Study of *BDNF* (rs6265) and *TPH2* (rs4570625) Gene Polymorphisms in Patients Suffering from Clinical Depression

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Dedication

My thesis is dedicated to the Teacher of mankind Prophet Muhammad Peace Be Upon Him (PBHU) Who set the base of education for the whole world.

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اَلْحَمْدُ لِلّٰہِ

"i commence with the Name of Allah – in Whom all excellences are combined and Who is free from all defects. The Compassionate – One Whose Blessings are extensive and unlimited. The Merciful – One Whose Blessings are inherent and eternal. Special Praise be to Allah (S.W.T), the Sustainer of the creation. The Compassionate, the Merciful. Lord of the Day of Judgement. You alone we worship, and to You alone we pray for help. Guide us to the straight path. The path of those whom You have Favoured – the Prophets and their successors. Not of those who have incurred Your Wrath, nor of those who have gone astray."

(Aameen Sum Aameen)

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Tunjeena Rehan

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LIST OF ACRONYMS

rs	Reference SNP
TPH	Tryptophan Hydroxylase
BDNF	Brain Derived Neurotropic Factor
EDTA	Ethylene-diamine Tetracetic Acid
µl	Micro-litre
SNP	Single Nucleotide Polymorphism
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
MADRS	Montgomery-Åsberg Depression Rating Scale
$MgCl_2$	Magnesium Chloride
NaCl	Sodium Chloride
PCR	Polymerase Chain Reaction
ARMS	Amplification Refractory Mutation System
BD	Beckton Dickinson
RCPR	Response-Contingent Positive Reinforcement

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ABSTRACT

Prevalence of clinical depression, a chronic disorder, in Pakistan is quite high as compared to other developing countries. Environmental factors and genetic predisposition enhance the risk of depression. Two important genes in this regard are *BDNF* and *TPH2*. The study is designed to find out any genetic association between two SNPs, rs6265 (*BDNF*) and rs4570625 (*TPH2*), with clinical depression in studied sample. Blood samples of patients with clinical depression from Rawalpindi, Pakistan were collected and analysed through ARMS-PCR. The results showed statistically significant association of *BDNF* SNP rs6265 with clinical depression in the sample. *In-silico* analysis suggested no alteration in siRNA binding pattern and alternative splicing pattern due to both SNPs. These results were obtained from a small sample size (87 patients vs 70 controls). Replication of these findings in larger population size from different ethnic groups may be performed in future to confirm these results. This is first report on association study of *BDNF* and *TPH2* gene polymorphisms and depression from Pakistan.

CHAPTER 1: INTRODUCTION

1.1 Depression

"A common and serious medical illness that negatively affects how you feel, the way you think and how you act" (American Psychiatric Association)[1].

The imprecise, non-technical term, depression, is not associated with a certain composition and cannot be considered as a syndrome as well. Depression originates from the Latin words *depressare* and *deprimere*. Geographically, the term is used to describe a depressed topography, and more commonly, it is referred to a low, sad mood as well as to the related symptoms. The first time this term was used was back in 1665 where it was merely considered as mood lowering or spirit. (Simpson and Weiner. 1989)[2].

. The pre-existing conditions of depression vary widely. Based on environmental and historical variables, depression is bound to symptomatic heterogeneity. Considering this variation, we can quite possibly target the potentially non-concerned variables involved in the common processes associated with depression. (Hayes & Follette, 1992)[3].

It has to be made sure that what is fixed to the wall as depression must not be related to certain emotional states or responding patterns. Sadness and other similar emotional states are basically co-occurring behavioural responses which apparently are linked together because they have common outcomes. In order to understand this better, let's take an example of a child who has somewhat strict parents. Due to his parents criticising him, he often ends up crying. But, at times when the parents are rather encouraging, the child attains the same co-occurring behavioural response of crying. This does not imply that the child is depressed rather it depicts the child's emotional state.

1.1.1 Traditional Behavioural Models of Depression

"If we remove a man from his characteristic surroundings, a large part of his social behaviour cannot be emitted and may therefore become more and more probable: he will return to his old surroundings whenever possible and will be particularly "sociable" when he does so. Other parts of his behaviour become strong because they are automatically reinforced under the prevailing deprivation; he will talk to anyone who will listen about his old surroundings, his old friends, and what he used to do. This is all a result of deprivation. But nostalgia is also an emotional condition in which there is a general weakening of other forms of behaviour—a "depression," which may be quite profound. We cannot classify this as the result of deprivation because the behaviour which is thus affected has not been specifically restrained" (p. 165, Skinner. 1953)[4].

Analysing Skinner's quote, three main aspects can be highlighted; firstly, the seriousness of depression was respected as he specifically quoted the term. Secondly, instead of discussing the emotional response of an individual he focused on the nostalgic behaviour of a person in depth. Thirdly, it was made clear that depression is bound to negativity as it minimizes positive reinforcement.

Lewinsohn's Theory (1974)[5] was developed on the basis of this conception. The theory proposed insufficient **response-contingent positive reinforcement (RCPR)**[i] as a symptom of depression (Lewinsohn, 1974 ; Lewinsohn , Sullivan , & Grosscup , 1980)[6]. Basically, Lewinsohn relates the consequential depression with environmental incidents such as losing a job or getting divorced. Following the footsteps of Skinner's perspective, Lewinsohn's theory

[i] Pleasure obtained through interaction with the environment.

did not pay much attention to the core responsive aspect. Additionally, the theory considers depressive symptoms such as fatigue as secondary ones.

To understand Lewinsohn's theory better, let's take an example of a person who just got fired from his job. To add to his problem, he is an introvert with almost no socializing skills. Consequentially, he gets depressed. In order to come out of this situation, he needs to find a new job. In turn, to find a new job he must develop a set of socializing skills. Minimized positive reinforcement makes things more complicated for the troubled individual. It's a mess[7].

1.1.2 An Adaptive Syndrome or Maladaptive Response? Genetics and Evolutionary Theories

If we consider the former part of the question i.e. depression as an adaptive syndrome, a theory holding this view has to overcome a couple of primary barriers. First, knowing that depression can result from a variety of symptoms, one has to determine that which set of symptoms is associated with the concerned syndrome. Alternately, it gets more complicated when there are multiple syndromes under focus (M. C. Keller & Nesse, 2006)[8]. This can be demonstrated by looking at the example of melancholic and atypical depression. It is highly impossible for both of these to be treated as adaptive syndrome. Why? Melancholic depression comprises of some symptoms such as loss of appetite and insomnia which are completely the opposite of few symptoms involved with atypical depression. One theory cannot take into account both aspects in such a case.

Considering the later, the nature of the symptoms associated with depression tends to be maladaptive. For example, a person's response to some negative event occurs in the form of a bad mood. This response can be considered adaptive as it draws out an empathetic behaviour. Such a response is natural and eventually helps a person normalize as support is attained. However, in clinical depression, the transient sad mood is more or less unresponsive to

supporting behaviour. Despite the fact that the evolutionary account proposes that the chronic response should gather social support, study shows that depressive behaviours concludes in reduced social help (Coyne, 1976; Gotlib & Lee, 1989; Joiner & Metalsky, 2001)[9]and worse psychosocial functioning in general (Barnett & Gotlib, 1988)[10]. Suicide is another example. Although suicidal gestures may be seen as operant attempts to garner support (Linehan, 1993)[11], completed suicide is difficult to conceive of as an operant (i.e., learned) behaviour (Hayes, Strosahl, & Wilson, 1999)[12] and is clearly not adaptive in terms of survival.

1.2 Clinical Depression

Clinical Depression or Major Depressive Disorder (MDD) is among the most common psychiatric disorders in the world. It is a complex medical illness which includes abnormalities of mood and affect cognition (such as feeling of worthlessness and guilt), psychomotor activity (such as retardation and agitation) and neurovegetative functions (such as sleep and appetite disturbances) (Flint and Kendler 2014)[13].

This leads to the question that what are its associated characteristics? Depression is not what people usually perceive it as i.e. a state of feeling sad, but it is an emotional breakdown comprising of tiredness, feeling annoyed, considering one-self worthless, lack of interest, inability to concentrate and so on. Depression leads to brain impairment and in doing so results in the individual being unable to function normally in different aspects of life. Dysphoria is the term used for depressed mood which is considered as the most basic symptom while diagnosing clinical depression. Suicidal ideation is another symptom usually associated with severe clinical depression. These symptoms together form a syndrome and the complexity emerges because these symptoms vary in nature and presence across patients (Líndal & Stefánsson, 1991)[14].

In order to fully understand depression, the first thing we need to do is to understand the term completely. We should be able to distinguish between clinical depression and how sometimes it can be used to denote a non-clinical situation. For example, a person who is trying to learn a new language might use the term depression incorrectly i.e. in some different context. Similarly, a person might be diagnosed with depression but it is later found that the individual was facing similar symptoms due to some other disease. More importantly, it must be assured that diagnoses should not be limited i.e. it is possible that a depressed person has all the symptoms to be diagnosed as clinically depressed except one. In such a case, the doctor as well as the client might not consider using the term at all[15].

1.2.1 Prevalence

It is very important to note that how many people are affected by depression (directly) around the globe. More than 322 million people i.e. 4.4 percent of the total population, around the globe have been diagnosed with depression. This includes 5.1 percent females and 3.6 percent males (below the age of 55). Considering the age bracket of 55 to 74, around 7.5 percent females and 5.5 percent males are diagnosed with depression. Additionally, during the decade period of 2005 to 2015, there was an increase in the diagnosis of depression by 18.4 percent (World Health Organization (WHO) – Global Health Estimates 2015)[16].

According to world health organization clinical depression is one of the most burdensome illnesses in the world (Organization 2002) Pakistan being the 9th most populous country has a very high incidence and prevalence rate for psychiatric disorders in general and clinical depression in particular (Gadit 2007)[17] [18].

The life time risk of developing clinical depression is higher in women than men. According to US Diagnostic and Statistical Manual of Mental Disorders 10 to 25 percent women are at risk of developing clinical depression whereas the number lies between 5 to 12 percent for men.

(Association 2013)[19]. Clinical depression is a complex heterogeneous disorder with a less understood aetiology. (Gao, Pan et al. 2012)[20]. Various genetic components have been identified and they account for 40 to 70 percent of the life time risk of developing clinical depression. The first degree relatives of probands have 2 to 4 time's higher risk of getting MDD than controls. (Weissman, Gershon et al. 1984)[21].

1.2.2 Pathophysiology of Clinical Depression

The pertinent question occurs as who is most likely to get depressed? What are the factors that cause depression? Who is most likely to get depressed? Although depression can and does affect people of all ages, from all walks of life, the risk of becoming depressed is increased by poverty, unemployment, life events such as the death of a loved one or a relationship break-up, physical illness and problems caused by alcohol and drug use[22].

The factors triggering depression can be genetic, environmental or a combination of the two. Oxytocin, Dopamine and Serotonin are few of several physiological genes which play a part in causing depression. Moreover, life stress has been reported to alter stress response leading to brain impairment and the consequential depression.

Biologically, abnormalities in the immune system cause alterations in the Central Nervous System (CNS) which in turn leads to depression. The immune system recognizes internal or external stress and initiates responses through neurotransmitters, neuroendocrine system, and CNS, and their interactions contribute to the expression and termination of depressive symptoms (Jeon and Kim, 2016)[23].

1.2.3 Genetics of Clinical Depression

Serotonin is the major neurotransmitter or central nervous system. It is involved in regulation of several vital functions of body which include neuroendocrine regulation, sleep wake cycle,

appetite and mood regulation. 5hydroxytriptamine (5 HT) is the precursor of serotonin. Tryptophan hydroxylase (TPH) is the rate limiting enzyme in the biosynthesis of 5 hydroxy tryptamine. *TPH1* is present in peripheral nervous system while *TPH2* is present in central nervous system. Due to its crucial role in synthesis of serotonin, TPH enzymes have largely been studied. The mutations in the genes of TPH enzymes have been explored. One such mutation, rs4570625, is in regulatory region of *TPH2* gene. Due to this single nucleotide polymorphism low level of serotonin precursor is produced in the brain which consequently leads to low level of serotonin in brain. The mutation rs4570625 is linked to several psychiatric disorders, particularly clinical depression.

Brain derived neurotrophic factor is a protein which is coded by *BDNF* gene. BDNF belongs to the family of neurotrophic proteins which are involved in growth, survival and protection of neurons. BDNF plays a major role in growth and survival of neurons and it is also crucial for the formation of new neurons from neuronal stem cells, in a process called neurogenesis. Although most of the neurons are formed prenatally in mammalian brain, however, the process of neurogenesis continuous throughout the life of an organism. BDNF is mainly expressed in prefrontal cortex and hippocampus. These areas of brain are linked with learning, memory and cognitive functioning. Adequate levels of BDNF in these areas are important for normal functioning. Different mutations leading to low level of BDNF in brain are linked to several psychiatric disorders. One such mutation is val66met in which at position 196 in BDNF gene, a guanine is replaced by adenine, hence the amino acid valine is replaced by methionine. This non synonymous mutation causes abnormal trafficking and secretion of BDNF protein. The mutant BDNF mRNA is prone to degradation. This leads to low levels of BDNF in brain. In the pathogenesis of mood related disorders. *BDNF* role has been largely studied. The evidence from structural imaging suggests the decrease in volume of hippocampus in the individuals carrying the Val/Met or Met/Met genotype (Montag, Weber et al. 2009)[24].

The primary objectives of our study are the following:

- To determine genotype and allele frequency of SNP rs6265 in *BDNF* and rs4570625 in *TPH2* genes in adult individuals with clinical depression.
- To predict the possible role of these variants through *in-silico* analysis.

CHAPTER 2: LITERATURE REVIEW

SECTION 1: *TRYPTOPHAN HYDROXYLASE 2 (TPH2)*

2.1 Introduction to Tryptophan Hydroxylase (TPH2)

Major Depression is associated with decreased bioavailability of tryptophan which is a precursor of serotonin or 5-hydroxytryptamine (5-HT). The biosynthesis of 5-HT is driven by a rate-restricting enzyme called tryptophan hydroxylase (TPH). It is further classified as TPH1, which is mostly abundant in periphery, rather than in the brain. And the concerned TPH2, which plays an important role in regulating serotonin neurotransmission (Invernizzi, 2007)[25]. According to a recent study amygdala function is affected by a single nucleotide polymorphism in the human *TPH2* gene's regulatory region. The T allele rs4570625 polymorphism was reported to have association with increased amygdala reactivity when compared with the G allele. In another study it was reported that T allele carriers tend to have higher reward dependence and considerably smaller hippocampus and amygdala volumes compared to G allele homozygotes.(Gao, Pan et al. 2012)[26]. This polymorphism is associated with other psychiatric disorders as well.

2.1.1 Role of TPH2 in Psychiatric Disorders

Zhang et al reported the involvement of *TPH2* gene rs4570625 polymorphism in developing the positive symptoms in schizophrenic patients of Han Chinese population (Zhang, Li et al. 2011)[27]. *Kim et al* identified that there could be a contribution of this particular polymorphism in developing the panic disorder (Kim, Lee et al. 2009)[28]. In addition to this, *Leppanen et al* discovered that infants with missing attention shifts are the T- allele carrier suggesting this polymorphism's involvement in pathogenicity of multiple psychiatric disorders.(Leppanen, Peltola et al. 2011)[29].

2.1.2 Role of TPH2 in Major Depressive Disorder (MDD)

Gao et al. (2012)[30] underwent a sequential general view and performed a meta-analysis on *TPH2* Gene Polymorphisms' affiliation with Major Depressive Disorder (MDD). They used random and fixed effects models to study the effect size of independent loci. The selection criterion was set as; at least 3 studies available on the specific subject and once selected, they were synthesized using the above mentioned models. Using **fixed effects models**[ii], data analysis shown that the under examination single nucleotide polymorphism (SNP) rs4570625 was one of the two SNPs, along with rs17110747, associated with the MDD. It was identified that SNP rs4570625 had low diversity and remained prominent using the **random effects model**[iii]. The conclusion clearly highlighted the significant epidemiological aspect of the SNP under discussion (rs4570625).

Lan et al. (2010)[31] analysed the relationship between tryptophan-hydroxylase 2 (*TPH2*) gene polymorphisms SNPs of loci rs4570625 and MDD. In addition to this, they also investigated the association of *TPH2* SNP rs4570625 with anti-depressant treatment response in Chinese people. For investigation purpose, a total of 693 Chinese Han people were enrolled. 346 out of those were patients with clinical depression and hence, formed the case group. The control group comprised of the other 347 psychiatrically healthy individuals. The case group was further categorized into treatment-resistant depression (TRD) and non-treatment-resistant depression (NTRD). On the basis of treatment response, the TRD consisted of 72 people while the NTRD contained the remaining 274 individuals in the case group. The analysis was done

[ii] Is a statistical model in which the model parameters are fixed or non-random quantities

[iii] Also called a variance components model is a kind of hierarchical linear model. It assumes that the data being analysed are drawn from a hierarchy of different populations whose differences relate to that hierarchy

post-genotyping and distributing gene type and allele in both groups. The results showed a clear distributive difference of rs4570625 gene type and allele between the respective groups. Significant differences in allocation of rs4570625 gene type and allele was observed between males of the two sub-groups i.e. TRD and NTRD. The conclusion of the study stated the facts-based opinion that TPH2 gene rs4570625 might be associated with MDD and that SNP of rs4570625 may indicate the anti-depressant treatment response in males with MDD. It must be noted again that the conclusion was based on the Chinese Han population.

2.1.3 SNPs of *TPH2* in Major Depressive Disorder (MDD)

Serretti et al. (2011)[32] poured significant light on the association between *TPH2* gene SNPs and Major Depression (MD) in their work on the 'Influence of *TPH2* variants on diagnosis and response to treatment in patients with major depression, bipolar disorder and schizophrenia'. 145 MD associated patients and 170 healthy individuals i.e. without any psychiatric disorder, formed the test group and control group, respectively. Both groups were genotyped with 6 *TPH2* SNPs, including the concerned rs4570625. Montgomery-Åsberg Depression Rating Scale (MADRS) was used as a clinical measuring scale in dealing with the MD patients. The analysis showed that rs4570625 G-A haplotype was notably affiliated with higher end-point MADRS extremity. The same conclusion was not extended to the response to treatment part of the exploration. The overall conclusion of the study did not support a positive influence.

SECTION 2: *BRAIN DERIVED NEUROTROPIC FACTOR (BDNF)*

2.2 Introduction to Brain-Derived Neurotropic Factor (BDNF)

The two mentioned methods i.e. use of anti-depressants and counselling, not being able to treat depression efficiently has led to the identification of brain-derived neurotropic factor (BDNF) as a potential treatment target of depression (Kuipers and Bramham, 2006)[33]. Several different studies have shown that BDNF is associated with depressive disorders on the basis of low BDNF level in people who are depressed and also that the expressive level can be altered using anti-depressants (Karege et al., 2002; Gonul et al., 2005; Aydemir et al., 2006)[34] [35] [36]. Additionally, studying people with a change from Val/Val to Val66Met i.e. a change from Valine to Methionine at position 66 has clearly shown a decreased amount of hippocampal production (Pezawas et al., 2004; Szeszko et al., 2005; Bueller et al., 2006)[37] [38] [39]. Recently, an examination resulted in the demonstration of genetic variation leading to depression and anxiety in a mice model (Chen et al., 2006)[40].

2.2.1 Role of BDNF in Pathophysiology of Clinical Depression

According to the BDNF hypothesis of depression the loss of brain derived neurotropic factor function directly contributes to the pathophysiology of depression and restoring its normal level may be one of the causes of antidepressant's therapeutic efficacy. The observation that BDNF concentration in depressed patient sample is decreased compared to the healthy human and increased after treatment with antidepressants supports the hypothesis (Lang, Hellweg et al. 2009)[41]. A common single nucleotide polymorphism causing non-synonymous mutations in human brain derived neurotropic gene has been identified and is associated with anxiety related disorders. An amino acid Methionine is replaced by Valine at 66 position (Val66Met) (dbSNP: rs6265)

In the pathogenesis of mood related disorders. BDNF role has been largely studied. The evidence from structural imaging suggests the decrease in volume of hippocampus in the individuals carrying the Val/Met or Met/Met genotype (Montag, Weber et al. 2009)[42].

While studying the complex behavioural disorders such as MDD it is crucial to consider multiple genes and their interaction with each other and how they are influenced by the environment. This approach will assist in making comprehensive models to identify individuals who are at risk of developing a particular psychiatric disease.

The pathophysiology of major depressive disorder (MDD) is significantly influenced by the variations in the brain-derived neurotropic factor (*BDNF*) – signalling. *Zhang K et al (2009)[43]* performed a study within a Chinese population to examine the effects of both single and combined *BDNF* genes which are involved in signalling pathways to MDD. Evidence-based study suggested susceptibility to MDD in the respective population.

2.2.2 SNPs of BDNF in Major Depressive Disorder (MDD)

The survival of neurons, their growth and **synaptic plasticity**[iv] all rely immensely on BDNF. In a study, 206 MDD - diagnosed patients were enrolled and genotyped with eight different BDNF SNPs, including rs6265, in order to examine and determine the susceptibility to MDD and respective response to treatment. The control group comprised of 76 people in this particular investigation. The conclusion made it clear that among other SNPs, rs6265 was not linked with MDD *(Kocabas NA et al 2011)[44]*.

[iv] Is the biological process by which specific patterns of synaptic activity result in changes in synaptic strength and is thought to contribute to learning and memory. Both pre-synaptic and post-synaptic mechanisms can contribute to the expression of synaptic plasticity

2.2.3 Role of *BDNF* with Bipolar Affective Disorders (BPAD)

Schumacher J et al (2005)[45] considered 2,376 individuals to test any sort of relationship between MDD and BDNF phenotypes. 465 out of these were MDD – diagnosed and 1,097 were part of the control group. The rest of the individuals were associated with Bipolar Affective Disorder (BPAD) and schizophrenia respectively. No such association came under light as a result of single-marker process. However, the haplotype analysis resulted in an unexpected finding in the form of a marker-combination rs988748-(GT) n-rs6265 showing some sort of bond with MDD.

2.3 An Epistasis Relationship between TPH2 and BDNF

According to the latest studies on mouse models and humans there is an epistasis relationship between serotoninergic regulatory genes such as *TPH2* and *BDNF*. It has been suggested that there is a novel interaction between *TPH2* and *BDNF* polymorphism. A combination of G/G genotype and Val/Val was found to be associated with compromised inhibition of the negative emotional content.(Latsko, Gilman et al. 2016)[46].

Psychiatric disorders such as depression result from decreased inhibitory control of negative emotional information. In addition to this, enhanced study has shown that inheritance serves as the main medium for such an emotion-inhibitory control, and in turn represents significant diseases such as the concerned depression. Recently an investigation on the relationship between TPH2 (rs4570625) and BDNF (rs6265) polymorphisms and their effect on emotion-inhibitory processing was done. Two separate examinations brought under light mixed evidence. The TPH2 T carriers were observed as having decreased inhibitory control, while relatively greater symptoms of depression were seen in the BDNF Met carriers (risk genotypes). Consistent results were seen in both groups; the healthy group (homozygous genotypes combination of Val/Val and G/G) and the case group (risk genotypes combination

of Met/Val and Met/Met with T/G and T/T). On the basis of this analysis, it was concluded that both combinations could lead to depression and that further examination is required. (Latsko, Gilman et al. 2016)[47].

CHAPTER 3: PATIENTS AND METHODS

3.1 Ethical approval

Ethical approval for the study was taken from following institutions prior to the start of research.

- Institutional Review Board (IRB) ASAB
- Department of Mental Health Ethical committee at Benazir Bhutto Hospital, Rawalpindi Medical University.

Informed consent in the written form was taken from the study participants. Participants included patients with their guardians and healthy controls. In the appendax-1 copies of sample informed consent and IRB letter are given.

3.2 Patient's Recruitment and clinical evaluation

The patients chosen for study were recruited from Benazir Bhutto Hospital Rawalpindi. 87 patients diagnosed with clinical depression were selected along with 70 control samples and blood samples (3ml approximately) was extracted from them. Confidentiality of participant's personal data was maintained. Blood samples were treated anonymously. Numbers were allotted to blood samples and their subsequent DNA extracts for identification.

3.3. Inclusion and exclusion criteria

3.3.1 Inclusion Criteria

Inclusion criteria are as follows:

- Diagnosed by professional psychiatrists, clinically depressed patients belonging to age group 18 to 50 years were included after written informed consent.
- Selected patients were either taking anti-depressants or had taken antidepressants in past.
- Both genders were included in this study.

3.3.2 Exclusion criteria

Following was the exclusion criteria for study based on medical history:

- Chronic diseases such as cancer, diabetes, hepatitis.
- Psychotic illnesses like anxiety disorders, schizophrenia.
- Drug induced depression and anxiety disorders.
- Pregnancy.
- Estrogen replacement therapy.
- Drug abuse.
- Anorexia nervosa.
- Depression symptoms because of bipolar disorder.

3.4 Blood sample collection

Blood sampling comprised of extraction from both healthy and effected people (control). In doing so, Becton Dickinson (BD) syringes (0.6 mm x 38 mm, 23 G x 1 Thin Wall) were used. This was followed by a transfer of samples to BD Vacutainer tubes (TM, Frankin Lakes, New Jersey, United States of America) coated with Ethylene Diamine Tetra-Acetic acid (EDTA). BD Vacutainer plastic tubes offer a safe method of blood collection and reduce the potential for tube breakage and specimen spillage, thereby reducing the potential for exposure to blood borne pathogens. For proper identification purpose, EDTA coated tubes were timely taken to the Genetic Research Laboratory (ASAB NUST, H-12 Campus Islamabad, Pakistan) after being labelled with respect to the numbers assigned to the control individuals. Sample storage was done at 4 degrees centigrade for the time being i.e. until further processing.

3.5 Extraction of DNA

3.5.1 Phenol-chloroform method

In order to draw out deoxyribonucleic acid (DNA) from the obtained blood samples, phenol-chloroform extraction method was taken aboard next. Such extraction is a liquid-liquid one. (A liquid-liquid extraction is a process that distinguishes molecules mixture based on the differential solubilities of the individual molecules in two different immiscible liquids). The method comprises of 4 different types of extraction solutions labelled A to D. Solution A is prepared by blending together Trizma HCL and Trizma Base to obtain the desired pH level of Tris, which in this case is 7.5. This in turn was added thoroughly to Magnesium Chloride (MgCl2) and Sucrose, and eventually autoclaved. Next, we have the addition of Triton X-100 [C14H22O(C2H4O)n(n=9-10)] in appropriate ratio. Similarly, Solution B was prepared through thorough mixing of Ethylene Diamine Tetra-Acetic acid (EDTA) , Tris (pH 7.5) and Sodium Chloride (NaCl) and again, autoclaved. A volatile white crystalline solid in its natural state, Phenol (C6H5OH) is used as Solution C. Chloroform (CHCl3) is efficiently mixed with a clear, colorless alcohol, Isoamyl (C5H12O) to form Solution D. Specific amounts of the two ingredients are used in preparing the last solution. Finally, we store these solutions at a temperature set at 4 degrees centigrade. The details of preparing the four solutions are given below.

First, 750µl of a blood sample was taken in a centrifuge tube and to that 750µl of Solution A was added. Next, the tube was inverted repeatedly to make sure the two liquids mixed together completely. The centrifuge machine (Spectrafuge 24D Labnet, Edison, New jersey, USA) was operated to centrifuge the tube at 13,000 revolutions per minute (rpm) for a period of one minute. After the centrifugation the liquid lying above the solid residue (supernatant) was discarded. Then, re-suspension of the nuclear pellet in 400µl of Solution A was done. Again, centrifugation took place in the same way i.e. 13,000rpm in one minute followed by the nuclear pellet being re-suspended in 400µl of solution B, 12µl of 20 percent sodium dodecyl sulphate

(SDS) and 5µl of proteinase K (20mg/ml) post supernatant removal. Overnight, the tube was left to incubate at 37 degrees centigrade. This was followed by the addition and thorough blending of 500µl of Solution C with the same volume of Solution D. Solution D comprised of 1 vol. of Isoamyl alcohol and 24 vol. of chloroform. The third centrifugation took place for one minute again at 13,000rpm. The layers have to be separated next starting off with the upper aqueous layer which contains the DNA. The respective layer was, after separation, added to the centrifuge tube and to that was added 500µl of Solution D (appropriate volumes of chloroform and Isoamyl alcohol). Next, centrifugation process took place again at 13.000 rpm for another minute. 55µl of 3M sodium acetate ($C_2H_3NaO_2$) and 55µl of isopropyl alcohol (C_3H_8O) or alternatively, 110µl of entirely pure ethanol was added. Inverting the tube resulted in DNA precipitation followed by the same centrifugation process. DNA pellet was washed with 70 percent ethanol and centrifuged at 13000rpm for 7 minutes now.

The supernatant was again discarded, followed by drying of nuclear pellet in the incubator at 37°C for 15 to 20 minutes. After completely drying the ethanol 150 microliter TE buffer (Tris (pH 8.0, 10mM), EDTA (0.1mM) was added in DNA. DNA dissolved in TE was stored at 4°C. To determine the quality of DNA isolated, Agarose gel electrophoresis was performed using 1 % Agarose-TAE gel. 4µl of loading dye (3.9ml of glycerol added in 500µl 10% (w/v) SDS and 200µl 0.5M EDTA along with 0.025g bromophenol blue to make final volume of 10ml dye by adding autoclaved distilled water) 6-7µl of ethidium bromide (stock 1mg/ml) were added during gel electrophoresis. To estimate size of DNA 1KB ladder was added (Biometra, Goettingen, Germany). Gel images were obtained by gel doc and analyzed by “Dolphin Doc” software (Wealtec Dolphin Doc, Sparks, USA).

3.6 SNP genotyping

The SNP rs26265 of *BDNF* gene and rs4570625 of TPH2 gene were genotyped by using tetra Amplification Refractory Mutation System also called ARMS-PCR.

This is an application of PCR to amplify point mutation in genome by allele specific primers. At 3' of primer, a mismatch dramatically reduces the annealing and consequently amplification of DNA. The reason is absence of proof reading activity of Taq polymerase in 3' to 5' direction. A homozygote or heterozygote is identified by using ARMS primers for polymorphic/mutant and the wild/normal alleles. The reaction for normal and mutant allele is often carried out in the separate tubes.

Tetra ARMS primer sequences were designed in the reaction. The details of used primers are given in the Table 3.2.

3.7 Polymerase Chain Reaction (PCR)

The amplification reaction was performed in 0.2 ml PCR tubes (Axygen, California, USA)

The constituents of reaction mixture are as follows.

- 2 µl of template DNA approximately 60 Nano grams.
- 2 µl (2mM) dNTPs
- 2.5 µl (2mM) of each forward and reverse primers
- 0.2µl (one unit) of Taq DNA polymerase (Thermoscientific, USA)
- 2.5µl of 10X PCR buffer (100mM Tris-HCl, pH 8.3, 500mM $(NH_4)_2SO_4$), (Thermoscientific, USA)
- 1.5µl of MgCl2 (25mM)
- 14.3 µl of PCR water

Total volume of reaction mixture was kept 25 µl and was vortexed. The amplification was performed using Thermo-cycler (Applied Bio system, Foster City USA).

The PCR was optimized on following conditions.

1) Denaturation of template (DNA) at 95°C for a period of 10 minutes. (one cycle).
2) DNA denaturation into single strands at 95°C for a period of 45 seconds (35 cycles).
3) Annealing of the primers to the complementary sequences at 53°C for BDNF rs 6265 and 62°C for TPH2 rs4570625 for a period of one minute (35 cycles).
4) Extension of the complementary DNA strands from their respective primer sequence at 72°C for period of one minute (35 cycles).
5) Final extension DNA at 72°C for a period of 10 minutes.

The final amplified PCR product was observed in 2% agarose gel electrophoresis; using TAE Buffer for thirty minutes at 75 volts. The reference ladder used was of 100bp (Thermo Scientific, EU, Lithuania).

3.8 Statistical Analysis

The statistical analysis of data was performed using SPSS software. Chi-squared Test was applied for SNPs association analysis. Hardy Weinberg Equilibrium Test (HWE) was employed for comparison of expected and observed genotype frequencies in the control samples to predict random mating in the selected population sample under study.

3.9 *In-silico* Analysis

Any variations in siRNA binding patterns at the SNP sites were identified using siDirect (http://sidirect2.rnai.jp/). In this study alternative Splice Site Predictor program (ASSP) was used to observe patterns of alternative splicing in both SNPs.

TABLES (CHAPTER 3)

Table 3.1 Composition of DNA extraction solutions

Solution A	Solution B	Solution C	Solution D
0.32M Sucrose 10mM Tris (pH 7.5) 5mM MgCl2 Autoclave and add Triton X100 1% v/v	10mM Tris (pH 7.5) 5mM MgCl2 Autoclave and add Triton X100 1% v/v 10mM Tris (pH 7.5) 400mM NaCl 2mM EDTA (pH 8)	Phenol	Chloropharm 24 volumes Iso-amyl alcohol 1 volume

Table 3.2 Tetra ARMS Primer for *BDNF* rs6265

Primers	Sequence	Amplicon Size	Melting Temp.
*BDNF*rs6265FG (Forward Primer for G Allele)	5`-GCTGACACTTTCGAACACG-3`	196bp	55
*BDNF*rs6265FA (Forward Primer for A Allele)	5`-GCTGACACTTTCGAACACA-3`	348bp	56
*BDNF*rs6265RG (Reverse primer for G Allele)	5`-CTCATGGACATGTTTGCAGC-3`	196bp	55
*BDNF*rs6265RA (Reverse Primer for A Allele)	5`-GCCTTTTGATACAGGGACC-3`	348bp	56

Table 3.3 Tetra ARMS Primers for *TPH2* rs4570625

Primers	Sequence	Amplicon Size	Melting Temp.
TPH2rs4570625FG (Forward Primer for G Allele)	5`-TTGCATGCACAAAATTAG-3`	203bp	54
TPH2rs4570625FT (Forward Primer for T Allele)	5`-TTGCATGCACAAAATTAT-3`	507bp	55
TPH2rs4570625RG (Reverse primer for G Allele)	5`-GCTCCCGAACACTAGAT-3`	203p	54
TPH2rs4570625RT (Reverse primer for T Allele)	5`-GATATCCATTGCCTCAA-3`	507bp	55

CHAPTER 4: RESULTS

4.1 Study Samples

Out of total 137 blood samples taken, 87 samples were taken from clinically depressed patients while 50 were healthy controls without any past and present record of clinical depression or any exposure to antidepressants. The patients were diagnosed by trained psychiatrists and were taking anti-depressant medications at the time of sample collection. In this study escitalopram, fluoxetine and paroxetine, some selective serotonin reuptake inhibitors were being given to 57%, 15.4% and 7.1% of patients respectively while a tricyclic antidepressant prothiaden was being used by 2.3% of patients. 1.1 % of patients were using specific serotonergic and noradrenergic atypical depressant mirtazapine. Anti-anxiety and sedative benzodiazepines was being given to 15.4% of patients (Copy of the Patient enrollment form has been mentioned in Appendix-1).

87 patients were genotyped, among them 50 were males and 37 were males. Whereas in cases 37 were males and 36 are females. (Fig 4.1)

The average ages of sample patients were 18 ± 30, while control samples had nearly same age. In this study Northern areas of Pakistan were included and samples included Rajpoot, Awaan, Abbasi and Mughal. There were 20.68 % Rajpoot, 19.5% Awan and 11.5% of Mughal, 6.9% Pathan, 5.74% Abbassi, 4.6% Sheikh and Gujjar, 2.3% Hazaara, Malik and Qureshi, 1.14% Gill, Bhatti, Jatt, Sudhan, Khatak, khokar, Sawati, Minhas, Fadons, Kiyani, Sardar, Satti, Khwaja, Manghs, Suddozai and Arain (Figure 4.2).

4.2 PCR Amplification

After ARMS PCR amplification, 196bp band size was formed for SNP rs6265 G allele while a 348bp was formed for A allele (Fig 4.3) Similarly for ARMS PCR of rs4570625 of TPH2

201bp band was formed for G allele and 507bp band was formed for T allele (Fig 4.5). PCR products were separated and analyzed on 2% TAE based agarose gel with 100bp ladder.

4.3 Statistical Analysis

For total 87 cases samples and 50 control samples allelic frequency and genotype frequency for rs6265 and rs4570625 were determined using the traditional counting method. The genotypic distribution for GA, GG, AA for rs6265 and GT, TT, GG for rs4570625 among controls and cases were determined using chi-squared test. The results showed that rs6265 with P value = 0.00014 is significantly associated with clinical depression. Whereas for rs4570625, P value = 0.2766, showing that this polymorphism is not significantly associated with clinical depression.

The genotypic distribution of rs6265 and rs4570625 SNPs in clinically depressed and controls is shown in Table 4.1 and 4.4 respectively

The absence or presence of A allele was analyzed by chi-square test, by, making a combination of the rare genotypes the homozygous GG and the heterozygous GA and were placed in the same group. These were then compared against the homozygous genotype AA and then then Chi-squared test was applied. The results showed a P value = 0.0026, which indicate that rs6265 is significantly associated with clinical depression. However, the rare genotype TT and TG in case of rs4570625 showed no significant association with clinical depression when chi-squared test was applied. The Chi-Square-Test results rs6265 and rs4570625 are given in Table 4.1 and 4.4 respectively.

Hardy Weinberg Equilibrium (HWE) was also calculated for the controls samples for both SNPs (rs6265 and rs4570625). The expected and observed genotype frequencies for rs6265 and rs4570625 are summarized in table 4.2 and 4.5 for rs6265 and rs4570625 respectively.

Further allelic frequency of rs6265 and rs4570625 were determined and their association with clinical depression was assessed in cases and controls. For that purpose, odd ratio with 95% confidence interval and chi-squared test were performed. For rs6266 minor allele G showed a significant association with clinical depression however the minor allele T in rs4570625 showed no association with clinical depression. Allelic frequencies are summarized in table 4.3 and table 4.6 respectively.

4.4 *In-silico* Analysis

The siRNA binding patterns was observed for rs6265 and rs4570625, no change in siRNA binding pattern was observed for both.

Additionally, splice site alteration for both SNPs were also observed.NO alteration in alternative splicing was observed for rs6265 and rs4570625 (Table 4.7).

TABLES AND FIGURES (CHAPTER4)

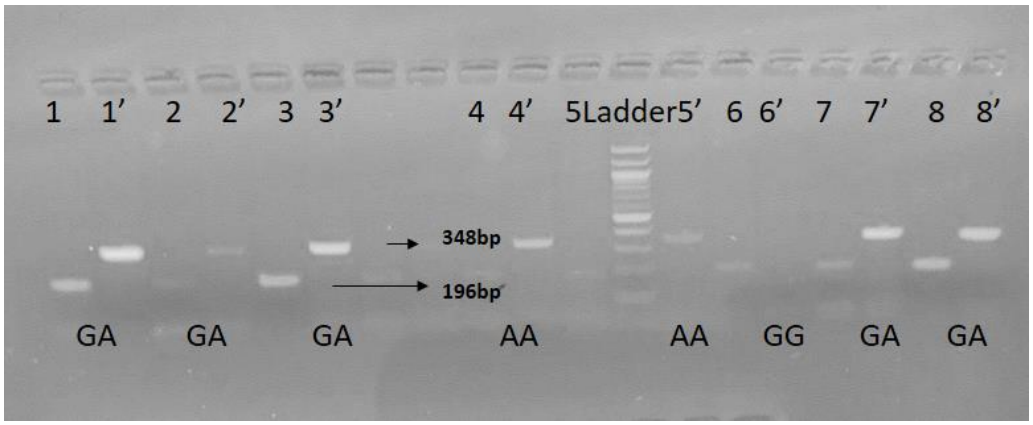

Fig. 1 ARMS PCR Amplification of BDNF rs6265 in Case Sample

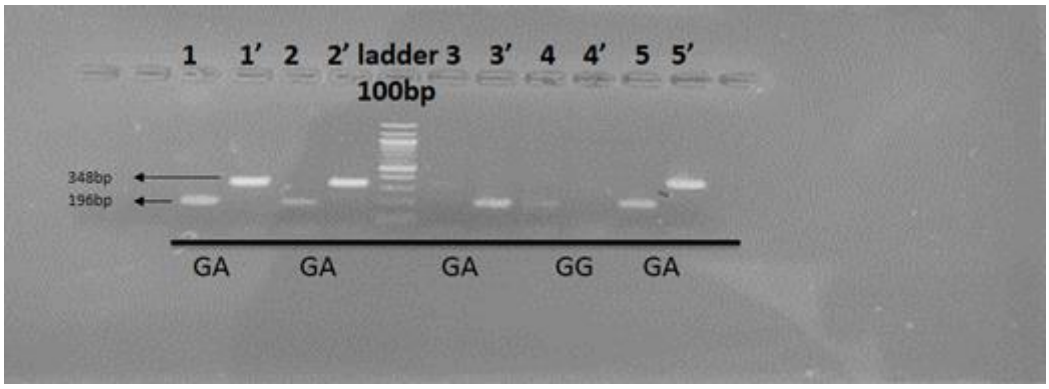

Fig. 2 ARMS PCR Amplification of BDNF rs6265 in Control Sample

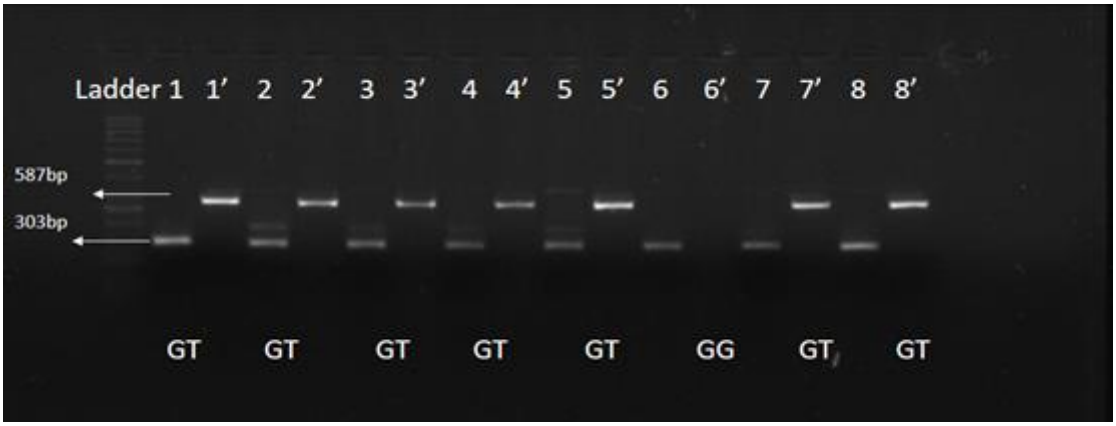

Fig. 3 ARMS PCR Amplification of TPH2 rs4570625 in Case Sample

Fig. 4 ARMS PCR Amplification of TPH2 rs4570625 in Control Sample

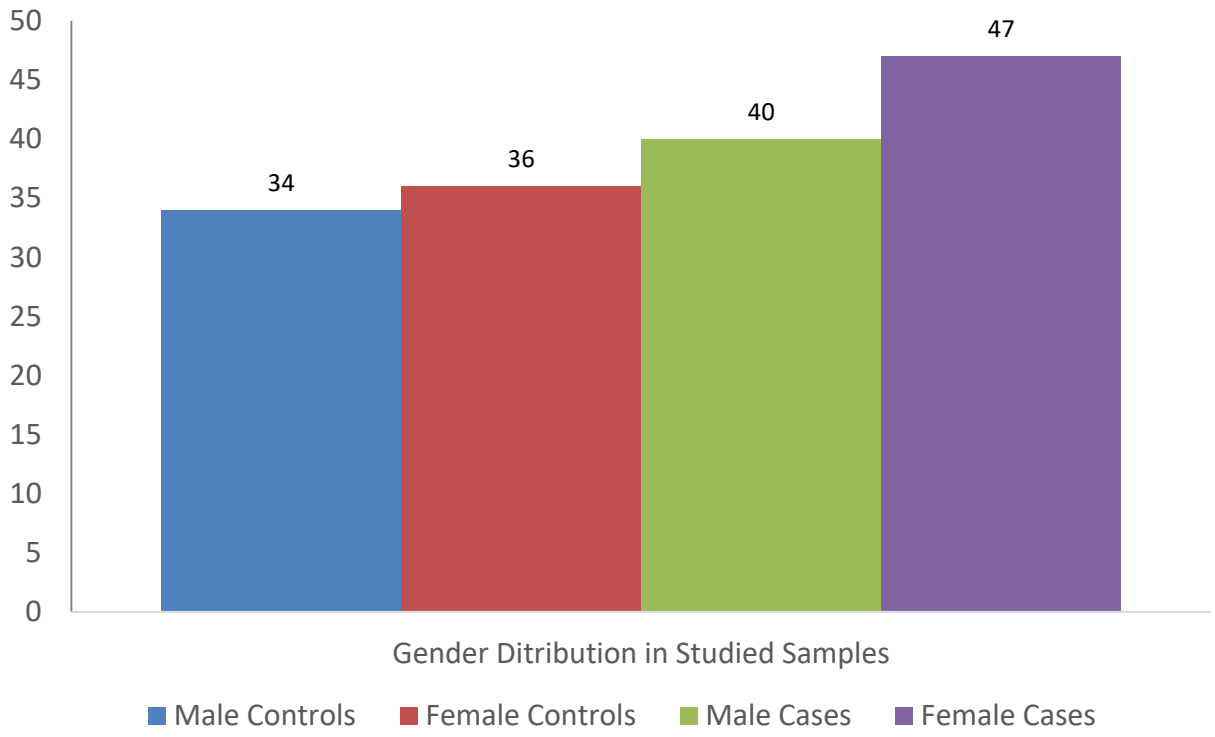

Fig. 5 Gender distributions in the cases and controls

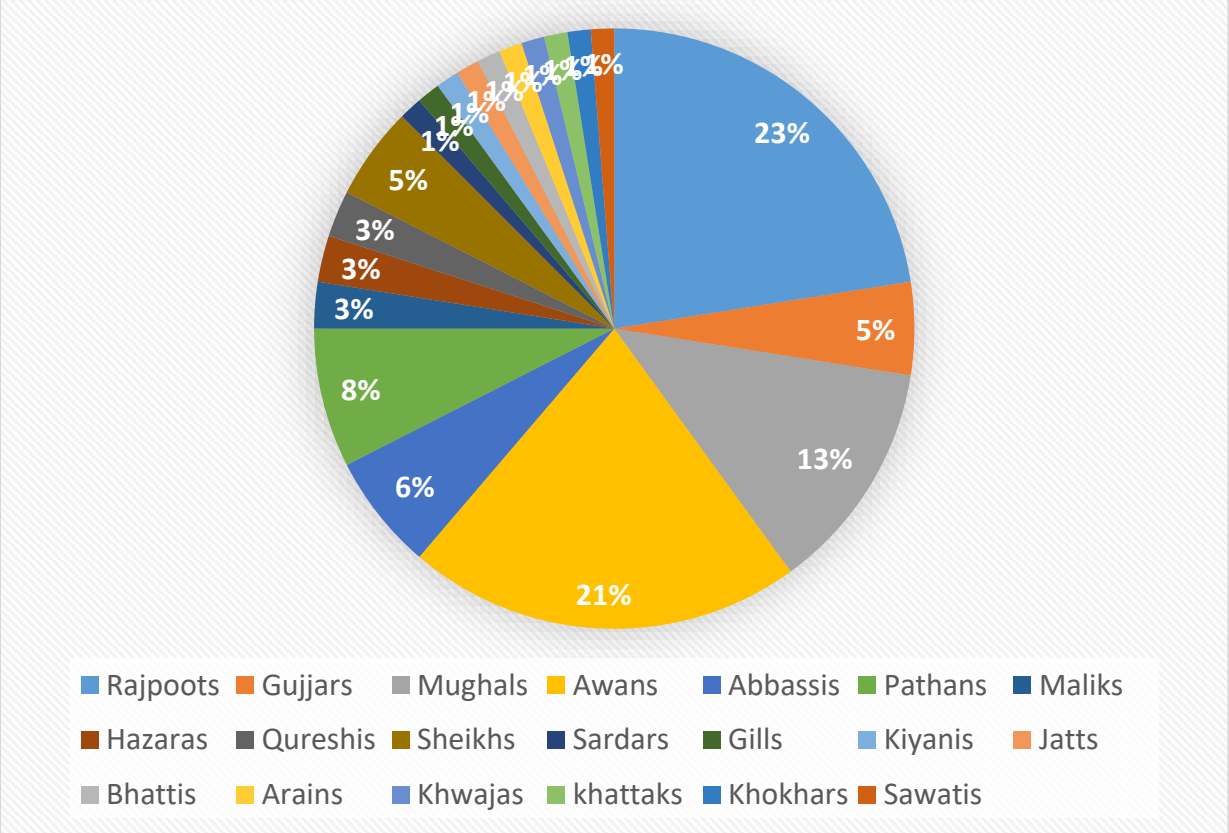

Fig. 6 Caste Distribution in the studied sample

Table 4.1 Genotype distribution for SNP rs6265 among clinically depressed patients and controls

SNP	Cases (n=87)	Controls (n=70)	Chi-Square (P value)
GG	15 (17.2%)	33 (47.1%)	Chi = 17.7 P = 0.00014
GA	32 (36.8%)	21 (30.0%)	
AA	40 (46.0%)	16 (22.9%)	

Table 4.2 Hardy Weinberg Equilibrium for control and case samples for *BDNF* rs6265

Sample Type	**Chi-Square (P-Value)** **Hardy Weinberg Equilibrium**
Cases (n=87)	Chi square =3.4, P = 0.0652
Controls (n=70)	Chi square = 9.1, P = 0.0026

Table 4.3 Allelic frequency of *BDNF* rs6265

Allele	**Cases (n=87)**	**Controls (n=70)**	
G	62 (35.6%)	87 (62.1%)	**OR = 2.9** **95%CI = 1.82-4.83** **P < 0.001**
A	112 (64.4%)	53 (37.9%)	

Table 4.4 Genotype distribution for *TPH2* rs4570625 among clinically depressed patients and controls

SNP	**Cases (n=87)**	**Controls (n=70)**	**Chi-Square (P value)**
GG	35 (40.2%)	21 (30.0%)	Chi = 2.6 P = 0.2766
GT	29 (33.3%)	23 (32.9%)	
TT	23 (26.5%)	26 (37.1%)	

Table 4.5 Hardy Weinberg Equilibrium for control and case samples for *TPH2* rs4570625

Sample Type	**Chi-Square (P-Value)** **Hardy Weinberg Equilibrium**
Cases (n=87)	Chi square = 8.9 , P = 0.0029
Controls (n=70)	Chi square = 8.0, P = 0.0047

Table 4.6 Allelic frequency of *TPH2* rs4570625

Allele	**Cases (n=87)**	**Controls (n=70)**	**P value**
G	99 (56.9%)	65 (46.4%)	**OR = 0.65** **95%CI = 0.40-1.05** **P = 0.065**
A	75 (43.1%)	75 (53.6%)	

Table 4.7 *In-silico analysis* of rs6265 and rs4570625 for siRNA binding pattern analysis and alternative splice site patterns

Sr. No.	***Insilico* analysis**	**rs6265** **G>A**	**Rs4570625** **G>T**
1.	**siRNA binding pattern** (siDirect)	No change	No change
2.	**Alternative splice site** (Alternative splice site predictor program)	No change	No change

CHAPTER 5: DISCUSSION

Skimming through the highlights of our study, we see that *BDNF* rs6265 showed significant association with clinical depression while it was the opposite in the case of *TPH2* rs4570625. For rs6265, minor allele G showed an association with clinical depression. However, the minor allele T in rs4570625 showed no association with clinical depression. Allelic frequency of rs6265 and rs4570625 were found to be less than 0.001 and equal to 0.065 respectively.

Different case-control tests have put forward the possibility of an affiliation between *TPH2* gene variants and Clinical Depression in Caucasian populations (Zill et al., 2004; Zhang et al., 2005; Zhou et al., 2005; Van Den Bogaert et al., 2006; Haghighi et al., 2008)[48 49 50 51 52] and a Chinese population (Tsai et al., 2009)[53] who are currently suffering from Major Depression.

Like our study, some studies agreed to disagree (Garriock et al., 2005; Gizatullin et al., 2008; Mann et al., 2008; Illi et al., 2009)[54 55 56 57]. Moreover, response to anti-depressants has been linked to several *TPH2* gene variants in some investigations as well (Peters et al., 2004; Zhang et al., 2005; Tzvetkov et al., 2008; Tsai et al., 2009)[58 59 60 61].

As far as *BDNF*'s association with depression is concerned, almost all the studies concluded in favour of the hypothesis. Possession of Met66 allele was found with increased risk of depression (Hwang et al.)[62]. Montag, Weber et al.[63] brought forward a very important insight that clinical depression is associated with a decrease in volume of hippocampus in the individual's carrying Val/Met or Met/Met genotype. Similarly, the infamous Zhang et al.[64] along with some other studies analysed the Chinese population's association with clinical depression with respect to the *BDNF* gene polymorphism. The examinations suggested an association of *BDNF* with clinical depression.

5.1 Conclusion

An association of rs6265 SNP with clinically depressed patients was observed. No association of rs4570625 SNP with clinically depressed patients was observed. *In-silico* analysis suggested no alteration in siRNA binding sites in case of both SNPs. No alternative splice site pattern was observed in case of both SNPs.

5.2 Future Prospects

Integrating multiple genetic influences on prominent underlying risk factors for emotional psychiatric diseases, such as poor inhibition of negative emotion, rather than associations with broader disease states, will help build more comprehensive models and identify groups at risk for psychiatric disease. We suggest the following:

- Determination of risk alleles in general population.
- A better insight into risk assessment in families of patients with clinical depression.
- Used as a potential target for pharmaceutical companies in terms of drug design.

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CHAPTER 7: APPENDIX

IRB Approval Letter

ASAB

ATTA-UR-RAHMAN SCHOOL OF APPLIED BIOSCIENCES
NATIONAL UNIVERSITY OF SCIENCES & TECHNOLOGY

Ref.No:25IRB-

Date: 20 April, 2017

IRB APPROVAL LETTER

Project Title: **Study of *BDNF* (rs6265) and *TPH2* (rs4570625) Gene Polymorphisms in Patients Suffering from Clinical Depression**

Name of Principal Investigator I:	Dr. M. Jawad Hassan
Name of Principal Investigator II:	–
Field and Subfield of Project:	Biosciences
Duration:	12 Month
Name of the Department:	Healthcare Biotechnology, ASAB, NUST

The aforesaid project has been reviewed by Institutional Review Board (IRB) Committee, ASAB, keeping in view the following selection criteria:

- Qualification, Expertise and Scientific Caliber of the Principal Investigators
- Proposed Goals of the Study
- Subject Selection
- Selection Criteria of Subjects
- Informed Consent Process
- Potential Problems
- Research Design and Methods
- Potential Benefits of the Study
- Risks of the Study
- Management of Risks
- Assessment of Risk
- Confidentiality
- Conflict of Interest

The committee thus **APPROVES** the project on **"Study of Oxytocin Receptor Gene Polymorphisms (Rs53576, Rs2254298) In Patients Suffering From Clinical Depression"** on the scales and criterion set by IRB.

Dr. Nasim us Sahar Zaidi
Member, IRB
ASAB, NUST

Dr. Muhammad Tahir
Member, IRB
ASAB, NUST

Dr. Hajra Sadia
HoD Research, Head of IRB
ASAB, NUST

Atta-Ur-Rahman School of Applied Biosciences, National University of Sciences & Technology, H-12, Islamabad, Pakistan.
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Informed Consent Form

INFORMED CONSENT FORM

AIMS AND OBJECTIVES
To identify and analyse disease associated gene variants in patients suffering from Clinical depression
SAMPLE COLLECTION
5ml blood sample will be collected from all participants

INFORMED CONSENT

You are being asked to participate in a research study to find the genetic risk of the disease. You will be asked to donate 5ml of blood. This will not cause any physical injury. Your samples will be preserved in the laboratory and will be tested for genetic polymorphism of specific genes involved in the disease. Your identity in this study will be protected. You can terminate your participation at any time in the course of this study. This research project will be carried out solely on a non commercial basis. Your participation is voluntarily. Further, if it becomes necessary, in your interest, counselling will be provided to you. The scientific information will only be shared among the collaborating scientists. The results of the study if novel or of medical interest will be published in scientific journals without disclosing your identity.

اجازت نامہ
مجوزہ تحقیقي منصوبہ آپ میں پائی جانے والي بیماري کي وجوہات معلوم کرنے کے لیے خالصتا غیر تجارتي بنیادوں پر ترتیب دیا گیا ہے. آپ سے ۵ ملي لیٹر خون کے عطیے کي درخواست کي جاتي ہے. خون دینے سے آپ کو کسي قسم کا درد ي زخم نہیں ہو گا. آپ کي شناخت کو مکمل طور پر صیغۂ راز میں رکھا جاۓ گا. آپ کا دیا ہوا عطیہ تجربہ گاہ میں محفوظ رکھا جاۓ گا اور ہم ان خاص جینیاتي تبدیلیوں کا مشاہدہ کرتے ہوۓ آپ کي بیماري کو سمجھنے کي کوشش کریں گے. جس سے آپ اور آپ جیسے دوسرے مریضوں کا بہتر علاج ممکن ہو سکے گا. آپ اس تحقیق کے دوران کسي بھي وقت اپني شمولیت سے دستبردار ہو سکتے ہیں. آپ کي شمولیت رضاکارانہ ہے. دوران تحقیق اگر ضروري ہوا تو آپ کو بیماري سے متعلق مشاورت بھي کي جاۓ گي. حاصل شدہ سائنسي معلومات کا تبادلہ صرف تحقیق میں شامل سائنسدانوں کے مابین کیا جاۓ گا.

I hereby confirm that I fully understand what has been stated above. I voluntarily donate blood sample from myself / and from my family for research purposes only.

میں تصدیق کرتا/ کرتي ہوں کہ جو کچھ بھي مجھ سے بیان کیا گیا ہے، میں اسے مکمل طور
پر سمجھ گیا/گي ہوں. نیز میں اپنے خون کا نمونہ رضاکارانہ طور پر صرف تحقیق کے لئے بطور عطیہ دیتا/دیتي ہوں مجھے میرے تمام سوالات کے جواب مل گئے ہیں اور في الوقت میرے ذہن میں کوئی اور سوالات نہیں.

Signature/Thumb impression of the patient: ----------

Name: ---------- **Participant ID**----------

Patient Enrolment Form

Patient's ID: ----------

Patient's name: ----------

Father's name: ----------

Age: ----------

Gender: Male/Female

Marital status: single/ married/ divorced

Annual income: ----------

Occupation: ----------

Cast/language: ----------

Address: ----------

Any trauma, family dispute, accident, natural disaster or chronic illness in early years of life: yes/ no

If yes, then describe the event

Is the tragic incident occurred in your close family members Yes/No

Have you witnessed the incident in public/ media Yes/No

Is the above mentioned incident, your personal experience Yes/No

Medication diagnosed:

Researcher name: **Signature:**

Electronic Data Base Information

- NCBI (http://www.ncbi.nlm.nih.gov/gene/5021)
- NEB cutter (http://nc2.neb.com/NEBcutter2/)
- SiDirect (http://sidirect2.rnai.jp/)
- PROMO-ALGGEN (Prediction of transcription factor binding sites)

(http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3)