plasticity of glutamatergic synapses during the development of neurons (Coley and Gao, 2017). At synapses, PSD-95 is bound to the ErbB4 and NMDA receptors, and *NRG1* signaling is increased by promoting ErbB4 dimerization. In addition, through synaptic activity, NRG1 has increased the C-terminal fragment (CTF) translocation to the nucleus (Savci, et al., 2022). Binding to the zinc-finger transcription factor, PSD-95 transcriptional activity is increased by *NRG1* (Bao, et al., 2004). However, overexpression of *NRG1* will reduce the synaptic signaling of the NMDA receptor. Thus, this is suggested to be an association with NMDA receptor dysfunction in schizophrenia (Coyle, et al., 2003). Figure 2.9 shows the

NRG1/ErbB4 signalling pathway that is involved in the pathogenesis of schizophrenia.

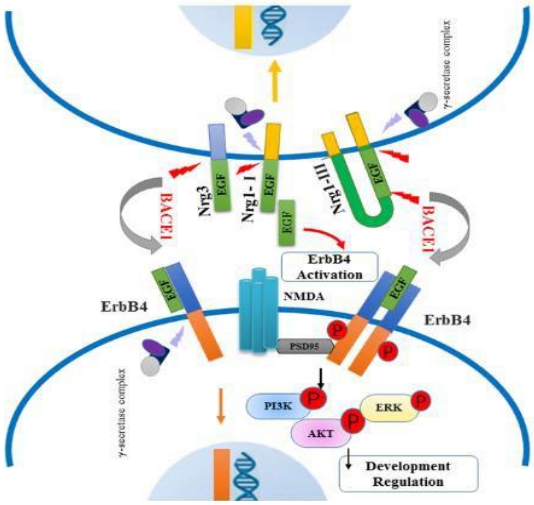


Figure 2.9: The Pathophysiology of Schizophrenia is Influenced by the NRG*1/ErbB4* Signaling Pathway (Zhang, et al., 2017).

2.3.4 Association of *NRG1* gene with Schizophrenia (Ethnic Group)

2.3.4.1 Genetic Marker

Genetic markers are DNA sequences or genes with a known location on a chromosome that can help link genetic disorders to the responsible genes. As genetic factors play an important role in the etiology of schizophrenia, genetic markers will be used to identify whether an individual is healthy or schizophrenic.

The genetic marker that is often used in identifying a schizophrenic or healthy individual is single nucleotide polymorphisms (SNPs), which are the most common type of genetic variation in humans (National Institutes of Health, 2019). The advantage of SNPs is that they have the ability to work on degraded DNA as a small target can be amplified (Butler, et al., 2007); thus, the products of polymorphism chain reaction (PCR) can only be prepared in a small amount.

2.3.4.2 *NRG1* and Schizophrenia

Various investigations to study an association of *NRG1* with schizophrenia were carried out, and a 7-marker *NRG1* 'high-risk' haplotype, termed HAPICE, was identified and found to be related to schizophrenia. The 7 marker haplotypes are 5 single nucleotide polymorphisms (SNPs) including

*NRG*221132, *NRG*221533, *NRG*241930, *NRG243177*, and *NRG*433E1006 and 2 microsatellite markers, which are 478B14-848 and 420M9-1395 (Tosato, et al., 2005) for schizophrenia in Iceland and Scotland.

In addition, Caucasian and Asian populations are also associated with schizophrenia. For the Caucasian and Asian populations, identification of 7-marker core risk haplotypes using only 3 markers includes SNP8NRG221533, 478B14-848, and 420M9-1395 (Tosato, et al., 2005). These 3 haplotype markers were chosen to genotype the schizophrenia cases and controls from unrelated Caucasians that were born in the United Kingdom (UK).

Chinese will be one of the major ethnic groups to be analysed for the association of the *NRG1* gene with schizophrenia. The first study analysed around 248 Chinese Han trios, but only 3 SNPs from *NRG1* were genotyped, which include SNP8NRG221533, exon 2 rs 3924999, and intron 5 rs 2954041 (Yang, et al., 2003). A strong relationship between these SNPs and schizophrenia is identified.

Not only that, but to study the association of the *NRG1* gene with schizophrenia in Asian populations Several case-control studies and family trios of Chinese Han samples are also studied by genotyping 25 microsatellite markers and 3 SNPs (Li, et al., 2004). The first haplotype is HAPCHINA1 (29H12-1 and D8S1711), upstream of HAPICE, which is significant in the comparison of case-control but not significant in family-based studies (Li, et al., 2004). The second haplotype HAPCHINA2, which is a four-marker haplotype (478B14-642, 487-2, 420M9-1395, D8S1810), is found to overlap HAPICE and is significant in family-based studies. Lastly, the third haplotype HAPCHINA3 (317J8-2123, 317J8-1, 317J8-2, and 317J8-4858), which is located at the 3' end of the *NRG1* gene, was found to be significant in the family-based studies but not significant in case-control comparisons. Based on these three haplotypes, we concluded that different haplotypes within the *NRG1* range might be associated with schizophrenia in the Chinese Han population.

Table 2.2: Summary of Association Study of SNPs, Microsatellite Markers and *NRG1* Gene.

High Risk Haplotype	Single Nucleotide Polymorphisms (SNPs) and Microsatellite Markers	Outcome	Reference
HAP_{ICE} (Icelandic)	SNP8NRG221132	Insignificant	Stefansson, et al., 2002
	SNP8NRG221533	Significant	
	SNP8NRG241930	Insignificant	
	SNP8NRG243177	Insignificant	
	SNPNRG433E1006	Insignificant	
	478B14-848	Insignificant	
	420M9-1395	Insignificant	
HAP_{ICE} (Scotland)	SNP8NRG221132	Insignificant	Stefansson, et al., 2003
	SNP8NRG221533	Significant	
	SNP8NRG241930	Significant	
	SNP8NRG243177	Significant	
	SNPNRG433E1006	Insignificant	
	478B14-848	Insignificant	
	420M9-1395	Insignificant	
HAP_{ICE} (UK)	SNP8NRG221533	Insignificant	William, et al., 2003
	478B14-848	Insignificant	
	420M9-1395	Insignificant	
HAP_{CHINA1} (Chinese Han)	29H12-1	Insignificant	Li, et al., 2004
	D8S11711	Insignificant	
HAP_{CHINA2} (Chinese Han)	478B14-642	Significant	Li, et al., 2004
	487-2	Significant	
	420M9-1395	Significant	
	D8S1810	Significant	

Table 2.2 (Continued)

HAP_{CHINA3} (Chinese Han)	317J8-1 317J8-2 317J8-4858	Insignificant Insignificant Insignificant	Li, et al., 2004
$HAP_{Iranian}$ (Iran)	SNP8NRG241930	Significant	Shariati, et al., 2011
HAP_{ICE} (Japnanese)	SNP8NRG243177 rs1081062 478B14-848 420M9-1395	Insignificant Insignificant Significant Significant	Shiota, et al., 2008

Based on the study carried out for different ethnic groups, it was found that the haplotype association replication is unsuccessful and the identification of the potential variants leading to schizophrenia remains contradictory. The differences in linkage disequilibrium between groups might cause the inconsistency. Thus, the SNP of rs3924999's association with schizophrenia in three ethnic groups in Malaysia, which are Malay, Chinese, and Indian, will be investigated. Meanwhile, meta-analysis will be used to combine all the results from the studies.

2.4 Gender and Age at Onset of Schizophrenia

It has been debatable whether there are gender disparities in the prevalence of schizophrenia. Generally, it has been accepted that the incidence and prevalence of schizophrenia are equal in men and women. According to studies that indicate gender differences in schizophrenia incidence, By using stricter diagnostic criteria for schizophrenia, it was found that men had a higher incidence of schizophrenia than women. However, a different set of diagnostic criteria is applied, and the manifestations found that the effect of different diagnoses on the gender ratio was profound. However, recent studies have shown that there is no gender difference in the prevalence of schizophrenia. In this situation, one of the possible explanations for the

difference between incidence and prevalence may be related to treatment adherence and higher suicide rates in men than in women. As there is a contradiction between gender differences in the incidence and prevalence of schizophrenia, a study of the gender differences in the Malaysian population is required.

Furthermore, "age at onset" (AAO) means the age at which an individual first acquires or experiences the symptoms of a disorder. Age at onset is considered one of the most important clues to understanding the etiology of schizophrenia. In addition, AAO in psychosis is generally recognized as an important clinical and prognostic factor.

Differences in AAO are the replicated findings to study the gender difference in schizophrenia. The AAO for men usually appears between the ages of 18 and 25 years, whereas the average AAO for women is between 25 and 35 years (Ochoa et al., 2012). Not only that, the incidence distribution curves for men and women are not isomorphic, and women appear to have two peaks in the AAO (the first time after menarche, the second time after age 40). Although the AAO distribution curve between men and women is different, the early-onset age distribution curve is similar between men and women. According to the schizophrenia estrogen hypothesis, the predominant prevalence in women after age 40 can be explained by postmenopausal estrogen reduction.

Furthermore, several studies indicate that the difference in AAO appears to depend on the presence or absence of family history, and there will be no gender difference if they have family history. (Albus, et al., 1994).

Thus, the study of age at onset for schizophrenia in the Malaysian population is required to be carried out to further identify whether there is any difference in the AAO for men and women.

CHAPTER 3

METHODOLOGY AND WORK PLAN

3.1 Introduction

This chapter summarizes the methodology that was used to gather the information and retrieve the literature in this review paper. For this study, it included lab-scale, which will be the Polymerase Chain Reaction Restriction-Fragment Length Polymorphism (PCR-RFLP) method, and also required the meta-analysis software to obtain the data. There are various journals, articles, or reports that will be analyzed or studied in this report; hence, to increase the reliability of the result, all the findings from different sources are required for comparison and summarization.

Furthermore, meta-analysis was used in this study as it is a quantitative technique, which is a research process used to summarize the results from single and multiple studies. As the meta-analysis provides a method for systematic review of the literature, it saves most of the time in analyzing the literature one by one, which this is helpful in this study as many articles or journals were included.

3.2 Participants

In this study, the participants were divided into two groups: schizophrenia patients and healthy participants, who serve as the controls. In this study, there will be a total of 44 participants involved. As ethnicity will be one of the studies, the participant will then be further subdivided into three ethnic groups, which are Malay, Chinese, and Indian. There are some criteria for the participants, as they must be free from smoking, drug abuse, mental illness, and a family history of mental illness.

For the patient with schizophrenia, they must be diagnosed through the Mini International Neuropsychiatric Interview (MINI), which was conducted by the doctor or an allied mental health specialist. According to Harm Research Institute, MINI is a brief and structured interview that was originally developed in 1990 by clinicians and psychiatrists in Europe and the United

States (US) for the Third Edition of Diagnostic and Statistical Manual of Mental Disorders (DSM III) and the International Classification of Diseases (ICD-10) for psychiatric disorders. The patient will only be recruited if he or she has been tested for schizophrenia by the MINI test.

3.3 DNA Isolation

DNA isolation is a method of purifying DNA that separates it from cell membranes and other cellular components by using physical or chemical methods. In this study, the GF-1 Viral Nucleic Acid Extraction Kit was used to perform the DNA isolation, which is shown in Figure 3.3.1 below.

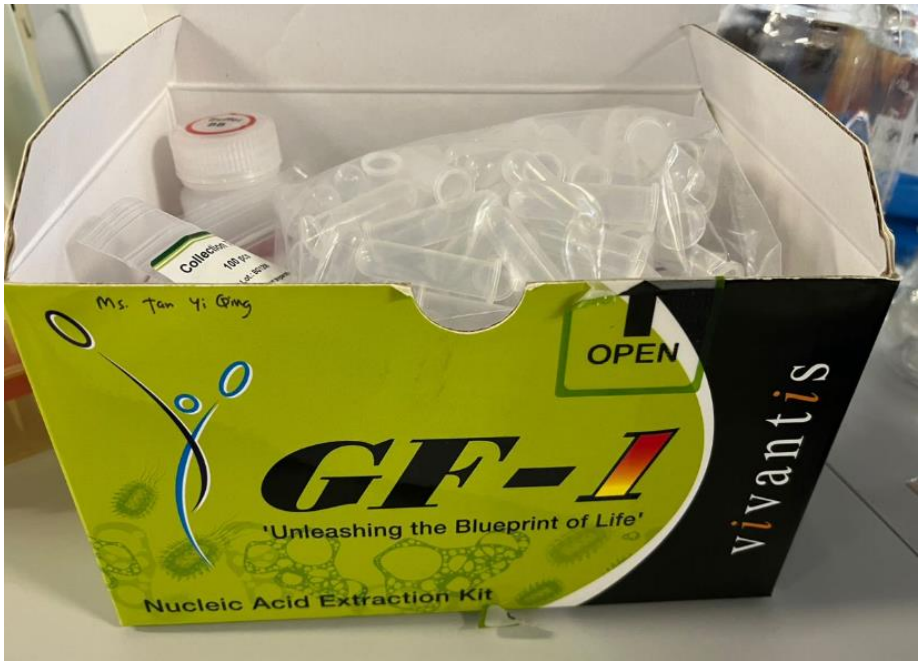


Figure 3.3.1: GF-1 Viral Nucleic Acid Extraction Kit.

The procedure for the GF-1 Viral Nucleic Acid Extraction Kit was to lyse, bind DNA, wash, and elute. The GF-1 Viral Nucleic Acid Extraction Kit contains buffer BB and proteinase K, which are optimal enzymes for use with the lysis buffer in the GF-1 Viral Nucleic Acid Extraction Kit. Meanwhile, the GF-1 Viral Nucleic Acid Extraction Kit also contained proteinase K, which is a preferred enzyme for sodium dodecyl sulphate (SDS) lysis buffer used in tissue, blood, and body fluid protocols.

The procedure for the GF-1 Viral Nucleic Acid Extraction Kit started with blood lysis which 200 µl was added into a 200 µl blood sample in a microcentrifuge tube. Mix the mixture by using the vortex mixer. After that, 20 µl of proteinase was added to the mixture and mixed by a vortex mixer. The next step was ethanol addition, 200 µl of absolute ethanol was added to the mixture and mixed immediately by using a vortex mixer to obtain a homogenous solution. After ethanol addition, it was proceeded to load the column, which transferred the sample to a column that had been put together in a tidy collection tube. Centrifuged the flow through at 5000xg for 1 minute. The next step was column washing 1, centrifuged the column at 5000xg for 1 minute after washing it with 500 µl wash buffer 1 then discarded the throughput. The step was followed by column washing 3, centrifuged the column at 5000xg for 1 minute after washing it with 500 µl wash buffer 2. Throw it away and let it flow through. Centrifuged the column once again for three minutes at maximum speed with 500 µl of wash buffer 2. The last step was DNA elution, and the column was placed into a new microcentrifuge tube. 100 µl preheated elution buffer is added directly onto the column membrane and left at room temperature for 2 minutes. To elute DNA, centrifuge 5000 xg for 1 minute and store the DNA at -20^{o}C.

3.4 Genotyping

Polymerase Chain Reaction Restriction-Fragment Length Polymorphism (PCR- RFLP) is a PCR method for adding enzymes after the amplification of enzymes, which will give a more specific result (Lubis, et al., 2018). To perform genotyping, the PCR-RFLP technique was applied for SNP genotyping as it is a more simple, accurate, and cheap technique (Ota, et al., 2007). PCR-RFLP is any genomic DNA that can be distinguished based on the absence or presence of the sites of restriction enzymes. The restriction enzyme will cut the sites once they are recognised (Saraswathy and Ramalingam, 2011). Once catted, the DNA fragments can be clearly visible as bands by using electrophoresis (Lee, et al., 2012), and this can be used for the analysis of genetics.

To perform genotyping, the PCR-RFLP technique was applied, as it can be used as a genetic marker. PCR was carried out to amplify the DNA or to multiply the copies of a segment of DNA in large amounts. Before the PCR process, a few materials need to be prepared, including magnesium dichloride (MgCl2), PCR buffer, Taq polymerase, free nucleotides (dNTPs), and a pair of primers. The primer used in this study will be Forward: 5'TGACATTATTCATTGTTTGTTGCT-3' and Reverse: 3'TGGGAACTCTCCATCTCTTTCA-5'.

Theoretically, the process started with incubating the extracted DNA at 94^{o}C for 2 minutes to separate the double strands of DNA. A pair of primers, a free nucleotide, and Taq polymerase are then added. The next step will be the annealing step, where the primers will attach to the single stranded DNA during incubation at 60^{o}C for 1 to 2 minutes. After that, incubate the DNA at 72^{o}C for 1 minute which is an extension process. The process from the beginning until the extension step was required to repeat 30 to 35 cycles before the final extension, which occurs at 72^{o}C for 6 minutes.

Before performing the PCR, the equipment and material used must be autoclaved first to avoid any contamination. The autoclave machine used is shown in Figure 3.4.

Figure 3.4.1: Autoclave Machine used in the Laboratory.

The PCR process was initiated by preparing the PCR reagents, which consisted of 17.3 µl of deionized water, 2.5 µl of 10x PCR buffer, 0.2 µl of Taq polymerase, 1.5 µl of magnesium dichloride, 0.5 µl of dNTPs, and 1 µl of a pair of primers. These reagents were added to a PCR tube and mixed well using a vortex mixer. Next, the DNA sample was added to the same PCR tube, and the contents were thoroughly mixed using a vortex mixer and minicentrifuge to ensure complete mixing of the DNA sample with the PCR reagents. The PCR tube was then placed into a thermal cycler for the PCR process. Once the PCR process was complete, the PCR tube was removed from the thermal cycler and subjected to enzyme incubation.

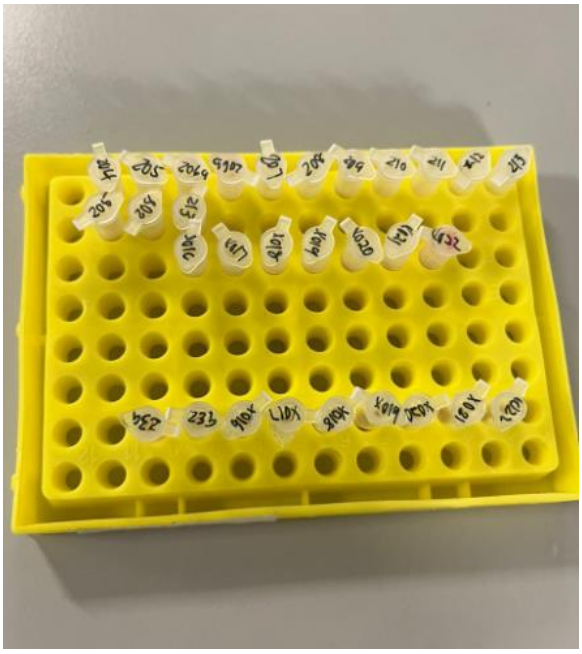


Figure 3.4.2: PCR Tubes with DNA Samples and PCR Reagents.

Figure 3.4.3: Vortex Mixer and Minicentrifuge that use to mix the DNA samples and PCR Reagents.

Figure 3.4.4: Thermal Cycle for PCR Process.

Following the completion of the PCR process, enzyme incubation was carried out to allow the restriction enzyme (*Msel*) to cut the specific part of the DNA. To prepare the enzyme incubation mixture, a microcentrifuge tube was used to combine 7.9 µl of water, 2 µl of enzyme buffer, and 0.1 µl of restriction enzyme (*Msel*), which was then thoroughly mixed using a vortex mixer. Next, 10 µl of the PCR samples were added to the same microcentrifuge tube and mixed well using the vortex mixer. The microcentrifuge tube was then incubated in a water bath at 65^{o}C for 1 hour. After the incubation period, the microcentrifuge was taken out and prepared for band viewing.

After incubation of the enzyme has been completed, the sample can be viewed through agarose gel electrophoresis by mixing the sample with 6x loading dye.

Figure 3.4.5: Water Bath for Incubation.

3.5 Gel Electrophoresis

The purpose of gel electrophoresis is to determine the number of nucleotides in a segment of DNA and the presence of DNA in the sample. According to their molecular sizes, a combination of DNA, RNA, or protein can be separated using the technique of gel electrophoresis. Electrical current will force the fragment of DNA to pass through the firm gel, which is a sieve with a small pore of a fixed size. The fragment of DNA is travelling through the pores in the gel based on their length. In other words, the longer fragments, which have more nucleotides, are bigger in size and have a larger molecular weight, so they are not able to pass through the small pores in the gel and get hung up at the beginning of the gel. Meanwhile, the shorter fragment can pass through the small pores and move further along the gel. Lastly, the intermediate-length fragment will travel to about the middle of the gel. The DNA fragments are then visualised in the gel with a dye.

To begin the process of gel electrophoresis, the gel was first prepared. This involved adding 0.8 g of agarose powder, 18 µl of water, and 2 µl of buffer to a beaker. The beaker was then heated in an oven for 40-50 seconds to dissolve the agarose powder in the solution. Next, 3 µl of gel stain was added to the beaker and stirred thoroughly. The resulting mixture was then poured into a mould and allowed to solidify.

After the gel has solidified, the gel electrophoresis procedure was carried out. The gel was placed into the electrophoresis tank containing 1x buffer, and 2 µl of the ladder was inserted into a well on the gel. To prepare the samples for visualization, 10 µl of each sample that had been incubated with enzyme was mixed with 2 µl of loading dye, and the resulting mixture was inserted into the well on the gel. The power supply was then connected to the electrophoresis tank and set to run at 80 voltage for 40 minutes. After 40 minutes, the gel was viewed using UV radiation to observe the band's existence.

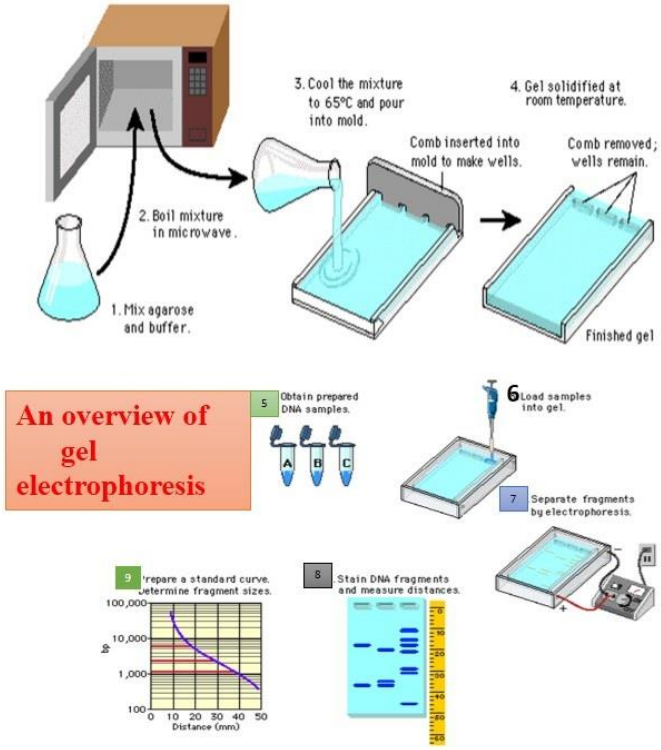


Figure 3.5.1: Step for Gel Electrophoresis (universe84a, nd).

3.6 Analysis

3.6.1 Statistical Analysis

Statistical analysis is the process of collecting and interpreting large amounts of data to identify patterns and trends. The Statistical Package for the Social Sciences (SPSS) software was used in this analysis. Chi-square (2) tests in the SPSS software were used to analyse the differences in allele and genotype frequencies between controls and patients. Not only that, a further analysis of

allele and genotype distribution will be carried out to inquire into ethnicity and gender for the SNP rs2954041.

3.6.2 Meta-Analysis

3.6.2.1.1 Search and Selection of Studies

In this review paper, the databases used will be the National Library of Medicine's PubMed and ScienceDirect. The searching keywords will be schizophrenia, *Neuregulin 1* (NRG1), and rs2954041 to collect the studies that were published up to 2019.

As each decision will influence the accuracy of the result, care must be taken when conducting the meta-analysis. There were several things to consider when carrying out the meta-analysis: (1) the way to search for the studies; (2) the selection of the studies based on some criteria. (3) deal with incomplete data; (4) prevent publication bias when analysing the data.

For more reliable meta-analysis results, the selection of the studies must be very careful. The are several criteria when selecting the studies (1) written in English (2) published in referee journal (3) shows positive or negative association between rs2954041 of *NRG1* and schizophrenia (4) provided enough information to calculate the odds ratio (OR) and *p*-value (5) show original data (6) included healthy control (7) show diagnosed method.

3.6.2.2 Data Analysis

All the data will be analyzed using a meta-analysis software called Comprehensive Meta Analyses Version 3.0. Several data are needed from each article or journal, including the odds ratio (OR), 95% confidence intervals (CIs) and haplotype *P*-value. Firstly, the odd ratio was required to be pooled by using the fixed effects and random effect methods, as it can be used to determine the association between risk factors and outcome while also comparing the magnitude of various risk factors for the particular outcome. A Z-test was used to determine the significance of the pooled odd ratio. Next, 95% CIs were calculated according to Woolf's method, which it used to figure out the precision or accuracy of the odd ratio. The haplotype *P*-value shows the association of the haplotype with schizophrenia, and when the *p*-value is

smaller than 0.05 ($p<0.05$) it is considered significant.

Moreover, the heterogeneity between the studies was tested by Cochran's chi-square-based Q-statistic and I-squared value (I2). The I^2 test was used to estimate the degree of the heterogeneity, where I^2 smaller than 25% (<25%) indicates low heterogeneity and I^2 bigger than 50% (>50%) indicates high heterogeneity.

Other than that, publication bias was evaluated by Egger's test with the aid of funnel plots (Egger et al, 1997). The value will be adjusted by the Fill and Trim test once publication bias was detected. Trim and Fill test was used to identify and correct the asymmetry of the funnel plot that was arising from the publication bias (Duval and Tweedie, 2000). The flow of the trim and fill method as following:

(i) To eliminate the smaller studies that contribute the imbalance of the funnel plot problem.

(ii) Estimate the trimmed funnel plot's real 'centre' to determine the funnel genuine shape.

(iii) Filling in the gaps left by the studies that were left out and their missing equivalents around the centre.

The trim and fill method not only provides the calculation for the adjusted confidence intervals but also offers an estimate of the absence of research via the asymmetry of the funnel plot.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

In this chapter, the results and discussions of laboratory work were discussed. The results of laboratory work were included Polymerase Chain Reaction (PCR) optimisation, Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP). Meanwhile, the results also included for statistically analysis of the data through meta-analyses.

In the PCR optimization process, the parameters such as annealing temperature and time were optimized to obtain the PCR product, The results of the PCR optimization showed that the PCR product was successfully amplified. In the PCR-RFLP analysis, the restriction enzyme was used to digest the PCR product, and the resulting fragment were visualized through gel electrophoresis.

The discussions on the results obtained were stated clearly in this chapter. The discussions highlighted the significance of the findings and their potential for future research.

4.2 Results

4.2.1 PCR Optimisation

The samples used for the laboratory work were collected from the hospital. The samples collected were divided into ethnic groups and genders. The characteristics of the patients and healthy controls are shown in Table 4.1 below.

Table 4.1: Characteristics of the Healthy Controls and Patients.

Characteristic	Patients	Controls
Gender		
Male	17	9
Female	11	7
Ethnic Group		
Malay	14	8
Chinese	10	6

Table 4.1 (Continued)

India	4	2
Age		
10-30 years	2	1
Above 30 years	26	15

The sample has to go through the PCR optimization in order to find out the annealing temperature. The annealing temperature is the temperature at which the primer adheres to the template DNA. The purpose of figuring out the annealing temperature is because the primer is a brief sequence of oligonucleotides that is used to kickstart DNA synthesis during PCR. Alongside the component/reagent required for PCR, the optimal temperature for annealing (annealing temperature) is important as it will affect the success of the PCR amplification process (Silalahi, et al., 2021). Figure 4.1 is the result of PCR optimization.

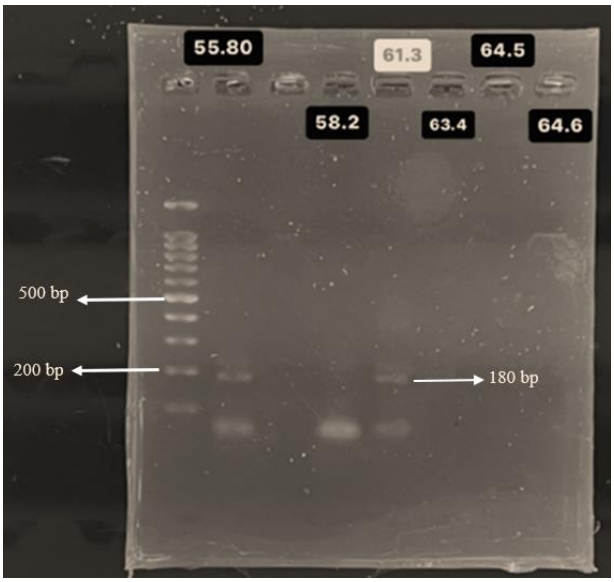


Figure 4.1: Gel Electrophoresis for PCR Optimisation.

It is clearly shown that the band is able to be visible at temperatures of 55.8^{o}C and 61.3^{o}C. The visible band indicates the annealing temperature. In this study, the temperature of 61.3^{o}C was chosen as the annealing temperature as it is more stable for PCR amplification.

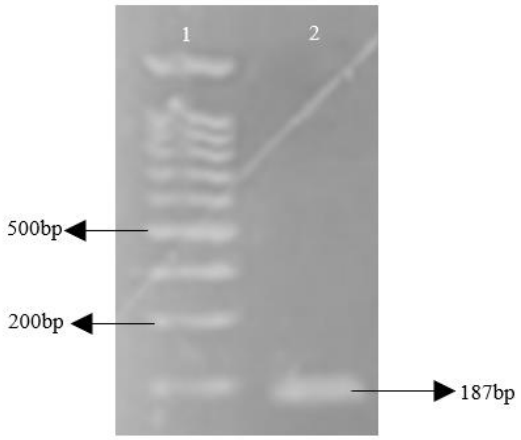
4.2.2 PCR-RFLP

The primary Polymerase Chain Reaction (PCR) product size, restriction enzyme used, and the Restriction Fragment Length Polymorphism (RFLP) product size are tabulated at the Table 4.2 below.

Table 4.2: Details of PCR Product Size.

SNP	Primer Sequence	PCR Product Size	Restriction enzyme	PFLP Product Size
rs 2954041	5’TGACATTATTCATTGTTTGTTGCT3’ (Forward) 5’GGATGCCATGGATATACTATGCAGA3’ (Reversed)	187bp	*Msel*	G 60,127 T 30,31,126

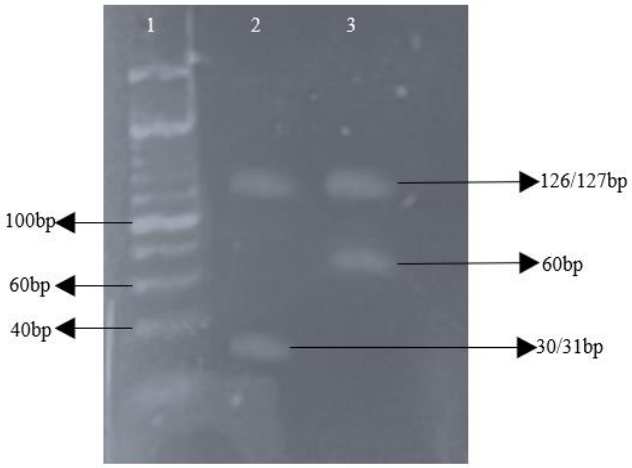
As shown in Table 4.2, the primary PCR product size for rs2954041 was 187 base pairs (bp) and included two *Mfel* sites, which resulted in five restriction fragments of 127 bp, 126 bp, 60 bp, 31 bp and 32 bp. Homozygous G/G genotypes were represented by two fragments (127 bp and 60 bp), whereas homozygous T/T genotypes were represented by three fragments (126 bp, 30 bp and 31 bp). In this study, Heterozygous G/T genotypes were not found. The base pairs of 31 and 30 are too close, and this causes the observation between the two base pairs to not show clearly. Meanwhile, similar reasons go to base pairs 126 and 127, which both base pairs could not observe clearly by agarose gel electrophoresis.



Lane 1: 100bp ladder (Vivantis)

Lane 2: 187 bp of PCR product of *NRG1* gene

Figure 4.2: PCR Product of *NRG1* (rs2954041) with 187 bp after running in 3.5 % Agarose Gel.



Lane 1: 20bp ladder (Vivantis)

Lane 2: TT homozygous (126 bp, 30 bp and 31 bp)

Lane 3: GG homozygous (127 bp and 60 bp)

Figure 4.3: RFLP for Screening of *NRG1* Gene (rs2954041) for G/G and T/T Allele polymorphism After Digesting by Restriction Enzyme with *Mfel* and running in 4% Agarose Gel.

Figure 4.2 above is the result after the DNA amplification while Figure 4.3 above is the result of the restriction fragment pattern of PCR products after digestion with restriction enzyme (*Mfel*). All 44 samples (healthy controls and patients) results can be found in Appendix 1. As mentioned earlier, there are only two genotypes (GG and TT) found in this study. However, among both genotypes, homozygous GG accounted for most of them, which took a majority in this study.

4.3 Analysis

4.3.1 Statistical Analysis

Allele and genotype frequency distributions of the SNP rs2954041 for Malaysian patients and healthy controls are tabulated at Table 4.3, Table 4.4 and Table 4.5 below.

Table 4.3: Allele and Genotype Frequency Distribution of SNP rs2954041 for Malaysian Patients and Controls (Ethnic Group).

	Allele (%)		Genotype (%)		
Malay Patients (rs2954041)					
Allele/Genotype	G	T	GG	TT	GT
	22 (78.57)	6 (21.43)	11(78.57)	3(21.43)	0(0)
Malay Controls (rs2954041)					
Allele/Genotype	G	T	GG	TT	GT
	10(62.5)	6(37.5)	5(62.5)	3(37.5)	0(0)
χ^2(df)	6.696(1)		6.691(1)		
P-value	0.01		0.01		
Odds Ratio (OR)	0.441		-		
95% Confidence Interval	0.235-0.825		-		
Chinese Patients (rs2954041)					
Allele/Genotype	G	T	GG	TT	GT
	14 (70)	6 (30)	7 (70)	3 (30)	0(0)

Table 4.3 (Continued)

Chinese Controls (rs2954041)					
Allele/Genotype	G	T	GG	TT	GT
	12 (100)	0 (0)	6 (100)	0(0)	0(0)
χ^2(df)	35.294(1)		35.294 (1)		
P-value	0.001		0.001		
Odds Ratio (OR)	1.429		-		
95% Confidence Interval	1.257-1.624		-		
India Patients (rs2954041)					
Allele/Genotype	G	T	GG	TT	GT
	8 (100)	0 (0)	4 (100)	0 (00)	0(0)
India Controls (rs2954041)					
Allele/Genotype	G	T	GG	TT	GT
	2 (50)	2 (50)	1 (50)	1(50)	0(0)
χ^2(df)	66.667(1)		35.294 (1)		
P-value	0.001		0.001		
Odds Ratio (OR)	0.5		-		
95% Confidence Interval	0.411-0.608			-	

Table 4.4: Allele and Genotype Frequency Distribution of SNP rs2954041 for Malaysian Patients and Controls (Gender).

	Allele (%)		Genotype (%)		
Female Patients (rs2954041)					
Allele/Genotype	G	T	GG	TT	GT
	14 (70)	6 (30)	7 (70)	3 (30)	0 (0)
Female Controls (rs2954041)					
Allele/Genotype	G	T	GG	TT	GT
	10 (71.43)	4 (28.57)	5 (71.43)	2 (28.57)	0 (0)
χ^2(df)	0.024 (1)		0.024 (1)		

Table 4.4 (Continued)

P-value	0.877		0.877		
Odds Ratio (OR)	1.049		-		
95% Confidence Interval	0.571-1.927		-		
Male Patients (rs2954041)					
Allele/Genotype	G	T	GG	TT	GT
	28 (82.35)	6 (17.65)	14 (82.35)	3 (17.65)	0 (0)
Male Controls (rs2954041)					
Allele/Genotype	G	T	GG	TT	GT
	14 (77.78)	4 (22.22)	7 (77.78)	2 (22.22)	0 (0)
χ^2(df)	0.500 (1)		0.500 (1)		
P-value	0.480		0.480		
Odds Ratio (OR)	0.778		-		
95% Confidence Interval	0.388-1.561		-		

Table 4.5: Allele and Genotype Frequency Distribution of SNP rs2954041 for Malaysian Patients and Controls.

	Allele (%)		Genotype (%)		
Patients (rs2954041)					
Allele/Genotype	G	T	GG	TT	GT
	40 (76.92)	12 (23.08)	20 (76.92)	6 (23.08)	0(0)
Controls (rs2954041)					
Allele/Genotype	G	T	GG	TT	GT
	24 (75)	8 (25)	12 (75)	4(25)	0(0)
χ^2(df)	0.110(1)		0.110(1)		
P-value	0.741		0.741		
HWE *p*-value	-		<0.001 (Control) <0.001 (Patient)		
Odds Ratio (OR)	0.896		-		

Table 4.5 (Continued)

95% Confidence Interval	0.468-1.716	-

By looking at Table 4.3, for the ethnicity groups of Malay, Chinese, and India in Malaysia, the allele frequency of rs2954041 for all of the ethnicity groups established significance (p =0.01 and p =0.001) in both patients and healthy controls. According to previous studies, rs2954041 was linked to schizophrenia found in family-based (Yang, et al., 2003). Next, as shown in Table 4.4 above, neither genotype nor allele is significantly associated with schizophrenia by gender ($p > .05$).

Next, as can be seen from Table 4.5, single locus analysis established that the genotype and allele of the SNP rs2954041 are insignificant (p =0.745) for the patient and healthy control populations in the Malaysian populations.

4.3.2 Meta-Analysis

Table 4.6: Different Studies of SNPrs2954041 in Association with Schizophrenia.

Study	Country/ Ethnicity	Sample Number	Diagnostic System	Odds Ratio (OR)	95% Confidence Interval (CI)
Benzel, 2007	UK Caucasian	396 cases 1342 controls	DSM-IV	1.100	0.57-2.12
Sander, 2008	USA Caucasian	1870 cases 2002 control	DSM-IV	1.160	0.79-1.70
Shiota, 2008	Japan Japanese	396 cases 491 controls	DSM-IV	1.210	0.99-1.48
Jonsson, 2009	Denmark Caucasians	837 cases 1473 controls		0.890	0.60-1.32

Table 4.6 (Continued)

Tee, 2012	Malaysia	153 cases 150 controls	MINI	1.00	0.56-1.78
	Asians (Malay)	183 cases	MINI	0.804	0.45-1.44
	Asians (Chinese)	179 controls			
	Asians (India)	81 cases' 100 controls	MINI	11.780	0.74-4.29
Rukhsana, 2014	Pakistan Pakistani	100 cases 70 controls	DSM-IV & ICD-10	4.429	1.816-10.801
This Study, 2023	Malaysia	14 cases 8 controls	MINI	0.441	0.235-0.825
	Asians (Malay)	10 cases	MINI	1.429	1.257-1.624
	Asians (Chinese)	6 controls			
	Asians (India)	4 cases 2 controls	MINI	0.500	0.411-0.608
Total (Pooled)		5517cases 5823 controls		1.053	0.967-1.146

Table 4.6 above shows the different studies for the association of rs2954041 with schizophrenia, including the current study for three ethnicities (Malay, Chinese, and Indian) in Malaysia. as to carry out the meta-analysis, at least five journals are required. The odds ratio (OR) and 95% Confidence Interval (Upper Limit and Lower Limit) are the data required for the meta-analysis. In this study, both the odds ratio and 95% confidence interval were calculated using Statistical Package for Social Sciences (SPSS) software.

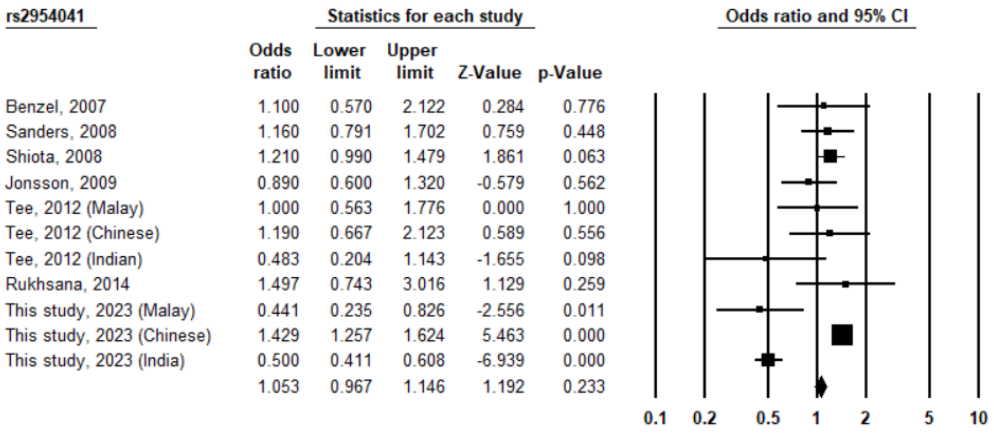


Figure 4.4: Meta Analysis Result (Forest Plot) of SNP rs2954051 Association with Schizophrenia Based on All Studies.

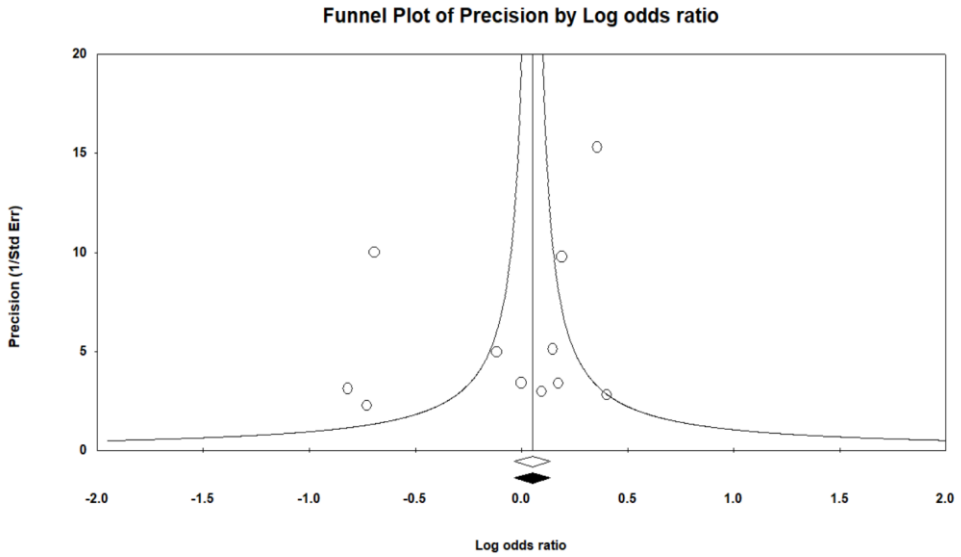


Figure 4.5: Meta Analysis Result (Funnel Plot) of SNP rs2954051 Association with Schizophrenia Based on All Studies.

In meta-analysis, the *p*-value shows the probability of gaining an even larger difference in the observed effect of the intervention (ignoring possible bias), assuming the null hypothesis is true (the null hypothesis means that there is no difference in the effect of the various interventions that were compared) (Jakobsen, et al., 2014). The observed effect is therefore exceedingly unlikely to have developed just by chance, and a *p*-value that is very small provides evidence against the null hypothesis (Higgins, et al., 2019). In simple words, when the *p*-value is greater than 0.05, the null hypothesis that

there is no difference between the means is rejected and it is concluded that there is a significant difference. Meanwhile, the presence of a significant difference cannot be inferred if the *p*-value is greater than 0.05 (Minitab Blog Editor, 2015).

No significant allelic associations were found when all population sources were included in a single analysis except for this study by ethnic groups and gender. As shown in Figure 4.5, all studies except for those on ethnic groups in this study have a *p*-value that is greater than 0.05. Although the ethnic group studies in this study showed a *p*-value less than 0.05, The different genders and pooled samples for this study have a *p*-value that is greater than 0.05 due to the size of the study's sample and its effect, both of which influence the *p*-value.

Furthermore, the odds are the ratio between the likelihood of something happening and its likelihood of not happening in each group. The odds ratio is the comparison of the probabilities of two groups. Next, the range of values surrounding the actual but unidentified population value is captured by the confidence intervals (Cls), which offer upper and lower limits. The commonly used confidence interval is 95% Cl, which is equivalent to the conventional 5% significance threshold used in the hypothesis tests (Israel and Richter, 2011).

The total sample sizes for both patients and controls were 28 and 22, respectively. The odds ratio and 95% confidence interval for the 12 population-based studies are shown in Figure 4.5. All the studies produce a combined risk, 95% confidence interval, and *p*-value of 1.050, 0.965 to 1.142, and 0.255, respectively, suggesting no conclusive evidence linking this polymorphism to schizophrenia.

Next, in meta-analysis, a funnel plot is a graphical representation that is used to evaluate potential publication bias, which happens when studies with statistically significant results are more likely to be published than studies with non-significant results (Sterne and Egger, 2001). If there is no publication bias, the plot should resemble an inverted funnel, with smaller research dispersed widely at the base and larger studies grouped near the top. As can be seen from Figure 4.6, the spots that are out of the range were a sign of

publication bias in the case of rs2954041. As a result of the publication bias, the Duval and Tweedie trim and fill method was used to recalculate the effect size (Shi and Ling, 2019). The funnel plot needed to have one study was lacking, according to the result from the Duval and Tweedie trim and fill method, which is shown in Figure 4.7.

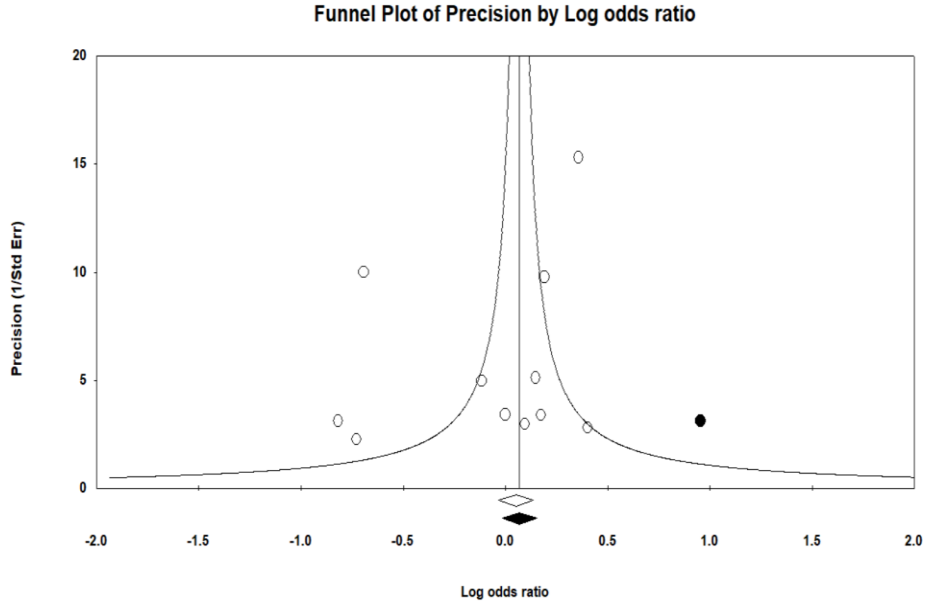


Figure 4.6: Meta Analysis Result (Funnel Plot After Trim and Filled) of SNP rs2954051 Association with Schizophrenia Based on All Studies.

4.4 Discussions

A complex biological disease with a 1% prevalence rate worldwide is schizophrenia. The risk of schizophrenia is increased by abnormal brain development. While it is true that non-genetic elements like hypertension, a stressful environment around the person, and traumatic life events also play a significant part in the development of the disorder, it is also discovered that schizophrenia is a genetic mental illness. There may be a connection between the pathophysiology of schizophrenia and the brain illness brought on by a confluence of genetic and environmental variables (Braff, et al., 2007).

Moreover, several susceptibility genes for schizophrenia play a crucial role in the disease's aetiology. One of the putative genes of interest, known as *NRG1*, encodes a protein with a variety of functions specific to

neurons. The *NRG1* gene, a schizophrenia locus, is situated on chromosome 8p22-12. *The NRG1* gene is a likely candidate gene for schizophrenia given its role in controlling the expression of glutamate, serotonin, and other neurotransmitters as well as synaptic plasticity (Fall, 2003).

The *NRG1* gene was selected to be studied as it has been linked to various important pathways that are involved in schizophrenia, such as the dopamine signalling pathway and the glutamate signalling pathway.

The prefrontal cortex, hippocampus, and striatum, which are all involved in the control of dopamine signalling, are some of the regions that express *NRG1*. The dopamine signalling system controls the release, reuptake, and communication of dopamine in the brain through a sophisticated network of neurons and receptors. Increased dopamine receptor expression, especially the D1 and D2 receptors, is one way that *NRG1* influences the dopamine signal. Increased dopamine release and signalling can be achieved by enhancing those receptors' expression, which improves the dopaminergic neurons' sensitivity to dopamine. The positive symptoms of schizophrenia, such as hallucinations and delusions, may be exacerbated by this increased expression of dopamine receptors (Brisch, et al., 2014). Furthermore, *NRG1* has been found to influence tyrosine hydroxylase (TH) activity, which is the enzyme in charge of dopamine production, in addition to its actions on dopamine receptors, hence enhancing dopamine release. The expression and function of *NRG1* and its receptors may change because of these mutations, which may then impact dopamine signalling and aid in the onset of schizophrenia (Paterson, et al., 2014). This pathway's deregulation has been linked to schizophrenia.

Next, the main excitatory neurotransmitter in the brain, glutamate, is involved in many critical activities, such as memory, cognition, and learning. *Dysbindin-1*, *NRG1*, G72, D-amino acid oxidase (DAAO), and regulator of G protein signalling are some of the genes associated with schizophrenia susceptibility that may influence glutamate (Glu) synaptic function (Owen et al, 2004). *NRG1* interacts with the postsynaptic density (PSD), is found in glutamate synaptic vesicles, controls N-methyl-D-aspartate (NMDA) receptor production, and triggers epidermal growth factor (*ErbB*) receptors, which

associate with NMDARs Schizophrenia's onset has been linked to abnormalities in glutamate signalling. *ErbB4*, which is mostly expressed in glutamatergic neurons, is the receptor that *NRG1* binds to to affect these neurons. The NMDA receptor, a subtype of glutamate receptor essential for many aspects of synaptic plasticity, learning, and memory, becomes more active because of *ErbB4* activation by *NRG1* (Carvajal, et al., 2016). Schizophrenia results in a disruption of *NRG1-ErbB4* signalling, which lowers NMDA receptor activation and modifies glutamate signalling. The cognitive and behavioural abnormalities seen in the condition may be a result of these alterations.

Using mapping and linkage investigations in Icelandic families, a relationship between the *NRG1* gene and schizophrenia has been investigated (Stefansson, et al., 2002). In this investigation, two microsatellites and five SNPs were examined for associations in the Scottish population as well as the Icelandic population. Three polymorphisms of the *NRG1* gene were genotyped in the Chinese population. One SNP from HAP_{ICE} (SNPNRG221533) and two others were chosen at random from a public database from exon 2 (rs3924999) and intron 5 (rs2954041). The link between these SNPs and schizophrenia showed a strong association. The same group quickly replicated the connection with a Scottish sample (Stefansson, et al., 2003). Yet, the outcomes of later replication trails were erratic, and no vulnerable variant has yet been found. Rukhsana et al. (2014) also studied the susceptibility of the haplotype consisting of rs2954041 to schizophrenia in the Pakistani population. Yet, there was no association between schizophrenia and the haplotype.

In the current research, there was a correlation between rs2954041 of *NRG1* and schizophrenia in the Malaysian population by ethnicity, with the p-value for three races, which are Malay, Chinese, and Indian, being less than 0.05, as shown in Table 4.4, where the genotype GG was more frequent in both patients and the controls, which is contrary to the Costa Rican population (Moon, et al., 2011). According to Haukvik, et al (2010), the study likewise discovered no association between the rs2954041 polymorphism and schizophrenia in Caucasians. The selection of patients may contribute to the variance in the association's result, as many countries have diverse patient

characteristics, such as subtypes, comorbidity, and the severity of their illnesses (Tsuang and Faraone, 1995). Although this study shows a significant association between rs2954041 and schizophrenia, the result is not reliable as the sample size is too small, which might lead to a false association.

Furthermore, male and female schizophrenia patients were studied separately to determine whether there was a different association based on gender. By looking at Table 4.5 above, it is clearly shown that no association between both genders was discovered, as the *p*-values for both the allele and genotype are greater than 0.05. The genotype GG was found to be most frequent in both patients and controls.

According to the study, males but not females were found to have an elevated risk of schizophrenia while carrying the rs2954041 risk allele. The study indicated that the males with the risk allele were around 1.5 times more likely than males without the risk gene to develop schizophrenia (Liu, et al., 2017). Nevertheless, the study also reported that there was no correlation between rs2954041 and schizophrenia in females (Liu, et al., 2017).

The result of no association of schizophrenia with gender in this study is contrary to the Chinese Han population (Chen, et al., 2011) who discovered a significant association of schizophrenia in both females and males. In fact, it seems that there may be gender differences in the complex link between schizophrenia and rs2954041. The gender issue has not been widely studied. There is no evidence to support the effect of different genders on schizophrenia. The mechanisms underlying this correlation and the potential gender-specific effects of rs2954041 on schizophrenia require further study.

Next, as shown in Table 4.6, there is no association between the genetic variant rs2954041 and schizophrenia. The null hypothesis—that there is no link between the genetic variant and the disease-cannot be rejected because the *p*-values for the allele and genotype in both the patient and control groups are greater than 0.05. This result is in contrast to the study by Yang, et al. (2003), which showed an association between rs2954041 and schizophrenia in a family-based sample. The genotype frequency of patients with homozygous G/G is 76.92%, homozygous T/T is 23.08%, and as there is no heterozygous genotype G/T found in this study, the frequency will be zero.

Meanwhile, the genotype frequency of control for homozygous G/G is 75%, homozygous T/T is 25%, and heterozygous G/T is 0%, as it is not found in this study. From the genotype frequency, it can be said that GG appears most frequent in the Malaysian population. The risk allele for rs2954041 contributing to schizophrenia is 'T' (Mostaid, et al., 2017), as the allele 'G' appears more in this study, hence the result will show an insignificant association.

According to earlier research, the gene rs2954041 was found to be significantly distributed in Chinese schizophrenia families and was related to schizophrenia in family-based analyses (Yang, et al., 2003). Hence, it can be said that the rs2954041 revealed that the relative risk was higher in instances with a feasible family history. Meanwhile, none of the *NRG1* markers demonstrated individual connections, as was the case for the Icelandic and Scottish populations (Stefansson, et al., 2003).

Next, the Hardy-Weinberg Equilibrium (HWE) *p*-value was calculated and shown in Table 4.6. HWE defines the connection between allele and genotype frequencies in a population that is not changing. It is a fundamental principle in population genetics. HWE asserts that in the absence of evolutionary processes like mutation, natural selection, migration, or genetic drift, the allele and genotype frequencies in a population will stay unchanged from generation to generation (Roux, 1974). The HWE principle is based on several assumptions: 1) There are no mutations in the population. 2) There is no population emigration or immigration. 3) The population size is sufficient to prevent genetic drift. 4) Mating is arbitrary. 5) Natural selection is not taking place (Roux, 1974).

For non-codominant loci that are in HWE, marker genotype testing was done. For the rs2954041 locus, the genotype distribution for both patients and controls were out of HWE as the HWE *p*-value was less than 0.05. When a HWE test results in a p-value of less than 0.05, it means that the observed genotype frequencies in a population are significantly different from the expected frequencies under HWE. There are several reasons that caused the patient and control groups to be out of HWE, such as the sample size being too small, which is affected by the random effects of genetic drift (Raymond and

Rousset, 1995), selection affecting patients and controls differently, or sampling error. A population can generally be out of equilibrium if the HWE's assumptions are violated in any way. For evaluating theories regarding the evolutionary factors influencing genetic variation in populations, the HWE is a suitable null model.

An extension section of the genome, like the *NRG1* locus, requires the examination of numerous combinations of markers to determine a haplotype. As there are fewer haplotypes observed than there should be, it is challenging to determine the statistical impact of running many tests with phase uncertainty. To determine the relationship between the Asian and Caucasian populations, a meta-analysis was conducted.

The meta-analysis conducted included 11 studies with a total of 5517 cases and 5823 controls for SNP rs2954041 produced the most thorough analysis of the relationship between schizophrenia and *NRG1* polymorphisms. In meta-analysis, the pooled data produced a pooled risk of 1.053, a 95% CI of 0.967–1.146, and a *p*-value of 0.233. The pooled data did not show any evidence linking rs2954041 to schizophrenia. For rs2954041, the majority of independent case-control studies (Benzel, et al., 2007, Sanders, et al., 2008, Shiota, et al., 2008, Jonsson, et al., 2009, Tee, et al., 2012) discovered a negative correlation between schizophrenia and rs2954041, and even after pooling the data with the current study, the results still showed no correlation between schizophrenia and rs2954041. These studies' sample sizes were sufficient to obviate type II error. The samples' ethnicity was substantially correlated with the odd ratios, according to a prior finding by Glatt, et al (2003). Because there was no discernible indication of heterogeneity among the odds ratios in the rs2954041 heterogeneity studies, separate analyses of Asian and Caucasian populations were not carried out.

The standard test for heterogeneity in meta-analyses is the Cochran's Q test. It creates a probability that when larger, implies greater variation among studies rather than within people within a study. This probability is based on a chi-squared distribution. The underlying null hypothesis assumes that variances are only due to chance and that the genuine treatment effect is the same across trials (West et al., 2010). The squared deviations of each study

effect size from the total meta-analytic effect size are added up to calculate the Q statistic, which is then weighted by the inverse of each deviation's variance.

As shown in Figure 4.3, the results of the Q statistic test of meta-analysis were not statistically significant, making it likely that the study's findings are homogeneous. In other words, the findings from the studies that were included in the meta-analysis are comparable, and any detected discrepancies are more likely the consequence of chance fluctuations than actual variations in the effect sizes (Higgins, et al., 2019). Due to the fact that all the patients in this study were inpatients with lengthy hospital stays at a single location, these cases were probably clinically typical, and the schizophrenia diagnoses were trustworthy. Hence, it is unlikely that the discrepancies in the clinical diagnosis and the demographics will result in heterogeneity. The adverse correlation between rs2954041 and schizophrenia is caused by variations in allele frequencies resulting from different ethnic backgrounds. Not only that, but external environmental factors, including social dynamics and stress, as well as the nutritional, hormonal, and chemical environment in the mother's womb during pregnancy, have a significant impact. There are several possibilities that the Q statistic test is not statistically significant, such as low inter-study variation, similar study populations, or studies' low degree of precision (Higgins and Thompson, 2002).

Next, as mentioned, early publication bias exists in the case of rs2954041; hence, the trim and fill method was used to test for publication bias at allelic rs2954041, which looks for symmetry in the funnel plot. This revealed that in the majority of the individual studies, rs2954041 was a low-risk allele (Duval and Tweedie, 2000). Sampling error, variations in the calibre of smaller research, and the existence of real heterogeneity are a few contributors to publication bias (Song, et al., 2010). The results of subsequent large multicentre studies and earlier meta-analyses may not agree due to publication bias. Meta-analyses frequently rely on published research, therefore, if any of those studies aren't published because of publication bias, the meta-analysis might not accurately reflect the information that is currently available (Dawn, et al., 2008).

In communities with a wide genetic diversity, such as those in Malaysia, population stratification could be troublesome. Typically, family-based association approaches are more trustworthy than case-control studies because they are less impacted by population stratification. In this study, the combined sample size did not produce enough statistical power to determine the nature of the correlation, and the difference between the case-control study and the family-based study could be caused by phenotypic selection.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

In conclusion, the present study shows an association (*p*-value < 0.05) of SNP rs2954041 from the *NRG1* gene with schizophrenia in Malaysian Malay, Chinese, and Indians. However, it shows no association (*p*-value > 0.05) of SNP rs2954041 from the *NRG1* gene with schizophrenia by gender or the pooled Malaysian samples.

There are only two genotypes appearing in this study, which are genotypes G/G and T/T. The risk allele associated with schizophrenia is the thymine (T) allele of SNP rs2954041, but the 'T' allele does not appear frequently in this study as most of the samples give a G/G genotype.

According to the result of the meta-analysis, the association between rs2954041 and schizophrenia, which was derived from Asian and Caucasian studies, was not statistically significant for either race. Thus, the rs2954041 of the *NRG 1* gene may not significantly contribute to the development of schizophrenia in Malaysian patients.

However, the SNP rs2954041 is located at the fifth intron of the *NRG1* gene, which is around 18kb from the type III promoter. The expression quantitative trait locus for type III expression for rs2954041 has not been evaluated. But considering that it is close to the type III promoter and that there is preclinical evidence that type III disruption causes phenotypes that are frequently linked to schizophrenia, rs2954041 could potentially be involved in the pathophysiology of schizophrenia.

In fact, the rs2954041 of the *NRG 1* gene would need further studies to understand its potential role. Even in this study, the association between rs2954041 and schizophrenia was found to have no significant correlation between the genetic variant and the risk of developing the disorder. It is critical to keep looking into the genetic variations and environmental factors that can influence the onset of schizophrenia.

5.2 Recommendations for future work

From the result of meta-analysis, publication bias was found which was due to the journals or articles only publishing the significant results instead of insignificant results. Whereby, this publication bias can influence the accuracy of the results. Therefore, the publication bias should be avoided in order to increase the accuracy of the results.

The sample sizes of the study are recommended to increase to above 100 samples. The recommendation is given as more samples will increase the accuracy of the result and avoid giving a false association which in this study, there is a significant association between rs2954041 and schizophrenia in Malaysian Malay, Chinese and India, the significant association in this study might be a false association as the sample size is too small which affected the result.

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APPENDICES

Appendix A: Figures

Label	Age	Race	Gender
X007		Chinese	Male
X008		Chinese	Male
X009		Chinese	Male
X010		Chinese	Female
X011		Chinese	Male
X012		Chinese	Male
X013	45	Malay	Male
X014	43	Malay	Female
X015	45	Malay	Male
X016		Malay	Female
X017		Malay	Female
X018	42	Malay	Male
X019	25	Malay	Female
X020	32	Malay	Female
X021	55	Indian	Male
X022	53	Indian	Female

Appendix A- 1: Demographic Characteristics of Healthy Control Samples.

Label	Age	Race	Gender
Y001	49	Malay	Male
Y002	37	Malay	Male
Y003	53	Malay	Female
Y004	39	Malay	Male
Y005	63	Malay	Female
Y006	35	Malay	Male
Y007	29	Indian	Male
Y008	42	Chinese	Male
Y009	56	Chinese	Female
Y010	32	Chinese	Male
Y011	43	Chinese	Female
Y012	34	Chinese	Female
Y013	29	Indian	Male

Appendix A- 2: Demographic Characteristics of Schizophrenia Patient Samples.

Label	Age	Race	Gender
Z001	37	Malay	Male
Z002	36	Malay	Male
Z003a	52	Malay	Male
Z003b	46	Malay	Male
Z004	45	Malay	Male
Z005	37	Malay	Male
Z006a	34	Malay	Female
Z006b	30	Malay	Male
Z007	28	Indian	Female
Z008	66	Chinese	Female
Z009	48	Chinese	Male
Z010	31	Chinese	Male
Z011	40	Chinese	Female
Z012	37	Chinese	Female
Z013	33	Indian	Female

Appendix A- 3: Demographic Characteristics of Schizophrenia Patient Samples.

Appendix B: Tables

Appendix B- 1: RFLP Results of Healthy controls and Patients.

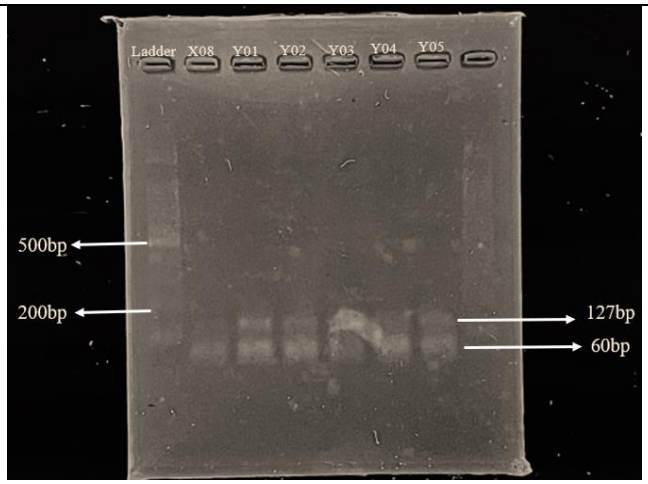
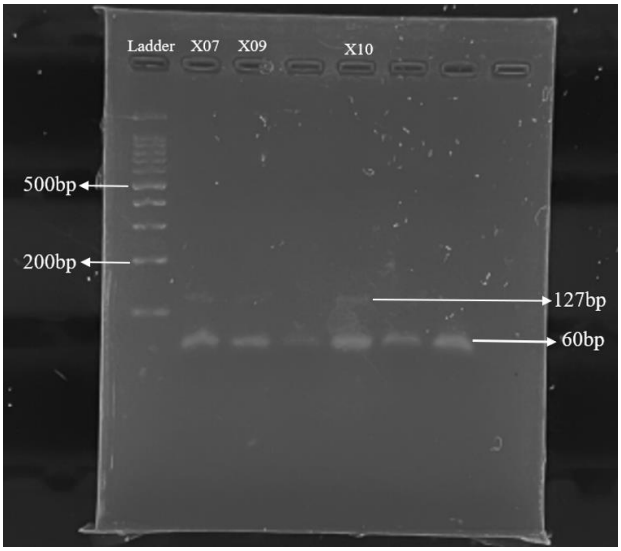
	RFLP Results
Controls and Patients	
Controls	

Table B-1 (Continued)

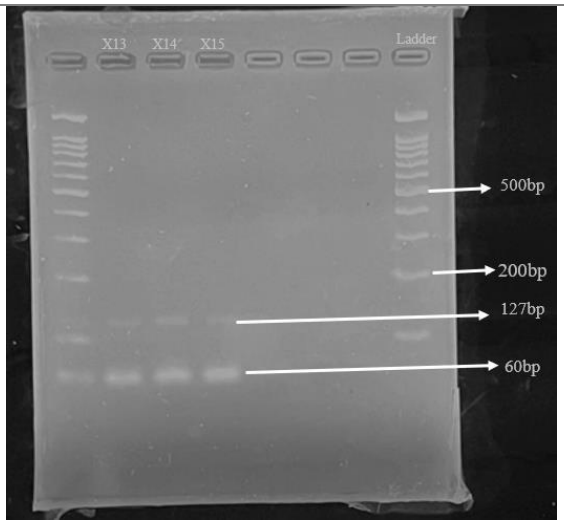
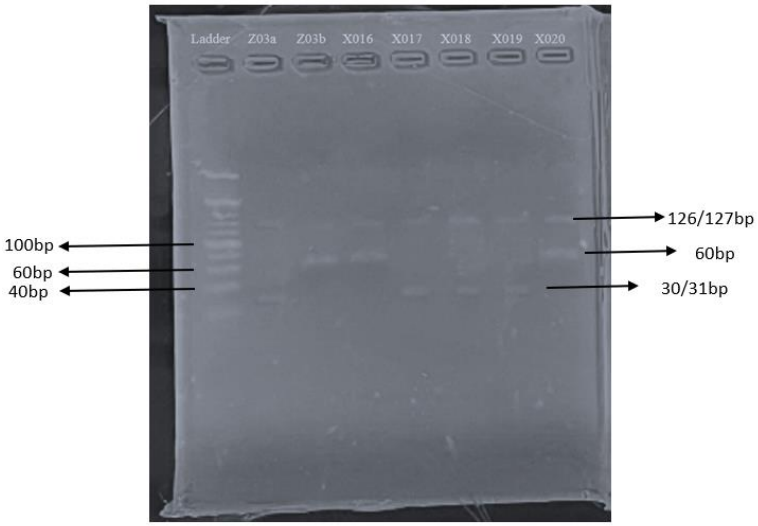
Controls	
Controls and Patients	

Table B-1 (Continued)

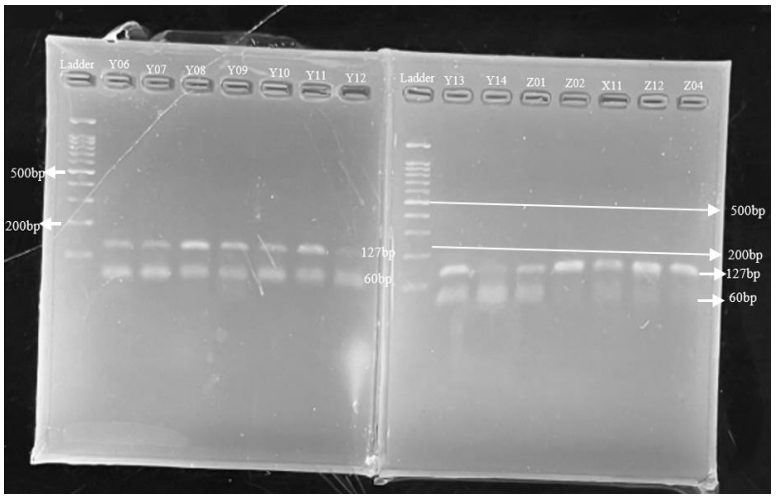
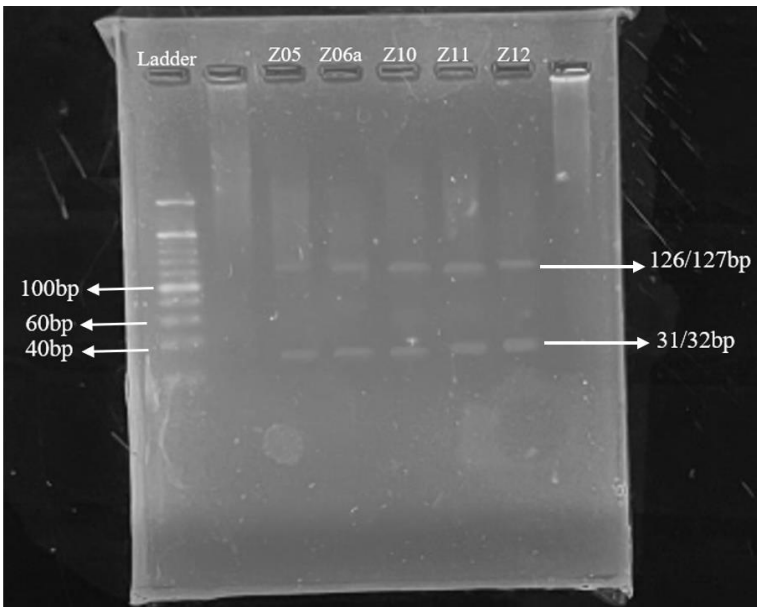
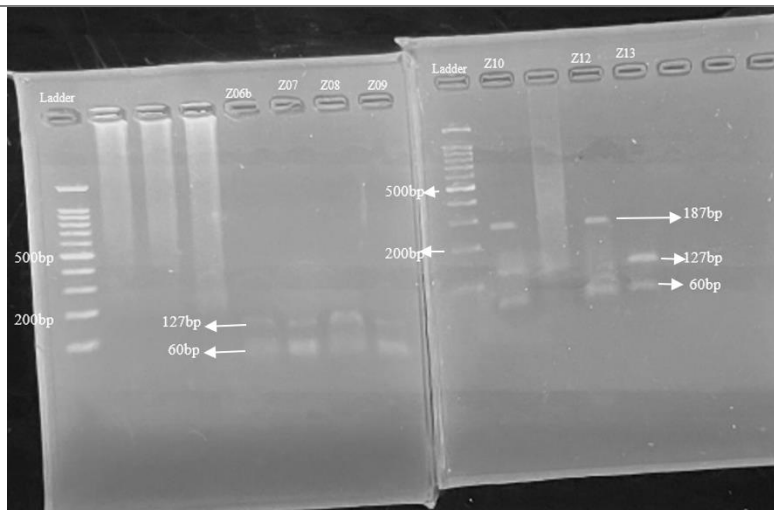
Controls	
Patients	
Patients	

Table B-1 (Continue)

Patients	

*X: Healthy Control; Y&Z: Patient