FUNCTIONAL ANALYSIS OF A SPLICE SITE MUTATION IN GABRG2 GENE AND ITS EFFECT ON GABAA RECEPTOR

by

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To those suffering from epilepsy

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ABSTRACT

FUNCTIONAL ANALYSIS OF A SPLICE SITE MUTATION IN GABRG2 GENE AND ITS EFFECT ON GABAA RECEPTOR

Epilepsy is a group of diseases produced by non-controlled discharge of neurons affecting more than 2% of the population and some forms include genetic determinants. The principal group of inherited epilepsy is Genetic Generalized Epilepsies (GGE) and Childhood Absence Epilepsy (CAE) is a common subtype of GGE. Gamma-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian brain. It acts through two classes of receptors: $GABA_A$ and $GABA_B$ receptors. Impairment of GABAergic transmission by genetic mutations cause epileptic seizures. $GABA_A$ receptor, gamma 2 which is located on 5q34, is encoded by GABRG gene. Besides mutations which are in coding region in *GABRG2* gene, mutations in intronic sites of the gene are also observed. It has been shown that a T-G transversion (*IVS6+2T-G)* mutation altered the *GABRG2* intron 6 splice donor site sequence, activated a cryptic splice site and generated partial intron retention. The aim of this study was to investigate the effect of an other splice site variant of *GABRG2* gene detected in a CAE patient on $GABA_A$ receptor. Scores of splice site tools showed that this mutation could create cryptic splice site. By site directed mutagenesis, the known mutation was created and cloned with the *GABRG2* variant in the patient by creating minigenes. After transfection, expressions were checked by RT- PCR. Under similar experimental conditions while the splicing defect of the known mutation was observed, *GABRG2* splice site variant in the CAE patient had no effect on splicing suggesting that it was a silent variant of the gene.

ÖZET

GABRG2 GENİNDE BULUNAN BİR KIRPILMA BÖLGE MUTASYONUNUN FONKSİYONEL ANALİZİ VE GABAA RESEPTÖRÜNE ETKİSİ

Epilepsi, nüfusun yüzde ikiden fazlasını etkileyen, kontrol edilemeyen nöron deşarjları şeklinde ortaya çıkan bir hastalıktır ve bazı formları genetik belirleyiciler içermektedir. Kalıtsal epilepsilerin ana grubu genetik jeneralize epilepsi (GJE) dir ve çocukluk çağı epilepsisi (ÇÇE), GJE'nin yaygın olan bir alt grubudur. Gama-aminobütirik asit (GABA), memeli beynindeki temel inhibitör nörotransmitterdir. Bu reseptör $GABA_A$ ve $GABA_B$ olmak üzere iki reseptör sınıfıyla görev yapar. GABAerjik iletimin genetik mutasyonlarla bozulması epileptik nöbetlere sebep olmaktadır. Beşinci kromozomun uzun kolunun 34. bölgesinde bulunan $GABA_A$ reseptör gama 2, GABRG geni tarafından kodlanır. *GABRG2* geninin kodlayıcı kısmında bulunan mutasyonların yanı sıra, bu genin intronik kısmında da mutasyonlar gözlenmektedir. T-G transversiyonunun (IVS6+2T-G) *GABRG2*'da 6. intronun kırpılma donör bölgesini değiştirdiği, kriptik kırpılma bölgesini aktive ettiği ve parsiyel intron eklenmesi yarattığı bilinmektedir. Bu çalışmanın amacı CAE hastasında tespit edilen *GABRG2* geni kırpılma varyantının $GABA_A$ reseptörüne etkisinin incelenmesidir. *In silico*-kırpılma bölgesi araçları bu mutasyonun kriptik kırpılma bölgesi yaratabileceğini göstermiştir. Yer yönlendirmeli mutagenez ile *GABRG2* geninde bilinen mutasyon yaratılmış ve hastadaki varyantla beraber klonlanarak mini genler yaratılmıştır. Transfeksiyon sonrası ekspresyonlar RT-PCR ile incelenmiştir. İlgili mutasyonun kırpılmaya etkisi benzer deneysel koşullarda daha önce gösterilmemiş olup ÇÇE hastasındaki *GABRG2* varyantının sessiz bir varyant olduğu ve kırpılma işlemini değiştirmediği gösterilmiştir.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iv
ABSTRACT v
ÖZET vi
LIST OF FIGURES x
LIST OF TABLES xiii
LIST OF SYMBOLS xv
LIST OF ACRONYMS/ABBREVIATIONS xvi
1. INTRODUCTION 1
1.1. Absence Epilepsies 1
1.2. Cellular and Network Mechanism of Absence Seizures 2
1.3. γ-aminobutyric Acid (GABA) 3
1.4. GABA Receptors 4
1.4.1. $GABA_A$ Receptors ($GABA_ARs$) 4
1.4.1.1. Gene Organization 4
1.4.1.2. Structure of $GABA_A$ Receptors 6
1.4.1.3. Mechanism 7
1.4.2. $GABA_B$ Receptors ($GABA_BRs$) 7
1.5. *GABRG2* Gene 10
2. PURPOSE 13
3. MATERIALS 14
3.1. CAE Patient 14
3.2. Primers 14
3.2.1. PCR Primers for *GABRG2* Exon 2 and 7 14
3.2.2. Primers for PCR Based Site Directed Mutagenesis 15
3.2.3. Primers Primers for Attachment of attB Recombination Sites 15
3.2.4. Primers for Reverse Transcription 16
3.3. Enzymes 16

3.4. Chemicals 17
3.5. Buffers and Solutions 18
3.6. Kits 18
3.7. Plasmid 19
3.8. Bacteria & Cell and Growth 20
3.9. Equipments 21
4. METHODS 23
4.1. DNA Analysis 23
4.1.1. DNA Extraction from White Blood Cells 23
4.1.2. Salt Extraction 23
4.1.3. Analysis by Agarose Gel Electrophoresis 24
4.1.4. Analysis by Spectrophotometer 24
4.1.5. Analysis by Splice Site Tools 24
4.2. PCR 25
4.2.1. PCR for *GABRG2* of the CAE Patient 25
4.2.1.1. Sequence Analysis of the CAE Patient 25
4.2.2. PCR for Site Directed Mutagenesis in *GABRG2* 26
4.3. Cloning 30
4.3.1. Preparation of the Insert 32
4.3.1.1. Attachment of attB Recombination sites by PCR 32
4.3.2. Preparation of the Vector 33
4.3.2.1. Plasmid Isolation 33
4.3.2.2. Concentration Measurement by Fluorometer 33
4.3.2.3. Recombination 34
4.3.3. Transformation into DH-5 alpha Cells 35
4.3.4. Purification of Plasmid DNA 36
4.3.5. Sequencing of the Plasmid DNA 36
4.4. Expression Analysis 37
4.4.1. Transfection into Shsy-5y Neuroblastoma Cells 37
4.4.2. RNA Isolation 38

4.4.3. Reverse Transcription 38
5. RESULTS 41
5.1. Mutational Analysis of the Patient 41
5.1.1. PCR Result of S1 and WT1 41
5.1.2. Confirmation of the Mutation by Re-sequencing 42
5.1.3. Splice Site Tool Results 43
5.2. Site Directed Mutagenesis 44
5.2.1. PCR Result of S2 and WT2 44
5.2.2. Sequencing Result After Mutagenesis 45
5.3. Cloning 46
5.3.1. Restriction Analysis 46
5.3.2. Sequencing of the Plasmids 48
5.4. Expression Analysis 49
5.4.1. Transfection into Shsy-5y Neuroblastoma Cells 49
5.4.2. RNA Isolation 49
5.4.3. Reverse Transcriptase PCR 50
5.4.4. Sequencing of RT-PCR Products 51
6. DISCUSSION 53
6.1. Conclusion 56
REFERENCES 57

LIST OF FIGURES

Figure 1.1. Cellular elements of thalamic and cortical network involved in spike-wave generation. 3

Figure 1.2. Phylogenetic tree analysis of the 19 known genes coding for human $GABA_A$ receptor subunits. 5

Figure 1.3. a) structure of a $GABA_A$ receptor subunit b) structure of a $GABA_A$ receptor. 6

Figure 1.4. $GABA_A$ receptor subunit gene mutations associated with genetic epilepsy syndromes. 8

Figure 3.1. The map of pSpliceExpress cloning vector. 19

Figure 3.2. The exonic map of pSpliceExpress cloning vector. 20

Figure 4.1. Amplified region for exon 2. 25

Figure 4.2. Amplified region for exon 7. 27

Figure 4.3. Exon 7 sequence. 27

Figure 4.4. Diagram of the PCR based site directed mutagenesis method. 29

Figure 4.5. Overview of the minigene method. 30

Figure 4.6. Illustration of mRNA structures that are expected to be expressed. 32

Figure 5.1. Amplified region for exon 2 and PCR products visualized on 2% agarose gel. 41

Figure 5.2. Reference sequence showing the mutation in the intronic area and chromatograms of the wild type and the patient showing that the T->C transition. 42

Figure 5.3. Results of ESEfinder analysis. 43

Figure 5.4. Amplified region for exon 7 and PCR products visualized on 2% agarose gel. 44

Figure 5.5. Reference sequence showing the mutation in the intronic area and chromatograms of the wild-type and the site directed mutagenesis product showing that the T->C transversion. 45

Figure 5.6. *Kpn I* sites placed in the vector. 46

Figure 5.7. Purified plasmids taken from different single colonies on 1% agarose gel. 47

Figure 5.8. Purified plasmids taken from different single colonies on 1% agarose gel. 47

Figure 5.9. Chromatogram showing the sequence of plasmid that contains T->C mutation in the patient. 48

Figure 5.10. Chromatogram showing the sequence of plasmid that contains T->G mutation in the site directed mutagenesis product. 48

Figure 5.11. Cell placement and morphology before and after the differentiation..... 49

Figure 5.12. Isolated RNAs on 2% agarose gel. 50

Figure 5.13. RT-PCR products on 2% agarose gel. 50

Figure 5.14. IVS6+2T->G mutation causes retention after the exonic region. 51

Figure 5.15. Chromatogram shows that the wild type 2 (WT2), the site directed mutagenesis that is changed the splicing and caused retention after the exonic region and alternative splicing that contains only rat exons 2 and 3. 51

Figure 5.16. Chromatogram shows that the wild type 1 (WT1), the patient and alternative splicing that contains only rat exons 2 and 3. 52

Figure 6.1. Basic mechanisms of alternative splicing. 54

LIST OF TABLES

Table 1.1. Table 1.1. $GABA_A$ receptor subunit mutation/variants and their structural features. 9

Table 3.1. Oligonucleotide primer pairs used in PCR and Sequencing. 14

Table 3.2. Oligonucleotide primer pairs used in PCR for Site Directed Mutagenesis. 15

Table 3.3. Oligonucleotide primer pairs used in PCR for attachment sites. 15

Table 3.4. Oligonucleotide primer pairs used in Reverse Transcription. 16

Table 3.5. Enzymes that are used in the study. 16

Table 3.6. Chemicals that are used in the study. 17

Table 3.7. Buffers and solutions that are used in the study. 17

Table 3.8. Kits that are used in the study. 18

Table 3.9. Bacteria, cell and growth materials that are used in the study. 20

Table 3.10. Equipments that are used in the study. 21

Table 4.1. Protocol of standard curve preparation. 34

Table 4.2. Required materials and their amounts for recombination process. 35

Table 4.3. Concentrations and required amount of samples for each plasmid. 37

Table 4.4. Composition of Reverse-Transcription Master Mix. 39

Table 4.5. Composition of RT-PCR reaction. 39

Table 5.1. Scores for ASSP analysis. 43

Table 5.2. Scores for ESEfinder analysis. 43

LIST OF SYMBOLS

α	alpha
β	beta
γ	gamma
ρ	rho
δ	delta
ε	epsilon
θ	theta
π	pi

LIST OF ACRONYMS/ABBREVIATIONS

5q	Long arm of chromosome 5
A	Adenine
bp	Base pair
C	Cytosine
CAE	Childhood Absence Epilepsy
Ca	Calcium
cDNA	Complementary Deoxyribonucleic acid
CNS	Central Nervous System
Cys	Cysteine
dH_2O	Distilled water
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleodites
DS	Dravet Syndrome
EEG	Electoensephalogram
EtBr	Ethidium Bromide
ETDA	Ethylenediaminetetraacetate

FC	Febrile convulsion
FS	Febrile Seizure
fmol	Femtomole
G	Guanine
GABA	γ–aminobutyric acid
$GABA_A$	γ–aminobutyric acid A receptor
$GABA_B$	γ–aminobutyric acid B receptor
$GABA_C$	γ–aminobutyric acid A receptor
GABRG2	γ–aminobutyric acid A receptor, gamma 2
GABRs	γ–aminobutyric acid receptors
GEFS+	Generalized Epilepsy with Febrile Seizures Plus
GTCS	Generalized tonic-clonic seizure
GPCR	G protein coupled receptor
IGE	Idiopathic Generalized Epilepsy
IPSC	Inhibitory postsynaptic current
IPSP	Inhibitory postsynaptic potential
JAE	Juvenile Absence Epilepsy
JME	Juvenile Myoclonous Epilepsy

K	Potassium
kb	Kilobase
µl	Microliter
µM	Micromolar
$MgCl_2$	Magnesium Chloride
mM	Milimolar
mRNA	Messenger Ribonucleic acid
NaCl	Sodium chloride
ng	Nanogram
nm	Nanometer
NMD	Nonsense mediated decay
nRT	Thalamic Reticular Nucleus
PCR	Polymerase Chain Reaction
PTC	Premature termination codon
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse Transcriptase PCR
sdm	Site directed mutagenesis

SWD	Spike wave discharge
TBE	Tris-Boric acid-EDTA
TE	Tris-EDTA
TM	Transmembrane
U	Unit
UV	Ulta Violet

1. INTRODUCTION

Epilepsy is a group of diseases produced by non-controlled discharge of neurons of either whole cortex which is called generalized epilepsies or localized brain areas which is called partial epilepsies (Moulard *et al.*, 2001). There are a few subtypes of epilepsy and according to the etiology, the classification contains generalized and symptomatic epilepsies. Symptomatic epilepsies include birth accidents, abnormal neurodevelopment, injection, vascular diseases, head trauma and brain tumors. In generalized epilepsy, genetics has an important role and is mainly related to genes encoding for ion channels, both voltage-gated and ligand-gated (Lemoine *et al.*, 2012, Gallentine and Mikati, 2012).

Epilepsies affect more than 2% of the population and the most common group of inherited epilepsy is genetic generalized epilepsies (GGE) which consist of three prevalent forms: childhood absence epilepsy (CAE), juvenile absence epilepsy (JAE) and juvenile myoclonous epilepsy (JME), but GGE has very complex inheritance (Wallace *et al.*, 2001).

1.1. Absence Epilepsies

Childhood absence epilepsy (CAE), also called as pyknolepsy is a common subtype of idiopathic generalized epilepsy (GGE) which is characterized by daily clusters of absence seizures appearing at an age of onset between 2 and 12 years. Absence seizures are a type of seizure that consists of the main symptom of a brief lapse of consciousness. The seizures are categorized into typical and atypical absences depending on their clinical and EEG characteristics (Ito *et.al.*, 2005). The most common seizure subtypes are febrile convulsions (FCs) which affects 3 to 5% of children younger than 6 years. JAE, on the other hand, is characterized by sporadic (non-pyknoleptic) occurrence of absence seizures generally associated with generalized tonic-clonic seizures (GTCS) and myoclonic jerks with an age of onset around puberty (Hughes, 2009).

CAE and FC have well established genetic basis. Due to the high incidence of FC in patients with CAE (10 to 15 percent) compared with general population, there is a proposed genetic overlap between both of these disorders. This common molecular basis is most obviously observed in the syndrome of generalized epilepsy with febrile seizures plus (GEFS+), a monogenic or oligogenic epilepsy that was first explained in 1997. GEFS+ is characterized by FCs that can continue after the age of 6 and usually gone along by generalized seizures including myoclonic and absence seizures (Kananura *et al.*, 2002).

1.2. Cellular and Network Mechanism of Absence Seizures

SWD (spike wave discharge) is defined as particular electroencephalographic pattern which has some characteristics like regular, symmetrical, generalized and transient observed in most of the types of epilepsy, especially in typical absence seizure (Akman *et al.*, 2010). SWDs include many cortical and subcortical structures like brainstem and basal ganglia. But, spike wave generation has a critical role for the highly interconnected circuitry of cortex and thalamus (Seidenbecher *et al.*, 1998, Blumenfeld, 2005).

Sensory and other inputs come to the thalamocortical relay cells (green) placed in the thalamus. Thalamocortical cells send excitatory connections to cortical layers III–IV, also to dendrites of layer V–VI pyramidal cells (blue). Cortical pyramidal cells, mostly from layer VI send reciprocal excitatory projections back to the same specific thalamic nuclei. Inhibitory GABAergic neurons (red) are placed throughout the cortex and thalamus. However, they make an almost pure layer of GABAergic cells in the thalamic reticular nucleus (nRT).

Axon collaterals from the reciprocal thalamocortical (green) and corticothalamic (blue) pathways send excitatory projections to nRT neurons passing through this nucleus. In turn, nRT neurons their send inhibitory GABAergic projections to reciprocal places of the thalamus, but not the cortex. nRT neurons are also attached to each other through inhibitory GABAergic synapses as together with gap junctions (McCormick and Contreras, 2001) (Figure 1.1).

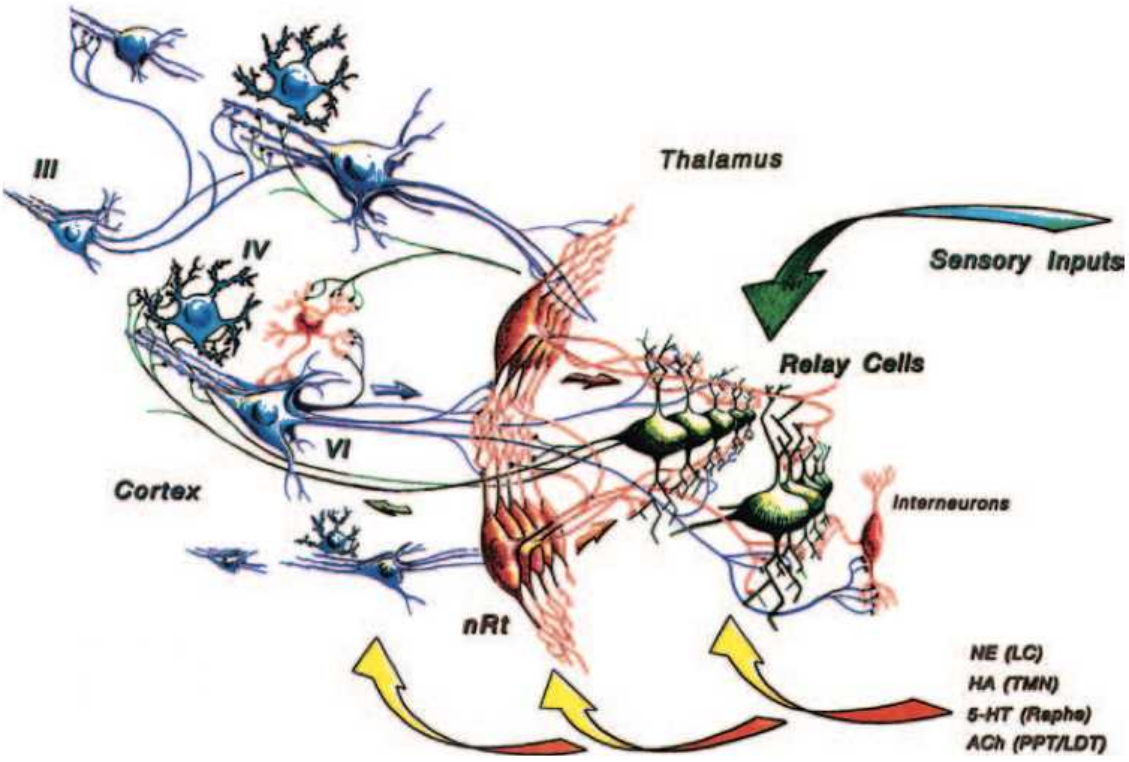

Figure 1.1. Cellular elements of thalamic and cortical network involved in spike-wave generation (Blumenfeld, 2005).

1.3. γ-aminobutyric Acid (GABA)

Some of the amino acids exist in high concentrations in brain, and some have been stated as neurotransmitters. L- Glutamic acid (glutamate) is the main neurotransmitter for fast excitatory synaptic transmission, whereas the γ–aminobutyric acid (GABA) is taken into account as a principal neurotransmitter for fast inhibitory synaptic transmission. Glycine which is placed mostly in the spinal cord, is a secondary rapid inhibitory neurotransmitter (Kumar and Kuppast, 2012).

GABA which is main inhibitory neurotransmitter in the vertebrate central nervous system (CNS), regulates every aspect of brain function and neurons that generate GABA as their product are named as GABAergic neurons (Fritschy and Brünig, 2003). GABA functions with binding to specific transmembrane receptors in the plasma membrane of

both pre and postsynaptic neuronal processes at inhibitory synapses in the brain. This binding leads to the opening of ion channels letting the flow of negatively charged chloride ions into the cell or positively charged potassium ions out of the cell. According to the preference of the opening of ion channels, the membrane potential is either hyperpolarized or repolarized. This phenomenon is concluded as a negative change in the transmembrane potential, generally inducing hyperpolarization (Kumar and Kuppast, 2012).

1.4. GABA Receptors

There are three principal classes of receptors that inhibitory neurotransmitter GABA activates, which are $GABA_A$, $GABA_B$ and $GABA_C$ receptors. $GABA_A$ and $GABA_C$ receptors are ligand-gated, which is also named as ionotorpic receptors; $GABA_B$ receptors are G protein-coupled receptors that is also named as metabotropic receptors (Chebib and Johnston, 1999).

1.4.1. $GABA_A$ Receptors ($GABA_ARs$)

1.4.1.1. Gene Organization. The $GABA_A$ receptors are part of a Cys loop-type ligand-gated ion channel superfamily that also contains the nicotinic cholinergic, glycine and serotonin 5-HT_3 receptors and the major mediators of fast inhibitory synaptic transmission in the CNS (Macdonald *et al.*, 2010).

All eukaryotic members of the Cys loop family carry the same motif consisting of two cysteine residues which are 13 amino acid residues apart from each other. In human, these receptors are separated into seven subunit classes that include six α subunits, three β subunits, three γ subunits, three ρ subunits (building blocks of the previously named $GABA_C$ receptors which is a subclass of $GABA_A$ receptors), and one each of the δ, ε , θ and π subunits. Within a subunit family, there is approximately 70% sequence identity; 20 % sequence identity or 50% sequence similarity exists between members of different families. The $GABA_A$ receptor subunit genes show a basic pattern consisting of nine coding exons and eight introns with two exceptions: δ contains 8 exons, and γ_3 contains 10

exons. For several subunits, there are some splice variants. However, their function and physiological role is not yet totally understood. As shown in Figure 1.2, there is a phylogenetic tree of the human $GABA_A$ receptor subunits and their chromosomal distribution and most of the genes that are coding the $GABA_A$ receptor subunits are separated into four clusters on chromosomes 4, 5, 15, and X in the human genome. This gene clustering is thought to provide organized gene expression. Two cluster groups contain four genes: α_2, α_4, β_1 and γ_1 on chromosome 4; α_1, α_6, β_2 and γ_2 on chromosome 5 and two cluster groups contain three genes, α_5, β_3 and γ_3 on chromosome 15 and α_3, ε and θ on chromosome X (Sigel and Steinmann, 2012, Arain *et al.*, 2012).

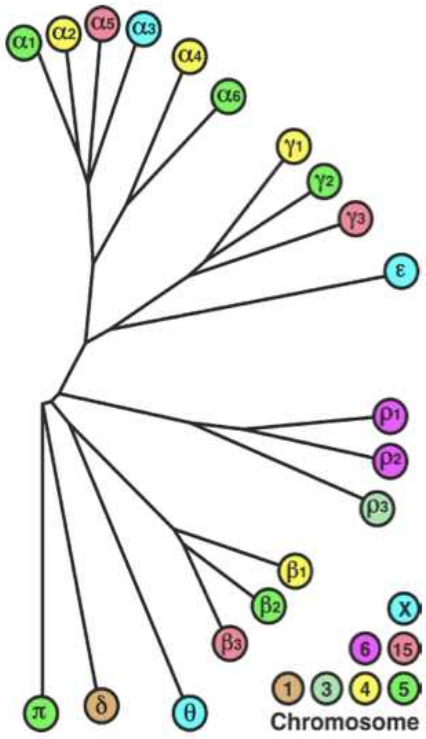

Figure 1.2. Phylogenetic tree and chromosomal distribution of the 19 known genes coding for human $GABA_A$ receptor subunits (Sigel and Steinmann, 2012).

1.4.1.2. Structure of $GABA_A$ Receptors. The $GABA_A$ receptors consist of multisubunit proteins and when examining the characteristics of a single mature subunit, its length is around 450 amino acid residues and it has a common topological organization. Around half of the subunit contains a hydrophilic extracellular N-terminal domain including the Cys loop, followed by four transmembrane sequences from TM1 to TM4 and the ion channel resides on TM2 (Figure 1.3). A large intracellular loop which is responsible in modulation by phosphorylation is placed between TM3 and TM4. There are also several proteins interacting with the intracellular loop between TM3 and TM4 of specific $GABA_A$ receptor subunits that have significant roles in receptor trafficking and anchoring of receptors in the cytoskeleton and in the postsynaptic membrane. Both N and C termini of the subunits are in extracellular region.

A big number of $GABA_A$ receptors may exist in a cell, even more than eight subunit isoforms have shown that has been expressed in a single cell. Moreover, alternative splicing and RNA editing provide to receptor diversity. The major adult isoform is mostly agreed to be consisted of α_1 , β_2, and γ_2 subunits. There is still high level of uncertainty for the number of different $GABA_A$ receptor isoforms that are existing in nature (Sigel and Steinmann, 2012).

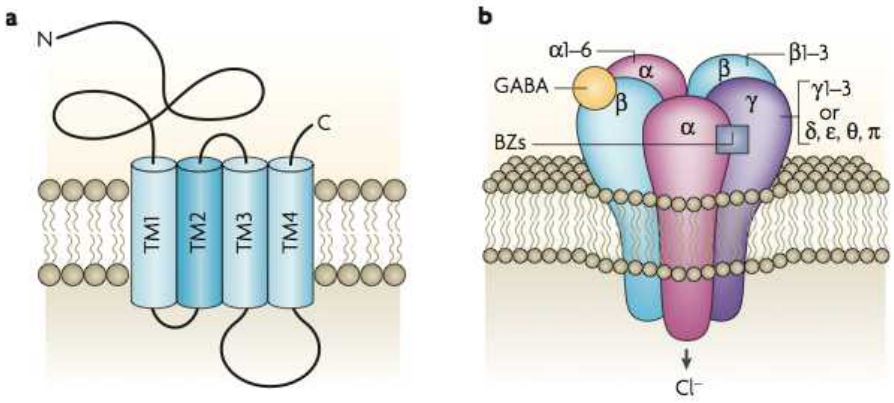

Figure 1.3. a) structure of a $GABA_A$ receptor subunit b) structure of a $GABA_A$ receptor (Jacob *et al.*, 2008).

1.4.1.3. Mechanism. Major subunit isoforms α, β and γ that are characterized the best are generally analyzed for mechanism. At first, the agonist GABA binds to its two binding sites placed at the α/β subunit interfaces, which shows a little different apparent affinities for channel opening. Agonist site occupancy is pursued by a conformational change locking the agonist in the binding pocket. The protein changes conformation and goes one or a few closed states which is termed "flipped states". Further conformational changes cause an opening of the ion pore. The open channel can then make short visits to a ligand-bound closed state (Sigel and Steinmann, 2012).

The $GABA_A$ receptor directly gates a Cl^- ionophore and has also modulatory binding sites for benzodiazepines, barbiturates, neurosteroids and ethanol (Bormann, 2000).

Several studies have proven the significance of the genetic changes of $GABA_A$ receptors in epilepsy and different epilepsy types have been linked with mutations and variants in several $GABA_A$ receptor subunit genes *GABRs* including *GABRA1, GABRB3, GABRD* and *GABRG2* (Figure 1.4, Table 1.1) (Lemoine *et al.*, 2012). In general, these mutations cause trafficking or channel gating defects (Kang and Macdonald, 2009).

1.4.2. $GABA_B$ Receptors ($GABA_B$Rs)

$GABA_B$ receptors are coupled proteins to Ca^{2+} and K^+ channels through G proteins and second messenger systems. They are seven transmembrane receptors and the functional $GABA_B$ receptor is produced by heterodimers of GABAB1 and GABAB2. The GABAB1 and GABAB2 subunits have 35% sequence homology and they are like a classical GPCR subunit including an extracellular N-terminal domain, seven-transmembrane domains, and a C-terminal intracellular domain (Padget and Slesinger, 2010).

$GABA_B$ receptors are activated by baclofen and are resistant to drugs which modulate $GABA_A$ receptors (Bormann, 2000). They generate slow, prolonged inhibitory signals and function to modulate the release of neurotransmitters (Chebib and Johnston, 1999).

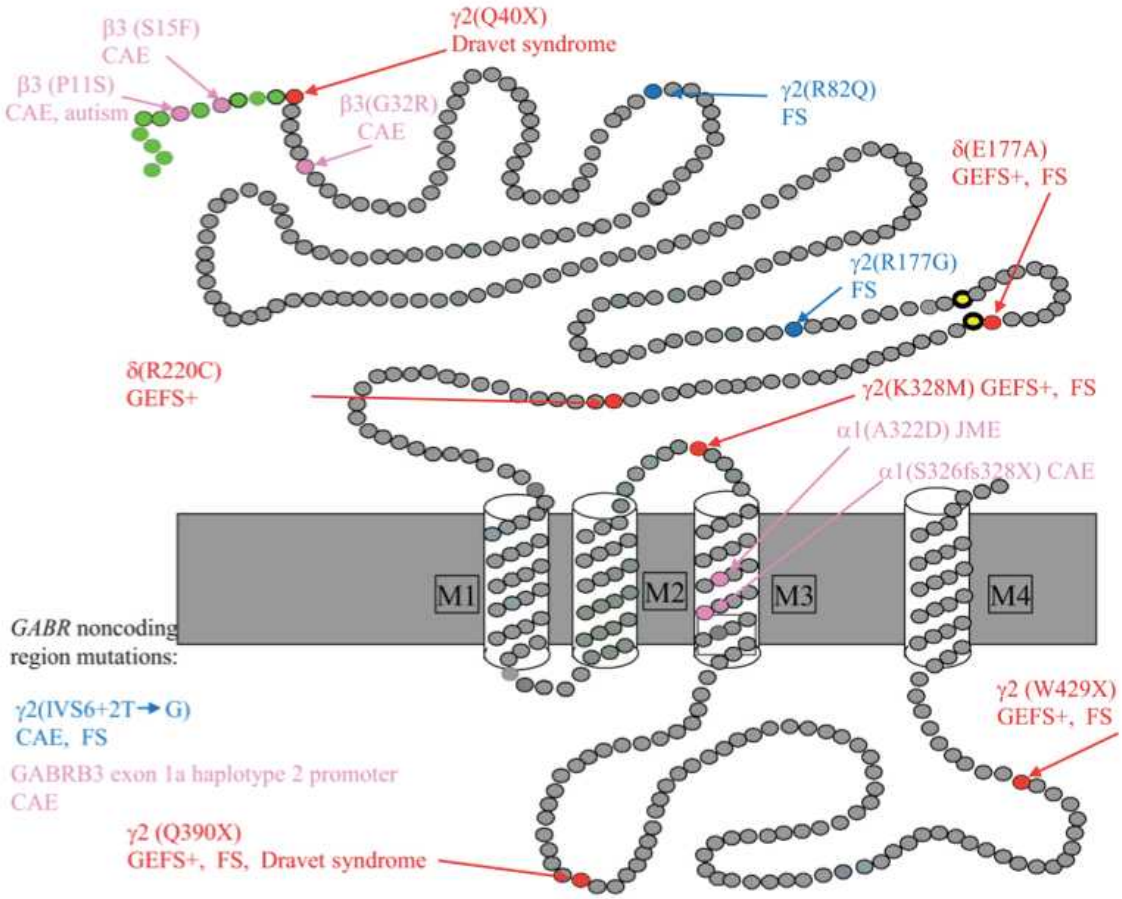

Figure 1.4. $GABA_A$ receptor subunit gene mutations associated with genetic epilepsy syndromes (Macdonald *et al.*, 2010).

Table 1.1. $GABA_A$ receptor subunit mutation/variants and their structural features.

Mutation/variant and Original Reference	IES	Structural Feature	$GABA_A$ receptor dysfunction
β3(P11S) Tanaka *et al.*, 2008	CAE	Signal peptide missense mutation	Abnormal N-linked glycosylation, reduced whole cell current
β3(S15F) Tanaka *et al.*, 2008	CAE	Signal peptide missense mutation	Abnormal N-linked glycosylation, reduced whole cell current
β3(G32R) Tanaka *et al.*, 2008	CAE	N-terminus missense mutation	Abnormal N-linked glycosylation, reduced whole cell current
GABRB3 haplotype 2 Urak *et al.*, 2006	CAE	Exon 1a haplotype 2 promoter	Impaired binding of N- Oct-3 transcription activator
α1(975delC, S326fs328X) Maljevic *et al.*, 2006	CAE	Frameshift mutation and PTC in M3	NMD of mRNA followed by ERAD of subunit protein
α1(A322D) Cossette *et al.*, 2002	JME	M3 missense mutation	Misfolded, reduced total and surface expression, reduced whole cell current
γ2(R82Q) Wallace *et al.*, 2001	FS	N-terminus missense mutation	Impaired oligomerization, ER retention, reduced surface expression, reduced current
γ2(R177G) Audenaert *et al.*, 2006	FS	N-terminus missense mutation	Altered whole cell current kinetics
GABRG2(IVS6+2 T→G) Kananura *et al.*, 2002	FS/ CAE	Intron 6 splice-donor site mutation	Predicted to cause PTC at 5th/7th exon junction
γ2(K328M) Baulac *et al.*, 2001	GEFS+	M2-M3 loop missense mutation	Reduced single channel mean open times, accelerated whole cell current deactivation
γ2(Q390X) Harkin *et al.*, 2002	GEFS+ /DS	M3/M4 loop missense mutation	ER retention, dominant negative reduction of wt receptors, reduced whole cell current

Table 1.1. $GABA_A$ receptor subunit mutation/variants and their structural features (cont.).

Mutation/variant and Original Reference	IES	Structural Feature	$GABA_A$ receptor dysfunction
γ2(W429X) Sun *et al.*, 2008	GEFS+	M3/M4 loop missense mutation	Unstudied, likely unaffected by NMD and therefore produces truncated peptide
δ(E177A) Dibbens *et al.*, 2004	GEFS+	N-terminus missense mutation	Reduced whole cell current, reduced single channel mean open time
δ(R220C) Dibbens *et al.*, 2004	GEFS+	N-terminus missense mutation	Reduced whole cell current, reduced single channel mean open time
γ2(Q40X) Hirose, 2006	DS	N-terminus nonsense mutation	Unstudied, likely triggers NMD

1.5. *GABRG2* Gene

Gamma-aminobutyric acid (GABA) A receptor, gamma 2 is encoded by *GABRG* gene, which is located on 5q34. Out of the fifteen *GABR* epilepsy-associated mutations or variants detected so far, seven of them in *GABRG2*, and these mutations have been shown to reduce channel function by changing receptor biogenesis or channel function (Kang and Macdonald, 2009).

The first mutation that was identified in *GABRG2* is a missense mutation, R82Q. It is in the distal N-terminus and is related with FS. Alignment of γ2 subunit and acetylcholine binding protein sequences showed that R82 is located at the γ2/β2 subunit-subunit interface, and it was observed that the mutation altered γ2 and β2 subunit oligomerization. This altered oligomerization is possibly caused by the reduced surface α1β2γ2 receptors, ER retention of unassembled γ2 (R82Q) subunits and decrease of $GABA_A$ receptor currents. Likewise, the R82Q mutation led to intracellular retention and decline in surface expression of $GABA_A$ receptors in cortical pyramidal neurons, decreased

miniature inhibitory postsynaptic currents (IPSCs) in layer II/III cortical neurons and electrographic and behavioral seizures in R82Q knock in mice.

The other *GABRG2* missense mutation, R177G, is placed in the N-terminus and also related to FS. The γ2 subunit R177 residue is preserved among γ2 subunits between species. Major residues are preserved among other γ subunits, and also in other cys-loop receptors, polar and charged amino acid residues exist at this position. Mutant α1β3γ2L receptors had changed current kinetics and decreased benzodiazepine susceptibility. However, the molecular mechanisms behind it for FSs related to this mutation are not fully understood.

The *GABRG2* missense mutation, K328M which is related to GEFS+, exists in the short extracellular loop between TM2 and TM3. There was no change of current amplitudes with GABA-evoked currents recorded from α1β3γ2L (K328M) receptors, but the deactivation was faster. When it was transfected to hippocampal neurons, the K328M mutation also caused accelerated deactivation of IPSCs, leading to decrease the time.

The *GABRG2* nonsense mutation, Q390X, which is in the intracellular loop between TM3 and TM4 was found in a family with GEFS+ and DS. The PTC is placed in the 9th exon which was the las exon, so that would not be thought to observe NMD. After the transfection with cDNAs that are carrying the mutant subunit to HEK293T cells, translation showed a truncated protein generation which is missing the C- terminal 78 amino acids. Eventually, it was retained in the ER and due to prevention of the cell surface trafficking of both α1β2γ2 (Q351X) and α1β2 receptors, there is no GABA-evoked currents recording from cells transfected with α1, β2, and γ2 (Q351X) subunits.

Q40X and W429X, the two *GABRG2* nonsense mutations were related to DS and GEFS+, respectively. The Q40X mutation is thought that it would result in NMD, but it has not yet been shown. In contrary, the W429X mutation that is producing a PTC in the last *GABRG2* exon, would not be predicted to activate NMD, it would be predicted to create a truncated protein lacking the C-terminal 39 amino acids (Macdonald *et al.*, 2010).

The last mutation that differs from others due to the placement in noncoding site, *GABRG2* splice-donor site mutation IVS6 + 2T→G, is placed in intron 6 and was identified in an Australian family with FS and CAE. It was found out that splice donor site mutated from GT to GG. In one study, wild-type and mutant γ2 subunit bacterial artificial chromosomes (BACs) driven by a CMV promoter was created and expressed in HEK293T cells. Wild-type and mutant γ2 subunit BACs containing the endogenous *hGABRG2* promoter was also expressed in transgenic mice. Wild-type and mutant *GABRG2* mRNA splicing patterns were observed in both BAC-transfected HEK293T cells and transgenic mouse brain, and both of them showed abolished intron 6 splicing at the donor site, led to activation of a cryptic splice site, created partial intron 6 retention and generated a frameshift in exon 7 that produced a premature translation termination codon (PTC). Produced mutant mRNA was either degraded partially by nonsense-mediated mRNA decay or translated to a stable, truncated γ2-PTC subunit including the first six *GABRG2* exons and a novel frameshifted 29 aa C-terminal tail. This subunit was retained in the ER and was not expressed on the surface membrane, but it still did oligomerize with α1 and β2 subunits. These results showed that the *GABRG2* mutation, IVS6 + 2T→G, decreased surface αβγ2 receptor levels, so that it led to a decline in GABAergic inhibition by decreasing *GABRG2* transcript level and generating a stable, nonfunctional truncated subunit (Tian and Macdonald, 2012).

Most of the human protein-coding transcripts are alternatively spliced and an inceasing number of human diseases are shown to be caused by aberrant splice site selection. One other way to test for aberrant splicing is known as a minigene system where a pSpliceExpress is used to create splicing reporter genes based on recombination. Also known as the Gateway system commercially, the system is a powerful method to study splice site selection *in vivo*. The system allows generation of minigenes and these minigenes produced by this new method has a similar splicing pattern without time-consuming construction and limited availability of restriction enzyme techniques (Kishore et.al., 2008). Minigene system with pSpliceExpress is quite useful for fast analysis of alternative splicing in transfection systems and widely used for splice variant analyses (Scott *et al.*, 2012, Listerman *et al.*, 2013, Rittore *et al.*, 2014).

2. PURPOSE

The principal group of inherited epilepsy is genetic generalized epilepsies (GGE) and Childhood Absence Epilepsy (CAE), also called as pyknolepsy is a common subtype of GGE. Up to now, it has been shown that almost half of the epilepsy-associated *GABR* mutations or variants in the *GABRG2* gene were found to cause CAE. Missense, nonsense and splice variants are among the mutation types predicted to cause the disease. Nonsense mutations truncate the protein and missense mutations substitute a different amino acid in the protein and therefore, the prediction of their effect on the function of protein is more direct. Ion channel function can experimentally be tested. However, splice variants can only be tested by in silico methods and their relation to CAE remains mostly unknown. The aim of this study is to functionally validate the effect of a mutation in the second exon intron boundary of *GABRG2* gene on $GABA_A$ receptor using a mini gene expression system to reveal the pathogenesis of the disease at the molecular level.

3. MATERIALS

3.1. CAE Patient

The CAE patient who had a *GABRG2* mutation (IVS2 + 6T→C) found in the context of the PhD thesis (Investigations on the susceptibility locus 2q36 in absence epilepsies) by Özlem Yalçın Çapan is included in this thesis work to study the effect of the mutation using the minigene approach. The study was approved by Ethics Committee of Boğaziçi University and informed consents were obtained from all analyzed subjects.

3.2. Primers

3.2.1. PCR Primers for *GABRG2* Exon 2 and 7

The primer sequences for the amplification of exon-intron regions of exon 2 and exon7 of *GABRG2* were purchased from Macrogen. The sequences of these primers were given in Table 3.1.

Table 3.1. Oligonucleotide primer pairs used in PCR and Sequencing.

Primer Name	Primer Sequence
Ex2i2-Forward	5'-AAAAAGCAGGCT-GCACAACCCTCAAGGGAGAAT-3'
Ex2i2-Reverse	5'-AGAAAGCTGGGT-TGTTGCTTCATAAAGGGGTCATTG-3'
Ex7i7-Forward	5'-AAAAAGCAGGCT-TGCCCTTTGGTCCAAGATCC-3'
Ex7i7-Reverse	5'-AGAAAGCTGGGT-TCCTGTGTGAAATGTGGCGA-3'

3.2.2. Primers for PCR Based Site Directed Mutagenesis

The mutagenesis primer sequences for the creation of the mutation of exon 7 of *GABRG2* were purchased from Macrogen. The sequences of these primers were given in Table 3.2.

Table 3.2. Oligonucleotide primer pairs used in PCR for Site Directed Mutagenesis.

Primer Name	Primer Sequence
Ex7i7Mut-Forward	5'-GACAACTTCCGG***G**AAGATGCACTGG-3'
Ex7i7mut-Reverse	5'-AGTGCATCTT***C**CCGGAAGTT-3'

* mutagenesis site

3.2.3. Primers for Attachment of attB Recombination Sites

The primer sequences for the attachment to the exon-intron regions of exon 2 and exon 7 of *GABRG2* were purchased from Macrogen. The sequences of these primers were given in Table 3.3.

Table 3.3. Oligonucleotide primer pairs used in PCR for attachment sites.

Primer Name	Primer Sequence
attB-Forward	5′-GGGG-ACAAGTTTCTACAAAAAAGCAGGCT-3'
attB-Reverse	5′-GGGG-ACCACTTTGTACAAGAAAGCTGGGT-3'

3.2.4. Primers for Reverse Transcription

The primer sequences for the Reverse Transcription for exon 2 and exon 7 of *GABRG2* were purchased from Macrogen. The sequences of these primers were given in Table 3.4.

Table 3.4. Oligonucleotide primer pairs used in Reverse Transcription.

Primer Name	Primer Sequence
pSE-RNEx3-Forward	5'-GCCCTGCCCAGGCTTTTGTCA-3'
pSE-RNEx3-Reverse	5'-GCAGAGGGGTGGACAGGGTAG-3'

3.3. Enzymes

Enzymes used in this study were given in Table 3.5.

Table 3.5. Enzymes that are used in the study.

Name	Product Information
Taq DNA Polymerase	5 U/µl, (Roche, Germany)
Pwo Superyield DNA Polymerase	5 U/µl, (Roche, Germany)
SapphireAmp Fast PCR Master Mix	2 X Premix, 1 U/µl (Takara, Japan)
Kpn I	10 U/µl (Promega, USA)
Gateway BP clonase Enzyme Mix	5 X, 1 U/µl (Invitrogen, USA)

3.4. Chemicals

Chemicals used in this study were given in Table 3.6.

Table 3.6. Chemicals that are used in the study.

Name	Product Information
Deoxyribonucleotides (dNTPs)	10 mM of each (Thermo, USA)
$MgCl_2$	25 mM in dH_2O (Thermo, USA)
DNA ladder	100 bp, 1kbp (Thermo, USA)
6 X Loading dye	(Thermo, USA)
Ethidium Bromide (EtBr)	10 mg/ml
Absolute Ethanol	(Sigma Aldrich, Germany)
BSA	10 mg/ml (Promega, USA)
Agarose	(Thomas, USA)
Lipofectamine	(Promega, USA)

3.5. Buffers and Solutions

Buffers and solutions used in this study were given in Table 3.7.

Table 3.7. Buffers and solutions that are used in the study.

Name	Product Information
Lysis Buffer	155 mM NH_4Cl 10 mM $KHCO_3$ 1 mM Na_2EDTA (pH 7.4)
Nuclease Buffer	10 mM Tris-HCl (pH 8.0) 400 mM NaCl 2 mM Na_2EDTA (pH 7.4)
Proteinase K	20 mg/ml in H_2O

Table 3.7. Buffers and solutions that are used in the study (cont.).

Name	Product Information
NaCl	5 M NaCl
TE Buffer	20 mM Tris-HCl (pH 8.0) 1 mM Na_2EDTA (pH 7.4)
10 X TBE Buffer	0.89 M Tris-Base 0.89 M Boric acid 20 mM Na_2EDTA (pH 8.3)
DMEM	(Gibco, USA)
OPTI-MEM	(Gibco, USA)
DPBS	(Gibco, USA)

3.6. Kits

Kits used in this study were given in Table 3.8.

Table 3.8. Kits that are used in the study.

Name	Product Information
DNA isolation kit	Roche DNA isolation kit from mammalian blood (Germany)
PCR purification kit	QIAGEN QIAquick purification kit (USA)
Gel extraction kit	QIAGEN QIAquick gel extraction kit (USA)
Plasmid isolation kit	Roche high pure plasmid isolation kit (Germany)
RNA isolation kit	Zymo quick RNA miniprep kit (USA)
RT-PCR kit	Qiagen LongRange 2Step RT-PCR kit (USA)

3.7. Plasmid

The plasmid pSpliceExpress contains the ccdB, colE1 ori, AmpR, SV40 ori and RS virus LTR that are indicated in Figure 3.1 and Figure 3.2. *Kpn I* restriction sites were chosen for restriction analysis. M13 site and its primers were used for sequencing. The selection cassette is flanked by attP sites.

pSpliceExpress + one *GABARG2* exon = 5kb

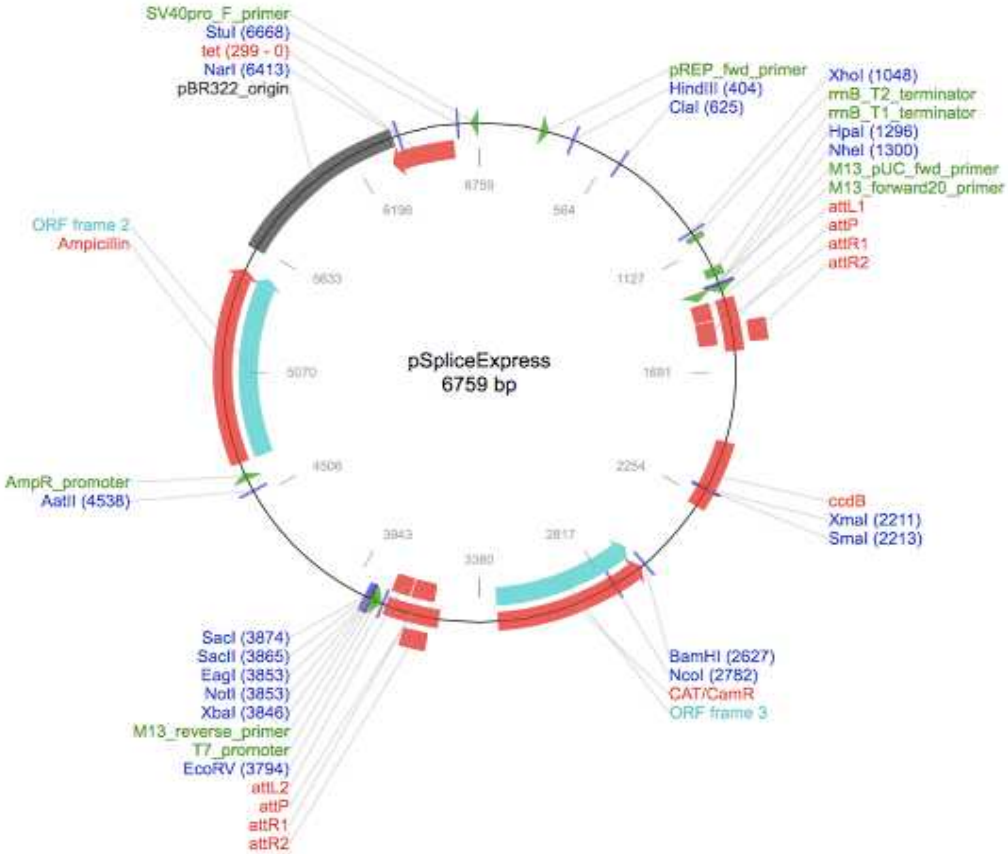

Figure 3.1. The map of pSpliceExpress cloning vector.

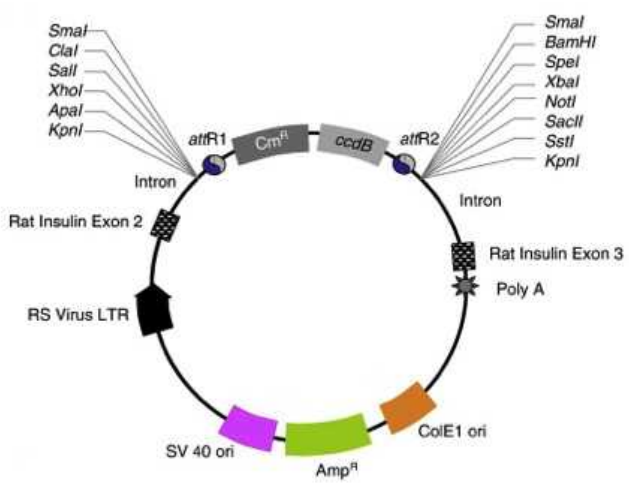

Figure 3.2. The exonic map of pSpliceExpress cloning vector.

3.8. Bacteria & Cell and Growth

Bacteria, cell and growth materials used in this study were given in Table 3.9.

Table 3.9. Bacteria, cell and growth materials that are used in the study.

Name	Product Information
DH-5 alpha cells	(Invitrogen, USA)
Shsy-5y neuroblastoma cells	(Sigma-Aldrich, Germany)
LB medium	10 g NaCl 10 g tryptone 5 g yeast extract deionized H_2O up to 1 L 100 mg filter-sterilized Ampicillin
LB-Agar	10 g NaCl 10 g tryptone 5 g yeast extract 20 g agar deionized H_2O up to 1 L 100 mg filter-sterilized Ampicillin

3.9. Equipments

Equipments used in this study were given in Table 3.10.

Table 3.10. Equipments that are used in the study.

Name	Product Information
Autoclaves	Model MAC-601 (Eyela, Japan) Model ASB260T (Astell, UK)
Balances	Electronic Balance Model VA124 (Gec Avery, UK) El ectronic Balance Model CC081 (Gec Avery, UK)
Centrifuges	Beckman J2-MC Centrifuge (USA) Beckman J2-21 Centrifuge (USA) Beckman Microfuge 18 (USA) VWR Galaxy MiniStar Microcentrifuge (USA)
Deep Freezers	-20ºC (Bosch, Germany) -20ºC (Arcelik, Turkey) -80ºC (Thermo, USA)
Documentation System	Gel Doc XR + System (BIO-RAD, USA)
Electrophoretic Systems	Owl D2 (BIO-RAD, USA) Owl Easycast B1 (BIO-RAD, USA) Mini-sub CELL-GT system (BIO-RAD, USA)
Fluorometer	QuantiFlor Single-Tube (Promega, USA)
Ice Machine	Brema Ice Maker (Italy)
Magnetic Stirrer	IKA Yellowline (Germany)
Oven	DBK (USA)
Pipettes	(Eppendorf, Germany)
Power Supplies	Owl EC300XL (Thermo, USA) EC250-90 (Thermo, USA)
Refrigerator	4ºC Sanyo Medicool (USA)
Spectrophotometers	Beckman DU730 (USA) Nanodrop 1000 (Thermo, USA)

Table 3.10. Equipments that are used in the study (cont.).

Name	Product Information
Thermocyclers	T-100 Thermal cycler (BIO-RAD, USA) Techne (Progene, UK) Techne Gradient (Progene, UK)
UV Transilluminator	UVP Benchtop Transilluminator Model 1TM-20UVP (USA)
Vortex	Fisons Whirlimixer (UK)
Water Bath	Köttermann, Laborteknik (Germany)

4. METHODS

4.1. DNA Analysis

4.1.1. DNA Extraction from White Blood Cells

In order to extract genomic DNA from white blood cells (leukocytes), Roche DNA Isolation Kit for Mammalian Blood was used. 10 ml of peripheral blood samples were collected in anticoagulant (K_2EDTA) containing tubes. To start with, the blood samples were treated with 30 ml lysis buffer to lyse the membrane of leukocytes. After incubation of the samples at room temperature for 10 minutes by inverting every 30 seconds, they were centrifuged at 3000 rpm for 10 minutes. Therefore, cellular nuclei were precipitated and lysed part was collected in suspension. Supernatant was removed and the pellet was resuspended in 5 ml white cell lysis buffer and vortexed for 1 minutes. Then, 2.6 ml protein precipitation solution was added and vortexed again for 25 seconds. The mixture was centrifuged at 9000 rpm for 10 minutes. After discartion of the proteins, supernatant containing the DNA was taken to the new tube and twice as much of absolute ethanol was added and inverted slowly in order to form and visualize DNA. The solution was centrifuged at 3000 rpm for 10 minutes. Supernatant was removed and the pellet was moved with 3 ml -20^{o}C 70% ethanol and centrifuged at 3000 rpm for 5 minutes. By fishing out with a pipette, DNA pellet was taken into an eppendorf tube and left into 56^{o}C for a few minutes to dry. When all the ethanol was dried, DNA was dissolved in appropriate amount of TE buffer.

4.1.2. Salt Extraction

For previously extracted DNA, NaCl extraction was used. First, 55 µl DNA was put in an eppendorf tube and 0.5 µl sodium acetate was added onto it. Then, 100 µl -20^{o}C 95% ethanol was added and mixed by up and down to visualize DNA. The solution was centrifuged at 13000 rpm for 1 minute and the pellet was rinsed with 100 µl -20^{o}C 70% ethanol. After that, it was centrifuged again at 13000 rpm for 1 minute and the pellet was left a few minutes at 56^{o}C to dry. Finally, DNA was dissolved in appropriate amount of TE buffer.

4.1.3. Analysis of DNA by Agarose Gel Electrophoresis

Extracted DNA was analyzed on 1% agarose gel which was prepared by dissolving 0.5 g of agarose in 50 ml 0.5 X TBE buffer. Agarose was dissolved in TBE buffer by boiling in microwave and kept at room temperature for a while to cool down. EtBr which is an intercalating agent for DNA and lead to visualization of DNA under UV light, was added into the mixture with a final volume of 5 %. Afterwards, the homogenous mixture was poured into electrophoresis plate and wait at room temperature until it polymerizes. Then, 1 µl of DNA was mixed with 1 µl of 6 X Loading dye for loading samples into the wells of agarose gel. The gel was run at 120 Volt for 30 minutes and visualized under UV light.

4.1.4. Analysis by Spectrophotometer

DNA concentration was measured by nanodrop spectrophotometer. After blanking with 1 µl TE buffer, 1 µl of DNA sample was loaded onto the machine and concentration of DNA was read at 260 nm. 260/280 and 260/230 ratios were measured to assess the purity of DNA.

4.1.5. Analysis by Splice Site Tools

The sequence of the patient and the reference sequence were uploaded to the tool and the results were analyzed by comparing the threshold value (ASSP, ESEfinder).

4.2. PCR

4.2.1. PCR for *GABRG2* of the CAE patient

The region that belongs to the second exon-intron region of *GABRG2* gene was amplified by using the specific primers (primer name given in Materials section) (Figure 4.1). PCR reaction was prepared in 50 µl volume containing SapphireAmp Fast PCR Master Mix, 0.2 µM final concentration for each primer, 50-75 ng genomic DNA and distilled water. The thermal cycle comprised an initial denaturation step of 1 minute at 94°C, 35 amplification cycles containing 10 seconds at 94°C and 1 minute at 68°C.

Initial Denaturation	94°C 1 min.
Amplification -35 cycles-	98°C 10 sec. 68°C 1 min.

PCR products were visualized on 2% agarose gels comparing and verified by sequencing.

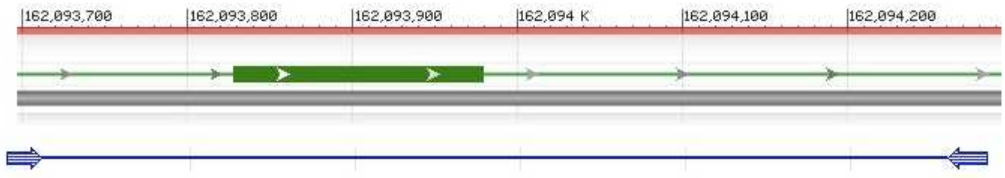

Figure 4.1. Amplified region for exon 2. Blue line shows the amplified region and green bar shows the exon.

4.2.1.1. Sequence Analysis of the CAE Patient. Exon 2 PCR product was purified by using QIAquick purification kit and run on 2% agarose gel. First, five volumes of PB buffer was added to 1 volume of the PCR sample and mixed. A QIAquick spin column was placed in a

2 ml collection tube for each sample. After that, the solutions were applied to the column and centrifuged for 1 minute. The flowthrough was discarded and 750 µl PE buffer was added to the column and centrifuged again for 1 minute. The flowthrough was discarded and the column was centrifuged again to get rid of all the flowthrough. Then, the column was placed into a new eppendorf tube with the lid cut and 50 µl Elution buffer was added to the center of the QIAquick membrane to elute DNA and centrifuged for 1 minute. Purified PCR products were checked on 2% agarose gel and visualized under UV light.

The sample was sent to Macrogen (Seuol, Korea) for automated sequencing. The result was obtained online as a pdf file in the form of a chromatogram.

4.2.2. PCR for Site Directed Mutagenesis in *GABRG2*

The primers and their placements used for site directed mutagenesis are shown in Figure 4.2 and Figure 4.3. In order to create mutation that is in the exon-intron boundary of exon 7, the region that belongs to the second exon-intron region of *GABRG2* gene was amplified first by forward and reverse primers (write primer names given in materials section) as described in Section 4.2.1 with similar conditions. Right flanking sequence was amplified using forward mutagenesis primer with regular reverse primer The PCR reaction was prepared in 50 µl volume containing 1 X reaction buffer, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 1.25 unit of Pwo polymerase, 0.3 µM final concentration for each primer, 75 ng genomic DNA and distilled water. The thermal cycle comprised an initial denaturation step of 2 minute at 95°C, 35 amplification cycles containing a denaturation step of 15 seconds at 92°C, an annealing step of 30 seconds at 60°C, an elongation step of 2 minutes at 72°C and a final extension step of 2 minutes at 72°C.

Initial Denaturation	95°C 2 min.
Amplification -35 cycles-	92°C 15 sec. 60°C 30 sec. 72°C 2 min.
Final Extension	72°C 2 min.

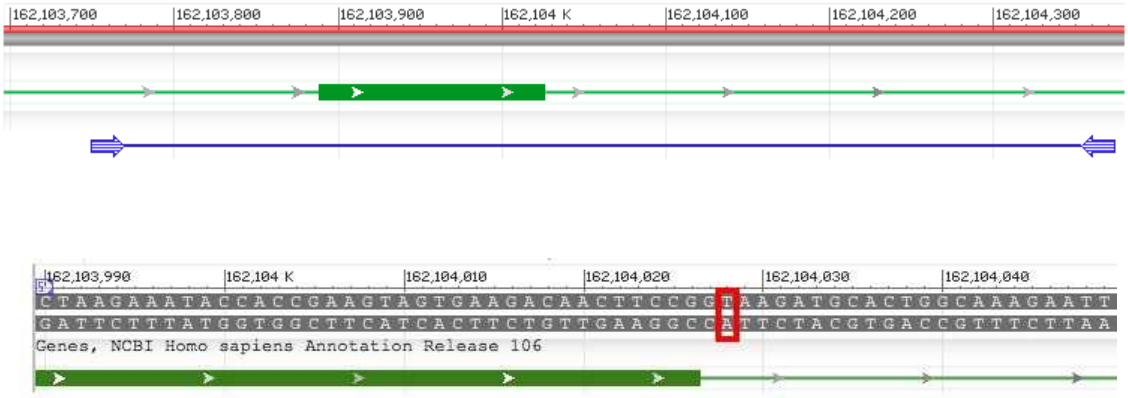

Figure 4.2. Amplified region for exon 7. The blue line in the upper diagram shows the amplified region and green bar shows the exon. The lower diagram shows the location of the mutation in the *GABRG2* sequence.

```
36061 acttcccatt aggatgcaaa gtcattgcaa tttatgttct cattgcaatg ccctttggtc
36121 caagatcctc atttagcttg taactatctt tctcagtctt tacctcagtg tcatgttcat
36181 agaagatggt tgctacatat gctaatttat aatcattttt aatgtgagct ttcctatctc
36241 acggcagatg gctatccacg tgaagaaatt gtttatcaat ggaagcgaag ttctgttgaa
36301 gtgggcgaca caagatcctg gaggctttat caattctcat ttgttggtct aagaaatacc
36361 accgaagtag tgaagacaac ttccggtaag atgcactggc aaagaatttc aagtgaccct
36421 tccagagttg aaattttggt atatatgaat tagaagattt ttcaatacct ctcaatgaat
36481 gataatcaga aaattaaatg aagtgtttta actttctaga gaaaattata attgaaattc
36541 aagtagaata gataaatata aatatattct accaatatgg gaaaattgga ttcaaattgg
36601 attagtaaat tattccctaa tttatttaag gaaaaatgaa tataattatg aaaaaataaa
36661 atatttatta gatgtacctg ttttagaaag tatgtttcat aatgtagtga aatcgccaca
36721 tttcacacag gaagagtgaa ttttttacaa atgcagaaaa gtaagcatag tggatatgga
```

Figure 4.3. Exon 7 sequence. PCR primers are shown in green. The mutagenesis primer is shown in red. Primer sites on the sequence (red: mutagenesis primer, green: forward and reverse primers).

The PCR product was visualized on 2% agarose gels and extracted from the gel using QIAquick gel extraction kit. First, the gel was cut as much as minimizing it and three volumes of QG buffer was added to 100 milligram of the gel. The mixture was incubated at 50°C for 10 minutes in order to resolve the gel. Then, 1 volume of isopropanol was added and the mixture was applied into a QIAquick column and centrifuged at 13000 rpm for 1

minute. The flowthrough was discarded and 500 µl of QG buffer was added to the column and centrifuged for 1 minute. The flowthrough was discarded and 750 µl of PE buffer was added to the column, waited for 5 minutes and centrifuged again for 1 minute. After discarding the flowthrough, the column was centrifuged again to get rid of all the flowthrough. Then, the column was placed into a new eppendorf tube with the lid cut and 30 µl Elution buffer was added to the center of the QIAquick membrane to elute DNA, waited for 5 minutes and centrifuged for 1 minute. The extracted product was checked on 2% agarose gel and visualized under UV light.

Then, left flanking sequence was amplified using reverse mutagenesis primer (primer name in materials section) with regular forward primer. The PCR reaction was prepared in 50 µl volume containing 1 X reaction buffer, 1 X GC rich solution, 1.5 mM of MgCl2, 0.2 mM of each dNTP, 1.25 unit of Pwo polymerase, 0.3 µM final concentration for each primer, 50 ng of PCR product DNA consisting of 624 bp of exon-intron region and distilled water. The thermal cycle comprised an initial denaturation step of 2 minute at 95^{o}C, 35 amplification cycles containing a denaturation step of 15 seconds at 92^{o}C, an annealing step of 30 seconds at 60^{o}C, an elongation step of 2 minutes at 72^{o}C and a final extension step of 2 minutes at 72^{o}C.

Initial Denaturation	95^{o}C 2 min.
Amplification -35 cycles-	92^{o}C 15 sec. 53.3^{o}C 30 sec. 72^{o}C 2 min.
Final Extension	72^{o}C 2 min.

The PCR product was visualized on 2% agarose gel and extracted from the gel similarly.

After extraction of PCR products from the gel, they were used as templates in order to create mutation in the center of the region by using regular forward and reverse primers of the amplification of exon-intron region for exon 7. The PCR reaction was prepared in 50

µl volume containing SapphireAmp Fast PCR Master Mix, 0.2 µM final concentration for each primer, 50 ng of each PCR product and distilled water. The thermal cycle comprised an initial denaturation step of 1 minute at 94°C, 35 amplification cycles containing 10 seconds at 94°C and 1 minute at 68°C.

Initial Denaturation	94°C 1 min.
Amplification -35 cycles-	98°C 10 sec. 68°C 1 min.

The PCR product was visualized on 2% agarose gel and extracted from the gel similarly. The extracted fragment containing mutation was sent to Macrogen for automated sequencing in order to confirm the presence of mutation.. The schematic illustration of the PCR based sited directed mutagenesis is shown in Figure 4.4.

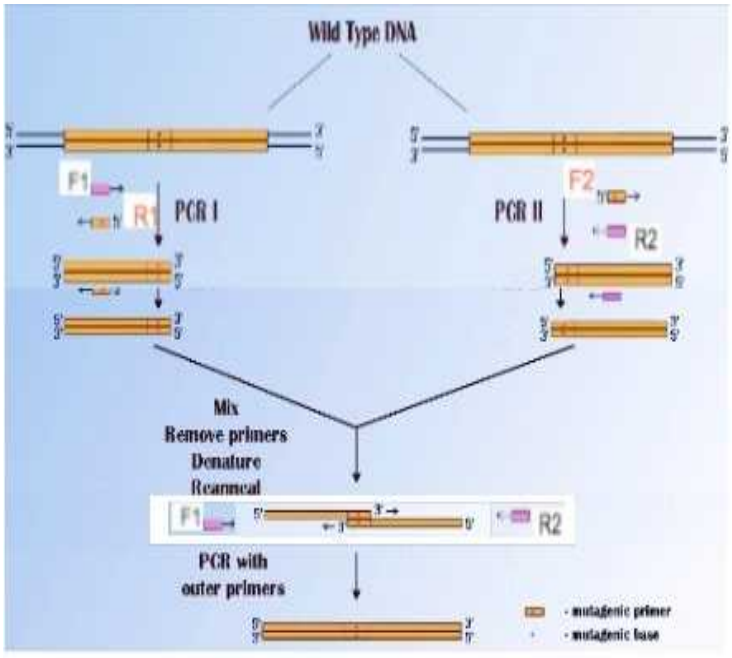

Figure 4.4. Diagram of the PCR based site directed mutagenesis method.

4.3. Cloning

Minigene, also named as gateway cloning system was applied for molecular cloning. The method was based on direct recombination between PCR product and modified exontrap vector (Figure 4.5).

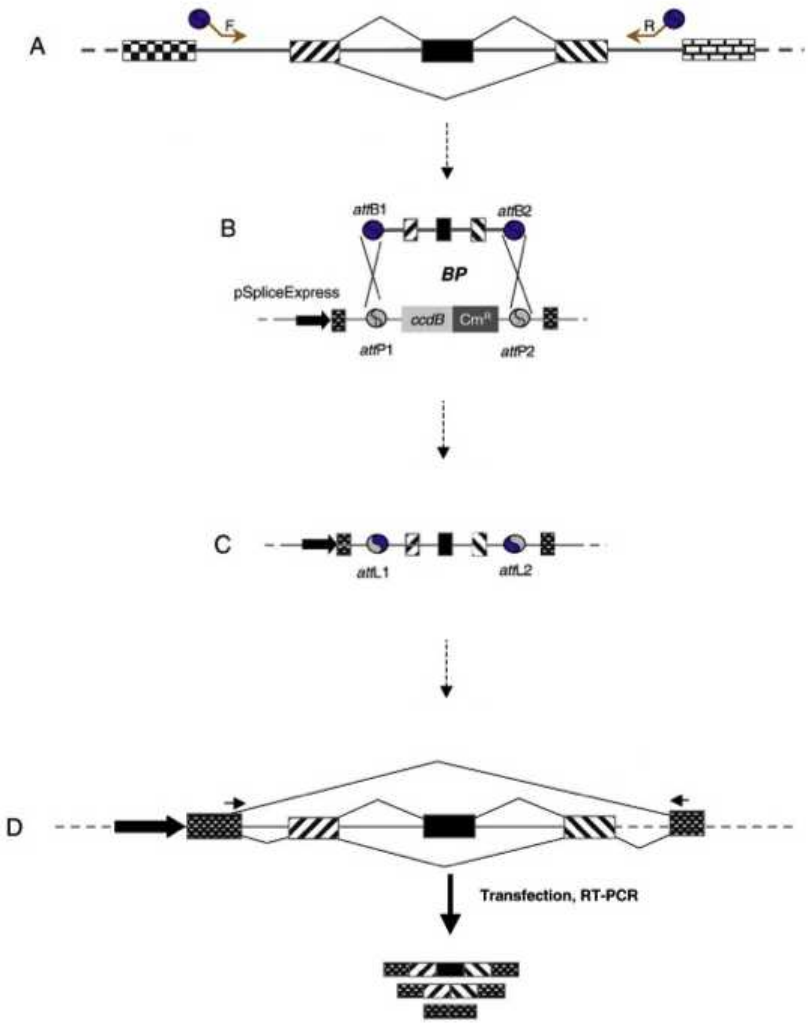

Figure 4.5. Overview of the minigene method (Kishore *et al.*, 2008).

In minigene method, PCR product is cloned into pSpliceExpress (Figure 4.5 A, B, C) in order to create the final construct (D). (A) Amplification of the interested area. F and R primers are used to amplify particular region of the genomic DNA that includes the alternative exon (black, splicing patterns are indicated). The primers carry recombination sites that are shown by circles. (B) Construction of the splicing reporter using pSpliceExpress. The PCR fragment is recombined in vitro with pSpliceExpress vector. The vector has Cm and ccdB selection markers which are used for isolation of recombined clones. (C) Final construct using (continued) pSpliceExpress. The inserted DNA is flanked by two constitutive rat insulin exons, shown by dotted pattern. The transcript is driven by a RSV LTR promoter (arrow) and the subcloned genomic fragment is flanked by attL sites, created by the recombination of attB and attP sites. (D) Analysis of the splicing reporter. The splicing reporter construct is transfected into a prefered cell line. The RNA is checked by RT-PCR, using the primers (small arrows) (Kishore *et al.*, 2008).

After RT-PCR, the mRNA structures are produced. The mRNA structures that are expected to be expressed are shown in Figure 4.6. If the mutation in the patient is effective, it is expected to be 6 bp retention in the CAE Patient's construct which is named as S1, and the mRNA product will be 458 bp long comparing with wild type (WT1) which is 452 bp long. For positive control created by site directed mutagenesis which is named as S2, there will be 53 bp retention and the mRNA construct will be 492 bp comparing with wild type (WT2) which is 439 bp long. mRNA constructs that are produced by only rat exons are 300 bp long (Figure 4.6).

Figure 4.6. Illustration of mRNA structures that are expected to be expressed. Black rectangles show the rat exons 3 and 2 respectively, white ones show the *GABRG2* exons and grey ones show the introns. (S1: patient, WT1: wild type 1, S2: site directed mutagenesis, WT2: wild type 2).

4.3.1. Preparation of the Insert

4.3.1.1. Attachment of attB Recombination Sites by PCR. Adaptor primers which are the primers that have recombination sites were added to each PCR product by a PCR reaction. The PCR reaction was prepared in 50 µl volume containing SapphireAmp Fast PCR Master Mix, 0.2 µM final concentration for each primer, 75 ng genomic DNA and distilled water. The thermal cycle comprised an initial denaturation step of 1 minute at 94°C, 35 amplification cycles containing 10 seconds at 94°C and 1 minute at 68°C. The PCR products were visualized on 2% agarose gel.

Initial Denaturation	94°C 1 min.
Amplification -35 cycles-	98°C 10 sec. 68°C 1 min.

4.3.2. Preparation of the Vector

4.3.2.1. Plasmid Isolation, 3 ml of LB media containing 3 µl Ampicillin (100 mg/ml) was prepared and put into a 15 ml falcon tube which has loosened caps letting air get into the tube. pSpliceExpress plasmid was taken from bacterial glycerol stock by scratching with a micropipette tip and put inside the tube. One LB media was put into a falcon tube without any plasmid in it in order to eliminate the possibility of contamination. The tube was placed in a shaker and left at 37°C for overnight.

After amplification of cells containing the pSpliceExpress plasmid, the plasmid DNA was isolated with Roche high pure plasmid isolation kit. First, the culture was taken and centrifuged at 4400 rpm for 15 minutes at 4°C. The supernatant was discarded and the pellet was resolved in 250 µl Suspension buffer which contains RNase. Then, 250 µl Lysis buffer was added, inverted slowly 6 times and incubated for 5 minutes at room temperature. After that, 350 µl chilled Binding buffer was added and inverted slowly 6 times again to make the DNA visible. It was incubated on ice for 5 minutes and centrifuged at 9200 rpm for 10 minutes at 4°C. The supernatant was taken, loaded onto the column and centrifuged at 13000 rpm for 1 minute. The flowthrough was discarded and 500 µl Wash buffer I added onto the column and centrifuged again at 13000 rpm for 1 minute. Afterwards, an additional washing step were applied by adding 700 µl Wash buffer II in order to eliminate high nuclease activity. After centrifugation at 13000 rpm for 1 minute and discartion of the flowthrough, column was centrifuged again to get rid of all the flowthrough. 1.5 ml eppendorf tube was taken, the lid was cut and the column was placed onto it. The appropriate amount of Elution buffer was added in the center of the column to elute the whole DNA from the column and centrifuged at 13000 rpm for 1 minute. Flowthrough was taken into a clean tube and stored at -20°C. To check the DNA, 1 µl of DNA was mixed with 1 µl of 6 X Loading dye and loaded into the wells of agarose gel. The gel was run at 100 Volt for 90 minutes and visualized under UV light.

4.3.2.2. Concentration Measurement by Fluorometer. On the day of experiment, working solution of the PicoGreen Reagent was prepared by making a 1:200 dilution by taking 5 µl of the reagent into the 995 µl distilled water. For the DNA standard curve, 2 µg/mL DNA

stock solution was diluted into acrylic cuvettes (Table 4.1). The mixtures were spinned down and incubated for 5 minutes at room temperature by protecting from light and used as standards.

1 µl of each DNA sample was added into 49 µl of distilled water and mixed with 50 µl of reagent. The fluorescence of the PCR products and the plasmid were measured using fluorometer and concentrations were calculated.

Table 4.1. Protocol of standard curve preparation.

Volume of 2 µg/ml DNA stock (µl)	Volume of TE buffer (µl)	Volume of diluted picogreen reagent (µL)	Final concentration in picogreen assay (ng/ml)
1000	0	1000	1000
500	500	1000	500
100	900	1000	100
10	990	1000	10
1	999	1000	1
0	1000	1000	blank

4.3.2.3. Recombination. According to the concentration of each sample and also regarding the number of bases in the samples, required amounts were calculated depending on the formula.

ng of DNA = (x fmol) X (N) X ((660fmol X 1ng)/(1fmol X 106 fg))

x = number of femtomoles desired
N = size of DNA in bp

Required amounts of PCR products added into 1 µl of plasmids in PCR tubes and completed to 8 µl with TE buffer (Table 4.2). Then, 2 µl of Gateway BP Clonase Enzyme Mix was added to the tubes and vortexed for 2 seconds. The mixtures were spinned down quickly and incubated at 25°C for overnight. After incubation, 1 µl Proteinase K solution which destroys clonase was added in order to stop the recombination reaction and incubated at 37°C for 10 minutes.

Table 4.2. Required materials and their amounts for recombination process.

Sample name	PCR product(µl)	Plasmid (µl)	TE buffer (µl)	BP Clonase (µl)
Sample 1 (patient)	1	1	6	2
Wild type 1	1	1	6	2
Sample 2 (sdm)	2	1	5	2
Wild type 2	1	1	6	2

4.3.3. Transformation into DH-5 alpha Cells

DH-5 alpha competent cells which were stored at -80°C were put on ice. 5 µl of recombinated products were added into 100 µl aliquots of competent cells, mixed gently and immediately put back on ice and waited for 5 minutes. Then, the cells were spreaded with a glass rod onto pre-warmed ampicillin selective plates (37°C) as the pSpliceExpress plasmid carried ampicillin resistance gene. The plates were incubated overnight at 37°C for overnight. After transformation, the cells that did not have recombination which has toxic ccdB (controlled cell death) gene instead of PCR product could not survive because of the lack of its antitoxin, ccdA.

4.3.4. Purification of Plasmid DNA

After growing the cells that has the desired plasmid, a single colony was taken from each plate with a micropipette tip and put into 500 ml autoclaved flasks that includes 100 ml of LB media containing 100 µl Ampicillin (100 mg/ml). One LB media was put into a falcon tube without any plasmid in it in order to eliminate the possibility of contamination. The flasks were covered with aluminum foil letting air get into each flask and placed in a shaker and left at 37^{o}C for overnight.

After amplification of cells containing the pSpliceExpress plasmid, the plasmid DNA was isolated and purified with Qiagen plasmid mini kit.

In order to verify the recombination, the plasmid DNA was checked with restriction enzyme assay. For this purpose, *Kpn I* restriction enzyme was used. In the plasmid, there are two sites outside of the attachment sites that *Kpn I* endonuclease recognizes and cleaves the phosphodiester bond in DNA. Digestion was carried out in 20 µl volume containing 2 µl enzyme buffer, 0.2 µl acetylated BSA, 10 units of *Kpn I* enzyme and 5 µl of plasmid DNA. The samples were mixed by pipetting up and down several times and incubated at 37^{o}C for 2 hours. After digestion, 5 µl of each sample was mixed with 1 µl of 6 X Loading dye and loaded on 1% agarose gel and run at 100V for 1 hour. The gel was checked under UV light.

4.3.5. Sequencing of the Plasmid DNA

Each purified plasmid DNA was sent to Macrogen for automated sequencing in order to confirm the presence of mutations.

4.4. Expression Analysis

4.4.1. Transfection into Shsy-5y Neuroblastoma Cells

Shsy-5y Neuroblastoma cells were seeded at $1.2x10^4$ cells/cm^2 density in a 12-well dish one day prior to transfection, the transfection procedure was started when the culture had reached 70% confluence. The media were removed and the cells were rinsed with DPBS. After that, 1 ml DMEM transfection media were added onto them.

3.5 µl Lipofectamine Reagent were added in 100 µl OPTI-MEM media and incubated for 5 minutes at room temperature. Then, plasmid concentrations were measured by nanodrop spectrophotometer and adjusted to final concentration of 800 ng/µl for each plasmid and added to the transfection mixture (Table 4.3). The mixtures were shaken slowly and horizontally and incubated for 25 minutes at room temperature. Finally they were added onto the cells and incubated at 37°C for 72 hours. After incubating the dish for 6 hours, the media were replaced by standard OPTI-MEM differentiation media. Transfection efficiency was assayed 72 hours after addition of the transfection mix. This procedure was done as duplicate samples. Finally, the cells were harvested by rinsing with PBS.

Table 4.3. Concentrations and required amount of samples for each plasmid.

Sample name	Concentration (ng/uL)	Added amount of DNA (uL)
Sample 1 (patient)	415.6	1.93
Wild type 1	571	1.4
Sample 2 (sdm)	1116.7	0.72
Wild type 2	543.6	1.47

4.4.2. RNA Isolation

After harvesting all the cells, total RNAs were isolated with RNA isolation kit (Zymo quick RNA miniprep). First, 600 µl of Lysis buffer was added onto the cells and the lyzates were cleared by centrifugating at 13000 rpm for 1 minute. The supernatants were transferred into filter in a collection tube and centrifuged at 13000 rpm for 1 minute to remove the majority of genomic DNA. The mixture was taken into new column in a collection tube and centrifuged at 13000 rpm for 30 seconds. Flowthrough was discarded. After that, 400 µl of RNA Wash buffer was added to the column, centrifuged at 13000 rpm for 30 seconds and flowthrough was discarded again. In order to get rid of the trace DNA, 80 µl of DNase I reaction mix was added directly to the column matrix and incubated at room temperature for 15 minutes, then centrifuged at 13000 rpm for 30 seconds. Afterwards, 400 µl RNA Prep buffer was added to the column and centrifuged at 13000 rpm for 30 seconds. After discarding the flowthrough, 700 µl RNA Wash buffer was added to the column. It was centrifuged at 13000 rpm for 30 seconds and flowthrough was discarded again. Then, 400 µl RNA Wash buffer was added to the column and centrifuged at 13000 rpm for 2 minutes in order to ensure complete removal of the wash buffer. The columns for each sample were placed into an Rnase free tube and appropriate amount of Dnase/RNase free water directly to the column matrix, then centrifuge at 13000 rpm for 30 seconds. Eluted RNA was stored at -80^{o}C.

4.4.3. Reverse Transcription

After RNA isolation, reverse transcription was done with QIAGEN LongRange 2 Step RT-PCR kit. First, cDNA synthesis procedure was applied. Template RNA was thawed on ice and reaction mix was prepared (Table 4.4). Master mix was mixed thoroughly and carefully by vortexing 5 seconds and centrifuged briefly and placed on ice. Then, RNA was added into the tube and vortexed again 5 seconds and incubated for 90 minutes at 42^{o}C. After that, the enzyme was inactivated by heating at 85^{o}C for 5 minutes and the samples were stored at -20^{o}C.

Table 4.4. Composition of Reverse-Transcription Master Mix.

Component	Volume in 20 µl reaction (µl)	Final concentration
LongRange RT Buffer 5 X	4	1 X
dNTP	2	1 mM
Oligo-dT	1	1 µM
RNase inhibitor	0.2	0.04 U/µl
Transcriptase	1	1 X
Water	6.8	
RNA	5	50-500 ng

Second, the procedure was continued with Reverse Transcriptase PCR (RT-PCR). The samples were thawed on ice and reaction mix was prepared (Table 4.5). Reaction mix was mixed thoroughly and cDNA samples were added each tube. The cycling protocol was comprised with a preheated lid at 93°C, an initial denaturation step of 3 minute at 93°C, 35 amplification cycles containing a denaturation step of 15 seconds at 93°C, an annealing step of 30 seconds at 62°C, an elongation step of 90 seconds at 68°C and a final extension step of 5 minutes at 68°C. The program was started using thermal cycler and the products were visualized on 2% agarose gel.

Table 4.5. Composition of RT-PCR Reaction.

Component	Volume in 50 µl reaction (µl)	Final concentration
LongRange PCR Buffer 10 X	4	1 X
dNTP	2.5	500 µM
Pse-RNex3-Forward Primer	2	0.4 µM
Pse-RNex2-Reverse Primer	2	0.4 mM
Water	34.1	
PCR enzyme mix	0.4	2
Template cDNA	4	

Hot start	93°C
Initial Denaturation	93°C 3 min.
Amplification -35 cycles-	92°C 15 sec. 62°C 30 sec. 68°C 90 sec.
Final Extension	68°C 2 min.

The products were visualized on 2% agarose gels and extracted from the gel using QIAquick gel extraction kit. Finally, they were sent to sequencing.

5. RESULTS

5.1. Mutational Analysis of the Patient

5.1.1. PCR Result of S1 and WT1

The region that covering the second exon-intron region of *GABRG2* gene was amplified successfully as described in Section 4.2.1. Fragment size was 595 bp and gel image of PCR product for the region of interest is shown in Figure 5.1.

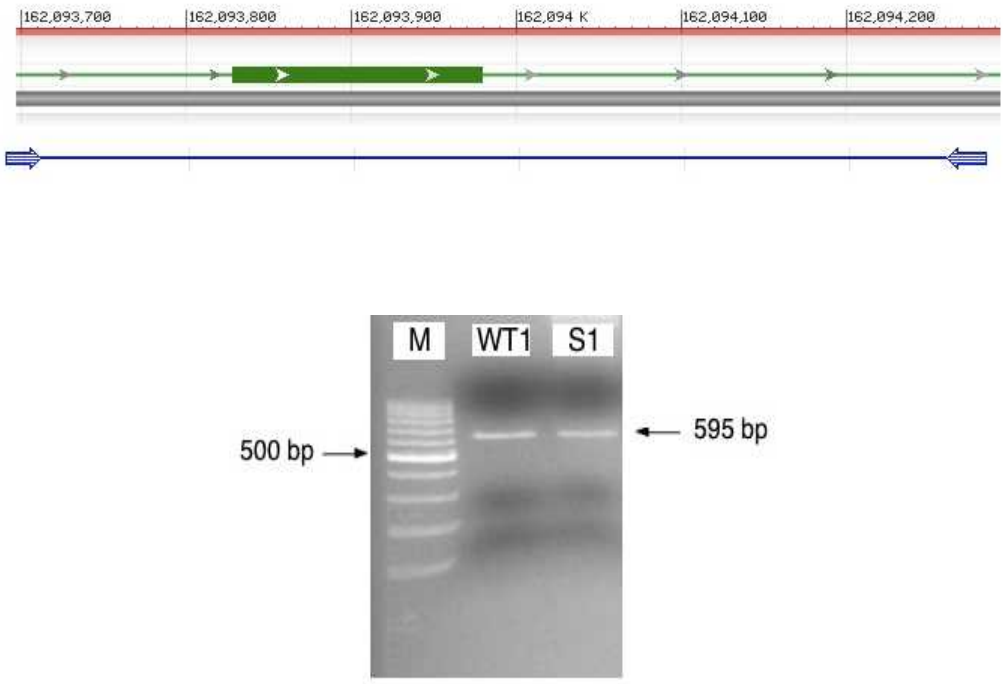

Figure 5.1. Amplified region for exon 2 (upper) and PCR products visualized on 2agarose gel (lower) (M: 100 bp DNA length marker, S1: patient, WT1: wild type 1).

5.1.2. Confirmation of the Mutation by Re-sequencing

Sequencing was repeated and the sample was sent to Macrogen for automated sequencing. The result was obtained and compared with the normal sequence. The result shows the T->C transition in intron 2 in the patient comparing to wild-type (Figure 5.2). The transition in intron 2, at 6th base cause a disruption at GU site and it could change the splicing process.

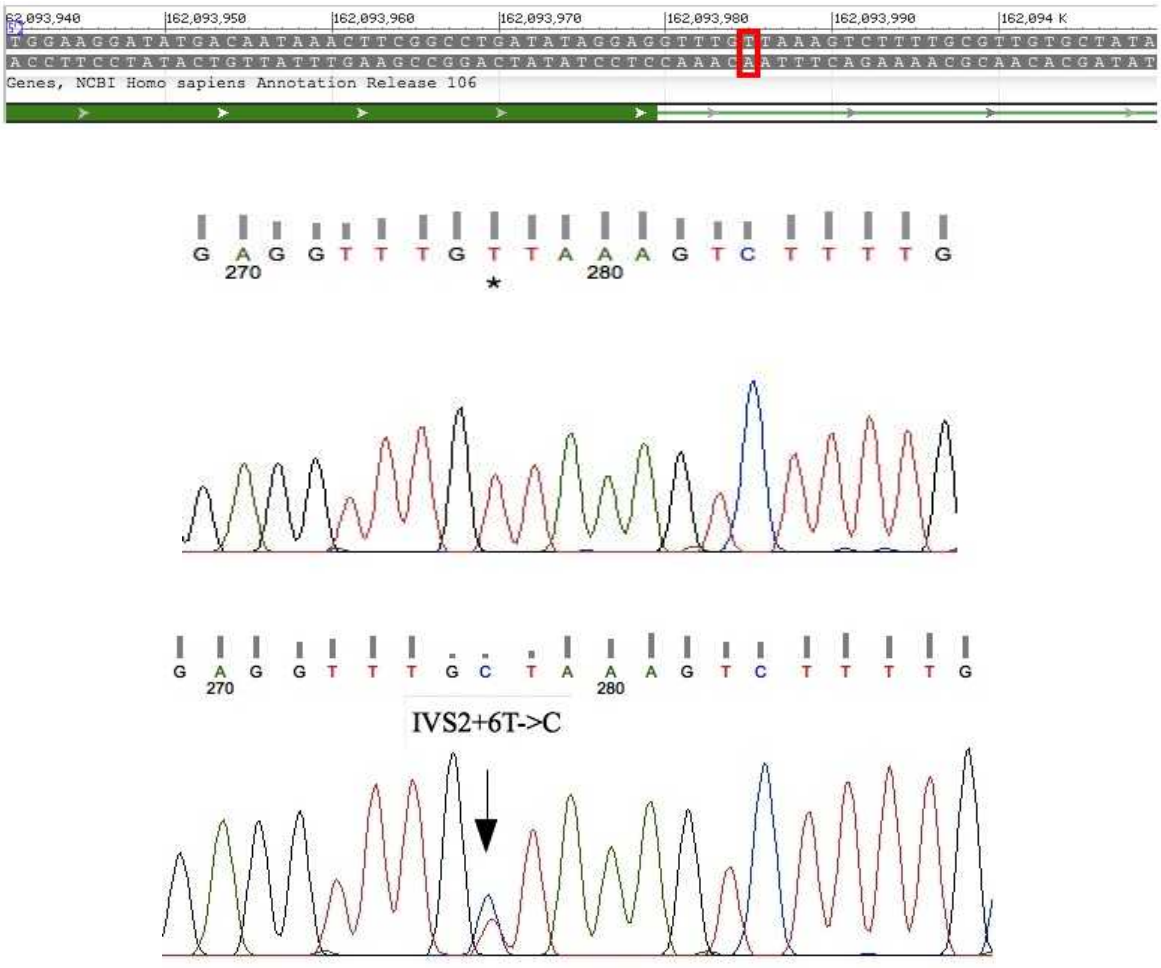

Figure 5.2. Reference sequence showing the mutation in the intronic area (top figure), chromatograms of the wild type (middle) and the patient (lower) showing that the T->C transition (indicated by the arrow).

5.1.3. Splice Site Tool Results

The threshold value and results of ASSP and ESEfinder analyses for the patient and a healthy individual are shown in Table 5.1, Table 5.2 and Figure 5.3, respectively. The comparison indicates that the mutation is likely to create a cryptic donor site.

Table 5.1. Scores for ASSP analysis.

				Activations**		
Putative splice site	Sequence	Score*	Intron GC*	Alt./Cryptic	Constitutive	Confidence**
Alt. isoform/cryptic donor	GATATAGGAGgtttgctaaa	5.312	0.329	0.878	0.088	0.900

Donor site cutoff score: 4.5

Table 5.2. Scores for ESEfinder analysis.

	Score
threshold	2.383
GTTG *C TA	3.657

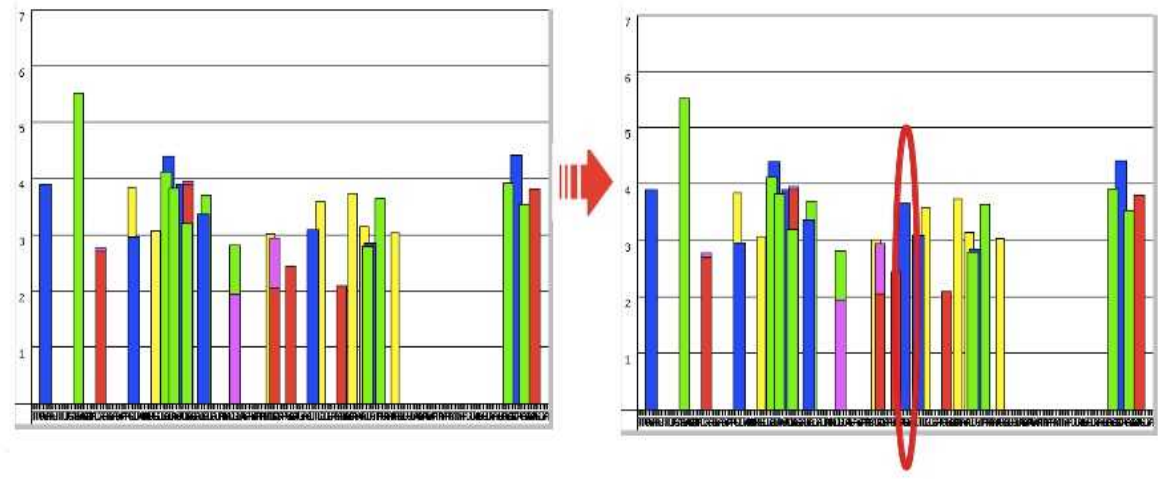

Figure 5.3. Result of ESEfinder analysis (red circle shows the increment comparing with the threshold value).

5.2. Site Directed Mutagenesis

5.2.1. PCR Result of S2 and WT2

In order to create the mutant splice site, the region that contains the site directed mutagenesis within seventh exon-intron region of *GABRG2* gene was amplified successfully as described in Section 4.2.1. Fragment size was 624 bp and gel image of PCR product for the region of interest is shown in Figure 5.4.

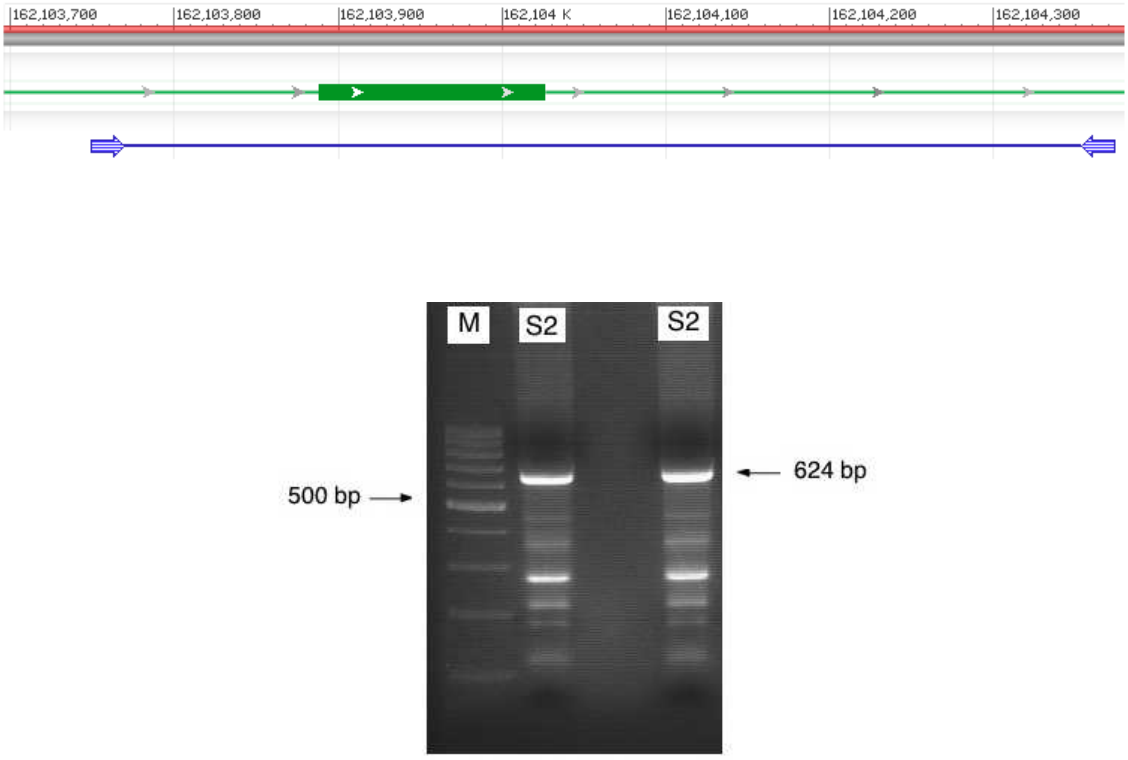

Figure 5.4. Amplified region for exon 7 (upper) and PCR products visualized on 2% agarose gel (lower) (M: 100 bp DNA length marker, S2: site directed mutagenesis).

5.2.2. Sequencing Result After Mutagenesis

The sample was sent to Macrogen for automated sequencing. The result was obtained and compared with the normal sequence. It was shown in Figure 5.5 that desired mutation was inserted successfully by site directed mutagenesis.

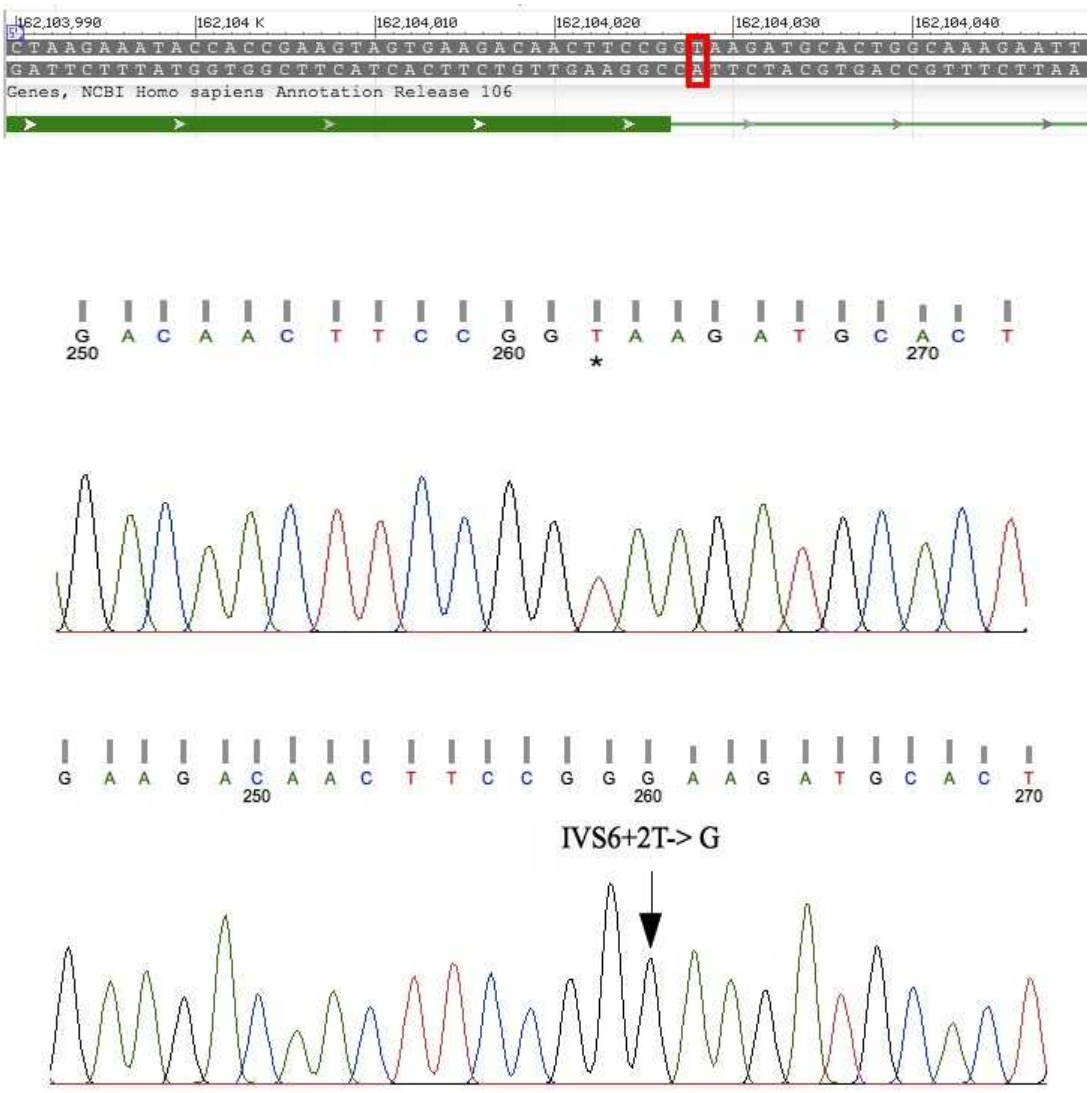

Figure 5.5. Reference sequence showing the mutation in the intronic area (top figure), chromatograms of the wild-type (middle) and the site directed mutagenesis product (lower) showing that the T->C transition (indicated by the arrow).

5.3. Cloning

PCR products were inserted by recombination and plasmids were isolated. After that, the results were checked by restriction analysis and sequencing.

5.3.1. Restriction Analysis

In order to see the results of molecular cloning, the plasmid DNAs were checked with restriction enzyme assay by using *Kpn I*. *Kpn I* sites are placed outside of the recombination sites as shown in Figure 5.6. *Kpn I* cuts the plasmid at two sites and produces a backbone with a length of 3920 bp; 1077 and 1106 bp inserts for each plasmid. For each plasmid, 6 different colonies were choosen and visualized on 1% agarose and the product lengths were observed as expected (Figure 5.7 and Figure 5.8).

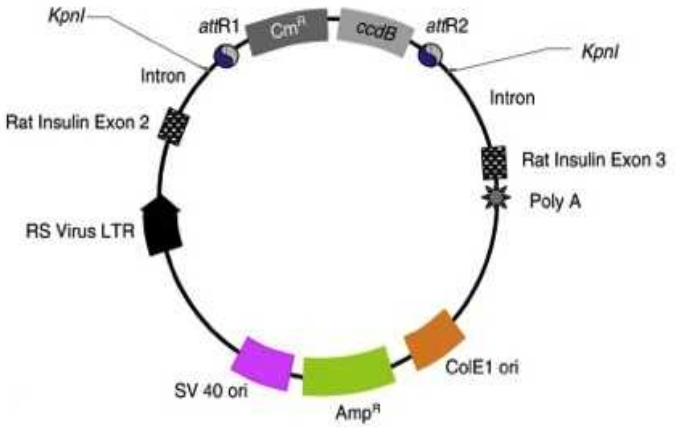

Figure 5.6. *Kpn I* sites placed in the vector.

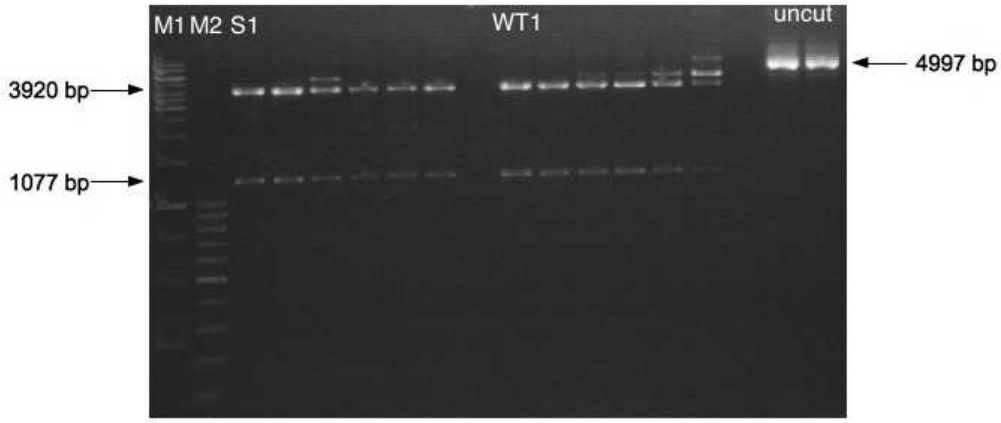

Figure 5.7. Purified plasmids taken from different single colonies on 1% agarose gel (M1: 1kb, M2: 100 bp DNA length marker; S1: patient, WT1: wild type 1).

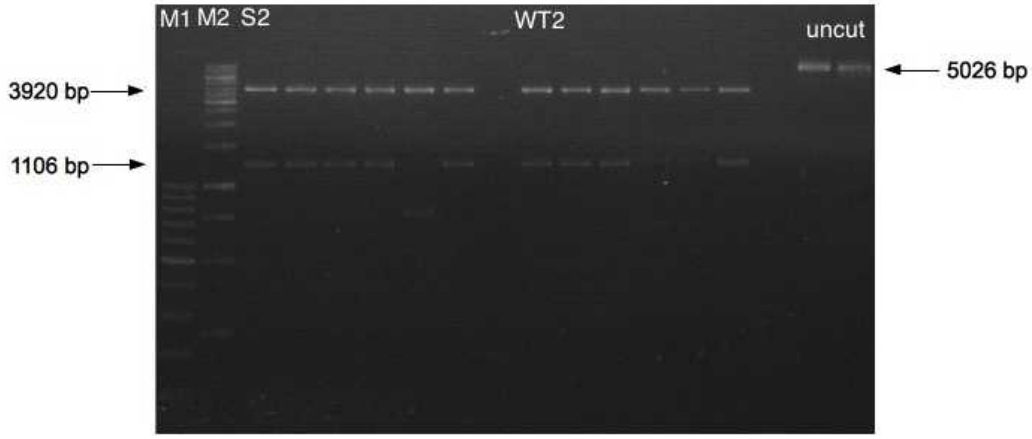

Figure 5.8. Purified plasmids taken from different single colonies on 1% agarose gel (M1: 1kb, M2: 100 bp DNA length marker; S2: site directed mutagenesis, WT2: wild type 2).

5.3.2. Sequencing of the Plasmids

Each purified plasmid DNA was sent to Macrogen for automated sequencing and sequences of the inserts were confirmed. The presence of mutations are shown in Figure 5.9 and Figure 5.10.

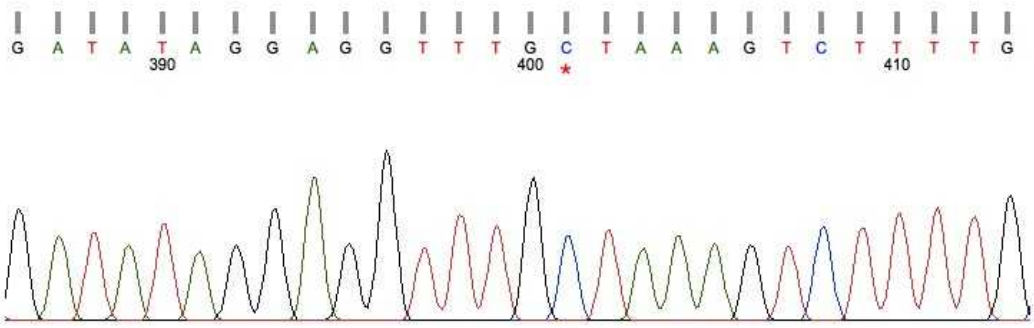

Figure 5.9. Chromatogram showing the sequence of plasmid that contains T->C mutation in the patient.

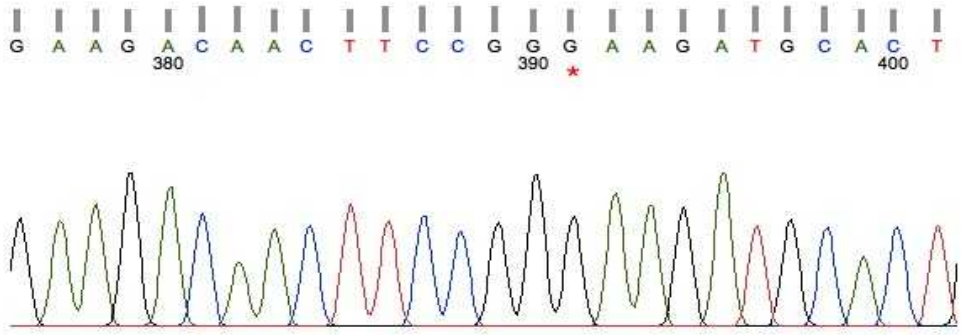

Figure 5.10. Chromatogram showing the sequence of plasmid that contains T->G mutation in the site directed mutagenesis product.

5.4. Expression Analysis

5.4.1. Transfection into Shsy-5y Neuroblastoma Cells

The transfection procedure was applied as explained in Section 4.4.1. Transfection was done after the cells were differentiated and the morphological changes are shown in Figure 5.11. After the cell culture had reached 70% confluence, transfection was started and all the cells incubated 72 hours in order to get more efficient transfection results Then, the cells were lysed in order to isolate all the RNAs.

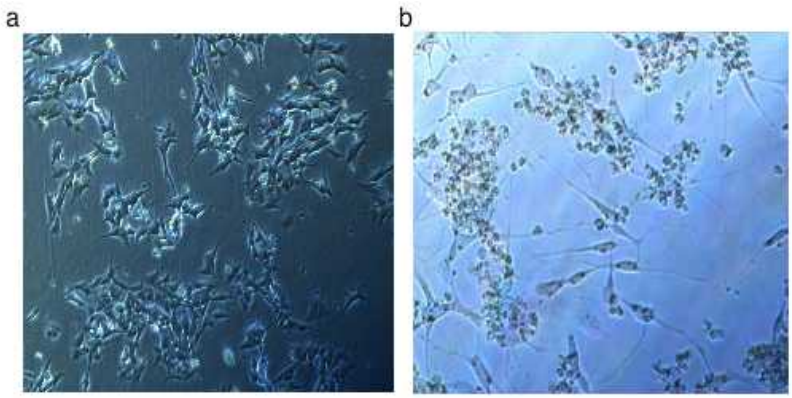

Figure 5.11. Cell placement and morphology a) before and b) after the differentiation.

5.4.2. RNA Isolation

After transfection, all the cells for each plasmid placed in different culture dish wells were harvested, total RNAs were isolated and stored at -80^{o}C for further analyses. Extraction results on 2% agarose gel as shown in Figure 5.12.

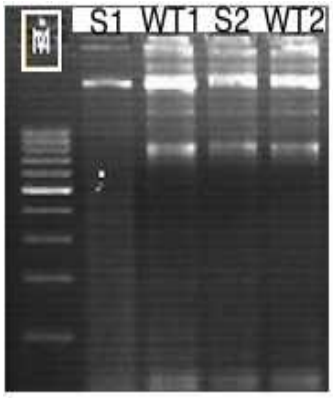

Figure 5.12. Isolated RNAs on 2% agarose gel (M: 100 bp DNA length marker, S1: patient, WT1: wild type 1, S2: site directed mutagenesis, WT2: wild type 2).

5.4.3. Reverse Transcriptase PCR

After RNA isolation, cDNAs were synthesized and amplified by RT-PCR. The products were visualized on 2% agarose gel as shown in Figure 5.13 as expected. Even the gel image, it can be observed that there is a retention for S2 with the 492 bp amplification comparing with 439 bp amplification in WT2. S1 and WT1 were expected to amplify closer to each other with the 458 and 452 bp long, respectively so that sequencing results for these will be decisive.

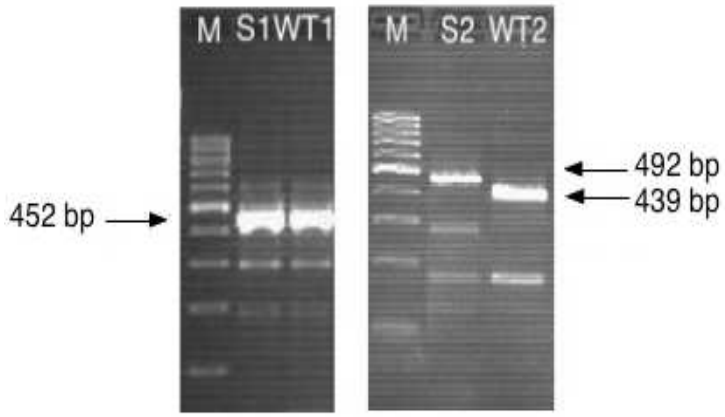

Figure 5.13. RT-PCR products on 2% agarose gel (M: 100 bp DNA length marker, S1: patient, WT1: wild type 1, S2: site directed mutagenesis, WT2: wild type 2).

5.4.4. Sequencing of RT-PCR Products

Each product was extracted and sent to Macrogen for automated sequencing (Figure 5.14 and Figure 5.15). Figure 5.14 proves that the positive control S2: IVS6+2T->G changed the splicing and there is a retention after the exonic region.

Figure 5.14. IVS6+2T->G mutation causes retention after the exonic region (Tian and Macdonald, 2012).

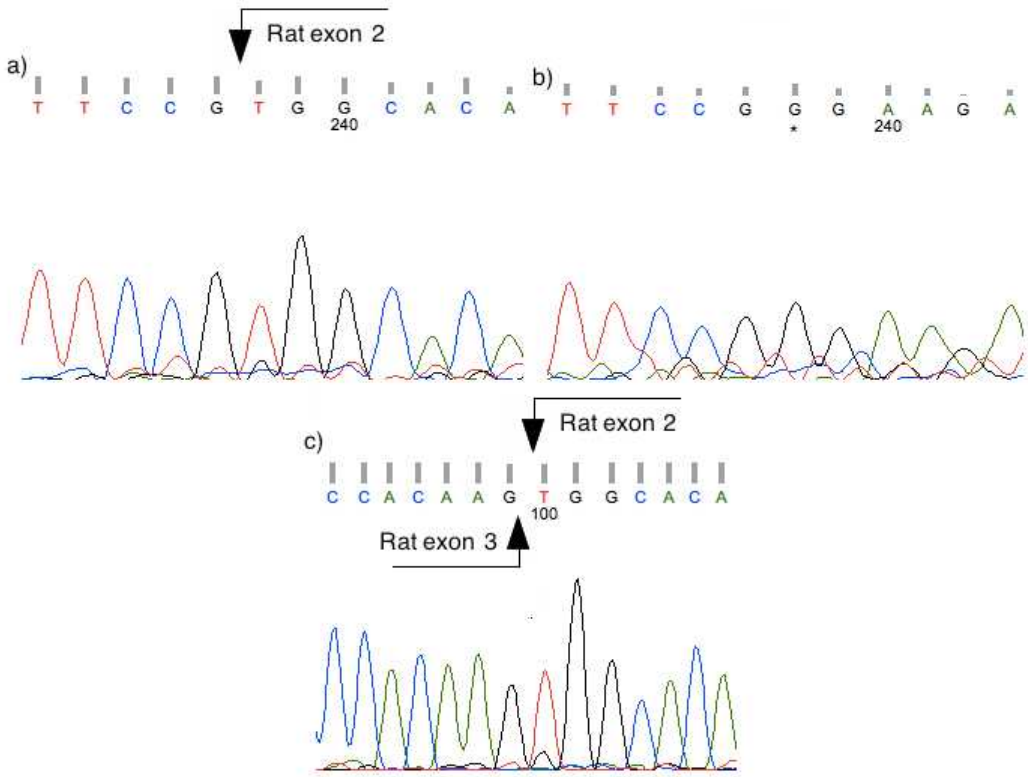

Figure 5.15. Chromatogram shows that a) the wild type 2 (WT2) b) the site directed mutagenesis (S2) (pointed out with an asterisk) that is changed the splicing and caused retention after the exonic region c) splice variant that contains only rat exons 2 and 3.

In Figure 5.16, it was shown that comparing with the WT1, there is no retention after the exonic region in the patient so that IVS2+6T->C mutation did not create a cryptic splice site.

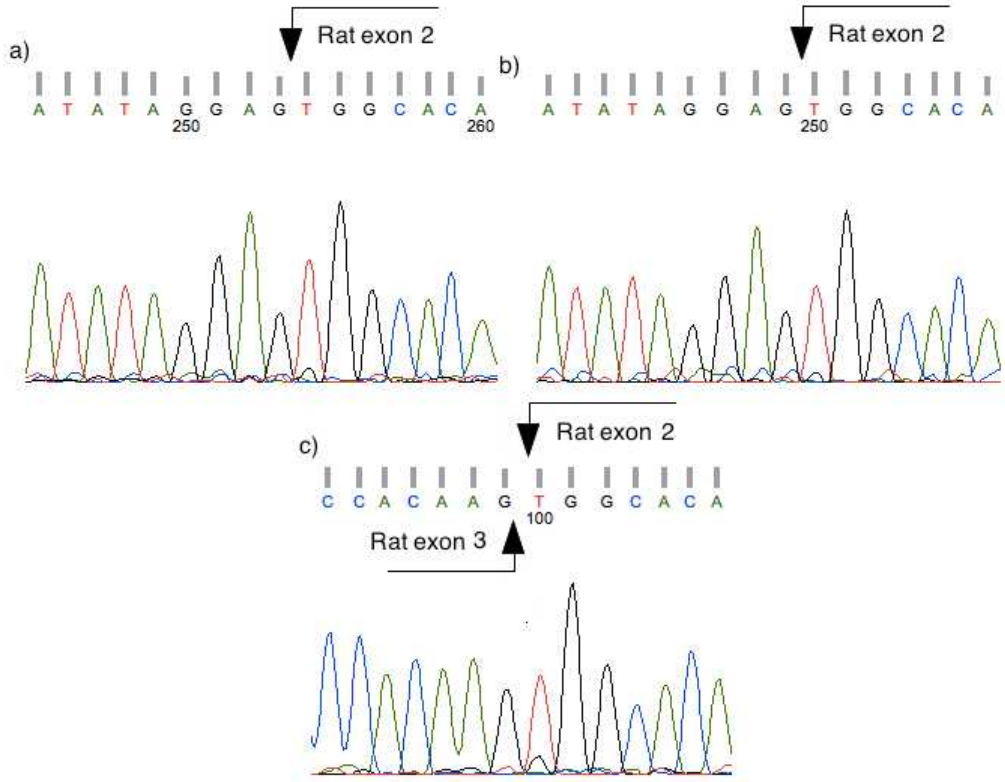

Figure 5.16. Chromatogram shows that a) the wild type 1 (WT1) b) the patient (S1) and c) splice variant that contains only rat exons 2 and 3.

6. DISCUSSION

Genetic Generalized Epilepsies which consist of around 40% of all epilepsies, are generally caused by genetic factors. But, idiopathic epilepsies inherit mainly oligogenic or multifactorially rather than monogenic inheritance. According to studies with large families with rare monogenic forms of disease showed that besides GABA receptors, there are mutations in different ion channels like voltage gated sodium channels and potassium channels causing epilepsy (Escayg and Goldin, 2010, Maljevic and Lerche, 2013). These channels are responsible for the regulation of excitability in central nervous system and any changes in ion channels lead to hyper- or hypoexcitability in brain which is stated as channelopathies.

One third of the all synapses use GABA as inhibitory neurotransmitter and related to *GABA* receptor epilepsy-associated mutations or variants, half of them in *GABRG2* which encode the subunits of GABA receptor were found to cause CAE. Despite of the all missense and nonsense mutations that are known to cause CAE, splice variants and the their relation with CAE is mostly unknown. One of our CAE patient has a T->C transition at 6th nucleotide in intron 2 in *GABRG2* gene and there are a few reasons to think that this mutation might be pathogenic. First, the mutation is sporadic, there is no mutation in the healthy family members and not observed in the population (tested on 180 individuals). Second, the splice site tools showed with relatively high score that the mutation in the intron could create a cryptic donor splice site and there will be an inclusion from the intron. Due to the new predicted splice site, there will be 6 bp retention from the intron, a premature stop codon will occur at the amino acid 89 thus truncated protein and there will be reduced inhibitory action that can cause seizure formation.

Alternative splicing is a key cellular mechanism for regulation of gene expression and protein diversity. In humans, almost 95% of multiexonic genes are alternatively spliced and a climbing number of human diseases are caused by aberrant splice site selection (Wang and Cooper, 2007). Splicing is done by the macromolecular machinery known as the spliceosome which contains U1, U2, U4/U6, and U5 small nuclear ribonucleoprotein particles (snRNPs) and a few protein factors. The process starts with binding of U1 snRNP

to the 5' splice site and continues with U2 snRNP binding to the branchpoint at the 3' splice site. Then, it is followed by the joining of U4/6 and U5 tri-snRNPs to produce the mature spliceosome for catalysis (Han *et al.*, 2011). It was predicted that T->C transition at 6th nucleotide in intron 2 can affect the binding of U1 snRNP so that splicing will be changed (Figure 6.1). Due to the change U1 snRNA may not bind to the gt site and there could be a intronic retention.

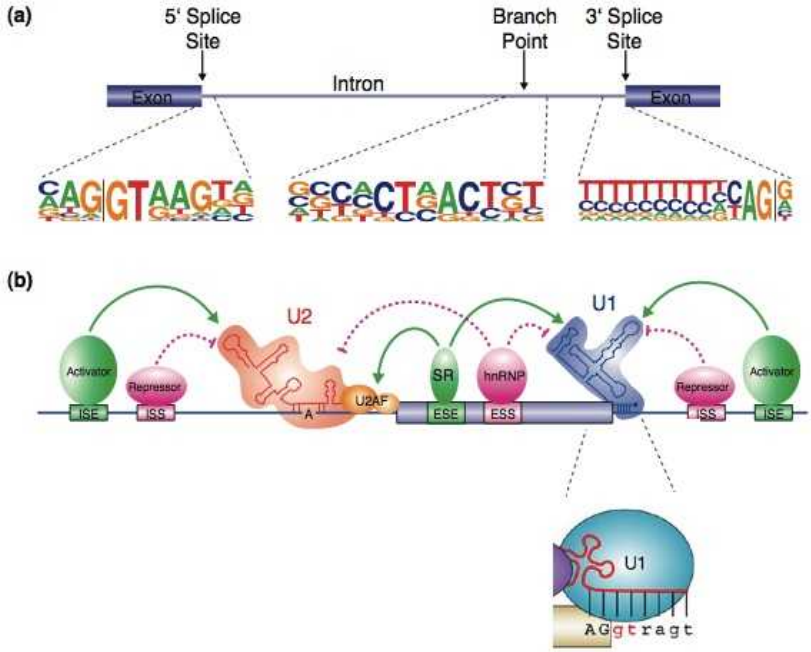

Figure 6.1. Basic mechanisms of alternative splicing. a) important sequence elements on pre-mRNA which is directing splicing reaction b) illustration of the sequences and proteins responsible from regulating alternative splicing. ISE: intronic splicing enhancer, ISS: intronic splicing silencer, ESE: exonic splicing enhancer, ESS: exonic splicing silencer (McManus and Graveley, 2011, Pagani and Baralle, 2004).

In order to investigate a mutation in the second exon intron boundary of *GABRG2* gene in functional level, minigene system is chosen. It is a recombination based method and compared with other restriction enzyme based cloning methods, minigene system is quite successful and efficient. In this method, pSpliceExpress plasmid has two rat insulin exons. When second exon intron region is inserted into plasmid, there could be three splice variants: first one is consisting of two rat exons, second one is normal spliced form of exon between two rat exons and if there is a splicing defect, there will be third variant which contains intronic retention after the interested exon within two rat exons. However, it was observed that the candidate has the same splicing pattern compared with the wild type sample implicating that IVS2+6T->C mutation did not create a cryptic splice site and not changing the splicing process. Therefore, *GABRG2* splice site variant in the CAE patient had no effect on splicing suggesting that it was a silent variant of the gene.

To show that the minigene system is working, an intronic mutation IVS6+2T->G in *GABRG2* gene previously known to create a cryptic splice site was choosen as positive control. The mutation abolished intron 6 splicing at the donor site, activated a cryptic splice donor site and created partial intron 6 retention. This mutation was generated by site directed mutagenesis and inserted into plasmid similary. In this case, the third splice variant exists and includes intronic retention after exon six leading to the conclusion that the system is working properly.

Most of the metazoan gene transcripts have neuron-specific splicing patterns and some splicing regulators are expressed in a tissue specific manner (Boutz *et al.*, 2007). Due to this, neuroblastoma cell line was choosen to get the most accurate results. As stated, neural differentiation is important and it can be thought that differentiation rate in neuroblastoma cells could affect the splicing pattern. But, it was shown that positive control IVS6+2T->G was working and proven that splicing is changed, possibility of low differentiation rate is eliminated. Similarly, in the case of nonsense mediated decay, our positive control is known to undergo NMD (Tian and Macdonald, 2012). So, if the mRNA of positive control could be amplified, the mRNA of the candidate mutation should be seen as well. Therefore, it could be concluded that normal splicing observed in IVS2+6T->C mutation is not caused by cellular and environmental factors.

GABAergic neurons mainly provide an exquisite balance between excitatory and inhibitory neurotransmission on functioning brain with highly diverse $GABA_A$ receptor subtypes. $GABA_A$ receptors are responsible for several diseases like anxiety, schizophrenia, insomnia and Alzheimer's disease besides epilepsy (Mochler, 2006). Specifically in terms of disease-associated alternative splicing patterns in ion channel genes, two common neurological conditions: Alzheimer's disease and epilepsy are related with extensive changes in gene splicing and importantly in GABA receptors (Heinzen *et al.*, 2007). Functional analysis of the gene is very informative and essential to be able to understand the pathogenic effect of the mutation and identification of pathogenic mutation and splicing abnormalities.

6.1. Conclusion

Genetic Generalized epilepsies are heterogenous group of disorders and causative gene mutations are likely to overlap with different brain disorders. Therefore, phenotype/genotype correlations are necessary for differential and accurate disease diagnosis. Mutations in noncoding regions could also be responsible for the diseases and traditional methods for mutation analysis overlook splicing defects that are placed at internal positions in coding exons. We showed that minigene method is quite suitable and useful to prioritize and identify mutations that lead to aberrant pre-mRNA splicing and reveal the pathogenesis of the disease at the molecular level.

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