ABSENCE EPILEPSY IN TURKISH PATIENTS: A NOVEL GENE AT SUSCEPTIBILITY LOCUS 2q36 AND THE ROLE OF GABRG2

by

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To my beloved parents

and

to Cengiz Çapan

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ABSTRACT

ABSENCE EPILEPSY IN TURKISH PATIENTS: A NOVEL GENE AT SUSCEPTIBILITY LOCUS 2q36 AND THE ROLE OF GABRG2

Idiopathic absence epilepsies (IAE) are complex disorders mainly caused by genetic factors. Two major whole-genome linkage studies performed on many IAE samples pointed to chromosome 2q36 as a susceptibility locus for absence epilepsies. Whole genome linkage study on absence epilepsy model Wag/Rij rats, also showed the syntenic 2q33-37 region to be linked to the quantative trait of absence seizures. Candidate ion channel genes at 2q36 were screened but causative mutation could not be identified. On the other hand, mutations have been found in the subunits of GABA receptors and Ca channels in a few patients. At present, therefore, the complete picture of pathogenesis of the absence seizures is not known. In this study, to assess the possible role of 2q36 region in absence epilepsy, 205 Turkish absence patients and 219 healthy controls were used in an association analysis. Based on the haplotype block structure of the Turkish population in this region 10 tagSNPs were selected to cover the 160kb region at 2q36. The patients were subgrouped according to the syndrome and seizure types. The results revealed a significant association of two neighboring SNPs (rs7588807 and rs2840128) with JAE syndrome and even higher significant association with GTCS with the same SNP, rs7588807 that resided in the INHA gene. The point mutation and qPCR analysis of the INHA gene revealed mutations/variations in several patients and a large deletion that covered 30-50 kb in at least seven JAE patients supporting the association of INHA with the epilepsy phenotype and its establishment as a novel gene involved in the pathogenesis of JAE. The presence of other candidate genes in the deleted region paves the way for further molecular genetic analysis to reveal the role of each candidate gene in the pathogenesis of epilepsies, if any. The study further supports the role of GABRG2 in the pathogenesis of absence seizures by the identification of novel variations/mutations especially affecting the splice sites in CAE patients.

ÖZET

TÜRK HASTALARINDA ABSANS EPİLEPSİ: YATKINLIK BÖLGESİ 2Q36 DA YENİ BİR GEN VE GABRG2'NİN ROLÜ

İdyopatik absans epilepsiler (IAE) genetik temeli olan kompleks hastalıklardır. Çok sayıda IAE hastasında yapılan bütün genomu kapsayan iki büyük bağlantı çalışması 2q36 kromozom bölgesinin absans nöbetlerine yatkınlık oluşturduğuna işaret etmektedir. Absans epilepsi modeli Wag/Rij sıçanlarında da bütün genomu kapsayan bağlantı çalışması sintenik 2q33-37 bölgesinin kantitatif karakterdeki absans nöbetlerine bağlı olduğunu göstermiştir. 2q36 da aday iyon kanal genleri tarandığında mutasyona rastlanmamıştır. Diğer taraftan, birkaç hastada GABA reseptör altüniteleri ve Ca kanallarında mutasyon bulunmuştur. Günümüzde absans nöbetlerinin tüm patojenezinin hala bilinmediği görülmektedir. Bu çalışmada, 2q36 bölgesinin absans nöbetlerinde olası rolünü sorgulamak için 205 Türk absans hastası ve 219 sağlıklı kontrol örnekleri bir ilişkilendirme çalışmasında kullanılmıştır. Türk toplumunun haplotip blok yapısına dayanılarak 2q36 bölgesinde 160 kb'lık bölgeyi kaplayacak şekilde 10 tag SNP seçilmiştir. Hastalar sendrom ve nöbet tiplerine göre alt gruplara ayrılmıştır. Sonuçlar iki komşu SNP'in (rs7588807 ve rs2840128) JAE sendromu ile anlamlı bir ilişkisi olduğunu ve INHA geni içinde bulunan SNP rs7588807'nin GTCS nöbet tipi ile daha da yüksek anlamda ilişkili olduğunu göstermiştir. INHA geninin nokta mutasyonu ve qPCR analizleri birkaç hastada mutasyon/varyasyonlar ve en az 7 JAE hastasında 30-50 kb'lık bölgeyi kapsayan büyük bir delesyonun varlığını göstererek INHA geni ile epilepsi fenotipi arasındaki ilişkiyi desteklemiş ve JAE patojenezinden sorumlu yeni bir genin varlığını ortaya koymuştur. Delesyonun olduğu bölgede başka aday genlerin varlığı ise her bir aday genin, eğer varsa, epilepsi patojenezindeki rolünü ortaya çıkaracak daha ileri moleküler genetik analizlerinin önünü açmıştır. Bu çalışmada ayrıca, GABRG2'nin absans nöbetleri patojenezindeki rolü CAE hastalarında özellikle yeni kırpılma bölge mutasyon/varyasyonlarının varlığı gösterilerek desteklenmiştir.

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2q	Long arm of chromosome 2
A	Adenine
ACCN4	Amiloride Sensitive Cation Channel
AED	Anti Epileptic Drug
Arg	Arginine
ASSP	Alternative Splice Site Predictor
Asp	Aspargine
bp	Base pair
BN	Brown Norway
C	Cytosine
Ca	Calcium
CACNA1A	Calcium Channel alpha 1A subunit
CACNA1G	Calcium Channel alpha 1G subunit
CACNA1H	Calcium Channel alpha 1H subunit
CACNA1I	Calcium Channel alpha 1I subunit
CAE	Childhood Absence Epilepsy
CEU	European
CHB	Chinese Han
CHPF	Chrondroitin Polymerizing Factor
CI	Chloride
CLCN2	Chloride Channel
CNV	Copy Number Variation
Cp	Cross point
C-terminus	Carboxyl Terminus
Del	Deletion
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate

EDTA	Ethylenediaminetetraacetate
EEG	Electroencephalogram
EMA	Eyelid Myoclonia with Absence Seizures
ESE	Exonic Splicing Enhancer
ESPS	Excitatory Post-synaptic Potentials
EtBr	Ethidium Bromide
EtOH	Ethanol
FSH	Follicle Stimulating Hormone
FXI	Factor XI
G	Guanine
GABA	Gamma-Amino Butyric Acid
GABRA1	Gamma-Amino Butyric Acid α1 Subunit
GABRA5	Gamma-Amino Butyric Acid α5 Subunit
GABRB2	Gamma-Amino Butyric Acid β2 Subunit
GABRG2	Gamma-Amino Butyric Acid γ2 Subunit
GABRG3	Gamma-Amino Butyric Acid γ3 Subunit
GAERS	Genetic Absence Rats from Strasbourg
GMPPA	GDP-mannose Phosporylase A
$GEFS^{+}$	Generalized Epilepsy with Febrile Seizures Plus
Glu	Glutamine
GTCS	Generalized Tonic Clonic Seizure
His	Histidine
Hz	Herzt
HRM	High Resolution Melting
HVA	High Voltage Activated
IAE	Idiopathic Absence Epilepsy
IGE	Idiopathic Generalized Epilepsy
INHA	Inhibin Alpha Precursor
IPSP	Inhibitory Postsynaptic Potentials
IVS	Intervening Sequence Variation
JAE	Juvenile Absence Epilepsy
JME	Juvenile Myoclonic Epilepsy
JPT	Japanese

kb	Kilo base
LD	Linkage Disequilibrium
Leu	Leucine
LVA	Low Voltage Activated
MAF	Minor Allele Frequency
Mb	Mega base
ME2	Malic Enzyme 2
Met	Methionine
$MgCl_2$	Magnesium chloride
min	Minute
mRNA	Messenger RNA
NaCl	Sodium chloride
ng	Nanogram
NMDA	N-Methly D-Aspartate
nRT	Thalamic Reticular Nucleus
OBSL-1	Obscruin-like
PCR	Polymerase Chain Reaction
Phe	Phenylalanine
Pro	Proline
qPCR	Quantative PCR
QTL	Quantative Trait Loci
RASE	Refractor Absence Status Epilepticus
RE	Restriction Enzyme
RNA	Ribonucleic acid
rpm	Revolutions per minute
rs	Reference sequence
RT-PCR	Reverse Transcriptase PCR
SDS	Sodium Dodecyl Sulfate
sec	Second
SLC4A3	Solute Carrier Family Anion Exchanger
SNP	Single Nucleotide Polymorphism
STK11IP	Serine/threonine Kinase 11 Interacting Protein
SWD	Spike Wave Discharges

T	Thymine
Taq	Thermus aquaticus
TBE	Tris-Boric acid-EDTA
TE	Tris-EDTA
TDT	Transmission Disequilibrium Test
TGF	Transforming Growth Factor
UTR	Untranslated Region
UV	Ultra Violet
Val	Valine
Wag/Rij	Wistar Albino Glaxo Rat
YRI	Yoruba

1. INTRODUCTION

Epilepsy, which affects more than 0.2 per cent of the general population, is a group of diseases caused by non-controlled discharge of neurons of either the whole cortex (generalized epilepsy) or localized brain areas (partial epilepsy). Idiopathic generalized epilepsies (IGE), that account for up to 40 per cent of all epilepsies are not preceded or occasioned by other disorders but mainly caused by genetic factors (Moulard *et al.*, 2001). Childhood absence epilepsy (CAE), juvenile absence epilepsy (JAE) and juvenile myoclonic epilepsy (JME) are the most common forms of IGEs however, in most cases of IGEs, there is no clear classification of each subtype as complex phenotypes overlap.

1.1. Clinical Features of Absence Epilepsy

Childhood absence epilepsy (CAE) is a subtype of IGEs and has a prevalence of 2-10 per cent among children with any type of epilepsy. It is characterized by non-convulsive epileptic seizures which has typical features of brief (14-20s) and sudden loss of consciousness with a generalized synchronous, bilateral, 2.5-4 Hz spike and slow wave discharge (SWD) in the electroencephalogram (EEG) shown in Figure 1.1 (Avoli *et al.,* 2001; Burgess and Noebels, 1999). The seizures which has a frequency of up to 200 per day (pyknoleptic), start at age between 3 and 8 and are not induced by visual or other sensory stimuli. In 70 per cent of the patients, the seizures often disappear spontaneously around adolescence (Crunelli and Lereche, 2002). JAE on the other hand, is characterized with non-pyknoleptic absence seizures with first occurrence after 10 years of age. Unlike CAE, the seizures are associated frequently with other epileptic symptoms such as generalized tonic-clonic seizures (GTCS) and myoclonic jerks.

1.2. Cellular and Network Mechanism of Absence Seizures

The experiments in animal models revealed that the thalamus and the cortex are both involved in the generation of SWDs. In an absence epilepsy cat model where the seizures generated by direct application of penicillin that is a weak gamma-aminobutyric acid

$(GABA)_A$ antagonist to the cortex is sufficient to induce SWDs, but the removal of the thalamus, cortex or interconnections abolish these seizures (Avoli and Gloor, 1982). On the other hand, by application of bicuculline which is an antagonist of $GABA_A$ receptors, into the thalamus of rats, SWDs can be generated (Castro-Alamancos, 1999). Therefore, there is a possibility that the onset of SWDs may vary between different species but also within individuals with the same or different absence syndromes showing the complexity of the disease.

As shown in Figure 1.2 there are three main components of thalamocortical network:

- Thalamocortical relay neurons which transfer inputs from a large number of sources to pyramidal neurons in III-IV and V-VI layers of the cortex through excitatory synaptic connections.
- Layer VI pyramidal cells of the cortex send back excitatory inputs to the thalamus
- Inhibitory GABAergic interneurons form a layer in the thalamic reticular nucleus (nRT) which receives excitatory inputs from axon collaterals of the reciprocal thalamocortical and corticothalamic pathways. When they are activated these GABAergic neurons send inhibitory inputs to the thalamus and also to each other but not to the cortex.

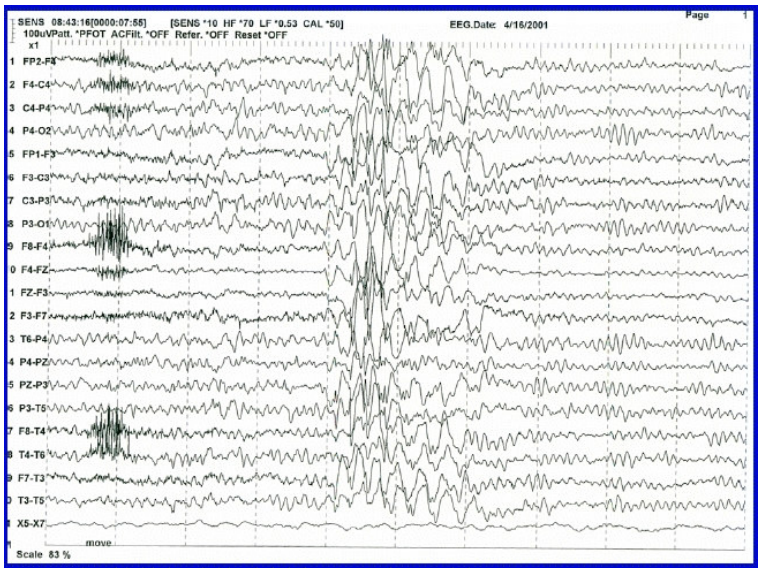


Figure 1.1 Spike wave discharges in EEG in absence epilepsy

(Babb and Brown, 1987)

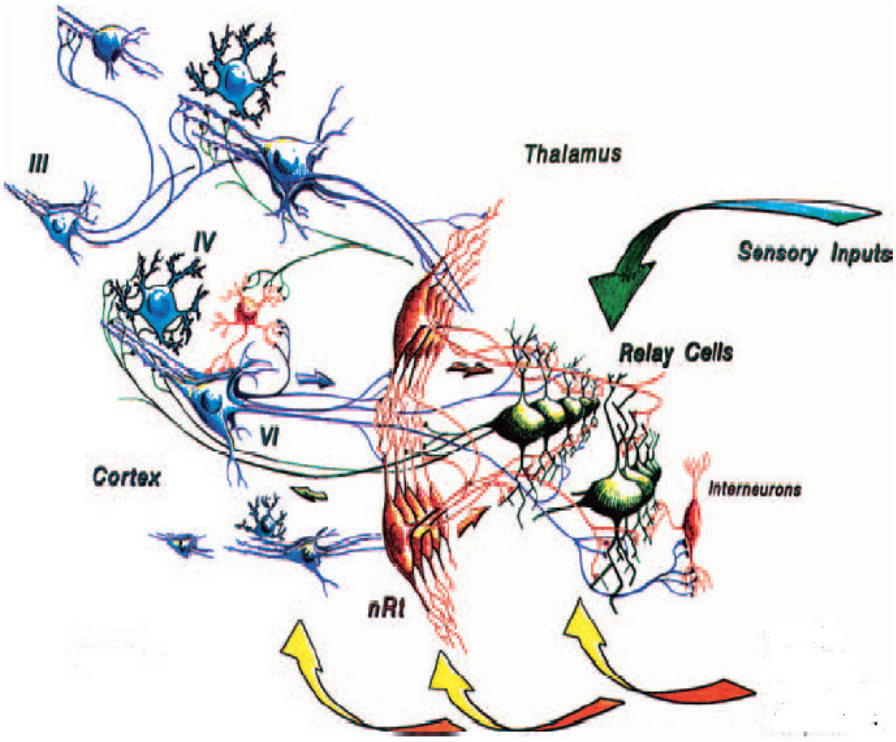


Figure 1.2. Cellular elements of thalamic and cortical network (Blumenfeld, 2005)

When this thalamocortical circuit works properly a burst of synchronized oscillitations with a frequency of ~10Hz occur. In this circuit thalamocortical neurons induce excitatory post-synaptic potentials (EPSPs) in GABAergic nRT neurons via NMDA and non-NMDA receptors. Low-threshold calcium channels in nRT neurons are activated that lead to the opening of Na channels and, therefore, initiate action potentials. The activations of these inhibitory neurons induce inhibitory postsynaptic potentials (IPSPs) in thalamocortical neurons via $GABA_A$ receptors. This inhibitory phase abolishes the burst firing in the circuit for a time. During this hyperpolarized state low-threshold calcium channels in thalamocortical neurons recover from inactivation. The calcium channels open and depolarize the membrane, thus, make the cell available for the next burst of action potentials. However, any abnormal acvity in this circuit would distrupt the alternating cycles of excitatory and inhibitory activity and lead to 3-4Hz spike wave discharges. For example, any increased excitatory activity in the cortex or thalamocortical region would lead to a longer burst of nRT neurons firing which causes longer IPSPs meadiated by $GABA_B$ receptors via G-protein coupled K channels in the thalamocortical region. During

this long hyperpolarized state calcium channels open and iniate several action potentials in every cycle causing paraxymal spike and slow wave discharges.

Besides this possible abnormal activity in the cortex or thalamocortical regions, there are other susggested mechanisms for the generation of abnormal spike wave discharges like the loss of $GABA_A$ receptor mediated inhibition between thalamic reticular cells, thus, these cells produce longer IPSPs on thalamortical cells mediated by $GABA_B$ receptors and enhancement of the low threshold Ca current (McCormick and Contreras, 2001).

1.3. Genetic Etiology of CAE

IGEs are known as channelopathies as in studies that explored the pathogenesis of the epilepsy, the first mutations identified were in ion channels in rare cases of IGEs. Up to date mutations and susceptibility alleles were found in GABA receptors, Ca and CI channels that generate spike wave discharges in absence seizures. However, in recent years novel genes and approaches like studying the copy number variations and epigenetic modifications draw more attention to understand the genetic components of the IGEs.

1.3.1. GABA Receptors

GABA is the main inhibitory neurotransmitter in the central nervous system. It interacts with two major subtypes of receptors $GABA_A$ and $GABA_B$ which are both involved in the generation of spike wave discharges in absence seizures.

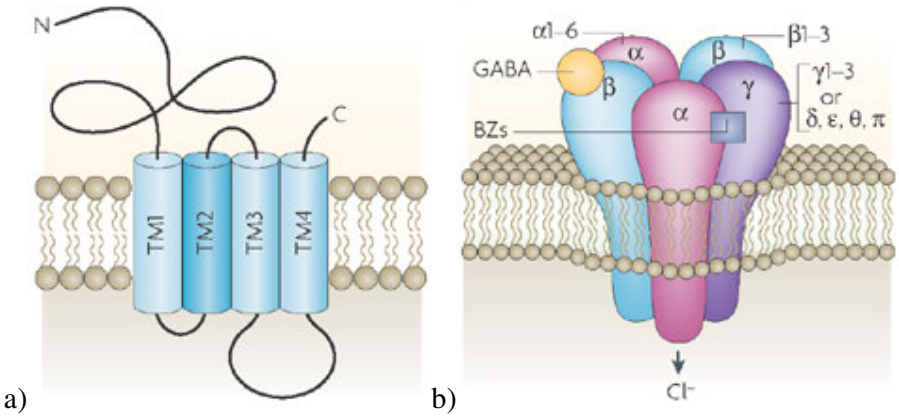
1.3.1.1. $GABA_A$ Receptors. Ionotropic $GABA_A$ receptors mediate fast synaptic inhibition in the central nervous system. Upon binding of GABA to the $GABA_A$ receptors, they allow the influx of CI^- ions causing hyperpolarization of the membrane and inhibition of action potentials (Fritschy and Brünig, 2003). $GABA_A$ receptors are pentameric structures that consist of five out of at least 18 subunits (α1-6, β1-3, γ1-3, δ, ε, θ, ρ1-3) (Frugier *et al*., 2007). Each subunit has four transmembrane domains with the second transmembrane of each subunit forming the channel pore (Chebib and Johnston, 1999). Figure 1.3 shows the genetic composition and GABA receptor stucture. The genes that code for these subunits are localized as clusters on different chromosomes. The most prevalent $GABA_A$ receptor

throughout the brain is formed by α1, β2 and γ2 subunits which are encoded by GABRA1, GABRB2 and GABRG2 genes localized on 5q34-35. Another GABA gene cluster (GABRA5, GABRB3 and GABRG3) resides on 15q11-12 coding for the α5, β3 and γ3 subunits. These receptors can be modulated by stereoids, barbiturates and benzodiazepines. The role of $GABA_A$ receptors in epileptic activity are well known as the $GABA_A$ receptor agonists such as barbiturates and benzodiazepines suppress seizures while the $GABA_A$ blockers such as bicuculline, penicillin and picrotoxin induces epileptic activity in model animals.

In the analysis of $GABA_A$ receptors in absence seizures, the focus has been mostly on the γ2 subunit as the first mutation was identified in the GABRG2 gene in a family with CAE and febrile seizures (Wallace *et al.*, 2001). The γ2 subunit is known to be responsible for modulation by benzodiazepine and for receptor targeting (Fritschy and Brünig, 2003). The mutation in the CAE family was arginine to glutamine substitution at amino acid 43 (Arg43Glu) leading to the loss of current through GABA receptors due to an impairment of trafficking of the receptor to the membrane. Functional studies pointed out that the subunit with the mutation is stuck in the endoplasmic reticulum so leading to the decreased surface expression (Kang and Macdonald, 2004). Arg43Glu mutation was also assessed in a mouse model of childhood absence epilepsy (Tan *et al.*, 2007). The mouse was constructed by insertion of Arg43Glu mutation in the heterozygous state. The mouse showed similar phenotype of childhood absence epilepsy confirming the causative role of γ2 subunit. A reduction of inhibition in the cortex of the mouse was measured pointing the start region of SWDs as cortex but not thalamus. However, thalamic bursting is subject to inputs from the cortical region, therefore, inhibition in the cortex would alter these inputs and trigger SWDs.

Second mutation in GABRG2 was found again in a CAE and febrile seizure family (Kananura *et al.*, 2002). IVS6+2T→G mutation disrupting a putative splice site and so, probably cause a truncated protein. Despite these findings there were some contradictory results in Japanese and Chinese populations as mutation analysis and association studies revealed negative linkage of CAE to GABRG2 (Ito *et al.*, 2005, Lu *et al.*, 2002). GABRG2 mutations were also identified in Generalized Epilepsy with Febrile seizures plus (GEFS+) suggesting that GABRG2 mutations can actually be responsible not for the whole CAE

syndrome but instead for febrile seizures as mutations were found in CAE families with febrile seizures.



a) b)

Figure 1.3. a) Genetic composition and b) receptor structure of $GABA_A$ receptor (Jakop *et al*., 2008)

The first study to reveal the role of GABRA1 in the pathogenesis of epilepsy included 61 JME, 38 JAE and 29 CAE patients. However, the results showed no linkage (Sander *et al*., 1996). Later mutations were identified in a JME family (Cossette *et al*., 2002) and in a CAE patient (Maljevic *et al*., 2006). In the CAE patient, single base pair deletion (975delC) caused a premature stop codon. Functional studies showed that the truncated receptor can not integrate into the membrane and causes no current. Therefore, because of the haploinsufficiency there is a loss of function of the inhibitory action of GABA channels in the thalamic neurons. In the Japanese population on other hand, attempts to find any causative mutation in typical and atypical absence patients revealed negative results (Ito *et al*., 2005). In the same study, β2 subunit of $GABA_A$ receptor was also screened, but no mutation was identified.

GABRB3 gene spans 250kb with ten exons and two alternative exon 1 for peptide signaling. The gene codes for the β3 subunit of the $GABA_A$ receptor (Glatt *et al*. ,1997). Association analysis carried out in different ethnic groups displayed possible associations in CAE patients (Urak *et al*., 2006; Lu *et al*., 2004; Feucht *et al*., 1999). In Urak's study a

common promoter haplotype was found to be at higher frequency in patients. A reporter gene assay is carried out for the possible effects of this haplotype on transcription and the results showed that this haplotype reduced the transcriptional level of GABRB3 gene by interfering with the binding site of neuron-specific transcriptional activator N-Oct-3. Reduced levels of β3 subunit would decrease the level of inhibitory $GABA_A$ receptors. This disease susceptible haplotype was also assessed in German IGE samples however, the results did not confirm that this was a common haplotype in absence patients with different ethnic groups (Hempelmann, 2007).

Mutations were found in GABRB3 in 4 families of 48 CAE Mexican families with American Indian and Spanish European ancestry. Three of the mutations resided in exon1a in signal peptide while Gly32Arg mutation in exon 2 affecting protein maturation, topology, assembly and subcellular localization of a GABA receptor by resulting in hyperglycosylation (Tanaka, 2008).

In animal models GABRB3 deficient mice showed abnormal EEG activity with generalized tonic clonic seizures, clonic and myoclonic seizures and also behaviour arrest during this abnormal EEG similar to absence seizures (DeLorey *et al*., 1998). However, the mice did not show pure epileptic seizures but also showed features of the Angelman syndrome which is known to have deletions on 15q11-13. Patients with this syndrome also have different epileptic seizures like atypical absence, myoclonic, atonic, tonic and tonic-clonic seizures. The mice were treated with carbamazepine which is a well-known antiepileptic drug (AED) for the treatment of focal epilepsies but the seizures aggravated seizures as in the case of human absence seizures (Liu *et al*., 2008)

α5-subunit was also subjected to analysis in 50 CAE patients but no causative mutation was identified (Feucht, 1999).

1.3.1.2. $GABA_B$ Receptors. Metabotropic $GABA_B$ receptors mediate their activity via G-coupled proteins by activating K^+ and Ca^{+2} ion channels, second messenger systems, phospholipase C and adenylate cyclase (Chebib and Johnston, 1999). These receptors are seven transmembrane receptors and the functional $GABA_B$ receptor is formed by heterodimers of $GABA_{(B1)}$ and $GABA_{(B2)}$ subunits. These receptors produce slow and

prolonged inhibitory signals and they are mostly located in presynaptic terminals so have an essential role in neurotransmitter release.

The mice models with knocked down $GABA_{(B1)}$ and $GABA_{(B2)}$ subunits display spontaneous SWDs indicating a possible role $GABA_B$ receptors in absence epilepsy (Gassman *et al*., 2004; Schuler *et al*., 2001). Also in the neocortex of Wag/rij rat, one of the best models of human absence seizures, there is reduced expression and function of $GABA_B$ receptor.

Although animal models emphasize the possible role of $GABA_B$ receptors, mutation and association analysis in Chinese CAE patients revealed negative results (Lu *et al*., 2003). However, as in the case of other candidate genes ethnic differences should be considered.

1.3.2. Calcium Channels

Calcium channels are voltage dependent channels whose conductance depends on changes in transmembrane potential. In excitable cells, they conduct Ca^{2+} ions and function in muscle contraction, hormone and neurotransmitter release by diverse calcium involved processes (Celesia, 2001). Calcium channels are composed of one main α1 subunit that is an integral membrane protein and smaller auxiliary subunits (β, α_2, δ and γ) (Perez-Reyez, 2006). The biological and physiological properties mostly depend on the α1 subunits which consist of four repeats of the six transmembrane domains. As in all other voltage gated channels, these domains include one S4 segment which functions as voltage sensor, a selective P-loop and S6 segment that forms the inner part of the channel. C-terminus of the α1 protein is also essential for interactions with the auxiliary subunits, Ca^{2+}-calmodulin-mediated inactivation and G-protein regulation (Catterall, 2000). Figure 1.4 shows the genomic composition, pore stucture and auxiliary subunits. There are at least two distinct classes of Ca^{2+} channels depending on the voltage requirement for activation. Low-voltage-activated (LVA) channels activate after small depolarizations of the membrane while high-voltaged-activated (HVA) channels function in the case of larger depolarizations. Calcium channels are possible targets for induction of SWDs because of their excitatory function in the thalamocortical region. Also ethosuximide which is an

essential AED to treat absence seziures is know to supress T-channel currents (Kostyuk *et al*., 1992). Attemps to find epilepsy genes in animal models and in absence patients revealed that both LVA and HVA channels have roles in the pathogenesis of the absence seizures.

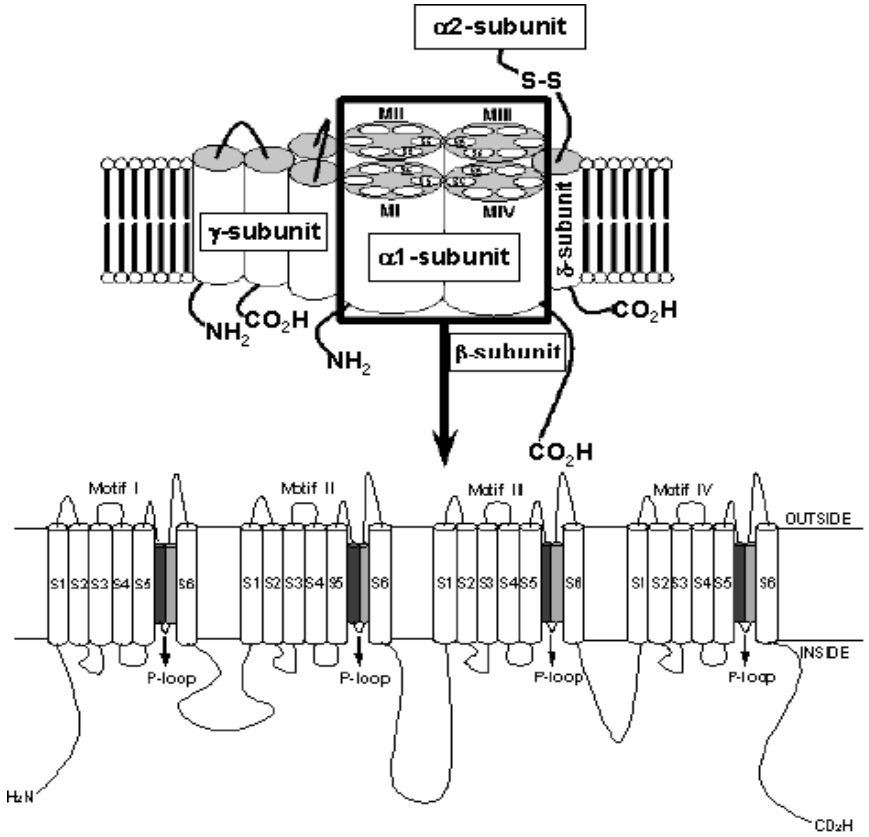


Figure 1.4. The genetic composition, pore structure and auxiliary subunits of calcium channels (Guimaraes et al., 2004)

1.3.2.1. HVA Calcium Channels. These include L-, P/Q-, N- and R- subtypes depending on their different electrophysiological, pharmacological properties and also amino acid identity (Perez-Reyes, 2003). The P/Q type calcium channels are mostly found in presynaptic terminal distribution and known to have an essential role in modulating neurotransmitter release (Regehr and Mintz, 1994). Therefore, dysfunction of these channels would impair the balance between neuronal inhibition and excitation leading to burst firing.

In a patient with severe phenotype of absence seizures and ataxia, a nonsense mutation was identified in the α1A subunit of P/Q channels (*CACNA1A*) for the first time (Jouvenceau *et al*., 2001). The gene resided on 19p13 and is composed of 47 exons spanning 300kb region. The mutation resided in C-terminus of the protein causing a premature stop codon. Functional studies showed that the mutation had a dominant negative effect which led to reduced Ca^{2+} current. Later a second mutation was identified in *CACNA1A* in a family with three generations of absence and ataxia clinical history (Imbrici *et al*., 2004). The phenotypes vary in the family with mild cases. 439G→A nucleotide transition in exon 3 in heterozygous state caused E147K amino acid substitution in the second transmembrane segment of domain I of the channel. Functional studies revealed a partial reduction in calcium channel function due to an impairment in the trafficking to the membrane.

Interestingly, in two animal models, tottering and leaner mouse which show absence and ataxia phenotype, mutations were found in *CACNA1A* (Cav2.1). In tottering mouse the mutation was located in the S4-S5 linker region of the third transmembrane domain near the pore-forming region of the channel (Fletcher *et al*., 1996). The mutation reduced the whole-cell current density and voltage dependent inactivation during prolonged depolarization in dissociated Purkinje somas (Wakamori *et al*., 1998). In the case of leaner mouse, the mutation in CACNA1A has more severe effects as it was located in C-terminus and reduced both current density and the open probability of single P/Q type channels (Doyle *et al*., 1997).

Although Cav2.1 channels appear to be good candidates for absence seizures both patients and mouse models do not show pure absence seizure but also other neurological disorders like ataxia and dystonia. Therefore, the mutations in CACNA1A could actually be the cause of other diseases, and another locus could be responsible for the epileptic phenotype. Actually, mutations in CACNA1A were identified in patients with ataxia. The other possibility is that the mutation has a secondary effect that leads to the seizures. Recent studies revealed that the second possibility is more likely as in mouse models with CACNA1A mutations, an increase in the current of LVA channels was measured in the thalamocortical region of the brain (Zhang *et al*., 2002). In mutant mice with null mutation

of CACNA1A LVA current was elevated in the thalamocortical region and also these mice were found to be more prone to have spike wave discharges (Song *et al*., 2004).

1.3.2.2.LVA Ca Channels. There are three genes that code for the α1 subunits which have low-voltage activation namely *CACNA1G* ($Ca_v3.1$), *CACNA1H* ($Ca_v3.2$) and *CACNA1I* ($Ca_v3.3$).

CACNA1G is located on 17q21.33 with 38 coding exons. $Ca_v3.1$ channel is mainly expressed in the thalamocortical neurons where the spike wave discharges occur. As a possible candidate gene, *CACNA1G* was screened in 73 Japanese and 50 non-Japanese patients and 13 variants were identified with 5 of them causing amino acid substitutions (Singh *et al*., 2007). One of these variants was found in a patient with sporadic case of JME and with a history of early childhood absence epilepsy. The mutation caused alanine to valine amino acid exchange at position 570 that was located in the intracellular portion of the protein within I-II loop. The mutation was not found in 360 control samples and when it was expressed in HEK cells, the mutated channels caused a larger Ca^{+2} current compared to the wild type, but the result was statistically not significant. The other variants on the other hand, may affect the alternative splicing of the gene as at least five different isoforms with different kinetics and steady-state properties were identified for Cav3.1 channel (Chemin *et al*., 2001).

Mutation analysis was also performed for 48 Chinese patients who were shown to have very common genomic structure with the Japanese in the HapMap project. Unfortunately, the research did not reveal any pathological change, however, six new variants were found. An association analysis was carried out to find out if there was a significant difference between controls, but it revealed negative association (Chen e*t al*., 2003a).

To assess the possible role of $Ca_v3.1$ channels in inducing spike wave discharges, *CACNA1G* was deleted in mice models. Interestingly, animals became resistant to have spike wave discharges as LVA T-type channel was abolished (Shin, 2006). In another study, *CACNA1G* is overexpressed in mouse with low and high transgene copy numbers. This led to an elevation in α1G expression and consequently in functional T-type currents

in thalamic neurons (Ernst, 2009). Both transgenic lines show spike wave discharges but, interestingly, they did not have much differences in the frequency of the seizures. The mouse also did not show any other neurological disorders, therefore, this is the direct evidence that an increase in Ca*v*3.1 led to pure absence seizures.

CACNA1H gene (Ca*v*3.2) is located on chromosome 16p13.3 with 36 coding exons (Zhong *et al*., 2006). The gene is extensively alternatively spliced and generates a family of variant transcripts. These different variants have shifted voltage-domain for gating, the kinetics of activation, inactivation and recovery from inactivation and the magnitutute and voltage midpoints for functional window currents. Therefore, changes that affect the ESE regulatory sites in exons or splicing in intronic regions could predispose seizures. CACNA1H is mainly expressed in the thalamic reticular nuclues (Shin, 2006).

The first mutation analysis that was carried out in 118 Chinese CAE patients revealed 12 missense mutations in 14 of the patients in heterozygous state (Chen Y *et al*., 2003b). The mutations were introduced into human $Ca_v3.2a$ cDNA and transfected into HEK-293 cells for whole-cell patch-clamp recordings (Vitko, 2007; Vitko *et al*., 2005; Khosravani *et al*., 2004). T-type channel activity was found to be increased in all mutant types causing SWD in absence seizures due to a shift in their activation potentials, therefore, the channels are activated in response to a smaller voltage change or a change in the rate of recovery of channels from the inactivated state (deinactivation) or an increase in surface expression of channels.

CACNA1H gene was also screened for 192 IGE patients for a common susceptibility allele (Heron *et al*., 2004). Four variants were found in patients but also in some unaffected family members indicating the polygenic inheritance of IGEs. These variants were also assessed for functional studies and they were shown to increase T-type currents with one exception, A480T variant did not lead different current compared to the wild type (Khosravani *et al*., 2005). However, considering the alternative splice variants, this variant may affect the regulation of transcription causing the expression of a channel which is more prone to excitation.

The possible role of CACNA1H was further confirmed in another Chinese population study. A SNP in exon 9 (rs9934839) was found to be highly associated with CAE by both a case-control study and a transmission disequilibrium test (Liang , 2006).

On the other hand, evaluation of CACNA1H in Caucasian origin populations revealed no linkage or mutation in this gene (Chioza *et al*., 2006). These contradictory results actually emphasize the presence of population specific susceptibility alleles to complex disorders.

In a polygenic rat model of absence epilepsy (GAERS), a mutation was found in exon 24 causing Arg158Pro (Powell *et al*., 2009). Further studies show that CACNA1H had two splice variants in the thalamus, one with exon 25 and one without. The mutation caused significantly faster recovery from channel inactivation and large Ca influx during high-frequency bursts only when it was on the variant with exon 25. Therefore, the mutations and the spliced variant should be considered together while studying the function of the channel. Actually, these splice variants could bring explanations to the mechanism of epileptic seizures in relation to the questions; why they are temporal and have cell type specific effects, why the seizures are present on certain ages but not before or later.

CACNA1I is located on the 22q13.1 with at least 36 exons. It is mainly expressed in the thalamic reticular nucleus (Shin, 2006). Mutational analysis was done on Chinese CAE patients, however, no mutations were found (Wang , 2006).

Non-pore forming modulatory subunits of Ca^{2+} channels are also possible candidates for burst firing as they can regulate the channel function, assembly and localization. β4 subunit can interact with both α1A (P/Q type) and α1B (N-type) subunits. A mutation was identified in β4 subunit gene in lethargic mouse which showed epileptic seizures and ataxia (Qian and Noebels, 2000). The mutation caused a truncated cytoplasmic protein and probably caused other β subunits to coassemble with α subunits to compensate the mutation at the hippocampal synapses (Zhang *et al*., 2002). However, in the thalamus β4 subunit is expressed highly whereas β1 –β3 subunits are not, suggesting that there may not

be a compansatory mechanism in the thalamus for the inhibitory function, therefore, the mutation may lead to spike wave discharges.

Mutations were identified in γ2 subunit gene in stargazer mouse (stg) (Letts *et al*., 1998). This subunit can interact with both HVA and LVA channel types. γ2 subunit has a role to elevate the inactivation of the calcium channels (Black, 2003). In both of these mutant mice there was an elevation of LVA current as in the case of tottering and leaner mutant mice which had mutations in *CACNA1A* (Zhang *et al*. ,2002).

In human studies on the other hand, γ3 subunit gene that is located on chromosome 16p13.1-p12 was found to be associated with CAE in the European population confirming the distinct roles of the regulatory subunits in channel function and consequently in epileptic seizures (Everett *et al*., 2007).

1.3.3. Chloride Channels

Chloride channel (CLCN2) gene is localized on 3q26 and codes for a channel which is responsible for maintaining the chloride hemeostatis of neurons and nonneuronal cells. These channels flow the CI ions out of the cell to establish a high transmembrane chloride gradient which is essential for inhibitory action of GABA receptors (Haug *et al*., 2003). The initial interest in CI channel was after a whole genome linkage study in which a susceptibility locus was identified at 3q36 (Sander *et al.*, 2000). Mutations have been identified in two individuals and in a family with different subtypes of epilepsy, a heterozygous 1bp insertion (597G) in a patient with grand mal seizures on awakeing (EMGA), heterozygous 11bp deletion in intron 2 of the channel with possible effect on splice site in a patient with childhood absence epilepsy and a heterozygous 2144G→A mutation in a family with juvenile absence epilepsy (Haug *et al*., 2003). The functional study of the 2144G→A mutation revealed that voltage dependent gating properties of the channels were changed leading to hyperexcitability of the cell.

1.3.4. Non-Ion Channel Genes

Although epilepsy has been considered as a channelopathy, novel non-ion channel genes were identified. 4-Malic enzyme 2 (ME2) which is located on chromosome 18 codes for the mitochondrial enyzme that converts malate to pyruvate and is involved in neuronal synthesis of the neurotransmitter GABA. In a patient group of 88 JME, 68 JAE+EGTCS, it was found be associated with all subtypes in a recessive model (Greenberg, 2005). 35 per cent cases were homozygous for nine SNPs that cover the ME2 gene and also its promoter while only 8 per cent of controls were homozygous.

In a family with hyperinsulinism/hyperammonemia (HI/HA), three children had photosensitive myoclonic absence epilepsy and had mutations in the glutamate dehydrogenase (GDH) gene which is located on 10q23.3. The patients have low levels of GABA compared to the controls (Bahi- Buisson *et al*., 2008).

In the mouse model of absence epilepsy named “jerky”, mutations have been identified in the JRK gene. The homolog of the gene (JRK/JH8) in human resides on 8q24 which is a candidate region based on linkage studies. The function of the gene is not clear but a rare mutation has been identified in a case of CAE patient evolving to JME (Moore *et al*., 2001).

Polymorphisms in the leucine-rich, glioma inactivated 4 (LGI4) gene were found to be associated with childhood absence epilepsy although the pathalogical effect of this variant was not clear (Gu *et al*., 2004). The gene resides on 19q13.11 and has a recessive model of inheritance in absence seizures.

1.4. Linkage Studies in Epilepsy

Linkage analysis is the classical method for identification of the causative genes in monogenic disorders which requires a precise genetic model, gene frequencies and penetrance of each genotype. Large families with many affected individuals, known as multiplex families or multiple families who has the same mutant gene are the key points for linkage studies.

However, in the case of complex disorders like epilepsy, linkage analysis is not a very powerful tool. Attempts to identify the causative mutations were not very successful for epilepsy, as patients do not show clearly distinct phenotypes and seizure types can change even within the same family members. Also, mutations in different genes may cause the similar epileptic phenotype in patients from different families. So difficulties in collecting multiple families with the same mutant gene, overlapping phenotypes and absence of a precise model bring obstacles in linkage studies for epilepsy.

To date, in idiopathic generalized epilepsies, two genome-wide linkage studies have been performed. One of the studies included the patients with JME, JAE and GTCS and the results support a strong disease locus on chromosome 18 and susceptibility loci on chromosome 6 for JME, on chromosome 8 for non-JME and two loci on chromosome 5 for absence seizures that may affect the seizure phenotypes (Durner *et al.*, 2001). In a previous linkage study in a large family affected with CAE from India, a common haplotype has also been identified in patients of the family on chromosome 8q24 (Fong *et al*, 1998). This candidate region was refined by Sugimoto *et al.* in 2000 and possible linkage was repeated (Sugimoto *et al*., 2000). However, association and linkage studies with candidate genes in this region did not support the possible linkage. Two loci on chromosome 5 (5p15 and 5q14-q22) were studied with 99 multiplex families in which there was at least one affected individual with typical absence seizures but the results did not support a possible susceptibility locus (Windemuth *et al.*, 2002) .

The other genome-wide linkage study included patients with absence seizures (CAE and JAE) and bilateral myoclonic seizures on awakening (JME), resulting in strong susceptibility locus on chromosome 3q26 and possible suggestive loci on chromosome 14q23 and chromosome 2q36 (Sander *et al.*, 2000a). Further studies on chromosome 3q26 lead to the identification of a novel epilepsy gene, a CI channel (Haug *et al.*, 2003). The mutations have been found in three different families with JME, epilepsy with grand seizures and JAE. For 2q36 region on other hand SLC4A3 gene that codes for an anion exchanger residing in this region was screened for possible mutations and susceptibility alleles but the results revealed only a slight contribution of a certain variant of this protein to IGE phenotype (Sander *et al*., 2002). In a recent study, 2q36 region was also found to confer susceptibility to IGE phenotype in a large IGE family (Klein *et al.*, 2008). In an

attempt to scan a candidate gene KNJ13 that codes for a potassium channel did not conclude in any causative mutation in the coding, promoter and regulatory regions.

Genome-wide scans were also performed for two animal models Wag/Rij and GAERS rats as they are the most appropriate models for human absence epilepsy. They show polygenic inheritance and do not have any other neurological malformations. The first study was carried out for the F_2 progeny of inbred Wistar Albino Glaxo rat (WAG/Rij) strain and ACI inbred strain (Gauguier *et al.*, 2004). These progenies show subphenotypes with different distinct types of spike wave discharges (SWDs-type I and type II) and the aim of the linkage study was to locate these quantative trait loci (QTLs) which affect these traits. The results showed that in rat chromosome 9, there was a locus which controls type I spike and a locus which controls type II spike in rat chromosome 5. The syntenic regions of these loci reside on chromosome 2q33-q37 for type I spike and on chromosome 1pter-p32 for type II spike in the human genome. This 2q33-q37 region overlaps with 2q36 region which was linked to epilepsy in CAE patients.

The other study conducted on the offsprings of GAERS and BN rats (Rudolf *et al*., 2004) concluded that chromosome 4, 7 and 8 regions include loci that affect the polygenic absence seizures. These regions corresponded to human chromosome 7q31-34, 7p14-15, 11q23, 22q12-q13 and 12q12-q13 which were not identified in any of the previous studies.

1.5. Association Studies in Epilepsy

In complex disorders, susceptibility is a result of a combination of mutations or polymorphisms which cause small changes in the expression of certain genes or in the function of the protein. Solely the subtle changes may not lead to any disease and they may be found in unaffected individuals. Moreover, diseased individuals may be carrying different susceptibility genes which cause changes in the disease phenotype and this explains the phenotypic difference within the same family (Tan *et al.*, 2004; Cardon and Bell, 2001).

As linkage study is not powerful enough to identify these small changes, association studies which aim to dissect the complex phenotype seem to be a better choice for complex

disorders like epilepsy. Association studies involve comparison of the frequency of a specific allele in patients against healthy controls. If the frequencies statistically differ, then the specific allele is said to be associated with the disease. However, detection of association is not a proof for the causation of the disease. Association could be observed because of population stratification which reflects the presence of multiple subgroups showing different allele frequencies in the same population. Population stratification is mostly a result of choosing inappropriate control group who should have similar genetic background except the disease allele with the affected group.

Secondly, association could be a result of usage of improper statistical test which may also lead to false positive results. Thirdly, the specific allele tested could be in linkage disequilibrium (LD) with true variant which affects the disease phenotype. Finally, association could be observed because the allele is the true susceptibility allele for the disease. However, causation has to be supported by mutational analysis of the linked gene and functional studies.

Association studies depend on the idea that two unrelated individuals in the same population who have the same disease allele most probably inherited their disease from a common ancestor, so they probably share the same loci close to this disease allele because of low recombination rates between close chromosomal regions. So, association study actually is a kind of linkage analysis in the population considering individuals as part of a large family.

Although association studies seem to be the most powerful method to identify susceptibility genes in complex disorders, attempts for association studies have accountered problems mainly as they can not be replicated in another patient group. However, there are several reasons for this failure in detecting a true association. One of them is inadequate sample size. Using small sample size would lead to false-negative results because of reduced statistical power. A minimum of 250 case-control pairs would be necessary to detect a true association. Another reason would be the differences in genetic contributions to the disease in the sample group. In order to avoid these differences, cases should be clearly defined with clinical and in the case of epilepsy, with EEG and neuroimaging data. Poorly matched control groups could be also another possible

reason in detecting a true association. Population stratification reflects the presence of differences in allele frequencies of subgroups in the same population. Different ethnic groups could carry different allele frequencies because of natural selection, migration patterns and random effects like natural disasters. If the control groups are not chosen from the same ethnic origin, a false-positive association can be detected at a loci unrelated to the disease. To overcome population stratification, two independent control groups could be used. An alternative and a more reliable strategy is the use of family-based controls such as parents and siblings like transmission disequilibrium test (TDT). However, family-based controls have less statistical power compared to case-control controls. Statistical tests could also give misleading results even all possible reasons for failures were eliminated. A positive result that is significant at 5 per cent level can be found by chance. The best approach could be the usage of multiple SNPs and testing them for association. Finally, although a positive association is observed in a study, the variant should be evaluated functionally. If the specific allele of a SNP that is tested does not have any effect on expression or function of the protein, the association could be false-positive or the variant could be in a strong LD with the true variant. In this case, other SNPs that are in the same LD blocks can be tested for association (Tan *et al.*, 2004).

In the case of absence epilepsy the studies that were carried out for mutation and association analysis revealed that SWDs may have different triggering mechanism thus, although the patients have similar phenotypes the seizures may result from mutations or subtle changes in inbitory or excitatory networks. The association analysis in different ethnic groups also confirm the presence of multiple causative mechanisms like many mutations have been identified in CACNA1H gene in Chinese population but no significant change could be found in european origin patients.

1.6. Haplotype Blocks and HapMap Project

Human genome is composed of chromosomal regions with high linkage disequilibrium (LD) between recombination hotspots (Cardon and Abecasis, 2003; Goldstein, 2001). These regions are called haplotype blocks which show limited diversity. If these blocks are identified, choosing a few “tagged” SNPs could be enough to differentiate the blocks. Actually, characterization of LD blocks is now the aim of the

HapMap project which as a start analyzes the haplotypes of four different populations with African, Asian, and European ancestry by using SNPs in every 1 kb. Now, the project involves other ethnic groups to identify and classify the similarities and differences. Up till now the data in the HapMap project revealed the large LD blocks in conserved populations like Chinese and Japanese and smaller blocks in older populations like African origin. Research groups study LD blocks from different ethnic origins and compare the transferability of HapMap data.

In a design of an association study haplotype block approach could reduce the complexity and cost of genome-wide association studies. However, the haplotype blocks may differ between populations, so the first step should be the determination of these blocks for the population that will be used in association studies.

2. PURPOSE

Idiopathic absence epilepsies (IAE) show a complex inheritance with a prevalence of 2-10 per cent among any other epilepsies. A previous whole genome linkage study which included IAE patients and another study conducted on a large IGE family revealed a susceptibility locus at 2q36. However, attempts to find causative mutations in candidate ion channel genes in the region did not bring out positive results to explain the role of these genes in the complex pathogenesis of absence seizures. In WAG/Rij rats which are pure absence epilepsy models the linkage analysis also pointed out to 2q33-37 syntenic region as one of the susceptibility loci for the polygenic absence phenotype. The linkage of absence seizures to the genes in the region may have been overlooked due to the technical drawbacks of the methodology employed earlier. Therefore, the possibility still exists that the region carries a yet unknown gene responsible for absence seizures or a strong susceptibility allele to IGE syndrome that manifests itself especially with typical absence seizures.

The aim of this study is to investigate the pathogenesis of absence seizures in the Turkish population utilizing 205 patients with absence seizures and 219 control samples by:

- Detecting the borders, possible hotspots for recombination and the pattern of haplotype blocks in the Turkish population in a 160 kb region on chromosome 2q36.
- Identifying a novel susceptibility gene for absence seizures at 2q36 through an association study.
- Identifying the role of the GABRG2 gene in the Turkish childhood absence patients by mutational screening.

3. MATERIALS

3.1. Blood Samples

The study was approved by the Ethics Committee of Boğaziçi University and informed consents were obtained from all analyzed subjects. The informed consent is shown in Appendix A.

3.1.1. Samples for the Hapmap Population Study

Blood samples were obtained from 38 unrelated Turkish families (mother-child-father trios) in İstanbul that receives immigrants from various regions of the country and is therefore, considered to represent the Turkish population as a whole. The sex ratio of the children were 1.4 (22/16) male:female.

3.1.2. Samples of Patients with Absence Epilepsy and Controls

Blood samples of 205 absence patients were provided according to certain criteria determined by the neurologists in the Genetics subcommitte of the Turkish League against Epilepsy Foundation. The names of the neurologists and the affiliations are listed in Table 3.1. According to these criteria, patients should have i) typical absence seizures; ii) 3-6Hz spike wave discharges (SWD) in their EEG; iii) normal neurological status and normal IQ level; 4) been diagnosed as IGE, including JME. For family based association studies internal control includes blood samples of mother and father of the 81 patients were also included in the study. Detailed phenotypical information of each patient was taken by the clinicians in the form prepared for patients with typical absence seizures as shown in Appendix B. Absence patients were classified according to the syndrome and seizure type based on the detailed informations as shown in Appendix C. According to the syndrome type, the study included 98 CAE, 70 JAE, 19 JME, 12 eyelid myoclonia with absence epilepsy (EMA), 2 refractor absence status epilepticus (RASE), 2 JAE-CAE and 2 JAE-JME patients. For the seizure type on the other hand, there were 81 patients with GTCS, 36

patients with myoclonic, 38 patients with febril seziures and as a feature of the seizures, 64 patients have photosensitive seizures.

For case control association studies 219 control samples with no epileptic seizures were collected.

Table 3.1. Neurologists and their affiliations involved in the association study

Name of the neurologists	University
Betül Baykan	İstanbul University
Nerses Bebek	İstanbul University
Zuhal Yapıcı	İstanbul University
Destina Yalçın	Şişli Etfal Hospital
Gülşen Dizdarerer	Tepecik Research Hospital
Dilşad Türkdoğan	Marmara University
Aycan Ünalp	Behçet Uz Hospital
Çiğdem Özkara	Cerrahpaşa Hospital
Günay Gül	Bakırköy Hospital
Derya Uludüz	Cerrahpaşa Hospital
Burak Tatlı	İstanbul University
Semih Ayta	Şanlıurfa Hospital
Sinan Çomu	
Kadriye Ağan	Marmara Hospital
Demet Kuşçu	Bakırköy Hospital
Ayşin Dervent	Cerrahpaşa Hospital
Kemal Tutkavul	Haydarpaşa Numune Hospital
Dilek Ataklı	Bakırköy Hospital
Cihan Meral	GATA
Kezban Arslan/Hacer Bozdemir	Çukurova Hospital
Aydan Özkaynakçı	Cerrahpaşa Hospital

3.2. Oligonucleotide Primers and Probes

3.2.1. Primers for Haplotype Block Analysis

The primer sequences used or the amplification of 29 SNPs at 2q36 are given in Table 3.2.

Table 3.2 Oligonucleotide primer pairs, allele frequencies and relative positions for each SNP

SNP name	Relative position (bp)	Alleles/ Frequency	PRIMER SEQUENCE (5'→3')
Rs4674403	**0**	G(0.593) T(0.407)	F-5'CATTCTCTCAAGGCCTCAGC R-5'TTACCTCCCTGGGAACACTG
Rs1397429	5470	T(0.563) C(0.437)	F-5'ATCTGTCCAAGTCACAGGGC R-5'ACACCCAAGCACACACACAT
Rs875097	7987	C(0.619) T(0.381)	F-5' CAGGAGTTGCTGCTGAGTGA R-5' CTTTTCCTCCAGACTTCCCC
Rs16859981	13469	G(0.800) A (0.200)	F-5' CTGGGTATTCCCCAACACAC R-5'GGGAACACGTGACAAGAGGT
Rs2010592	16369	C(0.570) T(0.430)	F-5' GTCCTTACTCCAGCGGATGA R-5' GTCCTCTTCCCTACCAAGCC
Rs12474050	17440	C(0.657) T (0.343)	F-5' GGTATTAGCCGGTGAGGTGA R-5' CCAGATTTCTGGGCTCAGTC
Rs2276640	24637	C(0.749) T(0.251)	F-5'CGAACCAGACCTGGAGAGAG R-5' CCTGCGGACTTGATCTGACT
Rs16860002	27516	T (0.859) C (0.141)	F-5' GGCCTTGTTATCAGCACCAG R-5' TCAGGACTGCTAAACCCCAC
Rs375062	31033	A(0.548) G(0.452)	F-5' GCACTTTGCCCAAACAAAAT R-5' CAGGTTCCTTTGCTCTCTGG
Rs907676	33216	C(0.726) G(0.274)	F-5' TCCACGAGAACACAGCAGAC R-5' CACTTTGCTTCCCCAAATGT
Rs3731909	34443	G(0.693) C(0.307)	F-5'GAAACCCAAGGAGAAGGAGG R-5' CAGCCAGCGAGGTGAGTAG
Rs746233	39009	G(0.550) A(0.450)	F-5' GCACACTACCTGGCACACAC R-5' ACTCCTGCACCTATGCACAC
Rs3755064	40103	C(0.545) T(0.455)	F-5' AGCTCTGAGGCACAGAAAGC R-5' AGTTGGGATGGAGTGTGGAG
Rs3770229	40826	A(0.655) G(0.335)	F-5' GGGAAGGGCAGTGACTGTTA R-5' GGCCATTTCTTCAAACGCTA
Rs3770234	46623	T(0.510) C(0.490)	F5'AAGGCAGAGAGAGAGGGAGG R-5' ACAAGGTCCCACAGGTTGTC
Rs2276643	54837	A(0.717) G(0.283)	F-5' TCTGTGTTGCAGACTCCCTG R-5' CCCACACACACGTACACACA

(F: Forward, R: Reverse)

Table 3.2 Oligonucleotide primer pairs, allele frequencies and relative positions for each SNP (continued)

SNP name	Relative position (bp)	Alleles/ Frequency	PRIMER SEQUENCE (5'→3')
Rs6436154	60113	G(0.600) A(0.400)	F-5' CTCCTCTAGAGCTGTCCCCA R-5' GATCCAGTGGGAGATCCAGA
Rs1983211	76300	C(0.260) A(0.440)	F-5' AGCCTCGCAAGGTTAAGATG R5'GGAGGGGATGAAAAGGAGAG
Rs2241526	82563	T(0.532) C(0.468)	F-5' AGAGGGACCTTCCTGAGACC R-5' GGCAAAAACATCTTGGAGGA
Rs3731920	88928	C(0.811) T(0.189)	F-5' GTCCAGATCAGCACCAACCT R-5' AAAGCACACCCCAAACTCAC
Rs7588807	93985	G(0.519) T(0.481)	F-5': CACGCCTGTGGTCTCAGTTA R-5' ATGCCCATCAGCAGTGTGTA
Rs6729914	98399	T(0.525) C(0.475)	F-5' GCCAAGGCAGGAGAATTGTA R-5' TCTACCCCACCTTTTTGTGG
Rs2840128	106784	T(0.746) A(0.254)	Primers for melting curve analysis
Rs12694468	113088	A(0.847) T(0.153)	F-5' CACCATGCCCAGCTAAAGAT R-5' TGATACCTTGAACCATGCGA
Rs681747	117523	T(0.857) G(0.143)	F-5'GAGACATGTTTTCCGGTCGT R5'GGAAAGAGGAAGCGGAAGAG
Rs673951	128238	C(0.559 T(0.450)	F-5' AGCTGCTTGGAGTTCCACCT R-5' AAGTGGAAGGTGAGCCCTTT
Rs668034	143599	A(0.868) G(0.132)	F-5' AGGGTTGTTGTGAGGAACCA R-5' TGCTAGAATCAGGGGAATGG
Rs2305055	155204	C(0.796) G(0.204)	F-5' CAATACACACTCCCCCATCC R-5' CTTCAGGGGTTGCTTCAGAG
Rs684428	160148	G(0.801) A(0.199)	F-5' CTCCCAGGTCACAGCTTCTC R-5' CATTGGCTGTGCTCTTTGG

(F: Forward, R: Reverse)

3.2.2. Primers and Hybridization Probes for the Association Study

For ten SNPs primers and hybridization probes were designed. Figure 3.1-10 shows the sequences of the two alternative primer pairs and probes, positions of each primer and

probe and also Tm of each oligonucleotide. The primers and probes were obtained from TIBMOLBiol, Germany.

927625	hu chromosome 2 rs1397429		AC053503	Tm
F-5&#8217	ATCTgTCCAAgTCACAgggC	S	143571-590	56,6°C
Primer S	CTgAAgTTggACTgTgTgACCT	S	143606-627	56,2°C
Primer A	AAgTACAgCCAgTTACAgAAACATTC	A	143733-708	56,2°C
R-5&#8217	ACACCCAAgCACACACACAT	A	143804-785	57,3°C
Sensor C	CACCACTTACgTCTgTCgggC-FL	S	143660-680	62,0°C
Anchor	640-ggCCTCCTCTCTCCTgggAgACCCT p	S	143682-706	70,3°C

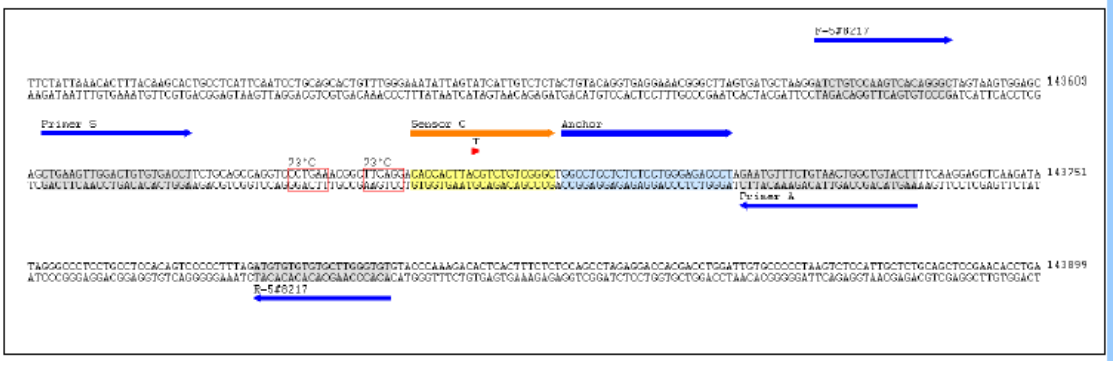


Figure 3.1. The design of primer and probes for rs1397429

930902	hu LOC729871, rs2010592, intron 45		rs2010592	Tm
2010592_F	gAgTCAgggCCCAgAgTT	S	391-408	54,8°C
2010592_S	AgggCCCAgAgTTCCTAA	S	396-413	54,2°C
2010592_A	CTATCAgggCAgCAgTTCA	A	537-519	53,6°C
2010592_R	ggAgggTCgTTACTgAggTAT	A	587-567	54,0°C
2010592_Anc	ggAgATggTgCgCTCgCggTAAgg--FL	A	455-432	71,2°C
2010592_[A]	LC-CTgTggCCAggCCTgTCT-PH	A	430-413	60,3°C
2010592_[G]	LC-CTgTggCCgggCCTgTCT-PH	A	430-413	64,7°C

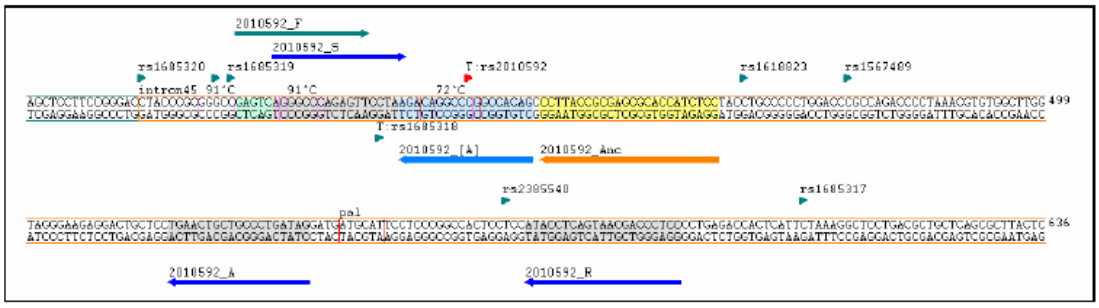


Figure 3.2. The design of primer and probes for rs2010592

933381	hu ACCN4 (rs907676)		AC053503	Tm
ACCN4 F	ATTCgCTCCCATTgACg	S	171305-321	53,7°C
ACCN4 S	TCTTCCTCTCCCTCCATTg	S	171364-382	53,1°C
ACCN4 A	AATCCCTTgCCACAACC	A	171491-475	53,5°C
ACCN4 R	TTTgCTTCCCCAAATgTTC	A	171514-496	54,4°C
Sensor C	ATAgAgCACCCCCgAAgC-FL	S	171403-420	56,7°C
Anchor ACCN4	640-CACCTCCCCTTTgCTCCCTgg p	S	171422-442	66,3°C

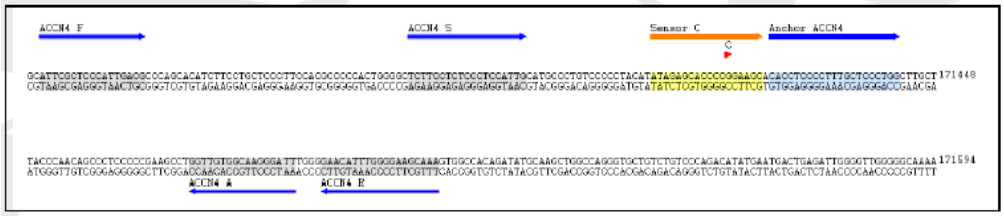


Figure 3.3. The design of primer and probes for rs907676

928567	hu ACCN4 (rs3770234)		AC139723	Tm
ASIC4 mis	CACgACCTTCCCCTTTACg	S	3174-3192	57,4°C
ASIC4 A	CCACAggTTgTCCACTgCTC	A	3280-3261	58,6°C
ASIC4 R	gCACAgCCCACAgCTTCTT	A	3334-3316	58,9°C
LNA ASIC4	gTATgCAAAggAgTTgAg-FL	A	3227-3210	64,0°C
ASIC4 mut	640-gTCCAgggTCCAgTCACg p	A	3208-3191	58,6°C
ASIC4 mut 2	640- TCCAgggTCCAgTCACg p	A	3207-3191	55,9°C

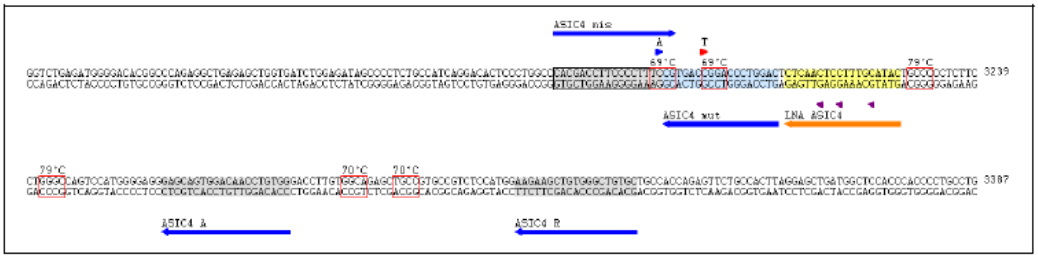


Figure 3.4. The design of primer and probes for rs3770234

928567	hu chondroitin polymerizing factor [H401H]		AC009955	Tm
CHPF F	CgTCggTAgCCATTCACC	S	11239-256	56,4°C
CHPF S	CCAgAACATCggCCACA	S	11321-337	56,0°C
iLC CHPF	CTgCgCTgggACTAC XTTC	A	11426-409	54,5°C
Sensor A	gCAggAgAAAgCATgCTgCTCCgTgA-FL	S	11385-410	70,5°C

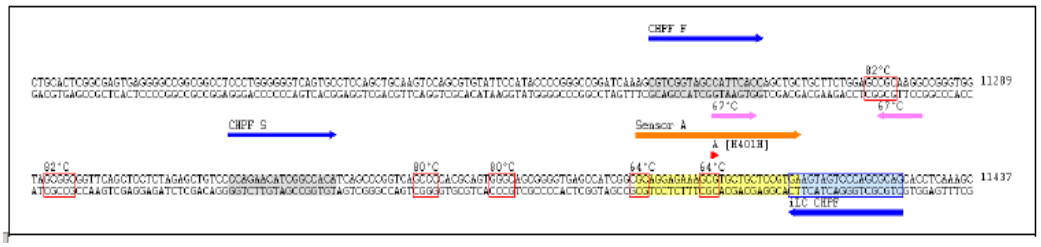


Figure 3.5. The design of primer and probes for rs6436154

930902	hu OBSL1 obscurin-like 1, rs2241526, intron 9		AC009955	Tm
2241526_F	ggACCTTCCTgAgACCAAAT	S	33670-689	54,4°C
2241526_S	ACTCACAgCTAgAgACACACgAA	S	33709-731	55,7°C
2241526_A	TgCAgggTAgggCAgAgT	A	33970-953	56,6°C
2241526_R	AATCTCTggggACAgTgAgTCTAT	A	33994-971	55,6°C
2241526_[C]	ggACAgggACggTATgAAgg--FL	S	33838-857	57,8°C
2241526_[T]	ggACAgggATggTATgAAgg--FL	S	33838-857	54,9°C
2241526_Anc	LC-TggAACTgATgAgAggAgggTTggg-PH	S	33859-883	66,0°C

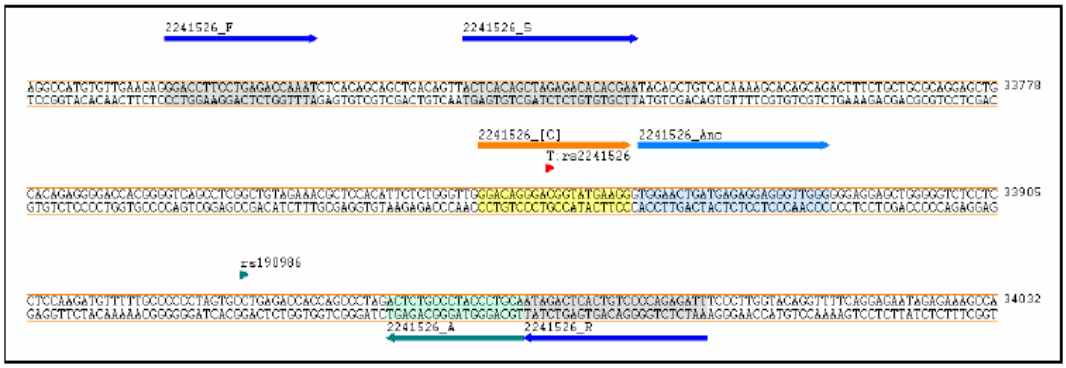


Figure 3.6. The design of primer and probes for rs2241526

928567	hu inhibin, alpha (INHA) (rs7588807)		AC009955	Tm
INHA F	CACTCCAgCCTgggTgAC	S	45132-149	57,3°C
INHA S	CATCCATgTAgACACCATTCAggA	S	45198-221	57,8°C
INHA A	CAATTAACATgCCCATCAgCAg	A	45330-309	58,1°C
INHA R	CCAACTTCTCTCTgAAgTggTTTTA	A	45356-332	56,6°C
INHA wt	CACCAgCACTAAgACTTTgTATTAgAC-FL	A	45283-257	55,7°C
Anchor INHA	640-CAATgCTTACTCACCAgATAgTgTgAAACA p	A	45254-225	62,1°C

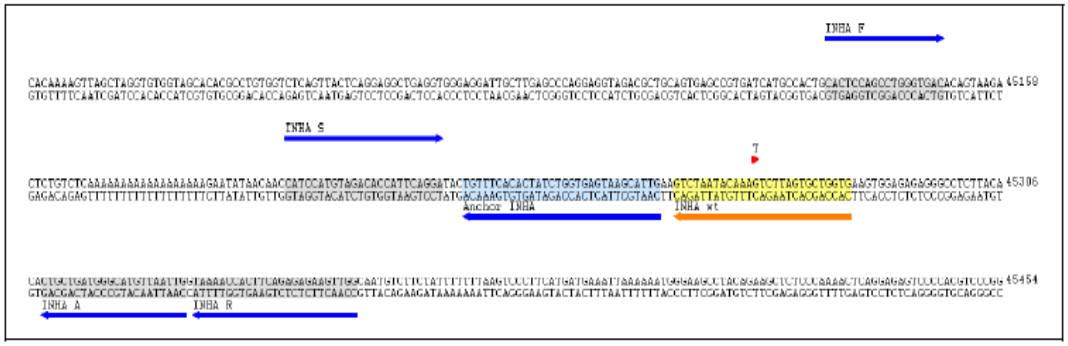


Figure 3.7. The design of primer and probes for rs7588807

932229	hu chromosome 2 (rs2840128)		AC009955	Tm
2840128 F	gAgTATgTTAAAATCCCTAAgACC	S	57970-993	51,1°C
2840128 S	gTTAAATAAAgTCTCCTgAAAATgAC	S	57995-020	52,4°C
2840128 A	CTCTTggCgAAgAATgg	A	58137-121	51,2°C
2840128 R	TCTTAATAAAAgggAAggACAC	A	58248-227	50,8°C
Sensor mut	TTAATCTgCCTTgACCTATTCACT-FL	A	58081-058	54,6°C
Anchor 2840128	640-CATgAATCAATTTATTggTgCTgTCTCTg p	A	58056-028	61,7°C

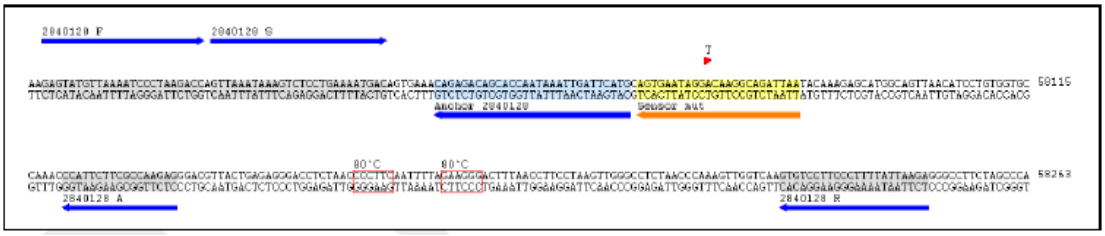


Figure 3.8. The design of primer and probes for rs2840128

932229	hu STK11IP [V563I]		AC009955	Tm
STK11IP F	gAgggCAgAgTgTgggTACT	S	79413-432	56,6°C
STK11IP S	CCTTTTCTCTTggTCTCTCCACA	S	79448-470	58,0°C
STK11IP A	gCTTggAgTTCCACCTCAAAC	A	79586-566	57,7°C
STK11IP R	gggCCTCCggCTCTATCT	A	79651-634	57,9°C
Sensor A	TgAgggCATACggggCAgg-FL	S	79515-533	64,6°C
Anchor SKL1IP	640-AATgCTTTCTCAgggTCACTTCTgCCCACC p	S	79535-564	70,3°C

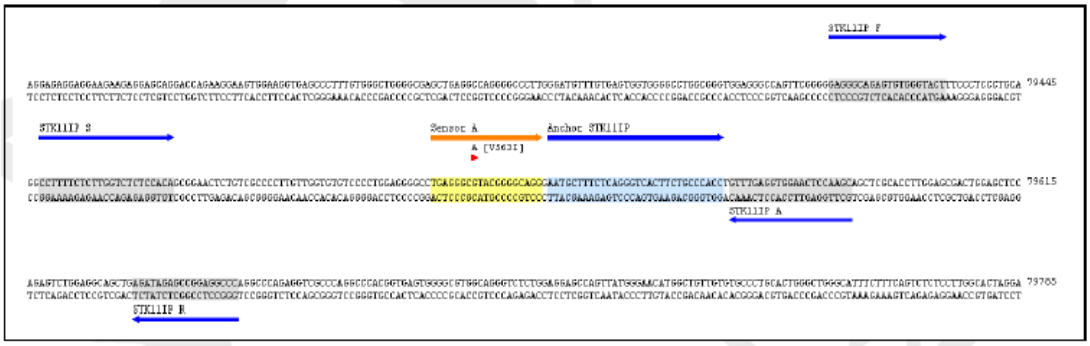


Figure 3.9. The design of primer and probes for rs673951.

928567	hu SLC4A3 (rs2305055)		AC009955	Tm
SLC4A3 F	AACAgACCAAAgTCgAgATgA	S	106332-352	53,2°C
SLC4A3 S	AgACCCTTggAggCTgAg	S	106389-406	55,0°C
SLC4A3 A	gCACAgACCACTCCCTCT	A	106537-520	53,4°C
SLC4A3 R	ACAgTTCTgggggAACAA	A	106567-550	53,3°C
Anchor SLC4A3	CCAgCCCCgTCCTgACTCCTgg-FL	S	106454-475	69,8°C
SLC4A3 C	640-gTCCTTTgCATCCCTgATCCCAAC p	S	106477-500	65,5°C

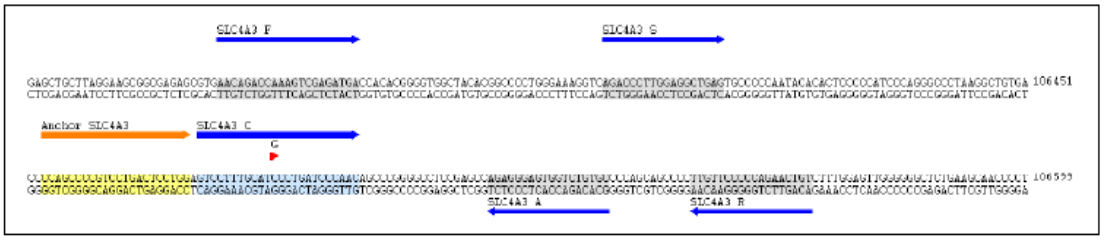


Figure 3.10. The design of primer and probes for rs2305055

3.2.3. Primers for the INHA Gene Analysis

The primer sequences that were used for the analysis of the promoter and two exons of the INHA gene are shown in Table 3.3. Exon 2 was separated into four regions to be analyzed by high resolution melting (HRM) analysis. Primers were purchased from Medek, İstanbul.

Table 3.3 Oligonucleotide primers for the INHA gene

Region	Forward primer	Reverse primer
Promoter1	5'-GCCTGGCTTCCTGCTCTTAG	5'-CATCACCCCTCAGACCTTCT
Promoter2	5'-CAGACTGGCTCCACTGGTCT	5'-TCAAGCGACCTGACTCACACA
Promoter3	5'-GGCTCCTGTCCCACCTGATGTC	5'-CCCACACCCACCCTCTTCTACCCTTTC
Exon1	5'-TAGGAGGTCTCAATGCCACG	5'-TTTCTCAAAGTCATCCTGCCGGTT
Exon2.1	5'-CTCCTGCTGAAGAGGAGGG	5'-GGTCCTCCCGGTGACAGT
Exon2.2	5'-AGCAGCCTCCAATAGCTCTG	5'- AGGACATCAGGGGAGTTGAG
Exon2.3	5'-CACACTCGGACCAGACC	5'-CAGGGACTGGAAGGGACA
Exon2.4	5'-TACTGTCATGGTGGTTGTGG	5'-CTCCCAGCTGATGATGGTG

3.2.4. Primers for the GABRG2 Gene Analysis

The primer sequences that were used in DNA analysis of ten exons of the GABRG2 gene are shown in Table 3.4.

Table 3.4 Oligonucletiode primers for the GABRG2 gene

Region	Forward primer	Reverse primer
Exon 1	5'-GAGCCACCATCAGATCATAAGC	5'-GTAAAGCCGCACATCCTAGGAG
Exon 2	5'- CAGTTAGTCTCCATCTATGCAG	5'-TCCTTGCTCTTGAACTACACTG
Exon 3	5'-TATGCGTGCTTGGTGCATGTGC	5'- GGATCTGGAAGACTATCTTTCAC
Exon 4	5'-GTGAGACAGTAACCTCCTCAGC	5'- GATAGCATGCCAACCCTGATGC
Exon 5	5'- CTTCATTGGGGATCACTCTGTG	5'-TCACCCTAATCGGAGCAAGCTG
Exon 6	5'- TGCCCTTTGGTCCAAGATCCTC	5'- CAACTCTGGAAGGGTCACTTG
Exon 7	5'-GGGATTCAGTTCAGGTTGTG	5'- GGGTTGGTTCCAAGTCTTTGC
Exon 8	5'- CATTGCTGAAACTGCCCATCAG	5'- CGTTATGGCCTGGCTAAACTC
Exon 9	5'- GCTCAGAACTCTCCTTCTGTG	5'- TAGCTTTTGGGCTTGGTGTAAG
Exon 10	5'- CATCACATTGGTGACATTGTGG	5'- ACATCTCTCCATGAGACTCAGT

3.3. Enzymes

Taq DNA polymerase : 5 U/µl, (Promega, USA)
5 U/µl, (Qiagen, Germany)

3.3.1. Restriction Enzymes

BsmI : New England BioLabs

HpyCH4 IV : New England BioLabs

BfaI : New England BioLabs

MspI : New England BioLabs

FokI	:	New England BioLabs
CfoI	:	Promega, USA
MaeIII	:	Roche, Germany
BccI	:	New England BioLabs
BsaH I	:	New England BioLabs
Bsp1286	:	New England BioLabs
BstO I	:	Promega, USA
TSp509 I	:	New England BioLabs
HinfI	:	New England BioLabs
BstF5 I	:	SibEnzyme
RsaI	:	Promega, USA
AlwN I	:	New England BioLabs

3.4. Chemicals

Ethidium Bromide (EtBr)	:	10 mg/ml
Absolute Ethanol	:	Riedel de-Häen, Germany
DNA Ladder	:	100 bp (Promega, USA)

3.5. Buffers and Solutions

3.5.1. DNA Extraction

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)
Sodiumdodecylsulphate (SDS):		10 per cent SDS (w/v) (pH 7.2)
Proteinase K	:	20 mg/ml in H_2O
TE Buffer	:	20 mM Tris-HCl (pH 8.0) 1 mM Na_2EDTA (pH 8.0)

3.5.2. Polymerase Chain Reaction

10 X $MgCl_2$ Free Buffer	:	100 mM Tris-HCl 500 mM KCl
$MgCl_2$	:	25 mM in dH_2O
Deoxyribonucleotides (dNTP)	:	100 mM of each dNTP (Promega, USA)

3.5.3. Agarose Gel Eletrophoresis

10 X TBE Buffer	:	0.89 M Tris-Base 0.89 M Boric acid

20 mM Na_2EDTA (pH 8.3)

1 or 2 per cent Agarose Gel	:	1 or 2 per cent (w/v) Agarose in 0.5 X TBE Buffer
10 X Loading Buffer	:	2.5 mg/ml Bromophenol Blue (BPB) 1 per cent SDS in glycerol

3.6. Kits

3.6.1. Kits for Light Cyler 480

Probes master	:	2X Faststart Taq DNA polymerase (Roche) Reaction Buffer dNTP mix, 6.4mM $MgCI_2$
High Resolution Melting Master:		2X Faststart Taq DNA polymerase (Roche) Reaction Buffer dNTP mix, 25mM $MgCI_2$ High resolution melting dye
Cyber Green I Master	:	2X Faststart Taq DNA polymerase (Roche) Reaction Buffer dNTP mix, $MgCI_2$ Cyber Green I dye

3.7. Equipments

Autoclave	:	Model MAC-601 Eyela, Japan
Balances	:	Electronic Balance Model CC081 Gec Avery, UK

Centrifuges	:	Centrifuge 5415C Eppendorf, Germany Universal 16R Beckman Coulter Hettrich, Germany
Deep Freezers (-20°C)	:	Bosch, Germany
Documentation System	:	BioDoc Video Documentation System Biometra, Germany
Electrophoretic Equipments	:	Horizon 58, Model 200 (BRL, USA) Multigel-Long (Biometra, Germany)
Light Cycler 480	:	Multiplate well 96 (Roche, Germany)
Magnetic Stirrer	:	Chiltern Hotplate Magnetic Stirrer, HS31 UK
Ovens	:	Microwave Oven (Vestel, Türkiye) EN400 (Nuve, Türkiye) 56°C (LEEC, UK)
Power Supplies	:	ECPS 3000/150 Constant Power Supply Pharmacia, Sweden Model 200 (BRL, USA)
Spectrophotometer	:	NanoDrop ND-1000, Thermo, USA
Thermocyclers	:	Techne (Progene, UK) Techne Gradient (Progene, UK)

UV Transilluminator	:	Chromato-Vue Transilluminator, Model 1TM-20UVP (USA
Water Bath	:	Köttermann, Laborteknik (Germany)

METHODS

4.1. Analysis of Genomic DNA

4.1.1. DNA Extraction from White Blood Cells and Saliva

In order to extract genomic DNA from white blood cells (leukocytes), 10 ml of peripheral blood samples were collected in anticoagulant (K_2EDTA) containing tubes. First the blood samples were treated with 30 ml cold lysis buffer to lyse the membrane of leukocytes. After incubation of the samples at 4 ^{o}C for 15 minutes, they were centrifuged at 5 K for 10 minutes at 4 ^{o}C. So, the cellular nuclei were precipitated and the lysed part was collected in suspension. After supernatant was removed, the pellet was resuspended in 10 ml cold lysis buffer and centifuged again at 5K for 10 minutes at 4 ^{o}C. The nuclear pellet was resuspended in lysis buffer and centrifuged at the same conditions two or three times until a clean pellet was obtained. In order to lyse the nuclear envelope, the nuclear pellet was resuspended in nuclease buffer. The nuclear proteins were degraded by treating the suspension with proteinase K (150 µg/ml) and SDS (0.14 per cent). Then the suspension was incubated either at 37 ^{o}C for overnight or at 56 ^{o}C for three hours. After incubation, the degraded proteins were precipitated by treating the suspension with 5 ml of cold distilled water and 5 ml of 5 M NaCI solution. The mixture was centrifuged at 5K for 25 minutes at room temperature, so the precipitated proteins were collected in the pellet. The supernatant part which contains the genomic DNA was removed and put into a new tube. In order to precipitate DNA, two volumes of absolute ethanol was added. By fishing out with a pipette, the DNA was taken into an eppendorf tube and left to dry. After all the ethanol was dried, DNA was dissolved in TE buffer.

Saliva was collected in Oragene tubes and mixed with Oragene DNA solution thus DNA is stabilized. The mixed saliva/DNA solution was incubated for at least 1 hour at 50 ^{o}C to let DNA release and to inactivate nucleases. 500 µl of this mixture was placed in 1.5 ml eppendorf and 20 µl of Oragene DNA purifier was added to precipitate the inhibitors and impurities. The mixture was incubated on ice for 10 minutes to enhance the precipitation. In order to separate the impurities the solution was centrifuged at 13.000 rpm

for 5 minutes. Clear supernatant was taken and mixed with 95-100 per cent ethanol to precipatite DNA. The solution was left at room temperature for 10 minutes and then centrifuged at 13.000 rpm for 2 minutes. The ethanol part was taken by a pipette and DNA pellet was washed with 70 per cent ethanol to remove residual inhibitors. The solution was centrifuged again at 13.000rpm for 5 minutes to remove all of the ethanol. DNA pellet was dissolved in TE buffer and stored at -20 ^{o}C.

4.1.2. Analysis by Agarose Gel Electrophoresis and Spectrophotometer

Genomic DNA was analyzed on 1 per cent agarose gel which was prepared by dissolving 1 g of agarose in 100 ml 0.5 X TBE buffer. Agarose was dissolved in TBE buffer by boiling in microwave and cooled down to 56 ^{o}C. EtBr, which intercalates DNA and causes DNA to be visualized under UV light, was added into the solution with a final concentration of 0.5 μg/ml. Then the homogeneous mixture was poured onto eletrophoresis plate and left to polymerize at room temperature. In order to load the DNA samples into the wells of the agarose gel, 1 μl of DNA was mixed with 9 μl of loading dye 1X BPB. The gel was run at 150 volts and visualized under UV light.

The concentration and purity of DNA was measured by the nanodrop spectrophotometer.

4.2. Haplotype Block Analysis

4.2.1. Selection of Single Nucleotide Polymorphisms

In the candidate region, 2q36, the Pubmed (GenBank database) data showed about 60 SNPs when this study was designed and 29 of those biallelic SNPs with a minor allele frequency (MAF) of at least 0.10 were selected (http://www.ncbi/nlm.nih.gov). The distance between two SNPs varied from 1 kb to 16 kb and on the average they were separated by 6.6 kb.

4.2.2. Oligonucleotide Design

For each SNP, primers were designed using the "workbench primer3" programme on the internet. Optimal primers were checked by BLAST not to have unspecific annealing. For rs2840128 hybrobe probe was designed as the alleles of this SNP could not be differentitated by restriction enzyme analysis.

4.2.3. Optimization for PCR Amplifications

For each SNP, PCR reactions were optimized by preparing the mixture in 25 µl volume containing 1 X Mg^{2+} free reaction buffer, 1, 1.5 or 2 mM of $MgCI_2$, 0.2 mM of each dNTP, 1.25 units of Taq DNA polymerase, 5 per cent DMSO, 5 pmol of each primer and 50 ng of genomic DNA. The optimization for amplification is done by the following PCR programme in a gradient annealing temperature :

95 ^oC for 5 minutes
95 ^oC for 30 seconds (denaturation)
50-65 ^oC for 25 seconds (annealing) } X 40
72 ^oC for 30 seconds (extension)
72 ^oC for 5 minutes

The PCR products were checked on 2 per cent agarose gel. 5 µl aliquot of each product were mixed with 5µl 1X loading dye and run at 150 V with a 100 bp DNA ladder. The products were visualized under UV light.

4.2.4. Restriction Enzyme Analysis

Endonuclease digestion was carried out in 20 µl volume containing 2 µl buffer, 10 units of restriction enzyme and 8 µl of each PCR product. The samples were left at optimal temperature (37 ^oC or 56 ^oC) in water bath for 3 hours or for overnight. In order to check the digestion results, the samples were run on 2 per cent agarose gel with 100 bp DNA

ladder and with uncut PCR products. Agarose gel is run at 150 V for 20-25 minutes and visualized under UV light.

4.2.5. Analysis of Linkage Disequilibrium and Haplotype Structure

Haploview version 4 was used for the analysis of LD, haplotype blocks and tagging SNP detection (Barret *et al*., 2005). For haploview two data files in notepad were prepared, one named ".pre" including the information of the patients and genotypes and the other named ".info" including marker locus informations. In ".pre" file the data should be in linkage pedigree format as shown in Table 4.1. In ".info" file on the other hand, name of the markers and the relative positions are loaded as separate columns.

A haplotype block was defined by solid spine where the first and the last markers are in strong LD (D'>0.8) with all intermediate markers but that intermediate markers are not necessarily in LD with each other. Estimated haplotypes were also calculated by Haploview 4 using an accelerated expectation-maximization (EM)-based algorithm similar to the partition/ligation method described in Qin *et al*. paper (Qin *et al*., 2002).

Haploview tagger selects the minimum set of markers that can capture the alleles that are correlated with each other greater than $r^2 > 0.8$. Chi square analysis for allele frequency comparison was performed by using Web Chi Square Calculator (http://www.georgetown.edu/faculty/ballc/webtools/web_chi.html).

Table 4.1. The format of the ".pre" file used in Haploview programme

Column							
1	2	3	4	5	6	7	8
Family Name	Individual ID	Father ID (0= unknown or not included)	Mother ID (0= unknown or not included)	Sex (male=1 Female=2)	Affection status (0=unknown 1=unaffected 2=affected)	The first allele of the first marker A=1 C=2 G=3 T=4	The second allele of the first marker A=1 C=2 G=3 T=4
Example							
1AE	1	3	2	2	2	1	2

4.3. Association Study

4.3.1. Selection of the SNPs at 2q36 Region for Association Study

Ten SNPs were selected based on the LD (Linkage Disequilibrium) block structure of the 160 kb region at 2q36 region. While selecting SNPs that would be used in association study certain criteria were considered. First of all, each block that was defined in the previous study should be represented by one or two SNPs which has the highest LD with other SNPs in the block. Secondly, heterozygozity of the SNP should be high in order to have more informative results. The SNPs that did not fall into any block were also included in the association study to cover the whole region.

4.3.2. Optimization of SNP Primer and Hybprobe Probes

A hybprobe probe consists of two flourescent hybridization probes called anchor and sensor. A sensor probe is specific to the variable target sequence while anchor is complementory to the adjacent sequence of the sensor. The sensor probe flouresences only when both probes bind to the right sequence, therefore, hybprobes are very specific. After the amplification process melting curve analysis is carried out. During the melting curve analysis the sensor detaches from the target sequence at higher Tm if the target sequence is wild type and at lower Tm if the sequence is mutant. Reactions were optimized by preparing mixture in 20 µl volume containing 1 X probe master mix with 3.2 mM Mg^{2+}, faststart taq DNA polymerase, reaction buffer and dNTP mix, 0.25 pmol of each primer pairs (F, R and S, A) 0.2 pmol of anchor and sensor probes and 60 ng of genomic DNA. In order to increase the flouresence of the peaks asymmetric primer pairs were also used for the optimization with 1:5 or 1:2 ratio of the reduced primer that is in the same orientation with the sensor. The optimization conditions for amplification and melting curve analysis is shown in Table 4.2.

Table 4.2. Optimization conditions for hybprobe probes in light cycler 480

Programme name	Tm	Acquisition mode	Time (hh:mm:ss)	Ramp rate (^{o}C/s)	Cycle
Pre-incubation	95 ^{o}C	None	00:05:00-00:10:00	4.4	1
Amplification	95 ^{o}C	None	00:00:10	4.4	45
	52-58 ^{o}C	None	00:00:15	2.2	
	72 ^{o}C	Single	00:00:01	4.4	
Melting curve	95 ^{o}C	None	00:02:00	4.4	1
	40 ^{o}C	None	00:02:00	2.2	
	80 ^{o}C	Continous	-	-	
Cooling	40 ^{o}C	None	00:00:30	1.5	1

4.3.3. Hardy-Weinberg Equilibrium for Controls

Before running the association test, frequencies of alleles of control samples were checked whether they were in Hardy-Weinberg equilibrium by the Haploview programme and they represent the population well. Under this equilibrium, the allele frequencies are expected to remain constant over time, so the genetic variation at each locus is ensured. Lack of consistency in the equilibrium should indicate potential complications like genotyping errors and population stratification. The Hardy-Weinberg equations are as follows:

- $p+q=1$ where p is the frequency of dominant allele and q is the frequency of recessive allele.
- $p^2 + 2pq + q^2 = 1$ where p^2 is the predicted frequency of homozygous dominant individuals and 2pq is the predicted frequency of heterozygous individuals, and q^2 is the predicted frequency of homozygous recessive ones in the population.

4.3.4. Case Control Association Study

The genotypes of patients and control samples were assessed by the Haploview programme in a case control association study. Chi-square and p values were calculated for each SNP and if the p value of the allele of a certain SNP was higher than 0.05 then the result was accepted to be significant and associated to the disease. If the results indicate a possible association with a specific allele, then the block that contains the associated SNP would be analyzed in more detail.

4.3.5. Family-Based Association Study

In order to avoid population stratification in the case control study, association studies with internal controls (family-based) were carried out by transmission/disequilibrium test (TDT). TDT is used for couples who have one or more affected children. For a biallelic marker with alleles M1 and M2, TDT tests whether one of the alleles is prefentially transmitted to the affected child and simply compares the transmitted and nontransmitted alleles with the following equation: $(a-b)^2 / (a+b)$, where a is the number of times a heterozygous parent transmits one of the alleles which is thought to be associated with the disease and b is the number of times the other allele. This equation has a chi-square distribution with 1 degree of freedom.

4.3.6. Haplotype Association Analysis

Haplotype association analysis was carried out by Haploview version 4. The test is carried out by summing the fractional likelihoods of each individual for each haplotype. Chi-square and p values are calculated for each haplotype for case control or TDT association.

4.4. DNA Analysis

4.4.1. DNA Analysis by High Resolution Melting Analysis

In high resolution melting analysis a flourecent dye specifically intercalates to doubled stranded DNA and flouresces. After amplification process a melting curve analysis follows where a sequence that has a variation melts at a different Tm and classified as a different group than the wild type. HRM reactions were optimized by preparing the mixture in 20 µl volume containing 1 X master mix with faststart taq DNA polymerase, reaction buffer, dNTP mix and high resolution melting dye, 0.2-0.5 mM of Mg^{2+}, 0.2-0.5 pmol of each primer pairs and 20-40 ng of genomic DNA. The optimization conditions for amplification and melting curve analysis is shown in Table 4.3.

Table 4.3 The optimized conditions for HRM analysis in light cycler 480

Programme name	Tm	Acquisition mode	Time (hh:mm:ss)	Ramp rate (^oC/s)	Cycle
Pre-incubation	95 ^oC	None	00:10:00	4.4	1
Amplification	95 ^oC	None	00:00:10	4.4	45
	Touchdown	None	00:00:15	2.2	
	72 ^oC	Single	00:00:10-00:00:16	4.4	
High resolution Melting	95 ^oC	None	00:01:00	4.4	1
	40 ^oC	None	00:01:00	2.2	1
	65 ^oC	None	00:00:01	1	1
	95 ^oC	Continous	-	-	25
Cooling	40 ^oC	None	00:00:10	2.2	1

4.4.2. DNA analysis by Direct Sequencing

The promoter sequence, exon 1 of the INHA gene and exon 1, exon 4, exon 5, exon 7 and exon 8 of the GABRG2 gene were analyzed by direct sequencing. PCR products were purified and sequenced at Macrogen, Korea. Exon2 of the INHA gene and exon 2,

exon 3, exon 6, exon 9 and exon 10 of the GABRG2 gene were first analyzed by HRM analysis. If any sample was classified as a different group than the wild type then, the sample was further analyzed by direct sequencing.

4.5. Copy Number Variation Analysis by Quantative PCR

An absolute and relative quantification analysis was carried out in real time using Light cycler 480 to detect the possible CNVs in the candidate region. Cyber green dye which binds to double stranded DNA was used in qPCR analysis. A standart curve was set up with control DNA with three different concentrations (5ng, 10ng and 20ng). In standart curve the concentration of standart samples were plotted against the cross points (CP) of the samples. CP is the cycle at which the amplified product is first visible in the data as the flouresence of the sample rises above the background flouresence. As the CP of the sample depends on the initial concentration, standart curve is used to determine the initial concentration of the unknown sample by determining where the CP of the unknown sample falls on the standart curve. Each standart and unknown sample was studied with three replicas and the experiment for each region was repeated at least three times. The efficiency of the experiments varied between 1.54-2.2 and the error which is the measure of the accuracy of the quantification result based on the standart curve was less than 0.2. In the experiments the initial concentrations of the unknown samples were set to 20ng by nanodrop spectrophotometer for both target and reference genes. The results of the target gene was normalized with the results of the reference gene which is the FXI gene on chromosome 4q35. The ratio of target gene to reference gene is 1 if the unknown sample has two alleles but the ratio is 0.5 is one of the alleles in target region is deleted. SNPs rs3770234, rs1983211, rs2241526, rs7588807 abd rs6729914 were subjected to CNV analysis in JAE patients and patients with GTCS. Prior to the absolute quantification analysis each region was also analyzed by melting curve analysis to confirm that there is no unspecific amplification. Table 4.4 shows the optimization conditions of CNV analysis in light cycler 480.

Table 4.4. The optimized conditions for qPCR analysis in light cycler 480

Programme name	**Tm**	**Acquisition mode**	**Time (hh:mm:ss)**	**Ramp rate (^{o}C/s)**	**Cycle**
Pre-incubation	95 ^{o}C	None	00:10:00	4.4	1
Amplification	95 ^{o}C	None	00:00:10	4.4	32
	60-64 ^{o}C	None	00:00:20	2.2	
	72 ^{o}C	Single	00:00:10	4.4	
High resolution Melting	95 ^{o}C	None	00:00:05	4.4	1
	40 ^{o}C	None	00:01:00	2.2	1
	65 ^{o}C	None	00:00:01	1	1
	97^{o}C	Continous	-	-	10
Cooling	40 ^{o}C	None	00:00:10	2.2	1

5. RESULTS

5.1. Haplotype and Linkage Disequilibrium Structure Analysis in Turkish Population at 2q36

In order to carry out an association study at 2q36 region tagSNPs should be identified. For this purpose 29 SNPs were selected and genotyped for 38 Turkish healthy trios by restriction enzyme analysis to detect the LD structure of the region.

5.1.1. PCR Amplification and Digestion Products:

Table 5.1 shows the expected PCR product of each SNP and digestion product of each SNP allele. For rs2840128 there was no restriction enzyme cutting site difference therefore, a hybprobe probe was designed and optimized in the light cyler.

5.1.2. Optimization of PCR Amplification of SNPs

Table 5.2 shows the optimized PCR conditions for 29 SNPs.

5.1.3. SNP Genotyping

Thirty-eight trios representing healthy population were genotyped for 29 SNPs and data were analyzed using Haploview version 4. Four of the SNPs (rs2276640, rs 6729914, rs12694468, rs652509) that had at least 0.1 MAF in the NCBI data were non-polymorphic in the Turkish population and were excluded from LD block analysis. The remaining 25 SNPs were successfully genotyped and used in the LD pattern and haplotype block analysis. All 25 SNPs were in Hardy-Weinberg equilibrium and their MAF ranged between 0.017-0.473. Table 5.3 shows the name, relative position, observed and predicted heterozygozity, p-value for Hardy-Weinberg equilibrium, minor allele frequency (MAF) and alleles of each SNP.

Table 5.1. PCR products, alleles, specific restriction enzymes and digestion products of 29 SNPs

SNP Name	PCR Product(bp)	Alleles	Restriction Products (bp)	Restriction Enzyme
Rs4674403	260	G	260 (No cutting)	BsmI GAATG_Cn'
		T	71 + 189	
Rs1397429	234	T	234 (No cutting)	HpyCH4 A'CG_T
		C	99 + 135	
Rs875097	299	C	299 (No cutting)	BfaI C'TA_G
		T	99 + 200	
Rs16859981	218	G	71 + 147	MspI C'CG_G
		A	218 (No cutting)	
Rs2010592	277	C	28 + 50 + 90 + 109	MspI C'CG_G
		T	28 + 109 + 140	
Rs12474050	292	C	67 + 225	FokI GGATGnnnnnnnnn'nnnn
		T	21 + 67 + 204	
Rs2276640	267	C	38 + 69 + 160	FokI GGATGnnnnnnnnn'nnnn
		T	69 + 198	
Rs16860002	210	T	210 (No cutting)	MspI C'CG_G
		C	60 + 150	
Rs375062	285	A	22 + 42 + 105 + 116	FokI GGATGnnnnnnnnn'nnnn
		G	22 + 105 + 158	
Rs907676	296	G	104 + 192	MspI C'CG_G
		C	296 (No cutting)	
Rs3731909	191	G	40 + 62 +83	CfoI G_CG'C
		C	40 + 145	
Rs746233	247	G	120 + 147	MaeIII 'GTnAC_
		A	247 (no cutting)	
Rs3755064	205	C	2 + 203	BccI CCATCnnnn'n
		T	2 + 41 +162	
Rs3770234	240	T	240 (no cutting)	MspI C'CG_G
		C	91 + 149	
Rs2276643	222	A	222 (no cutting)	CfoI G_CG'C
		G	72 + 150	
Rs6436154	235	G	235 (no cutting)	Hsp92II _CATG'
		A	97 + 138	

Table 5.1. PCR products, alleles, specific restriction enzymes and digestion products of 29 SNPs (continued)

SNP Name	PCR product(bp)	Alleles	Restriction Products (bp)	Restriction Enzyme
Rs1983211	261	C	55 + 58 +148	Bsp1286 G_dGCh'C
		A	58 + 203	
Rs2241526	258	T	82 + 176	BccI CCATCnnnn'n
		C	258 (no cutting)	
Rs3731920	239	C	27 + 68 + 144	BstQI 'CCwGG_
		T	68 + 171	
Rs7588807	285	G	285 (no cutting)	Tsp509I 'AATT
		T	55 + 230	
Rs2840128	250	A	Hybprobe probe/Melting curve analysis	
		T		
Rs6729914	298	T	44 + 111 +143	BstQI 'CCwGG_
		C	44 + 51 + 60 + 143	
Rs12694468	265	A	265 (no cutting)	HinfI G'AnT_C
		T	(109 + 156)	
Rs681747	278	T	53 + 110 + 115	BstF5I GGATG_nn’
		G	110 +168	
Rs673951	280	C	68 + 94 + 118	RsaI GT'AC
		T	118 + 162	
Rs652509	253	G	29 + 109 +115	MspI C'CG_G
		A	109 + 144	
Rs668034	296	A	18 + 278	SduI G_dGCh'C
		G	18 + 114 +164	
Rs2305055	191	C	7 +57 +127	FokI GGATGnnnnnnnnn'nnnn
		G	7 + 184	
Rs684428	244	G	70 + 174	AlwNI CAG_nnn'CTG
		A	244 (no cutting)	
		T	28 + 109 + 140	

Table 5.2. Optimized PCR conditions for 29 SNPs at 2q36

SNP name	Mg concentration (mmol)	Primer Concentration (mmol)	Annealing Tm (^{o}C)
Rs4674403	1.5	0.2	54.2
Rs1397429	1.5	0.2	55.4
Rs875097	2	0.2	58.2
Rs16859981	1	0.2	54.2
Rs2010592	1.5	0.2	54.2
Rs12474050	1.5	0.4	55.4
Rs2276640	1.5	0.2	55.4
Rs16860002	2	0.2	54.2
Rs375062	2	0.2	54.2
Rs907676	1.5	0.24	55.4
Rs3731909	1.5	0.12	55
Rs746233	1.5	0.2	54.2
Rs3755064	1.5	0.2	58.2
Rs3770234	2	0.2	60
Rs2276643	4	0.4	60
Rs6436154	1.5	0.2	58.2
Rs1983211	1.5	0.2	52.5
Rs2241526	2	0.2	55.4
Rs3731920	1.5	0.2	58.2
Rs7588807	1.5	0.24	55.4
Rs2840128	Hybrobe probe/ Melting curve analysis		
Rs6729914	2	0.2	58.2
Rs12694468	1.5	0.2	52.4
Rs681747	1.5	0.2	55.4
Rs673951	1.5	0.2	60
Rs652509	2	0.2	58.2
Rs668034	1.5	0.2	54.2
Rs2305055	1.5	0.4	55.4
Rs684428	2	0.2	58.2

Table 5.3 Analysis of 25 SNP genotypes on 38 trios of Turkish origin

No	Name	Position	ObsHET	PredHET	HWpval	MAF	Alleles
1	4674403	0	0.521	0.411	0.0448	0.289	G:T
2	1397429	5470	0.486	0.472	1.0	0.382	T:C
3	875097	7987	0.392	0.315	0.0647	0.196	C:T
4	16859981	13469	0.429	0.415	1.0	0.294	G:A
5	2010592	16369	0.431	0.478	0.5066	0.396	C:T
6	12474050	17440	0.385	0.443	0.3964	0.331	C:T
7	16860002	27516	0.147	0.136	1.0	0.073	T:C
8	3755062	31033	0.397	0.47	0.2585	0.377	A:G
9	907676	33216	0.333	0.44	0.0591	0.327	C:G
10	3731909	34443	0.431	0.49	0.4275	0.431	G:C
11	746233	39009	0.478	0.491	0.9703	0.433	G:A
12	3755064	40130	0.461	0.496	0.6545	0.454	T:C
13	3770234	46623	0.52	0.499	0.9344	0.473	C:T
14	2276643	54837	0.316	0.301	1.0	0.184	A:G
15	6436154	60113	0.427	0.474	0.4908	0.387	G:A
16	1983211	76300	0.268	0.282	0.9687	0.17	C:A
17	2241526	82563	0.56	0.461	0.1148	0.36	T:C
18	3731920	88928	0.197	0.199	1.0	0.112	C:T
19	7588807	93985	0.466	0.492	0.7753	0.438	G:T
20	2840128	106784	0.343	0.353	1	0.229	T:A
21	681747	117523	0.049	0.048	1.0	0.025	T:G
22	673951	128238	0.421	0.441	0.8321	0.329	C:T
23	668034	143599	0.033	0.033	1.0	0.017	A:G
24	2305055	155204	0.439	0.403	0.7345	0.28	C:G
25	684428	160148	0.392	0.388	1.0	0.264	G:A

(ObsHet: Observed Heterozygozity, PredHet:Predicted Heterozygozity, HW: Hardy-Weinberg Equilibrium, MAF: Minor Allele Frequency)

5.1.4. Linkage Disequilibrium and Haplotype Analysis

Solid spine analysis revealed seven possible blocks 3kb-37kb in length covering 55% of the region (Figure 5.1). The blocks included 21 SNPs while four SNPs did not show strong LD with any other SNP. Out of 21 SNPs all were tagged except rs668034. The analysis of the haplotype structures indicated that three major haplotypes in blocks numbered 1, 4, 5 and 7 represented 79, 92, 96 and 97 per cent of the population, respectively (Figure 5.2). Block 3 on the other hand, had only two common haplotypes covering more than 85 per cent of the population. All SNPs within the blocks were tag SNPs except the third SNP (rs668034) in block 7 showing that four observed haplotypes of the block can be represented by three SNPs.

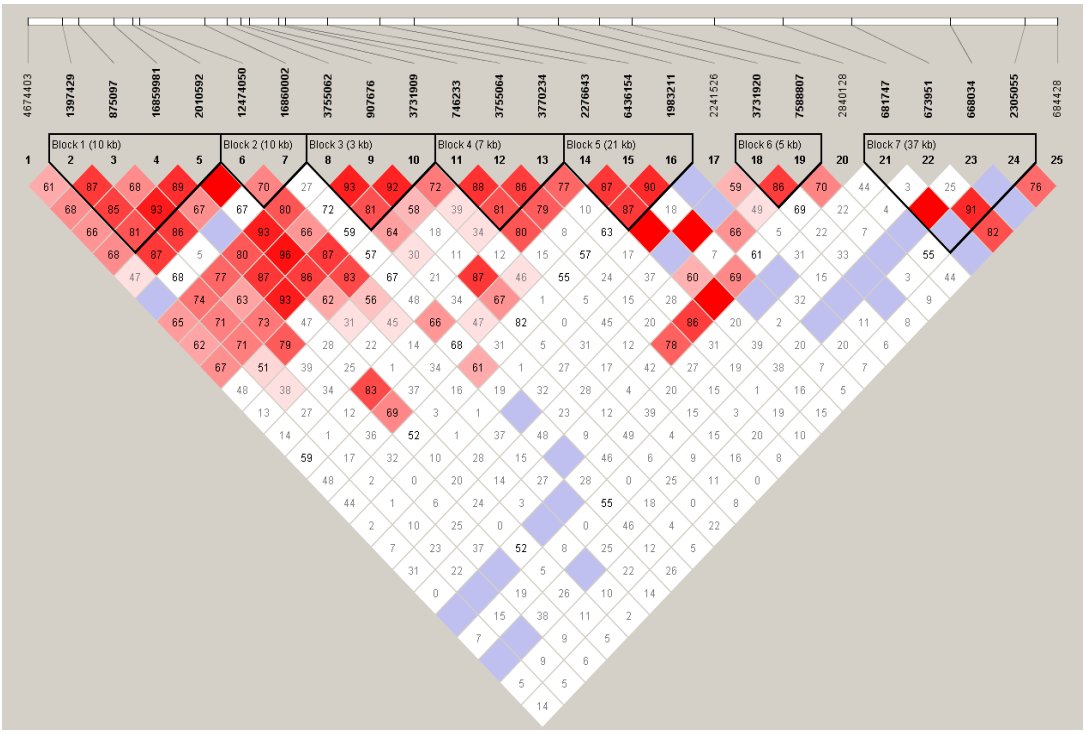


Figure 5.1 LD patterns of 25 SNPs and the block structure of 2q36 in the Turkish Population

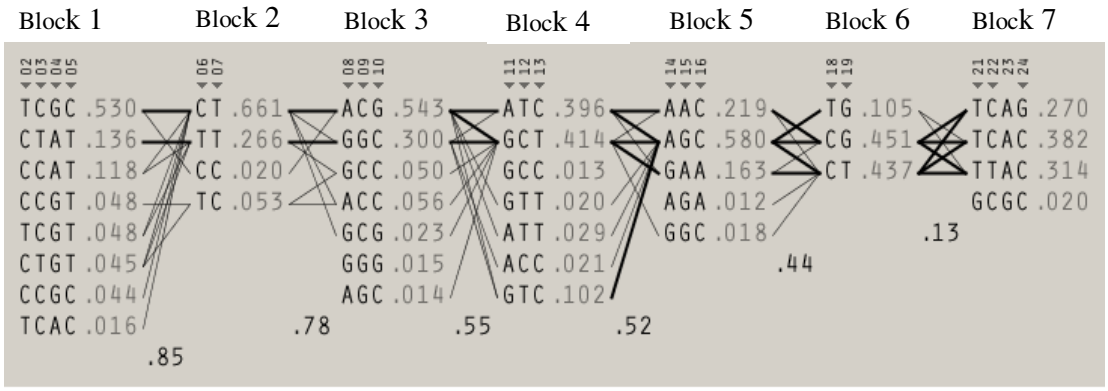


Figure 5.2 Haplotype block structures of the blocks at 2q36. The number below indicates the level of recombination between blocks. The triangles on SNPs are possible tagSNPs for each block.

5.1.5. Comparison of the Turkish Population Data with Hapmap Data

In order to assess whether any of the HapMap population could represent the Turkish population in association studies designed on Turkish samples, the LD patterns of 160kb at 2q36 of five populations were compared (Figure 5.3). The HapMap data showed that the reference populations were genotyped for more than 130 SNPs in the 160kb region that roughly corresponded to one SNP in every 1000 bases. However, approximately half of the SNPs were not polymorphic in European, Asian and Japanese populations, 29 SNPs were not polymorphic in the African population and some of the SNPs that were used in this study were not genotyped in all four of the reference populations. Therefore, the comparative analysis of the LD patterns and block structures were based on 15 SNPs common to all populations (Figure 5.3). Five blocks were present in the Turkish, African (YRI), and European (CEU) populations. The largest block observed in the Japanese (JPT) was split into 2 blocks in the Chinese (CHB) and into 4 blocks in both the Turkish and CEU populations. YRI had a different LD structure in blocks 4 and 5. However, the blocks seemed to have more LD breaks in general in the Turkish population and covered 53 per cent of the 160kb region whereas 65 per cent of the same region was within blocks in the CEU. The last block was common to all populations except YRI. The blocks were shorter in length in the YRI compared to other populations and covered only 34 per cent of the region which was in agreement with the expectation of low LD in the African population.

The highest LD was observed in the CHB and JPT populations covering 69 per cent and 74 per cent of the region, respectively (Table 5.4).

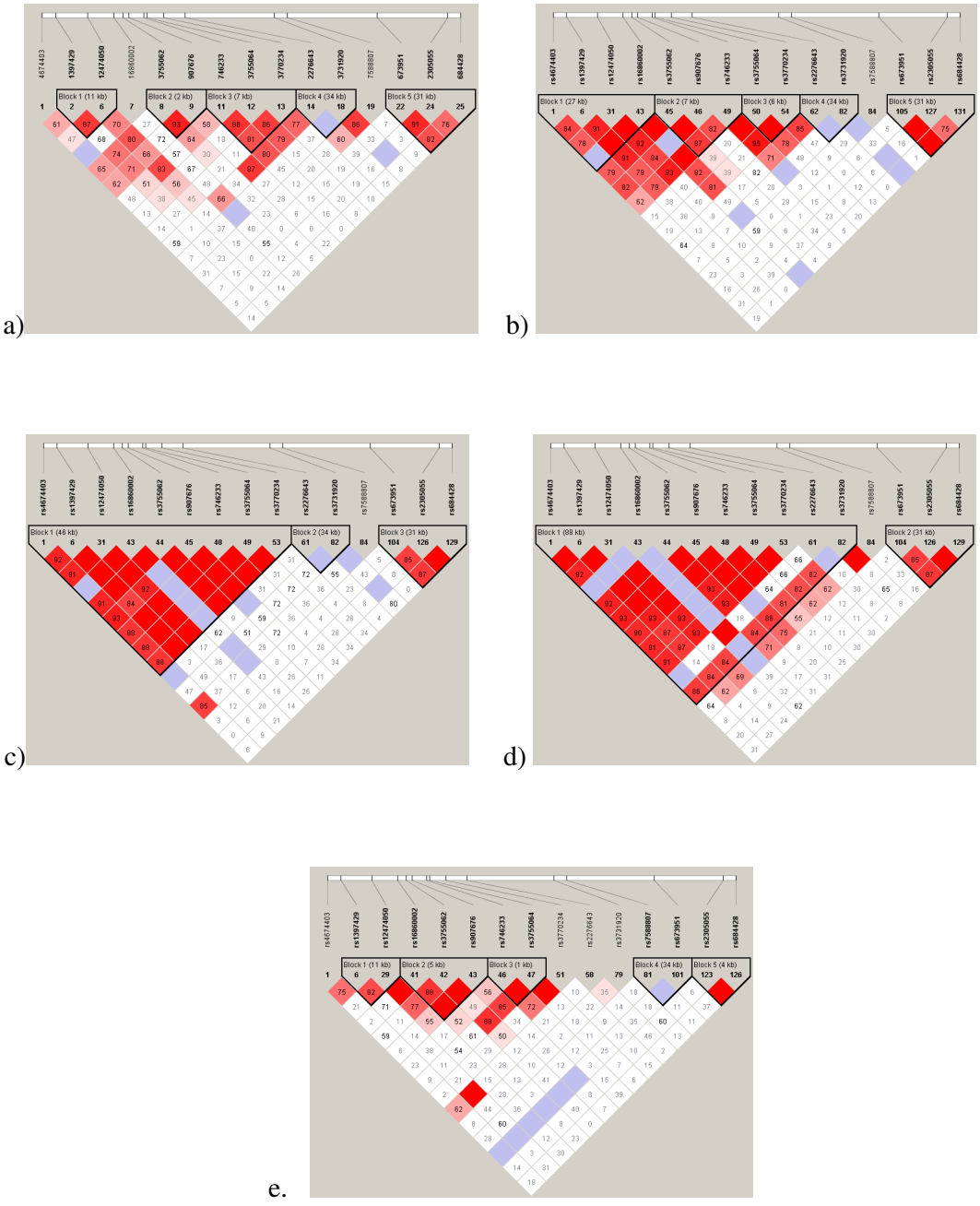


Figure 5.3. LD patterns and block structures of the Turkish and HapMap populations at 2q36, using 15 SNPs common to a) Turkish b) CEU c) CHB d) JPT and e) YRI populations between rs4674403 and rs684428 (160 kb)

In order to assess the similarities of the haplotype structures of the two most common blocks (blocks 4 and 5) haplotype frequencies were compared (Table 5.5). The frequencies of the common haplotypes were the closest between the Turkish and CEU populations. Pairwise comparisons of the allelic frequency of 15 SNPs were also carried out by Chi-square analysis (Table 5.6). There were 2-5 SNPs that differed significantly between Turkish-CEU, Turkish-CHB and Turkish-JPT comparisons, while Turkish-YRI comparison on the other hand revealed significant differences in the frequency of almost all SNP alleles.

Table 5.4. Comparative analyses of LD patterns and block structures in five populations using 15 common genotyped SNPs at 2q36

	Turkish		**CEU**		**CHB**		**JPT**		**YRI**	
Block	Lenght (kb)	No. of SNPs	Length (kb)	No. of SNPs	Lenght (kb)	No. of SNPs	Lenght (kb)	No. of SNPs	Lenght (kb)	No. of SNPs
1	11	2	27	4	46	9	88	11	11	2
2	2	2	7	3	34	2	31	3	5	3
3	7	3	6	2	31	3	-	-	1	2
4	34	2	34	2	-	-	-	-	34	2
5	31	3	31	3	-	-	-	-	4	2
Cover	53 per cent		65 per cent		69 per cent		74 per cent		34 per cent	

(CEU: European Caucasian, CHB: Chinese Han, JPT: Japanese and YRI: African Yoruba populations)

5.1.6. Tag SNP Analysis

In order to compare the transferability of HapMap data for Turkish population in an association study, tagger analysis in the Haploview program were applied to detect tagSNPs that would be representative for 160kb region at 2q36. For the Turkish population the results indicated that all 15 SNPs should be chosen in a design of an association study

not to miss a positive association in the Turkish population. Tagger analysis resulted in 12 tagSNPs in the CEU possibly due to more homogenous genetic background of CEU compared to the Turkish population. On the other hand, it showed that 9 SNPs would suffice to cover the region in CHB and JPT due to high LD and low diversity of haplotype structure in these populations.

Table 5.5. The frequency of haplotypes in blocks 4 and 5 in HapMap and Turkish populations

Haplotypes	**Haplotype Frequency**				
Block 4 (34 kb)	**Turkish**	**CEU**	**CHB**	**JPT**	**YRI**
AC	0.704	0.726	0.634	0.739	0.211
GC	0.184	0.207	0.194	0.136	0.381
AT	0.112	0.067	0.122	0.125	0.273
Block 5 (31 kb)	**Turkish**	**CEU**	**CHB**	**JPT**	**YRI**
CCG	0.372	0.324	0.247	0.151	0.450
TCG	0.305	0.444	0.576	0.694	0.017
CGA	0.218	0.125	0.164	0.142	0.154
CGG	0.047	0.074	0	0	0
CCA	0.034	0.033	0	0	0.371
TCA	0.016	0	0	0	0

(CEU: European Cauasian, CHB: Chinese Han, JPT: Japanese, YRI: African Yoruba)

Table 5.6 Comparative analysis of allele frequencies of 15 SNPs

	Turkish- CEU		Turkish-CHB		Turkish-JPT		Turkish-YRI	
SNP	X^2	p-value	X^2	p-value	X^2	p-value	X^2	p-value
Rs4674403	0.1732	0.68	1.3143	0.25	1.3143	0.25	74.6966	<0.001
Rs1397429	0.6772	0.41	0.5686	0.45	1.6509	0.20	81.3019	<0.001
Rs12474050	0.0487	0.82	0.0116	0.91	0.4210	0.51	24.9873	<0.001
Rs16860002	0.1131	0.74	0.2132	0.64	0.7581	0.38	32.9466	<0.001
Rs3755062	0.0624	0.80	0.0452	0.83	0.5667	0.45	51.9860	<0.001
Rs907676	1.2351	0.27	2.4928	0.12	1.9313	0.16	17.6017	<0.001
Rs746233	0.0678	0.79	2.7544	0.10	10	0.001	4.5161	0.03
Rs3755064	0.5680	0.45	0.0005	0.98	2.7955	0.09	58.7041	<0.001
Rs3770234	0.8813	0.35	0.0746	0.78	3.8622	0.04	24.0441	<0.001
Rs2276643	0.2487	0.62	0.0081	0.93	1.0605	0.30	33.4771	<0.001
Rs3731920	1.6396	0.20	0.0595	0.80	0.0595	0.80	32.0779	<0.001
Rs7588807	0.1812	0.68	1.9413	0.16	1.9413	0.16	0.1591	0.68
Rs681747	0.2894	0.59	For chinese and Japanese, MAF=0				91.9391	<0.001
Rs673951	3.6213	0.06	15.6243	<0.001	31.2683	<0.001	39.4884	<0.001
Rs668034	0.0350	0.85	For chinese and Japanese ,MAF=0				74.9460	<0.001
Rs2305055	5.9122	0.01	4.1906	0.04	6.0923	0.01	22.7240	<0.001
rs684428	4.3379	0.04	2.9969	0.08	3.7756	0.05	20.7273	<0.001

(CEU: European Cauasian, CHB: Chinese Han, JPT: Japanese, YRI: African Yoruba,

MAF: Minor allele frequency)

5.2. Association Study

5.2.1. Design and Optimization of SNP Primer and Hybrobe Probes

For the association study 10 SNPs were selected according to the haplotype block structure at 2q36 region in the Turkish population. As shown in Figure 5.4 one or two SNPs were chosen to represent each of the six blocks. For the second block which included rs12474050 and rs16860002, neither of them were selected as the MAF of rs16860002 was very low (0.073) and as the the region that included rs12474050 was not appropriate for a probe design. However, rs2010592 in block 1 is in high LD with rs12474050 therefore, it could be representative of the second block as well. Hybrobe probes were also designed for rs2241526, rs2840128 and rs673951 which did not fall into any block in order not to overlook any positive association in the region. Table 5.7 shows name, relative position and the optimized amplification and melting curve conditions of the ten selected SNPs.

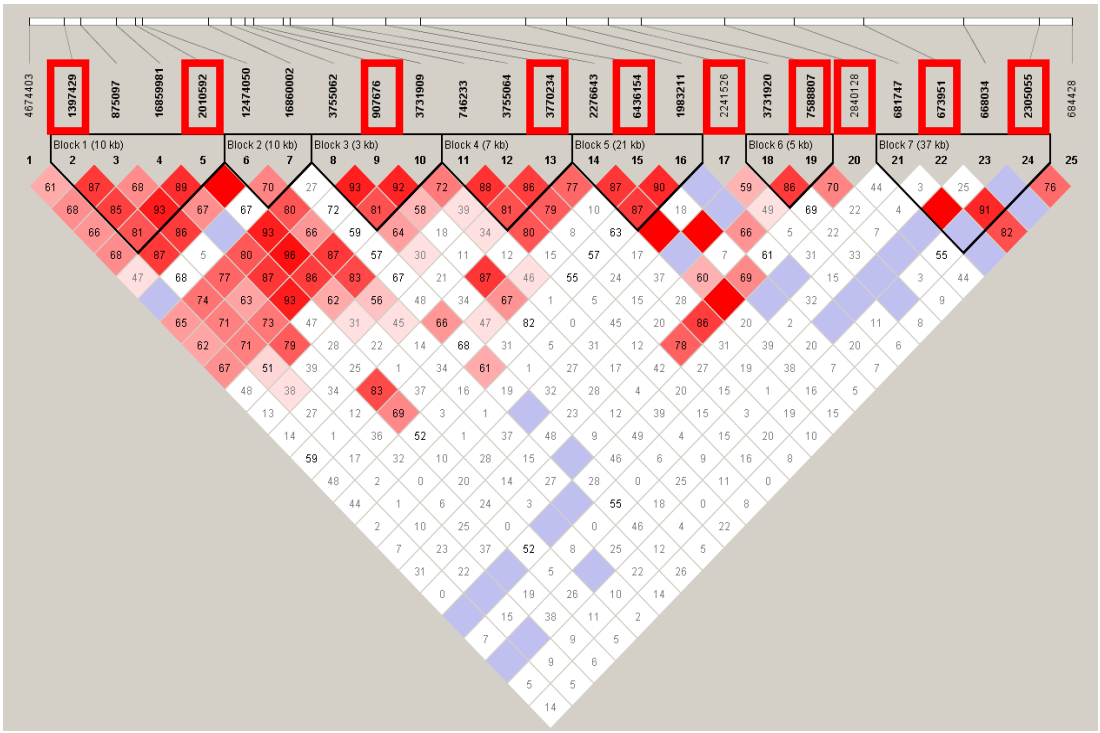


Figure 5.4. The SNPs that were chosen for association study are indicated with red boxes.

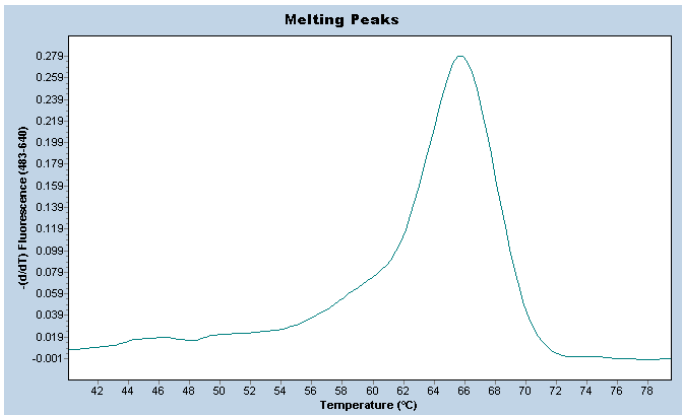
In melting curve analysis, wild type and mutant alleles are melted at different temperatures thus the florourescent peak for the wild type is observed at high Tm while

mutant allele is at lower Tm. In Figure 5.5 the optimized melting curve results for rs907676 is depicted. Melting curves for other SNPs are given in Appendix D.

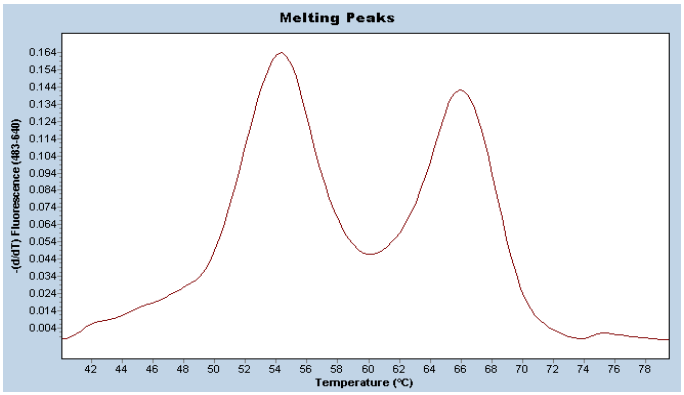
Table 5.7. Optimized conditions for 10 SNPs in Light cycler 480.

SNP name	Relative Position	Primer concentration (mM)	Sensor Concentration (mM)	Anchor Concentration (mM)	Annealing Tm (^{0}C)
Rs1397429	0	Primer S: 0.1 Primer A:0.5	Sensor C: 0.2	Anchor: 0.2	53
Rs2010592	10899	2010592_S: 0.5 2010592_A: 0.1	2010592_(A): 0.2	2010592_Anc: 0.2	52
Rs907676	27746	ACCN4 S: 0.1 ACCN4 A: 0.5	Sensor C: 0.15	Anchor ACCN4: 0.15	52
Rs3770234	41153	ASIC4 mis:0.5 ASIC A: 0.1	ASIC 4 mut2: 0.2	LNA ASIC 4: 0.2	55
Rs6436154	54643	CHPF S: 0.05 iLC CHPF: 0.25	Sensor A: 0.25	iLC CHPF: 0.25	54
Rs2241526	77093	2241526_S: 0.1 2241526_A: 0.5	2241526_(C): 0.2	2241526_Anc: 0.2	55
Rs7588807	88515	INHA S: 0.25 INHA A: 0.05	INHA wt: 0.2	Anchor INHA: 0.2	58
Rs2840128	101314	2840128F: 0.25 2840128R: 0.05	Sensor mut: 0.1	Anchor 2840128: 0.1	51
Rs673951	122768	STK11IP S: 0.05 STK11IP A: 0.25	Sensor A: 0.15	Anchor SKL1IP: 0.15	56
Rs2305055	149734	SLC4A3 F: 0.25 SLC4A3 R: 0.05	SLC4A3: 0.2	Anchor SLC4A3: 0.2	54

a)



b)



c)

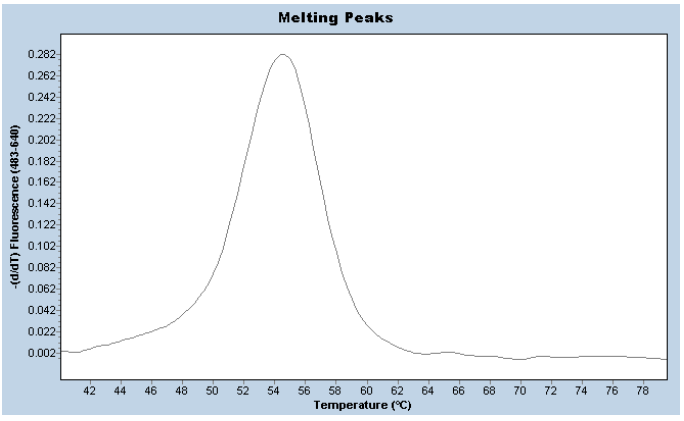


Figure 5.5 Melting peaks observed for rs907676 a) Homozygous wild type genotype C-C (66 ^{0}C), b) heterozygous genotype C-G (54 ^{0}C ve 66 ^{0}C) and c) homozygous mutant genotype G-G (54 ^{0}C).

Tm for melting peaks for homozygous wild type, heterozygous and homozygous mutants for 10 SNPs that were used in the association study are shown in Table 5.8.

Table 5.8 Tm for melting peaks of wild type and mutant allele of 10 SNPs

SNP name	Tm for wild type (^{o}C)	Tm for mutant type (^{o}C)
Rs1397429	69	60
Rs2010592	70	58
Rs907676	66	54
Rs3770234	63	57
Rs6436154	74	68
Rs2241526	64	55
Rs7588807	66	59
Rs2840128	63	58
Rs673951	68	61
Rs2305055	68	61

5.2.2. Case Control and TDT Association Analysis

205 Turkish absence patients and 219 healthy controls were genotyped for 10 SNPs and the results were assessed in Haploview 4 programme. First, the SNPs were checked in patient and control population samples separately to see whether they were in Hardy-Weinberg equilibrium. As shown in Table 5.9 and 5.10 all SNPs were found to be in Hardy-Weinberg equilibrium and also have a minor allele frequency higher than 0.26 for each.

Table 5.9. SNP marker analysis for 219 control samples.

SNP name	ObsHET	PredHET	HWpval	MAF	Alleles
Rs3731920	0.467	0.462	1	0.362	T:C
Rs2010592	0.469	0.47	1	0.378	C:T
Rs907676	0.425	0.439	0.714	0.325	C:G
Rs3770234	0.517	0.495	0.6361	0.448	C:T
Rs6436154	0.455	0.47	0.7256	0.378	G:A
Rs2241526	0.479	0.453	0.528	0.347	T:C
Rs7588807	0.502	0.498	1	0.469	G:T
Rs2840128	0.308	0.389	0.0049	0.264	T:A
Rs673951	0.355	0.423	0.0279	0.304	C:T
Rs2305055	0.422	0.415	0.9962	0.294	C:G

(ObsHet: Observed Heterozygozity, PredHet:Predicted Heterozygozity,

HW: Hardy-Weinberg Equilibrium, MAF: Minor Allele Frequency)

Table 5.10. SNP marker analysis for 205 patients with absence seizures

SNP name	ObsHET	PredHET	HWpval	MAF	Alleles
Rs3731920	0.423	0.461	0.2698	0.36	T:C
Rs2010592	0.423	0.479	0.1075	0.396	C:T
Rs907676	0.407	0.467	0.0747	0.371	C:G
Rs3770234	0.534	0.5	0.3998	0.498	C:T
Rs6436154	0.538	0.478	0.0887	0.396	G:A
Rs2241526	0.473	0.469	1	0.376	T:C
Rs7588807	0.452	0.481	0.4361	0.403	G:T
Rs2840128	0.339	0.363	0.4102	0.239	T:A
Rs673951	0.455	0.427	0.4478	0.309	C:T
Rs2305055	0.402	0.394	0.9286	0.269	C:G

(ObsHet: Observed Heterozygozity, PredHet:Predicted Heterozygozity,

HW: Hardy-Weinberg Equilibrium, MAF: Minor Allele Frequency)

For case control association analysis genotypes of Turkish absence patients and healthy controls for 10 SNPs were assessed in Haploview 4. As shown in Table 5.11 G allele of rs7588807 showed a significant association with the patient population (p-value=0.0235), while for the other SNPs p-values were higher than 0.05.

Table 5.11. Chi-square and p-values for the case control association study

SNP name	Associated allele	Chi-square	P value
Rs1397429	C	0.334	0.5632
Rs2010592	T	1.65	0.199
Rs907676	G	3.583	0.0584
Rs3770234	T	2.155	0.1421
Rs6436164	A	0.295	0.5869
Rs2241526	C	0.454	0.5005
Rs7588807	G	5.128	0.0235
Rs2840128	T	1.015	0.3136
Rs673951	T	0.824	0.3639
Rs2305055	C	1.324	0.2499

In order to confirm the positive association transmission disequilibrium test (TDT) was carried out for 81 trios by Haploview version 4. As shown in Table 5.12, p-value was higher than 0.05 thus did not conforme a positive association.

Table 5.12. TDT results for rs7588807

SNP name	Overtransmitted Allele	Transmitted/ untransmitted	Chi-square	p-value
Rs7588807	G	51:40	1.33	0.2489

5.2.3 Subgroup Analysis

The patients were subgrouped according to their syndrome and seizure type to investigate the possible difference in locus impact of either the syndrome or seizure type.

5.2.3.1. Association Analysis According to the Syndrome Type. Patients subgrouped according to the syndrome type included 98 CAE and 70 JAE patients. As shown in Table 5.13, p-values in the analysis of JAE patients for two neighbouring SNPs (rs7588807and rs2840128) turned out to be significant with p-values of 0.0030 and 0.0275, respectively.

Table 5.13 Chi-square and p-values for syndrome type analysis

SNP name	CAE		JAE	
	Chi-sqaure	P value	Chi-sqaure	P value
Rs1397429	0.425	0.5143	0.158	0.694
Rs2010592	2.631	0.1048	0.046	0.8293
Rs907676	2.608	0.1064	0.61	0.4348
Rs3770234	1.674	0.1957	1.423	0.2328
Rs6436164	0.0030	0.9533	0.861	0.3534
Rs2241526	0.286	0.5928	1.066	0.3019
Rs7588807	0.024	0.8765	8.802	0.0030
Rs2840128	0.489	0.4844	4.858	0.0275
Rs673951	2.636	0.1045	0.293	0.5886
Rs2305055	1.613	0.2041	0.089	0.7649

A TDT analysis on 26 JAE trios resulted in a p-value of 0.0201 for rs2840128, confirming the possible association of the locus with JAE syndrome (Table 5.14).

Table 5.14. TDT results for rs7588807 and rs2840128 in JAE patients

SNP name	Overtransmitted Allele	Transmitted/ Untransmitted	Chi-square	p-value
Rs7588807	G	22:13	2.314	0.1282
Rs2840128	T	12:3	5.4	0.0201

When these two SNPs (rs7588807 and rs2840128) were considered together and analyzed for haplotype associations, "GT" haplotype was found be significantly higher in patients while "TA" haplotype in control samples as shown in Table 5.15.

Table 5.15. Haplotype association analysis for 7588807 and rs2840128 in JAE patients

Haplotype	Frequency	Case: control frequencies	Chi-square	P value
GT	0.548	0.688, 0.514	10.285	0.0013
TA	0.225	0.141, 0.245	5.156	0.0232
TT	0.211	0.166, 0.222	1.156	0.2157
GA	0.017	0.004, 0.020	1.253	0.2629

5.2.3.2. Association Analysis According to the Seizure Type. When absence patients were subgrouped according to the seizure type there were 81 patients with GTCS, 36 patients with myoclonic, and 38 patients with febril seziures. 64 patients had photosensitivity as an additional feature to the seizures. For GTCS patients case control association analysis resulted in highly significant p-values for rs7588807, rs2840128 and significant value for rs907676 (0.0002, 0.0092 and 0.04, respectively) (Table 5.16). TDT analysis on 34 trios with GTCS confirmed the possible association of rs7588807 and rs2840128 with p-values of 0.0114 and 0.0343, respectively but did not confirm the association of rs907676 as shown in Table 5.17. A significant association of Rs907676 to patients with myoclonic seizures was also observed but TDT was not carried out as trios with myoclonic seziures were not enough. For other seizure types p-values are not significant.

Table 5.16. Chi-square and p-values for seziures type analysis

SNP name	GTCS		Myoclonic		Febril		Photosensitive	
	Chi-sqaure	P value	Chi-sqaure	P value	Chi-sqaure	P value	Chi-square	P value
Rs1397429	0.484	0.4867	0.311	0.5768	0.03	0.8616	0.18	0.6716
Rs2010592	1.429	0.232	3.643	0.0563	0.046	0.8299	0.069	0.793
Rs907676	4.054	0.0441	5.331	0.021	0.076	0.7829	0.215	0.6429
Rs3770234	1.505	0.2199	0.642	0.423	0.801	0.3709	0.061	0.8053
Rs6436164	0.337	0.5616	0.744	0.3883	0.202	0.6535	0.18	0.6716
Rs2241526	1.043	0.3072	0.286	0.5925	0.0020	0.968	0.968	0.3251
Rs7588807	13.91	0.0002	3.194	0.0739	0.844	0.3583	3.054	0.0805
Rs2840128	6.78	0.0092	0.586	0.4439	0.684	0.4083	0.595	0.4403
Rs673951	0.615	0.4329	0.813	0.3674	0.126	0.7229	0.0030	0.9539
Rs2305055	1.555	0.2123	0.586	0.4441	0.192	0.6614	0.08	0.7778

Table 5.17. TDT results for rs907676, rs7588807 and rs2840128 in absence patients with GTCS

SNP name	Overtransmitted Allele	Transmitted/ untransmitted	Chi-square	p-value
Rs907676	G	15:11	0.615	0.4328
Rs7588807	G	28:12	6.4	0.0094
Rs2840128	T	19:8	4.481	0.0343

The two SNPs (rs7588807 and rs2840128) were analyzed for haplotype associations, “GT” haplotype was also found be significantly higher in patients with GTCS while “TA” haplotype in control samples as shown in Table 5.18.

Table 5.18. Haplotype association analysis for 7588807 and rs2840128 in absence patients with GTCS.

Haplotype	Frequency	Case: control ratios	Chi-square	P value
GT	0.559	0.682, 0.514	13.293	0.0003
TA	0.217	0.142, 0.245	7.153	0.0075
TT	0.211	0.165, 0.222	2.253	0.1334
GA	0.017	0.011, 0.020	0.588	0.4431

5.2.4. HRM and DNA Sequence Analysis of the INHA Gene

The two SNPs rs7588807 and rs2840128 associated with both JAE and GCTS are located 12kb away from each other. Rs7588807 resides in the intron of inhibin alpha precursor gene (INHA) while rs2840128 does not fall into any gene. Figure 5.6 shows the positions of the two associated SNPs, INHA gene and the other SNPs within these two SNPs genotyped in the HapMap project. INHA codes for the alpha subunit of the inhibin protein which is known to be a gonadal glycoprotein that regulates the secretion of follicule-stimulating hormone (FSH).

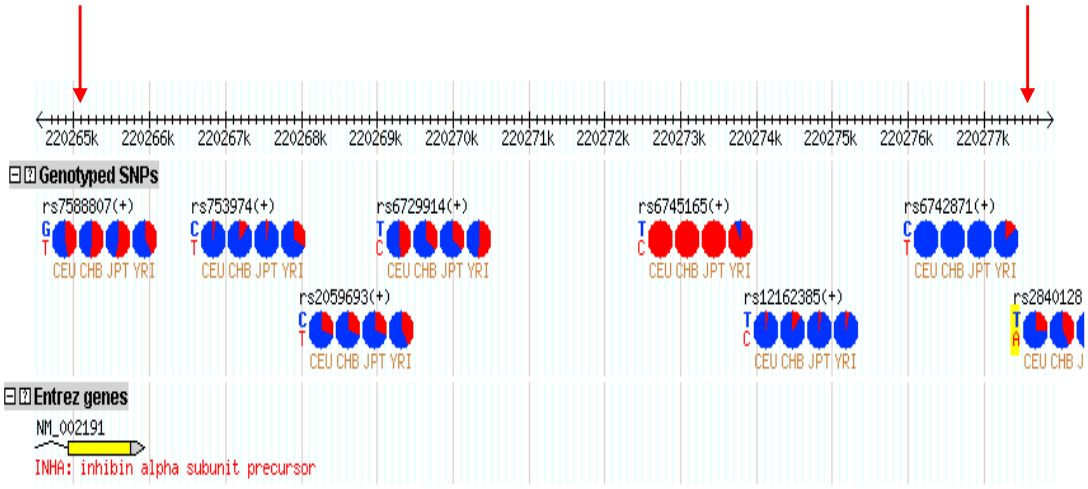


Figure 5.6. The positions of two associated SNPs (rs7588807 and rs2840128)

INHA is composed of two exons comprising approximately 1424 bp coding region. The two exons and the promoter region of the INHA gene in 70 JAE patients and 81 absence patients with GTCS were analyzed for the presence of a causative mutation and/or a susceptibility allele. The promoter region and exon 1 of the INHA gene was directly sequenced while exon 2 was divided into four regions and analyzed by HRM in the Light cycler 480. Table 5.19 shows the optimized conditions for each region.

Table 5.19. Optimized conditions for amplification of the promoter and exons of the INHA gene

Region	Mg Concentration (mM)	Primer concentration	Annealing Tm (^{0}C)	Length of the PCR product (bp)	Analysis type
Promoter 1	1.5	0.2Mm	58.2	346	Sequencing
Promoter 2	1.5	0.2Mm	58.2	299	Sequencing
Promoter 3	1.5	0.2Mm	58.2	350	Sequencing
Exon 1	1.5	0.2Mm	60	507	Sequencing
Exon 2.1	3	0.3Mm	Touchdown 71→65	291	HRM/ sequencing
Exon 2.2	3	0.5Mm	Touchdown 71→66	349	HRM/ sequencing
Exon 2.3	3	0.3Mm	Touchdown 71→65	274	HRM/ sequencing
Exon 2.4	3	0.3Mm	Touchdown 71→66	300	HRM/ Sequencing

DNA sequence analysis of the INHA gene in JAE patients (70) and patients with GCTS (81) and controls (49) revealed several novel nucleotide changes according to pubmed data. In the promoter region two novel nucleotide changes were identified in JAE patients. In patient 301AE710, -n.560G→A transition (Figure 5.7) and in patient 21AE69 n.-658A→T transversion (Figure 5.8) were detected in heterozygous state. These changes were not present in 49 control samples.

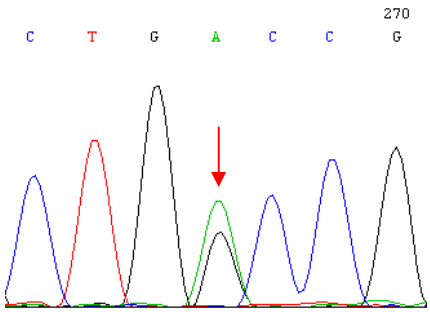


Figure 5.7. n.-560G→A transition in patient 301AE710 is indicated with an arrow

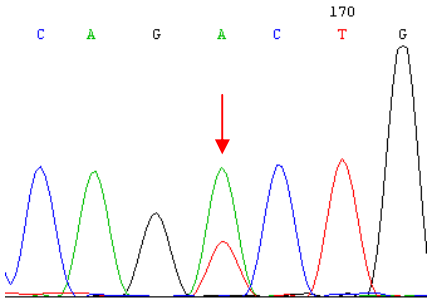


Figure 5.8. n.-658A→T transversion in patient 21AE69
is indicated with an arrow.

Patient 109AE319 had n.-106G→C transversion in 5'UTR of exon1 as shown in Figure 5.9 and this novel variation was not present in 49 control samples.

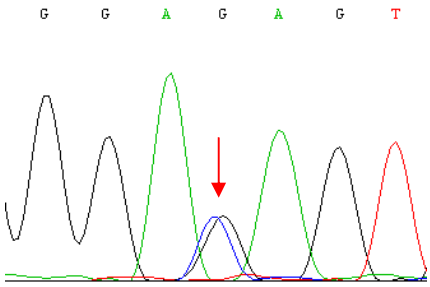


Figure 5.9. n.-106G→C transversion in patient 109AE319
is indicated with an arrow

In patient 60AE189 C→T transition is observed at 370. nucleotide which caused amino acid substitution, Arg124Cys (Figure 5.10). This novel variation also exited in the mother and healthy brother of the patient but was not detected in 70 control samples.

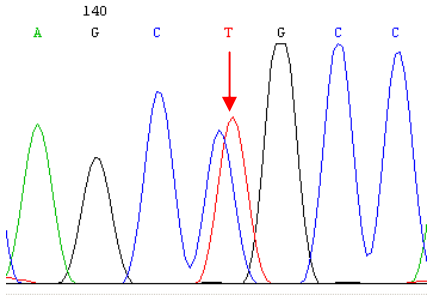


Figure 5.10. n.370C→T transition in patient 60AE189
is indicated with an arrow

In patient 122AE342 and control sample 77TR, a novel variation was detected in heterozygous state as shown in Figure 5.11 n.478G→A transition in exon 2 caused Val163Met susbtitution in these individuals. Its presence in one control sample indicated that the amino acid substitution did not have an effect on disease phenotype at least in JAE patients or patients with GTCS.

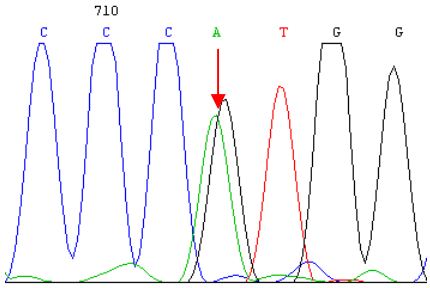


Figure 5.11. n.478G→A transition in patient 122AE342 and control 77TR is indicated with an arrow

In patient 132AE352 who was classified as JAE but also have GTCS, n747G→A exchange was detected in exon2 of the INHA gene as shown in Figure 5.12. This exchange resided in the third nucleotide of the codon thus did not cause an amino acid change (Leu249Leu). However, this new variation was not detected in 70 control samples.

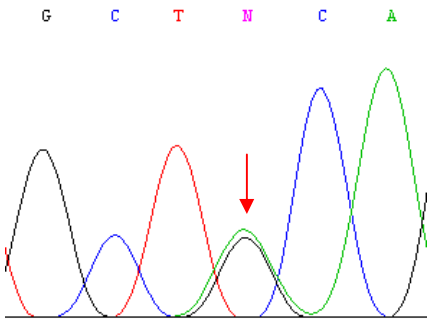


Figure 5.12. n.747G→A transition in patient 132AE352 is indicated with an arrow

In patient 11AE35 who had both absence seizures and GTCS, n.525C→G transversion was oberved as shown in Figure 5.13. This exchange caused His175Gln substitution and was not detected in 70 control samples.

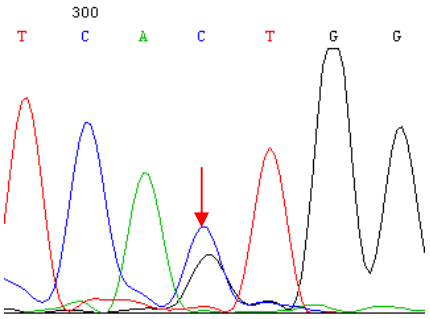


Figure 5.13 n.525C→G transversion in patient 11AE35
is indicated with an arrow

Patient 320AE7105 with absence seizures and GTCS has n.315G→C substitution leading to Glu105Asp substitution (Figure 5.14). This new variation was not detected in 70 control samples.

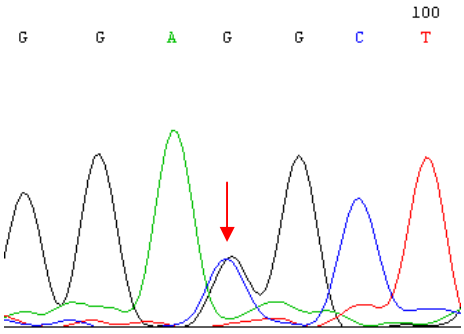


Figure 5.14 n.315G→C transversion in patient 320AE7105
is indicated with an arrow

Interestingly novel variations were also found in control samples in the promoter and exon 2 regions of the INHA gene. Control sample 119TR had a n.-542A→G transition in heterozygous state in the promoter region as shown in Figure 5.15.

In exon 2 of 124TR n.498C→T transition was found in the third nucleotide of the codon thus does not change the amino acid (Figure 5.16).

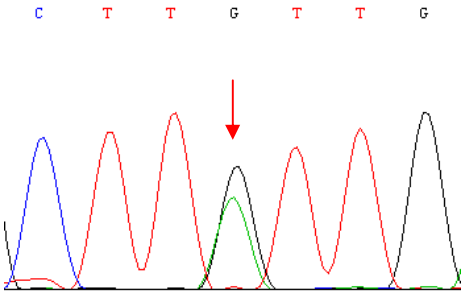


Figure 5.15 n.-542A→G transition in control sample 119TR is indicated with an arrow

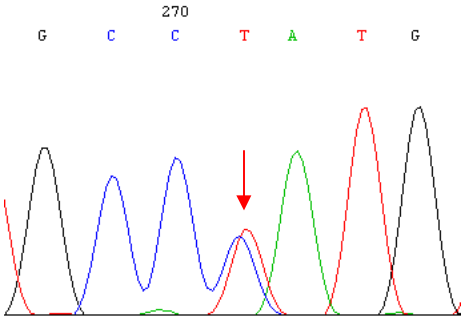


Figure 5.16 n.498C→T transition in control sample 124TR is indicated with an arrow

The summary of the novel variations in patients and control samples are shown in Table 5.20. Risk analysis of the novel variations were carried by "Polyphen" for amino acid substitutions, "ESEfinder" for exonic enhancer sequences and "Splice Site Predictor" and "Alternative Splice Site Predictor (ASSP) for any possible cryptic site (Wang and Marin, 2006; Cartegni *et al*., 2003; Ramenski *et al.*, 2001; Reese *et al*., 1997). According to these analysis, R124C and H175Q are highly damaging while V163M, L249L and E105D are benign substitutions.

Table 5.20. Novel INHA gene variations identified in absence patients and controls

JAE Patients	**Position of the SNP**	**Nucleotide change/ Position**	**Amino acid**	**Risk analysis (Polyphen, ESE Finder, ASSP, Splice Site Predictor)**
301AE710	Promoter	n.-560G→A	-	Conserved sequence change
21AE69	Promoter	n.-658A→T	-	Conserved sequence change
109AE319	Exon1	n.-106G→C	-	Non-conserved sequence change
60AE189	Exon 2	n.370C→T	R124C	Missense non-conservative change /Highly Damaging
122AE342	Exon 2	n.487G→A	V163M	Missense non-conservative change/ Benign effect
Patients with GTCS				
132AE352	Exon 2	n.747G→ A	L249L	Splicing regulation / medium risk
11AE35	Exon 2	n.525C→G	H175Q	Missense conservative change/ damaging
320AE7105	Exon 2	n.315G→C	E105D	Missense conservative change / Benign
Controls				
119TR	Promoter	n.-542A→G	-	Non-conservative sequence change
77TR	Exon 2	n.487G→A	V163M	Missense conservative change / Low Risk
124TR	Exon 2	n.498C→T	P166P	Sense change / very Low Risk

5.2.5. Haplotype Analysis of the INHA Gene

DNA analysis of the INHA gene revealed the presence of common variations in addition to novel variations shown in the previous section. Along with rs7588807 which resided in the intron of INHA gene, three more known SNPs were also identified with high heterozygozity. The frequency of these SNPs were calculated based on the DNA sequence

analysis of 49 control samples. To assess the possible role of these SNPs, an association test was carried out. The p- values for the two SNPs (rs11893842 and rs1270063) confirmed further the possible association of this locus to the JAE syndrome as shown in Table 5.21. Only one of the SNPs (rs11893842) in the INHA gene was associated with GTCS.

Table 5.21. Association analysis of the known SNPs in the INHA gene for JAE syndrome and GTCS

SNP name	Location of the SNP in INHA gene	Associated allele	JAE		GTCS	
			Chi-square	P value	Chi-square	P value
Rs11893842	5'UTR	G	10.821	0.0010	8.573	0.0034
Rs35118453	5'UTR	T	1.167	0.2801	0.262	0.609
Rs1270063	Exon 2	T	5.767	0.0163	2.384	0.1226

To see if there was a common haplotype comprising the whole INHA gene in patients compared to the controls, the four genotyped SNPs (rs11893842, rs35118453, rs7588807 and rs1270063) were used in a haplotype association test. In JAE patients and patients with GTCS haplotype associations pointed to the presence of a common haplotype (ACTC) in higher frequency in control samples as shown in Table 5.22. For GTCS on the other hand, another haplotype (GCGC) was found to be significantly higher in patient samples.

Table 5.22. Haplotype association analysis in the INHA gene

	Haplotype	Frequencies of Case and Control	Chi-square	P value
JAE	ACTC	0.244: 0.468	12.058	0.00005
	GCGC	0.310: 0.197	3.602	0.0577
	GTGT	0.266: 0.163	3.343	0.0675
GTCS	ACTC	0.268: 0.471	10.218	0.0014
	GCGC	0.333: 0.197	5.215	0.0224
	GTGT	0.234:0.163	1.745	0.1865

5.2.6. CNV Analysis of the INHA Gene

The heterozygozity of the four common SNPs (rs11893842, rs35118453, rs7588807 and rs1270063) in the INHA gene was high in general but in twelve JAE and in four absence patients with GTCS these SNPs were found to be homozygous indicating a possible loss of heterozygozity. Therefore, absolute and relative quantification analysis were carried out in real time. In initial experiments rs7588807 SNP region was selected as the target and exon 3 of the FXI gene as the reference region. FXI codes a glycoprotein which has a role in blood coagulation thus, it has no relevance to epileptic seizures. In order to quantify the unknown samples in target and reference genes standart curves were set up by 5ng, 10ng and 20ng (three replicates of each dilution) of a control DNA sample. As shown in Figure 5.17 Cp of the sample increases as the template amount decreases in the amplification curve.

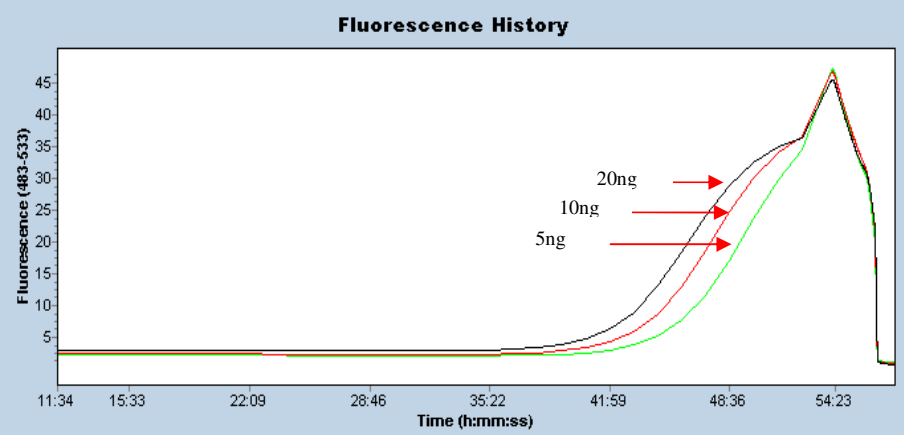


Figure 5.17. Amplification curves for standart samples

For the quantification of the unknown samples for rs7588807, 20ng of each sample were run with the control sample and standart curve dilutions. Six of the JAE patients (24AE79, 7GE43, 12AE37, 25AE82, 28AE93 and 52AE165) and one of the patients with GTCS (31AE99) were found to have a reduced copy number by half compared to the 20ng control sample and the reference gene. Figure 5.18 shows the

amplication curves of the 24AE79 and 7GE43 samples with standart dilutions in target region rs7588807. The Cp values of two samples correspond to 10ng of standart dilution indicating the loss of one allele.

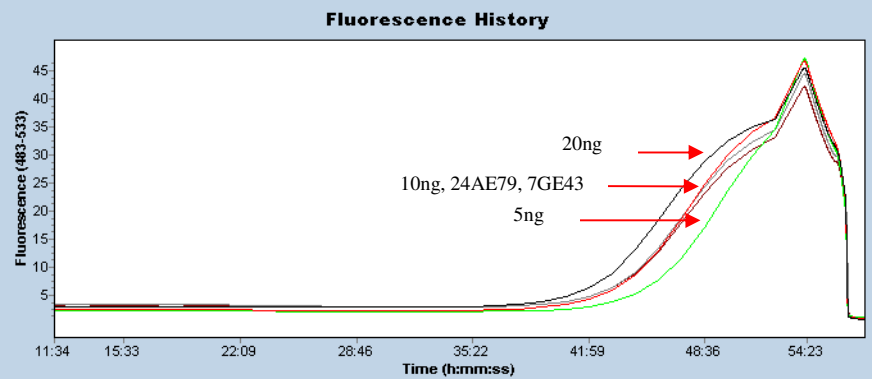


Figure 5.18. Amplification curves for patients 24AE79 and 7GE43.

qPCR analyses for target and reference gene were repeated three times with three replicas in each reaction for these patients and the results were normalized by calculating the mean concentration ratio of target to reference gene. As shown in Table 5.21 mean value of the normalizations were 0.45-0.58 for seven patients indicating the loss of one allele. In order to see whether the sequences in close proximity were also deleted, qPCR analysis was carried out for other SNP locations, rs1983211, rs2241526 and rs3770234 regions which were located in the 5' of the rs7588807 and rs6729914 region which was located in the 3' of the rs7588807. For rs1983211 mean normalization values ranged from 0.48 to 0.63 and for rs2241526 from 0.53 to 0.62 also indicating the loss of one allele corresponding to these SNP locations (Table 5.23). On the other hand, normalization values for rs3770234 varied 0.97 to 1.19 for the six patients except 7GE43, as the mean normalization value was 0.56 suggesting this SNP was also deleted in this patient. For rs6729914 which is located 4.4kb away from the 3'of the rs7588807 the values ranged from 0.79 to 1.01. Normalization values, mean concentrations, Cp values, standart deviations (STD) for target loci (rs7588807, rs1983211, rs2241526, rs3770234 and rs6729914) and reference gene (FXI) for three assays are shown in Appendix D.

Table 5.23. Normalization results for rs7588807, rs1983211, rs2241526, rs3770234 and rs6729914

	Target/reference (rs7588807)				
Patients	**Assay 1**	**Assay 2**	**Assay 3**	**Mean**	**STD**
24AE79	0.39	0.63	0.63	0.55	0.14
7GE43	0.32	0.53	0.50	0.45	0.11
12AE37	0.40	0.70	0.65	0.58	0.16
25AE82	0.37	0.58	0.58	0.51	0.12
28AE93	0.43	0.62	0.62	0.55	0.10
31AE99	0.40	-	0.56	0.48	0.11
52AE165	0.41	0.68	0.54	0.54	0.13
122TR	1.01	10.9	0.92	1	0.08
	Target/reference (rs1983211)				
24AE79	0.51	0.52	0.67	0.56	0.08
7GE43	0.40	0.36	0.69	0.48	0.18
12AE37	0.59	0.67	0.61	0.62	0.04
25AE82	0.58	0.47	0.47	0.50	0.06
28AE93	0.62	0.56	0.55	0.57	0.03
31AE99	0.62	-	0.55	0.58	0.04
52AE165	0.62	0.65	-	0.63	0.02
122TR	1.03	1.09	0.93	1.01	0.08
	Target/reference (rs2241526)				
24AE79	0.46	0.52	0.61	0.53	0.07
7GE43	0.46	0.52	0.64	0.54	0.09
12AE37	0.56	0.60	0.67	0.61	0.05
25AE82	0.54	0.61	-	0.57	0.04
28AE93	0.48	0.68	-	0.58	0.14
31AE99	0.56	0.62	-	0.59	0.04
52AE165	0.59	0.66	-	0.62	0.04
122TR	1.03	1.06	0.95	1.01	0.05

(STD: Standart Deviation)

Table 5.23. Normalization results for rs7588807, rs1983211, rs2241526, rs3770234 and rs6729914 (continued)

Patients	**Target/reference (rs3770234)**				
24AE79	1	1.08	0.83	0.97	0.12
7GE43	0.61	0.66	0.41	0.56	0.13
12AE37	1.04	1.32	0.92	1.09	0.2
25AE82	1.10	1.19	0.77	1.02	0.2
28AE93	1.21	1.30	0.83	1.1	0.2
31AE99	1.11	1.26	-	1.18	0.10
52AE165	1.23	1.35	1.01	1.19	0.17
164TR	1.30	1.18	1.09	1.19	0.10
	Target/reference (rs6729914)				
24AE79	0.92	0.73	0.82	0.82	0.9
7GE43	1.02	0.92	1.09	1.01	0.08
12AE37	0.94	0.84	-	0.89	0.07
25AE82	1.12	1	0.88	1	0.12
28AE93	0.86	0.74	0.79	0.79	0.06
31AE99	0.99	0.75	1.01	0.91	0.14
52AE165	0.96	0.69	1.07	0.90	0.19
122TR	1.26	1.08	1.3	1.22	1.11

(STD: Standart Deviation)

Among the fourty-nine control samples that were also sequenced for the INHA gene and four (56TR, 57TR, 58TR, 104TR) of them were also homozygous for all SNPs quantified in seven patient samples. Therefore, qPCR analysis was also carried out for these samples to assess whether these deletions were common copy number variations (CNV). Control sample 164TR was also included in the assay as a control as this sample was heterozygous for rs7588807 thus had no possibility for carrying a deletion. Table 5.24 shows the qPCR results of these five control samples for rs7588807. The ratio of the target

and reference gene is 1 indicating that the homozygozity in these control samples is not due to a loss of heterozygozity. Table 5.24 also includes the qPCR results for healthy siblings of the patients with deletion. 24AE81 is healthy brother of 24AE79, 28AE242 healthy sister and 28AE243 healthy brother of 28AE93 while 31AE102 is healthy sister of 31AE99. The normalization results for these siblings also revealed that they did not carry this deletion except 31AE102.

Table 5.24. qPCR results for healthy siblings and control samples

Rs7588807	**Target/reference**				
CONTROLS	**Assay 1**	**Assay 2**	**Assay 3**	**Mean**	**STD**
56TR	1.3	1.1	1	1.1	0.15
57TR	1.4	0.9	0.8	1	0.32
58TR	1.01	1.01	-	1.01	0
104TR	0.9	1.4	1.1	1.2	0.25
164TR	0.7	0.9	0.8	0.8	0.1
HEALTY SIBLINGS					
28AE242	0.9	1	0.8	0.9	0.1
28AE243	0.9	0.9	0.8	0.9	0.05
31AE102	0.7	0.8	0.6	0.7	0.1
24AE81	0.8	1	0.8	0.9	0.11

(STD: Standart Deviation)

The relative position of all SNPs used initially in the association study, revealed by the mutational analysis of the INHA gene and used in the analysis of the large deletion is shown in Figure 5.19.

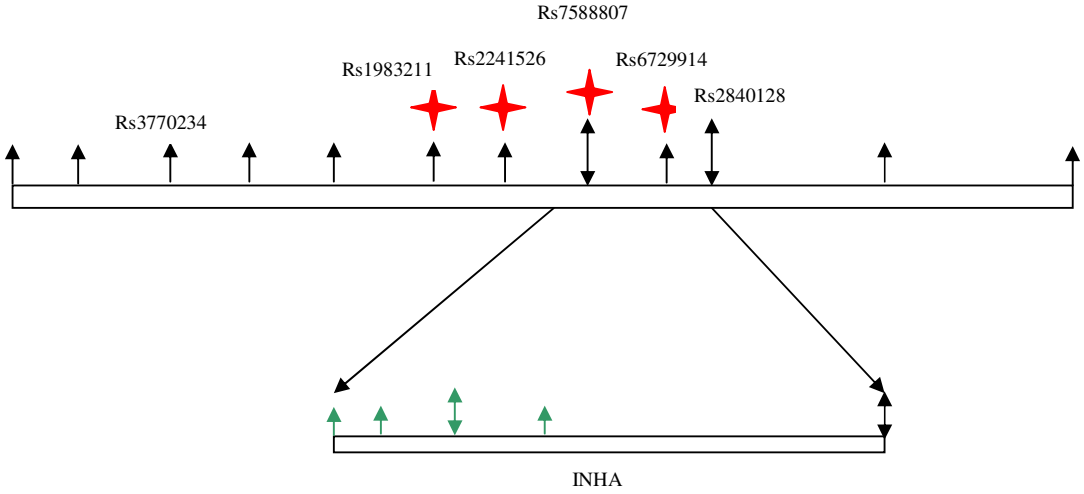


Figure 5.19. Schematic representation of SNPs at 2q36 in 160kb region. Double headed arrows indicate the SNPs that were found to be associated in case control and TDT studies. Green arrows shows the SNPs in the INHA gene that constituted the associated haplotype. Red stars indicated the SNP locations analyzed in qPCR analysis and homozygozity in deleted patients.

5.3 Analysis of the GABRG2 Gene for CAE Patients

In the search to further clarify the pathogenesis of absence seizures mutational analysis of GABRG2 gene was carried on for 85 CAE patients. GABRG2 gene has three transcript variants with the longest isoform composed of 10 coding exons. In order to search for a causative mutation exon 2, 3, 6, 9 and 10 were analyzed by HRM analysis while exon 1, 4, 5, 7 and 8 were sequenced directly. Table 5.25 shows the optimized amplification conditions, lenght of the PCR products and analysis type for each exon.

Table 5.25. Optimized amplification conditions for 10 exons of GABRG2

Region	Mg Concentration (mM)	Primer Concentration (mM)	Annealing Tm (^{0}C)	Additional Solution	PCR product (bp)	Analysis type
Exon1	1.5	0.2	62	DMSO	323	Sequencing
Exon2	2.5	0.2	Touchdown 64→54	-	415	HRM/ sequencing
Exon3	2.5	0.2	Touchdown 64→56	-	420	HRM
Exon4	1.5	0.2	58.2	DMSO	520	Sequencing
Exon5	1.5	0.2	60	_	395	Sequencing
Exon6	2.5	0.2	Touchdown 64→54	-	322	HRM/ sequencing
Exon7	1.5	0.2	60	Q solution	524	Sequencing
Exon8	1.5	0.2	58.2	DMSO	461	Sequencing
Exon9	2.5	0.2	Touchdown 64→58	-	397	HRM/ sequencing
Exon10	2.5	0.2	Touchdown 64→58	-	419	HRM/ sequencing

Mutation screening revealed novel variations, rare polymorphisms and common SNPs in CAE patients. By direct sequencing novel variations were identified in intron 1, 3, 5, 7 and exon 8. Intron 1 of patient 33AE106 included IVS1+12C→T transition in heterozygous state as indicated in Figure 5.20. The novel variation was in close proximity to the donor site of the exon1 thus may affect the splicing process.

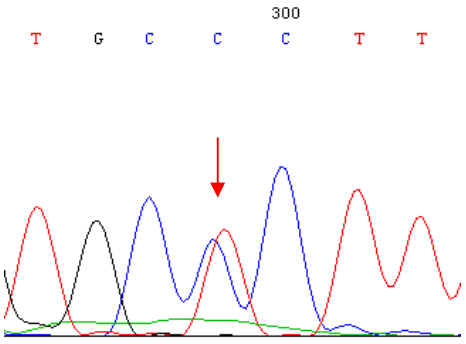


Figure 5.20 IVS1+12C→T transition in patient 33AE106 is indicated with an arrow

In intron 3 patient 18AE63 had IVS4-18A→G nucleotide transition which was located close to the acceptor site of exon 4. The variation was in heterozygous state as shown in Figure 5.21.

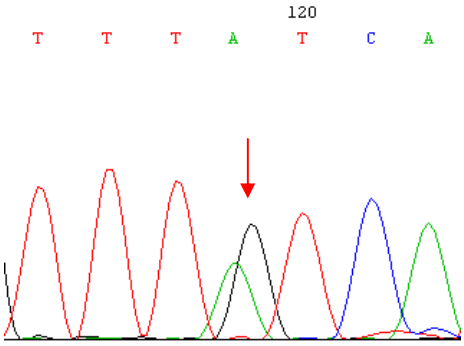


Figure 5.21 IVS4-18A→G transition in patient 18AE63
is indicated with an arrow

Patient 26AE86, 45AE142, 54AE172 and 136AE356 had a novel variation in intron 5 causing T→G transversion as shown in Figure 5.22. The nucleotide resides 55 bp away from the coding region thus not affecting the splice-site. The new SNP was submitted to pubmed data and named as rs55712126.

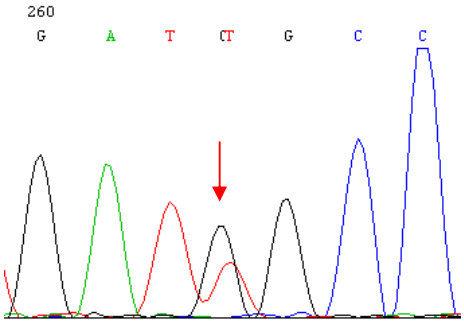


Figure 5.22 IVS5+55T→G transversion in patient 45AE142
is indicated with an arrow

Patient 136AE356 also had a novel variation in heterozygous state in exon 8 as shown in Figure 5.23. The transversion did not cause any amino acid substitution (Phe299Phe) but may affect ESE splice site.

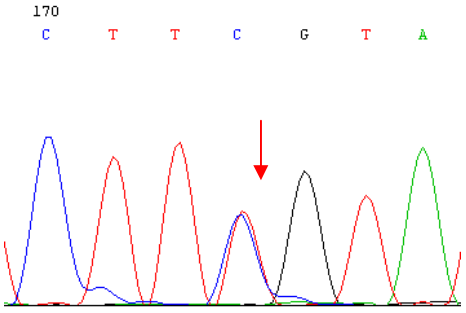


Figure 5.23 n.897T→C transition in patient 136AE356
is indicated with an arrow

By HRM analysis on the other hand, 129AE349, 86AE272 and 33AE106 were classified as different groups other than the wild type for exon 2, exon 3 and exon 6, respectively. Thus, they were subjected to DNA analysis. In patient 86AE272 C→T transition was detected in the intron 3 as shown in Figure 5.24. Splice site was not deleted as the nucleotide change is located 21 bp away from exon 3.

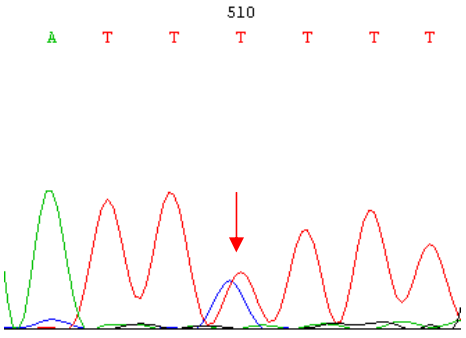


Figure 5.24 IVS3+21C→T transition in patient 86AE272
is indicated with an arrow

33AE106 had IVS6+10A→G transition in intron 6 as shown in Figure 5.25. Although this nucleotide exchange is in close proximity to the sequences that affect the splice site, this variation also does not change the splicing process.

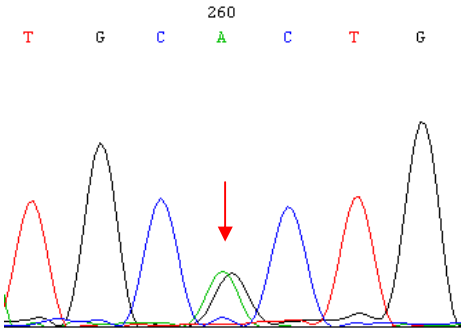


Figure 5.25 IVS6+10A→G transition in patient 33AE106
is indicated with an arrow

Patient 129AE349 has novel variation in intron 2 of GABRG2 gene (Figure 5.26). The mutation resides 6 bp away from the exon2 thus probably affecting the splicing process.

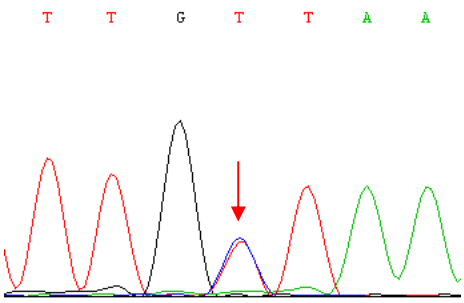


Figure 5.26 IVS2+6T→G transversion in patient 129AE349
is indicated with an arrow

The summary of the novel variations, their position and possible effects are shown in Table 5.26. Risk analysis of the novel variations were carried by "ESEfinder" for exonic enhancer sequences and "Splice Site Predictor" and "Alternative Splice Site Predictor (ASSP) for any possible cryptic site (Wang and Marin, 2006; Cartegni *et al.*, 2003; Ramenski *et al.*, 2001; Reese *et al.*, 1997).

Table 5.26. Variations in GABRG2 gene in CAE patients

CAE Patients	SNP Position	Nucleotide change/ position	Amino Acid	Risk analysis (ASSP, ESEfinder)
33AE106	Intron 1	IVS1+12C→T	-	No effect on splice site
129AE349	Intron 2	IVS2+6T→G	-	Cryptic splice site
86AE272	Intron 3	IVS3+21C→T	-	May create cryptic acceptor site
18AE63	Intron 3	IVS4-18A→G	-	No effect on splice site
26AE86	Intron 5	IVS5+55T→G	-	No effect on splice site
45AE142	Intron 5	IVS5+55T→G	-	No effect on splice site
54AE172	Intron 5	IVS5+55T→G	-	No effect on splice site
136AE356	Intron 5	IVS5+55T→G	-	No effect on splice site
33AE106	Intron 6	IVS6+10A→G	-	May affect the splice site
136AE356	Exon 8	n.897T→C	F299F	Effect on ESE sequence

6. DISCUSSION

6.1. LD and Haplotype Structure of the Turkish Population at 2q36 and HapMap Comparison

The first part of this study involves the construction of LD structure of 160kb region at 2q36 in the Turkish population to reveal the possible tag SNPs that would be used in an association study. Utilization of tag SNPs is essential because first of all association studies aim to find the closest loci to the disease in a group of unrelated individuals in the same population with a common ancestor. Due to recombinations in the disease loci over time the target region would be reduced, therefore, adequate number of SNPs should be utilized to have the highest resolution. As tag SNPs represent all SNPs in a LD block which is bounded by recombination hotspots, tag SNP approach would result with the least number of SNPs with the highest resolution. LD block structure of European, Chinese, Japanese and African origin populations were already constructed in the framework of the International HapMap project. The Hap Map data revealed that there were population specific LD structures, haplotypes and SNP frequencies, therefore, it is best to study the LD structure of the specific population in which the association study would be carried out.

At present there are approximately 120 SNPs covering the 160kb region at 2q36 in the pubmed data, however, the number of SNPs was 60 at the time this study was planned. By genotyping 25 SNPs, seven blocks tagged with 20 SNPs were revealed suggesting that genetic recombinations have been frequent and a significant reduction in the number of SNPs that would cover major haplotypes for association studies was not possible. Nevertheless, this study indicated that initial association studies could be conducted with at least 10 SNPs to cover seven LD blocks and the regions between blocks.

The comparative analysis of LD at 2q36 among the Turkish and HapMap populations using 15 SNPs common to all populations revealed that JPT and CHB populations had longer blocks with less diversity in haplotype structure. The analysis of LD structure of Turkish and YRI populations reflects the diversity of these populations in contrast to CEU, CHB and JPT populations where LD is stronger between alleles as

historical recombination rate is lower. The block structure of the Turkish population showed similarities to both the YRI and CEU data. The Turkish population was closer to CEU, however, when the frequencies of common haplotypes and SNP alleles were compared. The allele frequency comparison of the Turkish-YRI resulted in significant differences showing that the two populations had very distant common ancestry. In the second common block, there were six possible haplotypes in the Turkish population while CEU had five, YRI four and CHB and JPT had only three haplotypes. Interestingly, Turkish population as a reflection of the demographic nature of the region was more diverse and heterogenous in haplotype structure even when compared to the older YRI population. Haplotype structure and allele frequencies analysis indicate that genomic structure of the Turkish population is similar to CEU but show more haplotype diversity and lower LD. Our data was supported by a previous study involving that compared the polymorphic regions on Y chromosome. In this study, Turkish population was demonstrated to have similar patterns with Middle Easterners and Europeans. The genetic contribution of the Europeans to the population admixture in Turkey probably dated back to the migration to Europe through Anatolia in Neolithic times (Jarjanazi, 1999).

Four of the SNPs genotyped in the Turkish population did not show any heterozygosity and were excluded from further analysis. One of those, rs 12694468 had a MAF of 0.0080 in CEU and 0.475 in YRI according to the HapMap data. On the other hand, rs2276640 had MAF of 0.267 in the CEU, 0.233 in CHB, 0.227 in JPT but it was not genotyped in the YRI. The two other SNPs rs6729914 and rs652509 were not genotyped in HapMap populations. It was interesting to observe that Turkish population was not polymorphic for these 4 SNPs when the admixture and observed diversity of the Turkish population was considered.

TagSNPs transferability across populations is also another aim for researchers who design association studies. In a recent study, linkage disequilibrium of chromosome 7p15 was compared between Korean samples, and Japanese and Han Chinese samples from HapMap data (Lim *et al*., 2006). Although there are some differences in the boundaries of LD blocks, the data supported the efficiency of tagSNPs transferability across these three populations. In this study, tagSNP analysis indicated that in terms of genetic heterogeneity, the Turkish population was comparable to YRI; however, its genetic make-up was closer to

CEU. It can be argued that CEU HapMap data could be used in association studies involving Turkish samples with less precision however. The possible transferability of all HapMap data for all loci remains to be seen as more comparative LD and haplotype analysis are done.

6.2. Association Study

In a previous whole genome linkage study which included 116 CAE, 59JAE, 95JME and 6 idiopathic absence epilespy (IAE) patients revealed a susceptibility locus at 2q36 (Sander *et al*., 2000). In WAG/Rij rats which are pure absence epilepsy models the linkage analysis also pointed out to 2q33-37 syntenic region as one of the susceptibility loci for the polygenic absence phenotype. As a candidate gene in this locus SLC4A3 which codes for an anion exchanger was sequenced for IGE patients but revealed no causative mutation, only a slight contribution of a variation to the gene. In a large IGE family including patients with absence seizures and GTCS, 2q36 region was also found to confer susceptibility to IGE phenotype. However, attempts to find causative mutation in an ion channel gene KNJ13 that coded for a potassium channel in the region did not bring out positive results to explain the role of the gene in the complex pathogenesis of absence seizures (Klein *et al.*, 2008). Therefore, the possibility exists that the region still carries an unknown causative mutation or a strong susceptibility allele to IGE syndrome that manifests itself especially with typical absence seizures.

In this study the aim was to find a susceptibility allele in 160 kb region at 2q36 in Turkish patients with absence seizures. The selected 160 kb region included genes that could be candidates for absence seizures and that code for two ion channels; amiloride-sensitive cation channel (ACCN4) and anion exchanger carrier (SLC4A3), and also others like a transmembrane protein with unknown function (THEM198), GDP-mannose phosphorylase A (GMPPA), obscruin-like 1 gene (OBSL-1), chrondroitin polymerizing factor (CHPF), inhibin alpha subunit precursor (INHA) and serine/threonine kinase 11 interacting protein (STK11IP).

Ion channels were the most relevant candidates causing epilepsy pheontypes until recently but the current view is that mutations leading to prolongation in depolarization

thereby causing hyperexcitability of the network or changing the neurotransmitter release from the presynaptic terminals or sensitivity of the postsynaptic neuron may also cause epileptic seizures. Therefore, epilepsy is possibly not only caused by alteration in the function or structure of the ion channels but also by an imbalance of neurotransmission in the synaptic cleft (Kapur J, 2008; Noebels J. L, 2003). Ion channels, receptors or transporters may directly be involved in affecting membrane excitability or neurotranmitter release but also the subunits that change the function of the channels and receptors, transcription factors, hormones that alter the structure of the brain reducing the threshold of the epileptic seizures are part of the pathogenesis of the epilepsy. Identification of non-ion channel genes in recent years actually supports this approach to clarify the pathogenesis of the epilepsy. Therefore, this study aimed to analyze the 2q36 region found to be linked to epileptic seizures in various studies through an association analysis, rather than targeting on candidate genes.

Association study is the most convenient method to get close to the causative allele in complex disorders such as epilepsy. However, the design of the association study is critical as there are many drawbacks that may lead to false positive or negative results. First of all, the clinical features of the patients differ in a wide range within different individuals with the same syndrome or even within the same family members pointing out to the genetic heterogeneity of the disease. For this purpose in this study the patient samples were selected according to a carefully determined clinical criteria to have a homogenous sample group as much as possible. Patients with brain damage due to accidents, tumors or surgery and also with low IQ were not included to be certain that the cause of epileptic seizure is idiopathic. Whenever possible DNA samples of parents, healthy and sick siblings were also collected but only one of the affected siblings were included in the association test not to cause stratification which is the second main drawback in association studies. Choosing an inappropriate control group may lead to a false positive result due to population stratification and this is the main reason of the failure of replication of many associations studies. In this study control individuals had origin from different parts of the country and have no family history of epileptic seizures and other heritable disorders. As a second precaution DNA sample of parents were utilized in TDT as internal controls to check the accuracy of case control association results. Another major drawback of association studies is the selection of SNPs that fail to

represent the haplotypes in the region properly. The selection of tag SNPs based on the haplotype block structure of the Turkish population made it possible to overcome the above drawback and cover the region with a resolution of 5 kb on the average.

Association study with 205 absence patients and 219 control samples revealed a slight association to rs7588807 with a p value of 0.04. In order to increase the homogeneity of the samples, patients were classified according to seizure and syndrome type to carry out an subgroup association test. According to the syndrome type 98 CAE and 70 JAE patients were analyzed and the result revealed that the possible association at the prelimenary test was due to JAE patients but not CAE as the p-value of JAE was significantly lower while p-value of CAE was 0.87 showing absolutely no difference between control samples. Moreover, in analysis of JAE patients SNP rs2840128 which resided in 12kb away from 3' of rs7588807 was also found to be significantly associated to JAE syndrome with a p-value of 0.02. Association of two neigbouring SNPs further supported the linkage of the locus to the syndrome. Haplotype analysis revealed that "GT" haplotype was found to be significantly higher in patients. In order to avoid stratification TDT was carried out with 26 JAE trios, and the results confirmed the association of rs2840128 but not rs7588807. It is very likely that the association with rs7588807 would also have been observed if the trio number were higher.

Subgroup analysis according to seizure type on the other hand also revealed significant results with GTCS with three SNPs. The p-values for rs7588807 and rs2840128 indicated more significant association of this locus to the seizure type rather than to the JAE syndrome. TDT also confirmed that these associations were not due to the population stratification. Haplotype analysis with these two SNPs was also more significant as "GT" was found to be higher in patients with a p-value of 0.0003 and "TA" haplotype in controls with a p-value of 0.0075. Rs907676, on the other hand, which was also found to be slightly associated to GTCS is located 60kb away from the 5' of rs7588807 but TDT did not confirm the possible association. Rs907676 was also significant in 36 patients with myoclonic seizures, however, the number of patients should be increased to confirm the result.

6.3. Analysis of the INHA Gene

Rs7588807 is located within the inhibin alpha precursor gene (INHA) while rs2840128 does not fall into any gene, and is about 12 kb away upstream to the INHA gene. INHA gene codes for inhibin alpha subunit of inhibin A and inhibin B proteins which are the glycoproteins belonging to the TGF-β superfamily. Inhibin alpha subunit precursor protein consists of 366 a.a which include 18 a.a. for signal peptide sequence, 43 a.a. for proregion, 171 a.a. for N-terminus (αN) and 134 a.a for C-terminus (αC) mature region as shown in Figure 6.1 (Antenos *et al*., 2007). Alpha-subunit protein has three sites for glycosylation on asparagines at positions 146, 268 and 302 which are thought to be essential for the secretion of the mature protein from the cell. There are twelve cysteine residues on the protein which are essential for the formation of sulfur bridges between β-subunits and also within the protein itself for the proper three dimensional structure. Alpha subunit dimerizes with either β_A-subunit to form inhibin A or β_B-subunit to form inhibin B proteins. After dimerization of two subtypes the protein is transferred to endoplasmic reticulum for the addition of oligosaccarides and formation of the sulfure bridges between the two subunits. Then these dimeric protein is cleaved between amino acids 229-232 to have the mature proteins. However, uncut inhibin A is also found to be active and may have diverse functions. In the absence of inhibin alpha subunit, β_A-subunit on the other hand dimerizes as monomer to form another glycoprotein activin which is inhibited by inhibin activity.

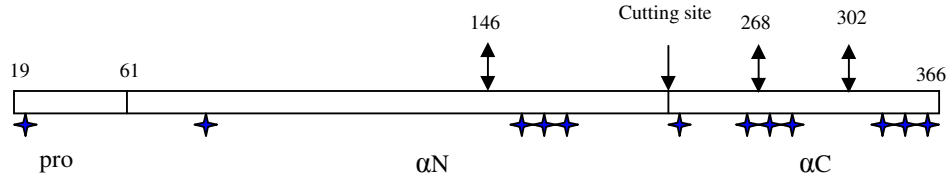


Figure 6.1. Schematic representation of inhibin alpha subunit precursor protein. Arrow shows the cutting site for mature protein, double-headed arrows shows the glycosylation sites and stars the cysteine residues through out the protein.

The novel candidate gene for JAE and/or GTCS, INHA was analyzed by DNA sequencing and the novel variations in promoter and two coding exons were shown in Table 5.21 for JAE patients, patients with GTCS and control samples. Although the variations in promoter regions were found in conserved regions, these are not on the binding sites for any of the transcription factors (Debieve and Thomas, 2002). However, these novel variations were not present in 49 control samples therefore, the possible effects of the change could be evaluated by functional studies.The patient 109AE319 who is classified as JAE has a novel variation in the 5'UTR of the INHA gene. This variation was also absent in 49 control samples but homolog INHA gene in mice also carries a "C" at this position showing that the nucleotide is not conserved and probably has a benign or no effect on transcription.

The novel variations in exon 2 on the other hand corresponded to the N-terminal region of the protein which was not found in mature dimeric protein but important for the dimerization. Patient 60AE189 who was classified as JAE and had pure absence seizures had an amino acid substitution of Arg124Cys which was analyzed by Polyphen and found to be highly damaging supported by the fact that arginine (R) which is a polar amino acid is exchanged with a nonpolar amino acid, cysteine (C) (Ramenski at al., 2001). Comparison of homolog genes in other species as shown in Table 6.1 revealed that the amino acid is not conserved but was always replaced with a polar amino acid like histidine. Cysteine residues are essential in the formation of true structure of the protein through the sulfur bonds. An extra cyteine residue may cause additional and unproper sulfur bonds within the protein and this may interfere with dimerization with β subunits. If the dimerization is not possible then βA subunit dimerizes as monomers to form more activin. This variation has a maternal inheritance and the healthy brother also has this variation. However, expression of epileptic phenotype does not solely depend on one gene but the cumulative effect of many changes in different genes should exceed the threshold level for a seizure. Therefore the presence of the variation in mother and healthy brother would not eliminate the possibility of the pathogenic effect of the variation. Actually the possible causative effect of this variation was further supported by the absence of this exchange in 70 control samples.

Table 6.1. Conservation of arginine residue (in bold) affected in the Arg124Cys mutation in homolog protein in different species.

Species	Amino acid sequence
Homo sapiens	FRYMFRPSQHTRS**R**QVTSAQ
Macca mulatta	FTYMFRPSQHTRS**R**QVTSAH
Mus musculus	FTYVFRPSQHIRS**H**QVTSAQ
Rattus norvegicus	FTYVFRPSQHIRS**H**QVTSAQ
Pan troglodytes	FRYMFRPSQHTRS**R**QVTSAQ

Another possible pathologic variation was the substitution of histidine with glutamine at non-conservative position 175 in patient 11AE35 who was classified as CAE-JAE and has GTCS, myoclonic and photosensitive seizures. Histidine which is a basic polar amino acid with essential imidazole ring is replaced with polar glutamine with neutral side chain. As shown in Table 6.2 this residue is exchanged with another basic amino acid arginine in some homolog proteins. This possible pathologic variation is also absent in 70 control samples. Patient 122AE342 who was classified as JAE and has pure absence seizure and a control sample carry the same novel variation causing V163M which was shown to have a benign effect by polyphen search. The facts that nonpolar valine is exchanged with nonpolar methionine and some of the homolog genes carry methionine instead of valine emphasize the mild effect of this variation.

Table 6.2. Conservation of histidine residue (in bold) affected in the His175Glu mutation in homolog protein in different species

Species	Amino acid sequence
Homo sapiens	GPVAVPMSLGHAPP**H**WAVLH
Macca mulatta	GPVAVPMSLGHAPP**R**WAVLH
Mus musculus	GPMAVPVSLGQGPP**R**WAVLH
Rattus norvegicus	GPMAVPVSLGQSPP**R**WAVLH
Monodelphis domestica	GPVAVPVLVGSAPR**H**WVVFH

Another patient 320AE7105 who is classified as JME and has GTCS and myoclonic seizures carries a substitution of polar glutamic acid with polar aspartic acid at position 105 indicating a benign effect of this variation. However, the variation was not present in 70 control samples pointing out that this may not be a common polymorhism.

Patient 132AE352 who has both GTCS and absence seizures and classified as JAE carries G to A exchange at the third codon of amino acid 249 thus not changing the amino acid. However, this new variation could not be found in 70 control samples therefore it was suspected whether this variation could create a cryptic acceptor or donor site in splicing process. This question was evaluated by two software programmes, "Splice Site Predictor" and "Alternative Splice Site Predictor (ASSP)". However, this nucleotide exchange did not create alternative splice sites. Whether this variation resided on exonic splicing enhancer sequences was also checked by a software programme "ESEfinder" developed by Cartegni *et al.* and found that a consensus motif for one of the SR proteins, SRp55 is lost by the exchange of G to A allele (Cartegni *et al.*, 2003). However, the presence or absence of putative enhancer should be experimentally evaluated.

When the clinical features of patients with novel variations are considered, they mostly show pure absence seizures as shown in Table 6.3.

Table 6.3. Clinical features of patients with novel variations in INHA gene

Patient ID	Birth date	Sex	syndrome	Seizures	Age of onset	Family history	Additional
60AE189	1980	F	JAE	Absence	12	-	Automatism absence
122AE342	1989	F	JAE	Absence	12	-	
132AE352	1995	F	JAE	Absence, GTCS	8	-	
11AE35	1991	F	CAE-JAE	Absence, GTCS, Photosensitivity	9	-	
109AE319	1994	F	JAE	Absence	11	-	Convulsions at age 1.5
21AE69	1996	F	JAE	Absence, GTCS	20	-	
710	1992	M	JAE	Absence, myoclonic	11	Epilepsy in father's family	
320AE7105	1985	M	JME	Absence, myoclonic, GTCS	14	-	

Besides these possible pathological variations, single marker and haplotype analysis of the four common SNPs (rs11893842, rs35118453, rs7588807 and rs1270063) in the

INHA gene further supported the association of the gene to this disease phenotype. According to the estimated frequencies of each haplotype in JAE and GTCS patients almost half the control samples have "ACTC" haplotype pointing out to a protective haplotype and the majority of the patients have "GCGC" with a frequency of ~0.320 and "GTGT" allele with frequency of ~0.250. In a research study the transcriptional activity of "AC", "GC" and "GT" haplotypes in 5'UTR of the INHA gene in human granulose cell was compared and the levels were found to be not different (Harris *et al*., 2005). However, expression levels could be examined in neuronal cells in order to assess the effects of the differences in patient and control sample.

In conclusion, point mutations and haplotype associations in the novel candidate gene INHA, support its association with the manifestation of the JAE phenotype especially in patients with GTCS.

6.3. CNV/Deletion Analysis of the Region

Absolute and relative quantification analysis was carried out in Light Cycler for twelve JAE and four patients with GTCS in order to assess whether the loss of heterozygozity in all of the common SNPs in the INHA gene was due to loss of one allele. Initial qPCR analysis was carried out for rs7588807 as the first association was found with this SNP. For seven patients the experiments were carried out in triplicate and found to have a deletion in the region carrying rs7588807. For four of the homozygous patients normalization results revealed that there is no deletion in the region and for five of them there was not enough DNA samples to carry out the experiments. In order to evaluate whether this deletion in seven patients is also present in the neighboring SNPs (rs2241526 and rs2840128) qPCR analysis was also carried out for these regions. Interestingly, the results of qPCR revealed that all seven patients had a deletion for the region including rs2241526 but for rs2840128 amplification process was not efficient enough to carry out an absolute quantification analysis therefore the results were inconclusive. For this reason another SNP (rs6729914) which resides in 5kb away from 3' of rs7588807 was studied and found to have no deleted allele for seven patients. In order to capture the boundary of the deletion two more SNPs (rs1983211 and rs3770234) in the 5' of rs7588807 were evaluated and found to have deletions for rs1983211 but not for rs3770234 regions. Figure 6.2 shows the positions of these SNPs and the genes that they cover. The region between rs1983211

and rs3770234 was 29.6 kb thus need to be further analysis to figure out the exact boundary of the deletion. Two more SNPs (rs6436154 and rs22766443) between these two SNPs were also subject to qPCR analysis but amplification efficiency was also not inadequate. For patient 7GE43 on the other hand rs3770234 is also deleted but further analysis was not carried out to find a boundary.

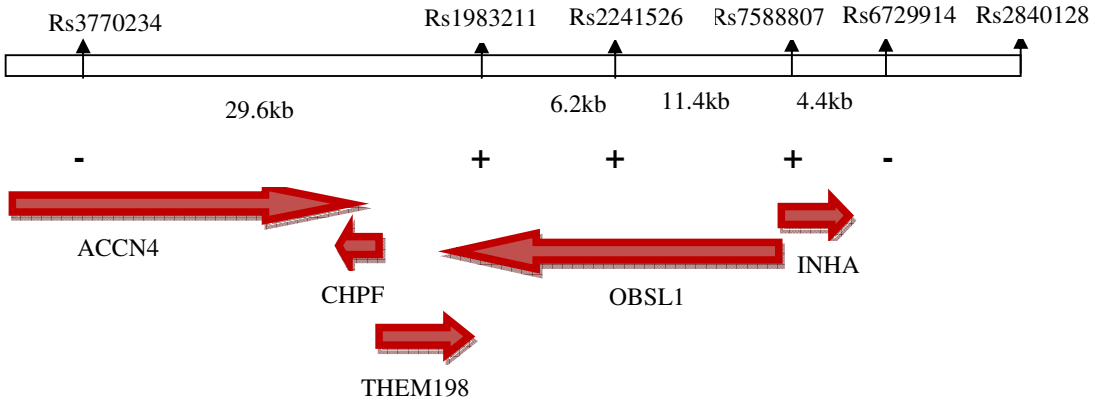


Figure 6.2. Positions of the SNPs and the genes in deleted region
(+ indicates a deletion while – indicates no deletion)

In conclusion it was shown that six JAE patients had large deletion compassing ~30-50kb and one patient even a larger deletion supported by qPCR. The deletion causes the loss of one allele of at least four genes, ACCN4 (ion channel), CHPF, OBSL-1, INHA and THEM198. Chondroitin polymerizing factor (CHPF) is expressed through out the nervous system and is known to have a role as a specific activator for chondroitin synthase for polymerization of chonroitin sulfate proteoglycans which have important roles in neuronal network formation in developing brain (Kitagawa *et al*., 2003). This is remarkable as epileptic seizures have a temporal expression at certain ages. Amiloride sensitive cation channel (ACCN4) is also notable as this protein reduces the membrane expression of another amiloride sensitive channel 1 (ACCN1a) which was shown to be involved in epileptic seizures (Donier *et al*., 2008; Zha *et al*., 2006). Therefore, deletion of one allele of ACCN4 would lead to less functional protein and higher amounts of ACCN1a that

would generate epileptic activity. THEM198 on the other hand is known to have transmembrane segments and have expression in brain but the function is unknown.

Recent studies show that large deletions identified in epilepsy patients are associated with mental retardations, schizophrenia and other genetic disorders (Helbig *et al*., 2009; Sharp *et al*., 2008). When the clinical features of our patients with deletion are considered as shown in Table 6.4, they do not show pure absence epilepsy, but six of them have also GTCS, four of them have myoclonic seizures and two of them have photosensitive seizures. The frequency of 15q13.3 deletion is about 1 per cent, however, seven patients in the INHA gene among 104 JAE+GTCS patients gives a frequency of 7 per cent. In addition as only 36 of the our total absence patients have myoclonic seizures, having this seizure in four of the seven patients with deletion indicate a significant difference (p-value=0.008). In order to evaluate whether this deletion was CNV or pathological alteration, five of the control samples who are homozygous for the common SNPs in the INHA gene and healthy siblings of the patients were also analyzed by qPCR. These samples were shown to have two alleles for the region including rs7588807 except the healthy sibling of 31AE99 as the mean value for the normalization result was 0.7 indicating a possible deletion. However, as shown in Table 6.4 the age of onset of patient 31AE99 is 26 therefore the unaffected sibling may have seizures in the years to come as she is 25 years old now. As one of the siblings may carry this deletion, this variation may not be a de novo mutation but the patients may have inherited from one of the parents. In order to asses this question DNA samples of parents should also be analyzed.

Interestingly, four of the patients with the large deletion have “GTGT” haplotype for the four common SNPs in INHA gene and two of them have “GCGC” and one of them has “ACTC” while five healthy control samples have “ACTC” haplotype. Therefore, the initial association of G allele of rs7588807 should be due to the deletion of “ACTC” haplotype in patients. Moreover, all of the seven patients have “T” allele and three of the five healthy controls have “A” allele for rs2840128 that is the other associated SNP in initial analysis.

Table 6.4. Clinical features of patients with deletion at 2q36

Hasta Nosu	Date of Birth	Sex	Syndrome	Seizures	Age of onset	Family story	Additional
12AE37	1978	M	JAE	Absence, myoclonic, GTCS	19		
24AE79	1981	F	JAE	Absence, GTCS	11	Sister and her son have FC	
25AE82	1987	F	JAE	Absence, myoclonic, GTCS, Photosensitivity	10		
28AE93	1992	F	JAE	Absence, myoclonic, Photosensitivity	12		
31AE99	1970	F	JME	Absence, myoclonic, GTCS	26	Daughter has GTCS	
52AE165	1990	F	JAE	Absence, GTCS, FC	11		Temporal Epileptic activity in EEG
7GE43	1988	F	JAE	Absence, myoclonic, GTCS	11	Brother is JME, another brother has pathologic EEG	

(GTCS: Generalized tonic clonic seizures, FC: Febrile convulsion, JME: juvenile myoclonic epilepsy)

6.5. The Possible Role of Inhibin Protein in Epileptic Seizures

There are some possible mechanisms by which inhibin alpha protein could lead to decreased seizure susceptibility in absence patients. First of all inhibin proteins are expressed in diverse tissues mostly in ovaries, testis, kidney and brain. The wellknown function of inhibins in the reproductive endocrine system is to inhibit the secretion of follicle stimulating hormone (FSH) from the pituitary gland. Inhibins act by antagonizing the function of activin which is the activator of FSH by competing with the activin type II receptors. As shown in Figure 6.3 they interact with betaglycan which is a cell surface proteoglycan that acts as a co-receptor to bind to type II receptor and blocks the activation of type I receptor that normally starts a signal cascade in the cell upon binding of activin (Harrison *et al*., 2005).

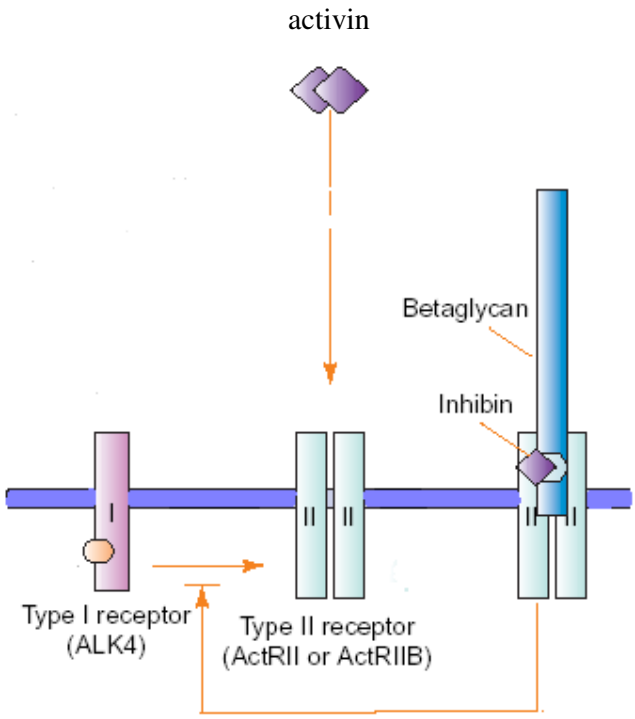


Figure 6.3 Inhibitory effect of inhibins through binding to type II receptor (adapted from Harrison *et al*., 2005)

In the normal cycle FSH is released from the anterior pituitary gland upon activation by activin. Then FSH induces ovary and testis to produce progesterone and estradiol. Progesterone is critical in the possible mechanism of absence epileptic seizures as this steroid hormone was shown to enhance the spike wave discharges through allopregnanolone which is metabolized from progesterone (Luijtelaar *et al*., 2001). Allopregnanolone is a positive modulator of $GABA_A$ receptors thus enhancing SWDs through activation of $GABA_A$ receptors. Therefore, if there is a deficiency as in the case of mutated or deleted inhibin alpha subunit, the amount of inhibin proteins would be lower and activin protein higher thus FSH level would be higher leading to a production of more progesterone and enhanced SWD. As a remarkable point, inhibin A levels in serum is not detectable until the reproductive age approximately 10 years. As the juvenile absence epilepsy has an age of onset after 10 years, this could be a good explanation for the time related expression of the disease. Also the fact that the serum progesterone level of females is always higher than males would explain why the females are more prone to have

absence seizures (Vadakkadath Meethal *et al*., 2005). In our study there were 134 female and 71 male absence patients with a ratio of approximately by 2:1.

The second possible mechanism could be more direct as the inhibin alpha subunit and activin expression and protein localization in different parts of the brain were also shown in previous studies (Fujimura *et al*., 1999; Roberts *et al*., 1996). Transgenic mice with dominant negative type I receptor shows decreased glutamic current and enhanced GABA release and $GABA_B$ receptor activation (Zheng *et al*., 2009; Müller *et al*., 2006). Therefore, decreased level of inhibin alpha subunit would reduce the formation of dimers with β subunits and increase in activin proteins and enhance the excitability effect of activin in brain.

Moreover, in the astrocytes alpha subunits were found to form monomers but the function is unknown (Fujimura *et al.*, 1999). Involvement of astrocytes in absence epilepsy is a new approach as the astrocytes have a function to remove the excitatory glutamate from the synaptic cleft and coverts it to glutamine which is later released back to excitatory neurons. In GAERS an upregulation of transport of the glutamine from astrocytes to neurons were detected in the cortex therefore, this is a proposed mechanism of the absence seizures in GAERS (Melø *et al.*, 2007). The cause of the increased astrocyte metabolism is unclear, but the role of inhibin alpha subunit could be considered.

6.6. Analysis of the GABRG2 Gene in CAE Patients

DNA sequencing of the GABRG2 gene revealed novel variations as shown in Table 6.6. Most of the variations reside in intronic region but splice site analysis pointed out that they do not change the splicing process except the IVS2+6T→G variation. This exhange on the other hand, created a crytic splice site with a higher score compared to the wild type site as calculated by AASP. As shown in Table 6.5 variation causes “gt” donor site. In order to observe the effect of the variation in mRNA, cDNA of GARG2 gene was extracted from blood of a control sample succesfully, unfortunately blood sample of the patient could not be obtained for comparison. Cyrptic splice site causes a premature stop codon at the amino acid 60 thus a truncated protein. The loss of one allele in gamma

subunit may cause a reduced inhibitory action between thalamic reticular nucleus thus they exert longer IPSPs on thalamocortical neurons that leads to activation of $GABA_B$ receptors. The patient shows absence and myoclonic seizures although the previous mutations have been identified with febrile seizures as shown in Table 6.6. Therefore, GABRG2 analysis should be considered for other seziure types.

Interestingly, four of the patients carry the same novel variation although they are not related and also not from the same part of Turkey. However, this variation seems to have no effect on splice site process. When the clinical features of the patients were compared as shown in Table 6.6 one has GTCS, one has myoclonic and two of them have pure absence seizures, thus the only intersection feature is absence seizures. But the variation should be checked in control samples and should be questioned whether this variation is actually in close proximity of the causative mutation which may reside in intronic regions or in the promoter.

Table 6.5. Comparison of the splice site variation (in red) in intron 2

	Intron 2 Donor site	**Score**
Wild type	**ataggag**gtttgttaaagtcttttggttgtgc	5.509
Mutant type	**ataggag**gtttg**g**taaagtcttttgcgttgtgc	6.433

(Exonic sequences are shown in bold)

Exonic variation in patient 136AE356 on the other hand does not change the amino acid but "C" allele seem to add a new binding site for an exonic splice enhacer Srp55. However, this assumption should be evaluated by further experimental data.

Table 6.6. Clinical features of CAE patients wtih variations in the GABRG2 gene

Patient ID	**Date of Birth**	**Sex**	**Syndrome**	**Seizures**	**Age of onset**	**Family story**	**Additional**
18AE63	1992	1	1	Absence	10	-	-
26AE86	1995	1	1	Absence, GTCS	9	Brother has epilepsy	-
33AE106	1998	2	1	Absence, photosensitivity	6	Aunt has epilepsy	-
45AE142	2001	2	1	Absence, myoclonic	3.5	-	-
54AE172	1996	2	1	Absence	4	-	-
86AE272	1995	1	1	Absence	6	-	Also have partial epilepsy
129AE349	2000	2	1	Absence, myoclonic	5	-	-
136AE356	1997	2	1	Absence	8	-	-

(GTCS: Generalized tonic clonic seizures)

7. CONCLUSION

The utilization of haplotybe blocks was a successful approach as the region could be covered with high resolution and a novel susceptibility gene and a deletion covering approximately 30-50 kb could be defined. Deletion of the INHA and additional four genes seems to cause a susceptibility to not only absence seizures but also myoclonic seizures mostly observed in JAE. The deletion should be confirmed by long PCR analysis, southern blotting or FISH techniques and should be screened in other absence patients and more control samples who are also homozygous for the deleted region. Similar genetic analysis of the INHA gene could be carried out or the presence of deletions in rat models could be analyzed. In order to evaluate the possible effect of these variations and deletions mRNA and protein levels of the inhibin alpha subunit could be studied in the thalamocortical region of WAG/Rij rats. In order to assess the indirect effect of inhibin protein, serum inhibin, progesterone, FSH and activin levels can be compared both in patients with deletion and variations and also in WAG/Rij rats with healthy controls.

Large number of mutations have not been identified in the vast majority of investigations in the genes found to be linked or associated with epilepsy phenotype. The frequency of large deletion is about 7 per cent which is the most striking finding higher than any mutation including deletions observed in epilepsy genes, except SCN1A, the most relevant gene for epilepsy phenotype.

The deletion covers other genes that may as well candidates for the epileptic phenotype. This study further opens up an area of molecular genetic analysis of these novel candidate genes. Mutational analysis of INHA gene supports its involvement in the pathogenesis of JAE and the contributing effects of the other genes to the phenotype should be the next question to be answered by a more complete genotyping of the patients with respect to these genes.

The mutations identified in ion channels do not clarify the whole pathogenesis of absence seizures as in our study only one causative mutation could be found in 85 CAE patients. Previously analyzed ion channels for point mutations should be covered for copy

number variations as deletion or duplication of the gene product may lead to exceeding the excitability threshold for seizures. Therefore, novel non-ion channel genes, copy number variations (deletions/duplications) and epigenetic changes should be the new targets for the researches.

APPENDIX A: INFORMED WRITTEN CONSENT

KATILIMCILAR İÇİN BİLGİ ve OLUR FORMU

Proje yürütücüsü: Prof. Dr. S. Hande Çağlayan,
Moleküler Biyoloji ve Genetik Bölümü,
Boğaziçi Üniversitesi, Bebek , 34342 İstanbul
Tel: 212 359 6881 veya 532 652 0461
Faks: 212 287 2468
eposta: hande@boun.edu.tr

Proje başlığı: Tipik absans (bilinç kaybı) nöbetli epilepsi hastalarında 2q36 bölgesini içeren ilişkilendirme çalışması

Proje konusu: Epilepsi gibi karmaşık kalıtım gösteren hastalıklarda, hastalıktan sorumlu birden çok gen bulunmaktadır. Bu çalışma kapsamında tipik absans nöbetli epilepsi hastalarında kromozom 2q36 bölgesine ilişkilendirme çalışması yapılması planlanmakta ve sonucunda absans nöbetlerinden sorumlu bir gen bulunması hedeflenmektedir. Bu projenin gerçekleşmesi için yaklaşık 250 tipik absans nöbetli hasta ve 250 kontrol örneğine ihtiyaç vardır. Tercih edilen anne, baba ve hasta şeklindeki üçlüler olmakla birlikte anne veya baba yoksa veya kan veremiyorsa sadece hastadan kan alınabilir. Bu çalışmaya katılabilecek hastalarda aranan kriterler Türk Epilepsi Derneği Genetik Komisyonu'na katılan nörologlar ile birlikte saptanmıştır. Deneysel çalışmalar Boğaziçi Üniversitesi etik kurulu onayı ile Moleküler Biyoloji ve Genetik bölümünde yapılacaktır.

Onay: Ailenizde görülen kalıtsal epilepsi hastalığı üzerine yapmak istediğimiz genetik araştırmaya katılmaya sizi davet ediyoruz. Bu çalışma kapsamında hastalıktan sorumlu genlerden birini bulmayı umuyoruz.

Araştırmaya katılmayı kabul ettiğiniz takdirde sizlerden, çocuğunuzdan ve ailenizin diğer bireylerinden 10 mililitre kan örneği alacağız. Ayrıca, ekteki formda istenen bilgileride sağlamanızı rica ediyoruz. İsminiz ve bu bilgiler tamamen gizli tutulacaktır.

Çalışmaya katılmanız tamamen isteğe bağlıdır. Sizden ücret talep etmiyoruz ve size herhangi bir ödeme yapmayacağız. Sizden alınan örnek ileride başka çalışmalar için de kullanılabilir. İstediğiniz zaman çalışmaya katılmaktan vazgeçebilirsiniz. Bu durumda sizden almış olduğumuz örnek imha edilecektir.

Yapmak istediğimiz araştırmanın size risk getirmesi beklenmemektedir. Kan aldırmanın genelde hiçbir zararı olmamasına karşın, nadiren ve çok az kanama ve morarmaya yol açabilir. Araştırma sonucunda aranan bilgi elde edildiği takdirde, ailenizin fertlerine genetik danışma hizmeti verebileceğiz. Hastalıktan sorumlu genlerden birini bulursak hastalığın moleküler temeli kısmen aydınlanacaktır. Ama bunun ailenize bir yarar getirip getirmeyeceğini şimdiden söylemek mümkün değildir ve size bu konuda söz veremeyiz. Araştırmanın ileride başka ailelere de yarar sağlaması muhtemeldir. Genetik çalışmalarımızın kalıtsal hastalıkların temelini anlamamıza katkıda bulunarak insan sağlığına yarar sağlamasını beklemekteyiz.

Bu formu imzalamadan önce, çalışmayla ilgili sorularınız varsa lütfen sorun. Daha sonra sorunuz olursa Prof. Dr. Betül Baykan'a (Telefon: 212 414 2000/32393) ya da Prof. Dr. S. Hande Çağlayan'a sorabilirsiniz (Telefon: 212 359 6881). Araştırmayla ilgili haklarınız konusunda Boğaziçi Üniversitesi İnsan Araştırmaları Etik Kuruluna danışabilirsiniz (Telefon: 212 – 359 6472, Prof. Dr. Aslı Tolun, başkan).

Adres ve telefon numaranız değişirse, bize haber vermenizi rica ederiz.

Bu formun bir kopyasını aldım.

Yukarıda yazılanları okudum, anladım ve çalışmaya katılmayı kabul ediyorum.

Tarih:

İsim: İmza:

Hastayla akrabalığı: Tel:

(katılımcının velisi/vasisi) Adres:

Şahit ismi: İmza:

Tel:

Adres:

Klinisyen ismi: İmza:

Tel:

Adres:

APPENDIX B: CLINICAL FORM FOR TYPICAL ABSENCE PATIENTS

TİPİK ABSANS NÖBETLİ EPİLEPSİLER İÇİN HASTA FORMU

ÖNEMLİ NOT:

- Bu çalışmaya absans nöbeti olan hastaların bulunduğu aileler dahil edilecektir.
- Çalışma için gerekli örnekler (önem sırasına göre):
  1- anne-baba- hasta çocuk üçlüsü (ve mümkünse sağlıklı kardeş)
  2- sağlıklı veya hasta diğer aile bireyleri
  3- anne- baba ve aile bireylerine ulaşılamaması durumunda sadece hasta bireyin örneği

Adı Soyadı: **Doğum Tarihi:** **Doğum yeri:** **Cinsiyet:** **Tel:** **Adres:**	**Gönderen Doktor:** **Çalıştığı Kurum:** **Tarih:** **Tel:** **Adres:** **Eposta:**

AİLE BİLGİLERİ

Anne Adı/Doğum Yeri:		**Anne Tarafının Kökeni (Yaşadığı Yöre, Göçmenlik Durmu vs.) :**
Baba Adı/Doğum Yeri:		**Baba Tarafının Kökeni (Yaşadığı Yöre, Göçmenlik Durmu vs.) :**
Akraba Evliliği:	**Var:** ☐	**Akrabalık Derecesi:**
	Yok: ☐	

HASTALIK BİLGİLERİ	
Hastalığın Hikayesi:	
Sendrom Tipi:	
Febril Konvülsiyon:	
Nöbet Tipi:	
Hastalığın Başlama Yaşı:	
Absans Nöbetlerin Süresi/ Sıklığı:	
Diğer nöbetler; JK-miyo ve Başlama Zamanları:	
Aktivasyon Yönteminin Etkisi:	
Fotosensitivite:	
EEG:	
İktal Kayıt Var mı?/ Süresi:	
İnteriktal Kayıt:	
Kullanılan Tedavi ve Tedaviye Verdiği Cevap:	
Mental Durum:	

Aynı veya Benzer Bulgular Başka Aile Bireylerinde Var mı? Kimler?	
Ailede Belirtilmesi Gereken Diğer İlgili Özellikler:	
Alınan Örneklerin Gönderileceği Adres ve Ulaştırma Yöntemi	Prof. Dr. S Hande Çağlayan Moleküler Biyoloji ve Genetik Bölümü Boğaziçi Üniversitesi Etiler, İstanbul Tel: 0212 359 6881 Faks:0212 287 2468 Eposta:hande@boun.edu.tr GSM: 0 532 652 04 61 (Hande Çağlayan) 0 532 346 37 93 (Özlem Yalçın) Ulaşım ödemeli olarak yurtiçi kargo ile yapılabilir. Yurtiçi Kargo çağrı merkezi : 444 9999

DNA Örneği Alınan Aile Bireyleri:

	Adı Soyadı	**Doğum Yılı**	**Akrabalık Derecesi**	**Mevcut Genetik/ Kronik Hastalığı**
1				
2				
3				
4				
5				

AİLE AĞACI:

APPENDIX C: PATIENT PHENOTYPE CHART

Table C.1. Detailed clinical informations for absence patients

		1: Female									1:typic	
		2:Male	**Syndrome**			**seizures**					2:atypic	
Patient ID	**Date of Birth**	**Sex**	**JAE**	**EMA**	**CAE**	**A**	**M**	**G**	**P**	**FC**	**EEG**	**Age of onset**
1AE1	1983	1	0	1	0	1	0	1	1	0	2	10
2AE5	1996	2	0	0	1	1	0	0	1	1	2	8
3AE8	1991	2	0	0	1	1	0	1	1	0	2	11
4AE12	1981	2	1	0	0	1	0	1	0	0	1	14
5AE15	1985	1	1	0	0	1	0	1	1	0	1	11
5AE16	1987	1	1	0	0	1	0	0	1	0	1	15
6AE19	1981	1	0	1	0	1	0	1	1	0	?	8
7AE23	1985	1	0	0	1	1	0	1	0	0	1	7
8AE26	1984	1	1	0	0	1	0	1	0	0	1	14
9AE29	1996	2	0	0	1	1	0	0	0	0	1	8
10AE32	1989	1	1	0	0	1	1	1	1	0	2	11
11AE35	1991	1	?	?	?	1	0	1	1	0	2	9
12AE37	1978	2	1	0	0	1	1	1	0	0	?	19
13AE40	1991	1	0	1	0	1	0	0	0	0	?	3
14AE44	1998	2	0	0	1	1	0	0	0	0	1	5
15AE49	1998	1	0	1	0	1	0	0	0	0	1	6
16AE53	1993	2	0	0	1	1	0	0	1	1	?	3
16AE54	1997	2	0	0	1	1	0	0	0	0	?	3
17AE59	1997	1	0	0	1	1	0	0	1	0	?	5
18AE63	1992	1	0	0	1	1	0	0	0	0	1	10
19AE67	1977	1	1	0	0	1	0	1	0	0	?	12
20AE68	1985	1	0	0	1	1	1	1	1	1	?	7
21AE69	1996	1	1	0	0	1	0	1	0	0	1	20
22AE71	1986	2	0	0	1	1	1	0	1	1	?	5
23AE75	1988	1	?	?	?	1	1	1	1	0	?	15
24AE79	1981	1	1	0	0	1	0	1	0	0	?	11
25AE82	1987	1	1	0	0	1	1	1	1	0	?	10
26AE85	1989	2	0	1	0	1	0	1	1	0	1	10
26AE86	1995	1	0	0	1	1	0	1	0	0	2	9
27AE89	2000	1	0	0	1	1	0	0	?	0	1	4,5
28AE93	1992	1	1	0	0	1	1	0	1	0	1	12
29AE94	1994	2	0	0	1	1	0	0	1	0	1	4,5
30AE95	1994	1	0	0	1	1	0	0	1	0	1	11
31AE99	1970	1	0	0	0	1	1	1	0	0	1	26
32AE103	2001	2	0	0	1	1	0	0	0	0	1	7
33AE106	1998	2	0	0	1	1	0	0	1	0	1	6
34AE109	1998	2	0	0	1	1	0	0	1	1	1	4
34AE110												

(JAE: juvenile absence epilepsy, CAE: childhood absence epilepsy, EMA: Eyelid myoclonia with absence epilepsy, A: Absence, M: Myoclonic, G: Generalized Tonic Clonic Seizure, P: Photosensitive, FC: Febril Convulsion)

Table C.1. Detailed clinical informations for absence patients (continued)

		1: Female									1:typic	
		2:Male	Syndrome			Seizures					2:atypic	
Patient ID	**Date of Birth**	**Sex**	**JAE**	**EMA**	**CAE**	**A**	**M**	**G**	**P**	**FC**	**EEG**	**Age of onset**
35AE113	1997	1	0	0	1	1	0	0	0	0	2	5,5
36AE114	1994	1	0	0	1	1	0	0	0	0	1	6
37AE117	1989	1	1	0	0	1	0	1	0	1	1	11
38AE120	1989	2	0	0	1	1	0	0	1	0	1	7
39AE123	1984	1	?	?	?	1	1	1	1	0	1	11
39AE124	1984	1	?	?	?	1	1	1	1	0	1	11
40AE125	1990	1	0	0	1	1	0	0	1	0	1	12
41AE126	1979	1	1	0	1	1	0	1	1	0	1	13
41AE127	1984	1	1	0	0	1	0	1	0	0	1	15
42AE131	1985	1	0	0	1	1	0	0	1	0	1	11
43AE135	1990	1	?	?	?	1	0	0	0	0	?	3
44AE138	1995	1	0	0	1	1	0	0	0	0	?	7
45AE142	2001	2	0	0	1	1	1	0	0	0	1	3,5
46AE145	1999	2	0	0	1	1	0	0	0	0	2	7
47AE149	1997	1	0	0	1	1	0	0	0	0	?	4
48AE154	1992	2	0	0	1	1	0	0	0	0	2	5
49AE157	1993	1	0	0	1	1	0	0	0	0	1?	10
50AE160	1992	2	0	0	1	1	0	0	1	1	1	5
51AE163	1994	2	0	0	1	1	0	0	0	0	1	6
52AE165	1990	1	?	?	?	1	0	1	0	1	2	11
53AE167	1993	1	0	0	1	1	0	0	1	1	1	7
54AE172	1996	2	0	0	1	1	0	0	0	0	?	4
55AE176	1993	2	0	0	1	1	0	0	0	0	2	10
56AE179	1992	1	0	0	1	1	0	1	0	0	1	8
57AE181	2001	2	0	0	1	1	1	0	0	0	2	5,5
58AE184	1992	1	0	0	1	1	0	0	0	1	1	8
59AE188	1993	1	1	0	0	1	0	0	?	?	?	113
60AE189	1980	1	1	0	0	1	0	0	0	0	1	12
61AE193	2001	1	0	0	1	1	0	0	0	0	1	4
62AE198	1991	1	0	0	1	1	0	1	0	1	1	7
63AE201	1993	1	0	0	1	1	0	0	0	0	1	6
64AE204	1997	2	0	0	1	1	1	0	0	0	1	8
65AE207	1994	1	0	0	1	1	0	0	1	0	1	11
66AE212	1994	2	0	0	1	1	0	0	0	0	1	10,5
67AE213	1993	2	0	1?	1?	1	0	0	0	0	2	4 month
68AE217	1989	2	0	0	1	1	0	1	0	0	2	11
69AE219	1994	2	0	0	1	1	0	1	0	0	1	10
70AE224	1982	1	0	0	0	1	1	1	0	0	?	18
71AE225	1988	1	1	0	0	1	1	1	1?	0	1	13
72AE229	1989	2	1	0	0	1	0	1	1	0	1	13
73AE233	1990	1	0	0	1	1	0	0	0	1	1	10
74AE237	1984	1	1	0	0	1	0	1	1	0	1	13
75AE244	1993	1	0	0	1	1	0	0	0	1	1	5
76AE245	1998	1	0	0	1	1	0	0	1	1	1	7
77AE246	2000	1	0	0	1	1	0	0	0	0	1	4
78AE247	1992	1	1	0	0	1	0	1	1	0	1	12
79AE248	1984	2	0	0	1	1	0	0	0	0	1	7
80AE252	1999	2	0	0	1	1	0	0	?	?	1	2,5

(JAE: juvenile absence epilepsy, CAE: childhood absence epilepsy, EMA: Eyelid myoclonia with absence epilepsy, A: Absence, M: Myoclonic, G: Generalized Tonic Clonic Seizure, P: Photosensitive, FC: Febril Convulsion)

Table C.1. Detailed clinical informations for absence patients (continued)

		1: Female									1:typic	
		2:Male	Syndrome			Seizures					2:atypic	
Patient ID	**Date of Birth**	**Sex**	**JAE**	**EMA**	**CAE**	**A**	**M**	**G**	**P**	**FC**	**EEG**	**Age of onset**
81AE255	2000	1	0	0	1	1	0	0	0	0	1	4
82AE259	1999	1	0	0	1	1	0	0	1	1	1	5,5
83AE262	1997	1	0	0	1	1	0	1	1	0	1	8
84AE266	1998	1	0	0	1	1	0	0	1	?	1	3
85AE268	1994	2	1	0	0	1	0	0	0	0	1	12
86AE272	1995	1	0	0	1	1	0	0	0	?	1	6
87AE275	1998	1	0	0	1	1	0	0	?	?	1	7
88AE278	1993	1	1	0	0	1	0	0	0	1	1	11,5
89AE279	1995	2	0	0	1	1	0	0	0	?	1	6
90AE284	1996	1	?	0	?	1	0	0	?	?	1	9
91AE285	1999	1	0	0	1	1	0	0	?	?	1	7
92AE286	1994	1	1	0	0	1	0	0	0	0	1	11
93AE287	1997	2	0	1	0	1	0	0	0	0	1	3
94AE288	1996	1	0	0	1	1	0	0	0	1	1	4
95AE289	-											
96AE290	1997	1	0	0	1	1	0	0	0	1	1	7
97AE294	1988	1	1	0	0	1	0	1	1	0	1	10
98AE296	1985	2	?	?	?	1	1	1	1	0	?	18
99AE298	1993	2	0	1	0	1	1	0	1	0	1	8
100AE301	1996	1	0	0	1	1	0	0	0	1	1	8
101AE305	1998	1	0	0	1	1	0	0	0	0	2	7
102AE308	2000	1	0	0	1	1	0	0	1	0	1	3
103AE309	-											
104AE310	1996	2	0	0	1	1	0	0	1	0	1	4
104AE311												
105AE315	1994	2	0	0	1	1	0	0	0	0	1	10
106AE316	1996	1	0	0	1	1	0	0	0	0	1	10,5
107AE317	1991	2	1	0	0	1	0	1	?	1	1	14
108AE318	1997	1	0	0	1	1	0	0	0	0	1	10
109AE319	1994	1	0	0	1	1	0	0	0	0	1	11
110Ae332	1995	1	0	0	1	1	0	0	?	?	?	9,5
111AE323	2001	2	0	0	1	1	0	0	0	0	1	4
112AE324	1999	2	0	0	1	1	0	0	0	0	1	5
113AE325	1987	1	1	0	0	1	0	0	1	0	1	12
114AE 326	1997	2	0	0	1	1	0	0	0	0	1	7
115AE327	1995	1	0	0	1	1	0	0	0	?	?	5
116AE328	1991	1	0	0	1	1	0	1	1	1	1	9
117AE329	1994	2	0	0	1	1	0	1	0	1	1	9
118AE332	1996	1	0	0	1	1	0	0	0	0	1	11
119AE336	1995	1	0	0	1	1	0	0	0	0	1	10
120AE337	1976	1				1	1	0	?	0	1	17
120AE338	-											
121AE341	1999	2	0	0	1	1	0	0	0	0	1	6,5
122AE342	1989	1	1	0	0	1	0	0	0	0	1	12
123AE343	1996	1	0	0	1	1	0	0	0	0	1	7
124AE344	1998					1						
125AE345	1976	1	1	0	0	1	0	1	0	0	1	14

(JAE: juvenile absence epilepsy, CAE: childhood absence epilepsy, EMA: Eyelid myoclonia with absence epilepsy, A: Absence, M: Myoclonic, G: Generalized Tonic Clonic Seizure, P: Photosensitive, FC: Febril Convulsion)

Table C.1. Detailed clinical informations for absence patients (continued)

		1: Female									1:typic	
		2:Male	Syndrome			Seizures					2:atypic	
Patient ID	**Date of Birth**	**Sex**	**JAE**	**EMA**	**CAE**	**A**	**M**	**G**	**P**	**FC**	**EEG**	**Age of onset**
126AE346	1971	1	1	0	0	1	1	0	0	0	1	15
127AE347	2000	1	0	0	1	1	0	0	0	0	1	3
128AE348	2004	2	0	0	1	1	0	0	0	1	1	?
129AE349	2000	2	0	0	1	1	1	0	0	0	1	5
130AE350	1996	1	0	0	1	1	0	0	0	0	1	7
131AE351	1999	1	?	?	?	1	0	1	0	0	?	6
132AE352	1995	1	1	0	0	1	0	1	0	0	1	8
133AE353	1998	2	0	0	1	1	0	0	0	0	1	7
134AE354	1996	2	?	?	?	1	0	0	0	1	1	11
135AE355	1995	1	0	0	1	1	0	0	0	1	1	7
136AE356	1997	2	0	0	1	1	0	0	0	0	1	8
137AE357	1997	1	1	0	0	1	0	1	1	0	1	8,5
138AE358	1988	1	?	?	?	1	1	1	0	0	1	16
139AE361	1994	1	0	0	1	1	0	0	0	0	1	10
140AE362	1992	2	0	0	1	1	0	0	0	1	1	8
141AE363	1988	2	0	0	1	1	0	0	0	1	1	8,5
142AE364	1997	1	0	0	0	1	0	0	1	0	1	7
143AE367	1994	1	?	?	?	1	1	1	1	0	1	9
144AE370	1984	1	1	0	0	1	0	1	1	0	1	16
145AE371	-											
146AE372	-											
147AE373	2002	1	0	0	1	1	0	0	0	0	1	5
148AE374	1983	2	0	0	0	1	1	1	0	0	1	15
149AE377	1984	2	1	0	0	1	0	1	0	0	1	12
150AE378	1981	1	1	0	0	1	0	1	0	0	1	16
151AE379	1992	1	1	0	0	1	0	1	1	1	1	7
152AE380	1985	1	1	0	0	1	0	1	0	0	1	16
153AE383	1998	2	0	1	0	1	0	1	1	0	1	15
154AE386	1993	2	0	0	0	1	1	1	1	0	1	11
155AE387	1997	1	0	0	1	1	0	1	0	0	1	3
156AE388	1988	2	1	0	0	1		1		1		15
157AE389	2001	1	0	0	1	1	0	0	0	0	1	6,5
158AE390	2001	1	0	0	1	1	0	0	0	0	1	5
159AE391	1980	2	0	0	0	1	1	1	0	0	1	14
159AE392	1983	2	0	0	0	1	1	1	0	0	?	17
160AE395	2000	1	0	0	1	1	0	0	1	0	1	6.5
161AE396	1993	1	1?	0	1?	1	0	0	0	0	1	11
162AE397	1994	2	0	0	1	1	0	0	0	?	1	8
163AE398	1998	1	0	0	1	1	0	0	0	0	1	9
164AE399	1989	2	0	0	0	1	1	1	0	0	1	15
165AE400	2000	2	0	0	1	1	0	0	0	0	2	3
166AE401	1984	1	0	0	0	1	0	1	1	0	?	14
167AE402	2001	1	0	0	1	1	0	0	0	0	1	5
168AE403	1995	1	0	0	1	1	0	0	0	0	1	6
169AE404	1970	1	?	?	?	1	1	1	1	0	1	6
170AE405	1996	2	0	0	1	1	0	0	0	0	1	6

(JAE: juvenile absence epilepsy, CAE: childhood absence epilepsy, EMA: Eyelid myoclonia with absence epilepsy, A: Absence, M: Myoclonic, G: Generalized Tonic Clonic Seizure, P: Photosensitive, FC: Febril Convulsion)

Table C.1. Detailed clinical informations for absence patients (continued)

		1: Female									1:typic	
		2:Male	Syndrome			Seizures					2:atypic	
Patient ID	**Date of Birth**	**Sex**	**JAE**	**EMA**	**CAE**	**A**	**M**	**G**	**P**	**FC**	**EEG**	**Age of onset**
1GE3	-	2	0	0	1							
4GE31	1980	1	1	0	0	1	0	0	0	0	1	13
7GE43	1988	1	1	0	0	1	1	1	0	0	1	11
03-47	1987	1	1	0	0	1	0	0	0	1	2	13
03-113	1984	1	0	0	1	1	0	1	0	0	1	6
03-150	1989	1	0	0	1	1	0	0	0	0	1	7
03-300	1927	1	0	0	0	1	1	1	0	?	1	16
03-1176	1973	2	0	0	0	1	0	1	0	0	1	22
03-1241	1983	1	0	0	1	1	0	0	0	0	1	9
04-70	1987	1	0	0	1	1	0	0	1	1	1	?
04-73	1985	1	0	0	0	1	0	1	1	0	2	15
04-47	1969	2	0	0	0	1	0	1	1	0	1	10
04-48	1944	1	1	0	0	1	0	1	1	1	1	9
04-113	1986	2	1	0	0	1	0	1	0	1	1	17
04-273	1977	1	1	0	0	1	1	1	0	1	2	14
04-347	1982	2	1	0	0	1	0	1	0	0	1	14
04-648	1988	1	0	0	0	1	1	0	0	0	1	14
04-1034	1990	2	1	0	0	1	0	1	0	0	1	11
04-1035	1977	1	0	0	0	1	0	1	0	0	1	7
E06-15	1988	1	1	0	0	1	0	1	0	0	1	14
E06-54	1989	1	1	0	0	1	0	1	0	0	1	13
E06-104	1973	2	0	1	0	1	0	1	0	1	1	9
4403	1988	1	0	0	1	1	0	1	1	0	1	8
4455	1976	1	0	0	0	1	0	1	0	0	2	20
4410	1986	1	1	0	0	1	0	1	0	0	1	12
E06-43	1986	1	1	0	0	1	0	1	1	0	1	17
E05-27	1985	1	0	0	1	1	0	0	0	0	1	7
E06-27	1995	1	0	0	1	1	0	0	0	0	1	5
E06-31	1986	2	0	0	0	1	1	1	0	0	1	10
07-01A	1978	1	1	0	0	1			1			22
07-10A	1992	2	1	0	0	1	1	0	0	0	1	11
07-12A	1971	2	0	0	0	1	0	1	0	0	1	22
07-13A	1995	2	0	0	1	1	0	0	0	0	1	10
07-14A	1988	1	0	0	1	1	0	0	0	0	?	5
07-15A	1980	1	0	0	0	1			0	0		14
07-16A	1990	2	1	0	0	1	0		0	0	1	11
07-17A	1969	1	1	0	0	1	0	1	0	0	1	15
07-18A	1952	1	1	0	0	1	0	1	0	1	1	17
07-66A	1996	1	0	0	1	1	0	0	0	0		9
07-72A	1976	1	0	0	0	1	0	1	0	0	1	9
07-74A	1984	2	0	0	0	1	1		1	0		16
07-75A	1985	1				1			1	0		11
07-77A	1990	1	1	0	0	1	0		0	0	1	14
07-80A	?											
07-83A	1994	1	1	0	0	1	0	0	0	0	2	12

(JAE: juvenile absence epilepsy, CAE: childhood absence epilepsy, EMA: Eyelid myoclonia with absence epilepsy, A: Absence, M: Myoclonic, G: Generalized Tonic Clonic Seizure, P: Photosensitive, FC: Febril Convulsion)

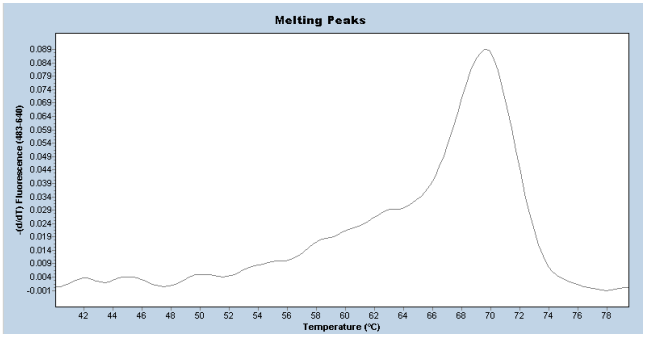
Table C.1. Detailed clinical informations for absence patients (continued)

		1: Female									1:typic	
		2:Male	Syndrome			Seizures					2:atypic	
Patient ID	**Date of Birth**	**Sex**	**JAE**	**EMA**	**CAE**	**A**	**M**	**G**	**P**	**FC**	**EEG**	**Age of onset**
07-84A	?											
07-88A	?											
07-89A	1992	1	0	0	1	1	0	0	0	0	1	8
07-105A	1985	2	0	0	0	1	1	1	0	0	1	14
07-115A	1993	1	0	0	0	1	1	0	0	0	1	4
07-118A	1981	1	0	0	0	1	1	1	0	?	1	16
07-123A	1983	1	0	0	0	1	1	0	1	0	1	14
07-128A	1961	2	0	0	0	1	0	1	1	1	1	13
06-106	1977	1	0	0	0	1	1	1	1	0	1	17
06-120	2002	1	?	?	?	?	?					
06-121	1995	2	0	0	1	1	0	0	0	0	1	10
02-589	1977	1	0	0	0	1	1	1	0	0	2	16
4423	1963	2	0	0	0	1	1	1	0	1?	1	16
07-19A	1997	2	0	0	1	1	0	1	0	0	1	6
04-235	1971	1	1	0	0	1	0	1	0	1	2	12
02-796	1958	1	1	0	0	1	0	1	0	0	1	13
07-20A	1989	1	0	0	0	1	1	1	0	0	1	15
07-187A	1947	1	?	?	?	1	0	0	1	0	1	13
07-198A	1979	1	1	0	0	1	0	0	1	1	1	10

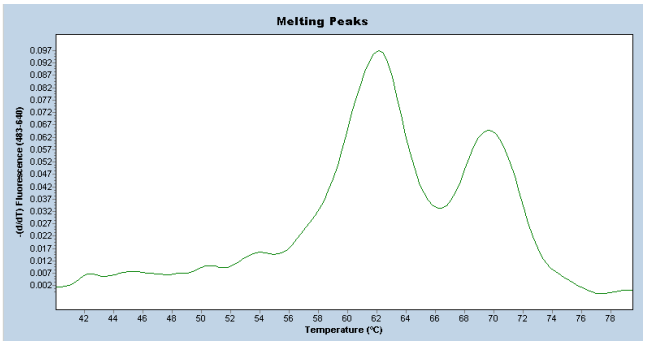
(JAE: juvenile absence epilepsy, CAE: childhood absence epilepsy, EMA: Eyelid myoclonia with absence epilepsy, A: Absence, M: Myoclonic, G: Generalized Tonic Clonic Seizure, P: Photosensitive, FC: Febril Convulsion)

APPENDIX D: PROBE OPTIMIZATIONS

a) Homozygous wild type (C/C)



b) Heterozygous (C/T)



c) Homozygous mutant (T/T)

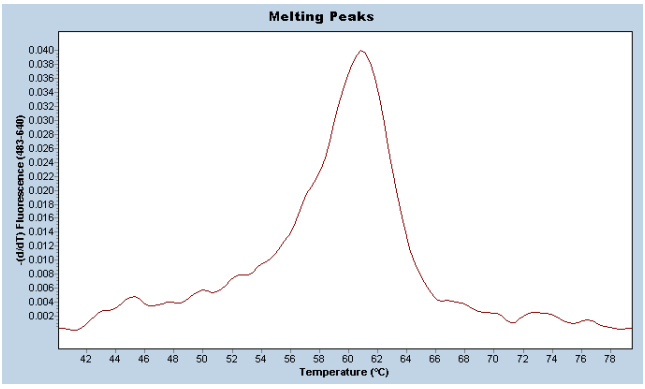
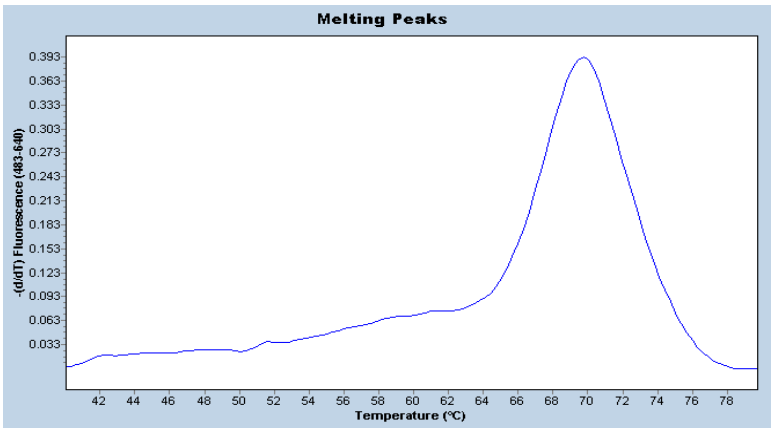
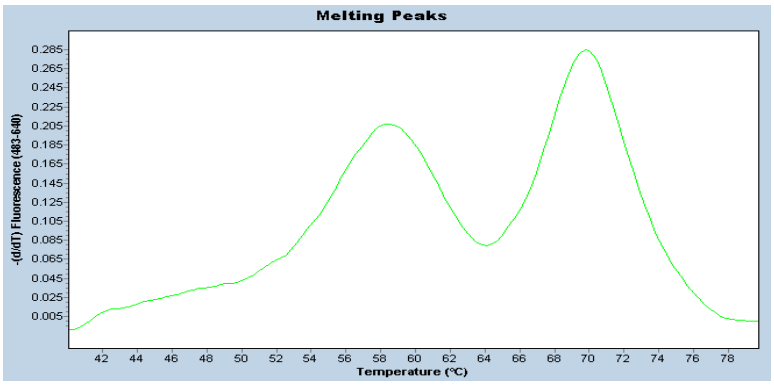


Figure D.1. Melting peaks observed for rs1397429 a) Homozygous wild type genotype (69^0C), b) heterozygous genotype (62^0C ve 69^0C) and c) homozygous mutant genotype (52^0C).

a) Homozygous wild type (T/T)



b) Heterozygous (C/T)



c) Homozygous mutant (C/C)

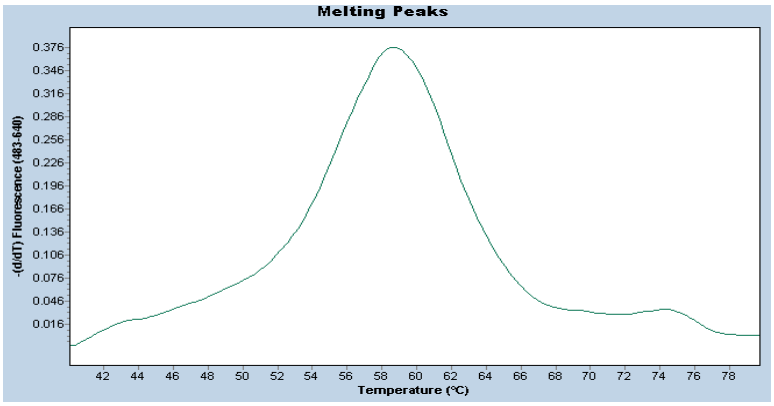
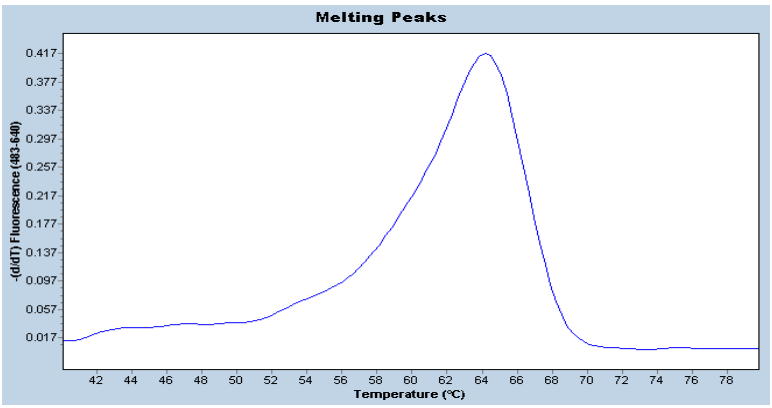
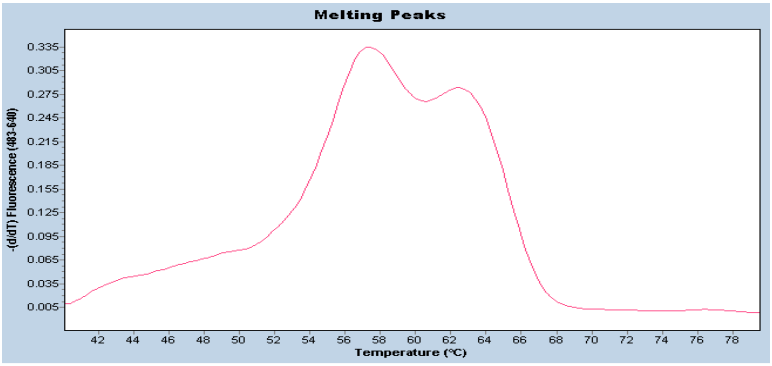


Figure D.2. Melting peaks observed for rs2010592 a) Homozygous wild type genotype (70^0C), b) heterozygous genotype (58.5^0C ve 70^0C) and c) homozygous mutant genotype (58.5^0C).

a) Homozygous wild type (T/T)



b) Heterozygous (C/T)



c) Homozygous mutant (C/C)

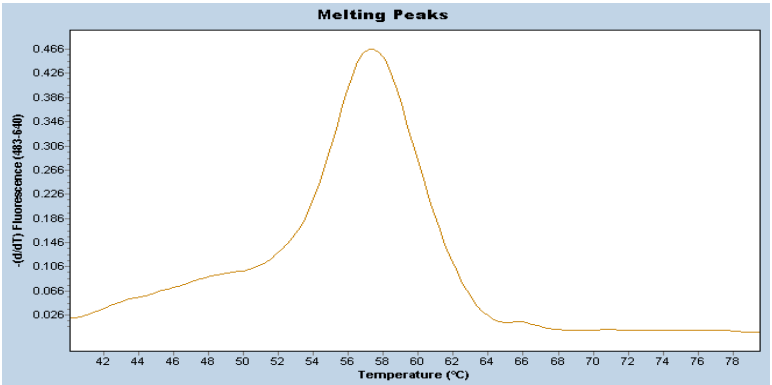
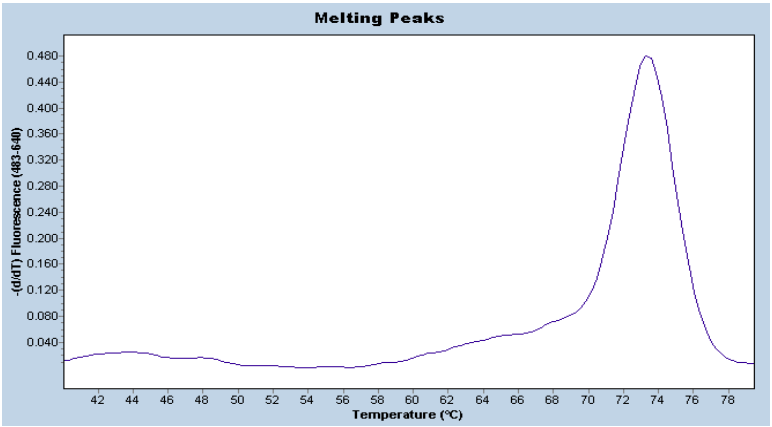
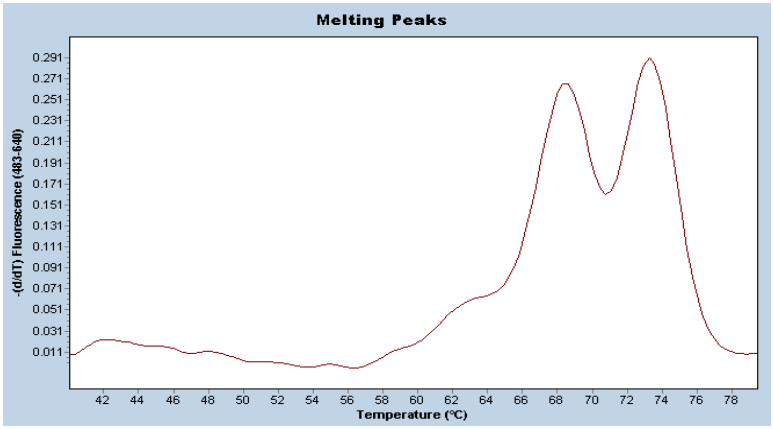


Figure D.3. Melting peaks observed for rs3770234 a) Homozygous wild type genotype (63^0C), b) heterozygous genotype (57.5^0C ve 63^0C) and c) homozygous mutant genotype (57.5^0C).

a) Homozygous wild type (A/A)



b) Heterozygous (A/G)



c) Homozygous mutant type (G/G)

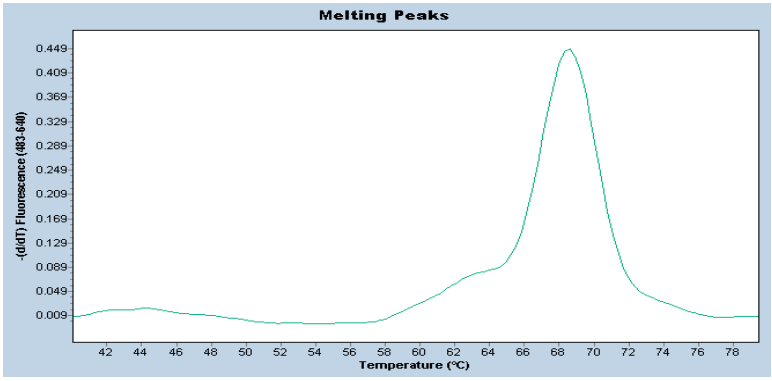
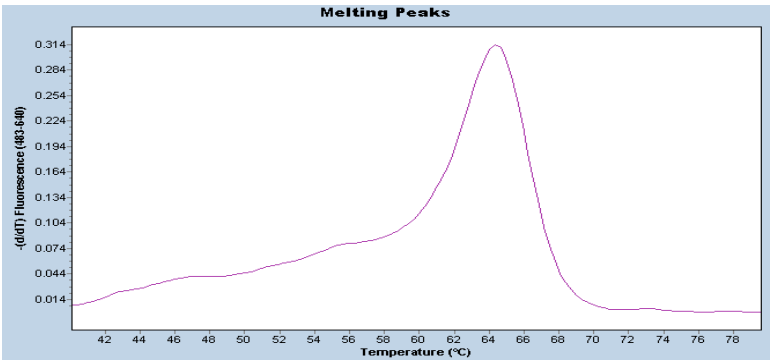
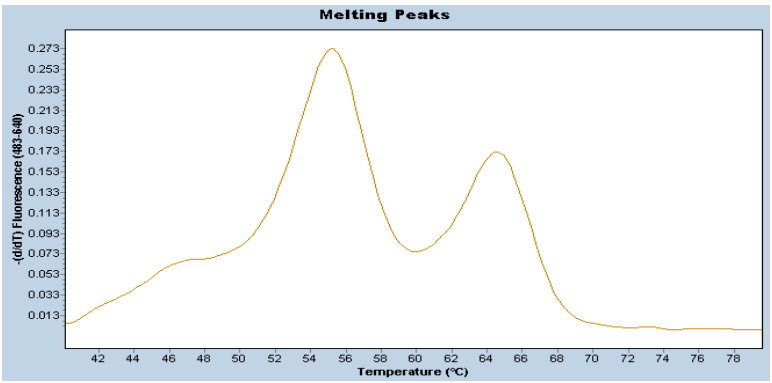


Figure D.4. Melting peaks observed for rs6436154 a) Homozygous wild type genotype (73^0C), b) heterozygous genotype (68^0C ve 73^0C) and c) homozygous mutant genotype (68^0C).

a) Homozygous wild type (C/C)



b) Heterozygous (C/T)



d) Homozygous mutant type (T/T)

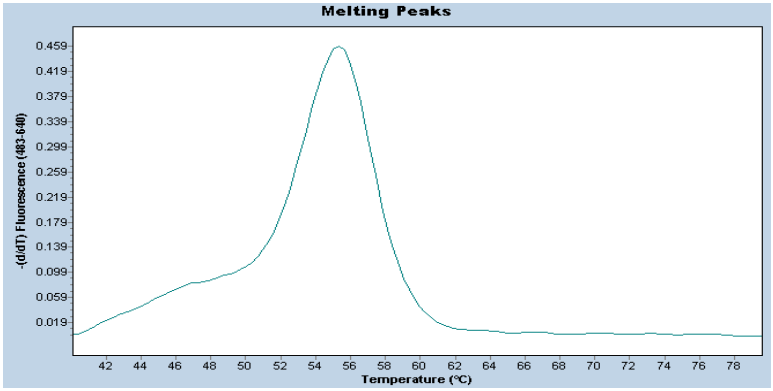
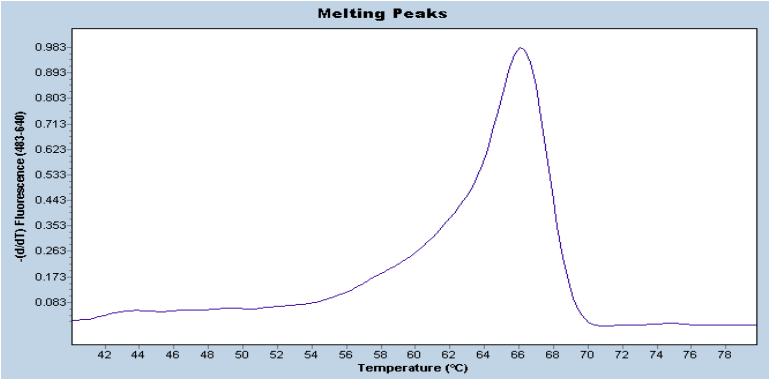
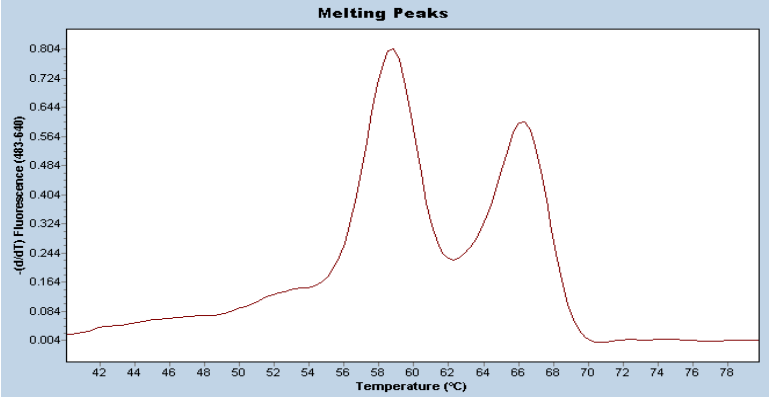


Figure D.5. Melting peaks observed for rs2241526 a) Homozygous wild type genotype (64^0C), b) heterozygous genotype (55^0C ve 64^0C) and c) homozygous mutant genotype (55^0C).

a) Homozygous wild type (G/G)



b) Heterozygous(G/T)



c) Homozygous mutant type (T/T)

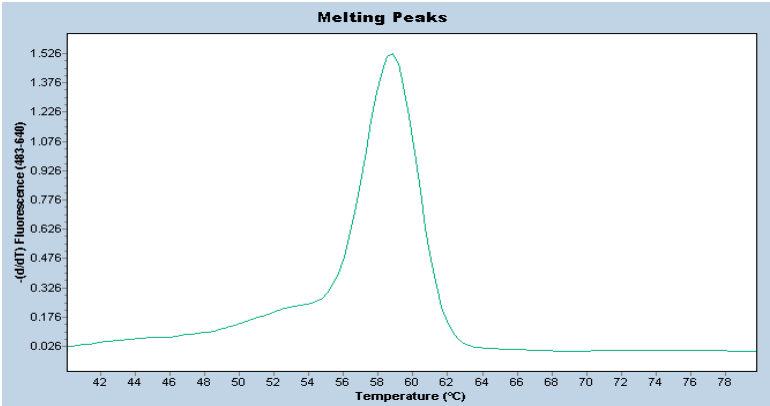
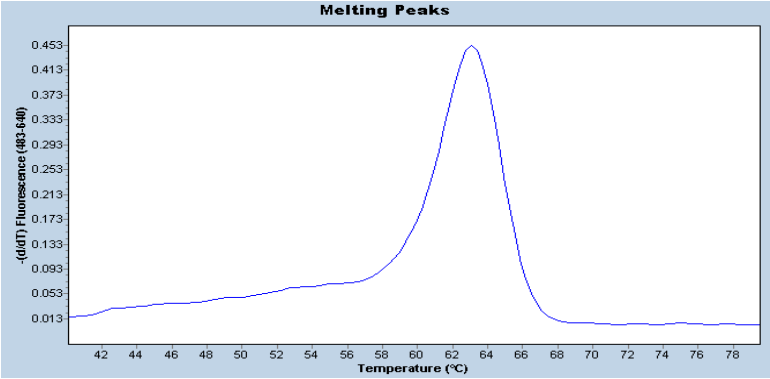
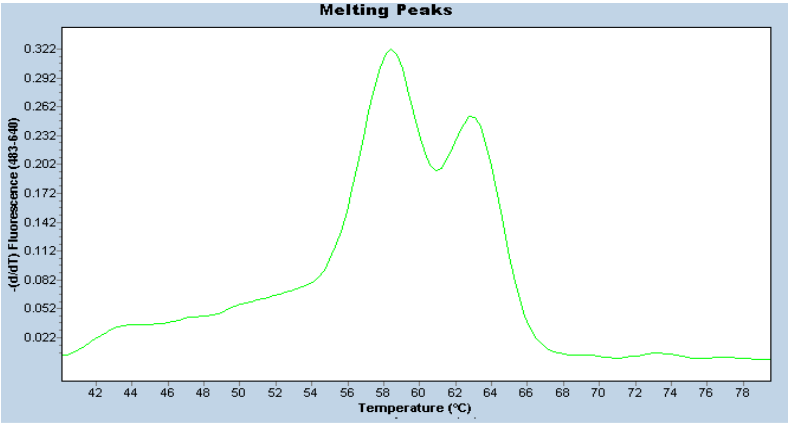


Figure D. 6. Melting peaks observed for rs7588807 a) Homozygous wild type genotype (66^0C), b) heterozygous genotype (59^0C ve 66^0C) and c) homozygous mutant genotype (59^0C)

a) Homozygous wild type (A/A)



b) Heterozygous(A/T)



c) Homozygous mutant type (T/T)

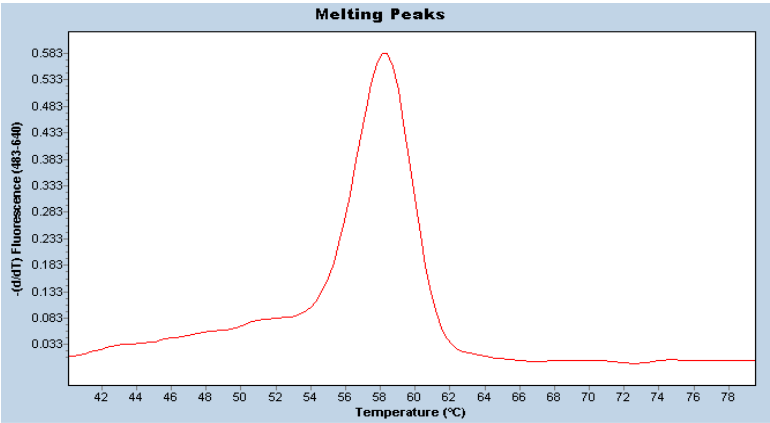
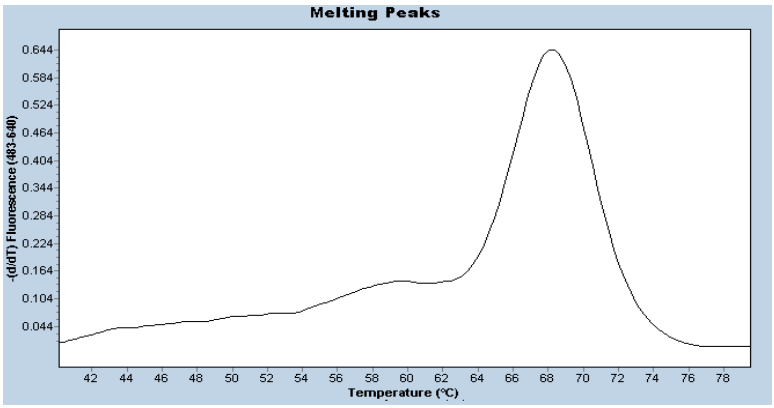
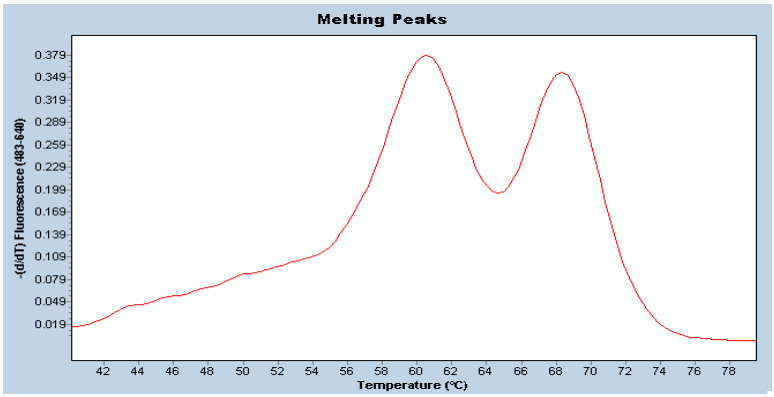


Figure D.7. Melting peaks observed for rs2840128 a) Homozygous wild type genotype (63^0C), b) heterozygous genotype (58^0C ve 63^0C) and c) homozygous mutant genotype (58^0C)

a) Homozygous wild type (T/T)



b) Heterozygous (C/T)



c) Heterozygous mutant type (C/C)

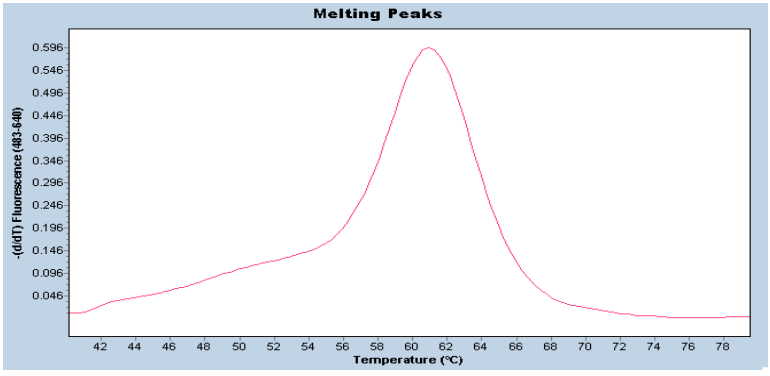
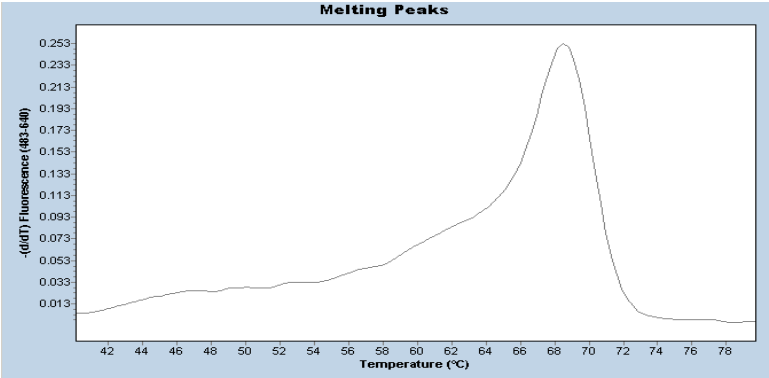
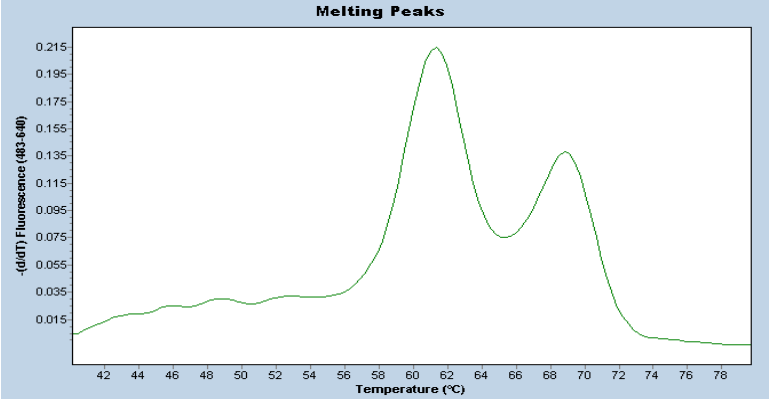


Figure D.8. Melting peaks observed for rs673951 a) Homozygous wild type genotype (68^0C), b) heterozygous genotype (61^0C ve 68^0C) and c) homozygous mutant genotype (61^0C)

a) Homozygous wild type (C/C)



b) Heterozygous(C/G)



c) Homozygous mutant type (T/T)

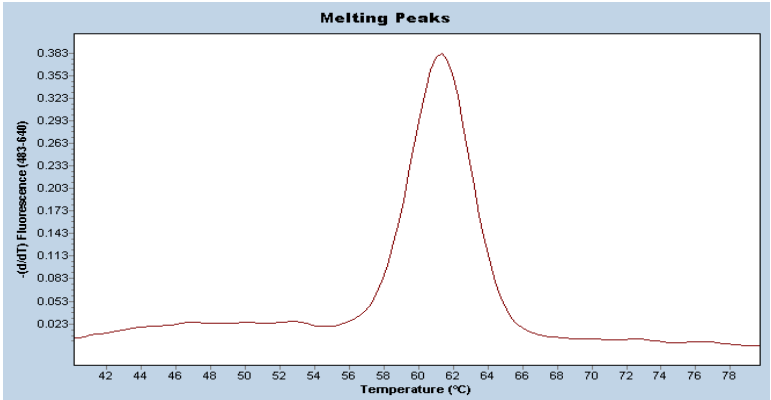


Figure D.9. Melting peaks observed for rs2305055 a) Homozygous wild type genotype (68^0C), b) heterozygous genotype (61^0C ve 68^0C) and c) homozygous mutant genotype (61^0C)

APPENDIX E: QUANTATIVE PCR RESULTS

Table E.1. qPCR results for rs7588807

ASSAY 1	Target				Reference				Normalization
	Error: 0.0017		Efficiency: 2.141		Error: 0.1270		Efficiency: 1.54		
Patient	Mean	STD	Cp	STD	Mean	STD	Cp	STD	Target/reference
24AE79	8.14	0.745	23.13	0.12	20.9	0.38	23.54	0.04	0.39
7GE43	6.15	0.576	23.5	0.12	19.2	0.58	23.74	0.07	0.32
12AE37	8.62	0.556	23.03	0.08	21.2	0.51	23.51	0.06	0.40
25AE82	8.34	0.0547	23.1	0.01	22	0.65	23.43	0.32	0.37
28AE93	9.27	0.16	22.96	0.02	21.3	0.0002	23.50	0.02	0.43
31AE99	8.09	0.232	23.14	0.04	20.2	0.18	23.62	0.02	0.40
52AE165	8.11	0.188	23.13	0.03	22	3.18	23.43	0.32	0.41
122TR	19	0.00818	21.95	0.08	18.6	0.48	23.81	0.06	1.01
10 ng	10	0.335	23.77	0.04	11.30	0.59	24.96	0.12	0.88
ASSAY 2	Target				Reference				Normalization
	Error: 0.0542		Efficiency: 2.011		Error: 0.0763		Efficiency: 1.763		
patient	mean	STD	Cp	STD	Mean	STD	Cp	STD	Target/reference
24AE79	12.5		24.08		19.6		23.98		0.63
7GE43	10.8		24.28		20.1		23.94		0.53
12AE37	12.10	0.768	24.22	0.09	17.3	0.21	23.76	0.02	0.70
25AE82	11.1		24.55		19.1		23.9		0.58
28AE93	12.4		24.36		20.1		23.86		0.62
31AE99	-	-	-	-	20.2	0.18	23.62		-
52AE165	12.9	0.522	24.13	0.06	22	3.18	23.43	0.32	0.68
122TR	18.8	0.289	23.59	0.02	18.8	0.675	23.6	0.675	1.09
ASSAY 3	Target				Reference				Normalization
	Error: 0.0000404		Efficiency: 2.087		Error: 0.1270		Efficiency: 1.54		
Patient	mean	STD	Cp	STD	Mean	STD	Cp	STD	Target/reference
24AE79	13.3	0.00123	24	0	20.9	0.38	23.54	0.04	0.63
7GE43	9.74	0.776	24.53	0.12	19.2	0.58	23.74	0.07	0.50
12AE37	13.8	0.444	23.96	0.05	21.2	0.51	23.51	0.06	0.65
25AE82	12.8	2.61	22.63	0.29	22	0.65	23.43	0.32	0.58
28AE93	13.4	2.42	22.57	0.25	21.3	0.0002	23.50	0.02	0.62
31AE99	11.4	0.675	24.23	0.09	20.2	0.18	23.62	0.02	0.56
52AE165	12	0.975	24.16	0.12	22	3.18	23.43	0.32	0.54
122TR	17.2	1.73	23.65	0.14	18.6	0.48	23.81	0.06	0.92

Table E.2. qPCR results for rs1983211

ASSAY 1	Target				Reference				Normalization
	Error: 0.0461		Efficiency: 1.99		Error: 0.1270		Efficiency: 1.54		
Patient	Mean	STD	Cp	STD	Mean	STD	Cp	STD	Target/reference
24AE79	10.7	0.81	24.07	0.02	20.9	0,38	23,54	0,04	0.51
7GE43	7.74	0.74	24.55	0.14	19.2	0,58	23,74	0,07	0.40
12AE37	12.5	0.57	23.86	0.07	21.2	0,51	23,51	0,06	0.59
25AE82	12.7	0.65	23.84	0.08	22	0,65	23,43	0,32	0.58
28AE93	13.3	1.05	23.77	0.12	21.3	0,0002	23,50	0,02	0.62
31AE99	12.6	0.96	23.84	0.11	20.2	0,18	23,62	0,02	0.62
52AE165	13.6	1.46	23.74	0.16	22	3,18	23,43	0,32	0.62
122TR	19.2	1.92	23.24	0.15	18.6	0,48	23,81	0,06	1.03
10 ng	10.6	0.78	24.09	0.11	11,30	0,59	24,96	0,12	0.94
ASSAY 2	Target				Reference				Normalization
	Error: 0.0099		Efficiency: 2.03		Error: 0.1270		Efficiency: 1.54		
patient	mean	STD	Cp	STD	Mean	STD	Cp	STD	Target/reference
24AE79	10.9	0.81	24.35	0.13	20.9	0,38	23,54	0,04	0.52
7GE43	6.93	1.06	24.99	0.17	19.2	0,58	23,74	0,07	0.36
12AE37	14.1	1.58	23.99	0.15	21.2	0,51	23,51	0,06	0.67
25AE82	10.3	2.08	24.44	0.30	22	0,65	23,43	0,32	0.47
28AE93	12	0.68	24.20	0.08	21.3	0,0002	23,50	0,02	0.56
31AE99	-	-	-	-	20.2	0,18	23,62	0,02	-
52AE165	14.4	1.9	23.96	0.19	22	3,18	23,43	0,32	0.65
122TR	20.2	1.22	24.34	0.08	18.6	0,48	23,81	0,06	1.09
ASSAY 3	Target				Reference				Normalization
	Error: 0.05		Efficiency: 1.99		Error: 0.1270		Efficiency: 1.54		
Patient	mean	STD	Cp	STD	Mean	STD	Cp	STD	Target/reference
24AE79	9.57	1.42	24.31	0.18	19.6	0.525	23.98	0.06	0.67
7GE43	7.55	0.0823	23.96	0.01	20.1	0.204	23.94	0.02	0.69
12AE37	12.60	0.229	23.46	0.02	20,7	0.71	23.8	0.07	0.61
25AE82	10.40	1.03	24.31	0.14	22	0,65	23,43	0,32	0.47
28AE93	11.90	0.99	24.12	0.12	21.3	0,0002	23,50	0,02	0.55
31AE99	11.30	0.95	24.19	0.12	20.2	0,18	23,62	0,02	0.55
52AE165	-	-	-	-	22	3,18	23,43	0,32	-
122TR	18.70	0.29	23.46	0.02	18.9		24.06		0.93

Table E.3. qPCR results for rs2241526

ASSAY 1	Target				Reference				Normalization
	Error: 0.000732		Efficiency: 1.766		Error: 0.0861		Efficiency: 1.571		
Patient	Mean	STD	Cp	STD	Mean	STD	Cp	STD	Target/reference
24AE79	9.6	0.678	22.57	0.12	20.9	0.234	23.72	0.02	0.46
7GE43	8.91	0.0028	22.7	0.01	19.5	0.375	23.88	0.04	0.46
12AE37	12	0.149	23.85	0.02	21.4	0.325	23.67	0.03	0.56
25AE82	12	0.149	22.18	0.02	22.3	0.369	23.58	0.04	0.54
28AE93	10.3	1.9	22.46	0.33	21.4	0.405	23.67	0.04	0.48
31AE99	11.3	-	22.29	-	20.2	0.24	23.8	0.03	0.56
52AE165	11.8	0.232	22.2	0.03	20	0.539	23.82	0.06	0.59
122TR	19.5	1.44	21.33	0.13	18.9	0.256	23.95	0.03	1.03
ASSAY 2	Target				Reference				Normalization
	Error: 0.00995		Efficiency: 2.034		Error: 0.0861		Efficiency: 1.571		
patient	Mean	STD	Cp	STD	Mean	STD	Cp	STD	Target/reference
24AE79	10.8	0.00685	24.97	0.01	20.9	0.234	23.72	0.02	0.52
7GE43	10.2	0.622	25.05	0.09	19.5	0.375	23.88	0.04	0.52
12AE37	12.8	0.222	24.73	0.02	21.4	0.325	23.67	0.03	0.60
25AE82	13.5	0.226	24.65	0.02	22.3	0.369	23.58	0.04	0.61
28AE93	14.5	1.08	24.56	0.1	21.4	0.405	23.67	0.04	0.68
31AE99	12.5	1.5	24.76	0.17	20.2	0.24	23.8	0.03	0.62
52AE165	13.2	0.159	24.68	0.02	20	0.539	23.82	0.06	0.66
122TR	20	0.43	24.1	0.03	18.9	0.256	23.95	0.03	1.06
ASSAY 3	Target				Reference				Normalization
	Error: 0.119		Efficiency: 1.557		Error: 0.101		Efficiency: 1.604		
Patient	Mean	STD	Cp	STD	Mean	STD	Cp	STD	Target/reference
24AE79	12	0.496	24.88	0.00	19.6	0.525	23.98	0.06	0.61
7GE43	12.8	0.0792	24.77	0.01	20.1	0.204	23.94	0.02	0.64
12AE37	13.9	0.659	24.55	0.11	20,7	0.71	23.8	0.07	0.67
25AE82									
28AE93									
31AE99									
52AE165									
122TR	18	0.83	23.96	0.1	18.9	1.04	24.06	0.11	0.95

Table E.4. qPCR results for rs3770234

ASSAY 1	Target				Reference				Normalization
	Error: 0.0594		Efficiency: 1.659		Error: 0.0861		Efficiency: 1.571		
Patient	Mean	STD	Cp	STD	Mean	STD	Cp	STD	Target/reference
24AE79	20.9	0.164	24.24	0.02	20.9	0.234	23.72	0.02	1
7GE43	11.9	0.58	25.36	0.1	19.5	0.375	23.88	0.04	0.61
12AE37	22.3	1.14	24.12	0.1	21.4	0.325	23.67	0.03	1.04
25AE82	24.5	0.78	23.94	0.06	22.3	0.369	23.58	0.04	1.10
28AE93	25.8	0.827	23.83	0.06	21.4	0.405	23.67	0.04	1.21
31AE99	22.4	1.69	24.11	0.15	20.2	0.24	23.8	0.03	1.11
52AE165	24.5	1.19	23.93	0.1	20	0.539	23.82	0.06	1.23
122TR	24.5	0.319	23.94	0.03	18.9	0.256	23.95	0.03	1.30
10 ng	9.29	0.0079	25.85	0.02	11	0.2	25.14	0.04	0.94
ASSAY 2	Target				Reference				Normalization
	Error: 0.0659		Efficiency: 1.721		Error: 0.0595		Efficiency: 1.785		
patient	Mean	STD	Cp	STD	Mean	STD	Cp	STD	Target/reference
24AE79	21.9	0.0563	24.36	0	20.3	0.884	23.58	0.08	1.08
7GE43	11.9	0.445	25.48	0.07	18.1	0.551	23.78	0.05	0.66
12AE37	23.5	0.279	24.09	0.02	19.2	1.33	23.68	0.12	1.32
25AE82	25.5	0.0741	24.08	0.01	21.4	0.838	23.49	0.07	1.19
28AE93	26.7	1.93	24	0.13	20.6	0.14	23.55	0.01	1.30
31AE99	24	3.66	24.21	0.28	19.1	0.662	23.68	0.06	1.26
52AE165	26.5	3.85	24.02	0.27	19.6	0.423	23.64	0.04	1.35
164TR	24.5	0.082	24.02	0.05	20.7	0.394	23.55	0.03	1.18
ASSAY 3	Target				Reference				Normalization
	Error: 0.0594		Efficiency: 1.659		Error: 0.182		Efficiency: 1.552		
Patient	Mean	STD	Cp	STD	Mean	STD	Cp	STD	Target/reference
24AE79	17.1	0.42	24.2	0.05	20.9	0.234	23.72	0.02	0.83
7GE43	7.94	1.71	25.76	0.46	19.5	0.375	23.88	0.04	0.41
12AE37	19.6	0.301	23.94	0.03	21.4	0.325	23.67	0.03	0.92
25AE82	17.2	0.92	24.2	0.11	22.3	0.369	23.58	0.04	0.77
28AE93	17.8	0.796	24.13	0.09	21.4	0.405	23.67	0.04	0.83
31AE99	-	-	-	-	20.2	0.24	23.8	0.03	-
52AE165	20.4	1.35	23.87	0.13	20	0.539	23.82	0.06	1.01
122TR	18.9	0.139	23.82	0.01	18.9	0.256	23.95	0.03	1.09

Table E.5. qPCR results for 6729914

ASSAY 1	Target				Reference				Normalization
	Error: 0.127		Efficiency: 1.806		Error: 0.0449		Efficiency: 1.583		
Patient	Mean	STD	Cp	STD	Mean	STD	Cp	STD	Target/reference
24AE79	19.1	1.93	24.89	0.17	20.7	1.12	23.85	0.12	0.92
7GE43	19.2	1.16	24.88	0.1	18.8	0.534	24.06	0.06	1.02
12AE37	20.3	0.0188	24.78	0	21.5	1.52	23.78	0.15	0.94
25AE82	24.6	3.03	24.47	0.21	22	0.795	23.72	0.08	1.12
28AE93	18.5	0.441	24.94	0.04	21.5	1.53	23.77	0.15	0.86
31AE99	19.5	0.691	24.85	0.06	19.6	1.07	23.98	0.12	0.99
52AE165	19.2	0.586	24.88	0.05	19.9	1.3	23.95	0.14	0.96
122TR	23.3	1.32	24.55	0.1	18.5	0.582	24.1	0.07	1.26
10 ng	8.33	0.215	26.29	0.04	10.5	0.176	25.33	0.04	0.79
ASSAY 2	Target				Reference				Normalization
	Error: 0.128		Efficiency: 1.828		Error: 0.101		Efficiency: 1604		
Patient	Mean	STD	Cp	STD	Mean	STD	Cp	STD	Target/reference
24AE79	14.5	0.696	26.06	0.08	19.6	0.525	23.98	0.06	0.73
7GE43	18.5	2.52	25.66	0.23	20.1	0.204	23.94	0.02	0.92
12AE37	17.4	1.24	25.75	0.12	20,7	0.71	23.8	0.07	0.84
25AE82	22	0.374	25.37	0.03	22	0,65	23,43	0,32	1
28AE93	15.9	1.03	25.9	0.11	21.3	0,0002	23,50	0,02	0.74
31AE99	15.2	0.235	25.98	0.03	20.2	0,18	23,62	0,02	0.75
52AE165	13.9	1.28	26.13	0.15	19.9	1.3	23.95	0.14	0.69
122TR	20.5	0.214	25.48	0.02	18.9	1.04	24.06	0.11	1.08
ASSAY 3	Target				Reference				Normalization
	Error: 0.128		Efficiency: 1.828		Error: 0.1270		Efficiency: 1.54		
Patient	Mean	STD	Cp	STD	Mean	STD	Cp	STD	Target/reference
24AE79	17	1.81	26.4	0.17	19.6	0.525	23.98	0.06	0.82
7GE43	20.5	1.15	24.29	0.09	20.1	0.204	23.94	0.02	1.09
12AE37	-	-	-	-	20,7	0.71	23.8	0.07	-
25AE82	19.3	0.762	24.39	0.06	22	0,65	23,43	0,32	0.88
28AE93	17	0.601	24.6	0.12	21.3	0,0002	23,50	0,02	0.79
31AE99	21	0.199	24.25	0.02	20.2	0,18	23,62	0,02	1.01
52AE165	20.1	0.601	24.32	0.05	19.9	1.3	23.95	0.14	10.7
122TR	24	0.257	24.02	0.02	18.9	1.04	24.06	0.11	1.3

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