ASSOCIATION BETWEEN GAMMA AMINOBUTYRIC ACID (GABA) TYPE B RECEPTORS GENE POLYMORPHISMS AND IDIOPATHIC GENERALIZED EPILEPSY

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

EZGİ EROĞLU

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOLOGY

FEBRUARY 2012

Approval of the Thesis

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submitted by **EZGİ EROĞLU** in partial fulfillment of the requirements for the degree of **Master of Science in Biology Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Musa Doğan _____
Head of Department, **Biology**

Prof. Dr. Orhan Adalı _____
Supervisor, **Biology Dept., METU**

Dr. Birsen Can Demirdöğen _____
Co-Supervisor, **Food Safety and Nutrition Dept., Refik Saydam National Public Health Agency**

Examining Committee Members:

Prof. Dr. Meral Yücel _____
Biology Dept., METU

Prof. Dr. Orhan Adalı _____
Biology Dept., METU

Prof. Dr. Leyla Açık _____
Biology Dept., Gazi University

Assist. Prof. Dr. Çağdaş D. Son _____
Biology Dept., METU

Dr. Birsen Can Demirdöğen _____
Food Safety and Nutrition Dept.,
Refik Saydam National Public Health Agency

Date: 08.02.2012

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Ezgi EROĞLU

Signature :

ABSTRACT

ASSOCIATION BETWEEN GAMMA AMINOBUTYRIC ACID (GABA) TYPE B RECEPTORS GENE POLYMORPHISMS AND IDIOPATHIC GENERALIZED EPILEPSY

Eroğlu, Ezgi

M.Sc., Department of Biology

Supervisor: Prof. Dr. Orhan Adalı

Co-supervisor: Dr. Birsen Can Demirdöğen

February 2012, 127 pages

Epilepsy is neurological disorder affecting 0.5 to 1% of the population all around the world. It is characterized by the seizures, which are the sudden alterations of behavior due to a temporary change in electrical functioning of the brain. Idiopathic generalized epilepsy (IGE) accounts for one-fifth of all the other epilepsy types, and several gene mutations were identified as the causes of IGE. In general, voltage-gated and ligand-gated ion channel mutations are linked with seizure formation. Gamma amino butyric acid (GABA), the most important inhibitory neurotransmitter of the central nervous system, and its receptors are commonly mentioned in the pathophysiology of epilepsies. Decrease in the inhibitory effect of GABA in neurons causes epileptic discharges resulting in seizure development.

The study population consisted of a total of 176 idiopathic generalized epilepsy (IGE) patients, 83 subjects having psychogenic non-epileptic seizures (PNES), 86 non-epileptic control subjects from Turkey. Total blood samples were obtained from Gülhane Military Medical Academy Hospital Neurology Department, Ankara. There was no statistically difference between the patient and control groups in terms of age. Genomic DNA isolations were performed and genotyping of G1465A and C59T polymorphisms of $GABA_{B1}$ gene; rs1999501, rs967932, rs3780428 and rs944688 polymorphisms of $GABA_{B2}$ gene were determined by PCR-RFLP technique.

In this study, $GABA_{B1}$ G1465A polymorphic allele was not observed in Turkish population. For $GABA_{B1}$ C59T polymorphism, polymorphic allele frequencies were found as 0.097 in IGE patients; 0.072 in PNES subjects and 0.105 in non-epileptic control subjects. No significant difference is identified for C59T polymorphism in all three groups.

Four SNPs of $GABA_{B2}$ were studied; rs967932 was found to increase the risk of IGE 3.6-fold (P=0.031) compared to PNES subjects, polymorphic allele frequencies were found as 0.060 in IGE patients; 0.018 in PNES subjects and 0.035 in non-epileptic control subjects. For rs1999501 polymorphism, polymorphic allele frequencies were found as 0.077 in IGE patients; 0.048 in PNES subjects and 0.093 in non-epileptic control subjects. For rs3780428 polymorphism, polymorphic allele frequencies were found as 0.267 in IGE patients; 0.235 in PNES subjects and 0.256 in non-epileptic control subjects. For rs944688 polymorphism, polymorphic allele frequencies were found as 0.196 in IGE patients; 0.260 in PNES subjects and 0.227 in non-epileptic control subjects. No significant difference was identified for rs1999501, rs3780428 and rs944688 polymorphisms among IGE patients, PNES subjects and non-epileptic control groups.

IGE risk was 6.54-fold higher for subjects having combined GA genotype for rs967932 and GG genotype for rs3780428 when compared with PNES subjects (*P*=0.042). The combination of CC genotype for rs1999501, GG genotype for rs967932 and TT genotype for rs944688 had around 9-fold protective effect against IGE when both compared with PNES subjects (*P*=0.038) and non-epileptic control subjects (*P*=0.041).

Key words: $GABA_B$ receptor, idiopathic generalized epilepsy, genetic polymorphism, G1465A, C59T, rs1999501, rs967932, rs3780428, rs944688.

ÖZ

GAMA AMİNOBUTİRİK ASİT (GABA) TİP B RESEPTÖRLERİNİN GENETİK POLİMORFİZMLERİ İLE İDİYOPATİK JENERALİZE EPİLEPSİ ARASINDAKİ İLİŞKİNİN ARAŞTIRILMASI

Eroğlu, Ezgi
Yüksek Lisans, Biyoloji Bölümü
Tez Yöneticisi: Prof. Dr. Orhan Adalı
Ortak Tez Yöneticisi: Dr. Birsen Can Demirdöğen
Şubat 2012, 127 sayfa

Epilepsi tüm dünya nüfusunun 0.5 ila 1%'ini etkileyen bir nörolojik hastalıktır. Epilepsi hastalığı epileptik nöbetlerle karakterize edilir. Nöronların artmış uyarılabilirliğinden kaynaklanan, ani, hipersenkron, yüksek voltajlı, anormal elektriksel deşarjları epileptik nöbetlere neden olur. İdiyopatik jeneralize epilepsy (İJE), tüm epilepsy türlerinin beşte birini oluşturur ve pek çok mutasyonun İJE'ye neden olduğu bilinmektedir. Genellikle voltaj-kapılı ve ligand-kapılı iyon kanallarındaki mutasyonların nöbet oluşumunda etkili olduğu bilinmektedir. Merkezi sinir sisteminin en önemli inhibitör nörotransmitteri olan gama aminobütirik asit (GABA) ve GABA reseptörleri epilepsi patofizyolojisinde sıkça anılmaktadır. Nöronlarda GABA'nın inhibitör etkisinin azalması epileptik deşarjlar meydana getirerek nöbet oluşmasına neden olmaktadır.

Bu kapsamda çalışmada Türkiye'den 176 idiyopatik jeneralize epilepsi hastası, 83 epileptik olmayan psikojenik nöbet (EOPN) geçiren birey ve 86 bayılma yakınması olmayan kontrol birey incelenmiştir. Tam kan örnekleri Gülhane Askeri Tıp Akademisi Hastanesi Nöroloji Bölümünce temin edilmiştir. Hasta ve kontrol grupları arasında yaş farkı açısından istatistiksel olarak anlamlı bir fark bulunmamaktadır. Genomik DNA izolasyonları yapılmış, $GABA_{B1}$ genindeki G1465A ve C59T polimorfizmlerinin, $GABA_{B2}$ genindeki rs1999501, rs967932, rs3780428 ve rs944688 polimorfizmlerinin genotiplemesi PZR-RFLP tekniği kullanılarak belirlenmiştir.

Bu çalışmada, Türk populasyonunda $GABA_{B1}$ G1465A polimorfizmine rastlanmamıştır. $GABA_{B1}$ C59T polimorfizmi için de polimorfik alel frekensı IJE hastalarında 0,097; EOPN bireylerde 0,072; epileptik olmayan kontrol bireylerinde 0,105 olarak hesaplanmıştır. C59T polimorfizmi için her üç grupta da anlamlı bir fark bulunmamıştır.

$GABA_{B2}$ geni üzerinde bulunan 4 SNP çalışılmıştır; bunların içinden rs967932 polimorfizminin İJE riskini EOPN bireylere göre 3.6 kat arttırdığı bulunmuştur (P=0.031) ve polimorfik alel frekensı IJE hastalarında 0,060; EOPN bireylerde 0,018; epileptik olmayan kontrol bireylerinde 0,035 olarak hesaplanmıştır. rs1999501 polimorfizmi için polimorfik alel frekensı IJE hastalarında 0,077; EOPN bireylerde 0,048; epileptik olmayan kontrol bireylerinde 0,093 olarak hesaplanmıştır. rs3780428 polimorfizmi için polimorfik alel frekensı IJE hastalarında 0,267; EOPN bireylerde 0,235; epileptik olmayan kontrol bireylerinde 0,256 olarak hesaplanmıştır. rs944688 polimorfizmi için polimorfik alel frekensı IJE hastalarında 0,196; EOPN bireylerde 0,260; epileptik olmayan kontrol bireylerinde 0,227 olarak hesaplanmıştır. rs1999501, rs3780428 ve rs944688 polimorfizmleri için her üç IJE hasta, EOPN bireyler ve epileptik olmayan kontrol bireyleri karşılaştırıldığında, IJE için anlamlı bir fark bulunmamıştır.

rs967932 polimorfizminde GA, rs3780428 polimorfizminde GG genotip kombinasyonu olan bireylerde IJE riskinin EOPN bireylere göre 6.54 kat arttığı bulunmuştur (*P*=0.042). rs1999501 polimorfizminde CC, rs967932 polimorfizminde GG ve rs944688 polimorfizminde TT genotip kombinasyonu, EOPN bireylere (*P*=0.038) ve epileptik olmayan kontrol bireylere (*P*=0.041) IJE'ye gore yaklaşık 9 kat koruyucu etki göstermiştir.

Anahtar kelimeler: $GABA_B$ reseptörü, idiyopatik jeneralize epilepsi, genetik polimorfizm, G1465A, C59T, rs1999501, rs967932, rs3780428, rs944688.

To my parents…

ACKNOWLEDGEMENTS

I would like to thank to my supervisor Prof. Dr. Orhan ADALI for his guidance, continued advice and support, critical discussions, and understanding throughout this study.

I am thankful for my co-supervisor Dr. Birsen CAN DEMİRDÖĞEN for her guidance and suggestions throughout the study and the preperation of thesis.

I wish to thank my examining committee members Prof. Dr. Meral YÜCEL, Prof. Dr. Leyla AÇIK and Assist. Prof. Dr. Çağdaş D. Son for their suggestions and criticism.

I would like to thank to Prof. Dr. Şeref DEMİRKAYA, Assist. Prof. Dr. Semai BEK and Dr. Güray KOÇ for providing the blood samples for this study. I also thank to all volunteers who gave blood to be used in this study.

I am so grateful to study with amazing lab mates. Especially, I would like to express my endless gratitude to my mentor Aysun TÜRKANOĞLU ÖZÇELİK for her patience to all my questions, for her guidance and for being a model in each area. I would like to thank Tuba ÇULCU for giving me amazing memories, Duygu YILMAZ to be next to me whenever I need, Tuğba ÖZAKTAŞ for making my mood positive all the time. For all those enjoyable moments and friendship, I am thankful for Serdar KARAKURT, Melike SEVER, Hasan Ufuk ÇELEBİOĞLU. Most of all, I would like to thank Yağmur MİÇOOĞULLARI for being such a good lab partner and for being next to me whenever I need throughout my university life.

I would like to thank Melda ARSLAN, Fulya ÇIRAY and Ayşegül Özgür GEZER for their help to the study and for their friendship.

I owe my special thanks to my intimate friend Damla GÜLDAĞ for being with me all time. I would like to thank Kevser GENÇALP for I wish to express my special thanks to Ezgi ERDOĞAN for her support and love in all conditions throughout my life.

I owe my special thanks to my dearest friends Gökhan ÜNLÜ, Alper MUTLU, Bahtiyar KURTULMUŞ, Ayça SEYHAN, Çağrı URFALI and Ahu İZGİ for their invaluable friendship and support during the course of my studies.

I would like to thank my sister Ela EROĞLU for being an amazing sister and believing me every time. Also I am so thankful to my brother-in-law Fred JOURDAN for giving encouragement for scientific study.

Finally, I would like to express my special thanks and loves to my mother Elif EROĞLU and my father İsmet EROĞLU for their endless patience, encouragement, support and love.

This study was supported by TUBİTAK (1002) Grant No: 210T117

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AD	Autosomal dominant
ADNFLE	Autosomal dominant nocturnal frontal lobe epilepsy
ADTLE	Autosomal dominant lateral temporal lobe epilepsy
AE	Absence epilepsy
BFNC	Benign familial neonatal convulsions
BFNIS	Benign familial neonatal-infantile seizures
CAE	Childhood absence epilepsy
CVD	Cerebrovascular disease
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EA1	Episodic ataxia type 1
EDTA	Ethylene diamine tetra acetic acid
EEG	Electroencephalography
EFMR	Epilepsy and mental retardation limited to females
FHM	Familial hemiplegic migraine
FS	Febrile seizures
GABA	Gamma-Aminobutyric acid
GABA-T	Gamma-Aminobutyric acid transaminase
GAD	Glutamate decarboxylase
GEFS	Generalized epilepsy with febrile seizures
GLUT-DS	Glucose transporter type 1 deficiency syndrome
HapMap	Haplotype Map
HLA	Human leukocyte antigen
IGE	Idiopathic generalized epilepsy
ILAE	International League Against Epilepsy
IPSC	Inhibitory postsynaptic current
JME	Juvenile myoclonic epilepsy
MERRF	Myoclonic epilepsy with ragged red fibres

MTLE	Mesial temporal lobe epilepsy
nAChR	Neuronal nicotinic acetylcholine receptor
NCL	Neuronal ceroid lipofuscinosis
OR	Odds ratio
PCR	Polymerase chain reaction
PED	Paroxysmal exercise-induced dyskinesia
PNES	Psychogenic non-epileptic seizures
RFLP	Restriction fragment length polymorphism
RE	Restriction endonuclease
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
SSA	Succinic semialdehyde
SSADH	Succinic semialdehyde dehydrogenease
TCA	Tricarboxylic acid
UTR	Untranslated region
χ^2	Chi-square

CHAPTER 1

INTRODUCTION

1.1 Epilepsy

Epilepsy is one of the oldest recognized disorders, known from the ancient times. According to World Health Organization, around 50 million people all around the world have epilepsy disorder at one time (http://www.who.int).

Epilepsy is a neurological disorder affecting 0.5 to 1% of the population all around the world. It is characterized by the seizures, which are the sudden alterations of behavior due to a temporary change in electrical functioning of the brain. At least one epileptic seizure is necessary to diagnose epilepsy. Family history or Electroencephalography (EEG) changes are not sufficient for epilepsy diagnosis (Fisher *et al.*, 2005).

A variety of damages to the brain may result in epilepsy such as head injury, prenatal injury, stroke, brain infections or brain tumors. Also epilepsy has a hereditary background. Some mutations in proteins coding for voltage-gated and ligand-gated ion channels are linked with seizure formation. Reduced inhibitory action and increased excitatory action lead to epileptic seizures (Figure 1.1). Release of large amounts of glutamate (excitatory neurotransmitter) and also ineffective GABA (inhibitory neurotransmitter) action triggers the seizure formation.

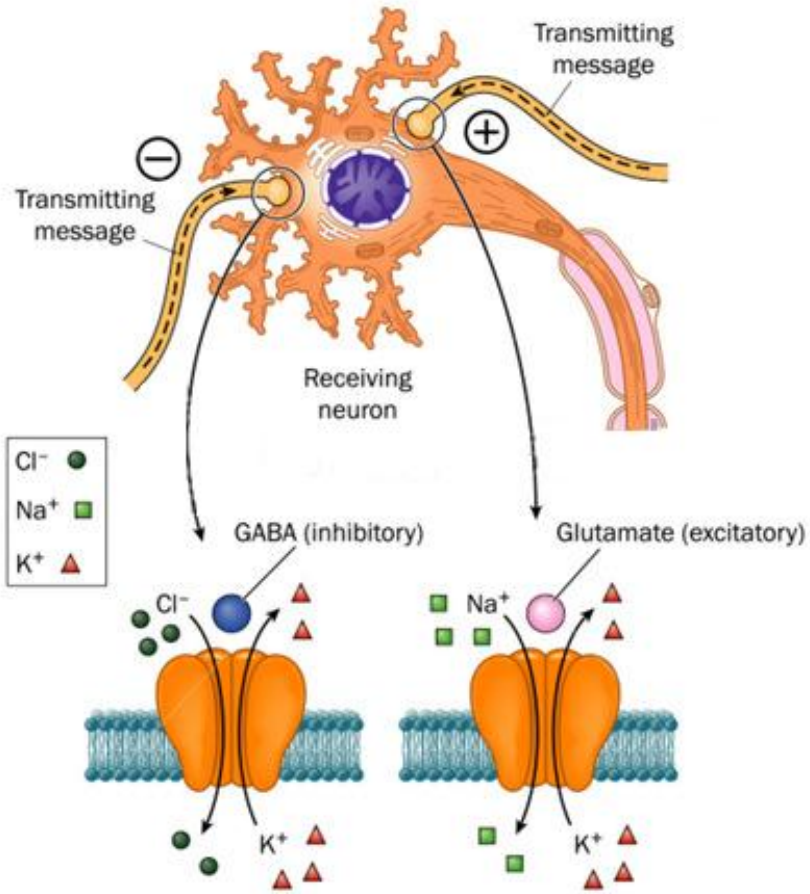

Figure 1. 1 GABA and Glutamate neurotransmitters involved in epilepsy (taken from http://www.shutterstock.com).

Epilepsy is classified according to the seizure types (generalized, focal) and the cause of the disease (idiopathic, symptomatic, provoked and cryptogenic) (Shorvon, 2011).

1.1.1 Prevalence and Incidence of Epilepsy

Epilepsy is one of the most common chronic neurological diseases. It is estimated that 3-5% of the world population have at least one seizure during their lifetime, and 0.5-1% of the world population have active epilepsy (Sridharan, 2002).

In Turkey, prevalence of epilepsy is reported to be 6.1 per 1000 in northeast region (Velioğlu *et al.*, 2010). In central Anatolia, prevalence is 7, while 4.5 in city centers and 8.7 per 1000 in urban areas (Güvener *et al.*, 1995). According to the study held on Silivri, age-adjusted prevalence was reported as 10.2 per 1000 (Karaağaç *et al.*, 1999). In European region of İstanbul, age-adjusted prevalence is lower with 7.0 per 1000 (Önal *et al.*, 2002). In rural area of Sivas, prevalence is 17.3 (Özdemir, 1995), and in the city center of Sivas, prevalence is decreased to 6.1 per 1000 (Topalkara *et al.*, 1999). In Bursa, epilepsy prevalence is reported as 12.2 per 1000 (Çalışır *et al.*, 2006).

Incidence of the epilepsy in industrial countries ranges from 26 to 70 (per 100,000 people-years). In the developing countries, newly diagnosed epilepsy patients were reported two to three times higher than the developing countries (Banerjee & Hauser, 2008).

1.1.2 Causes of Epilepsy

There are some factors that increase the risk for epilepsy identified by the epidemiological studies. Causes of epilepsy and their relative risks are shown in Figure 1.2.

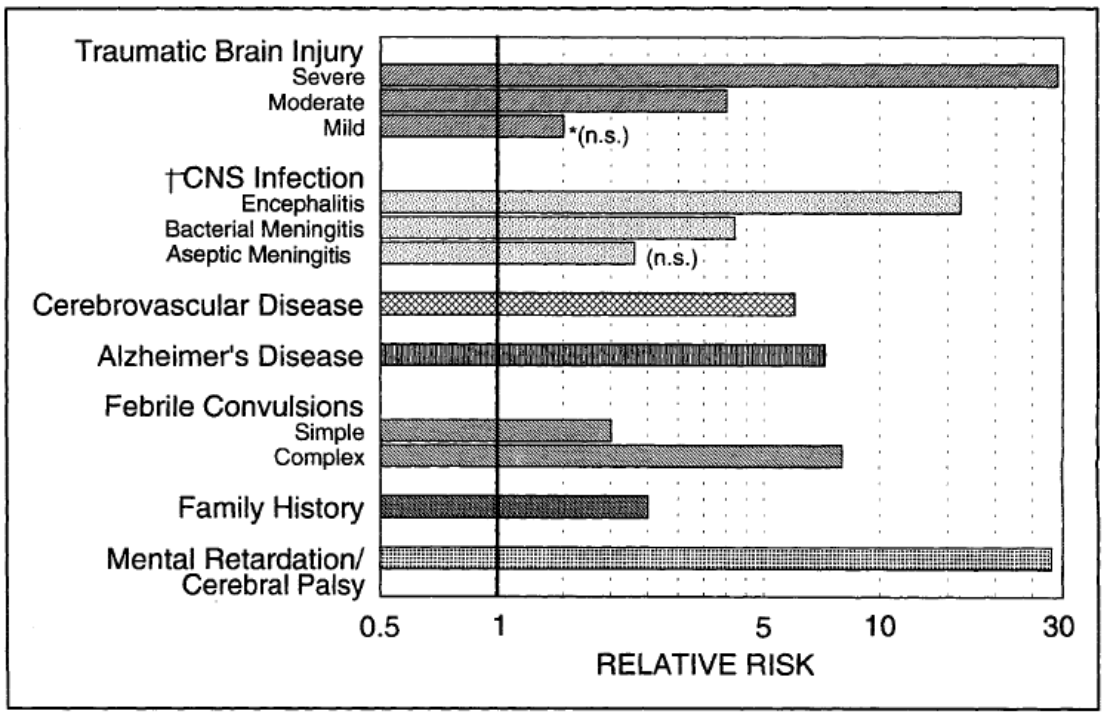

Figure 1. 2 Relative risk of epilepsy (log scale), shown by cause. *n.s.= not statistically significant (taken from Annagers *et al.*, 1996).

- **Traumatic Brain injury:** Post-traumatic epilepsy studies indicate that the risk of developing epilepsy increases with brain injuries. In Vietnam, 53% of the veterans developed post-traumatic epilepsy after the war (Salazar *et al.*, 1985). Civilian studies show that the traumatic brain injury increases the risk of epilepsy and the risk varies according to the severity and the time of the injury (Annagers *et al.*, 1998).

- **Central Nervous System (CNS) Infections:** CNS infections can cause a single seizure or turn into chronic epilepsy. The type of the infectious disease and its damage to central nervous system show different epileptic complications. Between 1935 and 1981, the survivors of encephalitis and meningitis patients were analyzed for the risk of having seizure. 22% of patients with viral encephalitis developed unprovoked seizures, and 13% of

the patients with bacterial meningitis developed seizures (Annegers *et al.*, 1988). Another study indicates the epilepsy incidence of the children having CNS infection is 12.2% (Baek *et al.*, 2010).

- **Cerebrovascular disease (CVD):** CVD can cause epileptic seizures with the neuronal damage balance between the excitation and inhibition of brain. This hyperexcitability may result in a seizure. 10% of stroke patients have seizures, and 3% to 4% stroke patients develop epilepsy (Olsen, 2001).

- **Brain tumors:** Brain tumors are one of the major causes of epilepsy. 12% of the acquired epilepsy occur as a result of brain tumors, and affect all ages (Annagers *et al.,* 1996). Seizures may occur due to the location of tumor hemispheric dysfunction and incomplete resection of tumor (Boarini *et al.*, 1985; Cohen *et al.*, 1988).

- **Degenerative CNS disease:** 2% of all epilepsy cases are associated with a degenerative CNS disease (Annagers *et al.,* 1996). Alzheimer's disease plays an important role in developing seizures in older adults (Romanelli *et al.*, 1990).

- **Developmental disabilities:** Abnormalities from birth can lead to epilepsy. Cerebral palsy and mental retardation increase the incidence of epilepsy (Nelson & Ellenberg, 1986), Down Syndrome increase the epilepsy prevalence 5% in age group between 18 to 29 year age group and 50% among the patients older than 50 years (McVicker *et al.*, 1994).

- **Febrile convulsions:** Febrile seizures increase the risk of epilepsy depending on having complex features at the same time. Patient having simple febrile seizures without repeated episodes within 24 hours have 2.4% risk for developing unprovoked seizure, however patients having febrile seizures with one or more complex features (repeated episodes of febrile convulsions,

focal or prolonged seizures) at the same time have 50% risk of developing unprovoked seizures (Annagers *et al.*, 1987).

- **Familial and genetic factors:** 40% of the epilepsy patients estimated to have genetic background. Different inheritance mechanisms can cause epilepsy; autosomal, mitochondrial, X-chromosomal and complex inheritance (Steinlein, 2004). Idiopathic epilepsies are generally caused as a result of the mutations in the genes coding for ion channels. Voltage-gated channels (sodium, potassium) and ligand-gated channels (GABA and acetylcholine) gene mutations are known to develop epilepsy (Steinlein, 2004). Mutations in the gene coding for neuronal nicotinic acetylcholine receptor cause familial nocturnal frontal lobe epilepsy (Steinlein *et al.*, 1995; De Fusco *et al.*, 2000). Benign familial neonatal convulsions can be caused by voltage-gated potassium channel gene mutations (Biervert *et al.*, 1998; Singh *et al.*, 1998). For generalized epilepsy with febrile seizures (GEFS), mutations in voltage-gated ion channel coding genes (Wallace *et al.*, 1998; Escayg *et al.*, 2000) and $GABA_A$ receptor gene (Baulac *et al.*, 2001; Wallace *et al.*, 2001) are identified. The genetic link between $GABA_B$ receptor gene and epilepsy was confirmed with the $GABA_{B1}$ knock-out mice studies. Null mice develop generalized epilepsy (Prosser *et al.*, 2001). Also G1465A polymorphism of $GABA_{B1}$ gene was reported to be associated with temporal lobe epilepsy (Gambardella *et al.*, 2003). Detailed information on $GABA_B$ receptors are given in Section 1.3.2. Some of the genes involved in epilepsy are shown in Table 1.1.

Table 1. 1 Some of the genes involved in epilepsy (taken from Steinlein, 2004).

Subtypes	Gene symbol	Phenotype
Ion channel genes in idiopathic epilepsy		
Nicotinic acetylcholine receptors	*CHRNA4/CHRNB2*	ADNFLE
Potassium channels	*KCNQ2/KCNQ3*	BFNC
Sodium channels	*SCN1A/SCN2A/SCN1B*	$GEFS^+$
Chloride channels	*CLCN2*	IGE
$GABA_A$ receptors	*GABRG2/GABRA1*	$GEFS^+$/IGE
Non-ion channel genes in idiopathic epilepsy		
Function unknown	*LGI1*	ADLTE
G-protein coupled receptors	*MASS1/VLGR1*	FS
Progressive myoclonus epilepsies		
Polyglucosan metabolism	*EPM2A/EPM2B (NHLRC1)*	Lafora disease
Cysteine protease inhibition	*CSTB*	Unverricht-Lundborg disease
Respiratory chain	*MTTK/MTTL1*	MERRF
Lipidoses	*PPT*	Infantile NCL
	CLN2	Late infantile NCL
	CLN3	Juvenile NCL
	CLN5	Late infantile NCL, Finnish variant
	CLN6	Late infantile NCL, Indian variant
	CLN8	Northern epilepsy
Glycopeptide/oligosaccharide	*NEU1*	Sialidosis metabolism

ADLTE, autosomal dominant lateral temporal lobe epilepsy; ADNFLE, autosomal dominant nocturnal frontal lobe epilepsy; BFNC, benign familial neonatal convulsions; FS, febrile seizures; $GEFS^+$, generalized epilepsy with febrile seizures plus; IGE, idiopathic generalized epilepsies; MERRF, myoclonic epilepsy with ragged red fibres; NCL, neuronal ceroid lipofuscinosis.

1.1.3 Seizures

In the brain, there is a balance between the factors inducing electrical activity and the factors restricting it. Due to break down of one of these factors, abnormal electrical discharges occur and spread to the neighboring cells. Seizures are the physical reactions given to the excessive amount of electrical discharges. Seizures are also known as attacks or convulsions (Parkinson & Johnson, 2006).

Seizures are classified depending on the localization and also according to the etiology.

1.1.3.1 Classification of seizures depending on localization

There are many factors which triggers the seizure. The triggerring factors of the seizures are listed by Richard Appleton and John Gibbs (1998);

- Photosensitivity
- Reading
- Startle reflex
- Physical activity
- Eating
- Immersion in hot or cold water
- Doing mathematics or calculations

As a result of triggering factors, electrical discharges occur. These electrical discharges may start in one part of the brain and then spread to whole brain, or directly affect the whole brain. According to this localization, seizure types are classified. Table 1.2 shows the classification of the seizures, which was reported by the International League Against Epilepsy (ILAE) (Berg *et al*, 2010).

Table 1. 2 Classification of seizures according to localization (taken from ILAE commission report, Berg *et al.*, 2010).

Generalized seizures
Tonic-clonic (in any combination)
Absence
Typical
Atypical
Absence with special features
Myoclonic absence
Eyelid myoclonia
Myoclonic
Myoclonic
Myoclonic atonic
Myoclonic tonic
Clonic
Tonic
Atonic
Focal seizures
Unknown
Epileptic spasms

1.1.3.1.1 Generalized Seizures

Generalized seizures, also known as bilateral seizures, affect both sides of the brain from the beginning of the seizure. Hereditary factors play an important role in generalized seizures. They are sub-categorized into six major groups: generalized, tonic-clonic, absence, myoclonic, clonic, tonic and atonic seizures (Parkinson & Johnson, 2006).

Generalized tonic-clonic (Grand Mal) seizures: This seizure is the most common type; therefore it is the one that people think of when they hear the word epilepsy. It has both characteristics of tonic and clonic seizures. In the tonic phase, muscles stiffen and patient loses consciousness. In the clonic phase, jerking movement of the arms and legs take place as a result of muscle relaxation and contractions. Clonic phase lasts less than a minute (Wyllie, 2007).

Absence seizures: This seizure type occurs as brief loss of consciousness, most generally seen in school-age children (Wyllie, 2007). It lasts less than 10 seconds; this may lead to escape from detection. The patient having absence seizure interrupts an activity and simply stares without speaking or hearing any thing for a few seconds. When the seizure ends, patient continues to the previous activity. They may not be aware of having seizure, and it can be experienced 50-100 times a day.

Myoclonic seizures: Patients have rapid muscle jerks. It can be mild and affect one part of the body, or it can be strong enough to throw the patient to the floor. They usually last for one or two seconds (Parkinson & Johnson, 2006).

Clonic seizures: Repetitive, jerking movements characterize this seizure. Patients have rhythmic jerking movements of arms and legs, mostly on both sides of the body. These movements cannot be stopped by restraining or repositioning.

Tonic seizures: This seizure is characterized by muscle stiffness and rigidity. Tone means the normal tension of a resting muscle. Tonic seizure starts with the increased tone in the body and leads to sudden stiffening movements.

Atonic seizures: This seizure is characterized by loss of muscle tone which means muscles suddenly lose strength. Patient may drop the things from his/her hand, or fall into the ground. Therefore, this seizure is also called as “drop seizure” (Parkinson & Johnson, 2006).

1.1.3.1.2 Partial (Focal) Seizures

Partial seizures affect just one side of the brain from the beginning of the seizure. They are sub-categorized into simple partial seizures, complex partial seizures and the secondarily generalized seizures (Parkinson & Johnson, 2006).

Simple Partial Seizures: There is no loss of consciousness during the seizure, and patient can remember what happened during the seizure. According to the part of the brain where the electrical discharge occurs, different responses are given.

> ***Motor seizures:*** It affects the muscle activity like jerking of stiffening of part of the body. Also weakness affecting the speech and coordinated actions are examples for the motor seizures.
>
> ***Sensory seizures:*** Changes in the senses; like hearing, seeing, feeling, smelling and tasting things that are not actually there. Patients may have some hallucinations and illusions.
>
> ***Autonomic seizures:*** This seizure type affects the autonomous system. Patients may feel some unpleasant sensations in the chest, head or stomach. Also heart rates and as a result breathing rates may change.
>
> ***Psychic seizures:*** People having this seizure may have problems with memory, speech and understanding the language. Without any reason, patients feel sudden emotions like depression, fear or happiness.

Complex Partial Seizures: It is characterized by unresponsiveness, stiffening, language and body disturbances, amnesia and automatisms, which are repetitive, coordinated movements like chewing, lip smacking and walking (Wyllie, 2007). These automatisms are mostly seen in the seizures start in the frontal lobe. It starts with a simple partial seizure, called aura. After this warning seizure, patient loses awareness and automatisms starts. At the end, patient may not remember before or after the seizure.

Secondarily Generalized Seizures: This seizure type starts as a partial seizure, but then suddenly it spreads throughout the brain and turn into a generalized seizure.

1.1.3.1.3 Psychogenic non-epileptic seizures (PNES)

Psychogenic non-epileptic seizures (PNES) are the episodic events, however there is no abnormal electrical activity seen in these patients. Its origins are psychological. 5-20% of the epilepsy population would be PNES patients due to misdiagnosis and the prevalence of PNES is estimated to be 2-33 cases per 100,000 (Benbadis & Hauser, 2000). The diagnosis of PNES have an average delay of 7.2 years due to be diagnosed for epilepsy (Reuber *et al.,* 2002).

The diagnosis of PNES is done by EEG monitoring. In the EEG readings of the patients having seizure, electrical disruptions in the brain are not detected. However, the PNES patient may not have a seizure during the EEG monitoring, which makes the PNES diagnosis more difficult. PNES last longer than the epileptic seizures, and changes characteristics.

Misdiagnose causes wrong usage of anti-epileptic drugs by the PNES patients (Abubakr *et al.,* 2003). Using anti-epileptic drugs may cause health and financial problems. Therefore, it is important to find new diagnostic parameters to distinguish PNES and epileptic seizures.

1.1.3.2 Classification of Epilepsy by Etiology

Underlying causes of epilepsy are divided into three groups in ILAE commission report; genetic, structural/metabolic and unknown cause (Berg *et al*, 2010). However, the etiological categories were not listed in ILAE commission report. The etiological classification of epilepsies is done by Shorvon (2011).

Epilepsies are divided into four categories depending on etiology; idiopathic, symptomatic, provoked and cryptogenic.

Idiopathic epilepsy: Type of epilepsy defined with genetic origins and shows no neuroanatomic or neuropathologic abnormality (Shorvon, 2011). It is sub-categorized into two; pure epilepsies due to single-gene disorder and pure epilepsies with complex inheritance (Shorvon *et al.,* 2011). Genetic abnormalities cause damage to brain nutrition and leads to epilepsy.

Symptomatic epilepsy: Two subcategories of symptomatic epilepsy are; predominantly genetic, and predominantly acquired causation. Genetic abnormalities, birth-injuries, cerebral trauma, tumors and infections are the causes of symptomatic epilepsies (Shorvon *et al*., 2011). Anatomical and pathological abnormalities are also seen in these patients (Shorvon, 2011).

Provoked epilepsy: There are no causative neuroanatomic or neuropathological changes. Factors like fever, sleep, alcohol or hot water induces this type of epilepsy (Shorvon *et al.,* 2011). Some of them may have genetic background but mostly they are not inherited.

Cryptogenic epilepsy: Cryptogenic epilepsy is presumed to have symptomatic nature but their causes have not been identified yet (Shorvon, 2011).

1.1.4 Idiopathic Generalized Epilepsy (IGE)

Idiopathic generalized epilepsy (IGE) was also named as primary generalized epilepsy. IGE accounts for one-fifth of all the other epilepsy types (Jallon & Latour, 2005). Idiopathic means the underlying cause is unknown, however underlying causes for this type of epilepsy are genetic (Gilman, 2007).

There are no neuroanatomical or neuropathological abnormalities seen in idiopathic generalized epilepsy patients. Age of onset, family history, response to epilepsy drugs, EEG results and seizure types are needed for the diagnosis of IGE (Jallon & Latour, 2005).

Idiopathic generalized epilepsy patients have at least one type of seizures from myoclonic, absence and generalized tonic-clonic seizures. Therefore, International League Against Epilepsy (ILAE) recognizes different idiopathic generalized epilepsy types (Nordli, 2005). Those syndromes are;

- Benign Myoclonic Epilepsy in Infancy
- Generalized Epilepsy with Febrile Seizures Plus (GEFS+)
- Epilepsy with Myoclonic Absences
- Epilepsy with Myoclonic-Astatic Seizures (Doose Syndrome)
- Childhood Absence Epilepsy (Pyknolepsy)
- Juvenile Absence Epilepsy
- Juvenile Myoclonic Epilepsy
- Epilepsy with Generalize Tonic-Clonic Seizures

Several genes mutations responsible for different types of idiopathic generalized epilepsy have been identified. Those genes encode ligand-gated or voltage-gated ion channels; some of them are given in Table 1.3. As a result, channelopathies are known as the most important causes of epilepsies.

Table 1. 3 Idiopathic Mendelian epilepsy genes identified by linkage analysis in extended pedigrees (taken from Shorvon *et al.*, 2011).

Class	Gene	Gene product	Epilepsy syndrome	Reference
Voltage-gated ion channel genes				
	SCN1A	Sodium channel α1 subunit	GEFS+, Dravet	Escayg et al, 2000
	SCN1B	Sodium channel β1 subunit	GEFS+	Wallace et al, 1998
	SCN2A	Sodium channel α2 subunit	BFNIS	Heron et al, 2002
	KCNQ2	Potassium channel subunit	BFNC	Singh et al, 1998
	KCNQ3	Potassium channel subunit	BFNC	Charlier et al, 1998
	KCNA1	Potassium channel subunit	EA1 and epilepsy	Zuberi et al, 1999
Ligand-gated ion channel genes				
	CHRNA4	Acetylcholine receptor α4 subunit	ADNFLE	Steinlein et al, 1995
	CHRNA2	Acetylcholine receptor α2 subunit	ADNFLE	Aridon et al, 2006
	CHRNB2	Acetylcholine receptor β2 subunit	ADNFLE	De Fusco et al, 2000
	GABRA1	$GABA_A$ receptor α1 subunit	AD JME, CAE	Cossette et al, 2002
	GABRG2	$GABA_A$ receptor γ2 subunit	GEFS+, CAE	Wallace et al, 2001; Baulac et al, 2001
Others				
	LGI1	Leucine-rich glioma inactivated	ADLTE	Kalachikov et al, 2002
	EFHC1	Protein with EF-hand motif	IGE, particularly JME	Suzuki et al, 2004
	PCDH19	Protocadherin 19	EFMR	Dibbens et al, 2008
	ATP1A2	Na/K ATPase pump	FHM and epilepsy (including BFNIC)	Vanmolkot et al, 2003
	POLGI	Mitochondrial DNA polymerase	Mixed epilepsy phenotypes	Engelsen et al, 2008

AD = autosomal dominant, ADNFLE = autosomal dominant frontal lobe epilepsy, ADLTE = autosomal dominant lateral temporal lobe epilepsy, AE = absence epilepsy, BFNC = benign familial neonatal convulsions, BFNIS = benign familial neonatal-infantile seizures, CAE = childhood absence epilepsy, EA1 = episodic ataxia type 1, EFMR = epilepsy and mental retardation limited to females, FHM = familial hemiplegic migraine, GEFS+ = generalised epilepsy with febrile seizures plus, GLUT-DS = glucose transporter type 1 deficiency syndrome, JME = juvenile myoclonic epilepsy, IGE = idiopathic generalised epilepsy, PED = paroxysmal exercise-induced dyskinesia

1.2 Gamma Aminobutyric Acid (GABA)

γ-Aminobutyric acid (GABA) is one of the major inhibitory neurotransmitters in central nervous system. GABA regulates the impulse transmission between the cells for the prevention of over-firing of nerve cells. GABA was firstly identified in brain extracts of the animals (Roberts and Frankel, 1950). Glutamate is found to be the precursor of GABA molecule; as glutamate is added in the brain extracts of animals, GABA amounts started to increase (Awapara *et al.*, 1950). Also brain extracts of mouse, frog, rat and rabbit were incubated with isotopically labeled glutamate, which then serve as a precursor of GABA (Roberts and Frankel, 1950).

The major GABA metabolism pathway is called GABA shunt, shown in Figure 1.3. In the first step of GABA shunt, glutamate is produced from α-ketoglutarate (generated by TCA cycle) with glutamate dehydrogenease. Glutamate decarboxylase (GAD) enzyme converts glutamate to GABA irreversibly. GABA is catabolized by GABA transaminase (GABA-T) and succinic semialdehyde (SSA) is generated. SSA is oxidized to succinate by succinic semialdehyde dehydrogenase (SSADH). Finally, succinate enters the TCA cycle (Watanabe *et al.*, 2002).

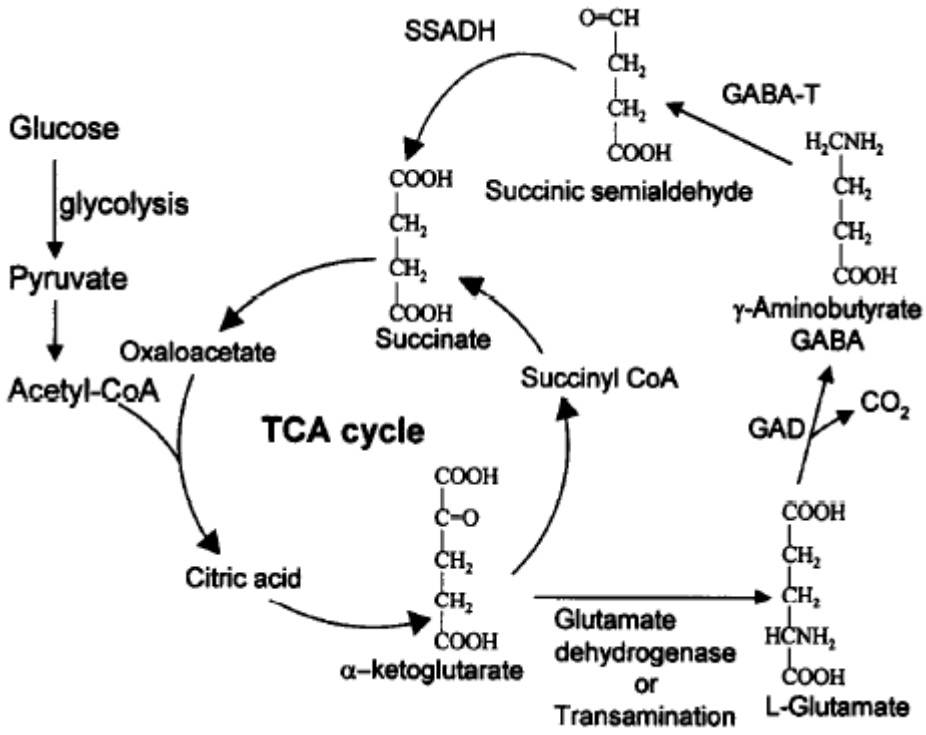

Figure 1. 3 Major metabolic pathway of GABA (taken from Watanabe *et al.*, 2002).

After the synthesis of GABA in pre-synaptic terminal of neurons, GABA is packaged in vesicles. With the arrival of an action potential, those vesicles are released from the post-synaptic terminal to synaptic cleft. GABA is recognized by GABA receptors; ionotrophic $GABA_A$ and metabotrphic $GABA_B$ receptors.

1.2.1 GABA Receptors

GABA mediates its inhibitory action by binding its receptors; $GABA_A$ and $GABA_B$. $GABA_A$ receptors are ionotrophic and mediate fast inhibitory action (Macdonald & Olsen, 1994). $GABA_B$ receptors are metabotropic receptors and mediate long-term inhibitory action (Kerr and Ong, 1995). GABA receptors have binding sites for both positive and negative allosteric modulators.

1.2.1.1 $GABA_A$ Receptors

$GABA_A$ receptors function in most of the physiological actions of GABA molecule. $GABA_A$ receptor structure is a heteropentamer, composed of various subunits. There are different subunit classes; α, β, γ, δ, ε, π, θ and ρ (previously known as $GABA_C$ receptor). They also have subtypes; α1-6, β1-3, γ1-3 and ρ1-3 (Macdonald *et al.*, 2004). Those subunits are composed of 450 amino acids, and they have 200 amino acid-length N-terminus. A cysteine disulfide bridge gives the shape of N-terminus (Engel *et al.*, 2008). Subunit classes show 60-80% amino acid sequence identity between its members, while 20-40% amino acid identity was found with different subunit families (Olsen & Tobin, 1990). Binding-site of GABA is located between α and β subunit interfaces (Smith & Olsen, 1995) (Figure 1.4).

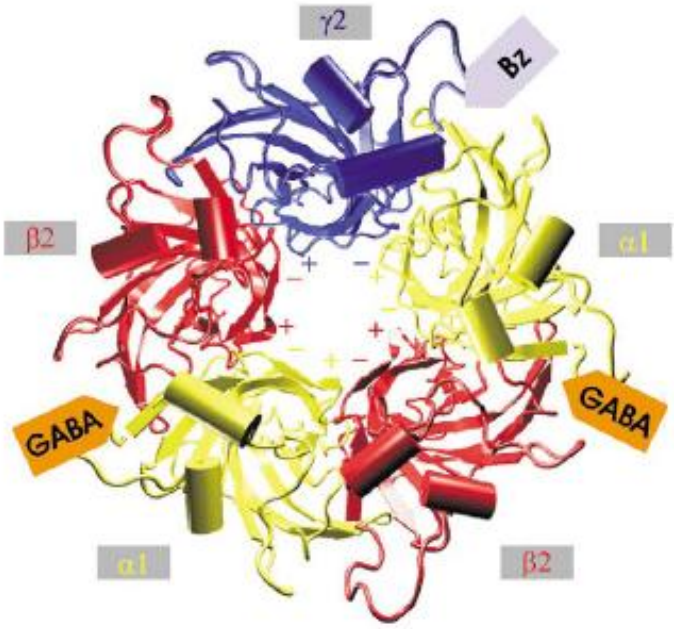

Figure 1. 4 Model structure of $GABA_A$ receptor containing α, β and γ subunits. Binding sites for GABA and benzodiazepine are indicated with arrows (taken from Ernst *et al.*, 2003).

$GABA_A$ receptors are ligand-gated and ionotrophic receptors. With binding of presynaptic GABA molecules to postsynaptic $GABA_A$ receptors, inhibitory postsynaptic currents (IPSCs) are triggered. $GABA_A$ receptors are activated and chloride channels are opened. At least two GABA molecules have to bind in order to fully activate the $GABA_A$ receptors (Sakmann *et al.*, 1983). Chloride ions (Cl^-) flow through the channels and enter the cell through the $GABA_A$ receptor complex shown in Figure 1.5. That leads hyperpolarization of the neuronal membrane. When the electrochemical potential of chloride ions are higher than the outside environment, depolarization occurs and chloride ions are pushed out of the cell (Bureau *et al.*, 1999). However, with the binding of GABA to $GABA_A$ receptor, it becomes difficult for excitatory neurotransmitters to depolarize the neurons.

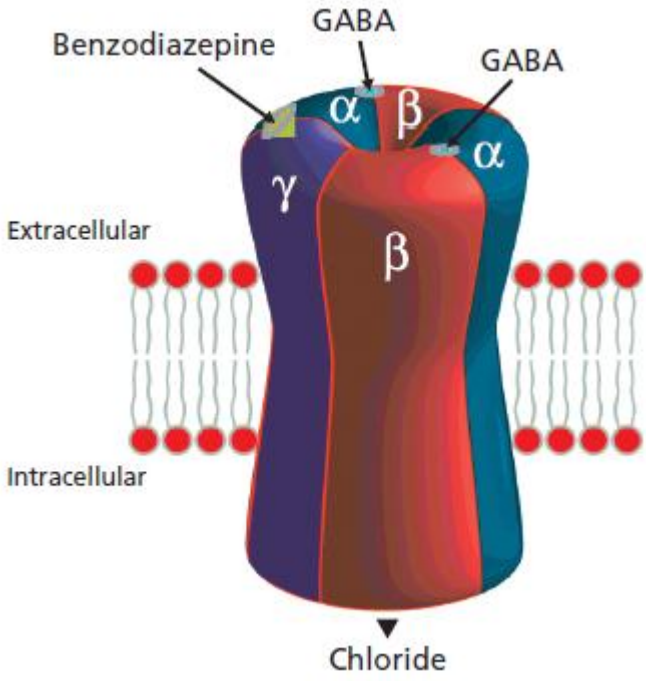

Figure 1. 5 A schematic representation of the $GABA_A$ receptor complex (taken from Dawson *et al.*, 2005).

$GABA_A$ receptor has specific binding sites for benzodiazepines, anesthetic steroids, picrotoxin and barbiturates other than GABA (Table 1.4) (Macdonald & Olsen, 1994). Different binding sites of $GABA_A$ receptor are formed by combination of subunits.

Table 1. 4 Agonists and antagonists of $GABA_A$ receptor (taken from Macdonald & Olsen, 1994).

Selective agonists	Muscimol, GABA
Competitive antagonist	Bicuculline
Non-competitive antagonists	Picrotoxin, PTZ, TBPS
Channel blocker	Penicillin
Benzodiazepine receptor agonists	Diazepam, clonazepam
Benzodiazepine receptor inverse agonists	DMCM, βCCM
Benzodiazepine receptor antagonist	Flumazenil
Barbitrate receptor agonists	Pentobarbitral, phenobarbitraş
Anesthetic steroid receptor agonist	Alphaxalone
Channel ion selectivity	Chloride ions

1.2.1.2 $GABA_B$ Receptors

$GABA_B$ receptors are metabotropic receptors and like the $GABA_A$ receptors, they are distributed throughout the mammalian brain and spinal cord (Bowery & Enna, 2000). They are Class C G-protein coupled receptors. While the general role of $GABA_B$ receptors is synaptic inhibition, they also act in muscle relaxation, slow wave sleep and hippocampal long-term potentiation (Jones *et al.*, 2000). $GABA_B$ receptor has two subunits; $GABA_{B1}$ and $GABA_{B2}$ and each of them have seven transmembrane domains. There are different isoforms of $GABA_{B1}$ and $GABA_{B2}$ proteins with approximately 800-900 amino acids. For the activation of the receptor, those

subunits have to form heterodimers. Co-expression of both receptor subunits generates functional receptor by transporting $GABA_{B1}$ to the cell surface. For the heterodimerization of subunits, a coiled-coil interaction between alpha-helices within the intracellular carboxy tails of the subunits occur (Jones *et al.*, 1998). A schematic representation of $GABA_{B1}$ and $GABA_{B2}$ receptors and their dimerization is given in Figure 1.6.

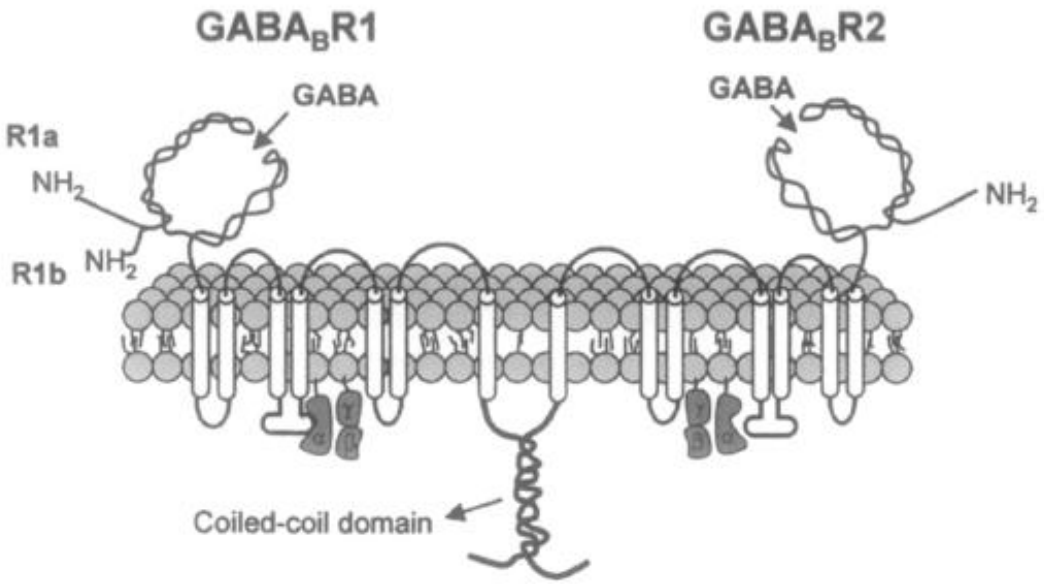

Figure 1. 6 A schematic representation of the $GABA_B$ receptors (taken from Watanabe *et al.*, 2002).

$GABA_{B2}$ subunit mediates the G-protein coupled signaling, while $GABA_{B1}$ subunit takes role in ligand activation of the receptor with the extracellular domain including GABA-binding site (Bowery *et al.*, 2002, Pin *et al.*, 2004). As a result of allosteric coupling between $GABA_{B1}$ and $GABA_{B2}$, $GABA_{B2}$ increases the $GABA_{B1}$ affinity for agonists, and $GABA_{B1}$ facilitates G proteins binding to $GABA_{B2}$ subunit (Pin *et al.*, 2004).

$GABA_B$ receptors function presynaptically and postsynaptically as shown in Figure 1.7. Presynaptic $GABA_B$ receptors suppress the high threshold calcium channels, and inhibit the neurotransmitter release with decreased Ca^{+2} conductance. Postsynaptic $GABA_B$ receptors activate the inward potassium channels (Jones *et al.*, 2000). They are associated with calcium and potassium channels via second messenger system. $GABA_B$ receptors bind to $G_i\alpha$ and $G_o\alpha$ type G proteins. $GABA_B$ receptors inhibit Ca^{+2} channels and so repress Ca^{+2} influx via $G\beta\gamma$ subunits. Also opening of the K^+ channels by postsynaptic $GABA_B$ receptors is activated by $G\beta\gamma$ subunits. This action results with the hyperpolarization of the postsynaptic neuron (Bettler *et al.*, 2004).

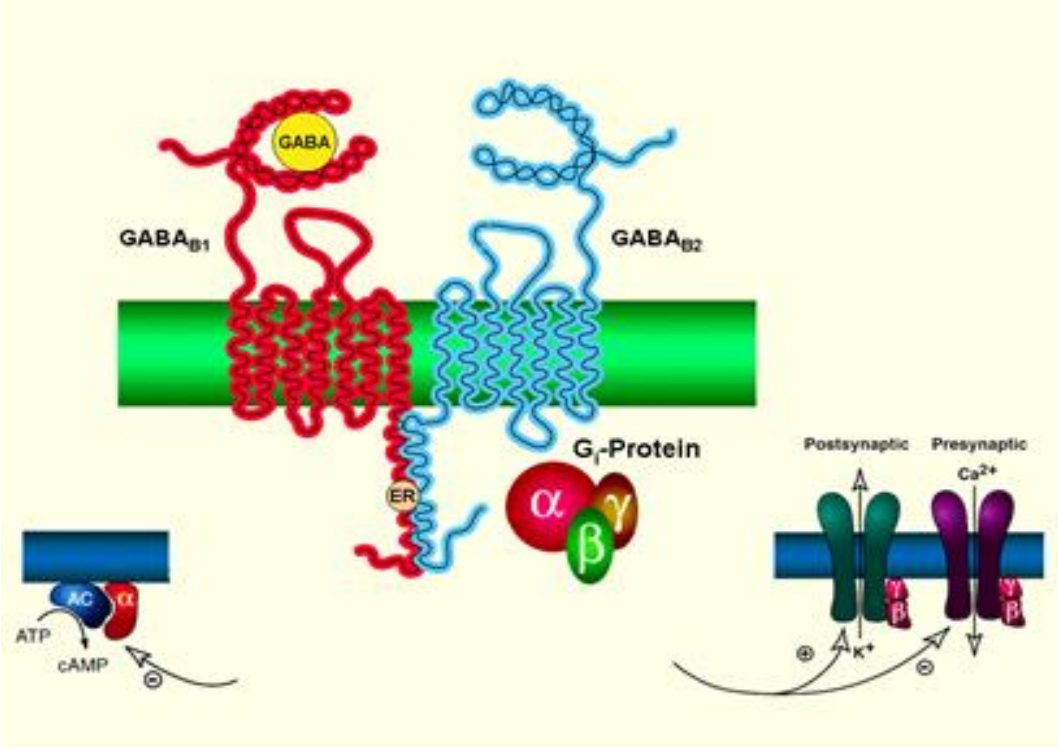

Figure 1. 7 A Schematic represantation of $GABA_{B1}$ and $GABA_{B2}$ receptor complex and their functions (taken form http://www.pharma.uzh.ch).

Baclofen, a well-known muscle relaxant, is a selective agonist of $GABA_B$ receptor. Saclofen, an analogue of baclofen, is the competitive antagonist whereas another analogue, phaclofen is aselective antagonist for $GABA_B$ receptor.

1.3 Genetic Polymorphism

Individual genomes show extensive variations. There are some differences among the DNA sequence of the individuals, which are called genetic polymorphism. Genetic polymorphims include insertions, deletions, sequence repeats, recombination and single nucleotide polymorphisms. In order to be defined as polymorphic, this allele must have a frequency of more than 1% of the population. When genetic polymorphism causes a change in the function of the gene, it could be detected phenotypically. However, most polymorphisms do not affect the function of the gene, differences remain at sequence level (Lewin, 2008).

1.3.1 Single Nucleotide Polymorphism (SNP)

Single nucleotide polymorphism is one nucleotide difference among individual genomes, with a frequency of more than 1% in the population. It is the most common form of the genetic polymorphisms. SNP types are divided as non-coding and coding SNPs. Non-coding SNP sites are; 5' UTR, 3' UTR, introns, intergenic regions, pseudogenes and regulatory sites. Coding SNPs could be synonymous (no amino acid change) or nonsynonymus (amino acid change), which can result with missense or nonsense polymorphisms. Missense polymorphism is the coding of different amino acid. Non-sense polymorphism is the case when amino acid change end up with premature stop codon.

For the detection of SNPs, the restriction maps of different individuals have to be compared, which is defined as restriction fragment length polymorphims (RFLP). RFLP is an SNP, located on the region that restriction enzyme recognizes. RFLPs and SNPs are important for genetic mapping (Lewin, 2008). International HapMap (haplotype map) Project is aimed to determine 10 million estimated SNPs. 1.6 milion

common SNPs have been genotyped by HapMap (International HapMap 3 consortium, 2010).

1.3.2 Polymorphisms of $GABA_{B1}$ gene

$GABA_{B1}$ receptor gene is located in 6p21.3 within the HLA class I region, have 23 exons. GABBR1 is 961 amino acid-long, and 108320 Da. $GABA_{B1}$ receptor gene has multiple transcript variants as a result of alternative splicing.

Animal experiments show that $GABA_B$ receptors play a significant role in absence seizures (Caddick & Hosford, 1996). Antagonist-agonist studies were held on this receptor type. As a general knowledge, a decrease in the GABA action triggers seizure formation. However, there are contradictory results were reported with antagonist-agonist experiments. In one study, while antagonists of $GABA_B$ receptor suppressed the seizures in lethargic mice (lh/lh), animal model for absence seizures, agonists of $GABA_B$ receptor increase the seizure development (Hosford *et al.*, 1992). Mares & Slamberova (2006) explained this situation as the GABA antagonists have opposite effects in epileptic seizures depending on the type of seizure and the stage of maturation.

The link between $GABA_{B1}$ receptors and idiopathic generalized epilepsy was shown with $GABA_{B1}$ null mice. $GABA_{B1}$ knock-out mice were reported to develop generalized epilepsy (Prosser *et al.*, 2001).

G1465A polymorphism is located in exon 11 of $GABA_{B1}$ gene. $GABA_{B1}$ G1465A changes glycine amino acid to serine (Gly489Ser) in a highly conserved region and its frequency in healty population was reported as 0.027 in NCBI database. G1465A polymorphism is reported to be associated with temporal lobe epilepsy (Gambardella *et al.*, 2003; Xi *et al.*, 2011). On the other hand, some studies could not confirm G1465A association with temporal lobe epilepsy (Wang *et al.*, 2008; Salzmann *et al.*, 2005; Kauffman *et al.*, 2008).

C59T polymorphism of $GABA_{B1}$ gene also leads to amino acid change like $GABA_{B1}$ G1465A. C59T nucleotide exchange causes a substitution of alanine to valine (Ala20Val) in exon1a1 of $GABA_{B1}$. The frequency of C59T in healty population was reported as 0.001 in NCBI database. C59T polymorphism was first identified in idiopathic generalized epilepsy patients, however the healthy relatives of these patients also carry C59T polymorphism (Peters *et al.,* 1998). Another study analyze the association between three exonic polymorphisms of $GABA_{B1}$ gene; G1465A, C59T, T1974C, and idiopathic generalized epilepsy and no association reported between those polymorphisms and IGE (Sander *et al.,* 1999).

1.3.3 Polymorphisms of $GABA_{B2}$ gene

As mentioned in Section 1.3.3, $GABA_B$ receptors play important role in seizure development. $GABA_{B2}$ receptor gene is located in 9q22.1-q22.3 chromosome region and contains 19 exons. GABBR1 is 941 amino acid-long, and 105821 Da.

There is one study investigating the association between $GABA_{B2}$ gene polymorphisms and epilepsy. Association between four single nucleotide polymorphisms (rs3780428, rs1999501, rs967932 and rs944688) and mesial temporal lobe epilepsy (MTLE) in Han Chinese population were analyzed (Wang *et al.*, 2008). Among these polymorphisms, rs967932 was found to increase the MTLE risk. Also haplotype of G-C-A-C (rs3780428-rs1999501-rs967932-rs944688) was found to be higher in MTLE patients, compared to control subjects in Han Chinese population (Wang *et al.,* 2008). According to the NCBI database, the frequencies of the SNPs in healty population are; 0.320 for rs3780428; 0.200 for rs1999501; 0.177 for rs967932; 0.303 for rs944688. This is the first study to search for the association between $GABA_{B2}$ rs3780428, rs1999501, rs967932, rs944688 SNPs and idiopathic generalized epilepsy.

1.4 Aim of the study

Epilepsy is characterized by the seizures, which occur due to the decrease in inhibition of neurons or due to a total disinhibition of neurons. A modification in the GABAergic inhibition results in epileptic discharges. Animal experiments prove that the drugs blocking the GABAergic inhibition lead to partial seizures (Schwartkroin *et al*, 1980). Also many loss-of-function studies show the relation between GABA and epilepsy.

Previous studies indicate the association between epilepsy and $GABA_{B1}$ polymorphisms G1465A (Gambardella *et al.,* 2003) and C59T (Peters *et al.,* 1998). For $GABA_{B2}$ receptor, G-C-A-C haplotype (rs3780428-rs1999501-rs967932-rs944688) was found to increase the risk of mesial temporal lobe epilepsy (MTLE) (Wang *et al.*, 2008).

Idiopathic generalized epilepsy is one of the most common types of epilepsy. GABA receptor mutations are identified in the pathogenesis of idiopathic generalized seizures. This is the first study to search for the association between $GABA_{B2}$ rs3780428, rs1999501, rs967932, rs944688 SNPs and idiopathic generalized epilepsy. Also the frequencies of these $GABA_{B2}$ SNPs in Turkish population were reported for the first time in this study. It is well known that frequencies of genetic polymorphisms show wide variation among different ethnic groups. Therefore, the aim of this study was to investigate the association between the $GABA_{B1}$ and $GABA_{B2}$ polymorphisms and idiopathic generalized epilepsy in Turkish population.

Psychogenic non-epileptic seizures (PNES) mimic any kind of epileptic seizures, therefore wrong diagnosis causes unnecessary medical expenditures and the origin of the problem cannot be saught. Thus, in this study, determination of the role of the differences found in the GABA receptor genes in differential diagnosis of PNES is also aimed.

To achieve this aim, following steps were designed;

- Collection of blood samples of idiopathic generalized epilepsy patients, subjects having psychogenic non-epileptic seizures (PNES) and non-epileptic control subjects,
- Genomic DNA isolation of blood samples,
- Amplification of the region including SNP of interest by polymerase chain reaction,
- Digestion of PCR products with specific restriction enzymes or the determination of SNP genotypes,
- Determination of allele and genotype frequencies of G1465A and C59T polymorphisms of $GABA_{B1}$ gene; rs1999501, rs967932, rs3780428 and rs944688 polymorphisms of $GABA_{B2}$ gene for all subjects,
- Analysis of the association between $GABA_{B1}$ and $GABA_{B2}$ single nucleotide polymorphisms and idiopathic generalized epilepsy and PNES in Turkish population, using biostatistic techniques.

CHAPTER 2

MATERIALS & METHODS

2.1 Materials

2.1.1 Population and Blood Sampling

Population of this study includes a total of 176 idiopathic generalized epilepsy patients, 83 subjects having psychogenic non-epileptic seizures, 86 non-epileptic control subjects from Turkey. Total blood samples were collected with the collaboration of Gülhane Military Medical Academy Hospital, Neurology Department, Ankara. Before the blood collection, informed consent was signed by all the participants of the study (see Appendix A). The study has the ethical committee approval according to the principles of Declaration of Helsinki by the ethical committee of Gülhane Military Medical Faculty (see Appendix B).

2.1.2 Chemicals

The chemicals used in this study are given in Appendix C, including their code numbers and suppliers. All the chemicals were of molecular grade or obtained from commercial sources at the highest grade of purity.

2.1.3 Primers

Six primer pairs used in this study were obtained from Iontek (Iontek, İstanbul, Turkey) in order to be used in the amplification of the regions including single nucleotide polymorphim of interest (Table 2.1). At first, ultrapure water added on those primers to make the concentration 100 pmol/μL. Then, primers were stored as 10 pmol/μL concentration, at -20°C.

Table 2. 1 Primer sequences used for the amplification of single nucleotide polymorphism of interest

Gene	SNP	Primers
$GABA_{B1}$	G1465A (rs1805057)	F5' AACAGTAACACAAACCATCC 3' R5' GCATGTTTGTAGAAGGTGCC 3'
	C59T (rs1805056)	F5' GGGTGCGGGCCGCGCCG 3' F5' AGAAATGAGGAGATGCAGG 3'
$GABA_{B2}$	rs1999501	F5' TTCTCTAACTCCAGGTCCAA 3' R5' TCAGGGTCACAAACACAAAG 3'
	rs967932	F5' AGAACGGGCTCCAACTAAAA 3' R5' GCTGAGATATTCACCCACCA 3'
	rs3780428	F5' AGACTTTCATCTGGGCTCAA 3' R5' AAACTTGGCTGATGCTTGGT 3'
	rs944688	F5' CCTAATGTCTGCTATCACCA 3' R5' AGGGTAAGTTTTTAACAGAGTT 3'

2.2Methods

2.2.1 Preparation of Genomic DNA for PCR

2.2.1.1 Isolation of Genomic DNA from Human Whole Blood Samples

Principle

For the isolation of genomic DNA, salting-out method described by Lahiri and Schnabel (1993) was used with some modifications. This method is preferred in order to avoid toxic chemical usage. In saturated salt solution, other than DNA, proteins and other cellular contaminants are precipitated due to hydrophobicity. Whole blood samples were collected into tubes containing EDTA in order to prevent blood clotting, and maintained at -20°C until isolation performed.

Reagents

1. **TKM Buffer (pH 7.6, 200 mL)**

   10 mM Trizma base (pH 7.6)

   10 mM KCl

   4 mM $MgCl_2$

   2 mM EDTA.H_2O

   242 mg Trizma Base, 149 mg KCl, 163 mg $MgCl_2$ and 149 EDTA.H_2O were measured and put into a beaker. Distilled water less than 200 mL, were added onto the chemicals and dissolved by using magnetic stirrer. The pH of the solution was adjusted to 7.6 with HCl, and the volume was completed to 200 mL. Buffer was autoclaved before use and stored at 4°C.

2. **Tris-HCl (pH 8.0, 100 mM, 100 mL)**

   1.21 g Trizma base was measured and put into a beaker. Distilled water less than 100 mL, were added onto Trizma base and dissolved by using magnetic stirrer. The pH of the solution was adjusted to 8.0 with HCl, and

the volume was completed to 100 mL. Buffer was autoclaved before use and stored at 4°C.

3. **EDTA (pH 8.0, 500 mM, 100 mL)**

   18.61 g EDTA.H_2O was weighed and put into a beaker. Distilled water less than 100 mL, were added onto EDTA and dissolved by using magnetic stirrer. The pH of the solution was adjusted to 8.0 with NaOH, and the volume was completed to 100 mL. Buffer was autoclaved before use and stored at 4°C.

4. **TE Buffer (pH 8.0, 100 mL)**

   100 mM Tris-HCl

   500 mM EDTA

   0.2 mL of 500 mM EDTA is mixed with 10 mL of Tris-HCl solution, and the final volume is completed to 100 mL. TE buffer was stored at 4°C.

5. **10% SDS Solution**

   0.1 g of molecular grade SDS detergent was dissolved in 1 mL distilled water, stored at 4°C.

6. **Saturated NaCl Solution (100 mL)**

   35.06 g of molecular grade NaCl was dissolved in 100 mL of distilled water, stored at 4°C.

Procedure

750 µl of whole blood was treated with 750 µl of low salt TKM buffer (10 mM Tris-HCl, pH 7.6, 10 mM KCl, 4 mM $MgCl_2$ and 2 mM EDTA). After 20 µl Triton X-100 was added into the blood-buffer mixture, tubes were inverted several times for the red blood cell lysis. The suspension was centrifuged at 1,000 g for 10 min at room temperature by using Sigma 1-15 benchtop microcentrifuge (Sigma, Postfach 1713-D-37507, Osterode) in order to separate leukocytes from red blood cell lysates. The

pellet including leukocytes was washed with TKM two or more times, until a clear white pellet obtained. The final clear pellet was resuspended in 200 µl of TKM buffer. 10 µl of 10 % sodium dodecyl sulfate (SDS) was added to suspension and mixed until more viscous and clear pellet obtained. The suspension was incubated at 58°C for 10 min. 75 µl of cold saturated NaCl (~6 M) was added and suspension mixed well and centrifuged at 14,000 g for 10 min at 4°C, in order to precipitate proteins and other cellular contaminants. The supernatant, which contains DNA, was taken and 2X volume of absolute ethanol, which is maintained at -20°C before use, was added in order to precipitate DNA. Solution was inverted several times and kept in -20°C for 30 min, and centrifuged at 10,000 g for 10 min at 4°C. Ethanol was removed as supernatant, and DNA pellet was dried for the complete removal of ethanol. DNA was dissolved in 100 µl of TE buffer, pH 8.0 and incubated at 37°C overnight.

2.2.1.2 Quantification of DNA concentration by Spectrophotometry

Quantification of genomic DNA was performed with NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA). Software measures the concentration at 260 nm for DNA samples.

2.2.1.3 Qualification of Genomic DNA by Spectrophotometry

In order to control the purity of DNA sample, absorbance at 280 nm was also determined, in addition to 260 nm. The 1.8 ratio between the absorbance values at 260 nm and 280 nm (A_{260}/A_{280}) indicates pure DNA, while the higher values indicate RNA and lower values indicate protein contaminations in DNA sample.

2.2.1.4 Qualification of Genomic DNA by Agarose Gel Electrophoresis

Principle

Quality of genomic DNA samples was determined by looking at the intactness of the DNA samples on 0.5% agarose gel electrophoresis, using Scie-Plas HU13W horizontal gel electrophoresis device.

Reagents

Tris-Borate-EDTA Buffer (5X TBE Buffer, pH 8.3, 1L)

450 mM Trizma Base
450 mM Boric acid
10 mM EDTA.H_2O

54 g Trizma Base and 27.5 g boric acid were weighed and put into a beaker. 20 µL of 500 mM EDTA solution and distilled water less than 1 L were added onto the chemicals and dissolved by using magnetic stirrer. The pH of the solution was adjusted to 8.3, and the volume was completed to 1 L. Buffer was autoclaved before use and stored at 4°C.

Procedure

0.5 % agarose gel was prepared by mixing 1 g agarose and 200 mL of 0.5X TBE buffer, pH 8.3, inside an Erlenmeyer flask. Suspension was heated in microwave oven, until agarose completely dissolved and a colorless gel solution obtained. Gel solution is cooled to approximately 60°C on a magnetic stirrer to obtain a homogenous mixture, and then 10 µL of ethidium bromide (10 mg/mL) was added and mixed well. Before pouring the gel solution, gel tray, combs and the stoppers were cleaned with 70% ethanol. Gel tray was placed on a smooth surface, enclosed with the stoppers and the combs were placed 0.5-1.0 mm above the plate. The molten agarose gel solution was poured into the gel tray, and air bubbles were removed by

using micropipette. Gel was allowed to cool and solidify for 20-40 min at room temperature. Combs and the stoppers were removed and the gel tray was placed in gel tank, which was filled with 300 µL of 0.5X TBE buffer. 5 µL of DNA sample (0.25- 0.5 ng) was mixed with 1 µL of gel loading buffer (0.25% bromophenol blue and 40% sucrose in dH_2O) on a parafilm piece and loaded into the wells. The lid of the gel tank was closed and the electric wires were attached to the power supply. The wire placed on top of the DNA containing wells was attached to negative pole; the wire at the bottom of the gel was attached to the positive pole on the power supply. Power supply was set to 100 volts, and the gel was run for approximately 45 min until the bromophenol blue was reached to the end of the gel. Gel was examined under UV light, and the picture of the gel was taken by using Bio-Capture (Version 99.03) in the Vilber Lourmat Gel Imaging System (Marre La Vallee, Cedex, France). Pure DNA runs as a single band on the agarose gel electrophoresis, while the samples with RNA contamination run as two bands and the samples with DNA degradation run as a smear.

2.2.2 Genotyping of Single Nucleotide Polymorphisms

PCR protocols and the restriction enzyme digestions used in genotyping the $GABA_{B1}$ and $GABA_{B2}$ single nucleotide polymorphisms are given in Table 2.2.

Table 2. 2 Genotyping of Single Nucleotide Polymorphisms; Table showing the gene, SNP number, the regions of amplification, size of PCR products and restriction endonuclease (RE) used for digestion of PCR products.

Gene	SNP	Region of amplification	PCR product size	RE
$GABA_{B1}$	G1465A (rs1805057)	Coding region	441	*Eag*I
	C59T (rs1805056)	Coding region	212	*Hin*6I
$GABA_{B2}$	rs1999501	Intronic region	370	*Bsa*AI
	rs967932	Intronic region	348	*Scr*FI
	rs3780428	Intronic region	255	*Sau*96I
	rs944688	Intronic region	137	*Hpa*I

For the PCR amplification, Techne TC-4000 thermal cycler (Techne Ltd., Duxford, Cambridge) was used. For the digestion procedure, samples were incubated in Biosan TDB-120 Heat Block (Biosan Ltd., Latvia).

2.2.2.1 Genotyping of $GABA_{B1}$ gene SNPs

2.2.2.1.1 $GABA_{B1}$ G1465A Single Nucleotide Polymorphism

2.2.2.1.1.1 Polymerase Chain Reaction for G1465A of $GABA_{B1}$ gene

The region including the G1465A polymorphism was amplified using primer pairs given by Wang *et al.* (2008) (Table 2.1). The sequence of the amplified region including G1465A single nucleotide polymorphism is given in Figure 2.1.

-29581471 AGCTAATACCTACTCTAGTCTAGTAGCTTCCGATCTAAGGCAGACACATG
-29581421 GGTATAGTTAAAGATTTTGAATGTACATGTGTCCAATCTGAC**AACAGTAA**
-29581371 **CACAAACCATCC**ATTCAAGTAGAAGTGATTGAGTCAGAATTGGATTGCAC
-29581321 CCCTTCCCCCACACCCACACACATTTCAGTTCTTTCCTCATGATTTTTTC
-29581271 CTCCCAAGACATCCCAGGAATTTGTGGAGAAACTAACCAAGCGACTGAAA
T S Q E F V E K L T K R L K
-29581221 AGACACCCTGAGGAGACAGGAGGCTTCCAGGAGGCACCGCTGGCCTATGA
R H P E E T G G F Q E A P L A Y D
-29581171 TGCCATCTGGGCCTTGGCACTGGCCCTGAACAAGACATCTGGAGGAGGCG
A I W A L A L A L N K T S G G G
-29581121 GCCGTTCTGGTGTGCGCCTGGAGGACTTCAACTACAACAACCAGACCATT
G R S G V R L E D F N Y N N Q T I
-29581071 ACCGACCAAATCTACCGGGCAATGAACTCTTCGTCCTTTGAGGGTGTCTC
T D Q I Y R A M N S S S F E G V S
-29581021 TGTGAGTTAAAACTTCCTTCATACTCCCCTGTCTTCCCAATCTTGAGAGA
-29580971 GACTCCCAAGA**GGCACCTTCTACAAACATGC**ATTCTCTGTTTTTCTCAGT

Figure 2. 1 Sequence of amplified fragment of $GABA_{B1}$ gene including G1465A single nucleotide polymorphism. The forward and reverse primers are written in bold and underlined. Location of recognition sequence for *Eag* I restriction endonuclease is marked with yellow box. The polymorphic nucleotide is written in red (the nucleotide sequence is taken from http://www.ncbi.nlm.nih.gov).

Optimization of PCR procedure was done by several modifications on the concentrations of $MgCl_2$, primers and DNA template. Components of the optimized PCR mixture for the amplification of G1465A SNP are given in Table 2.3. In a total of 50 µl PCR reaction, approximately 200 ng genomic DNA, 1.0 mM $MgCl_2$, 200 µM dNTPs, 0.6 pmol/µL of forward and reverse primers and 3 Unit *Taq* polymerase were included.

Table 2. 3 Components of PCR mixture for $GABA_{B1}$ G1465A SNP.

Component	**Stock concentration**	**Volume added**	**Final Concentration in 50 µL reaction mixture**
Sterile Apyrogen H_2O		Up to 50 µL	
Amplification Buffer	10X	5 µL	1X
$MgCl_2$	25 mM	2 µL	1 mM
dNTP mixture	10 mM	1 µL	200 µM
Forward Primer	10 pmol/µL	3 µL	0.6 pmol/µL
Reverse Primer	10 pmol/µL	3 µL	0.6 pmol/µL
Template DNA	varies	Varies	~200 ng
Taq DNA Polymerase	5 U/µL	0.6 µL	3 U

The program of the thermalcycler used for the amplification of G1465A SNP region of $GABA_{B1}$ was taken from those published (Wang *et al*., 2008) and given in Table 2.4.

Table 2. 4 PCR program used for the amplification of G1465A SNP region of $GABA_{B1}$ gene.

Initial denaturation	95°C	5 min.	
Denaturation	95°C	30 sec.	30 cycles
Annealing	58°C	30 sec.	
Extension	72°C	30 sec.	
Final extension	72°C	10 min.	

PCR products were analyzed on 2.0% agarose gel, which was prepared as described in section 2.2.1.4. Ten μL of PCR product was mixed with 3 μL of gel loading buffer and then 10 μL from this mixture and 6 μL of DNA ladder (50-1000 bp) were loaded to the wells of the gel. The agarose gel run was performed at 100V, for 1 hour

2.2.2.1.1.2 Restriction Endonuclease Digestion of PCR Products for Determination of $GABA_{B1}$ G1465A SNP

Schematic representation of the digestion procedure for the determination of G1465A genotypes is given in Figure 2.2. Wild type allele including G nucleotide can be recognized by *Eag*I restriction enzyme, while the polymorphic allele including A nucleotide cannot be recognized by *Eag*I restriction enzyme. Recognition site of the *Eag*I restriction enzyme is as given below;

5'...C^G G C C G...3'
3'...G C C G G^C...5'

Eag I cuts 441-bp PCR product into two fragments of 258-bp and 183-bp. Undigested 441-bp fragment indicates the polymorphic allele. Heterozygotes contain both wild type and polymorphic alleles, and indicated with three bands (Figure 2.2).

For the determination of G1465A genotypes, 10 μl of 441-bp PCR product was incubated with 4U *Eag*I at 37°C for 24 hours, in a restriction mixture as given in Table 2.5. The digestion products were analyzed on 3% agarose gel.

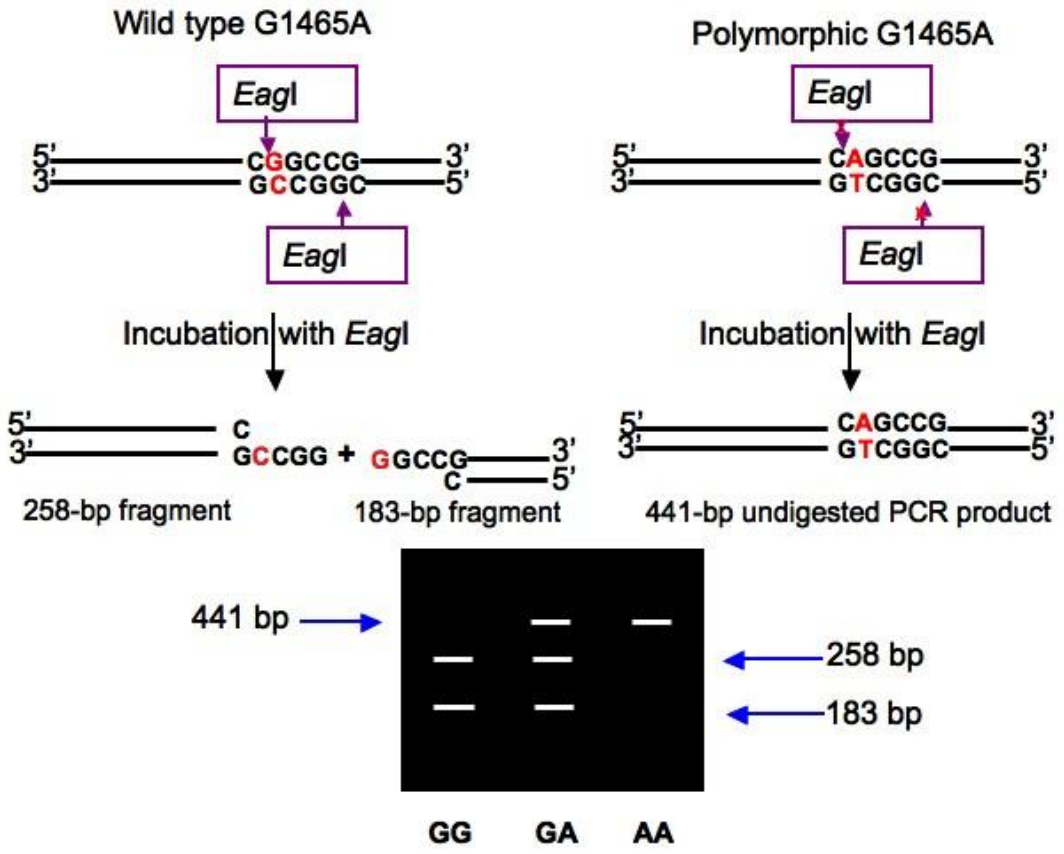

Figure 2. 2 Schematic representation of G1465A genotype determination. Wild type G nucleotide shown in the left panel is replaced with A in the polymorphic allele which is shown in right panel. *Eag*I restriction enzyme recognizes (5' CGGCCG 3') site within wild type allele and cuts 441-bp PCR product into two fragments of 258-bp and 183-bp. The polymorphic allele is not recognized by *Eag*I enzyme, thus undigested 441-bp fragment implies polymorphic allele. Three of these bands are seen in the heterozygote individuals.

Table 2. 5 Components of reaction mixture for restriction endonuclease (*Eag*I) digestion of PCR products for the determination of $GABA_{B1}$ G1465A SNP.

Component	Concentration	Volume added	Final concentration in 30 μL reaction mixture
Buffer Tango	10 X	2 μL	0.66 X
Sterile Apyrogen dH_2O	---	Up to 30 μL	---
*Eag*I	10 U/μL	0.4 μL	4 U
PCR product	---	10 μL	---

2.2.2.1.2 $GABA_{B1}$ C59T Single Nucleotide Polymorphism

2.2.2.1.2.1 Polymerase Chain Reaction for C59T of $GABA_{B1}$ gene

The region including the C59T polymorphism was amplified using primer pairs given by Peters *et al.* (1998) (Table 2.1). The sequence of the amplified region including C59T single nucleotide polymorphism is given in Figure 2.3.

Optimization of PCR procedure was done by several modifications on the concentrations of $MgCl_2$, primers and DNA template. Components of the optimized PCR mixture for the amplification of C59T SNP are given in Table 2.6. In a total of 50 μl PCR reaction, approximately 200 ng genomic DNA, 2 mM $MgCl_2$, 200 μM dNTPs, 0.6 pmol/μL of forward and reverse primers and 3 Unit *Taq* polymerase were included.

Figure 2. 3 Sequence of amplified fragment of $GABA_{B1}$ gene including C59T single nucleotide polymorphism. The forward and reverse primers are written in bold and underlined. Location of recognition sequence for *Hin*6I restriction endonuclease is marked with yellow box. The polymorphic nucleotide is written in red (the nucleotide sequence is taken from http://www.ncbi.nlm.nih.gov).

Table 2. 6 Components of PCR mixture for $GABA_{B1}$ C59T SNP.

Component	**Stock concentration**	**Volume added**	**Final Concentration in 50 μL reaction mixture**
Sterile Apyrogen H_2O		Up to 50 μL	
Amplification Buffer	10X	5 μL	1X
$MgCl_2$	25 mM	2 μL	1 mM
dNTP mixture	10 mM	1 μL	200 μM
Forward Primer	10 pmol/μL	3 μL	0.6 pmol/μL
Reverse Primer	10 pmol/μL	3 μL	0.6 pmol/μL
Template DNA	varies	Varies	~200 ng
Taq DNA Polymerase	5 U/μL	0.6 μL	3 U

The program of the thermalcycler used for the amplification of C59T SNP region of $GABA_{B1}$ was taken from those published (Sander *et al*., 1999) and given in Table 2.7.

Table 2. 7 PCR program used for the amplification of C59T region of $GABA_{B1}$ gene

Denaturation	95°C	30 sec.	35 cycles
Annealing	58°C	30 sec.	
Extension	72°C	30 sec.	

PCR products were analyzed on 2.0% agarose gel, which was prepared as described in section 2.2.1.4. Ten μL of PCR product was mixed with 3 μL of gel loading buffer and then10 μL from this mixture and 6 μL of DNA ladder (50-1000 bp) were loaded to the wells of the gel. The agarose gel run was performed at 100V, for 1 hour.

2.2.2.1.2.2 Restriction Endonuclease Digestion of PCR Products for Determination of $GABA_{B1}$ C59T SNP

Schematic representation of the digestion procedure for the determination of C59T genotypes is given in Figure 2.4. Wild type allele including C nucleotide can be recognized by *Hin*6I restriction enzyme, while the polymorphic allele including T nucleotide cannot be recognized by *Hin*6I restriction enzyme. Additionally there are three more recognition sites in the PCR product for *Hin*6I. Recognition site of the *Hin*6I restriction enzyme is as given below;

5'...G^C G C...3'
3'...C G C^G...5'

*Hin*6I cuts PCR products at three more sites other than the RFLP site. Therefore, three of these bands (67-bp, 25-bp and 12-bp) are found in all genotypes. For the recognition of C59T SNP genotype, 108-bp band cuts into 97-bp and 11-bp bands in wild type individuals. So, undigested 108-bp band indicates the polymorphic allele. Heterozygotes contain both wild type and polymorphic alleles, and indicated with total six bands in the agarose gel (Figure 2.4).

For the determination of C59T genotypes, 10 μl of 212-bp PCR product was incubated with 3U *Hin*6I at 37°C for 16 hours, in a restriction mixture as given in Table 2.8. The digestion products were analyzed on 3% Nu-micropore agarose gel.

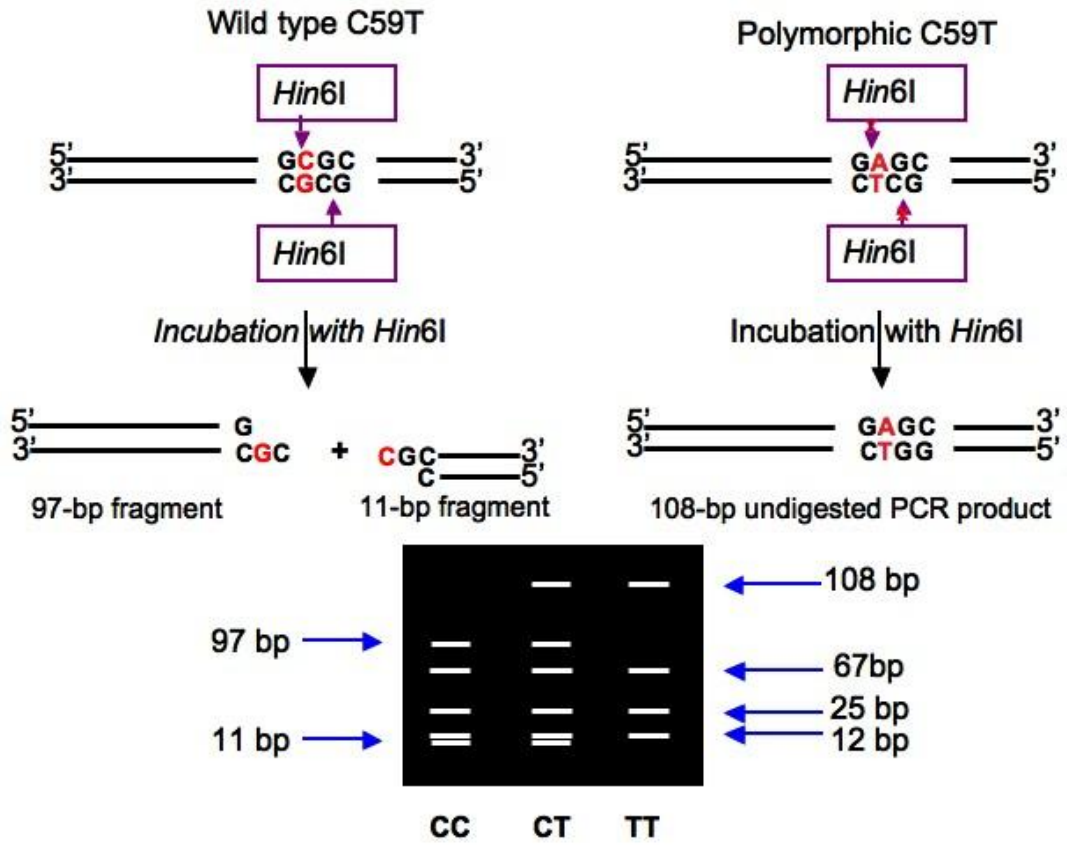

Figure 2. 4 Schematic representation of C59T genotype determination. Wild type C nucleotide shown in the left panel is replaced with T in the polymorphic allele which is shown in right panel. *Hin*6I restriction enzyme recognizes four (5' GCGC 3') sites within wild type allele carrying PCR product and cuts 212-bp PCR product into five fragments of 97-bp, 67-bp, 25-bp, 12-bp and 11-bp in wild type individuals. The polymorphic allele containing PCR product is recognized by *Hin*6I enzyme from three sites, thus undigested 108-bp fragment implies polymorphic allele. Six of these bands are seen in the heterozygote individuals.

Table 2. 8 Components of reaction mixture for restriction endonuclease (*Hin*6I) digestion of PCR products for the determination of $GABA_{B1}$ C59T SNP.

Component	**Concentration**	**Volume added**	**Final concentration in 30 μL reaction mixture**
Buffer Tango	10 X	2 μL	0.66 X
Sterile Apyrogen dH_2O	---	Up to 30 μL	---
*Hin*6I	10 U/μL	0.3 μL	3 U
PCR product	---	10 μL	---

2.2.2.2 Genotyping of $GABA_{B2}$ gene SNPs

2.2.2.2.1 $GABA_{B2}$ rs1999501 Single Nucleotide Polymorphism

2.2.2.2.1.1 Polymerase Chain Reaction for rs1999501 of $GABA_{B2}$ gene

The region including the rs1999501 polymorphism was amplified using primer pairs given by Wang *et al.* (2008) (Table 2.1). The sequence of the amplified region including rs1999501 single nucleotide polymorphism is given in Figure 2.5.

Optimization of PCR procedure was done by several modifications on the concentrations of $MgCl_2$, primers and DNA. Components of the optimized PCR mixture for the amplification of rs1999501 SNP are given in Table 2.9. In a total of 50 μl PCR reaction, approximately 200 ng genomic DNA, 0.75 mM $MgCl_2$, 200 μM dNTPs, 1 pmol/μL of forward and reverse primers and 3 Unit of *Taq* polymerase were included.

-70952721 TGCGTTTATGGGATGAATGAATA**TTCTCTAACTCCAGGTCCAA**CCCTCCA

-70952671 TTACACCATTTACTACATGTTGCTAAATTATTTCCATGTCTCTCACCCAC

-70952621 TCCACATTATAAATCCCATGAGAGCAAGGACCCAGTCTTACCCATCTCAT

-70952571 CTTCCCCTACCTAGCTCAGGCGCCCAGCCAATGTTGAACCAATGTGCCCC

-70952521 ACAAATGTGGAATGGAAAGGAGTGGCGATGGCTTGGTGGCACACGTAGTT

-70952471 GGAGTTTGGTATCTCTTAAGAGGGCGATCCAGCCCCCACCCCCGGAGAAG

-70952421 GGCTGCCAACATGTGATATTTGCACAAACCTGCAGAAAAGAGCACCCCTC

-70952371 TCCTGATCGGCAGCCCGAGTACA**CTTTGTGTTTGTGACCCTGA**CTTTTAA

Figure 2. 5 Sequence of amplified fragment of $GABA_{B2}$ gene including rs1999501 single nucleotide polymorphism. The forward and reverse primers are written in bold and underlined. Location of recognition sequence for *Bsa* AI restriction endonuclease is marked with yellow box. The polymorphic nucleotide is written in red (the nucleotide sequence is taken from http://www.ncbi.nlm.nih.gov).

Table 2. 9 Components of PCR mixture for $GABA_{B2}$ rs1999501 SNP.

Component	**Stock concentration**	**Volume added**	**Final Concentration in 50 μL reaction mixture**
Sterile Apyrogen H_2O		Up to 50 μL	
Amplification Buffer	10X	5 μL	1X
$MgCl_2$	25 mM	1.5 μL	0.75 mM
dNTP mixture	10 mM	1 μL	200 μM
Forward Primer	10 pmol/μL	5 μL	1 pmol/μL
Reverse Primer	10 pmol/μL	5 μL	1 pmol/μL
Template DNA	varies	Varies	~200 ng
Taq DNA Polymerase	5 U/μL	0.6 μL	3 U

The program of the thermalcycler used for the amplification of rs1999501 SNP region of $GABA_{B2}$ was taken from those published (Wang *et al*., 2008) and given in Table 2.10.

Table 2. 10 PCR program used for the amplification of rs1999501 SNP region of $GABA_{B2}$ gene.

Initial denaturation	95°C	5 min.	
Denaturation	95°C	30 sec.	30 cycles
Annealing	56°C	30 sec.	
Extension	72°C	30 sec.	
Final extension	72°C	10 min.	

PCR products were analyzed on 2.0% agarose gel, which was prepared as described in section 2.2.1.4. Ten μL of PCR product was mixed with 3 μL of gel loading buffer and then 10 μL from this mixture and 6 μL of DNA ladder (50-1000 bp) were loaded to the wells of the gel. The agarose gel run was performed at 100V, for 1 hour.

2.2.2.2.1.2 Restriction Endonuclease Digestion of PCR Products for Determination of $GABA_{B2}$ rs1999501 SNP

Schematic representation of the digestion procedure for the determination of rs1999501 genotypes is given in Figure 2.6. Wild type allele including C nucleotide, can be recognized by *Bsa*AI restriction enzyme, while the polymorphic allele including T nucleotide cannot be recognized by *Bsa*AI restriction enzyme. Recognition site of the *Bsa*AI restriction enzyme is as given below;

5'...Y A C^G T R...3'
3'...R T G^C A Y...5'

*Bsa*AI cuts 370-bp PCR product into two fragments of 221-bp and 149-bp in wild type individuals. Undigested 370-bp fragment indicates the polymorphic allele. Heterozygotes contain both wild type and polymorphic alleles, and indicated with three bands (Figure 2.6).

For the determination of rs1999501 genotypes, 10 μl of 370-bp PCR product was incubated with 4U *Bsa*AI at 30°C for 16 hours, in a restriction mixture as given in Table 2.11. The digestion products were analyzed on 3% agarose gel.

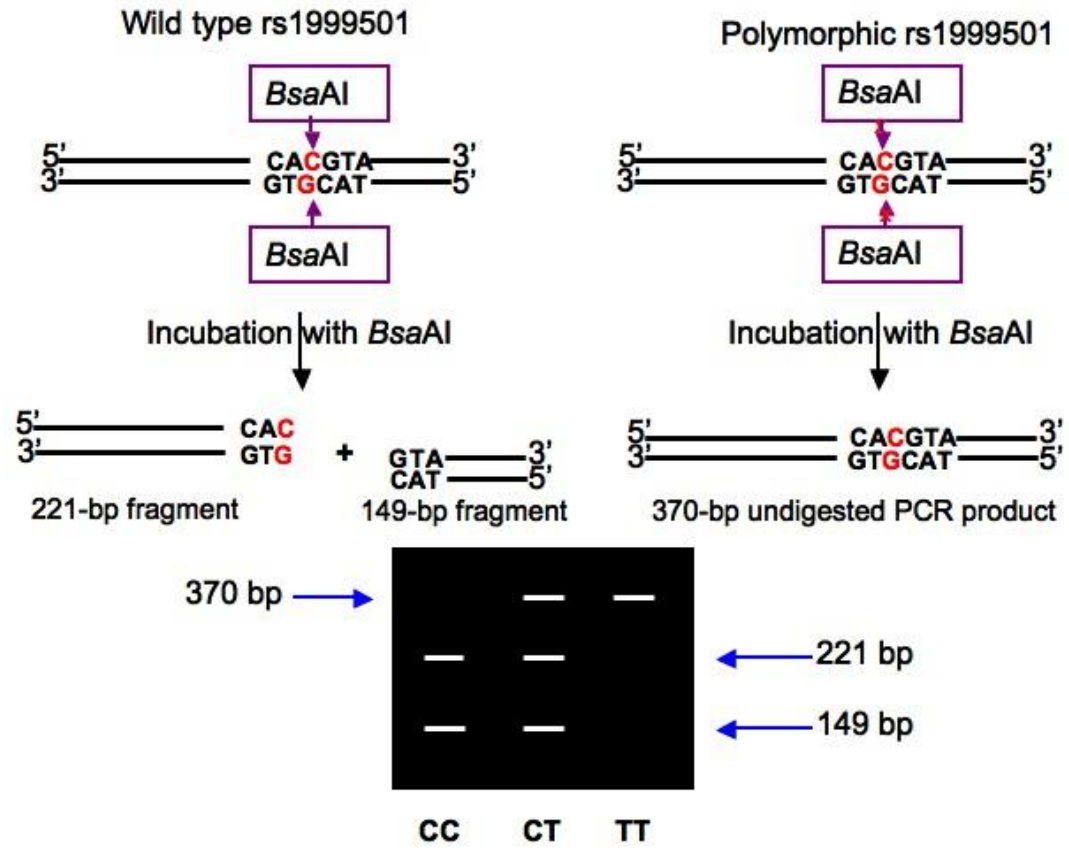

Figure 2. 6 Schematic representation of $GABA_{B2}$ rs1999501 genotype determination. Wild type C nucleotide shown in the left panel is replaced with T in the polymorphic allele which is shown in right panel. *Bsa*AI restriction enzyme recognizes (5' CACGTA 3') site within wild type allele and cuts 370-bp PCR product into two fragments of 221-bp and 149-bp. The polymorphic allele is not recognized by *Bsa*AI enzyme, thus undigested 370-bp fragment implies polymorphic allele. Three of these bands are seen in the heterozygote individuals.

Table 2. 11 Components of reaction mixture for restriction endonuclease (*Bsa*AI) digestion of PCR products for the determination of $GABA_{B2}$ rs1999501 SNP.

Component	**Concentration**	**Volume added**	**Final concentration in 30 μL reaction mixture**
Buffer B	10 X	2 μL	0.66 X
Sterile Apyrogen dH_2O	---	Up to 30 μL	---
*Bsa*AI	10 U/μL	0.4 μL	4 U
PCR product	---	10 μL	---

2.2.2.2.2 $GABA_{B2}$ rs967932 Single Nucleotide Polymorphism

2.2.2.2.2.1 Polymerase Chain Reaction for rs967932 of $GABA_{B2}$ gene

The region including the rs967932 polymorphism was amplified using primer pairs given by Wang *et al.* (2008) (Table 2.1). The sequence of the amplified region including rs967932 single nucleotide polymorphism is given in Figure 2.7.

Optimization of PCR procedure was done by several modifications on the concentrations of $MgCl_2$, primers and DNA template. Components of the optimized PCR mixture for the amplification of rs967932 SNP are given in Table 2.12. In a total of 50 μl PCR reaction, approximately 200 ng genomic DNA, 1.0 mM $MgCl_2$, 200 μM dNTPs, 1 pmol/μL of forward and reverse primers and 3 Unit *Taq* polymerase were included.

-71005171 ACAGACATAGAAAGAAA**AGAACGGGCTCCAACTAAAA**TAAAGGCAGAATA

-71005121 GGCTCTCTTCTCTCTTAAAATCTTTCTATCAGTGAATTTCAGGAACAAAG

-71005071 ACAGTGGAAAGAATGTCCACTATGCAACTGGAACCATGACACCTCTGTGT

-71005021 GCCTGTGTGGGCACGTGAGTATTGAGCATGGCAGAGCAGAAAAGCAGATT

-71004971 CCATGGAGGAAATGACTTTTGTCTAGAACCTGTGTAGCCACCCCTGTCGC

-71004921 TGCCCCATCCATCGCTCTTGGGCACTCTCTATGCCGTTGCATGCTGCCCT

-71004871 GGCAATTTCCCTCTGCTAGGATCTCACAGCAGTGGCTGGCAGGGT**TGGTG**

-71004821 **GGTGAATATC**TCAGCTCCCTCTCCCCTCCGAGTGCCCAGTGGGAACTGCA

Figure 2. 7 Sequence of amplified fragment of $GABA_{B2}$ gene including rs967932 single nucleotide polymorphism. The forward and reverse primers are written in bold and underlined. Location of recognition sequence for *Scr* FI restriction endonuclease is marked with yellow box. The polymorphic nucleotide is written in red (the nucleotide sequence is taken from http://www.ncbi.nlm.nih.gov).

Table 2. 12 Components of PCR mixture for $GABA_{B2}$ rs967932 SNP.

Component	**Stock concentration**	**Volume added**	**Final Concentration in 50 μL reaction mixture**
Sterile Apyrogen H_2O		Up to 50 μL	
Amplification Buffer	10X	5 μL	1X
$MgCl_2$	25 mM	2 μL	1 mM
dNTP mixture	10 mM	1 μL	200 μM
Forward Primer	10 pmol/μL	5 μL	1 pmol/μL
Reverse Primer	10 pmol/μL	5 μL	1 pmol/μL
Template DNA	varies	Varies	~200 ng
Taq DNA Polymerase	5 U/μL	0.6 μL	3 U

The program of the thermalcycler used for the amplification of rs967932 SNP region of $GABA_{B2}$ was taken from those published (Wang *et al*., 2008) and given in Table 2.13.

Table 2. 13 PCR program used for the amplification of rs967932 SNP region of $GABA_{B2}$ gene.

Initial denaturation	95°C	5 min.	
Denaturation	95°C	30 sec.	30 cycles
Annealing	58.5°C	30 sec.	
Extension	72°C	30 sec.	
Final extension	72°C	10 min.	

PCR products were analyzed on 2.0% agarose gel, which was prepared as described in section 2.2.1.4. Ten μL of PCR product was mixed with 3 μL of gel loading buffer and then 10 μL from this mixture and 6 μL of DNA ladder (50-1000 bp) were loaded to the gel. The agarose gel run was performed at 100V, for 1 hour.

2.2.2.2.2.2 Restriction Endonuclease Digestion of PCR Products for Determination of $GABA_{B2}$ rs967932 SNP

Schematic representation of the digestion procedure for the determination of rs967932 genotypes is given in Figure 2.8. Wild type allele including G nucleotide can be recognized by *Scr*FI restriction enzyme, while the polymorphic allele including A nucleotide cannot be recognized by *Scr*FI restriction enzyme. Recognition site of the *Scr*FI restriction enzyme is as given below;

5'...C C^N G G...3'
3'...G G N^C C...5'

*Scr*FI cuts 348-bp PCR product into two fragments of 282-bp and 66-bp. Undigested 348-bp fragment indicates the polymorphic allele. Heterozygotes contain both wild type and polymorphic alleles, and indicated with three bands (Figure 2.8).

For the determination of rs967932 genotypes, 10 μl of 348-bp PCR product was incubated with 10U *Scr*FI at 37°C for 16 hours, in a restriction mixture as given in Table 2.14. The digestion products were analyzed on 3% agarose gel.

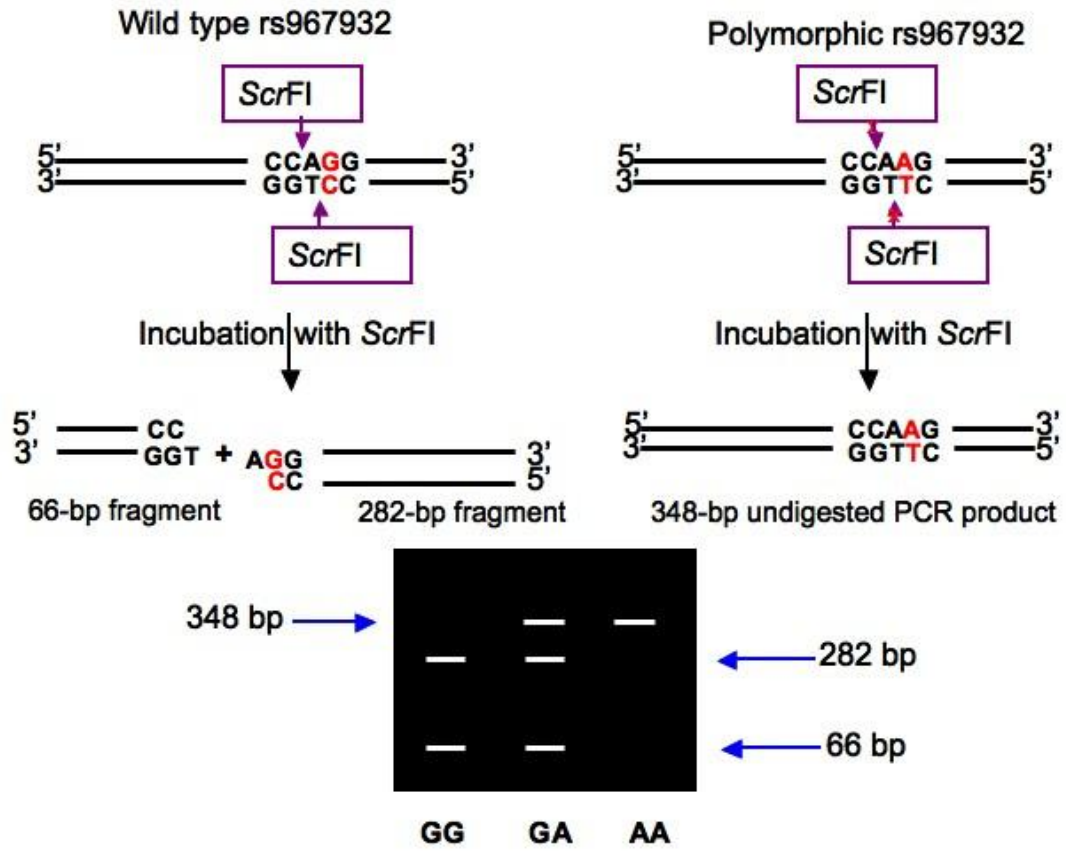

Figure 2. 8 Schematic representation of rs967932 genotype determination. Wild type G nucleotide shown in the left panel is replaced with A in the polymorphic allele, which is shown in right panel. *Scr*FI restriction enzyme recognizes (5' CCTGG 3') site within wild type allele and cuts 348-bp PCR product into two fragments of 282-bp and 66-bp. The polymorphic allele is not recognized by *Scr*FI enzyme, thus undigested 348-bp fragment implies polymorphic allele. Three of these bands are seen in the heterozygote individuals.

Table 2. 14 Components of reaction mixture for restriction endonuclease (*Scr*FI) digestion of PCR products for the determination of $GABA_{B2}$ rs967932 SNP.

Component	**Concentration**	**Volume added**	**Final concentration in 30 μL reaction mixture**
Buffer Tango	10 X	2 μL	0.66 X
Sterile Apyrogen dH_2O	---	Up to 30 μL	---
*Scr*FI	10 U/μL	1 μL	10 U
PCR product	---	10 μL	---

2.2.2.2.3 $GABA_{B2}$ rs3780428 Single Nucleotide Polymorphism

2.2.2.2.3.1 Polymerase Chain Reaction for rs3780428 of $GABA_{B2}$ gene

The region including the rs3780428 polymorphism was amplified using primer pairs given by Wang *et al.* (2008) (Table 2.1). The sequence of the amplified region including rs3780428 single nucleotide polymorphism is given in Figure 2.9.

Optimization of PCR procedure was done by several modifications on the concentrations of $MgCl_2$, primers and DNA template. Components of the optimized PCR mixture for the amplification of rs3780428 SNP are given in Table 2.15. In a total of 50 μl PCR reaction, approximately 200 ng genomic DNA, 0.75 mM $MgCl_2$, 200 μM dNTPs, 0.6 pmol/μL of forward and reverse primers and 3 Unit *Taq* polymerase were included.

-70729181 AGACAATAACATGAGTGGGAAAAGCACCTGAGGATAAACCCTC**AGACTTT**

-70729131 **CATCTGGGCTCAA**CTTAAGGAAAATGCTGAGGGCTTTTAGCAGCTGTCAT

-70729081 GAAGCATCTGCCATCTAAAGACGACAGGAGAGCTCAGCCCCAGGGGCACC

-70729031 ATCCATGCTGTATTCCTCCTGAATGGTGTTCCCTGAAGAAATGAGTTGGC

-70728981 CCTAGTGGAAGGCCAGTCTGAATAGGTCAGAAAATCTCAAGTTTTTTGAA

-70728931 AAAATGAAGAAATGCCCTTTTCTCTTA**ACCAAGCATCAGCCAAGTTT**TTC

Figure 2. 9 Sequence of amplified fragment of $GABA_{B2}$ gene that includes rs3780428 single nucleotide polymorphism. The forward and reverse primers are written in bold and underlined. Location of recognition sequence for *Sau* 96I restriction endonuclease is marked with yellow box. The polymorphic nucleotide is written in red (the nucleotide sequence is taken from http://www.ncbi.nlm.nih.gov).

Table 2. 15 Components of PCR mixture for $GABA_{B2}$ rs3780428 SNP.

Component	**Stock concentration**	**Volume added**	**Final Concentration in 50 μL reaction mixture**
Sterile Apyrogen H_2O		Up to 50 μL	
Amplification Buffer	10X	5 μL	1X
$MgCl_2$	25 mM	1.5 μL	0.75 mM
dNTP mixture	10 mM	1 μL	200 μM
Forward Primer	10 pmol/μL	3 μL	0.6 pmol/μL
Reverse Primer	10 pmol/μL	3 μL	0.6 pmol/μL
Template DNA	varies	Varies	~200 ng
Taq DNA Polymerase	5 U/μL	0.6 μL	3 U

The program of the thermalcycler used for the amplification of rs3780428 SNP region of $GABA_{B2}$ was taken from those published (Wang *et al*., 2008) and given in Table 2.16.

Table 2. 16 PCR program used for the amplification of rs3780428 SNP region of $GABA_{B2}$ gene.

Initial denaturation	95°C	5 min.	
Denaturation	95°C	30 sec.	30 cycles
Annealing	55.5°C	30 sec.	
Extension	72°C	30 sec.	
Final extension	72°C	10 min.	

PCR products were analyzed on 2.0% agarose gel, which was prepared as described in section 2.2.1.4. Ten μL of PCR product was mixed with 3 μL of gel loading buffer and then 10 μL from this mixture and 6 μL of DNA ladder (50-1000 bp) were loaded to the gel. The agarose gel run was performed at 100V, for 1 hour.

2.2.2.2.3.2 Restriction Endonuclease Digestion of PCR Products for Determination of $GABA_{B2}$ rs3780428 SNP

Schematic representation of the digestion procedure for the determination of rs3780428 genotypes is given in Figure 2.10. Wild type allele including G nucleotide, can be recognized by *Sau*96I restriction enzyme, while the polymorphic allele including A nucleotide cannot be recognized by *Sau*96I restriction enzyme. Recognition site of the *Sau*96I restriction enzyme is as given below;

5'...G^G N C C...3'
3'...C C N G^G...5'

*Sau*96I cuts 255-bp PCR product into two fragments of 156-bp and 99-bp. Undigested 255-bp fragment indicates the polymorphic allele. Heterozygotes contain both wild type and polymorphic alleles, and indicated with three bands (Figure 2.10).

For the determination of rs3780428 genotypes, 10 μl of 255-bp PCR product was incubated with 3U *Sau*96I at 37°C for 16 hours, in a restriction mixture as given in Table 2.17. The digestion products were analyzed on 3% agarose gel.

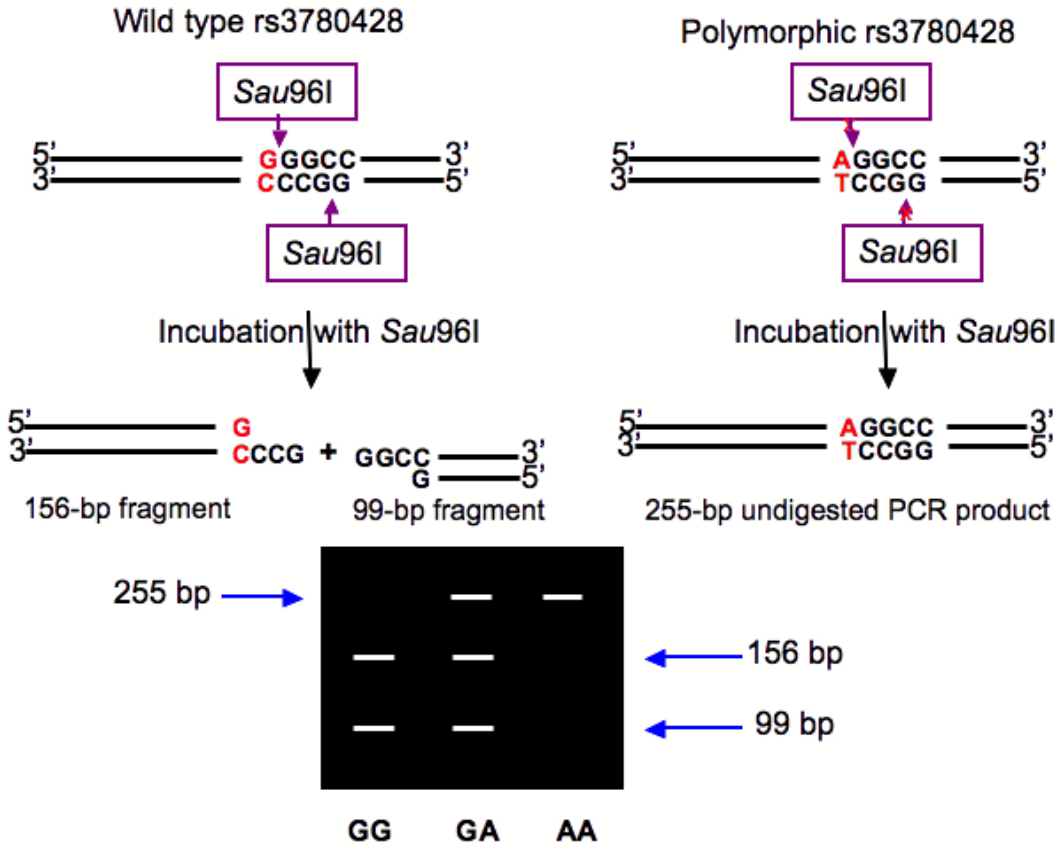

Figure 2. 10 Schematic representation of rs3780428 genotype determination. Wild type G nucleotide shown in the left panel is replaced with A in the polymorphic allele which is shown in right panel. *Sau*96I restriction enzyme recognizes (5' CCCGG 3') site within wild type allele and cuts 255-bp PCR product into two fragments of 156-bp and 99-bp. The polymorphic allele is not recognized by *Sau*96I enzyme, thus undigested 255-bp fragment implies polymorphic allele. Three of these bands are seen in the heterozygote individuals.

Table 2. 17 Components of reaction mixture for restriction endonuclease (*Sau*96I) digestion of PCR products for the determination of $GABA_{B2}$ rs3780428 SNP.

Component	Concentration	Volume added	Final concentration in 30 µL reaction mixture
Buffer Tango	10 X	2 µL	0.66 X
Sterile Apyrogen dH_2O	---	Up to 30 µL	---
*Sau*96I	10 U/µL	0.3 µL	3 U
PCR product	---	10 µL	---

2.2.2.2.4 $GABA_{B2}$ rs944688 Single Nucleotide Polymorphism

2.2.2.2.4.1 Polymerase Chain Reaction for rs944688 of $GABA_{B2}$ gene

The region including the rs944688 polymorphism was amplified using primer pairs given by Wang *et al.* (2008) (Table 2.1). The sequence of the amplified region including rs944688 single nucleotide polymorphism is given in Figure 2.11. With a nucleotide change inside the reverse primer, recognition site is created for *Hpa*I restriction enzyme.

Primer sequence: AACTCTGTTAAAAACTTACCCT
DNA sequence: AGCTCTGTTAAAAACTTACCCT

Optimization of PCR procedure was done by several modifications on the concentrations of $MgCl_2$, primers and DNA template. Components of the optimized PCR mixture for the amplification of rs944688 SNP are given in Table 2.18. In a total of 50 µl PCR reaction, approximately 200 ng genomic DNA, 1.5 mM $MgCl_2$, 200 µM dNTPs, 1 pmol/µL of forward and reverse primers and 3 Unit *Taq* polymerase were included.

30528291 CTGCACTCCAACCTGGAGGATGAAGCGAGACCCTGTCTCTTAAAAAAAGA

30528341 AAATAATAAATGAAATAAATAAATCAATAAACAAAT**CCTAATGTCTGCTA**

30528391 **TCACCA**CAATTTAATTTTTTAAAGGACATAAGAAGGAAGGAGGAGGAGGG

30528441 AAGGGAAGGCACCATCTCAAAGTGAAGGGTGTTCTTTAAAACAACAAAGT

30528491 C**AG*CTCTGTTAAAAACTTACCCT**TATTTTCCTCAGGGCACCATGGTCTCT

Figure 2. 11 Sequence of amplified fragment of $GABA_{B2}$ gene that includes rs944688 single nucleotide polymorphism. The forward and reverse primers are written in bold and underlined. Location of recognition sequence for *Hpa* I restriction endonuclease is marked with yellow box. Guanine* nucleotide is changed into adenine in the primer sequence in order to be recognized by the restriction enzyme. The polymorphic nucleotide is written in red (the nucleotide sequence is taken from http://www.ncbi.nlm.nih.gov).

Table 2. 18 Components of PCR mixture for $GABA_{B2}$ rs944688 SNP.

Component	**Stock concentration**	**Volume added**	**Final Concentration in 50 μL reaction mixture**
Sterile Apyrogen H_2O		Up to 50 μL	
Amplification Buffer	10X	5 μL	1X
$MgCl_2$	25 mM	3 μL	1.5 mM
dNTP mixture	10 mM	1 μL	200 μM
Forward Primer	10 pmol/μL	5 μL	1 pmol/μL
Reverse Primer	10 pmol/μL	5 μL	1 pmol/μL
Template DNA	varies	Varies	~200 ng
Taq DNA Polymerase	5 U/μL	0.6 μL	3 U

The program of the thermalcycler used for the amplification of rs944688 SNP region of $GABA_{B2}$ was taken from those published (Wang *et al*., 2008) and given in Table 2.19.

Table 2. 19 PCR program used for the amplification of rs944688 SNP region of $GABA_{B2}$ gene.

Initial denaturation	95°C	5 min.	
Denaturation	95°C	30 sec.	30 cycles
Annealing	51°C	30 sec.	
Extension	72°C	30 sec.	
Final extension	72°C	10 min.	

PCR products were analyzed on 2.0% agarose gel, which was prepared as described in section 2.2.1.4. Ten μL of PCR product was mixed with 3 μL of gel loading buffer and then 10 μL from this mixture and 6 μL of DNA ladder (50-1000 bp) were loaded to the gel. The agarose gel run was performed at 100V, for 1 hour.

2.2.2.2.4.2 Restriction Endonuclease Digestion of PCR Products for Determination of $GABA_{B2}$ rs944688 SNP

Schematic representation of the digestion procedure for the determination of rs944688 genotypes is given in Figure 2.12. Polymorphic allele including T nucleotide, can be recognized by *Hpa*I restriction enzyme, while the wild type including C nucleotide cannot be recognized by *Hpa*I restriction enzyme. Recognition site of the *Hpa*I restriction enzyme is as given below;

5'...G T T^A A C...3'
3'...C A A^T T G...5'

*Hpa*I cuts 137-bp PCR product into two fragments of 115-bp and 22-bp. Undigested 137-bp fragment indicates the wild type allele. Heterozygotes contain both wild type and polymorphic alleles, and indicated with three bands (Figure 2.12).

For the determination of rs944688 genotypes, 10 μl of 137-bp PCR product was incubated with 3U *Hpa*I at 37°C for 16 hours, in a restriction mixture as given in Table 2.20. The digestion products were analyzed on 3% Nu- micropore agarose gel.

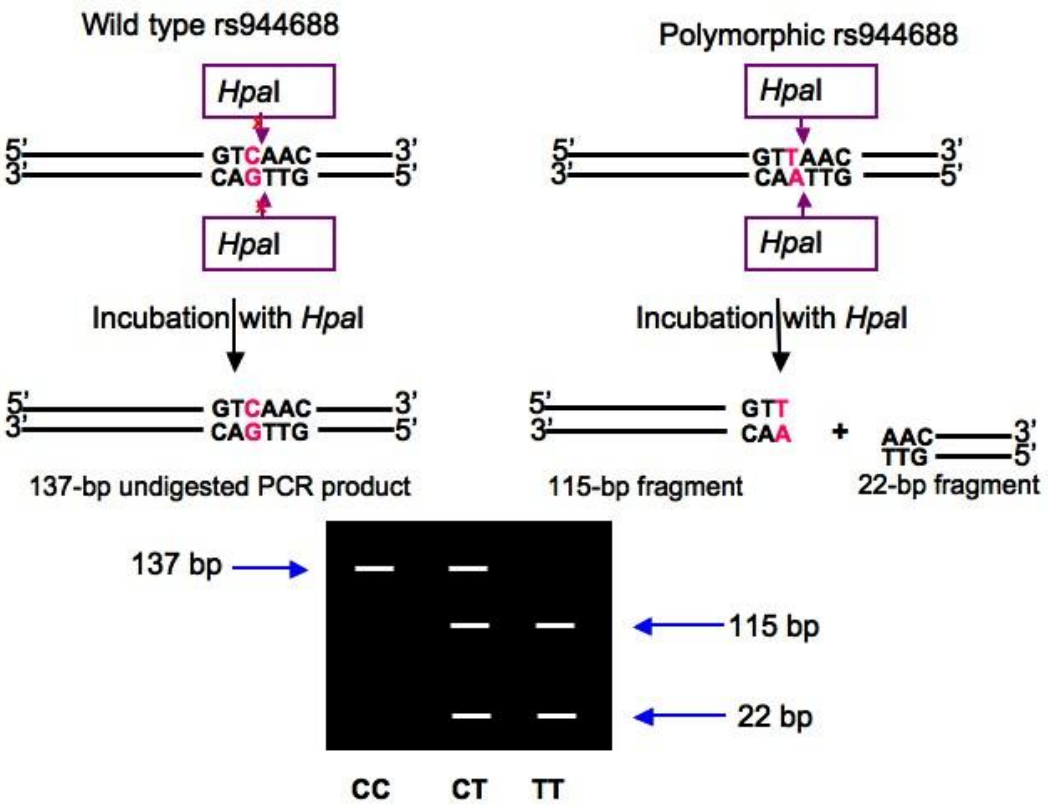

Figure 2. 12 Schematic representation of rs944688 genotype determination. Wild type C nucleotide shown in the left panel is replaced with T in the polymorphic allele which is shown in right panel. *Hpa*I restriction enzyme recognizes (5' GTTAAC 3') site within polymorphic allele and cuts 137-bp PCR product into two fragments of 115-bp and 22-bp. The wild type is not recognized by *Hpa*I enzyme, thus undigested 137-bp fragment implies wild type allele. Three of these bands are seen in the heterozygote individuals.

Table 2. 20 Components of reaction mixture for restriction endonuclease (*Hpa*I) digestion of PCR products for the determination of $GABA_{B2}$ rs944688 SNP.

Component	**Concentration**	**Volume added**	**Final concentration in 30 μL reaction mixture**
Buffer Tango	10 X	2 μL	0.66 X
Sterile Apyrogen dH_2O	---	Up to 30 μL	---
*Hpa*I	10 U/μL	0.3 μL	3 U
PCR product	---	10 μL	---

2.2.3 Statistical Analyses

PASW Statistics 20 software package (SPSS, Chicago, IL, USA) was used for the statistical analyses. Gene counting and combined genotype analyses were performed by using PASW software.

Allele and genotype frequencies were determined by the gene counting method and their departure from the Hardy-Weinberg equilibrium was evaluated by the χ^2 test. Comparisons of genotype distribution and allele frequencies were assessed by χ^2 statistics with 2 and 1 df, respectively. *P* value less than 0.05 was evaluated as statistically significant.

CHAPTER 3

RESULTS

3.1 Study Participants

The study population was composed of 176 idiopathic generalized epilepsy (IGE) patients, 83 subjects having psychogenic non-epileptic seizure (PNES) and 86 non-epileptic control individuals. Total blood samples were obtained from Gülhane Military Medical Academy Hospital Neurology Department, Ankara. All the individuals were male and they came from different parts of Turkey. There was no statistically significant difference between the patient and control groups in terms of age.

3.2 Genotyping of Single Nucleotide Polymorphisms

Genotyping of single nucleotide polymorphisms was performed by restriction fragment length polymorphism technique. The region of interest was amplified with polymerase chain reaction, and followed by restriction with the specific enzyme that includes the SNP in its recognition site.

3.2.1 Genotyping of $GABA_{B1}$ gene SNPs

3.2.1.1 Genotyping for G1465A Single Nucleotide Polymorphism of $GABA_{B1}$

G1465A is located in exon 7 of $GABA_{B1}$ gene in human genome. The region including G1465A polymorphism in $GABA_{B1}$ gene was amplified as described in section 2.2.2.1.1.1 Ten μL of PCR product was mixed with 3 μL of gel loading buffer and 10 μL from this mixture and 6 μL of DNA ladder (50-1000 bp) were loaded to the gel. The agarose gel run was performed at 100V, for 1 hour. Figure 3.1 shows the agarose gel electrophoresis of PCR products of G1465A, with expected 441-bp band size.

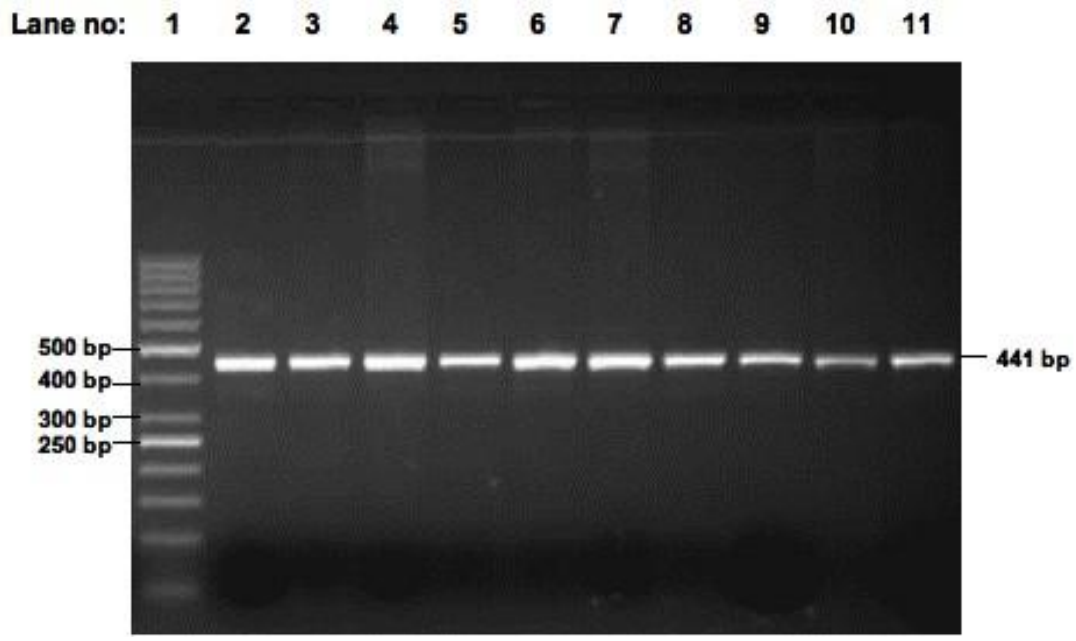

Figure 3. 1 2% Agarose gel electrophoresis of PCR products for G1465A SNP region of $GABA_{B1}$. First lane was loaded with DNA ladder (50-1000 bp), following lanes were loaded with PCR products of G1465A. Expected size of G1465A PCR product was 441-bp.

PCR products were digested with *Eag* I enzyme for the determination of G1465A genotypes. Digestion procedure was performed as described in section 2.2.2.1.1.2 Wild type allele including G nucleotide, can be recognized by *Eag* I restriction enzyme, while the polymorphic allele including A nucleotide cannot be recognized by *Eag* I restriction enzyme. Figure 3.2 shows the agarose gel electrophoresis of digestion products, with 258-bp and 183-bp bands indicating the wild type allele.

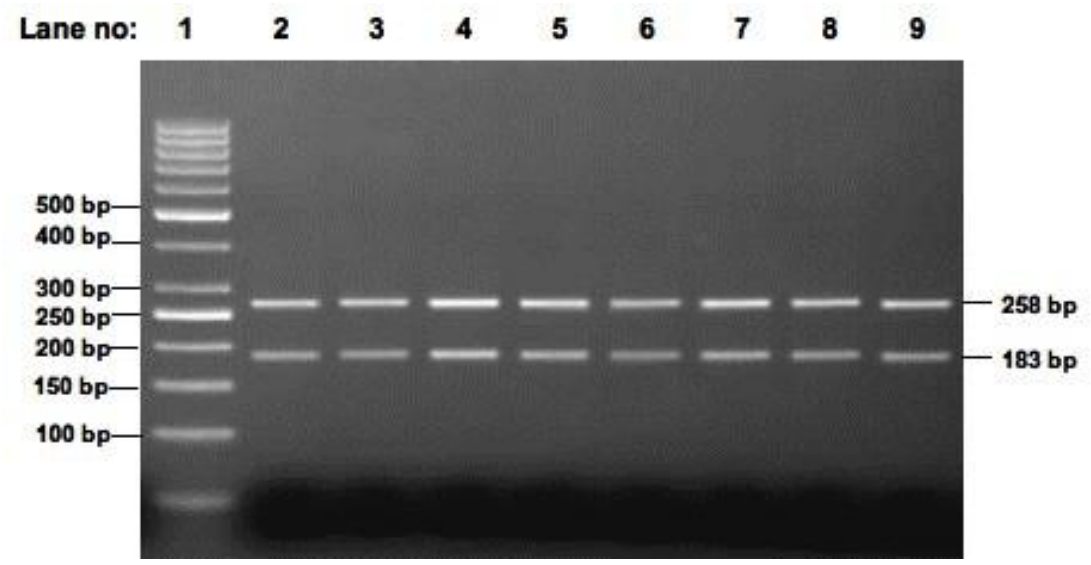

Figure 3. 2 3% Agarose gel electrophoresis of *Eag* I digestion products for G1465A SNP region of $GABA_{B1}$. First lane was loaded with DNA ladder (50-1000 bp), following lanes were loaded with digestion products of the subjects. All the individuals were heterozygous (GA) with 258-bp and 183-bp bands, for this SNP.

3.2.1.2 Genotyping for C59T Single Nucleotide Polymorphism of $GABA_{B1}$

C59T is located in exon 1a1 of $GABA_{B1}$ gene in human genome. The region including C59T polymorphism in $GABA_{B1}$ gene was amplified as described in section 2.2.2.1.2.1 Ten μL of PCR product was mixed with 3 μL of gel loading buffer and 10 μL from this mixture and 6 μL of DNA ladder (50-1000 bp) were loaded to the gel. The agarose gel run was performed at 100V, for 1 hour. Figure 3.3 shows the agarose gel electrophoresis of PCR products of C59T, with expected 212-bp band size.

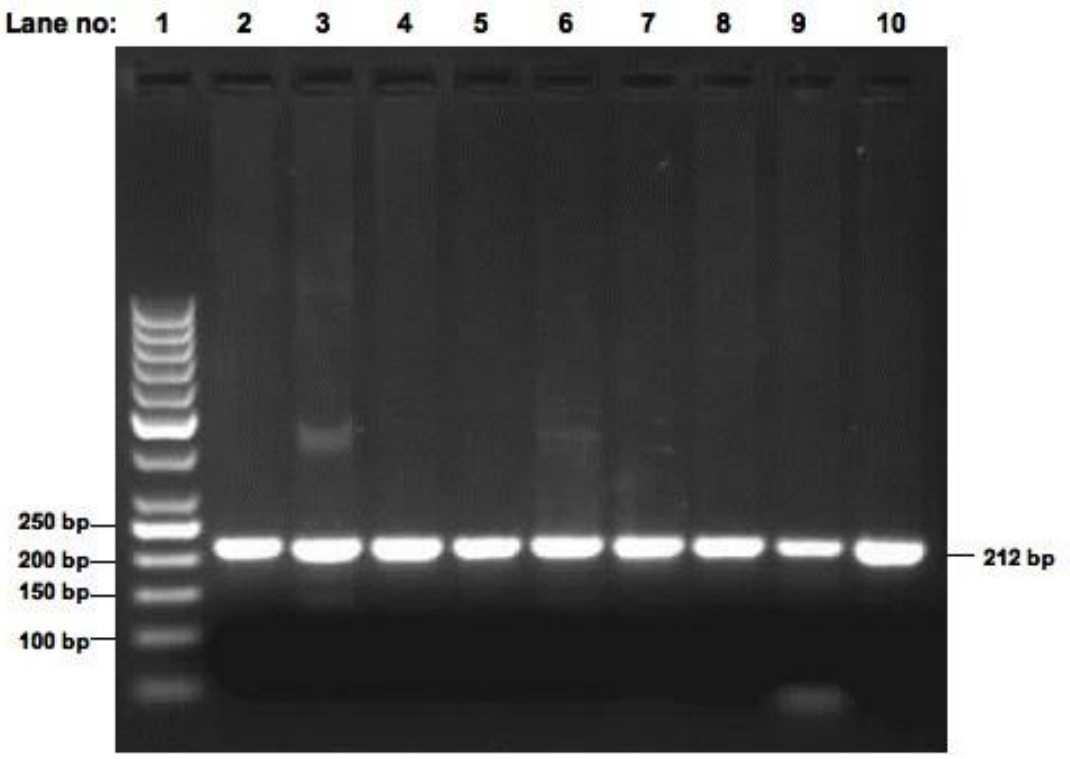

Figure 3. 3 2% Agarose gel electrophoresis of PCR products for C59T SNP region of $GABA_{B1}$. First lane was loaded with DNA ladder (50-1000 bp), following lanes were loaded with PCR products of C59T. Expected size of C59T PCR product was 212-bp.

PCR products were digested with *Hin* 6I enzyme for the determination of C59T genotypes. Digestion procedure was performed as described in section 2.2.2.1.2.2

Hin 6I restriction enzyme recognizes four (3' CGCG 5') sites within wild type allele and cuts 212-bp PCR product into five fragments of 97-bp, 67-bp, 25-bp, 12-bp and 11-bp in wild type individuals. The polymorphic allele is recognized by *Hin* 6I enzyme from three sites and cuts 212-bp PCR product into four fragments of 108-bp, 67-bp, 25-bp, 12-bp implies polymorphic allele. Figure 3.4 shows the Nu-micropore agarose gel electrophoresis of digestion products of C59T SNP.

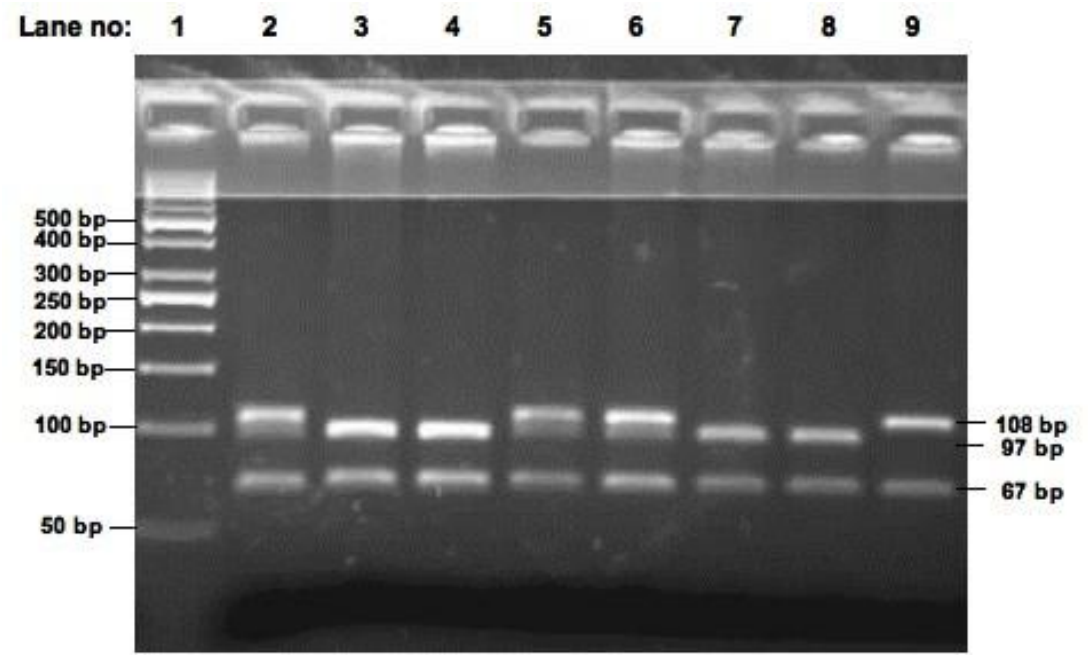

Figure 3. 4 3% Nu-micropore agarose gel electrophoresis of *Hin* 6I digestion products for C59T SNP region of $GABA_{B1}$. First lane was loaded with DNA ladder (50-1000 bp), following lanes were loaded with digestion products of the subjects. Lane 2, 5 and 6 shows heterozygous genotype (CT) with 108-bp, 97-bp and 67-bp bands. Lane 3, 4, 7 and 8 show wild type genotype (CC) with 97-bp and 67-bp bands. Lane 9 shows polymorphic genotype (TT) with 108-bp and 67-bp bands.

3.2.2 Genotyping of $GABA_{B2}$ gene SNPs

3.2.2.1 Genotyping for rs1999501 Single Nucleotide Polymorphism of $GABA_{B2}$

rs1999501 is located in intronic region of $GABA_{B2}$ gene, which is on chromosome 5 in human genome. The region including rs1999501 polymorphism in $GABA_{B2}$ gene was amplified as described in section 2.2.2.2.1.1 Ten μL of PCR product was mixed with 3 μL of gel loading buffer and 10 μL from this mixture and 6 μL of DNA ladder (50-1000 bp) were loaded to the gel. The agarose gel run was performed at 100V, for 1 hour. Figure 3.5 shows the agarose gel electrophoresis of PCR products of rs1999501, with expected 370-bp band size.

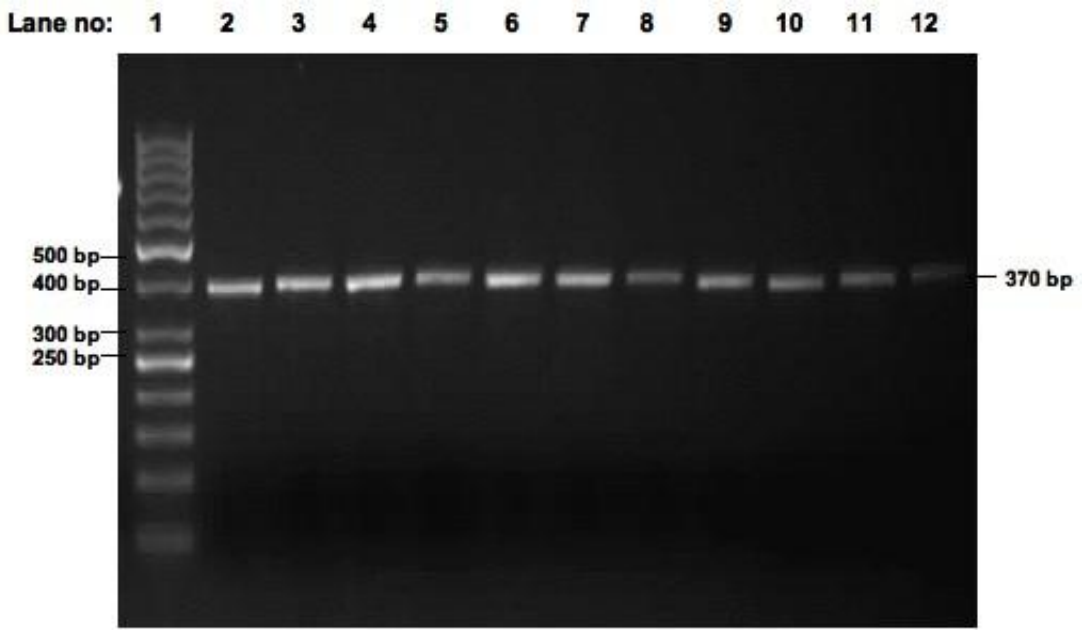

Figure 3. 5 2% Agarose gel electrophoresis of PCR products for rs1999501 SNP region of $GABA_{B2}$. First lane was loaded with DNA ladder (50-1000 bp), following lanes were loaded with PCR products of rs1999501. Expected size of rs1999501 PCR product is 370-bp.

PCR products were digested with *Bsa* I enzyme for the determination of rs1999501 genotypes. Digestion procedure was performed as described in section 2.2.2.2.1.2 Wild type allele including C nucleotide, can be recognized by *Bsa* I restriction enzyme, while the polymorphic allele including T nucleotide cannot be recognized by *Bsa* I restriction enzyme. Figure 3.6 shows the agarose gel electrophoresis of digestion products with undigested 370-bp fragment indicating the polymorphic allele, while 221-bp and 149-bp bands indicating the wild type allele.

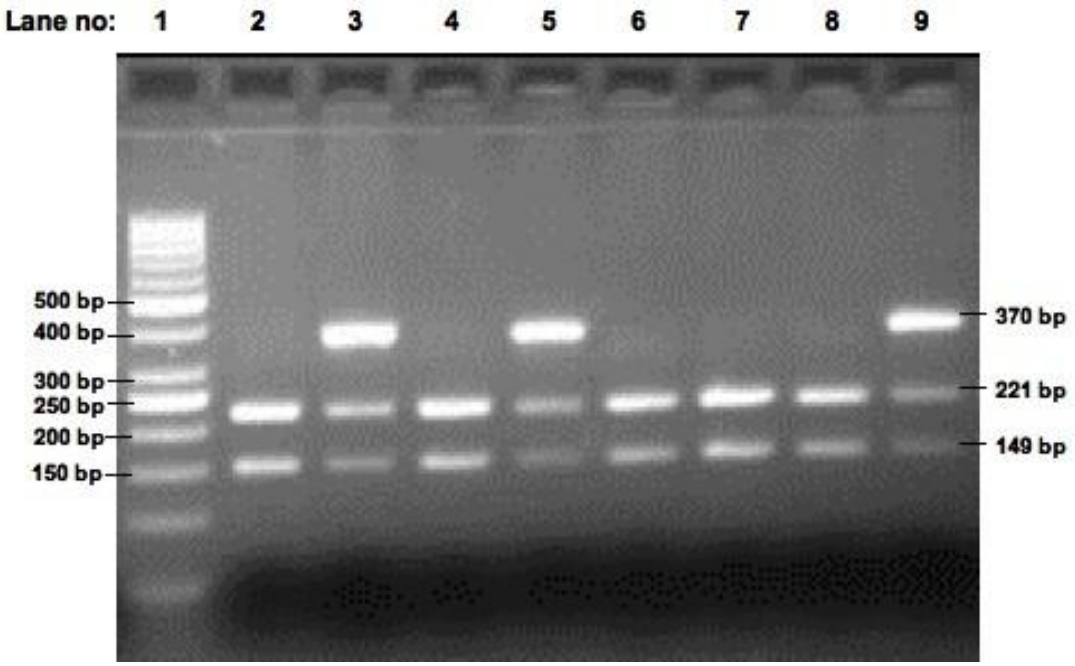

Figure 3. 6 3% Agarose gel electrophoresis of *Bsa* I digestion products for rs1999501 SNP region of $GABA_{B2}$. First lane was loaded with DNA ladder (50-1000 bp), following lanes were loaded with digestion products of the subjects. Lane 2, 4, 6, 7 and 8 show wild type genotype (CC) with 221-bp and 149-bp. Lane 3, 5 and 9 show heterozygous genotype (CT) with 370-bp, 221-bp and 149-bp.

3.2.2.2 Genotyping for rs967932 Single Nucleotide Polymorphism of $GABA_{B2}$

rs967932 is located in intronic region of $GABA_{B2}$ gene, which is on chromosome 5 in human genome. The region including rs967932 polymorphism in $GABA_{B2}$ gene was amplified as described in section 2.2.2.2.2.1 Ten μL of PCR product was mixed with 3 μL of gel loading buffer and 10 μL from this mixture and 6 μL of DNA ladder (50-1000 bp) were loaded to the gel. The agarose gel run was performed at 100V, for 1 hour. Figure 3.7 shows the agarose gel electrophoresis of PCR products of rs967932, with expected 348-bp band size.

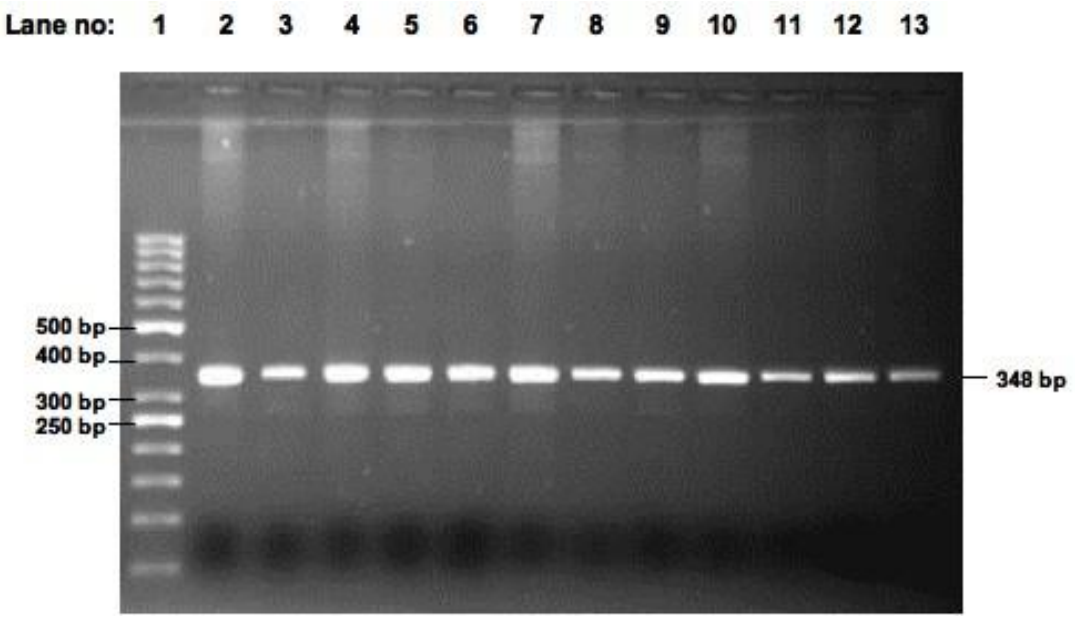

Figure 3. 7 2% Agarose gel electrophoresis of PCR products for rs967932 SNP region of $GABA_{B2}$. First lane was loaded with DNA ladder (50-1000 bp), following lanes were loaded with PCR products of rs967932. Expected size of rs967932 PCR product is 348-bp.

PCR products were digested with *Scr* FI enzyme for the determination of rs967932 genotypes. Digestion procedure was performed as described in section 2.2.2.2.2.2 Wild type allele including G nucleotide, can be recognized by *Scr* FI restriction enzyme, while the polymorphic allele including A nucleotide cannot be recognized by *Scr* FI restriction enzyme. Figure 3.8 shows the agarose gel electrophoresis of digestion products with undigested 348-bp fragment indicating the polymorphic allele, while 282-bp and 66-bp bands indicating the wild type allele.

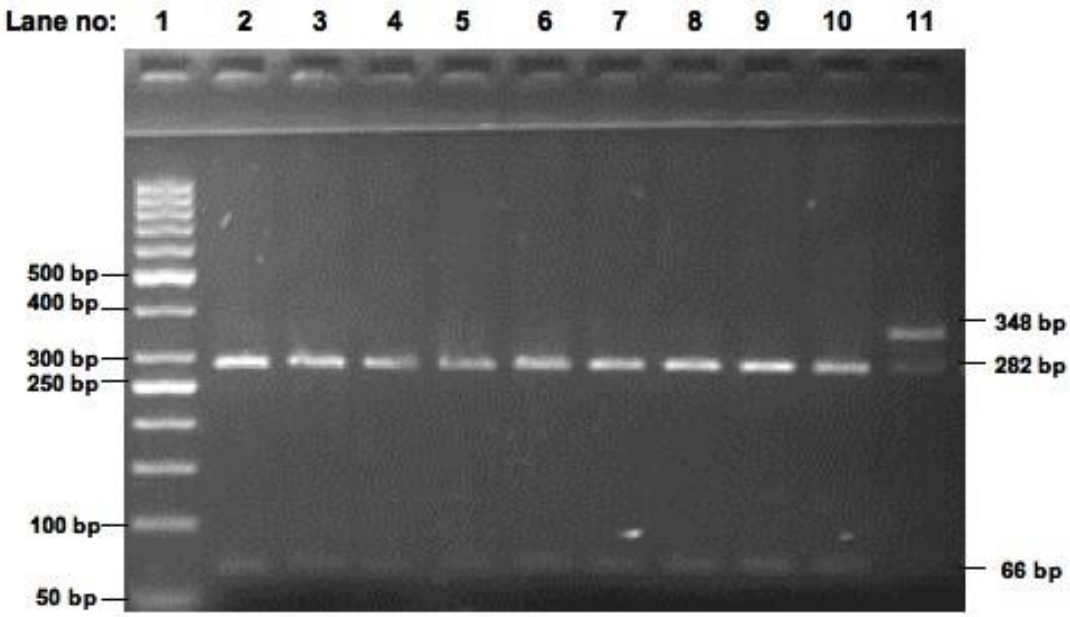

Figure 3. 8 3% Agarose gel electrophoresis of *Scr* FI digestion products for rs967932 SNP region of $GABA_{B2}$. First lane was loaded with DNA ladder (50-1000 bp), following lanes were loaded with digestion products of the subjects. Lane 11 shows heterozygous genotype (GA) with 348-bp, 282-bp and 66-bp bands. Lane 2-10 show wild type genotype (GG) with 282-bp and 66-bp bands.

3.2.2.3 Genotyping for rs3780428 Single Nucleotide Polymorphism of $GABA_{B2}$

rs3780428 is located in intronic region of $GABA_{B2}$ gene, which is on chromosome 5 in human genome. The region including rs3780428 polymorphism in $GABA_{B2}$ gene was amplified as described in section 2.2.2.2.3.1 Ten μL of PCR product was mixed with 3 μL of gel loading buffer and 10 μL from this mixture and 6 μL of DNA ladder (50-1000 bp) were loaded to the gel. The agarose gel run was performed at 100V, for 1 hour. Figure 3.9 shows the agarose gel electrophoresis of PCR products of rs3780428, with expected 255-bp band size.

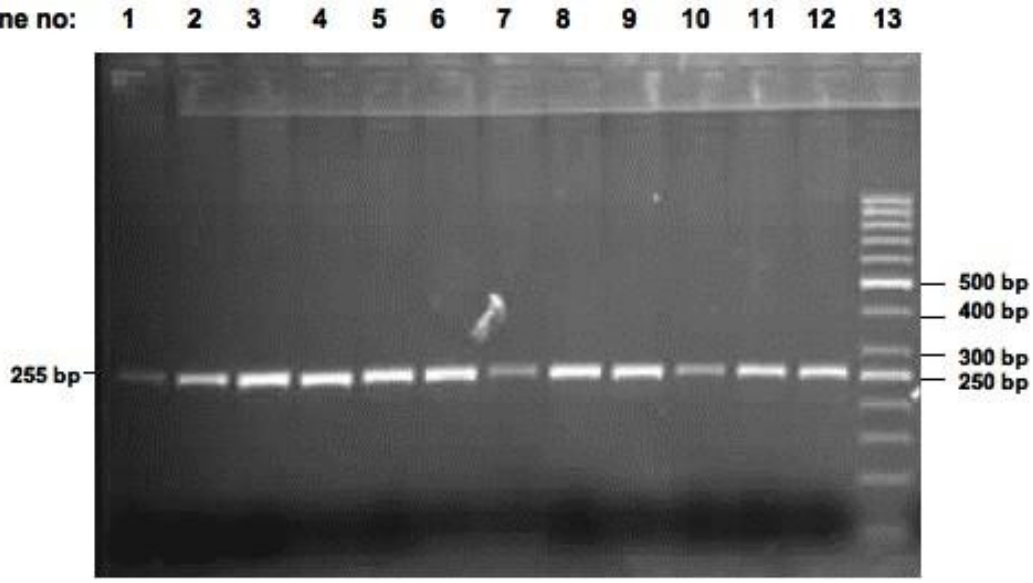

Figure 3. 9 2% Agarose gel electrophoresis of PCR products for rs3780428 SNP region of $GABA_{B2}$. Last lane was loaded with DNA ladder (50-1000 bp), first twelve lanes were loaded with PCR products of rs3780428. Expected size of rs3780428 PCR product was 255-bp.

PCR products were digested with *Sau* 96I enzyme for the determination of rs3780428 genotypes. Digestion procedure was performed as described in section 2.2.2.2.3.2 Wild type allele including G nucleotide, can be recognized by *Sau* 96I restriction enzyme, while the polymorphic allele including A nucleotide cannot be recognized by *Sau* 96I restriction enzyme. Figure 3.10 shows the agarose gel electrophoresis of digestion products with undigested 255-bp fragment indicating the polymorphic allele, while 156-bp and 99-bp bands indicating the wild type allele.

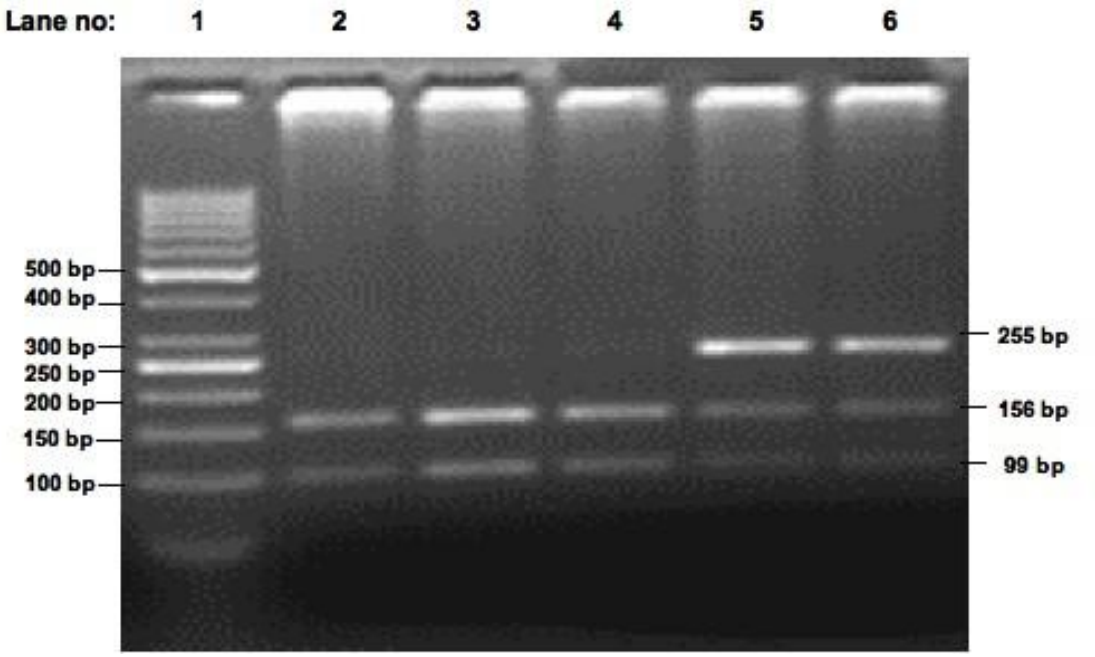

Figure 3. 10 3% Agarose gel electrophoresis of *Sau* 96I digestion products for rs3780428 SNP region of $GABA_{B2}$. First lane was loaded with DNA ladder (50-1000 bp), following lanes were loaded with digestion products of the subjects. Lane 2, 3 and 4 show wild type (GG) with 156-bp and 99-bp. Lane 5 and 6 show heterozygous genotype (GA) with 255-bp, 156-bp and 99-bp.

3.2.2.4 Genotyping for rs944688 Single Nucleotide Polymorphism of $GABA_{B2}$

rs944688 is located in intronic region of $GABA_{B2}$ gene, which is on chromosome 5 in human genome. The region including rs944688 polymorphism in $GABA_{B2}$ gene was amplified as described in section 2.2.2.2.4.1 Ten μL of PCR product was mixed with 3 μL of gel loading buffer and 10 μL from this mixture and 6 μL of DNA ladder (50-1000 bp) were loaded to the gel. The agarose gel run was performed at 100V, for 1 hour. Figure 3.11 shows the agarose gel electrophoresis of PCR products of rs944688, with expected 137-bp band size.

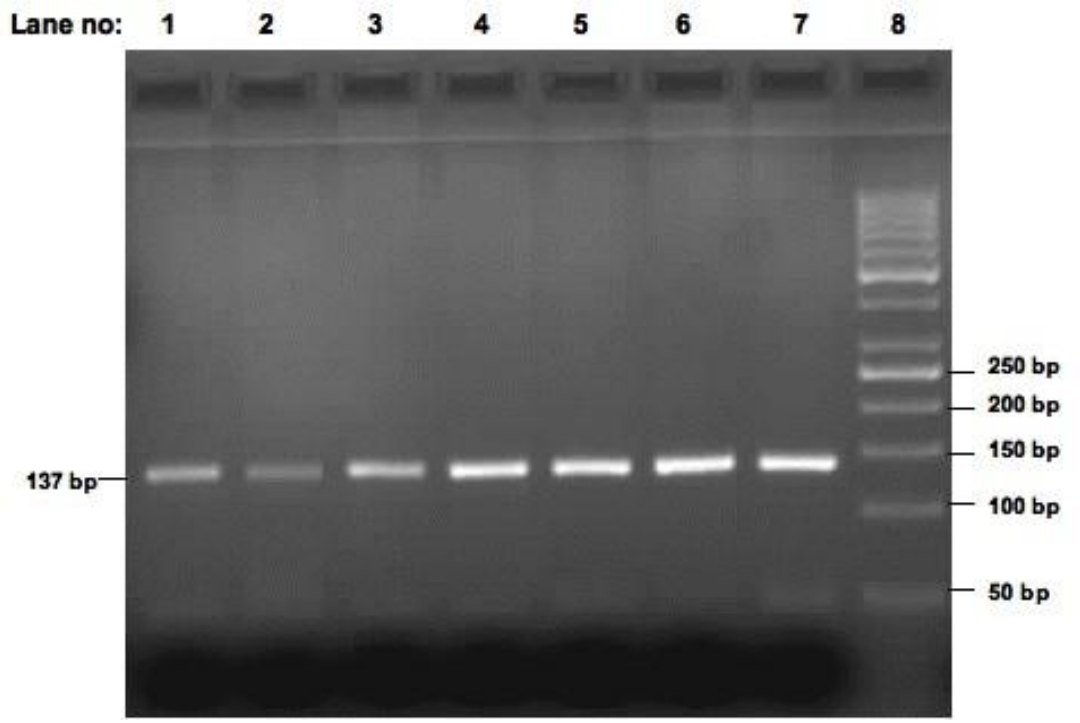

Figure 3. 11 2% Agarose gel electrophoresis of PCR products for rs944688 SNP region of $GABA_{B2}$. Last lane was loaded with DNA ladder (50-1000 bp), first seven lanes were loaded with PCR products of rs944688. Expected size of rs944688 PCR product was 137-bp.

PCR products were digested with *Hpa* I enzyme for the determination of rs944688 genotypes. Digestion procedure was performed as described in section 2.2.2.2.4.2 Wild type allele including C nucleotide, cannot be recognized by *Hpa* I restriction enzyme, while the polymorphic allele including T nucleotide can be recognized by *Hpa* I restriction enzyme. Figure 3.12 shows the Nu-micropore agarose gel electrophoresis of digestion products with undigested 137-bp fragment indicating the wild type allele, while 115-bp and 22-bp bands indicating the polymorphic allele.

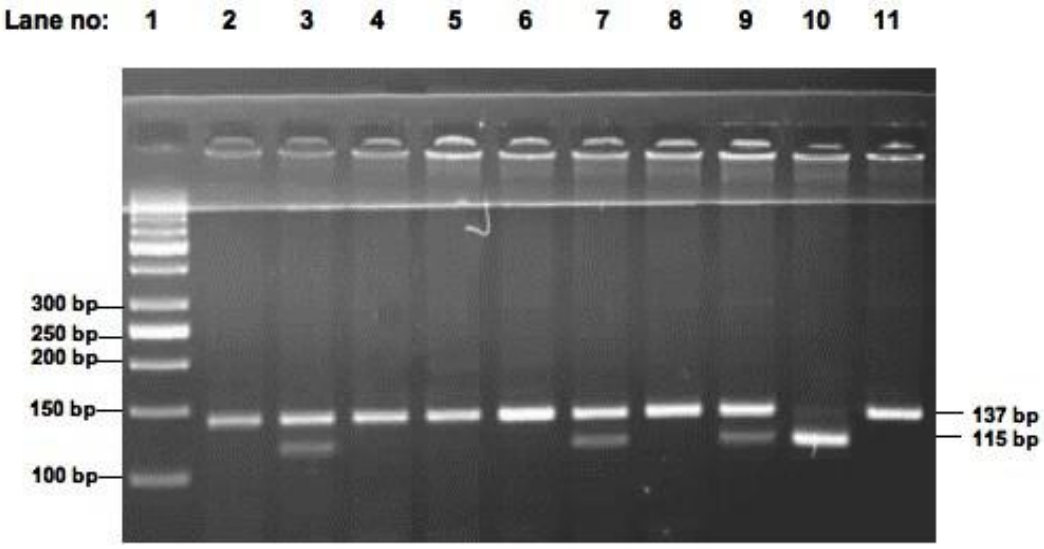

Figure 3. 12 3% Nu-micropore agarose gel electrophoresis of *Hpa* I digestion products for rs944688 SNP region of $GABA_{B2}$. First lane was loaded with DNA ladder (50-1000 bp), following lanes were loaded with digestion products of the subjects. Lane 2, 4, 5, 6, 8 and 11 show wild type genotype (CC) with 137-bp band. Lane 3, 7 and 9 show heterozygous genotype (CT) with 137-bp and 115-bp bands. Lane 10 shows polymorphic genotype (TT) with 115-bp band. (50-bp band, at the bottom of the gel, is an indicator of primer dimer).

3.3 Genotype and Allele Frequencies of Single Nucleotide Polymorphisms

3.3.1 Genotype and Allele Frequencies of $GABA_{B1}$ gene SNPs

3.3.1.1 Genotype and Allele Frequencies of G1465A Single Nucleotide Polymorphism of $GABA_{B1}$

Genotype and allele frequencies of $GABA_{B1}$ G1465A single nucleotide polymorphism in IGE patients, PNES subjects and non-epileptic control individuals are given in Table 3.1. There was no deviation of genotype frequencies from Hardy-Weinberg equilibrium. All the individuals in each groups was genotyped as wild type. Therefore, no risk analysis was performed.

Table 3. 1 Genotype distribution and allele frequencies of $GABA_{B1}$ G1465A SNP in IGE patients, PNES subjects and non-epileptic control subjects.

G1465A	**Patients (n=176)**	**PNES (n=83)**	**Control (n=86)**	**OR (95% CI)**	***P***
Genotypes, n (%)					
GG	176 (100.0)	83 (100.0)	86 (100.0)		
GA	0	0	0	-	-
AA	0	0	0	-	-
Alelles					
G	1	1	1	-	-
A	0	0	0	-	-

3.3.1.2 Genotype and Allele Frequencies of C59T Single Nucleotide Polymorphism of $GABA_{B1}$

Genotype and allele frequencies of $GABA_{B1}$ C59T single nucleotide polymorphism in 176 IGE patients, 83 PNES subjects and 86 non-epileptic control individuals are given in Table 3.2. There was no deviation of genotype frequencies from Hardy-Weinberg equilibrium. Homozygous wild type genotype was seen in 144 (81.8%) patients, 71 (85.5%) PNES subjects and 70 (81.4%) control individuals. 30 (17.0%) patients, 12 (14.5%) PNES subjects and 14 (16.3%) control individuals were heterozygous genotype. Homozygous polymorphic genotype was seen 2 (1.1%) IGE patients and 2 (2.3%) control individuals. Genotype and allele frequencies were close to each other in all groups, therefore no significant difference was observed.

Table 3. 2 Genotype distribution and allele frequencies of $GABA_{B1}$ C59T SNP in IGE patients, PNES subjects and non-epileptic control subjects.

C59T	Patients (n=176)	PNES (n=83)	Control (n=86)	OR	*P*
Genotypes, n (%)					
CC	144 (81.8)	71 (85.5)	70 (81.4)		
CT	30 (17.0)	12 (14.5)	14 (16.3)	0.972[a] (0.500-1.890)	1.0[a]
TT	2 (1.1)	0 (0.0)	2 (2.3)	1.315[b] (0.639-2.706)	0.456[b]
Alleles					
C	0.903	0.928	0.895	0.915[c] (0.501-1.672)	0.772[c]
T	0.097	0.072	0.105	1.372[d] (0.691-2.724)	0.364[d]

[a]CT+TT vs. CC, IGE patients vs. control
[b]CT+TT vs. CC, IGE patients vs. PNES subjects
[c] T vs C, IGE patients vs. control
[d] T vs C, IGE patients vs. PNES subjects

3.3.2 Genotype and Allele Frequencies of $GABA_{B2}$ gene SNPs

3.3.2.1 Genotype and Allele Frequencies rs1999501 Single Nucleotide Polymorphism of $GABA_{B2}$

Genotype and allele frequencies of $GABA_{B2}$ rs1999501 single nucleotide polymorphism in 176 IGE patients, 83 PNES subjects and 86 non-epileptic control individuals are given in Table 3.3. There was no deviation of genotype frequencies from Hardy-Weinberg equilibrium. There is no homozygous polymorphic genotype in all three groups. Heterozygous genotype was found to increase the IGE risk 1.699-fold compared to PNES subjects. However, this result was not statistically significant (*P*=0.210). Polymorphic allele frequencies were; 0.077 in IGE patients, 0.048 in PNES subjects, 0.093 in non-epileptic control group. There is no significant difference among these allele frequencies.

Table 3. 3 Genotype distribution and allele frequencies of $GABA_{B2}$ rs1999501 SNP in IGE patients, PNES subjects and non-epileptic control subjects.

rs 1999501	Patients (n=176)	PNES (n=83)	Control (n=86)	OR (95% CI)	*P*
Genotypes, n (%)					
CC	149 (84.7)	75 (90.4)	70 (81.4)		
CT	27 (15.3)	8 (9.6)	16 (18.6)	0.793[a] (0.401-1.566)	0.503[a]
TT	0	0	0	1.699[b] (0.736-3.921)	0.210[b]
Alelles					
C	0.923	0.952	0.907	0.810[a] (0.424-1.547)	0.523[c]
T	0.077	0.048	0.093	1.641[b] (0.729-3.694)	0.228[d]

[a]CT+TT vs. CC, IGE patients vs. control,
[b]CT+TT vs. CC, IGE patients vs. PNES subjects
[c] T vs C, IGE patients vs. control,
[d] T vs C, IGE patients vs. PNES subjects

3.3.2.2 Genotype and Allele Frequencies of rs967932 Single Nucleotide Polymorphism of $GABA_{B2}$

Genotype and allele frequencies of $GABA_{B2}$ rs967932 single nucleotide polymorphism in 176 IGE patients, 83 PNES subjects and 86 non-epileptic control individuals are given in Table 3.4. There was no deviation of genotype frequencies from Hardy-Weinberg equilibrium. There was no homozygous polymorphic genotype seen in all three groups. When heterozygous individuals were taken as risky group for IGE and PNES subjects compared with IGE patients, $GABA_{B2}$ rs967932 was found to increase the risk of IGE 3.612-fold and the risk was statistically significant (*P*=0.031). Frequency of polymorphic allele was found to be significantly (*P*=0.036) increasing IGE risk 3.447-fold, when patients and PNES subjects compared. Polymorphic allele also increased the IGE risk 1.755-fold, when IGE patients and controls compared. However, this result was not found to be statistically significant.

Table 3. 4 Genotype distribution and allele frequencies of $GABA_{B2}$ rs967932 SNP in IGE patients, PNES subjects and non-epileptic control subjects.

rs 967932	**Patients (n=176)**	**PNES (n=83)**	**Control (n=86)**	**OR (95% CI)**	***P***
Genotypes, n (%)					
GG	155 (88.1)	80 (96.4)	80 (93.0)		
GA	21 (11.9)	3 (3.6)	6 (7.0)	1.806[a] (0.701-4.655)	0.215[a]
AA	0	0	0	3.612[b] (1.046-12.478)	0.031[b]
Alelles					
G	0.940	0.982	0.965	1.755 [c] (0.695-4.432)	0.228[c]
A	0.060	0.018	0.035	3.447 [d] (1.013-11.725)	0.036[d]

[a]GA+AA vs. GG, IGE patients vs. control
[b]GA+AA vs. GG, IGE patients vs. PNES subjects
[c] A vs G, IGE patients vs. control
[d] A vs G, IGE patients vs. PNES subjects

3.3.2.3 Genotype and Allele Frequencies of rs3780428 Single Nucleotide Polymorphism of $GABA_{B2}$

Genotype and allele frequencies of $GABA_{B2}$ rs3780428 single nucleotide polymorphism in 176 IGE patients, 83 PNES subjects and 86 non-epileptic control individuals are given in Table 3.5. There was no deviation of genotype frequencies from Hardy-Weinberg equilibrium. Risky genotypes (GA+GG) were found to increase the IGE risk 1.335-fold compared to PNES subjects. However, this result was not statistically significant (*P*= 0.290). Polymorphic allele frequencies were; 0.267 in IGE patients, 0.235 in PNES subjects, 0.256 in non-epileptic control group. No significant difference was found between these alleles.

Table 3. 5 Genotype distribution and allele frequencies of $GABA_{B2}$ rs3780428 SNP in IGE patients, PNES subjects and non-epileptic control subjects.

rs 3780428	Patients (n=176)	PNES (n=83)	Control (n=86)	OR (95% CI)	*P*
Genotypes, n (%)					
GG	98 (55.7)	52 (62.7)	47 (54.7)		
GA	62 (35.2)	23 (27.7)	34 (39.5)	0.960[a] (0.571-1.611)	0.874[a]
AA	16 (9.1)	8 (9.6)	5 (5.8)	1.335[b] (0.782-2.28)	0.290[b]
Alelles					
G	0.733	0.765	0.744	1.060 [a] (0.699-1.607)	0.784[c]
A	0.267	0.235	0.256	1.186[b] (0.772-1.823)	0.435[d]

[a]GA+AA vs. GG, IGE patients vs. control
[b]GA+AA vs. GG, IGE patients vs. PNES subjects
[c] A vs G, IGE patients vs. control
[d] A vs G, IGE patients vs. PNES subjects

3.3.2.4 Genotype and Allele Frequencies of rs944688 Single Nucleotide Polymorphism of $GABA_{B2}$

Genotype and allele frequencies of $GABA_{B2}$ rs944688 single nucleotide polymorphism in 176 IGE patients, 83 PNES subjects and 86 non-epileptic control individuals are given in Table 3.6. There was no deviation of genotype frequencies from Hardy-Weinberg equilibrium in the PNES and non-epileptic control subjects, however genotype frequencies differed significantly from the expected values calculated according to the Hardy-Weinberg equilibrium in the IGE patient group. There were no significant differences in terms of genotype distributions between IGE patients and controls, or PNES subjects and controls. Polymorphic allele frequencies were; 0.196 in IGE patients, 0.260 in PNES subjects, 0.227 in non-epileptic control group; however the differences between allele frequencies were not statistically significant.

Table 3. 6 Genotype distribution and allele frequencies of $GABA_{B2}$ rs944688 SNP in IGE patients, PNES subjects and non-epileptic control subjects.

rs 944688	**Patients (n=176)**	**PNES (n=83)**	**Control (n=86)**	**OR (95% CI)**	***P***
Genotypes, n (%)					
CC	108 (61.4)	45 (54.2)	52 (60.5)		
CT	67 (38.1)	33 (39.8)	29 (33.7)	0.963[a] (0.568-1.633)	1.0[a]
TT	1 (0.6)	5 (6.0)	5 (5.8)	0.746[b] (0.440-1.264)	0.275[b]
Alelles					
C	0.804	0.740	0.773	0.831[c] (0.534-1.295)	0.414[c]
T	0.196	0.260	0.227	0.697[d] (0.451-1.078)	0.104[d]

[a]CT+TT vs. CC, IGE patients vs. control
[b]CT+TT vs. CC, IGE patients vs. PNES subjects
[c] T vs C, IGE patients vs. control
[d] T vs C, IGE patients vs. PNES subjects

3.4 Combined Genotypes of Single Nucleotide Polymorphisms

3.4.1 Combined Genotypes of $GABA_{B1}$ SNPs

In this study, two SNPs of $GABA_{B1}$ gene was studied; G1465A and C59T. All the individuals were found to be wild type for G1465A SNP, therefore combined genotypes could not be studied for $GABA_{B1}$ gene.

3.4.2 Combined Genotypes of $GABA_{B2}$ SNPs

Four different SNPs of $GABA_{B2}$ gene was studied; rs1999501, rs967932, rs3780428 and rs944688. All the genotype combinations of these SNPs were performed. Statistically significant results are given.

Distribution of double combined genotypes in IGE patients and PNES subjects are given in Table 3.7. IGE risk was 6.54-fold higher for subjects having GA genotype for rs967932 and GG genotype for rs3780428 when compared with PNES subjects (P=0.040).

Table 3. 7 Distribution of double combined genotypes in IGE patients and PNES subjects.

	Patient (n=176)	PNES (n=83)	OR (95% CI)	*P*
rs1999501(C/T) and rs967932(G/A)				
CCGG	141 (80.1%)	75 (90.4%)	0.430 (0.190-0.973)	0.039
rs1999501(C/T) and rs944688(C/T)				
CCTT	1 (0.6%)	4 (4.8%)	0.113 (0.012-1.026)	0.038*
rs967932(G/A) and rs3780428(G/A)				
GAGG	13 (7.4%)	1 (1.2%)	6.540 (0.841-50.862)	0.042*
rs967932(G/A) and rs944688(C/T)				
GGTT	1 (0.6%)	4 (4.8%)	0.113 (0.012-1.026)	0.038*

*Fisher's exact test was used. Chi-square test was used for other comparisons.

Distribution of double combined genotypes in IGE patients and non-epileptic control subjects are given in Table 3.8. IGE risk was 6.22-fold higher for subjects having AA genotype for rs3780428 and CC genotype for rs944688 when compared with non-epileptic control subjects (*P*=0.048).

Table 3. 8 Distribution of double combined genotypes in IGE patients and non-epileptic control subjects.

	Patient (n=176)	Control (n=86)	OR (95% CI)	*P*
rs1999501(C/T) and rs944688(C/T)				
CCTT	1 (0.6%)	4 (4.7%)	0.117 (0.013-1.065)	0.041*
rs967932(G/A) and rs944688(C/T)				
GGTT	1 (0.6%)	5 (5.8%)	0.092 (0.011-0.805)	0.016*

*Fisher's exact test was used. Chi-square test was used for other comparisons.

Distribution of triple combined genotypes in IGE patients and PNES subjects are given in Table 3.9. CCGGTT genotype of rs1999501-rs967932-rs944688 had around 9-fold protective effect against IGE when compared to PNES subjects (*P*=0.020).

Table 3. 9 Distribution of triple combined genotypes in IGE patients and PNES subjects.

	Patient (n=176)	PNES (n=83)	OR (95% CI)	*P*
rs1999501(C/T) and rs967932(G/A) and rs3780428(G/A)				
CCGGGG	78 (44.3%)	49 (59.1%)	0.552 (0.325-0.937)	0.027
rs1999501(C/T) and rs967932(G/A) and rs944688(C/T)				
CCGGTT	1 (0.6%)	4 (4.8%)	0.113 (0.012-1.026)	0.038*

*Fisher's exact test was used. Chi-square test was used for other comparisons.

Distribution of triple combined genotypes in IGE patients and non-epileptic control subjects are given in Table 3.10. The combination of CC genotype for rs1999501, GG genotype for rs967932 and TT genotype for rs944688 had around 9-fold protective effect against IGE when both compared with non-epileptic subjects having pseudoseizures (*P*=0.020) and non-epileptic control subjects (*P*=0.023).

Table 3. 10 Distribution of triple combined genotypes in IGE patients and non-epileptic control subjects.

	Patient (n=176)	**Control (n=86)**	**OR (95% CI)**	***P***
rs1999501(C/T) and rs967932(G/A) and rs944688(C/T)				
CCGGTT	1 (0.6%)	4 (4.7%)	0.117 (0.013-1.065)	0.041*

*Fisher's exact test was used. Chi-square test was used for other comparisons.

Distribution of quadruple combined genotypes in IGE patients and PNES subjects are given in Table 3.11. The combination of GG genotype for rs3780428, CC genotype for rs1999501, GG genotype for rs967932 and CT genotype for rs944688 gave a significant result with 2-fold protective effect against IGE when compared with PNES subjects (*P*=0.058).

G-C-A-C combined haplotype (rs3780428-rs1999501-rs967932-rs944688), reported to be associated with MTLE in Wang *et al.* (2008) study, was also analyzed. There were just 3 IGE patients and no PNES or non-epileptic control subjects having the possibility to produce the G-C-A-C combined haplotype, therefore no significant difference was found (Table 3.11 and Table 3.12).

Table 3. 11 Distribution of quadruple combined genotypes in IGE patients and PNES subjects.

	Patient (n=176)	PNES (n=83)	OR (95% CI)	*P*
rs3780428(G/A) and rs1999501(C/T) and rs967932(G/A) and rs944688(C/T)				
GGCCGACC	3 (1.7%)	0	-	0.553*
GGCCAACC	0	0	-	-

*Fisher's exact test was used. Chi-square test was used for other comparisons.

Table 3. 12 Distribution of quadruple combined genotypes in IGE patients and non-epileptic control subjects.

	Patient (n=176)	Control (n=86)	OR (95% CI)	*P*
rs3780428(G/A) and rs1999501(C/T) and rs967932(G/A) and rs944688(C/T)				
GGCCGACC	3 (1.7%)	0	-	0.553*
GGCCAACC	0	0	-	-

*Fisher's exact test was used. Chi-square test was used for other comparisons.

CHAPTER 4

DISCUSSION

Epilepsy is characterized by the seizures, which occur due to the decrease in inhibition of neurons or due to a total disinhibition of neurons. The most important known causes of seizures are channelopathies. Mutations in proteins coding for voltage-gated and ligand-gated ion channels are linked with seizure formation. Therefore, inhibitory neurotransmitter GABA and its receptors are known to affect seizure formation. Animal experiments prove that the drugs blocking the GABAergic inhibition lead to partial seizures (Schwartkroin *et al*, 1980). Also many loss-of-function studies show the relation between GABA and epilepsy.

GABA mediates its inhibitory action by binding its receptors; $GABA_A$ and $GABA_B$. $GABA_A$ receptors are ionotrophic and mediate fast inhibitory action (Macdonald & Olsen, 1994) while $GABA_B$ receptors are metabotropic receptors and mediate long-term inhibitory action (Kerr and Ong, 1995). Many experiments show that $GABA_B$ receptors play a significant role in seizures formation. $GABA_{B1}$ knock-out mice were reported to develop generalized epilepsy (Prosser *et al.*, 2001). Animal experiments show that $GABA_B$ receptors play a significant role in absence seizures (Caddick & Hosford, 1996). Drugs that inhibit GABA synthesis are known to trigger seizure formation (Treiman, 2001).

In 40% of the epilepsy patients, the pathology is estimated to have genetic background. Idiopathic epilepsies generally occur as a result of the mutations in the genes coding for ion channels. Nocturnal frontal lobe epilepsy (ADNFLE) is linked with neuronal nicotinic acetylcholine receptor (nAChR) mutations (Steinlein, 1995).

Benign familial neonatal convulsions (BFNC) were found to be caused by the voltage-gated potassium channel gene mutations which lead to slow opening and closing of the pores (Wang *et al.*, 1998). Voltage-gated sodium channel subunit (SCN1A, SCN1B, SCN2A) mutations were identified to cause generalized epilepsy with febrile seizures plus ($GEFS^{+}$) by subtle gate defect (Wallace *et al.*, 1998).

Voltage-gated channels (sodium, potassium) and ligand-gated channels (GABA and acetylcholine) gene mutations are known to develop epilepsy (Steinlein, 2004). With this knowledge, GABA and GABA receptors are widely studied for epilepsy. The linkage between GABA and epilepsy was pointed by experimental and clinical studies; animal models of epilepsy show abnormalities on GABAergic function; reduced GABA-mediated inhibition in human epileptic brain tissues; drugs inhibiting GABA synthesis trigger seizure formation; GABA agonists suppress seizure formation while GABA antagonists produce seizures (Treiman, 2001). Some antagonist-agonist studies showed contradictory results. This has been explained as the GABA action could have opposite effects on seizure formation, according to the type of seizure and stage of maturation (Mares & Slamberova, 2006).

Many GABA mutations are identified which lead to epileptic seizures. Also recent studies point that some single nucleotide polymorphisms on GABA receptor genes are associated with epilepsy types. In this study, the association between $GABA_{B1}$ and $GABA_{B2}$ gene polymorphisms and idiopathic generalized epilepsy were studied in Turkish population.

$GABA_{B1}$ genetic polymorphisms

$GABA_{B1}$ subunit takes role in ligand activation of the receptor with the extracellular domain including GABA-binding site. G1465A polymorphism is located in exon 11 of $GABA_{B1}$ gene. $GABA_{B1}$ G1465A changes glycine amino acid to serine (Gly489Ser) in a highly conserved region. This region contains ligand-binding site of GABA receptor (French, 2003). Therefore $GABA_{B1}$ G1465A polymorphism could

affect the receptor function. The frequency of G1465A in healthy population was reported as 0.027 in NCBI database.

At first, Gambardella and colleagues reported the association between $GABA_{B1}$ G1465A polymorphism and temporal lobe epilepsy (TLE). Polymorphic allele was detected in 17% of TLE patients and in 0.5% of controls. After this study, seven different groups studied $GABA_{B1}$ G1465A polymorphism and TLE (shown in Table 4.1), however none of them could show an association. Also, no polymorphic allele was reported in the studies held on Chinese (Ren *et al.*, 2005), Han Chinese (Wang *et al.*, 2008), Middle European (Stogmann *et al.*, 2006) and French (Salzmann *et al.*, 2005) populations. Sander *et al.* (1999) studied the association between $GABA_{B1}$ G1465A and IGE, however they failed to demonstrate a significant association.

The $GABA_{B1}$ G1465A polymorphism was studied in Turkish population in an association study with obstructive sleep apnea syndrome; all the individuals were reported to be wild type for $GABA_{B1}$ G1465A polymorphism (Bayazit *et al.*, 2007). In our study, all the individuals were also wild type for $GABA_{B1}$ G1465A, which shows similar results with previous study held on Turkish population. Allele frequencies of $GABA_{B1}$ G1465A polymorphism in different populations are given at Table 4.1.

Table 4. 1 Allele frequencies of $GABA_{B1}$ G1465A polymorphism in different populations.

Populations	G1465A Polymorphism		N	Reference
	Wild Type Allele	Polymorphic Allele		
Turkish	**1.00**	**0.0**	**86**	**This study**
Turkish	1.00	0.0	99	Bayazit *et al.* 2007
American	0.90	0.10	50	Hisama *et al.* 2001
Argentina	0.96	0.04	71	Kauffman *et al.* 2007
Caucasians	0.996	0.004	218	Ma *et al.* 2005
Chinese	1.00	0.0	124	Ren *et al.* 2005
French	1.00	0.0	145	Salzmann *et al.* 2005
German	0.946	0.054	130	Sander *et al.* 1999
Han Chinese	1.00	0.0	315	Wang *et al.* 2008
Italian	0.998	0.002	372	Gambardella *et al.* 2003
Japanese	1.00	0.0	100	Imai *et al.* 2002
Middle Europe	1.00	0.0	259	Stogmann *et al.* 2006

C59T polymorphism of $GABA_{B1}$ gene also leads to amino acid change like $GABA_{B1}$ G1465A. $GABA_{B1}$ C59T nucleotide exchange causes a substitution of alanine to valine (Ala20Val) in exon1a1. The frequency of C59T in healthy population was reported as 0.001 in NCBI database. In the study of Peters *et al.* (1998) all the IGE patients of two families were found to carry $GABA_{B1}$ C59T polymorphism; however their unaffected relatives did also carry the same polymorphism. Similarly another association study held on German population for $GABA_{B1}$ C59T failed to demonstrate any association between $GABA_{B1}$ C59T and IGE (Sander *et al.*, 1999).

In our study, no significant difference was observed between IGE patients and PNES subjects or non-epileptic control subjects. The frequency of $GABA_{B1}$ C59T polymorphism was found as 0.105 in Turkish population (this study). In Bayazıt *et*

al. (2007) study, the frequency of $GABA_{B1}$ C59T polymorphism was reported as 0.120 in Turkish population, which is very close to our result. Allele frequencies of $GABA_{B1}$ C59T polymorphism in different populations are given at Table 4.2.

Table 4. 2 Allele frequencies of $GABA_{B1}$ C59T polymorphism in different populations.

Populations	C59T Polymorphism		N	Reference
	Wild Type Allele	Polymorphic Allele		
Turkish	**0.895**	**0.105**	**86**	**This study**
Turkish	0.88	0.12	99	Bayazit *et al.* 2007
German	0.921	0.079	127	Sander *et al.* 1999
Japanese	1.00	0.0	100	Imai *et al.* 2002

G1465A and C59T polymorphisms of $GABA_{B1}$ gene are both missense polymorphisms, which could affect the receptor function. Therefore those two SNPs were chosen for the association study. However, no significant association was identified for G1465A and C59T polymorphisms in this study.

$GABA_{B2}$ genetic polymorphisms

$GABA_{B2}$ receptor gene is located in 9q22.1-q22.3 chromosome region. $GABA_{B2}$ subunit is responsible for G-protein coupled signaling. In the study of Wang *et al.* (2008) $GABA_{B2}$ rs967932 SNP was found to be associated with mesial temporal lobe epilepsy (MTLE). Also haplotype of G-C-A-C (rs3780428-rs1999501-rs967932-

rs94688) found to be higher in MTLE patients, compared to control subjects. Therefore those four SNPs were chosen in this study.

All four $GABA_{B2}$ SNPs in this study are located in intron site, and their allele frequencies are given in Table 4.3. The polymorphisms in the intron site could create alternative splice sites, which compete with the normal splice site. Also intronic polymorphisms could be located on the binding sites of regulatory proteins and affect the amount of protein product.

Table 4. 3 Allele frequencies of $GABA_{B2}$ polymorphisms in Turkish population found in this study.

SNPs	**$GABA_{B2}$ Polymorphism**	
	Wild Type Allele	**Polymorphic Allele**
rs1999501	0.907	0.093
rs967932	0.965	0.035
rs3780428	0.744	0.256
rs944688	0.773	0.227

This is the first study to search for the association between $GABA_{B2}$ rs3780428, rs1999501, rs967932, rs94688 SNPs and idiopathic generalized epilepsy. Previously, there was just one group that studied these $GABA_{B2}$ polymorphisms. Wang *et al.* (2008) investigated these polymorphisms in MTLE and gave frequencies in Han Chinese population.

For $GABA_{B2}$ rs1999501 single nucleotide polymorphism, there was no homozygous polymorphic genotype seen in all three groups. Heterozygous genotype was found to increase the IGE risk 1.699-fold compared to PNES subjects. However, this result

was not statistically significant (*P*=0.210). $GABA_{B2}$ rs1999501 SNP was also found to be not associated with mesial temporal lobe epilepsy (MTLE) in Wang *et al.* (2008) study. The frequency of $GABA_{B2}$ rs1999501 SNP was found as 0.093 in Turkish population (this study), which was reported as 0.441 in Han Chinese population (Wang *et al.,* 2008), and given as 0.200 in NCBI database.

For $GABA_{B2}$ rs967932 single nucleotide polymorphism, there was no homozygous polymorphic genotype seen in all three groups. When heterozygous individuals are taken as risky group for IGE and PNES subjects compared with IGE patients, $GABA_{B2}$ rs967932 was found to increase the risk of IGE 3.612-fold (*P*=0.031). Frequency of polymorphic allele was found to be significantly (*P*=0.036) increasing IGE risk 3.447-fold, when patients and PNES subjects compared. When IGE and non-epileptic control subjects compared, no statistically significant results could be found; heterozygous genotype was found to increase the IGE risk 1.806-fold (*P*=0.215) compared to non-epileptic control subjects; polymorphic allele also increased the IGE risk 1.755-fold, but insignificantly (*P*=0.228). $GABA_{B2}$ rs967932 SNP was also found to be associated with MTLE in Wang *et al.* (2008) study. The frequency of $GABA_{B2}$ rs967932 SNP was found as 0.035 in Turkish population (this study), which was reported as 0.416 in Han Chinese population (Wang *et al.,* 2008), and given as 0.177 in NCBI database.

For $GABA_{B2}$ rs3780428 single nucleotide, risky genotypes (GA+GG) were found to increase the IGE risk 1.335-fold compared to PNES subjects. However, this result was not statistically significant (*P*=0.290). No significant difference was found between polymorphic alleles. $GABA_{B2}$ rs3780428 SNP was also found to be not associated with MTLE in Wang *et al.* (2008) study. The frequency of $GABA_{B2}$ rs3780428 SNP was found as 0.256 in Turkish population (this study), which was reported as 0.175 in Han Chinese population (Wang *et al.,* 2008), and given as 0.320 in NCBI database.

For $GABA_{B2}$ rs944688 single nucleotide polymorphism, no significant results were obtained in genotype distributions. Polymorphic allele frequencies were; 0.196 in

IGE patients, 0.260 in PNES subjects, 0.227 in non-epileptic control group; however the differences between allele frequencies were not statistically significant. $GABA_{B2}$ rs944688 SNP was also found to be not associated with MTLE in Wang *et al.* (2008) study. The frequency of $GABA_{B2}$ rs3780428 SNP was found as 0.227 in Turkish population (this study), which was reported as 0.105 in Han Chinese population (Wang *et al.*, 2008), and given as 0.303 in NCBI database.

Combined genotypes of $GABA_{B2}$ SNPs

In this study, four different single nucleotide polymorphisms were studied on the same gene. Therefore, combined genotype analysis of those SNPs were performed in order to find if the combination of any genotype could make a significant difference between IGE patients and controls, or between IGE patients and PNES subjects.

Double, triple and quadruple combinations of $GABA_{B2}$ rs1999501, rs967932, rs3780428 and rs944688 polymorphisms were performed for all three groups. In Section 3.4.2, just the significant results were given.

$GABA_{B2}$ rs967932 SNP was found to be associated with IGE when compared to PNES subjects. There was no homozygous polymorphic genotype seen in all three groups individuals. GA genotype of rs967932 was found to increase the risk of IGE 3.612-fold (P=0.031) when compared to PNES subjects. This risk increases to 6.54-fold when GA genotype of rs967932 was considered together with the wild type GG genotype for rs3780428 (P=0.042); however rs3780428 SNP showed no significant affect on IGE when it is considered alone.

Polymorphic allele of rs944688 SNP showed a protective effect in some genotype combinations. Polymorphic T allele of rs944688 SNP was found to have around 2-fold protective effect against IGE when compared to PNES subjects, however this result was not statistically significant (P=0.104). When rs944688 TT genotype came together with CC genotype for rs1999501, GG genotype for rs967932; the triple

genotype combination had around 9-fold protective effect against IGE when both compared with PNES subjects (*P*=0.038) and non-epileptic control subjects (*P*=0.041). Also, among the quadruple genotype combinations; the combination of wild type GG genotype for rs3780428, wild type CC genotype for rs1999501, wild type GG genotype for rs967932 and heterozygous CT genotype for rs944688 showed 2-fold protective effect against IGE when compared with PNES subjects; however the result was not statistically significant (*P*=0.058).

In the study of Wang *et al.* (2008), G-C-A-C combined haplotype (rs3780428-rs1999501-rs967932-rs944688) was found to significantly increase the risk of MTLE 12.4-fold when compared to the control group. However, in our study there were just 3 IGE patients and no PNES or non-epileptic control subjects having the possibility to produce G-C-A-C combined haplotype, no significant difference was found.

CHAPTER 5

CONCLUSION

Epilepsy is a seizure disorder. The most important known causes of seizures are channelopathies. Mutations in genes coding for voltage-gated and ligand-gated ion channels are linked with seizure formation. Also several ion-channel mutations were identified as the causes of idiopathic generalized epilepsy (IGE) which accounts for one-fifth of all the other epilepsy types. Among those ion-channels, gamma amino butyric acid (GABA) receptor mutations were also identified as the causes of epilepsy.

GABA mediates its inhibitory action by binding its receptors; $GABA_A$ and $GABA_B$. $GABA_B$ receptors were reported to play a significant role in seizures formation in animal experiments. Therefore in this study, two polymorphisms of $GABA_{B1}$ gene and four polymorphisms of $GABA_{B2}$ gene were tested as the risk factors for IGE. Also misdiagnosis of phsycogenic non-epileptic seizures (PNES) cause health and financial problems. It is aimed to determine the role of the differences found in the GABA receptor genes to be used for the diagnosis of PNES.

The study population consisted of a total of 176 idiopathic generalized epilepsy (IGE) patients, 83 subjects having psychogenic non-epileptic seizures (PNES), 86 non-epileptic control subjects from Turkey. There was no statistically significant difference between the patient and control groups in terms of age.

Two missense polymorphisms in $GABA_{B1}$ gene were studied; G1465A and C59T. The association between those SNPs and IGE were previously studied; in this study this association were analyzed in PNES subjects for the first time. $GABA_{B1}$ G1465A polymorphic allele was not observed in Turkish population in our study. For $GABA_{B1}$ C59T polymorphism, polymorphic allele frequencies were found as 0.097 in IGE patients; 0.072 in PNES subjects and 0.105 in non-epileptic control subjects. No significant difference is identified for C59T polymorphism in all three groups.

This is the first study to search for the association between $GABA_{B2}$ rs3780428, rs1999501, rs967932, rs944688 SNPs and idiopathic generalized epilepsy. Also the frequencies of these $GABA_{B2}$ SNPs in Turkish population were reported for the first time in this study. rs967932 was found to increase the risk of IGE 3.6-fold (P=0.031) compared to PNES subjects. No significant difference was identified for rs1999501, rs3780428 and rs944688 polymorphisms among IGE patients, PNES subjects and non-epileptic control groups in Turkish population. The frequencies of the polymorphisms were found as; 0.035 for rs967932, 0.093 for rs1999501, 0.256 for rs3780428 and 0.227 for rs944688 in Turkish population.

In the combined genotypes; the IGE risk increases to 6.54-fold when GA genotype of rs967932 was considered together with the wild type GG genotype for rs3780428 (P=0.042) when compared with PNES subjects. Also, the genotype combinations indicated that the polymorphic allele of rs944688 SNP showed a protective effect in some cases. When rs944688 TT genotype came together with CC genotype for rs1999501, GG genotype for rs967932; the triple genotype combination had around 9-fold protective effect against IGE when both compared with PNES subjects (P=0.038) and non-epileptic control subjects (P=0.041).

In conclusion, this was the first study to analyze the roles of $GABA_{B2}$ SNPs in IGE and PNES and the roles of $GABA_{B1}$ SNPs in PNES. Some significant conclusions were drawn which will add to the growing body of information on genetic factors having role in IGE pathology.

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http://www.who.int, November 2011

http://www.shutterstock.com/pic-44426533/stock-vector-neurotransmitters-involved-in-epilepsy.html, December 2011

APPENDIX A

INFORMED CONSENT FOR CONTROLS

Bu çalışmaya katılmak için karar vermeden önce sizi bilgilendirecek olan bu belgeyi incelemeniz önemlidir. Yine de bu konuya ilişkin sorunuz olduğu takdirde lütfen doktorunuzla görüşmekten çekinmeyiniz.

Araştırmada nöroloji kliniğinde yatırılarak takip edilen hastalarda diğer gerekli tetkikler için alınan kan örneklerinizde epilepsi ile ilgili olabilecek "Gama Amino Butirik Asit (GABA)" diye bilinen bir maddeyle ilgili genetik farklılıklar incelenecektir. Bu araştırma sonucunda sebebi bilinmeyen (idiyopatik) jeneralize epilepsi hastalarında genetik yatkınlık konusunda bilgi edinilecektir. Kaydedilen bilgiler bilimsel amaçlarla kullanılacaktır. Ek kan alınmayacak ve ücret talep edilmeyecektir.

Bu araştırma için hastalığınızın standart tedavi şeklinde herhangi bir değişiklik yapılmayacaktır. Çalışmayla ilgili ayrıntılı bilgi alma isteminiz olursa aşağıdaki numaraları arayabilirsiniz.

Dr. Güray KOÇ Tel: (312) 304-4481
Dr. Semai BEK Tel: (312) 304-4489

Eğer bu çalışmada yer almak istemiyorsanız bunu belirtmeniz yeterlidir. Bu durum bundan sonraki tedavinizi etkilemeyecektir. Çalışmaya katılmaya karar verirseniz kimliğinizin gizli kalması koşuluyla bu araştırmadan elde edilecek bilgi ve bulguların

istendiğinde ilgili makamlara verilebileceğini ve yayınlanabileceğini önceden kabul etmek durumundasınız.

Tarih: .....

Yukarıda gönüllüye araştırmadan önce verilmesi gereken bilgileri gösteren metni okudum. Bunlar hakkında tarafıma yazılı ve sözlü açıklamalar yapıldı. Bu koşullarda söz konusu klinik araştırmaya kendi rızamla, hiçbir baskı ve zorlama olmaksızın katılmayı kabul ediyorum. Bu formun bir nüshası da bana veriliştir.

Gönüllünün
Adı-Soyadı :
Adresi :
Telefon :
İmzası :

(gerekliyse)
Veli veya Vasinin
Adı-Soyadı :
Adresi :

Telefon :
İmzası :

Rıza alınma işlemine başından sonuna kadar tanıklık eden kuruluş görevlisinin
Adı-Soyadı :
İmzası :
Tarih :

APPENDIX B

ETHICAL COMMITTEE APPROVAL FORM

HİZMETE ÖZEL

T.C.
GENELKURMAY BAŞKANLIĞI
GÜLHANE ASKERİ TIP AKADEMİSİ KOMUTANLIĞI
A N K A R A

Ağustos 2010

Y. ETİK KRL. : 1491 -982 - 10/1539

KONU : GATA Etik Kurulu

Dr. Birsen CAN DEMİRDÖĞEN

"Gama Amino Butirik Asit (GABA) Reseptörlerinin Genetik Polimorfizmleri İle İdiyopatik Jeneralize Epilepsi Arasındaki İlişkinin Araştırılması" başlıklı, çok merkezli, klinik ve laboratuar çalışması olan araştırma projeniz ile ilgili, GATA Etik Kurulu'nun kararı EK'tedir.

Rica ederim.

Ali Uğur URAL
Prof. Tbp. Kd. Alb.
GATA Etik Kurulu Başkanı

EK :
1 Adet Etik Kurul Raporu

HİZMETE ÖZEL

T.C.
GENELKURMAY BAŞKANLIĞI
GÜLHANE ASKERİ TIP AKADEMİSİ KOMUTANLIĞI
ETİK KURUL TOPLANTI RAPORU

OTURUM NO : 157
OTURUM TARİHİ : 04 Ağustos 2010
OTURUM BAŞKANI : Prof. Tbp. Kd. Alb. Ali Uğur URAL
OTURUM SEKRETERİ : Doç. Dr. Ecz. Kd. Alb. Adnan ATAÇ

GATA Etik Kurulu'nun 04 Ağustos 2010 günü yapılan 157. oturumunda, Refik Saydam Hıfzısıhha Merkezi Başkanlığı'ndan Dr. Birsen CAN DEMİRDÖĞEN'in sorumlu araştırmacılığını yaptığı **"Gama Amino Butirik Asit (GABA) Reseptörlerinin Genetik Polimorfizmleri İle İdiyopatik Jeneralize Epilepsi Arasındaki İlişkinin Araştırılması"** başlıklı, çok merkezli, klinik ve laboratuar çalışması olan araştırma dosyası değerlendirildi.

Araştırma dosyasının amaç, yöntem ve yaklaşım bakımından etik ilkelere UYGUN olduğuna karar verildi.

BAŞKAN	**ÜYE**	**ÜYE**	
Ali Uğur URAL Prof.Tbp.Alb.	Adnan ATAÇ Prof.Dr.Ecz.Alb.	**Toplantıya Katılmadı** Ali İhsan UZAR Prof.Hv.Tbp.Alb.	
ÜYE	**ÜYE**	**ÜYE**	**ÜYE**
Tunçer HAZNEDAROĞLU Prof.Dz.Tbp.Alb.	Ayhan KUBAR Prof.Tbp.Alb.	Yalçın ÖZKAN Prof. Dr. Ecz. Alb.	**Toplantıya Katılmadı** Nalan AKBAYRAK Prof. Dr. Sağ. Alb.
ÜYE	**ÜYE**	**ÜYE**	**ÜYE**
Toplantıya Katılmadı Mükerrem SAFALI Doç.Tbp.Alb.	K. Metin AKAY Doç.Tbp.Alb.	**Toplantıya Katılmadı** Ergun TOZKOPARAN Doç.Tbp.Alb.	Muharrem UÇAR Doç. J. Tbp. Alb.

APPENDIX C

LIST OF CHEMICALS AND THEIR SUPPLIERS

Chemical	**Catalog No**	**Supplier**
Absolute ethanol	32221	Riedel de Haen, Germany
Agarose	A5093	Sigma-Aldrich, Germany
Boric acid	A949265	Merck KgaA, Germany
Bromophenol blue	B8026	Sigma-Aldrich, Germany
*Bsa*AI (*Ppu*21I)	ER1971	MBI Fermentas, USA
dNTP mix	R0192	MBI Fermentas, USA
*Eag*I (*Eco*52I)	ER0332	MBI Fermentas, USA
Ethidium bromide	E1510	Sigma-Aldrich, Germany
EDTA	A5097	Applichem GmbH, Germany
Gene RulerTM 50 bp DNA Ladder	SM0371	MBI Fermentas, USA
*Hin*6I (*Hin*P1I)	ER0481	MBI Fermentas, USA
*Hpa*I (*Ksp*AI)	ER1032	MBI Fermentas, USA
Magnesium chloride	Art5833	Merck KgaA, Germany
Nu-micropore agarose	Hs-8065A	Prona
Potassium chloride	A2939	Applichem GmbH, Germany
Primers		Iontek, İstanbul
*Sau*96I (*Cfr*13I)	ER0191	MBI Fermentas, USA
*Scr*FI (*Bme*1390I)	ER1422	MBI Fermentas, USA
SDS	L4390	Sigma-Aldrich, Germany
Taq polymerase	EP0402	MBI Fermentas, USA
Trizma base	T1503	Sigma-Aldrich, Germany

APPENDIX D

LIST OF STUDY POPULATION

Table D.1 List of study population consisted of 176 idiopathic generalized epilepsy patients, 83 subjects having psychogenic non-epileptic seizures, 86 non-epileptic control subjects. IGE: idiopathic generalized epilepsy, PNES: psychogenic non-epileptic seizures.

No	Patients&Controls	Age	rs967932 (G/A)	rs3780428 (G/A)	rs1999501 (C/T)	rs944688 (C/T)	G1465A (G/A)	C59T (C/T)
1	PNES	20	GG	GG	CC	CC	GG	CC
2	IGE	19	GG	GA	CC	CT	GG	CT
3	IGE	21	GG	GA	CC	CT	GG	CT
4	IGE	22	GA	GG	CT	CC	GG	CC
5	IGE	21	GG	GG	CC	CC	GG	CT
6	IGE	21	GG	GG	CC	CT	GG	CC
7	PNES	21	GG	GA	CC	CC	GG	CC
8	IGE	21	GG	AA	CT	CC	GG	CC
9	PNES	22	GG	GG	CC	CT	GG	CC
10	PNES	27	GG	GG	CC	CC	GG	CC
11	IGE	21	GG	GA	CC	CC	GG	CC
12	PNES	21	GG	GA	CC	CT	GG	CC
13	IGE	21	GG	GA	CC	CT	GG	CT
14	PNES	21	GG	GG	CC	CC	GG	CC
15	IGE	21	GG	GG	CC	CC	GG	CC
16	IGE	22	GG	GG	CC	CC	GG	CC
17	IGE	20	GG	GA	CC	CT	GG	TT
18	IGE	20	GG	AA	CC	CC	GG	CC

Table D.1 (continued).

No	Patients&Controls	Age	rs967932 (G/A)	rs3780428 (G/A)	rs1999501 (C/T)	rs944688 (C/T)	G1465A (G/A)	C59T (C/T)
19	IGE	20	GG	GG	CC	CT	GG	CC
20	PNES	27	GG	GG	CC	CT	GG	CT
21	IGE	34	GG	GG	CC	CC	GG	CC
22	IGE	21	GG	GA	CC	CT	GG	CC
23	IGE	23	GG	GG	CC	CC	GG	CC
24	IGE	23	GG	GG	CC	CC	GG	CC
25	IGE	21	GG	GG	CC	CT	GG	CC
26	IGE	27	GG	GA	CT	CC	GG	CC
27	PNES	21	GG	GG	CC	CC	GG	CC
28	IGE	21	GA	GG	CT	CT	GG	CC
29	IGE	20	GG	GA	CC	CT	GG	CT
30	IGE	20	GG	GA	CC	CT	GG	CC
31	IGE	20	GG	GG	CC	CC	GG	CC
32	IGE	20	GA	GA	CT	CC	GG	CT
33	IGE	21	GA	GG	CT	CC	GG	CC
34	IGE	20	GG	AA	CC	CC	GG	CC
35	PNES	21	GG	AA	CC	CC	GG	CC
36	IGE	20	GG	GG	CC	CC	GG	CC
37	IGE	21	GG	GA	CC	CT	GG	CC
38	IGE	20	GG	GG	CT	CT	GG	CC
39	IGE	22	GG	GA	CC	CT	GG	CC
40	IGE	20	GG	GG	CC	CC	GG	CT
41	IGE	20	GG	GG	CC	CC	GG	CC
42	IGE	22	GA	GG	CT	CT	GG	CC
44	IGE	25	GG	GA	CC	CC	GG	CC
45	IGE	25	GG	AA	CC	CC	GG	CT
46	IGE	22	GG	GA	CC	CC	GG	CC
47	PNES	22	GG	AA	CC	CC	GG	CC
48	IGE	28	GG	GG	CC	CC	GG	CC
49	IGE	26	GG	GA	CC	CT	GG	CC
50	IGE	20	GA	GG	CT	CT	GG	CC
51	IGE	20	GG	GG	CC	CT	GG	CC
52	IGE	27	GG	GA	CC	CT	GG	CC
53	IGE	21	GG	GA	CC	CC	GG	CC
54	IGE	21	GG	GG	CC	CC	GG	CC
55	IGE	21	GG	GG	CC	CT	GG	CT
56	PNES	22	GG	GG	CC	CC	GG	CC
57	PNES	20	GG	GG	CC	CC	GG	CC

Table D.1 (continued).

No	Patients&Controls	Age	rs967932 (G/A)	rs3780428 (G/A)	rs1999501 (C/T)	rs944688 (C/T)	G1465A (G/A)	C59T (C/T)
58	PNES	22	GG	GA	CC	CT	GG	CC
59	PNES	21	GG	GA	CC	CT	GG	CC
60	IGE	20	GG	GG	CC	CC	GG	CC
61	IGE	21	GG	GG	CC	CC	GG	CC
62	IGE	20	GG	GA	CC	CC	GG	CC
63	IGE	20	GG	GG	CC	CT	GG	CC
64	IGE	20	GG	GG	CC	CC	GG	CC
65	IGE	21	GG	GA	CC	CC	GG	CT
66	PNES	22	GG	GG	CC	CT	GG	CC
67	IGE	21	GG	GG	CC	CC	GG	CC
68	IGE	20	GG	GG	CC	CT	GG	CT
69	IGE	21	GG	AA	CC	CC	GG	CT
70	IGE	20	GA	GA	CT	CC	GG	CC
71	IGE	22	GG	GG	CC	CC	GG	TT
72	IGE	21	GG	GG	CC	CC	GG	CC
73	PNES	20	GG	GG	CC	CC	GG	CC
74	PNES	20	GG	GG	CC	CT	GG	CC
75	IGE	20	GG	GG	CC	CC	GG	CC
76	IGE	20	GA	GG	CT	CC	GG	CT
77	IGE	20	GG	GG	CC	CT	GG	CT
78	IGE	20	GG	GA	CC	CC	GG	CC
79	IGE	20	GG	GG	CC	CT	GG	CC
80	IGE	21	GA	GG	CC	CC	GG	CC
81	IGE	27	GG	GG	CC	CC	GG	CC
82	PNES	22	GG	GA	CC	CC	GG	CC
83	PNES	21	GG	GG	CC	CT	GG	CC
84	IGE	22	GG	GA	CC	CT	GG	CC
85	IGE	20	GG	GG	CC	CT	GG	CC
86	IGE	23	GG	AA	CC	CC	GG	CC
87	IGE	21	GG	GA	CC	CT	GG	CC
88	PNES	20	GG	GA	CC	CC	GG	CC
89	IGE	25	GG	GA	CC	CC	GG	CC
90	PNES	20	GG	GA	CC	CC	GG	CC
91	IGE	20	GG	GA	CC	CC	GG	CC
92	IGE	20	GG	AA	CC	CT	GG	CC
93	PNES	21	GG	GG	CC	CT	GG	CT
94	IGE	20	GA	GA	CC	CT	GG	CC
95	IGE	20	GG	AA	CC	CT	GG	CT
96	IGE	20	GG	GG	CC	CT	GG	CC

Table D.1 (continued).

No	Patients&Controls	Age	rs967932 (G/A)	rs3780428 (G/A)	rs1999501 (C/T)	rs944688 (C/T)	G1465A (G/A)	C59T (C/T)
98	PNES	20	GG	GA	CC	CC	GG	CT
99	IGE	28	GG	GA	CC	CT	GG	CC
100	IGE	20	GG	GA	CC	CC	GG	CC
101	PNES	20	GG	GA	CC	CT	GG	CC
102	PNES	23	GG	GG	CC	CT	GG	CC
103	IGE	20	GG	GG	CC	CC	GG	CC
104	IGE	25	GG	GA	CC	CT	GG	CC
105	IGE	21	GG	GG	CC	CC	GG	CC
106	PNES	21	GG	GG	CC	CT	GG	CT
107	PNES	20	GG	GA	CT	CC	GG	CC
108	PNES	27	GG	GG	CC	CT	GG	CC
109	IGE	20	GG	GA	CC	CC	GG	CC
110	IGE	24	GG	GG	CC	CC	GG	CT
111	IGE	25	GG	GG	CC	CT	GG	CC
112	IGE	21	GG	GG	CC	CT	GG	CC
113	IGE	28	GG	GG	CC	CT	GG	CC
114	IGE	22	GG	GA	CT	CC	GG	CC
115	IGE	22	GG	GG	CC	CC	GG	CC
116	IGE	20	GG	GG	CC	CT	GG	CC
117	IGE	22	GG	GG	CC	CT	GG	CC
118	IGE	21	GG	GA	CC	CT	GG	CC
119	IGE	30	GG	GG	CC	CT	GG	CC
120	PNES	20	GG	GA	CT	CC	GG	CC
121	IGE	21	GG	GG	CC	CC	GG	CC
122	IGE	23	GG	GG	CC	CC	GG	CC
123	IGE	21	GG	GA	CC	CC	GG	CC
124	IGE	20	GA	GG	CC	CC	GG	CC
125	PNES	20	GG	GG	CC	CC	GG	CT
126	PNES	21	GG	GG	CC	CC	GG	CC
127	IGE	25	GG	GG	CC	CC	GG	CC
128	IGE	22	GG	GG	CC	CT	GG	CC
129	IGE	22	GG	GG	CC	CC	GG	CC
130	IGE	39	GG	GG	CC	CC	GG	CC
131	PNES	20	GG	GG	CC	CC	GG	CC
132	IGE	20	GG	GG	CT	CC	GG	CT
133	PNES	21	GG	GG	CC	CT	GG	CC
134	PNES	20	GG	GA	CC	CC	GG	CT
135	PNES	21	GA	GG	CT	TT	GG	CC
136	PNES	20	GG	AA	CT	CT	GG	CT

Table D.1 (continued).

No	Patients&Controls	Age	rs967932 (G/A)	rs3780428 (G/A)	rs1999501 (C/T)	rs944688 (C/T)	G1465A (G/A)	C59T (C/T)
137	IGE	20	GG	GG	CT	CT	GG	CC
138	PNES	20	GG	GA	CC	CC	GG	CC
139	IGE	21	GG	GA	CT	CC	GG	CC
140	PNES	21	GG	GG	CC	CC	GG	CC
141	PNES	21	GG	GG	CC	CC	GG	CT
142	PNES	23	GG	GG	CC	CT	GG	CC
143	IGE	29	GG	GG	CC	CT	GG	CC
144	IGE	31	GG	GA	CC	CC	GG	CC
145	IGE	21	GG	GG	CC	CT	GG	CC
146	PNES	20	GG	GG	CC	CT	GG	CC
147	IGE	-	GG	GA	CT	CT	GG	CC
148	IGE	-	GG	GG	CT	CC	GG	CT
149	PNES	21	GG	GA	CC	CT	GG	CC
150	IGE	21	GA	GG	CT	CC	GG	CT
151	PNES	20	GG	GG	CC	CT	GG	CC
152	PNES	20	GG	GG	CC	CT	GG	CC
153	PNES	21	GG	GA	CC	CT	GG	CC
154	IGE	20	GG	GA	CC	CC	GG	CC
155	IGE	20	GA	GG	CT	CC	GG	CT
156	PNES	24	GG	GG	CC	CC	GG	CC
157	IGE	20	GG	GG	CC	CC	GG	CC
158	IGE	-	GG	GG	CT	CC	GG	CC
159	IGE	-	GG	GG	CC	CC	GG	CC
160	IGE	20	GG	GG	CC	CC	GG	CC
161	PNES	-	GG	GG	CC	CT	GG	CC
162	IGE	23	GG	GG	CC	CC	GG	CC
163	IGE	20	GG	GG	CC	CC	GG	CC
164	PNES	-	GG	AA	CC	CC	GG	CC
165	PNES	-	GG	GG	CC	CT	GG	CC
166	IGE	20	GG	GA	CT	CC	GG	CT
167	IGE	20	GG	GG	CC	CC	GG	CC
168	IGE	20	GG	GG	CC	CC	GG	CC
169	IGE	20	GG	GG	CC	CT	GG	CC
170	IGE	20	GG	GG	CC	CC	GG	CC
171	IGE	20	GG	AA	CC	CT	GG	CC
172	IGE	20	GG	GG	CC	CC	GG	CC
173	IGE	22	GA	GG	CC	CC	GG	CC
174	IGE	20	GG	GA	CC	CC	GG	CC
175	PNES	21	GG	GG	CT	CC	GG	CC

Table D.1 (continued).

No	Patients&Controls	Age	rs967932 (G/A)	rs3780428 (G/A)	rs1999501 (C/T)	rs944688 (C/T)	G1465A (G/A)	C59T (C/T)
176	PNES	23	GG	GG	CC	TT	GG	CT
178	IGE	25	GG	GA	CC	CT	GG	CC
179	IGE	22	GG	GG	CC	CC	GG	CC
180	IGE	19	GG	GA	CC	CC	GG	CT
181	PNES	19	GG	GA	CC	CC	GG	CC
182	IGE	26	GG	GG	CC	CC	GG	CC
183	PNES	23	GG	AA	CC	TT	GG	CC
184	IGE	20	GA	GA	CC	CT	GG	CC
185	IGE	20	GG	GG	CC	CT	GG	CC
186	PNES	21	GG	GG	CC	CC	GG	CC
187	IGE	21	GG	GG	CC	CT	GG	CC
188	IGE	20	GG	GA	CC	CC	GG	CC
189	IGE	20	GG	GA	CC	CC	GG	CC
190	IGE	20	GG	GG	CC	CT	GG	CC
191	IGE	21	GG	GA	CC	CC	GG	CC
192	PNES	25	GG	GA	CC	CT	GG	CT
193	PNES	23	GG	GG	CC	CT	GG	CC
194	PNES	27	GG	GA	CC	CT	GG	CC
195	PNES	20	GG	GG	CC	CC	GG	CC
196	PNES	25	GG	GG	CC	CC	GG	CC
197	IGE	20	GG	GG	CC	CC	GG	CC
198	IGE	21	GG	GA	CC	CT	GG	CT
199	IGE	-	GG	GG	CC	CC	GG	CC
200	IGE	22	GG	GA	CC	CC	GG	CC
201	PNES	20	GG	GG	CC	TT	GG	CC
202	IGE	-	GG	GA	CC	CT	GG	CC
203	PNES	-	GG	GG	CT	CC	GG	CT
204	IGE	19	GG	AA	CC	CC	GG	CC
205	IGE	-	GG	GG	CC	CC	GG	CC
206	IGE	31	GG	AA	CC	CC	GG	CT
207	IGE	-	GG	AA	CC	CT	GG	CT
208	IGE	-	GG	GG	CC	CT	GG	CC
209	IGE	-	GG	GG	CC	CT	GG	CC
210	PNES	-	GG	AA	CC	TT	GG	CC
211	IGE	29	GG	GA	CC	CC	GG	CC
212	PNES	21	GG	GA	CC	CC	GG	CC
213	PNES	32	GG	GG	CC	CC	GG	CC
214	PNES	20	GG	GG	CC	CC	GG	CC
215	IGE	23	GA	GA	CC	CT	GG	CT

Table D.1 (continued).

No	Patients&Controls	Age	rs967932 (G/A)	rs3780428 (G/A)	rs1999501 (C/T)	rs944688 (C/T)	G1465A (G/A)	C59T (C/T)
216	IGE	21	GG	GA	CC	CC	GG	CC
217	CONTROL	19	GG	GG	CC	CC	GG	CC
218	CONTROL	21	GG	GA	CC	CT	GG	CC
219	CONTROL	21	GG	GG	CC	CC	GG	CT
220	CONTROL	20	GG	GG	CC	CC	GG	CT
221	CONTROL	21	GG	AA	CC	CT	GG	CC
222	CONTROL	28	GG	AA	CC	TT	GG	CC
223	PNES	21	GG	GA	CC	CT	GG	CT
224	CONTROL	21	GG	GG	CC	CT	GG	CT
225	CONTROL	21	GG	GG	CC	CC	GG	CC
226	IGE	23	GG	GA	CC	CT	GG	CC
227	CONTROL	21	GG	GA	CC	CT	GG	TT
228	PNES	21	GG	GG	CC	CT	GG	CC
229	IGE	20	GG	GG	CC	CT	GG	CC
230	CONTROL	22	GG	GG	CC	CT	GG	CC
231	CONTROL	22	GG	GG	CC	CC	GG	CC
232	IGE	22	GG	GA	CC	CC	GG	CC
233	IGE	22	GA	AA	CT	CC	GG	CC
234	CONTROL	32	GG	GA	CC	CC	GG	CC
235	IGE	20	GG	GA	CC	CT	GG	CC
236	CONTROL	30	GG	GA	CC	CT	GG	CC
237	PNES	23	GG	GG	CC	CT	GG	CC
238	CONTROL	27	GG	GG	CC	CC	GG	CC
239	IGE	23	GG	GA	CC	TT	GG	CC
240	CONTROL	21	GG	GG	CC	CT	GG	CC
241	CONTROL	26	GG	GG	CC	CC	GG	CC
242	IGE	23	GG	GA	CT	CT	GG	CC
243	IGE	23	GA	GA	CC	CC	GG	CC
246	CONTROL	24	GG	GG	CC	CT	GG	CC
247	CONTROL	19	GG	GG	CC	CT	GG	CC
248	CONTROL	-	GG	GG	CC	CC	GG	CC
249	IGE	21	GG	GA	CC	CC	GG	CC
250	PNES	21	GG	GG	CC	CC	GG	CC
251	IGE	27	GG	GG	CC	CC	GG	CC
253	IGE	21	GA	GA	CC	CT	GG	CC
254	CONTROL	21	GG	GA	CC	CC	GG	CC
255	CONTROL	21	GG	GA	CC	CC	GG	CC
256	CONTROL	21	GG	GG	CC	CC	GG	CT
257	PNES	32	GG	GG	CC	CC	GG	CC

Table D.1 (continued).

No	Patients&Controls	Age	rs967932 (G/A)	rs3780428 (G/A)	rs1999501 (C/T)	rs944688 (C/T)	G1465A (G/A)	C59T (C/T)
258	PNES	21	GA	AA	CT	CC	GG	CC
259	CONTROL	23	GG	GA	CC	CC	GG	CT
260	PNES	21	GA	GA	CT	CC	GG	CC
261	PNES	32	GG	GG	CC	CT	GG	CC
263	IGE	23	GG	GG	CC	CT	GG	CT
264	CONTROL	22	GG	AA	CC	TT	GG	CC
265	IGE	19	GG	GA	CC	CC	GG	CC
266	PNES	27	GG	AA	CC	CC	GG	CC
267	IGE	21	GG	GG	CC	CC	GG	CC
268	CONTROL	20	GG	GA	CC	CT	GG	CC
269	CONTROL	20	GA	GA	CC	CT	GG	CC
271	CONTROL	26	GG	GG	CC	CC	GG	CC
272	CONTROL	20	GG	GA	CC	CC	GG	CC
273	PNES	20	GG	GG	CC	CC	GG	CC
274	IGE	21	GA	GG	CT	CC	GG	CC
275	IGE	23	GG	GA	CC	CC	GG	CT
277	CONTROL	21	GG	GA	CC	CC	GG	CC
278	IGE	20	GG	GG	CT	CC	GG	CC
279	CONTROL	21	GG	GA	CC	CC	GG	CC
280	PNES	23	GG	GG	CC	CT	GG	CC
281	CONTROL	21	GG	GA	CT	CC	GG	CC
282	PNES	21	GG	GG	CC	CC	GG	CC
283	IGE	27	GG	GG	CC	CC	GG	CC
284	CONTROL	20	GA	GG	CT	CC	GG	CT
285	PNES	20	GG	GG	CC	CT	GG	CC
286	CONTROL	21	GG	GA	CC	CT	GG	CC
288	CONTROL	22	GG	GG	CC	CC	GG	CC
290	CONTROL	23	GG	GG	CC	CC	GG	CT
291	IGE	20	GG	GG	CC	CC	GG	CC
292	CONTROL	26	GG	GG	CC	CT	GG	CC
293	CONTROL	26	GG	GA	CC	CC	GG	CC
295	IGE	20	GG	AA	CC	CC	GG	CC
296	CONTROL	24	GG	GA	CC	CC	GG	CC
297	CONTROL	-	GG	GG	CC	CC	GG	CC
298	CONTROL	23	GG	GA	CT	CC	GG	CC
301	PNES	25	GG	GG	CC	CC	GG	CC
302	CONTROL	26	GA	GG	CT	CC	GG	CC
305	CONTROL	21	GG	GG	CC	CT	GG	CC
306	IGE	22	GA	GG	CT	CT	GG	CT

Table D.1 (continued).

No	Patients&Controls	Age	rs967932 (G/A)	rs3780428 (G/A)	rs1999501 (C/T)	rs944688 (C/T)	G1465A (G/A)	C59T (C/T)
309	PNES	20	GG	GA	CC	CT	GG	CC
310	CONTROL	26	GG	GG	CC	CC	GG	CC
311	CONTROL	-	GA	GA	CT	CC	GG	CC
313	CONTROL	24	GG	GG	CT	CT	GG	CC
315	CONTROL	20	GG	GG	CT	CC	GG	CC
316	CONTROL	22	GG	GG	CC	CT	GG	CT
318	CONTROL	24	GG	GG	CC	CC	GG	CC
320	CONTROL	24	GG	GA	CC	CT	GG	CC
321	CONTROL	21	GG	GG	CC	CT	GG	CC
322	CONTROL	20	GG	GG	CC	CC	GG	CC
323	CONTROL	21	GG	GA	CC	TT	GG	CC
324	CONTROL	30	GG	GA	CC	CT	GG	CT
325	CONTROL	29	GG	GA	CC	CC	GG	CT
329	IGE	20	GG	AA	CC	CC	GG	CC
330	CONTROL	22	GG	GG	CC	CC	GG	CC
331	CONTROL	22	GG	GA	CC	CC	GG	CT
332	PNES	20	GG	GA	CC	CC	GG	CC
334	CONTROL	21	GG	GA	CC	CT	GG	CC
335	CONTROL	21	GG	GA	CC	CT	GG	CC
337	CONTROL	20	GG	GA	CC	CC	GG	CC
342	CONTROL	20	GA	GA	CT	CC	GG	CT
347	IGE	20	GG	GG	CC	CC	GG	CT
348	CONTROL	20	GG	GG	CC	CC	GG	CC
350	CONTROL	21	GG	GA	CC	CC	GG	CC
354	CONTROL	22	GG	GA	CC	CC	GG	CT
355	CONTROL	20	GG	GG	CC	CC	GG	CC
356	CONTROL	20	GG	AA	CC	CT	GG	CC
360	IGE	21	GG	GG	CC	CC	GG	CC
361	CONTROL	20	GG	GG	CC	CC	GG	CC
362	CONTROL	24	GA	GG	CT	CT	GG	CC
363	PNES	21	GG	GG	CC	CC	GG	CC
364	CONTROL	23	GG	GA	CC	CT	GG	CC
365	CONTROL	24	GG	GG	CC	CC	GG	CT
366	CONTROL	32	GG	GG	CC	CC	GG	TT
368	CONTROL	26	GG	GG	CC	CC	GG	CC
369	IGE	25	GG	GG	CT	CT	GG	CC
370	CONTROL	21	GG	GA	CC	CT	GG	CC
371	IGE	24	GG	AA	CC	CC	GG	CC
372	PNES	19	GG	GG	CC	CC	GG	CC

Table D.1 (continued).

No	Patients&Controls	Age	rs967932 (G/A)	rs3780428 (G/A)	rs1999501 (C/T)	rs944688 (C/T)	G1465A (G/A)	C59T (C/T)
374	CONTROL	21	GG	GG	CT	CC	GG	CC
375	IGE	20	GG	GG	CC	CT	GG	CC
376	IGE	20	GG	GA	CC	CC	GG	CC
377	CONTROL	20	GG	GA	CC	TT	GG	CC
378	CONTROL	20	GG	GG	CC	CC	GG	CC
379	CONTROL	21	GG	GG	CT	CC	GG	CC
380	CONTROL	26	GG	GG	CC	CC	GG	CC
382	CONTROL	22	GG	GG	CC	CT	GG	CC
384	CONTROL	24	GG	GG	CC	CT	GG	CC
385	CONTROL	20	GG	GG	CT	CC	GG	CC
391	CONTROL	21	GG	GA	CC	CT	GG	CC
392	CONTROL	24	GG	GA	CT	CC	GG	CC
393	CONTROL	19	GG	GG	CC	CT	GG	CC
394	CONTROL	20	GG	GG	CT	CC	GG	CC
395	CONTROL	21	GG	AA	CT	CC	GG	CC
396	CONTROL	20	GG	GG	CT	TT	GG	CC