MOLECULAR GENETIC ANALYSIS OF LAFORA DISEASE AND DRAVET'S SYNDROME

SEDA SALAR

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MOLECULAR GENETIC ANALYSIS OF LAFORA DISEASE AND DRAVET'S SYNDROME

by

Seda Salar

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To my beloved grandparents,

Neriman & Hayri Dinçer

Mesude & Necati Salar

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ABSTRACT

MOLECULAR GENETIC ANALYSIS OF LAFORA DISEASE AND DRAVET'S SYNDROME

Lafora Disease (LD), a type of Progressive Myoclonus Epilepsy (PME) and Dravet's Syndrome (DS), the most severe form in Generalized Epilepsy with Febrile Seizure Plus (GEFS+) spectrum, are debilitating examples of epilepsy syndromes. Early diagnosis is critical for distinguishing these diseases from other epilepsy syndromes and for developing effective treatment strategies as patients with these diseases are generally resistant to drug treatment. Molecular genetic analysis of genes that are involved in the development of such severe forms of epilepsy is essential for early diagnosis. LD is associated with two genes, EPM2A and EPM2B encoding laforin, a dual specificity phosphatase and malin, an E3 ubiquitin ligase proteins, respectively. DS is frequently associated with SCN1A gene encoding α subunit of voltage-gated sodium channel type 1 ($Na_v1.1$). All molecular genetic analysis in the relevant genes were carried out by PCR amplifications of all coding regions followed by DNA sequence analysis. Mutational analysis of EPM2A and EPM2B genes on 4 patients suspected to have LD revealed a novel nonsense mutation in the EPM2B gene of two sisters and a recurrent nonsense mutation in the EPM2A gene in the third patient, confirming the clinical diagnosis. The fourth patient who did not have any changes in both EPM2A and EPM2B genes was analyzed for point mutations in the EPM1A gene by direct sequencing since LD and Unverricht-Lundborg disease (ULD) caused by mutations in the EPM1A gene can not be easily distinguished clinically. ULD diagnosis was also excluded in this patient. Mutational analysis of SCN1A gene on 4 patients, one suspected to have GEFS+ and 3 suspected to have DS revealed two novel mutations, a missense mutation and a dinucleotide deletion in two patients. The non-polymorphic nature and evolutinary conservation of the missense mutation indicated that the mutation may have a major effect on the phenotype and hence cause DS. The third patient suspected to have DS and the patient suspected to have

GEFS+ did not have any changes in the SCN1A gene. The DNA analysis results along with the information about the good prognosis of two DS patients suggested that they may also be considered in the GEFS+ spectrum. The GEFS+ patient on the other hand can be screened for other genes relevant to GEFS+ phenotype. In conclusion, mutational analysis of EMP1A, EPM2A, EPM2B and SCN1A genes were successfully implemented through direct DNA sequencing of the coding regions as an initial study to aid the clinical diagnosis and further to reveal the prevalence and molecular pathology of LD and DS in Turkey.

ÖZET

LAFORA HASTALIĞI VE DRAVET SENDROMUNUN MOLEKÜLER GENETİK ANALİZİ

Bir progresif miyoklonik epilepsi tipi olan Lafora Hastalığı (LH) ve ateşli nöbetli generalize epilepsiler (GEFS+) spektrumunun en ağır formu olan Dravet Sendromu (DS) ağır seyreden epilepsi sendromlarına örnektir. Bu hastalıkları diğer epilepsi sendromlarından ayırmada ve hastalar genellikle ilaç tedavilerine dirençli oldukları için etkili tedavi stratejileri geliştirmede, erken tanı kritiktir. Bu tip ağır epilepsi tiplerinin gelişiminde yer alan genlerin moleküler genetik analizi erken tanı için önemlidir. LH, sırasıyla bir çift-özgül fosfataz olan laforin ve bir E3 ubikuitin ligaz olan malini kodlayan EPM2A ve EPM2B genleri ile ilişkilidir. DS sıklıkla, tip 1 voltaj kapılı sodyum kanalının ($Na_v1.1$) α alt ünitesini kodlayan SCN1A geni ile ilişkilidir. Tüm ilgili genlerin moleküler genetik analizleri, tüm kodlayıcı bölgelerin PCR ile çoğaltılmaları ve takibinde DNA dizileme analizi ile yapılmıştır. LH olduğu şüphelenilen 4 hastada, EPM2A ve EPM2B genlerinin mutasyon analizi, klinik teşhisi onaylayarak, iki kızkardeşte EPM2B geninde yeni anlamsız bir mutasyon ve üçüncü hastada, başka hastalarda gösterilmiş anlamsız bir EPM2A mutasyonu ortaya çıkarmıştır. EPM2A ve EPM2B genlerinde herhangi bir değişiklik olmayan 4. hasta, LH ve EPM1A genindeki mutasyonların sebep olduğu Unverricht-Lundborg Hastalığı (ULH) erken dönemde klinik olarak ayrılamadıklarından, EPM1A genindeki nokta mutasyonu için de direkt dizileme ile incelenmiştir. Bu hastada ULH tanısı da dışlanmıştır. Birinin GEFS+ ve üçünün DS olduğu şüphelenilen 4 hastada, SCN1A geninin mutasyon analizi, 2 hastada, bir yanlış anlamlı ve bir dinükleotit delesyonu olmak üzere 2 yeni mutasyon ortaya çıkarmıştır. Yanlış anlamlı mutasyonun polimorfik olmayışı ve evrimsel olarak korunmuş olması, fenotipte ciddi etkisinin olduğunu, dolayısıyla DS'e sebebiyet verebileceğini göstermiştir. DS ve GEFS+ olduğu şüphelenilen üçüncü ve dördüncü hastalarda herhangi bir değişikliğe rastlanmamıştır. Bu iki DS hastasında, hastalığın iyi yönde gelişmesi ile birlikte DNA analiz sonuçları, hastaların GEFS+ spektrumu içinde de değerlendirebileceğini göstermektedir. Diğer taraftan, GEFS+ hastası, GEFS+ fenotipi ile ilgili olduğu bulunan diğer genler için incelenebilir.

Sonuçta, klinik tanıya yardımcı olacak ve ileride Türkiye'de LH ve DS'nin prevalansı ve moleküler patolojisini ortaya çıkarmaya yönelik bir ön çalışma olarak EPM1A, EPM2A, EPM2B ve SCN1A genlerinin mutasyon analizi, kodlayıcı bölgelerin doğrudan DNA dizilemesi ile başarıyla tamamlanmıştır.

TABLE OF CONTENTS

ACKNOWLEDGMENTS iv
ABSTRACT v
ÖZET vii
LIST OF FIGURES ix
LIST OF TABLES xi
LIST OF ABBREVIATIONS xiii
1. INTRODUCTION 1
    1.1. Epilepsies 1
        1.1.1. Progressive Myoclonus Epilepsies 1
            1.1.1.1. Lafora Disease 2
            1.1.1.2. Unverricht-Lundborg Disease 6
        1.2.1. Idiopathic Generalized Epilepsies 8
            1.2.1.1. Voltage-gated sodium channels 10
            1.2.1.2. GEFS+ 14
            1.2.1.3. SCN1A and DS 15
2. PURPOSE 17
3. MATERIALS 18
    3.1. Blood Samples 18
    3.2. Oligonucleotide Primers 18
    3.3. Enzymes 18
    3.4. Buffers and Solutions 19
        3.4.1. DNA Extraction 19
        3.4.2. Polymerase Chain Reaction 19
        3.4.3. Agarose Gel Electrophoresis 20
    3.5. Kits 21
    3.6. Equipment 21
4. METHODS 26
    4.1. Analysis of Genomic DNA 26
        4.1.1. DNA Extraction from White Blood Cells 27
        4.1.2. DNA Extraction from White Blood Cells by MagNaPure Compact

System 27
4.1.3. Analysis by Agarose Gel Electrophoresis 27
4.1.4. Analysis by Spectrophotometer 27
4.2. Investigation of Mutations 28
4.2.1. PCR 28
4.2.2. Purification of PCR Products 30
4.2.3. DNA Sequence Analysis 31
5. RESULTS 32
5.1. PCR Amplification 32
5.2. Mutational Analysis of Lafora Disease Patients 34
5.3. Mutational Analysis of SCN1A Gene in GEFS+ and DS Patients 41
6. DISCUSSION 55
6.1. DNA Diagnosis for LD 55
6.2. DNA Diagnosis for DS and GEFS+ 56
7. REFERENCES 60

LIST OF FIGURES

Figure 1.1. LBs in light microscopic examination of the axillary skin biopsy 3

Figure 1.2. Schematic representation of exons of EPM2A gene and their corresponding protein domains 4

Figure 1.3. Schematic representation of the exon of EPM2B gene and its corresponding protein domains 4

Figure 1.4. Proposed role of laforin-malin complex in glycogen synthesis regulation ... 6

Figure 1.5. Schematic representation of CSTB gene and dodecamer repeat region 7

Figure 1.6. Structure of VGSC $Na_v1.2$ 12

Figure 1.7. Chromosomal locations of rat VGSC genes.. 13

Figure 1.8. Localization of sequence differences in α subunits 13

Figure 1.9. Amino acid sequence similarity of rat VGSC α subunits 14

Figure 4.1. The gradient thermal cycler and optimization of annealing temperatures.... 28

Figure 5.1. PCR products and sizes of EPM1A exons shown on agarose gels.............. 32

Figure 5.2. PCR products and sizes of EPM2A exons shown on agarose gels.............. 32

Figure 5.3. PCR products and sizes of EPM2B exons shown on agarose gels.............. 32

Figure 5.4. PCR products and sizes of SCN1A exons shown on agarose gels.............. 33

Figure 5.5. PCR products and sizes of SCN1A IVS 9 ... 34

Figure 5.6. DNA sequence analysis of the EPM2B gene showing the mutation in family 3PME ... 36

Figure 5.7. The pedigree of 3PME family ... 37

Figure 5.8. DNA sequence analysis of the EPM2A gene showing the mutation in family 4PME ... 38

Figure 5.9. The pedigree of 4PME family ... 39

Figure 5.10. Dodecamer repeat region of EPM1A gene in patient 2PME4 ... 40

Figure 5.11. DNA sequence analysis of the SCN1A gene showing the mutation in family 1DS ... 43

Figure 5.12. Evolutionary conservation of S4 part in domain I of α subunit of VGSCs.. 44

Figure 5.13. The pedigree of 1DS family ... 44

Figure 5.14. DNA sequence analysis of the SCN1A gene showing the mutation in IVS 9 ... 45

Figure 5.15. Schematic representation of deletion in IVS 9 ... 46

Figure 5.16. DNA sequence analysis of the SCN1A gene showing the mutation in family 2DS ... 48

Figure 5.17. DNA sequence analysis of the SCN1A gene showing the silent mutation in patient 3DS7 ... 51

Figure 5.18. Alterations seen in exon 26b of SCN1A gene ... 53

LIST OF TABLES

Table 1.1. Types of PMEs.. 2

Table 1.2. Epilepsy syndromes caused by ion channel mutations 9

Table 1.3. Disorders caused by Mutations in VGICs and LGICs 10

Table 1.4. Voltage-gated sodium channels 11

Table 1.5. GEFS+ types and their related genes 14

Table 3.1. Oligonucleotide primer pairs used in PCR and sequencing of EPM1A exons 23

Table 3.2. Oligonucleotide primer pairs used in PCR and sequencing of EPM1A dodecamer repeats 23

Table 3.3. Oligonucleotide primer pairs used in PCR and sequencing of EPM2A exons 23

Table 3.4. Oligonucleotide primer pairs used in PCR and sequencing of EPM2B exons 24

Table 3.5. Oligonucleotide primer pairs used in PCR and sequencing of SCN1A exons 24

Table 3.6. Additional primer pairs 25

Table 5.1. DNA sequence analysis of EPM2A coding regions in patients 3PME5 and 3PME6 35

Table 5.2. DNA sequence analysis of EPM2B coding region in patients 3PME5 and 3PME6 35

Table 5.3. DNA sequence analysis of EPM2A coding regions in patient 4PME10 36

Table 5.4. DNA sequence analysis of EPM2A coding regions in patient 2PME4 38

Table 5.5. DNA sequence analysis of EPM2B coding region in patient 2PME4 40

Table 5.6. DNA sequence analysis of EPM1A coding regions in patient 2PME4 40

Table 5.7. DNA sequence analysis of SCN1A coding regions in patient 1DS1 41

Table 5.8. DNA sequence analysis of SCN1A coding regions in patient 2DS4 47

Table 5.9. DNA sequence analysis of SCN1A coding regions in patient 3DS7 49

Table 5.10. DNA sequence analysis of SCN1A coding regions in patient 1GEFS1 51

Table 5.11. Alterations found in patients with LD and DS 54

LIST OF SYMBOLS / LIST OF ABBREVIATIONS

A	Adenine
C	Cytosine
G	Guanine
T	Thymine
X	Stop codon
δ	Delta
$\gamma 2$	Gamma 2
NaCl	Sodium chloride
$MgCl_2$	Magnesium chloride

2q24	Region 24 of the long arm of the chromosome 2
6p22.3	Region 24.3 of the short arm of the chromosome 6
6q24	Region 24 of the long arm of the chromosome 6
21q22.3	Region 22.3 of the long arm of the chromosome 21
Arg	Arginine
BFIS	Benign familial infantile seizures
BFNIS	Benign familial neonatal infantile seizures
BFNC	Benign familial neonatal convulsions
bp	Base pair
c.	Coding
CA	Corpora amylacea
CACNB4	Voltage-gated calcium channel beta subunit
CACNA1A	Voltage-gated calcium channel alpha 1A subunit
CACNA1H	Voltage-gated calcium channel alpha 1 H subunit
CAE	Childhood absence epilepsy
CBM20	Carbonhydrate-binding module family 20
CLN3	Ceroid-lipofuscinosis neural 3
CLN5	Ceroid-lipofuscinosis neural 5
CLCN2	Chloride channel 2

CHRNA2	Cholinergic receptor, nicotinic, alpha 1
CHRNA4	Cholinergic receptor, nicotinic, alpha 4
CHRNB	Cholinergic receptor, nicotinic, beta 1
CSTB	Cystatin B
C-terISO	C-terminal isoform
D	Domain
dbSNP	Database SNP
del.	Deletion
DEKA	Aspartic acid-glutamic acid-lysine-alanine
DMSO	Dimethlysulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic acid triphosphate
DRPLA	Dentatorubral-pallidoluysian atrophy
DS	Dravet's Syndrome
DSPD	Dual specificity phosphatase domain
EDTA	Ethylenediaminetetraacetic acid
EEDD	Glutamic acid-glutamic acid-aspartic acid-aspartic acid
EEG	Electroencephelogram
EPM1A	Epilepsy myoclonus type 1 A
EPM2A	Epilepsy myoclonus type 2 A
EPM2AIP1	EPM2A interacting protein 1
EPM2B	Epilepsy myoclonus type 2 B
EtOH	Ethanol
FISH	Flouresan in situ hybridization
FS	Febrile seizure
GABRA1	Gamma-aminobutyric acid A receptor, alpha 1 subunit
GABRD	Gamma-aminobutyric acid A receptor, delta subunit
GABRG2	Gamma-aminobutyric acid A receptor, gamma 2 subunit
GEFS+	Generalized epilepsy with febrile seizure plus
GEFS+1-5	Generalized epilepsy with febrile seizure plus type 1 to 5
Glu	Glutamic acid
Gly	Glycine
HIRIP5	HIRA interacting protein 5

ICEGTC	Intractable childhood epilepsy with generalized tonic clonic seizures
IFM	Isoleucine-phenylalanine-methionine
IGE	Idiopathic Generalized Epilepsies
ILAE	International League Against Epilepsy
IVS	Intervening sequence
JME	Juvenile myoclonic epilepsy
kb	Kilobase
KCNQ2	Potassium voltage-gated channel, KQT-like, subfamily member 2
KCNQ3	Potassium voltage-gated channel, KQT-like, subfamily member 3
kDa	Kilodalton
LB	Lafora Body
LD	Lafora Disease
LGIC	Ligand-gated ion channel
ml	Mililiter
mM	Milimolar
MERRF	Mitochondrial Encephelomyopathy with Ragged Red Fibers
MGS	Muscle glycogen synthase
MTTK	Mitochondrially encoded tRNA lysine
ng	Nanogram
NCBI	National Center for Biotechnology Information
NHL	Conserved structural motif along Ncl1, HT2A and Lin-41 proteins
NHLRC1	NHL repeat containing 1
Na_v1.1-1.9	Voltage-gated sodium channel type 1, subtype 1 to 9
OMIM	Online Mendelian Inheritance in Man
PAS	Periodic-acid Schiff
PCR	Polymerase chain reaction
PP1	Protein phosphatase 1
PTG	Adaptor protein targeting glycogen
R5	Laforin interacting protein
rNa_v1.1-1.9	Rat voltage-gated sodium channel type 1, subtype 1 to 9
S	Subunit
SCN1A	Sodium channel voltage-gated type 1 alpha subunit
SCN1B	Sodium channel voltage-gated type 1 beta subunit

SCN2A	Sodium channel voltage-gated type 2 alpha subunit
SDS	Sodiumdodecylsulphate
SMEI	Severe Myoclonic Epilepsy of Infancy
SNP	Single nucleotide polymorphism
TPP1	Tripeptidyl peptidase I
U	Unit
ULD	Unverricht-Lundborg Disease
UTR	Untranslated region
UV	Ultraviolet
VGIC	Voltage-gated ion channel
VGSC	Voltage-gated sodium channel
y.	Years
µl	Microliter

1. INTRODUCTION

1.1. Epilepsies

Epilepsy is a common neurological disorder affecting more than 0.5 percent of the world's population (Lossin *et al.*, 2002; Kaneko *et al.*, 2002). Sporadic or recurrent seizures that result from a variety of causes such as acquired structural brain damage, genetic susceptibility or altered metabolic stress and that can cause motor, sensory, cognitive, psychic or autonomic disturbances are main features of epilepsies. Dispersion of hyperexcitability or abnormal synchronization of neurons throughout surrounding neural networks result in seizure generation (Steinlein, 2004).

According to the International League Against Epilepsy (ILAE), approximately thirty types of epilepsies have been defined depending on the clinical phenotype. In general, epilepsies can be divided into two groups: Symptomatic Epilepsies or Cryptogenic Epilepsies where epilepsy develops secondary to a disease or is likely to develop secondary to a disease and Idiopathic Generalized Epilepsies (IGEs) where principal etiological origin can not be clearly determined (Steinlein, 2001).

1.1.1. Progressive Myoclonus Epilepsies

PMEs are a group of symptomatic generalized epilepsies caused by rare disorders that are characterized by myoclonic seizures, generalized tonic-clonic or grand mal seizures, absences and progressive neurological deterioration, typically with cerebellar signs and dementia (Table1.1) (Delgado-Escueta *et al.*, 2001; Shahwan *et al.*, 2005; Joensuu *et al.*, 2007). Etiologically heterogeneous, PMEs are mostly autosomal recessive disorders with the exception of autosomal dominant dentatorubral-pallidoluysian atrophy and mitochondrial encephelomyopathy with ragged red fibers (MERRF) (Delgado-Escueta *et al.*, 2001). Myoclonus is often precipitated by posture, action or an external stimuli such as light, sound or touch (Shahwan *et al.*, 2005).

LD and ULD are the most frequently seen PMEs.

Table 1.1. Types of PMEs (Shahwan *et al.*, 2005)

PME type	**Etiology**
Unverricht-Lundborg Disease	CSTB (EPM1A) gene mutations
Lafora Disease	EPM2A and EPM2B gene mutation
Myoclonic Epilepsy with Ragged Red Fibers	MTTK mutation
Neuronal Ceroid Lipofuscinosis	TPP1,CLN3 and CLN5 gene mutation
Sialidoses	Neuraminidase deficieny in leukocytes or fibroblasts
Dentatorubral-pallidoluysian atrophy	Abnormal CAG repeats in DRPLA gene
Other PMEs with rare causes	Various causes

1.1.1.1. Lafora Disease. First described by Lafora and Gluelkin in 1911, LD (OMIM 254780) is an autosomal recessive, fatal disorder with pathognomonic periodic acid-schiff positive (PAS+) staining intracellular inclusion bodies called Lafora Bodies (LBs) (Gómez-Abad *et al.*, 2005; Ganesh *et al.*, 2006) (Figure 1.1). It occurs worldwide, but is particularly common in the Mediterranean countries of Southern Europe and Northern Africa, in Southern India and in the Middle East (Gómez-Abad *et al.*, 2005).

LD typically manifests itself during adolescence with generalized tonic-clonic seizures, myoclonus, absences, drop attacks or partial visual seizures. At onset it may be difficult to distinguish LD clinically from typical generalized epilepsy with no evidence of cognitive decline, particularly juvenile myoclonic epilepsy (Shahwan *et al.*, 2005). EEG shows discharges of fast spike wave and polyspike-wave complexes, photosensitivity, deterioration of background activity and the appearance of multifocal abnormalities (Gómez-Abad *et al.*, 2005). As the disease progresses myoclonus increases in frequency and becomes constant and

afflicted individuals suffer a rapidly progressive dementia with apraxia, aphasia and visual loss, leading to a vegetative state and death usually in the first decade from the onset of the initial symptoms (Gómez-Abad *et al*., 2005; Vilchez *et al*., 2007).

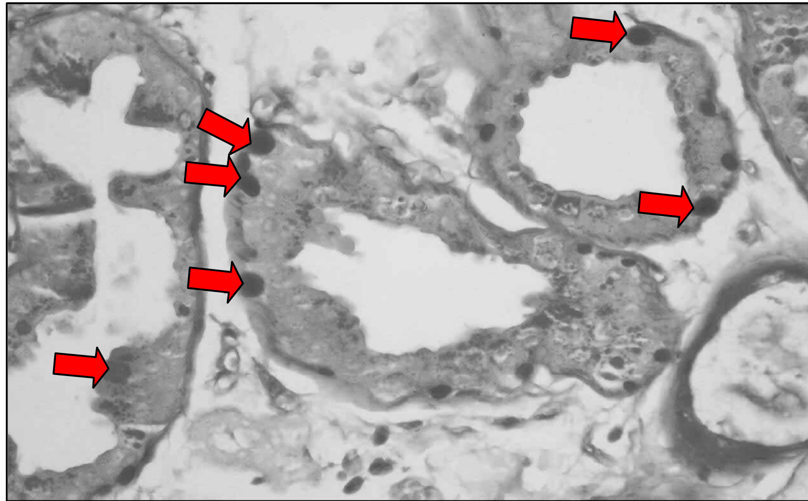

Figure 1.1. LBs in light microscopic examination of the axillary skin biopsy by PAS (Koç *et al*., 2004). Red arrows indicate LBs.

Pathognomonic LBs found in brain and in almost every organ stain strongly with periodic acid-Shiff indicating a major content of polysaccharides (Minassian *et al*., 2000). LBs are histochemically and chemically almost indistinguishable from corpora amylacea (CA), an inclusion body seen in normal aging cells in most tissues. In brain, CA are located almost exclusively in glial cells, whereas LB are found primarily in axons and dendrites of neurons (Minassian *et al*., 2000; Vilchez *et al*., 2007). However, it is not clear whether LBs have any direct role in the pathogenesis of the epileptic phenotype or not (Singh *et al*., 2005). In addition, a transgenic mouse overexpressing a dominant negative laforin mutant developed LBs but show no epileptic symptoms (Chan *et al*., 2004).

Mutations in EPM2A gene was found to be related with LD (Minassian *et al*. 1998). Localized on the long arm of chromosome 6 to 6q24 and spanning approximately 70 kilobases (kb), EPM2A gene consists of 4 exons. It encodes for laforin, a 37 kilodalton (kDa) dual specificity protein tyrosine phosphatase (Figure 1.2). Laforin is expressed from antisense strand and has two alternatively spliced forms. Composed of 331 amino acids, longer isoform,

isoform a, is localized on the rough endoplasmic reticulum. The other isoform, isoform b, also known as C-terISO, is localized in the nucleus. It has shorter and different C terminus when compared to isoform a (National Center for Biotechnology Information-NCBI, EPM2A). Physiological significance of this isoform b has not been determined yet.

Figure 1.2. Schematic representation of exons of EPM2A gene and their corresponding protein domains (CBM20: N-terminal carbonhydrate-binding domain, DSPD: C-terminal dual-specificity phosphatase domain) (Ganesh *et al*., 2006)

Mutations in EPM2B (NHLRC1) was also found to be associated with LD in patients with LD who did not carry mutations in EPM2A (Chan *et al*., 2003). Localized on the long arm of chromosome 6 to 6p22.3 and spanning approximately 1.2 kb, EPM2B gene consists of only one exon. It encodes for malin, a 42 kDa E3 ubiquitin ligase. (Figure 1.3). Malin is expressed from the antisense strand and composed of 395 amino acids (Gómez-Abad *et al*., 2005). It was shown that malin ubiquinates and promotes degradation of laforin (Gentry *et al*., 2005).

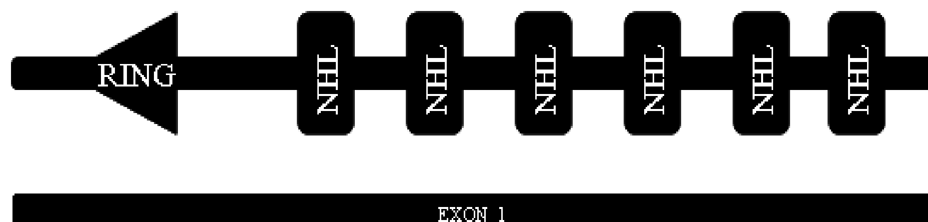

Figure 1.3. Schematic representation of the exon of EPM2B gene and its corresponding protein domains (RING: Characterictic domain of one class of E3 ubiquitin ligases , NHL: Domains involve in protein-protein interactions) (Ganesh *et al*., 2006)

Recent studies suggest that there is a third locus involved in pathogenesis of LD (Chan *et al*., 2004; Singh *et al*., 2005). Linkage analysis in a family whose members were clinically

diagnosed to have Lafora Disease, but revealed no mutations in EPM2A and EPM2B genes excluded linkage from these regions along with other regions as EPM2AIP1, HIRIP5 and R5, previously identified as laforin-interacting protein encoding genes (Chan *et al*., 2004).

Alterations in EPM2A and EPM2B genes are gathered in a locus specific database established in 2005 . It is available on the World Wide Web (http://projects.tcag.ca/lafora). Entries can be submitted via the curator of the database or via a web-based form (Ianzano *et al.*, 2005). To date, 43 mutations in the EPM2A gene and 47 mutations in the EPM2B gene have been reported. Half of the mutations are point mutations. Others involve insertions and deletions. Mutations are spread evenly across EPM2A exons, while most of the mutations in EPM2B is seen in the region encoding its RING domain (Ganesh *et al*., 2006).

Although LBs are the hallmark of Lafora disease, the mechanism by which these accumulations are formed could not be clearly understood as it was thought that neurons do not synthesize glycogen. However, it was shown that neurons have the enzymatic machinery for synthesizing glycogen. It is also now known that laforin and malin are members of a protein complex which suppresses glycogen synthesis by inducing the proteosome-dependent degradation of muscle glycogen synthase (MGS) and adaptor protein targeting to glycogen (PTG) which brings protein phosphatase 1 (PP1) to MGS for activation in normal physiological conditions. LD associated mutations disturbs this regulation which in turn results in abnormal glycogen synthesis in neurons (Figure 1.4). However, the exact mechanism have not been determined still (Vilchez *et al*., 2007).

It was reported that LD patients with EPM2B mutations have milder disease course than patients with EPM2A mutations; however, no preventive or curative treatment is available for LD (Ganesh *et al*., 2006).

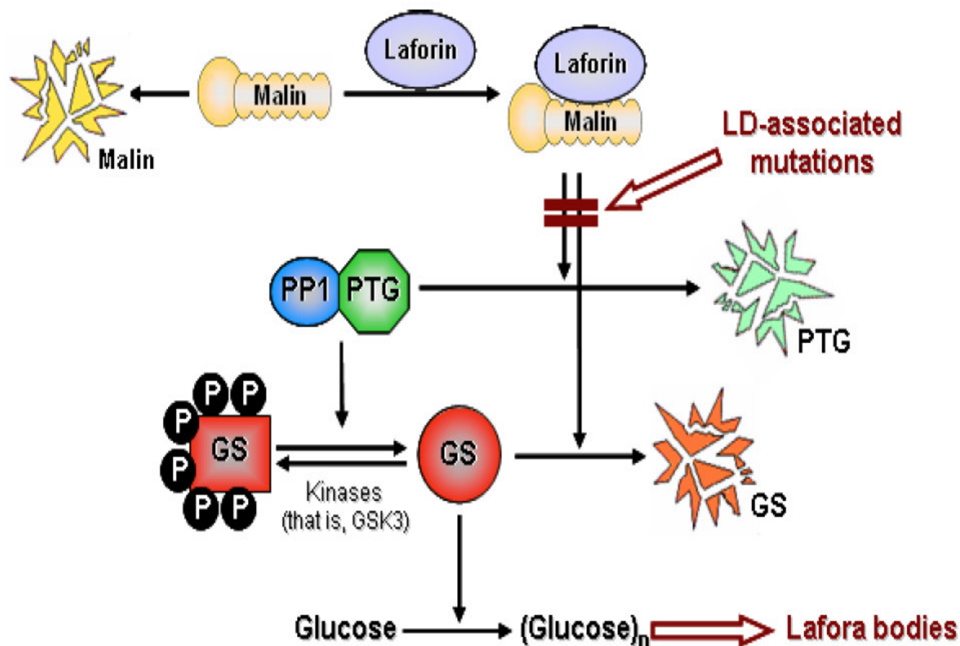

Figure 1.4. Proposed role of laforin-malin complex in glycogen synthesis regulation. P:phosphate , PP1:protein phosphatase 1, PTG: adaptor protein targeting to glycogen, GS: glycogen synthase (Vilchez *et al.*, 2007)

1.1.1.2. Unverricht-Lundborg Disease. ULD (OMIM 254800) is an autosomal recessive disorder that was described by Unverricht in 1891 and by Lundborg in 1903 (Shahwan *et al.*, 2005). The age of onset differs between 6 and 13 years with myoclonic and tonic-clonic seizures followed by variable progression to mental deterioration and cerebellar ataxia (Lalioti *et al.*, 1997a; Berkovic *et al.*, 2005; Shahwan *et al.*, 2005). Stimulus sensitive myoclonic jerks are important in diagnosis of the disease and are generally the first symptom in at least half of the patients. EEG shows generalized high-voltage spike and wave, and polyspike and wave paroxysms ranging from a slow to faster frequencies and photosensitivity is typical(Shahwan *et al.*, 2005). ULD is also known as Baltic Myoclonus Epilepsy as although a rare disorder, it is relatively common in Finland (1 in 20.000). ULD is also seen in Western Mediterranean countries (Lalioti *et al.*, 1997a).

Mutations in Cystatin B (CSTB or EPM1) gene was found to be related with ULD. EPM1A (CSTB) gene is localized on 21q22.3 and spans approximately 2.5 kb. It consists of three exons. The 5' UTR of CTSB gene contains two potential transcription start sites

localized at 67 and 78 nucleotides downstream of the 12-nucleotide, dodecamer repeat element (5'CCCCGCCCCGCG'3) (Figure 1.5). Normal alleles contain 2 or 3 copies of this repeat (Lalioti *et al*., 1997b). Patients with ULD may carry expansion of the dodecamer repeat on both alleles, an expansion of the dodecamer repeat on one allele and a deletion or a point mutation in the coding region or rarely point mutations in the coding region of both alleles (Shahwan *et al*., 2005). So far only one patient with two point mutations has been described (Lalioti *et al*., 1997a). Mutant alleles contain more than 30 dodecamer repeats. It was identified that alleles carrying 12 – 17 dodecamer repeat show instability while being transmitted to the offspring. It was shown that repeat expansion has no effect on either the age of onset or severity of the disease (Lalioti *et al*., 1998). Different from other repeat expansion diseases, it was found that methylation is not involved in the transcriptional regulation of CSTB gene (Lalioti *et al*., 1997b). Repeat expansion reduces CTSB promoter activity giving rise to decreased CTSB expression (Lalioti *et al*., 1999).

CSTB gene encodes for ubiquitiously expressed cystatin B, also known as stefin B, a 98 amino acids long, approximately 11 kDa cysteine protease. It has at least five isoforms with tissue specificity; however, physiological significance of these isoforms remains unclear. In addition, functional importance of CSTB has not been determined yet although it was shown that it tightly binds cathepsin B, H, L and S which are important in nonselective degradation of proteins and participate in antigen processing and apoptosis. It was shown that decreased CSTB activity in patients with ULD correlate with increased activiy of cathepsins B, L and S (Joensuu *et al*., 2007).

Figure 1.5. Schematic representation of CSTB gene and dodecamer repeat region indicated by the triangle.

Recent studies suggest that there is a second locus, EPM1B, involved in pathogenesis of ULD. Results of linkage analysis in a consanguineous Arab family whose members were clinically diagnosed to have ULD but carried no mutations in CSTB gene restricted the relevant locus at the pericentromeric region of chromosome 12. However, analysis of the known genes residing in that region show no obvious relationship to pathways related to cystatin B (Berkovic *et al.*, 2005).

With the advances in antiepileptic drug development and their use, the prognosis of the disease has improved significantly and ULD patients now live into their sixties (Shahwan *et al.*, 2005)

Although LBs are distinctive feature of LD, patients with LD and ULD can not always be clinically distinguished from each other as skin biopsy may result in both false negatives and false positives (Lohi *et al.*, 2007).

1.1.2. Idiopathic Generalized Epilepsies

IGEs constituting 40 percent of all epilepsies are a group of neurological disorders, that are common, paroxysmal and clinically heterogeneous with incompletely understood mechanisms (Steinlein, 2001; Lossin *et al.*, 2002).

Recently, it has been shown that ion channel mutations have a significant role in the pathogenesis of idiopathic epilepsies which in turn resulted in the definition of many of the idiopathic generalized epilepsies as channelopathies (Scheffer and Berkovic, 2003; Steinlein, 2004) (Table 1.2). Although mutations in ion channel genes show autosomal dominant inheritance pattern, mutations in different ion channels can lead to similar phenotypes (locus heterogeneity) or different mutations in the same gene can cause variable phenotypes (allelic heterogeneity) (Kullmann and Hanna, 2002). Therefore, apart from causing different epilepsy syndromes, mutations in these channels lead to various other disorders (Table 1.3).

The most relevant gene in idiopathic generalized epilepsies is SCN1A having the largest number of epilepsy related mutations (Mulley *et al.*, 2005).

Table 1.2. Epilepsy Syndromes caused by ion channel mutations

Voltage-gated Ion Channels (VGICs)	**Epilepsy Syndrome**
SCN1A (Voltage-gated sodium channel type 1, α subunit)	Generalized Epilepsy with Febrile Seizure Plus 2 (GEFS+2) Severe Myolonic Epilepsy of Infancy (SMEI) – Dravet's Syndrome (DS) Borderline Severe Myoclonic Epilepsy of Infancy (SMEB) Intractable Childhood Epilepsy with Generalized Tonic-Clonic Seizures (ICEGTC)
SCN2A (Voltage-gated sodium channel type 1, α subunit)	Benign Familial Neonatal-Infantile Seizures (BFNIS) GEFS +2
SCN1B (Voltage-gated sodium channel type 1, β subunit)	GEFS+1 Benign Familial Infantile Convulsions (BFIC)
KCNQ2 (Voltage-gated potassium channel type 2, α subunit)	Benign Familial Neonatal Convulsions (BFNC)
KCNQ3 (Voltage-gated potassium channel type 3, α subunit)	BFNC
CLCN2 (Voltage-gated chloride channel type 2, α subunit)	Juvenile Myoclonic Epilepsy (JME) Childhood Absence Epilepsy (CAE) Juvenile Absence Epilepsy (JAE)
CACNB4 (Voltage-gated calcium channel, β subunit)	JME IGE
CACNA1A (Voltage-gated calcium channel, α1A subunit)	IGE
CACNA1H (Voltage-gated calcium channel, α1H subunit)	IGE

Table 1.2. Epilepsy Syndromes caused by channelopathies (continued)

Ligand-gated Ion Channels (LGICs)	**Epilepsy Syndrome**
CHRNA2 (Nicotinic acetylcholine receptor α2 subunit)	Nocturnal Frontal Lobe Epilepsy Type IV
CHRNA4 (Nicotinic acetylcholine receptor α4 subunit)	Nocturnal Frontal Lobe Epilepsy Type I
CHRNB2 (Nicotinic acetylcholine receptor β2 subunit)	Nocturnal Frontal Lobe Epilepsy Type III
GABRA1 (GABA-A receptor α1 subunit)	JME
GABRG2 (GABA-A receptor γ2 subunit)	CAE GEFS+3
GABRD (GABA-A receptor δ subunit)	GEFS+5

Table 1.3. Disorders caused by Mutations in VGICs and LGICs (NCBI)

GENE	**DISORDER**
KCNQ2	• Myokymia • Myokymia with neonatal epilepsy
CACNA1A	• Familial hemiplegic migrane type 1 • Episodic ataxia type 2 • Spinocerebellar ataxia 6
CACNB4	• Episodic ataxia type 5
CHRNA4	• Protection against nicotine addiction (association)

1.1.2.1. Voltage-gated Sodium Channels. Voltage-gated sodium channels (VGSCs) are important in initiation and propagation of action potentials in excitable cells (Meisler and Kearney 2005). These channels are sensitive to ionic changes in the micro environment. When the threshold of these channels are reached, conformational change in the protein leads to opening of the pore. After a sudden flow of sodium ions, first, the inactivation gate and then, the main gate of the channel is closed.

VGSCs are composed of pore-forming α subunits and one or two modulatory β subunits (Koopmann *et al*., 2006). The pore-forming 260 kDa α subunits are sufficient for functional expression, but the kinetics and voltage dependence of channel gating are modified by the 33-

36 kDa β subunits (Goldin *et al*., 2000; Yu and Catterall, 2003). Nine types of α subunits and four types of β subunits have been identified depending on the tissue and cell type in which they are expressed (Yu and Catterall, 2003) (Table 1.4).

Table 1.4. Voltage-gated sodium channels

Protein Name	**Gene Name**	**Auxillary Subunits**	**Expression Profile**
Na_v 1.1	SCN1A	β1, β2, β3, β4	Central neurons and cardiac myocytes
Na_v 1.2	SCN2A	β1, β2, β3, β4	Central neurons
Na_v 1.3	SCN3A	β1, β3	Central neurons and cardiac myocytes
Na_v 1.4	SCN4A	β1	Skeletal muscle
Na_v 1.5	SCN5A	β1, β2, β3, β4	Central neurons, cardiac myocytes
Na_v 1.6	SCN8A	β1, β2	Central neurons, dorsal root ganglia, peripheral neurons
Na_v 1.7	SCN9A	β1, β2	Dorsal root ganglia, sympathetic neurons, schwann cells, neuroendocrine cells
Na_v 1.8	SCN10A	Unknown	Dorsal root ganglia
Na_v 1.9	SCN11A	Unknown	Dorsal root ganglia

Pore-forming α subunits are approximately 2000 amino acid residues, composed of four homologous domains (D) connected by intracellular loops (Meisler and Kearney, 2005). Each homologous domain consists of six transmembrane segments connected by intra and extracellular loops (Figure 1.6). The forth transmembrane segment of each domain is voltage sensing unit of the channel. Every third amino acid in protein sequence of voltage sensing unit of each domain is positively charged which is important in sensing environmental ionic changes. The fifth and sixth transmembrane segments of each domain creates the pore of α subunits. Amino acid sequence glutamic acid-glutamic acid-aspartic acid-aspartic acid (EEDD) in the outer domain and aspartic acid-glutamic acid-lysine-alanine (DEKA) the inner domain in re-entrant loops in the pore create ion selectivity filter. Inactivation gate resides on the intracellular loop between third and the forth domains. It has a hydrophobic sequence motif, isoleucine-phenylalanine-methionine (IFM) that can interact and block the inner mouth of the protein. Intracellular and extracellular loops also carry post-translational modification sites as phosphorylation and glycosylation sites which are subtype specific and important in modulation of channel kinetics (Yu and Catterall, 2003; Diss *et al*., 2004). Some of α subunit transcripts are also subjected to alternative splicing giving rise to several distinct variants; however, their functional roles have not been identified (Diss *et al*., 2004).

Modulatory β subunits are composed of a transmembrane segment, an intracellular domain and a N-terminal glycosylated extracellular domain (Koopmann *et al.*, 2006; Diss *et al.*, 2004). Their exact interaction motifs with α subunits have not been determined yet. They are thought to modulate the activation and inactivation rates, voltage dependance and gating modes of the VGSCs. They may also mediate functional channel expression and interaction with extracellular matrix and other modulatory molecules (Diss *et al.*, 2004) (Figure 1.6).

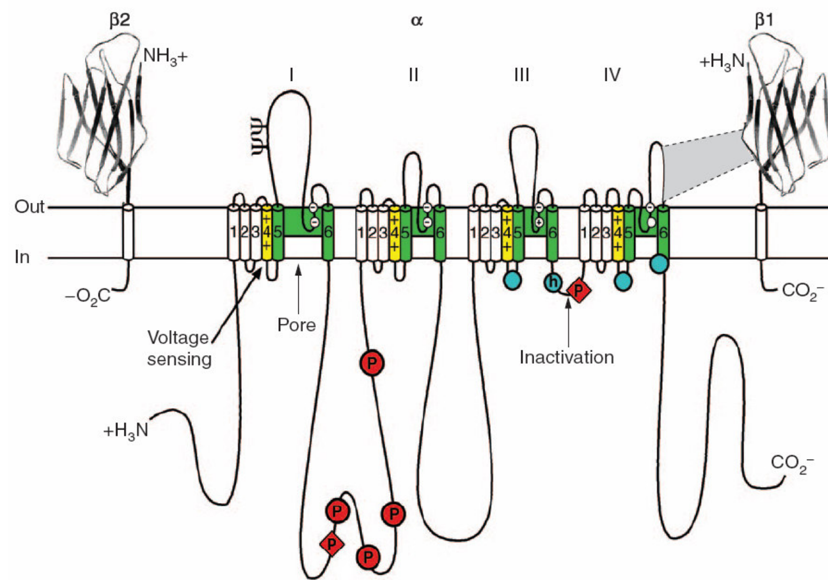

Figure 1.6. Structure of voltage-gated sodium channel Na_v1.2. Green: pore-forming parts, yellow: sensors, blue: inactivation gate, red: phosphorylation sites for phosphokinase A and C, Ψ: N-linked glycosylation site, white circles in the re-entrant loops: ion selectivity filter (Yu and Catterall, 2003)

VGSCs are members of a superfamily of ion channels that include voltage gated potassium, voltage-gated calcium and cyclic-nucleotide-gated channels. Evolutionary analysis showed that VGSC genes can be separated into four groups, clustered on a different chromosomes (Yu and Catterall, 2003) (Figure 1.7). All VGSC genes share highly conserved parts as voltage sensors, pore-forming regions and domain 3-4 linker regions, but all possess unique amino acid sequences in modulatory regions as N-terminus, D1 S5-6 linker, D1-2 linker, D2-3 linker and C-terminus (Diss *et al.*, 2004). Subtle changes in sequences creates differences in channel kinetics of distinct α subunits (Figure 1.8, 1.9).

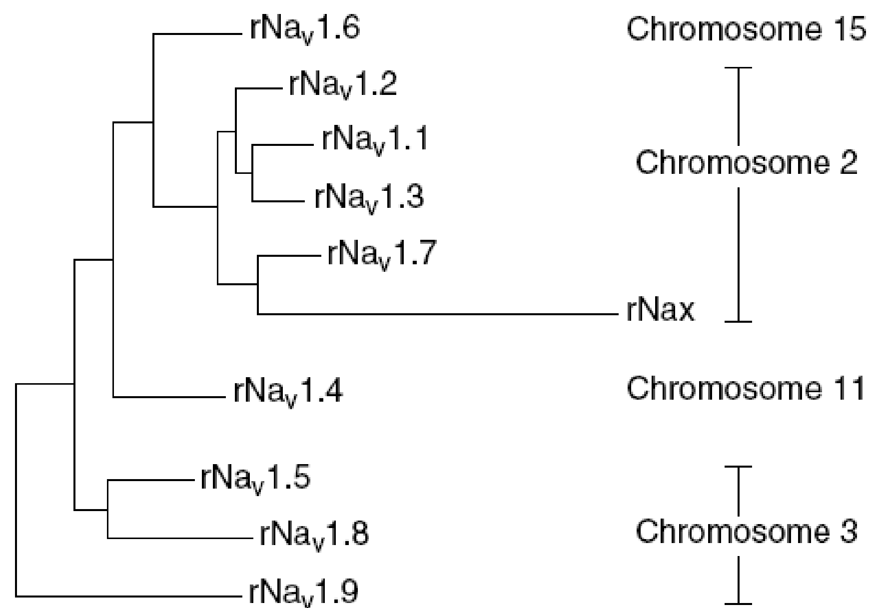

Figure 1.7. Chromosomal locations of rat VGSC genes. rNa_x is also located in chromosome 2; however, it is thought that it acts as a salt sensor rather than a voltage-gated sodium channel (Yu and Catterall, 2003)

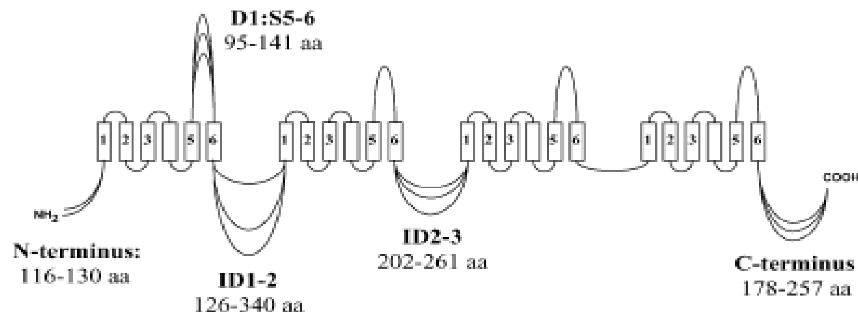

Figure 1.8. Localization of sequence differences in α subunits (Diss *et al*., 2004)

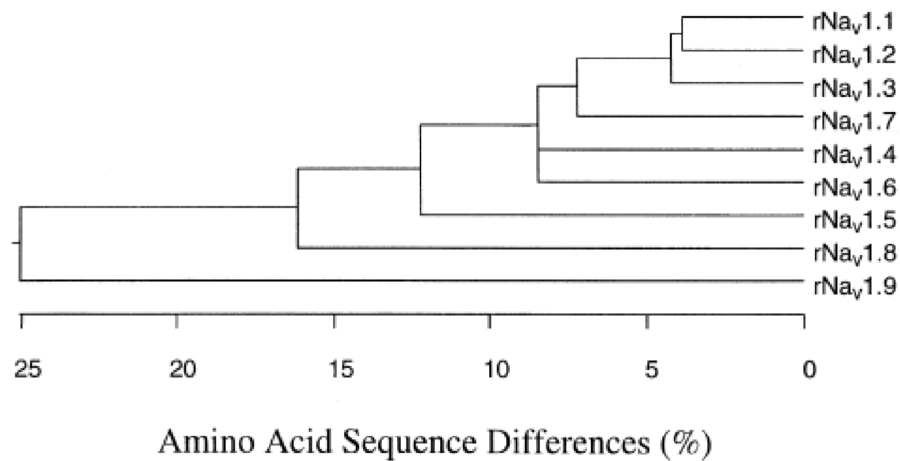

Figure 1.9. Amino acid sequence similarity of rat voltage-gated sodium channel α subunits (Goldin *et al.*, 2000)

1.1.2.2. GEFS+. First described by Scheffer and Berkovic in 1997, GEFS+ (OMIM 604233) is a group of familial autosomal dominant IGEs with febrile seizures that begin in early childhood and persist beyond 6 years of age as febrile seizures or afebrile generalized tonic-clonic seizures (Wallece *et al.*, 1998; Harkin *et al.*, 2002). Types of GEFS+ are classified according to mutations observed in different genes (Table 1.5) or by phenotypic differences such as mild in GEFS+ or severe in DS. The first mutation identified in a GEFS+ family was in the SCN1B gene (Wallace *et al.* 1998) and hence the GEFS+ phenotype was named as Type 1 or GEFS+1. However, only four mutations have been found to be associated with GEFS+1 so far (Sheffer *et al.*, 2007). SCN2A and GABRD mutations were also found in few cases and named Type 2 and Type 5, respectively (Sun *et al.*, 2008). 2p24 have been found to be a susceptibility locus for GEFS+4 (Audenaert *et al.*, 2005). SCN1A and GABRAG2 mutations are more frequently seen in GEFS+2 and GEFS+3 families, respectively. (Mulley *et al.*, 2005 and Sun *et al.*, 2008).

Table 1.5. GEFS+ types and their related genes

GEFS+ Type	Related Gene
GEFS+1	SCN1B
GEFS+2	SCN1A and SCN2A
GEFS+3	GABRAG2

Table 1.5. GEFS+ types and their related genes
(continued)

GEFS+4	2p24 (susceptibility locus)
GEFS+5	GABRD

Mutations in SCN1A was also found to be related with DS, the most severe disorder in the GEFS+ spectrum. (Claes *et al*., 2001). However, currently, there is no clear genotype-phenotype correlation.

1.1.2.3. SCN1A and DS. Spanning approximately 100 kb, SCN1A gene is localized on the long arm of chromosome 2 (2q24) and consists of 26 exons. It is transcribed from the antisense strand. Alternative splicing of SCN1A transcript gives rise to two isoforms, 1998 and 2009 amino acid-sodium channel protein α subunit subtype one (Na_v 1.1) that is approximately 227 kDa and the longer isoform carries 33 more nucleotide pairs in the 3' end of exon 11. Relative expression of these isoforms in neurons have not been determined. In addition, no mutations residing in this 11 amino-acid region in the full length isoform have been reported (Mulley *et. al*., 2005)

First described by Dravet in 1978, DS (OMIM 607208) also called as SMEI is an autosomal domiant disorder characterized by prolonged fever-sensitive and refractory generalized clonic, tonic-clonic seizures or unilateral seizures beginning in the first year of life (Ceulemans *et al*., 2004; Fujiwara, 2006). Early psychomotor development is normal but patients develop developmental stagnation and mental retardation. Patients become ataxic and speech development is delayed. EEG is normal at the beginning but later on positive intermittent light stimulation can be observed with epileptic features as multifocal spikes, spike waves and polyspike waves (Claes *et al*., 2001; Ceulemans *et al*., 2004).

The prevalance of SCN1A mutations in DS is eighty percent (Ceulemans *et al*., 2004). Ninety percent of the mutations causing DS are *de novo* mutations in affected individuals. Approximately half of the mutations in DS patients result in truncated protein showing haploinsufficiency for SCN1A. Some of the missense mutations give rise to loss of function of Na_v 1.1 protein. Depending on the mutation location, distinct physiological effects can be

seen in functional studies of particular mutations (Kearney *et al*., 2006). DS patients who do not carry a mutation in SCN1A were also examined for relevant genes in GEFS+ such as SCN1B, SCN2A, GABRG2 and SCN2B; however, no mutations were found except a GABRG2 nonsense mutation in a proband with DS in GEFS+ family suggesting polygenic etiology for DS in that family (Harkin *et al*., 2002; Mulley *et al*., 2005). Deletions covering the whole gene or several adjacent exons of the SCN1A were also found in several patients with DS who do not carry a point mutation or a microdeletion in SCN1A (Suls *et al*., 2006; Mulley *et al*., 2006). Recently, a balanced translocation truncating SCN1A gene in an ex-patient diagnosed with DS has been determined in cytogenetic analysis and postmortem flouresan in situ hybridization (FISH) analysis (Møller *et al*. 2008). In addition, further parental analysis in three independent families with sib pairs having DS with SCN1A mutations and with healty parents not having SCN1A mutations showed that one of the parents in each family is mosaic carrier of the particular SCN1A mutation suggesting the importance of further analysis of parents (Depienne *et al*., 2006; Morimoto *et al*., 2006).

In general patients with DS are resistant to all forms of pharmacotheraphy (Claes *et al*., 2001; Ceulemans *et al*., 2004). Long term follow up of patients and twin studies may shed light on the effect of mutations on the onset and prognosis of DS and genotype-phenotype relationships (Fujiwara *et al*., 2006).

2. PURPOSE

LD, a type of PME, and DS as part of the IGE spectrum diseases are the most devastating examples of epilepsy syndromes. These diseases are not easily diagnosed clinically and distinguished from other syndromes at early onset, which is very important for disease prognosis and treatment strategies. The current knowledge on their genetic etiology however, gives additional information and may lead to a distinguishable diagnosis when the relevant genes are screened for mutations. Until now, prevalance of these diseases is not known and mutational analysis of the caausative genes have not been carried out in the Turkish population. Although a rare disorder, LD caused by either EPM2A or EPM2B gene mutations is common in Mediterranean countries and may be a relatively common PME in Turkey, as well. Occuring worldwide, DS is the most severe form of epilepsy syndromes in the GEFS+ spectrum. Resistance to certain drugs hinders the therapy and prevention of epileptic seizures. The definition and classification of IGE syndromes is continuously re-evaluated and both DS and GEFS+ are relatively recently described syndromes without a well known molecular pathology. SCN1A gene mutations have been found to be the most relevant cause of both DS and GEFS+. However, there are no direct genotype-phenotype correlations due to the complexity of the epilepsy phenotypes. The correlation if there is any is generally irrespective of the mutation type or location but possibly due to whether the mutation has a major or minor effect on the phenotype as well as the genotypes of other genes responsible from the phenotype. As more data on the molecular pathology of these syndromes accumulate, the progress in defining and distinguishing epilepsy syndromes would certainly improve. The purpose of this thesis therefore, was to establish the methods for mutational analysis of the EPM1A, EPM2A, EPM2B and SCN1A genes and initiate the mutational analysis in patients with LD and DS to elicit the mutation profiles and the prevalence of these diseases in Turkey.

3. MATERIALS

3.1. Blood Samples

Blood Samples of patients with Lafora Disease and Dravet's Syndrome were provided by clinicians from the Department of Neurology, Cerrahpaşa Medical School, Istanbul University, İstanbul; Department of Pediatrics, GATA Haydarpaşa Teaching Hospital, İstanbul; Department of Neurology, Medical School, Ege University, İzmir; and Department of Neurology, Medical School, Kocaeli University, İzmit. Informed consent was obtained from all patients.

3.2. Oligonucleotide Primers

Primers for EPM1A exons were designed using SDSC Biology Workbench program available on the World Wide Web (http://workbench.sdsc.edu/) and obtained from Sigma Ark, Germany. Primer sequences for EPM1A dodecamer repeat were taken from Lalioti *et al*., 1998 and obtained from IDT, USA (Table 3.1 and Table 3.2). Primer sequences used in amplification of EPM2A, EPM2B and SCN1A were taken from Gómez-Garre *et al*. 2000, Singh *et al*. 2005 and Wallace *et al*., 2001 respectively (Table 3.3, Table 3.4 and Table 3.5). They were purchased from Operon, USA.

Primer pairs used in further identification of the molecular defect in patients were designed using SDSC Biology Workbench program available on the World Wide Web (http://workbench.sdsc.edu/) and purchased from Operon, USA (Table 3.6).

3.3. Enzymes

Taq DNA Polymerase	:	5 U/µl Roche, Germany and Qiagen, USA
Pfu DNA Polymerase	:	2-3U/µl Promega, USA

3.4. Buffers and Solutions

3.4.1. DNA Extraction

Ethanol (EtOH)	:	Absolute EtOH, Riedel de Haen, Germany
Lysis Buffer	:	155 mM NH_4Cl 10 mM $KHCO_3$ 1 mM Na_2EDTA (pH 7.4)
Nuclease Buffer	:	10 mM Tris-HCL (pH 8.0) 400 mM NaCl 2 mM Na_2EDTA (pH 7.4)
Proteinase K	:	20 mg/ml in dH_2O
Sodiumdodecylsulphate (SDS)	:	10 per cent SDS (w/v) (pH 7.2)
Sodium Acetate (CH_3COONa)	:	3 M CH_3COONa
Sodium Chloride (NaCl)	:	2.5 M NaCl
TE Buffer	:	20 mM Tris-HCl (pH 8.0) 1 mM Na_2EDTA (pH8.0)

3.4.2. Polymerase Chain Reaction (PCR)

10X $MgCl_2$ Free Buffer	:	100 mM Tris-HCl 500 mM KCl Roche, Germany

10X PCR Reaction Buffer	:	100 mM Tris-HCl 500 mM KCl 25 mM $MgCl_2$ Roche, Germany and Qiagen, USA
10X PCR Reaction Buffer	:	200 mM Tris-HCl (pH 8.8 at 25 °C) 100 mM KCl 100 mM $(NH_4)_2SO_4$ 20 mM $MgSO_4$ 1% Triton X-100 1mg/ml nuclease-free BSA Promega, USA
Magnesium Chloride ($MgCl_2$)	:	25 mM in dH_2O Roche, Germany
Deoxyribonucleotides (dNTP)	:	100 mM of each dNTP Promega, USA
Deoxyribonucleotides (7-deaza-dGTP)	:	Roche, Germany
Dimethylsulphoxide (DMSO)	:	Stock solution Sigma, Germany

3.4.3. Agarose Gel Electrophoresis

10X TBE Buffer	:	0.89 M Tris-Base 0.89 M Boric acid 20 mM Na_2EDTA (pH 8.3)
Ethidium Bromide (EtBr)	:	10 mg/ml Sigma, Germany
1 or 2 per cent Agarose Gel	:	1 or 2 per cent agarose in 0.5X TBE Buffer, containing 0.5ug/ml Ethidium bromide

10X Loading Dye	:	2.5 mg/ml Bromophenol Blue (BPB) 1 per cent SDS in glycerol
DNA Ladder	:	100 bp, MBI Fermentas, Lithuania 100 bp Roche, Germany

3.5. Kits

DNA extraction kit	:	MagNa Pure Compact Nucleic Acid Isolation Kit-I Large Volume Roche, Germany
PCR purification kit	:	QIAquick PCR purification kit Qiagen,USA High Pure PCR Product Purification kit Roche, Germany

3.6. Equipment

Autoclave	:	ASB270NT Astell, UK Midas 55, Prior Clave London,UK
Balances	:	Electonic Balance ModelVA124, Gec Avery, UK Electronic Balance Model CC081, Gec Avery, UK
Centrifuges	:	Centrifuge 5415C, Eppendorf, Germany Microfuge 18, Beckman Coulter, Germany Universal 16R, Hettich Zentrifugen, Germany Allegra X-22R, Beckman Coulter, Germany
Deep Freezers	:	(-20 ºC) Bosch, Germany (-70 ºC) Freezer Harris,UK

Documentation System	:	GelDoc Documentation System, BIO-RAD, USA
Electrophoretic Equipments	:	Horizon 58, Model 200, BRL, USA Thermo Minicell Primo E320 Electrophoretic Gel System, USA
Incubators	:	EN 400, Nuve, Turkey 56 ºC, LEEC, UK
Magnetic Stirrer	:	Chiltern Hotplate Magnetic Stirrer HS31, UK
MagNa Pure Compact	:	MagNa Pure Compact Nucleic Acid Purification System Roche, Germany
Ovens	:	Drying Oven, Maxo 156, UK Microwave Oven, Vestel, Turkey
Pipettes	:	Hamilton, USA Rainin, USA
Power Supplies	:	EC 250-90 Thermo, USA EC 1000-90 Thermo, USA Model 200, BRL, USA
Refrigerator	:	4ºC Medicool, Sanyo, Japan Arçelik 4042T, Turkey
Spectrophotometer	:	Nanodrop ND-1000, USA
Thermocyclers	:	Techne TC-512, UK Techne TC-3000, UK
UV Transilluminator	:	Chromato-Vue Transilluminator

		Model 1-TM-20 UVP USA
Water Bath	:	Memmert, Germany
Water Purification	:	WaTech Water Technologies, Turkey
Vortex	:	WhirliMixer Fisons, UK

Table 3.1. Oligonucleotide primer pairs used in PCR and sequencing of EPM1A exons

Primer Name	Primer Sequence
CSTB Ex.1f CSTB	F-5' GCCGAGTCCCCTCGCCAGATT R-5' CAAAGCGGCTTCTTTCGCTCC
CSTB-4R(Ex.1)	R-5' CGCCCTGAGGCTAAGGCAGG
CSTB Ex.2f CSTB Ex.2r	F-5' CAGAATCGTGTGTGTGCTCACAT R-5' GGCCTGCACAGGCGGTTTCCTA
CSTB Ex.3f CSTB Ez.3r	F-5' GTGACTTGGGATCAGAGGCTT R-5' CTGGTAGACGGAGGATGACTT

Table 3.2. Oligonucleotide primer pairs used in PCR of EPM1A dodecamer repeats

Primer Name	Primer Sequence
8L	F-5' CTGCAGGATTGCCCCTACTCCGACTG
17R	R-5' CTGGTCGGCGATGTGCTGGGTCTC
10R	R-5' GGGGTCACGTGACGCGCGGGCGGAA

Table 3.3. Oligonucleotide primer pairs used in PCR and sequencing of EPM2A exons

Primer Name	Primer Sequence
EPM2A.ex.1F EPM2A.ex.1R	F-5' AACAGCACGTGTTCTCCCCG R-5' AGGGACGCGGGCAAAAAGC
EPM2A.ex.2F EPM2A.ex.2R	F-5' GTATCAGCTGCTTGAGGATA R-5' CTTGTCCTACTTCTATGCCTA
EPM2A.ex.3F EPM2A.ex.3R	F-5' CTACATGTTTTATGCAGCTCC R-5' ATTTATTCCATTTCTACCATTCAT
EPM2A.ex.4F EPM2A.ex.4R	F-5' GAGAGAGCCTCTGGCCTC R-5' GGCTCCTTAGGGAAATCAGG

Table 3.4. Oligonucleotide primer pairs used in PCR and sequencing of EPM2B exon

Primer Name	Primer Sequence
EPM2BF1 EPM2BR1	F-5’ TGACCATGACTGTGACCGTGA R-5’ GCTGAGCCCAGGAGCTCTATG
EPM2BF2 EPM2BR2	F-5’ GGTGCTGCACCTCATAGAGCT R-5’ GACAACCACATGGCAGTCGTT
EPM2BF3 EPM2BR3	F-5’ ATGTCACCATCACCAACGACT R-5’ AGGTATCCACTTGGCCGACAA
EPM2BF4 EPM2BR4	F-5’ TCAAGTATGCAGCTTGTCGGC R-5’ AACAATTCATTAATGGCAGCACTAGTG

Table 3.5. Oligonucleotide primer pairs used in PCR and sequencing of SCN1A exons

Primer Name	Primer Sequence
SCN1A-EX1F SCN1A-EX1R	F-5’ CCTCTAGCTCATGTTTCATGA R-5’ TGCAGTAGGCAATTAGCAGC
SCN1A-EX2F SCN1A-EX2R	F-5’ CTAATTAAGAAGAGATCCAGTGACAG R-5’ GCTATAAAGTGCTTACAGATCATGTAC
SCN1A-EX3F SCN1A-EX3R	F-5’ CCCTGAATTTTGGCTAAGCTGCAG R-5’ CTACATTAAGACACAGTTTCAAAATCC
SCN1A-EX4F SCN1A-EX4R	F-5’ GGGCTACGTTTCATTTGTATG R-5’ GCAAACCTATTCTTAAAAGCATAAGCACTG
SCN1A-EX5F SCN1A-EX5R	F-5’ AGGCTCTTTGTACCTACAGC R-5’ CATGATGGGTCCGTCTCATT
SCN1A-EX6F SCN1A-EX6R	F-5’ CACACGTGTTAAGTCTTCATAGT R-5’ AGCCCCTCAAGTATTTATCCT
SCN1A-EX7F SCN1A-EX7R	F-5’ GAACCTGACCTTCCTGTTCTC R-5’ GTTGGCTGTTATCTTCAGTTTC
SCN1A-EX8F SCN1A-EX8R	F-5’ GACTAGGCAATATCATAGCATAG R-5’ CTTTCTACTATATTATCATCCGG
SCN1A-EX9F SCN1A-EX9R	F-5’ TTGAAAGTTGAAGCCACCAC R-5’ CCACCTGCTCTTAGGTACTC
SCN1A-EX10F SCN1A-EX10R	F-5’ GCCATGCAAATACTTCAGCCC R-5’ CACAACAGTGGTTGATTCAGTTG
SCN1A-EX11aF SCN1A-EX11aR	F-5’ TGAATGCTGAAATCTCCTTCTAC R-5’ CTCAGGTTGCTGTTGCGTCTC
SCN1A-EX11bF SCN1A-EX11bR	F-5’ GATAACGAGAGCCGTAGAGAT R-5’ TCTGTAGAAACACTGGCTGG
SCN1A-EX12F SCN1A-EX12R	F-5’ CATGAAATTCACTGTGTCACC R-5’ CAGCTCTTGAATTAGACTGTC
SCN1A-EX13aF SCN1A-EX13aR	F-5’ ATCCTTGGGAGGTTTAGAGT R-5’ CATCACAACCAGGTTGACAAC
SCN1A-EX14F SCN1A-EX14R	F-5’ CATTGTGGGAAAATAGCATAAGC R-5’ GCTATGCAGAACCCTGATTG
SCN1A-EX15aF SCN1A-EX15aR	F-5’ TGAGACGGTTAGGGCAGATC R-5’ AGAAGTCATTCATGTGCCAGC

Table 3.5. Oligonucleotide primer pairs used in PCR and sequencing of SCN1A exons (continued)

SCN1A-EX15bF SCN1A-EX15bR	F-5' CTGCAAGATCGCCAGTGATTG R-5' ACATGTGCACAATGTGCAGG
SCN1A-EX16aF SCN1A-EX16aR	F-5' GTGGTGTTTCCTTCTCATCAAG R-5' TCTGCTGTATGATTGGACATAC
SCN1A-EX16bF SCN1A-EX16bR	F-5' CAACAGTCCTTCATTAGGAAAC R-5' ACCTTCCCACACCTATAGAATC
SCN1A-EX17F SCN1A-EX17R	F-5' CTTGGCAGGCAACTTATTACC R-5' CAAGCTGCACTCCAAATGAAAG
SCN1A-EX18F SCN1A-EX18R	F-5' TGGAAGCAGAGACACTTTATCTAC R-5' GTGCTGTATCACCTTTTCTTAATC
SCN1A-EX19F SCN1A-EX19R	F-5' CCTATTCCAATGAAATGTCATATG R-5' CAAGCTACCTTGAACAGAGAC
SCN1A-EX20F SCN1A-EX20R	F-5' CTACACATTGAATGATGATTCTGT R-5' GCTATATACAATACTTCAGGTTCT
SCN1A-EX21aF SCN1A-EX21aR	F-5' ACCAGAGATTACTAGGGGAAT R-5' CCATCGAGCAGTCTCATTTCT
SCN1A-EX21bF SCN1A-EX21bR	F-5' ACAACTGGTGACAGGTTTGAC R-5' CTGGGCTCATAAACTTGTACTAAC
SCN1A-EX22F SCN1A-EX22R	F-5' ACTGTCTTGGTCCAAAATCTG R-5' TTCGATTAATTTTACCACCTGATC
SCN1A-EX23F SCN1A-EX23R	F-5' AGCACCAGTGACATTTCCAGC R-5' GGCAGAGAAAACACTCCAAGG
SCN1A-EX24F SCN1A-EX24R	F-5' GACACAGTTTTAACCAGTTTG R-5' TGTGAGACAAGCATGCAAGTT
SCN1A-EX25F SCN1A-EX25R	F-5' CAGGGCCAATGACTACTTTGC R-5' CTGATTGCTGGGATGATCTTGAATC
SCN1A-EX26aF SCN1A-EX26aR	F-5' CGCATGATTTCTTCACTGGTTGG R-5' GCGTAGATGAACATGACTAGG
SCN1A-EX26bF SCN1A-EX26bR	F-5' TCCTGCGTTGTTTAACATCGG R-5' ATTCCAACAGATGGGTTCCCA
SCN1A-EX26cF SCN1A-EX26cR	F-5' TGGAAGCTCAGTTAAGGGAGA R-5' AGCGCAGCTGCAAACTGAGAT
SCN1A-EX26dF SCN1A-EX26dR	F-5' CCGATGCAACTCAGTTCATGGA R-5' GTAGTGATTGGCTGATAGGAG
SCN1A-EX26eF SCN1A-EX26eR	F-5' AGAGCGATTCATGGCTTCCAATCC R-5' TGCCTTCTTGCTCATGTTTTTCCACA
SCN1A-EX26fF SCN1A-EX26fR	F-5' CCTATGACCGGGTGACAAAGCC R-5' TGCTGACAAGGGGTCACTGTCT

Table 3.6. Additional oligonucleotide primer pairs

Primer Name	Primer Sequence
SCN1A-exon10Fint	F-5' ATCTCCAAAAGCCTTCATTAGG
SCN1A-INTRON9-1F SCN1A-INTRON9-1R	F-5' TGAGTACCTAAGAGCAGGTGG R-5' CATGGGCATGGAATAAAACA
SCN1A-INTRON9-2F SCN1A-INTRON9-2R	F-5' GGTTACCCTGGGAACAATGA R-5' CCGTTGCTGCCTGCTATATT

4. METHODS

4.1. Analysis of Genomic DNA

4.1.1. DNA Extraction from White Blood Cells

Ten ml blood samples from patients and their relatives were collected into K’EDTA containing tubes to prevent coagulation before DNA extraction. They were stored at 4ºC or -20ºC. For DNA extraction, the samples were taken into sterile Falcon centrifuge tubes, and after addition of 30 ml ice cold lysis buffer they were kept at 4ºC for 15 minutes to allow lysis of leukocyte membranes. In order to collect the nuclei, the samples were centrifuged at 5000 rpm for 10 minutes. Centrifugation step was performed at 4ºC. The supernatant was discarded and centrifugation was repeated with 10 ml lysis buffer to wash the nuclear pellet. Then, the DNA was extracted using NaCl (salting out) extraction. In NaCl extraction method, nuclear pellet was resuspended in 3 ml of nuclei lysis buffer in order to lyse nuclear envelope of leukocytes. After the addition of 30 µl proteinase K (20 µg/ml) and 50 µl SDS (0.10 per cent, pH 7.2), the samples were incubated at 37ºC overnight or 56ºC for 3 hours for degradation of cellular proteins. The protein residues were salted out by adding 10 ml of 2.5 M NaCl. The samples were centrifuged at 5000 rpm at room temperature for 30 minutes. The supernatant was taken into a sterile Falcon tube and DNA was precipitated with two volumes of absolute EtOH. The precipitated DNA was fished out with a pipette. After evaporation of ethanol, DNA was dissolved in TE buffer in an eppendorf tube and stored at 4ºC (Miller *et al.*, 1988).

If DNA could not be fished out, further extraction methods were performed by the addition of absolute ethanol and DNA was kept at - 70 ºC for at least one day. Samples then were thawed at room temperature and were centrifugated at 5000 rpm for 30 minutes at 24 ºC. Supernatants were discarded and pellets were left to dry. They were dissolved in TE buffer and samples were taken into a sterile ependorf tube. As extracted DNA was generally precipitated with high amount of salt in this method, we carried out a purification step before using it. In this method, 55 µl DNA in TE extracted by -70 ºC protocol was taken to a sterile

ependorf tube. After addition of 5 µl 3M sodium acetate and 100 µl 95% ethanol, it was mixed and centrifuged at maximum speed for 1 minute. Supernatant was discarded and 100 µl 70% ethanol was added to wash it. Then, it was centrifugated at maximum speed for 1 minute. Liquid part was discarded and the sample was left to dry. It was dissolved in 50 µl TE buffer or 25 µl dH_2O.

4.1.2 DNA Extraction from White Blood Cells by MagNaPure Compact System

One ml blood samples from patients and their relatives collected into K'EDTA containing tubes were taken into sample tubes provided by the MagNa Pure Compact Nucleic Acid Isolation Kit I- Large Volume (Roche, Germany). Sample tubes, disposable tip trays, reagent cartridges, elution tubes were placed in the equipment after their barcodes were read by handheld barcode scanner. Pure DNA was extracted automatically in 45 minutes. Elution tube caps were placed and DNA was stored at 4ºC .

4.1.3. Analysis by Agarose Gel Electrophoresis

One per cent agarose gel was prepared by boiling in 100 ml of 0.5X TBE buffer. After the solution was cooled down to 55ºC, 5 µl EtBr (10 mg/ml) was added for visualization of DNA under ultra violet (UV) light. The gel was poured onto the electrophoresis plate and the comb was placed in the gel. The gel was left at room temperature to polymerize. For loading, DNA samples were mixed with 10X loading dye to a final concentration of 1X. The gel was run at 150 volts for 15-20 minutes.

4.1.4. Analysis by Spectrophotometer

The concentration of genomic DNA was also measured by a spectrophotometer. Nucleic acid application module and double stranded DNA measurement (DNA-50) were selected in the software of NanoDrop program and required cleaning was carried out by dH_2O. As NanoDrop accurately measures DNA concentrations up to 3700 ng/ µl, we directly apply DNA samples to the light path. 1 µl of the sample is sufficient for analysis. The program directly gives the concentration of the sample. Additionally, it also gives 260/280 and 260/230 absorbance ratios that are important in determining the purity and secondary purity values of

the sample respectively. The samples having an 260/280 ratio of 1.8 and 260/230 ratio of 1.8 to 2.2 were considered as being pure.

4.2. Investigations of Mutations

4.2.1. PCR

PCR amplification conditions of each exon in the EPM1A, EPM2A, EPM2B and SCN1A genes were optimized using a gradient thermal cycler. Depending on the melting temperatures of primer pairs, a temperature spectrum for each exon was determined using the program in the gradient thermal cycler. Several of these temperatures were then chosen and amplifications were done using them as annealing temperatures. Optimization of SCN1A gene exon 26f is given as an example in Figure 4.1. The gradient temperature spectrum was determined to be 60°C since the melting temperature (T_m) of primers were 66.4°C and 64.54°C. The products were visualized on 2% agarose gels. In general, the highest temperature was chosen as the annealing temperature when all amplifications gave similar results in order to prevent the amplification of unspecific products.

a) b)

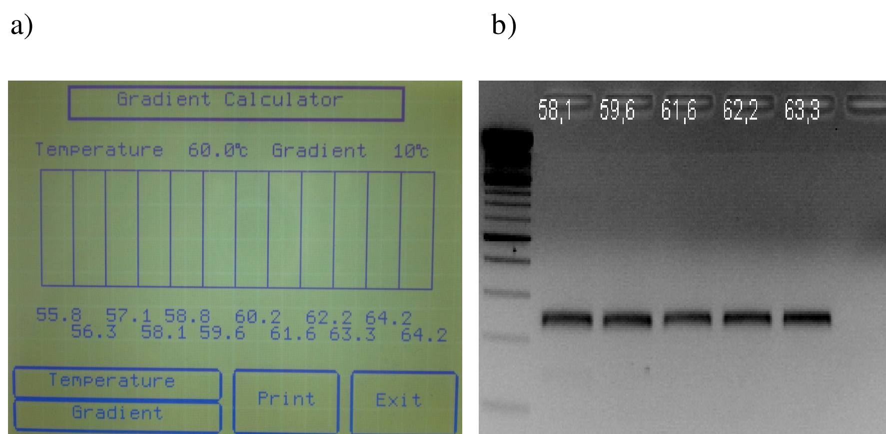

Figure 4.1. The gradient thermal cycler and optimization of annealing temperatures. (a) The gradient temperature spectrum. (b) 242 bp-PCR products of SCN1A exon 26f obtained at 5 temperatures selected. First lane contains 100 bp marker and last lane contains no DNA negative control.

After appropriate annealing temperatures were found, DMSO, DNA, primer and $MgCl_2$ concentrations were adjusted for optimum amplification of each exon. Different enzymes and its buffers were used for optimization if necessary. As some exons are much longer than the sequencing range, namely the only exon of EPM2B and exon 11, 13, 15, 16, 21, 26 of SCN1A, they were divided into pieces of appropriate lengths and amplified using overlapping primers in separate PCRs (Wallace et al., 2001) . PCR program for amplification of all exons were 35 cycles; denaturation at 95°C for 30 seconds, annealing with variable temperatures for 25 seconds and final extension at 72°C for 30 seconds.

All exons were optimized to be amplified in the presence of 1X PCR buffer with $MgCl_2$ and 0.2mM dNTP in total volume of 25 μl. Amount of dH_2O, primer pairs (Table 3.1 - 3.5), DMSO, $MgCl_2$ and DNA concentrations were variable between exons.

All exons of EPM1A was amplified in the presence of 0.2 mM of each primer, 10% DMSO, 0.5 U Qiagen Taq polymerase and 8 ng of genomic DNA. Annealing temperatures of exon 1-3 were 59.6°C, 62.2°C and 56.6°C, respectively.

Exon 1-4 of EPM2A required 0.2mM of each primer and 1U Roche Taq Polymerase. 8 ng genomic DNA was used in amplification of exon 1-3 of EPM2A, while 32 ng of genomic DNA was used in amplification of exon 4. Additional 1.5 mM $MgCl_2$ was used for amplification of exon 1 and exon 4 and exon 4 required 10% DMSO. Annealing temperatures of exon 2 and 3 were 55.2°C, while of exon 1 and exon 4 were 63.3°C and 61.3°C, respectively.

The only exon of EPM2B was amplified in 4 separate reactions. All fragments were amplified in the presence of 0.2 mM of each primer, 10% DMSO, 1 U Roche Taq polymerase and 8 ng of genomic DNA. Annealing temperature of the first two fragments of EPM2B exon were 58.2°C and temperatures of the last two fragments were 57.1°C and 59.6°C, respectively.

Twenty six exons of SCN1A were amplified in 36 separate reactions. All exons required 1U Roche Taq polymerase. 8 ng genomic DNA was used in all exons but for the fragments of exon 13 and exon 19, 7 ng of genomic DNA was used. While 0.16 mM of each primer was

added to the reaction of exons 3, 23 and 25, 0.2 mM of each primer was added to the rest. All exons but exon 3 required additional $MgCl_2$. The two fragments of exon 15 required additional 0.5 mM $MgCl_2$, while the other exons required additional 1.5 mM $MgCl_2$. 10% DMSO was used in amplification of Exon 5, 6, 9, 10, 21a, 23, 25, 26b, 26d, 26e and 26f. Annealing temperatures were 51.3°C for exon 3 and 24; 52.1°C for exon 12; 53.8°C for exon 5, 6, 9 and 11a; 54.6°C for exon 22, 26a, 26c and 26d; 54.9°C for exon 7; 55.1°C for exon 10, 13a, 16a and 19, 57.2°C for exon 8; 57.6°C for exon 15b, 58.3°C for exon 2, 4, 14, 18, 20, 21a, 21b, 25 and 26b; 59.2°C for exon 17; 59.6°C for exon 1, 11b and 16b; 60.2°C for exon 15a; 61.3°C for exon 13b and exon 23; 62.2°C for exon 26e and finally 63.3°C for exon 26f.

Amplifications for IVS 9 of SCN1A gene were carried out in three reactions in the presence of 1X PCR reaction buffer with $MgCl_2$, 1.5 mM additional $MgCl_2$, 0.2mM of each dNTP and 0.2 mM of each primer and 0.04U/µl Taq DNA polymerase in a total volume of 25 µl. 8 ng/ µl DNA was used in amplification of Part I and Part II of IVS 9, whereas 32 ng/ µl DNA was used in Part III. Part II and Part III required 10% DMSO for amplification. Annealing temperatures were 58.3°C for Part I and Part III and 60.2°C for Part II.

Amplification condition of EPM1A dodecamer repeat was previously optimized in our laboratory using a touchdown PCR program and specific amplification conditions (Lalioti et al., 1998).

4.2.2. Purification of PCR Products

The PCR products were purified using QIAquick PCR purification kit and Roche High Pure PCR Product Purification kit following the instructions of the manufacturer.

After elution, purified PCR products were checked on 2 per cent agarose gels, visualized under UV light. They were stored at -20°C until they were sent for sequencing.

4.2.3. DNA Sequence Analysis

Sufficient amount of purified products with 5 pmol forward or reverse primers were sent to İontek, İstanbul or Refgen, Ankara for automated sequencing. The results were obtained online as an ABI document.

The sequencing results were read according to the reference sequence in NCBI (EPM1A: P04080 - NC_000021.7 / 44018259-44020687; EPM2A: O95278-NC_000006.10 / 145988133 – 46098684; EPM2B: Q6VVB1 - NC_000006.10 / 18228697-18230830; SCN1A: P35498 - NC_000002.10 / 166553916-66638395). All of the coding regions in EPM1A, EPM2A, EPM2B and SCN1A genes were read successfully.

5. RESULTS

5.1. PCR Amplifications

PCR products of all amplified fragments of EPM1A, EPM2A, EPM2B and SCN1A genes are shown in Figure 5.1 – 5.5, respectively.

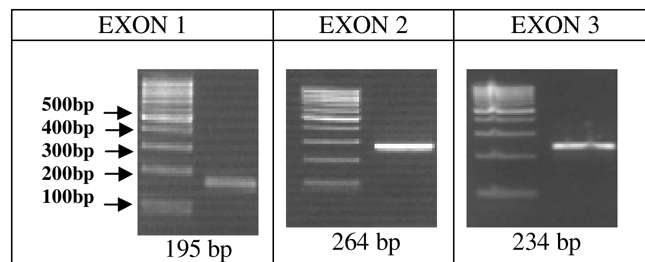

Figure 5.1. PCR products and sizes of EPM1A exons shown on agarose gels. First lane is 100 bp ladder DNA and the brightest band is 500 bp.

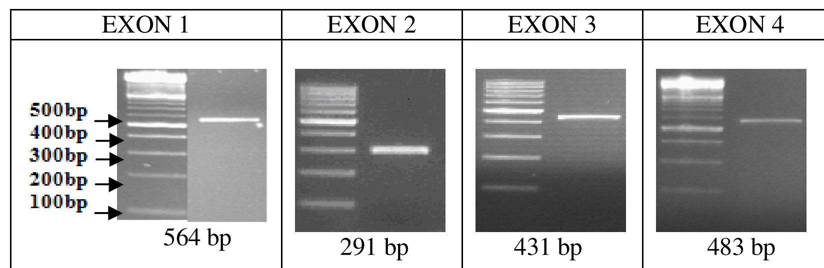

Figure 5.2. PCR products and sizes of EPM2A exons shown on agarose gels. First lane is 100 bp ladder DNA and the brightest band is 500 bp.

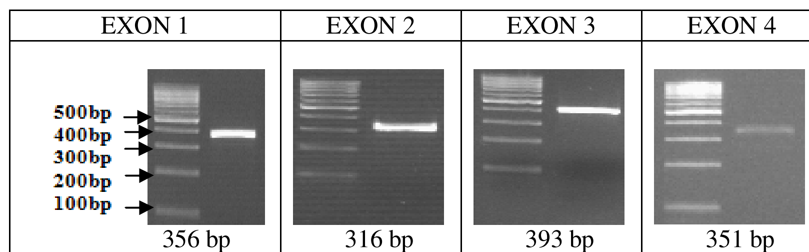

Figure 5.3. PCR products and sizes of EPM2B exon shown on agarose gels. First lane is 100 bp ladder DNA and the brightest band is 500 bp.

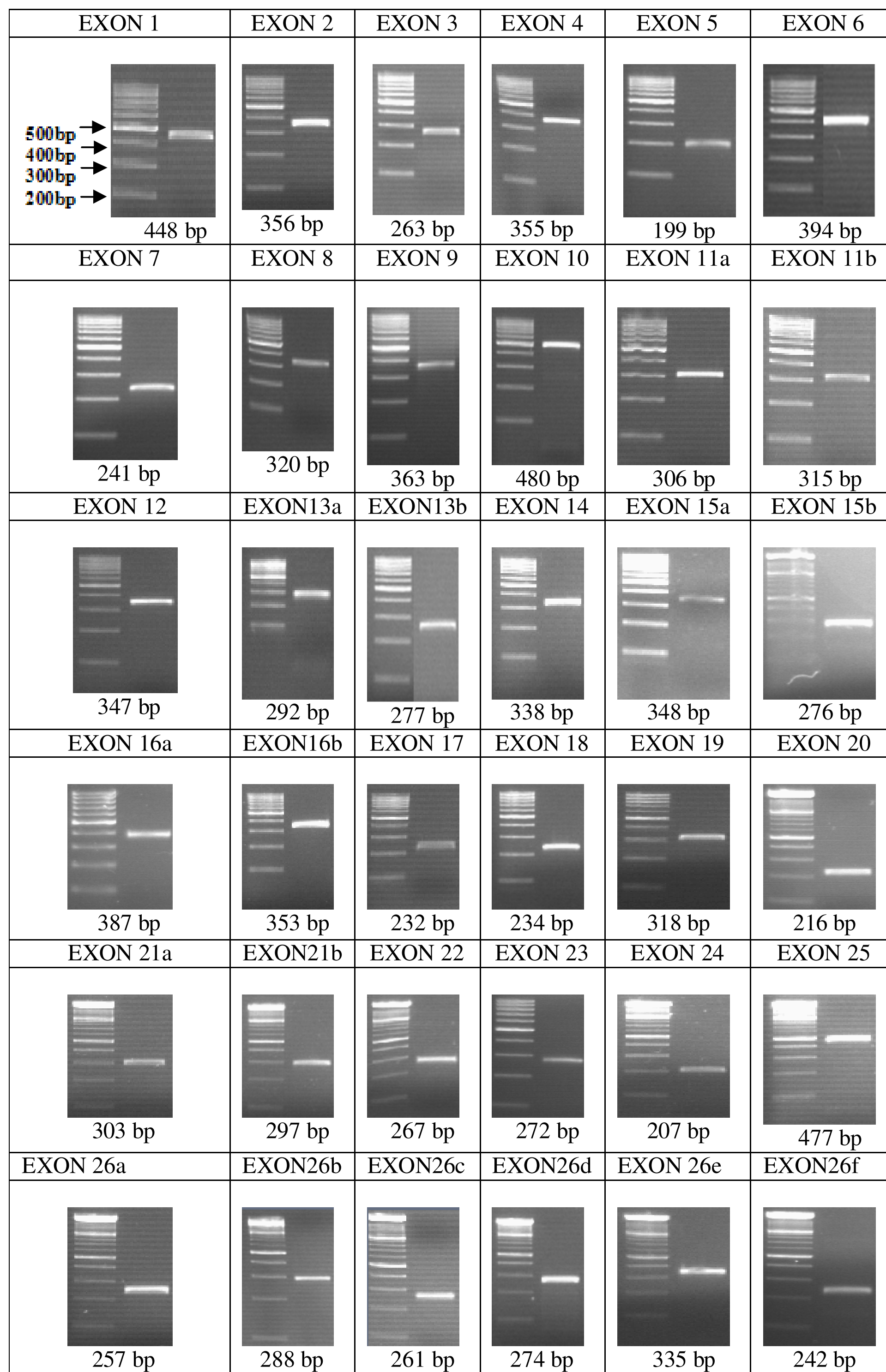

Figure 5.4. PCR products and sizes of SCN1A exons shown on agarose gels. First lane is 100 bp ladder DNA and the brightest band is 500 bp.

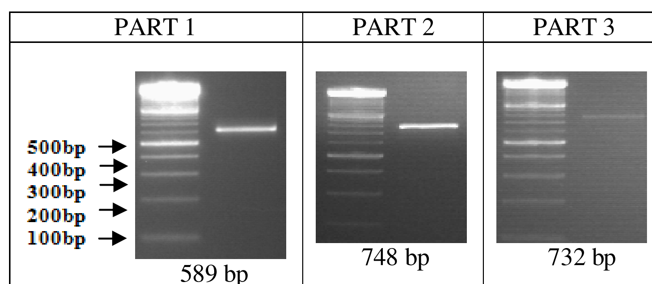

Figure 5.5. PCR products and sizes of SCN1A IVS9 shown on agarose gels.
First lane is 100 bp ladder DNA and the brightest band is 500 bp.

5.2. Mutational analysis of Lafora Disease Patients

Mutational analysis in the three genes EPM1A, EPM2A, EPM2B were carried out where appropriate for four patients suspected to have Lafora disease.

Patients 3PME5 (16 y.) and 3PME6 (10 y.) were siblings in a consanguineous family. Both had myoclonus, recurrent seizures, multifocal spike-wave discharges in EEG. The disease status was confirmed by Lafora bodies seen in skin biopsy in 3PME5. However, skin biopsy investigations of 3PME6 did not show any inclusion bodies. Parents and brother were phenotypically normal. Sequencing of EPM2A and EPM2B exons of both patients showed that their genotypes were identical (Table 5.1 and 5.2) and they both had a novel substitution (c.391 G>T) in homozygous condition in the EPM2B (Figure 5.6b and 5.6c). The mutation created a stop codon at position 131 (Gly131X) in the malin mRNA truncating the protein in the first NHL domain. The six NHL repeats in malin are important in protein-protein interactions. Parents and the brother were heterozygous carriers of this mutation (Figure 5.6d, 5.6e and 5.6f). The mutation was confirmed by sequencing the region with the reverse primer after re-amplification in both patients. In this consanguineous family, DNA analysis confirmed the clinical diagnosis of Lafora disease. The pedigree is shown in Figure 5.7.

Table 5.1. DNA Sequence Analysis of EPM2A coding region in patients 3PME5 and 3PME6

Exon	**Nucleotide (bp)**	**Nucleotides Read**	**SNPs**	**Genotype**	**Comments**
1	226 - 789	All	dbSNP2235482	G/C	-
2	49486 - 49776	All	dbSNP5014204 dbSNP61758155 dbSNP35230590 dbSNP61757376	T G G A	-
3	100293 - 100723	All	dbSNP1045816 dbSNP61758156	A C	-
4	107944 - 108484	All	-	-	-

Table 5.2. DNA Sequence Analysis of EPM2B coding region in patients 3PME5 and 3PME6

Exon	**Nucleotide (bp)**	**Nucleotides Read**	**SNPs**	**Genotype**	**Comments**
1a	(-64) - 301	All	dbSNP35660419 dbSNP28940575 dbSNP28940576	GA T C	-
1b	269 - 584	All	dbSNP10949483	C	A homozygous nonsense mutation c.391 G>T
1c	550 – 942	All	dbSNP34551044	CT	-
1d	909 – 1255	All	-	-	-

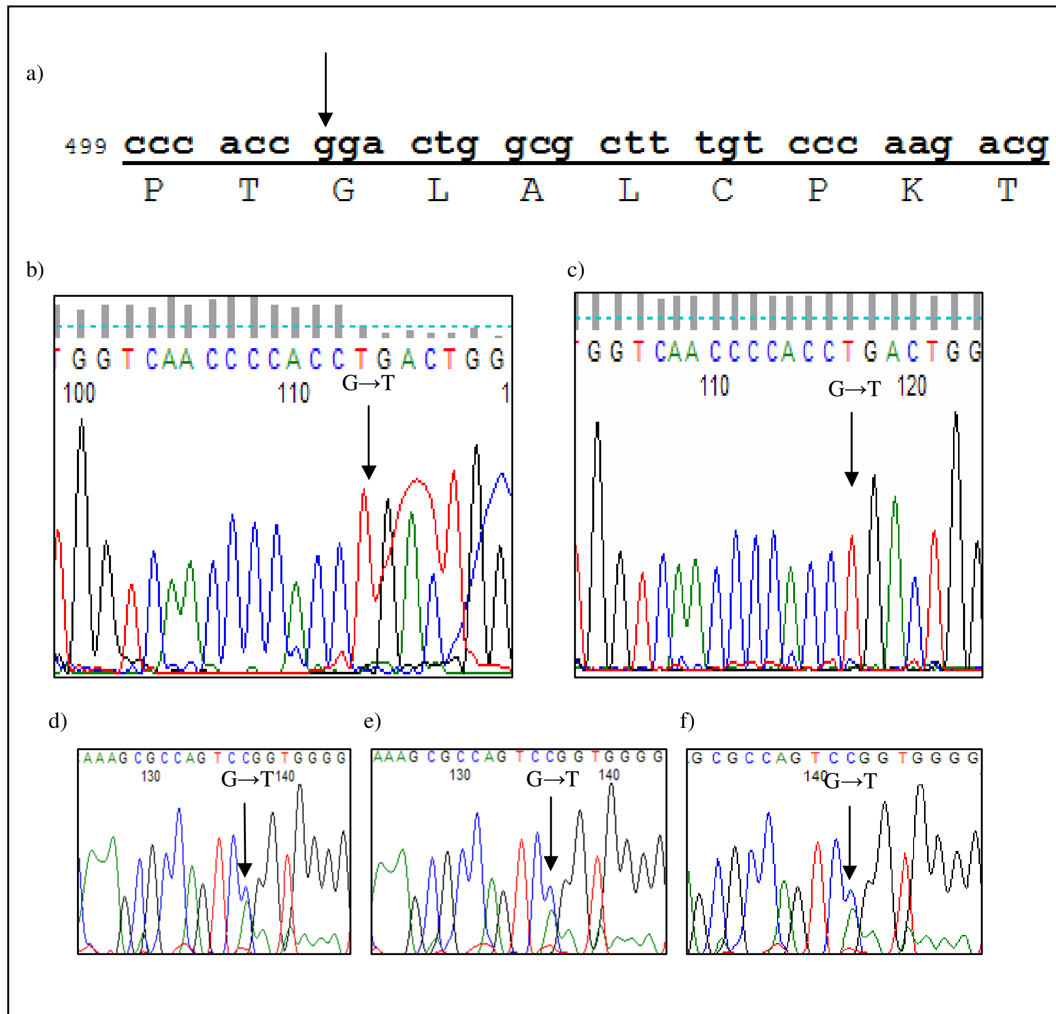

Figure 5.6. DNA sequence analysis of the EPM2B gene showing the mutation in family 3PME. (a) The partial reference sequence of the coding region in the EPM2B gene. Arrows indicate the position of the nucleotide change. Letters below the codons are the corresponding amino acids. (b,c) Chromatograms of patients 3PME5 and 3PME6, respectively. (d,e,f) Chromatograms of father, brother and mother, respectively.

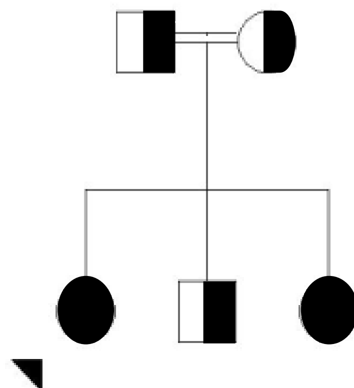

Figure 5.7. The pedigree of 3PME family.
Arrow indicates the index.

Patient 4PME10 had myclonus and atypical EEG. Lafora bodies were seen in skin biopsy. Other family members were phenotypically normal. DNA sequence analysis of exons 1-3 of EPM2A and all of the coding region of EPM2B were not successful due to sequencing problems. The EPM2A genotype is shown in Table 5.3. However, a recurrent transition was observed in exon 4 of the EPM2A gene (c.721 C>T) in homozygous condition (Figure 5.8b). The mutation created a stop codon at position 241 in the Laforin mRNA (Arg241X) corresponding to a part of DSP domain. This second frequently seen EPM2A mutation in Lafora Gene Mutation Database most probably led to impairment of phosphatase activity of laforin. Both parents were heterozygous for the mutation but the sister was homozygous normal (Figure 5.8c, 5.8d and 5.8e). The mutation was confirmed by sequencing the region with the reverse primer after re-amplification of the region in both patients. Clinical diagnosis of Lafora Disease was also confirmed by DNA analysis for patient 4PME10. The pedigree of the family is shown in Figure 5.9.

Table 5.3. DNA sequence analysis of EPM2A coding region in patient 4PME10

Exon	Nucleotide (bp)	Nucleotides Read	SNPs	Genotype	Comments
1	226 - 789	-	dbSNP2235482		Chromatogram not readable
2	49486 - 49776	-	dbSNP5014204 dbSNP61758155 dbSNP35230590 dbSNP61757376		Chromatogram not readable

Table 5.8. DNA sequence analysis of EPM2A coding region in patient 4PME10 (continued)

3	100293 - 100723	-	dbSNP1045816 dbSNP61758156		Chromatogram not readable
4	107944 - 108484	Partial	-		A homozygous nonsense mutation c.721 C>T

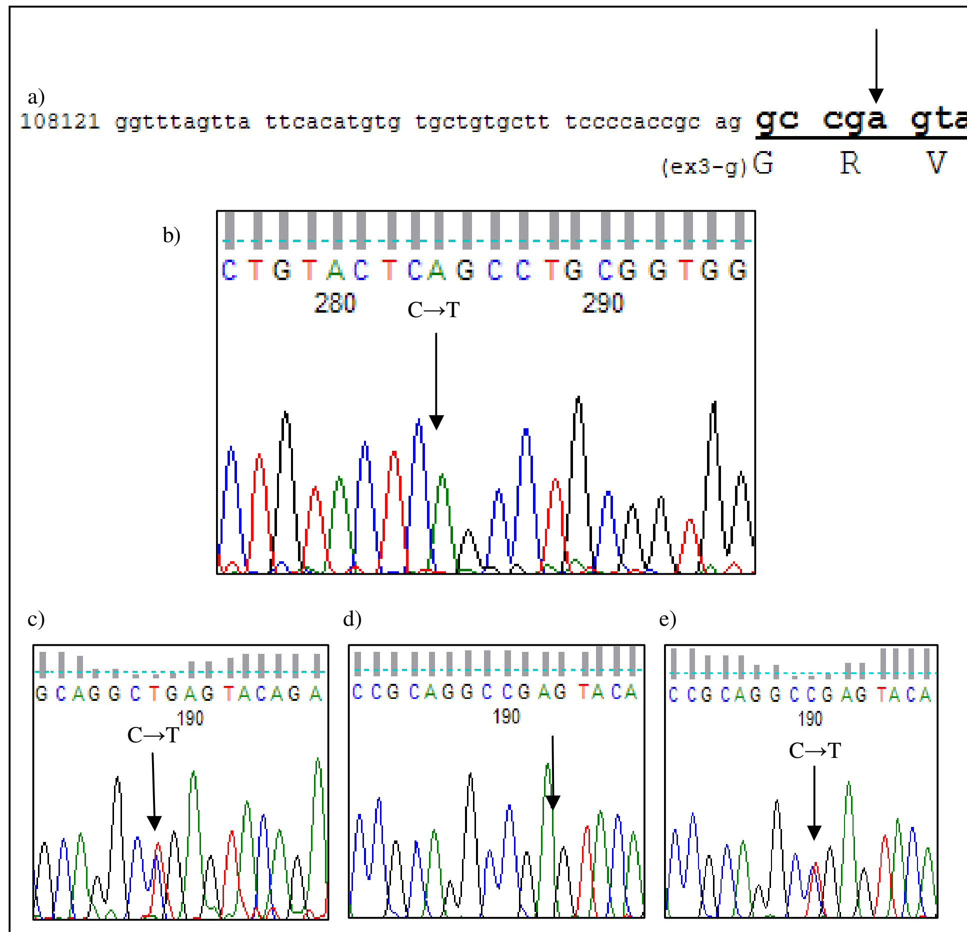

Figure 5.8. DNA sequence analysis of the EPM2A gene showing the mutation in family 4PME. (a) The partial reference sequence of exon 4 in the EPM2A gene. Arrows indicate the position of the nucleotide change. Normal letters and bold letters indicate IVS3 and codons, respectively. Letters below the codons are the corresponding amino acids. (b) Chromatogram of patient 4PME10. (c, d, e) Chromatograms of father, sister and mother, respectively.

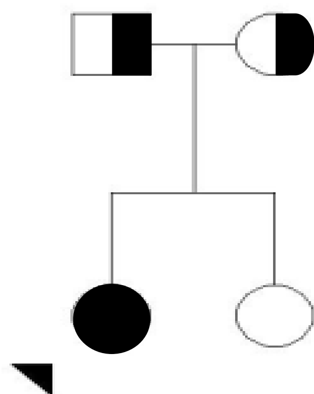

Figure 5.9. The Pedigree of 4PME family.
Arrow indicates the index.

In patient 2PME4, LBs were seen in electron microscopic investigations, but not in light microscopy. Sequencing results of EPM2A and EPM2B exons showed no alterations apart from the known SNPs. The EPM2A and EPM2B genotypes of the patient were as shown in Table 5.4 and 5.5. As skin biopsy may result in false negative or false positive results (Lohi *et al.* 2007) and ULD patients show similar clinical features as of Lafora Disease patients, EPM1A exons and dodecamer repeat were also screened in this patient. No alteration was found in EPM1A exons (Table 5.6). Normal repeat size was observed (Figure 5.10) suggesting that the patient was either misdiagnosed or LD was caused by a change in a yet unidentified genetic locus.

Table 5.4. DNA Sequence Analysis of EPM2A Coding Regions in patient 2PME4

Exon	Nucleotide (bp)	Nucleotides Read	SNPs	Genotype	Comments
1	226 - 789	All	dbSNP2235482	G/C	-
2	49486 - 49776	All	dbSNP5014204 dbSNP61758155 dbSNP35230590 dbSNP61757376	T G G A	-
3	100293 - 100723	All	dbSNP1045816 dbSNP61758156	A A	-
4	107944 - 108484	All	-	-	-

Table 5.5. DNA sequence Analysis of EPM2B coding region in patient 2PME4

Exon	Nucleotide (bp)	Nucleotides Read	SNPs	Genotype	Comments
1a	(-64) - 301	All	dbSNP35660419 dbSNP28940575 dbSNP28940576	GA T C	-
1b	269 - 584	All	dbSNP10949483	C	-
1c	550 – 942	All	dbSNP34551044	CT	-
1d	909 – 1255	All	-	-	-

Table 5.6. DNA Sequence Analysis of EPM1A Coding Regions

Exon	Nucleotide (bp)	Nucleotides Read	SNPs	Genotype	Comments
1	71 - 265	All	dbSNP4533 dbSNP9984466 dbSNP11553836 dbSNP9984388	G G G G	-
2	1524 - 1787	All	dbSNP6382 dbSNP6383	C T	-
3	2005 - 2238	All	dbSNP6384	C	-

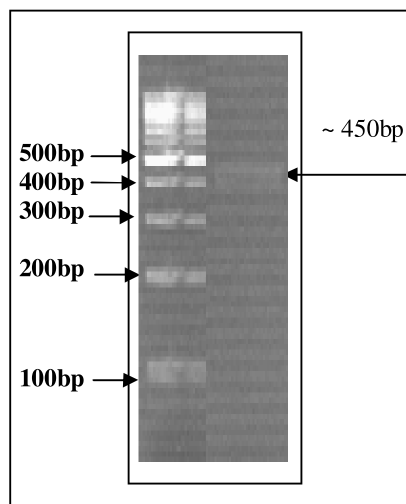

Figure 5.10. Dodecamer Repeat Region of EPM1A gene in patient 2PME4.

First lane is 100 bp ladder DNA and the brightest band is 500 bp.

5.3. Mutational Analysis of SCN1A Gene in GEFS+ and DS Patients

Mutational analysis of SCN1A gene was carried out on 4 patients, one with a mild form of epilepsy in GEFS+ spectrum and three were clinically suspected to have Dravet's Syndrome, the most severe form of epilepsy in GEFS+ spectrum.

Patient 1DS1 was approximately 2 years old male. His first convulsions were seen when he was 2 months old. He had myoclonus and cognitive decline. His parents were phenotypically normal. However, his mother's sister had febrile seizures (FS) until she was 7 years old and her brother died when he was four months old as a result of a convulsion. SCN1A genotype of patient 1DS1 suspected to have DS is shown in Table 5.7. A novel transversion was observed in exon 5 in heterozygous condition (c.655 A>G) (Figure 5.11b). The mutation resulted in arginine to glycine substitution at position 219 (Arg219Gly) and resided in the evolutionary conserved voltage-sensing part of Na_v 1.1 encoded by exon 5 (Figure 5.12). Every third amino acid in this region is a positively charged amino acid and the mutation possibly effects the channel kinetics by causing a neutral amino acid substitution. The mutation was confirmed by sequencing the region using reverse primer after re-amplification of the region. Exon 5 of both parents were also sequenced but no alteration was found. (Figure 5.11c and 5.11d). The pedigree of the family is shown in Figure 5.13.

SCN1A exon 5 was amplified in 60 healthy Turkish individuals and directly sequenced. None of these individuals had an epilepsy history and carried the c.655 A>G change confirming that it was not a rare variant or a polymorphism.

Table 5.7. DNA sequence analysis of SCN1A coding region in patient 1DS1

Exon	Nucleotide (bp)	Nucleotides Read	SNPs	Genotype	Comments
1	(-119) - 329	All	dbSNP566839 dbSNP41265141 dbSNP35595680	T G TC	-
2	14776 - 15131	All	dbSNP10930201 dbSNP61741123	T C	-
3	17034 - 17296	All	-	-	-
4	18725 - 19082	All	dbSNP10930200	T	-

Table 5.7. DNA sequence analysis of SCN1A coding region in patient 1DS1 (continued)

5	20627 - 20826	All	-	-	A heterozygous missense mutation c.655 A>G
6	21609 - 22002	All	-	-	-
7	24613 - 24853	All	dbSNP994399 dbSNP1542484	T C	-
8	25791 - 26110	All	-	-	-
9	26598 - 26960	All	dbSNP93335574 dbSNP7580482 dbSNP56112036 dbSNP6432861	$(AA)_5$ G C A	-
10	28232 - 28711	All	dbSNP13424709 dbSNP7559148	A C	del. C in IVS 9
11a	29491 - 29796	All	dbSNP6753355 dbSNP28934003	G A	-
11b	29726 - 30040	All	-	-	-
12	31129 - 31475	All	dbSNP59251406	A	-
13a	32001 - 32292	All	-	-	-
13b	32235 - 32511	All	dbSNP6432860	C	-
14	33944 - 34281	All	dbSNP490317 dbSNP2126152	G C	-
15a	35383 - 35730	All	dbSNP12479442 dbSNP563792 dbSNP12617205	T C G	-
15b	35678 - 35953	All	dbSNP10613159 dbSNP2020318	GG G	-
16a	36980 - 37366	All	dbSNP35620251 dbSNP7601520	GT T	-
16b	37266 - 37618	All	dbSNP2298771	A	-
17	57850 - 58081	All	-	-	-
18	59695 - 59928	All	-	-	-
19	61256 - 61573	All	dbSNP35420838 dbSNP36031496	TG T	-
20	63744 - 63959	All	-	-	-
21a	70806 - 71108	All	-	-	-
21b	71022 - 71318	All	-	-	-
22	73713 - 73979	All	-	-	-
23	75399 - 75669	All	-	-	-
24	77485 - 77691	All	dbSNP347077958 dbSNP56775989	TG GG	-
25	79108 - 79584	All	-	-	-
26a	81173 - 81419	All	dbSNP55690783	T	-
26b	81366 - 81653	All	-	-	further analysis required
26c	81609 - 81849	All	-	-	-
26d	81815 - 82088	All	-	-	-
26e	82035 - 82369	All	dbSNP3749029 dbSNP35735053	G T	-
26f	82319 - 82560	All	-	-	-

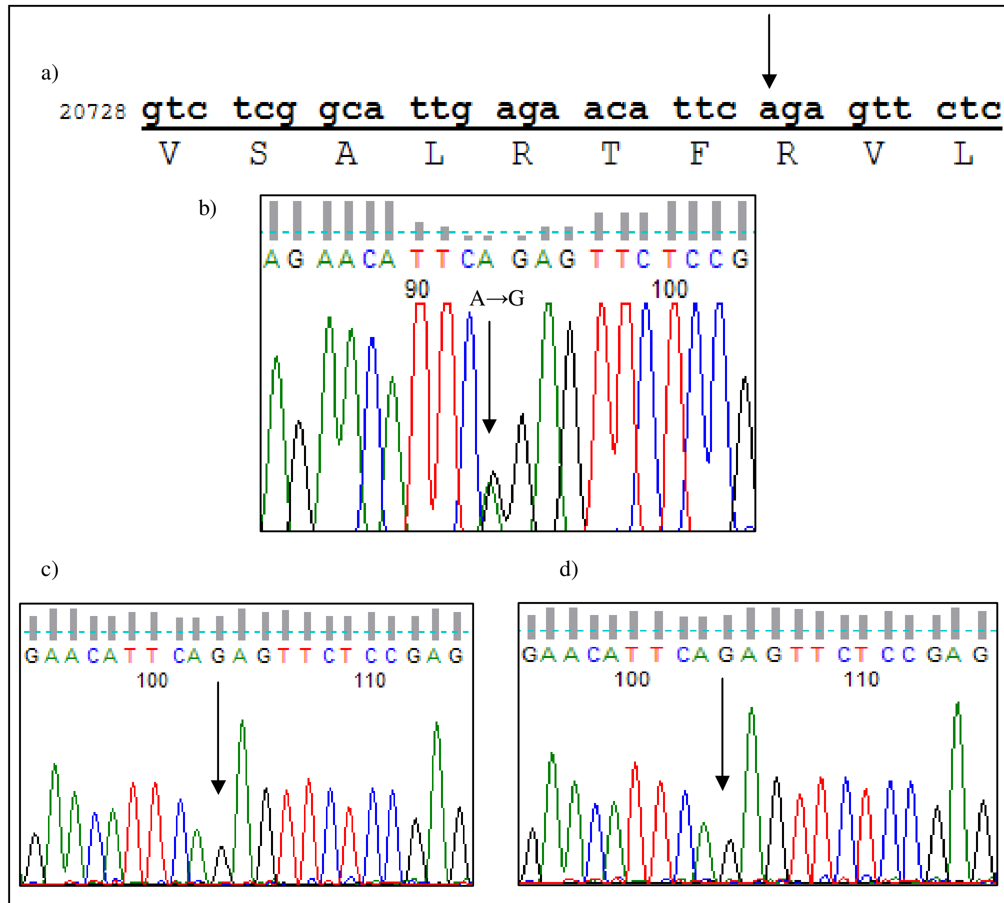

Figure 5.11. DNA sequence analysis of the SCN1A gene showing the mutation in family 1DS. (a) The partial reference sequence of exon. Arrows indicate the position of the nucleotide change. Letters below the codons are the corresponding amino acids. (b) Chromatogram of patient 1DS1. (c and d) Chromatograms of father and mother, respectively.

SCN1A_HUMAN	ALRTFRVLRALKTISVIPGL
Scn1a_RAT	ALRTFRVLRALKTISVIPGL
SCN2A_HUMAN	ALRTFRVLRALKTISVIPGL
SCN3A_HUMAN	ALRTFRVLRALKTISVIPGL
SCN4A_HUMAN	ALRTFRVLRALKTITVIPGL
SCN5A_HUMAN	ALRTFRVLRALKTISVISGL
SCN8A_HUMAN	ALRTFRVLRALKTISVIPGL
SCN9A_HUMAN	ALRTFRVLRALKTISVIPGL
SCN11A_HUMAN	PLRTFRVFRALKAISVVSRL
TAKI FUGU	ALRTFRVLRALKTITVIPGL
ELECTRIC EEL	RNVSALRTFRVLRALKTITIF
DROSOPHILA	ALRTFRVLRALKTVAIVPGL

Figure 5.12. Evolutionary conservation of S4 part in domain I of α subunit of voltage-gated sodium channels.

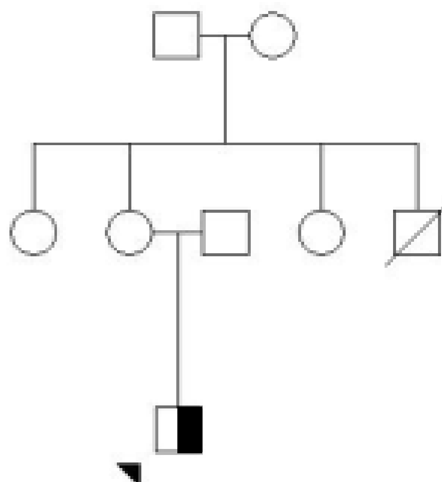

Figure 5.13. The pedigree of 1DS family. Arrow indicates the index.

In patient 1DS1 DNA sequence analysis of exon 10 using the forward primer indicated a shift in the pattern from the beginning of the chromatogram. Using reverse primer for sequencing, exon 10 was readable and the shift was at the last nucleotide of the forward primer binding site (Figure 5.14b). A new forward primer located approximately 90 nucleotides upstream of the previous one was designed in order to determine the position the shift occurred. Results of sequencing using the new forward primer showed a C deletion at the binding site of the former primer (Figure 5.14c). The schematic drawing showing the C deletion is given in Figure 5.15. The sequence of analysis of the region in the mother showed

the normal sequence, however, the father also had a frameshift and probably had the same deletion in IVS 9 (Figure 5.11d and 5.11e). The deletion is probably a polymorphism.

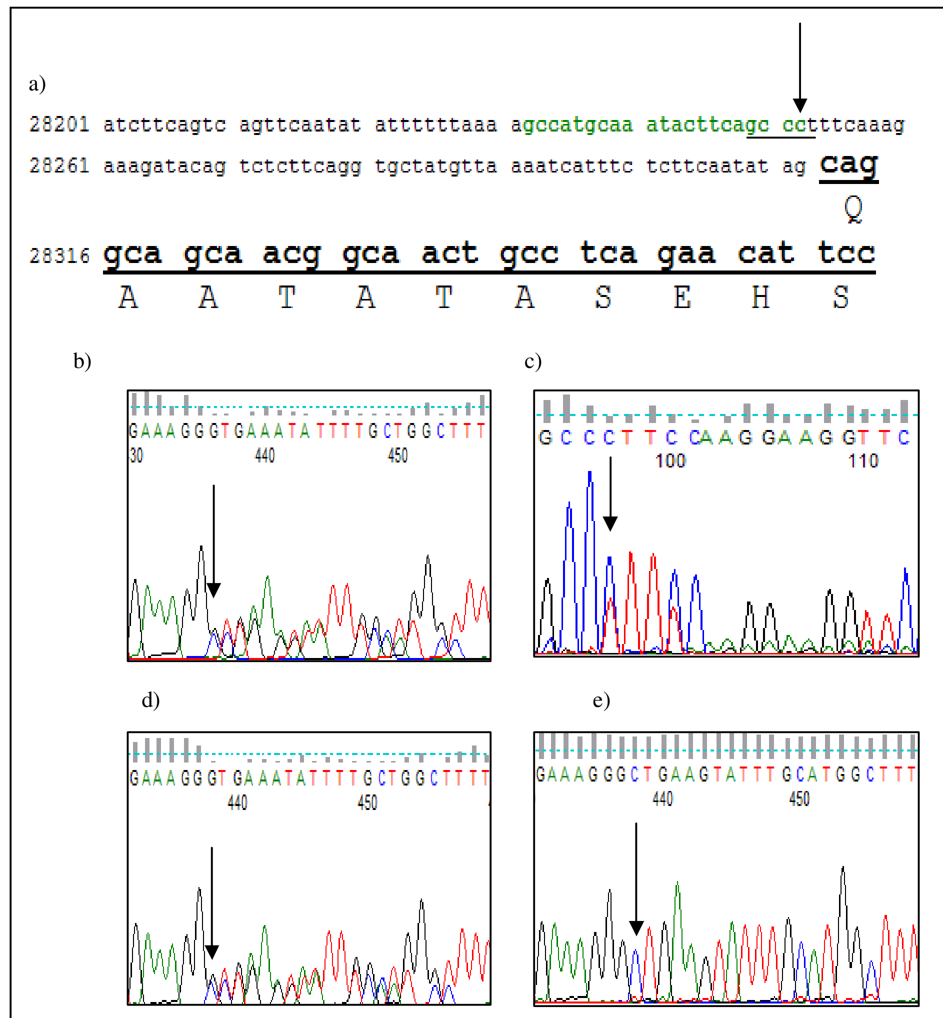

Figure 5.14. DNA sequence analysis of the SCN1A gene showing the mutation in IVS9. (a) The partial reference sequence of intron 9 and exon 10. Arrows indicate the position of the nucleotide change. Normal, bold and green letters indicate IVS9, codons of exon 10 and the first forward primer, respectively. Letters below the codons are the corresponding amino acids. (b and c) Chromatograms of 1DS1 sequenced by the new forward primer and the reverse primer, respectively. (d and e) Chromatograms of father and mother, respectively.

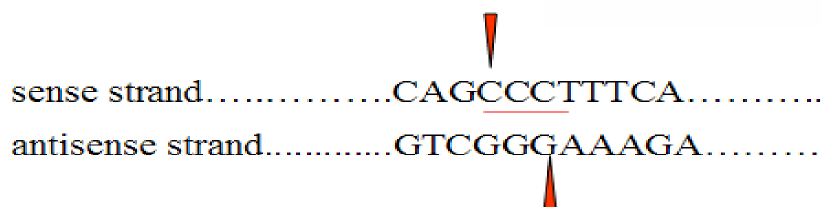

Figure 5.15. Schematic representation of deletion in IVS 9 in 1DS family members. Starting point of deletion by forward primer (sense strand) and reverse primer (antisense strand)

In patient 2DS4, 4 years old female suspected to have DS, a novel mutation in exon 16 was observed upon mutational analysis of the SCN1A gene. Table 5.8 shows the SCN1A genotype of the patient. The AG dinucleotide in exon 16 encoding linker between domain II-III of Na_v 1.1 were deleted in heterozygous condition (c.3428-3429 delAG) as shown by the frameshift in Figure 5.16b. The mutation corresponded to the domain II-III linker of Na_v 1.1. The AG dinucleotide deletion in the last amino acid of exon 16 might result in a defective splicing pattern. Both alleles, one with the AG deletion and one normal were readable following the reference sequence when forward primers were used for sequencing. It was not possible to correctly read the region using reverse primer for sequencing as it was very close to the exonic region. Parental analysis showed no alteration in this region (Figure 5.16c and 5.16d).

Table 5.8. DNA sequence analysis of SCN1A coding region in patient 2DS4

Exon	Nucleotide (bp)	Nucleotides Read	SNPs	Genotype	Comments
1	(-119) - 329	All	dbSNP566839 dbSNP41265141 dbSNP35595680	T G TC	-
2	14776 - 15131	All	dbSNP10930201 dbSNP61741123	T C	-
3	17034 - 17296	All	-	-	-
4	18725 - 19082	All	dbSNP10930200	T	-
5	20627 - 20826	All	-	-	-
6	21609 - 22002	All	-	-	-
7	24613 - 24853	All	dbSNP994399 dbSNP1542484	C T/C	-

Table 5.8. DNA sequence analysis of SCN1A coding region in patient 2DS4 (continued)

8	25791 - 26110	All	-	-	-
9	26598 - 26960	All	dbSNP93335574 dbSNP7580482 dbSNP56112036 dbSNP6432861	$(AA)_5$ G C G/A	-
10	28232 - 28711	All	dbSNP13424709 dbSNP7559148	A C	-
11a	29491 - 29796	All	dbSNP6753355 dbSNP28934003	G A	-
11b	29726 - 30040	All	-	-	-
12	31129 - 31475	All	dbSNP59251406	A	-
13a	32001 - 32292	All	-	-	-
13b	32235 - 32511	All	dbSNP6432860	T	-
14	33944 - 34281	All	dbSNP490317 dbSNP2126152	G C	-
15a	35383 - 35730	All	dbSNP12479442 dbSNP563792 dbSNP12617205	T C G	-
15b	35678 - 35953	All	dbSNP10613159 dbSNP2020318	GG G	-
16a	36980 - 37366	All	dbSNP35620251 dbSNP7601520	GT T	-
16b	37266 - 37618	All	dbSNP2298771	A	c.3428-29 del.AG
17	57850 - 58081	All	-	-	-
18	59695 - 59928	All	-	-	-
19	61256 - 61573	All	dbSNP35420838 dbSNP36031496	TG T	-
20	63744 - 63959	All	-	-	-
21a	70806 - 71108	All	-	-	-
21b	71022 - 71318	All	-	-	-
22	73713 - 73979	All	-	-	-
23	75399 - 75669	All	-	-	-
24	77485 - 77691	All	dbSNP347077958 dbSNP56775989	TG GG	-
25	79108 - 79584	All	-	-	-
26a	81173 - 81419	All	dbSNP55690783	T	-
26b	81366 - 81653	All	-	-	further analysis required
26c	81609 - 81849	All	-	-	-
26d	81815 - 82088	All	-	-	-
26e	82035 - 82369	All	dbSNP3749029 dbSNP35735053	G T	-
26f	82319 - 82560	All	-	-	-

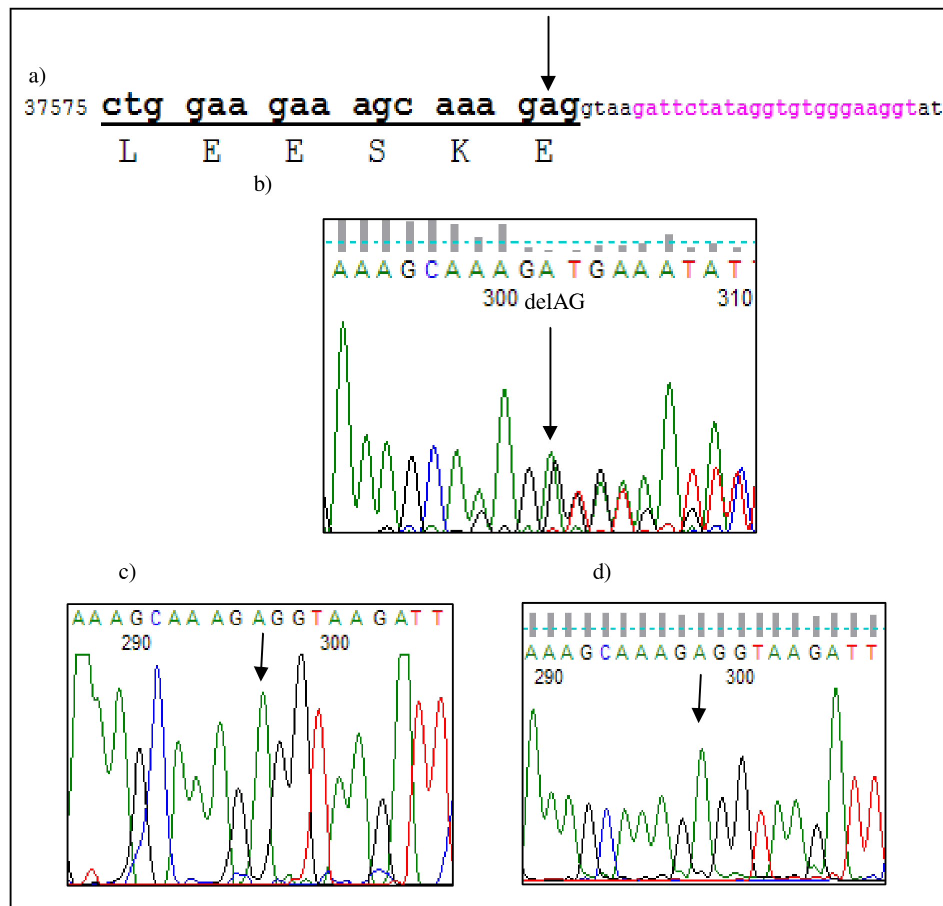

Figure 5.16. DNA sequence analysis of the SCN1A gene showing the mutation in family 2DS. (a) The partial reference sequence of exon 16. Arrows indicate the position of the nucleotide change. Normal, bold and pink letters indicate IVS16, codons and reverse primer, respectively. Letters below the codons are the corresponding amino acids. (b) Chromatogram of patient 2DS4. (c and d) Chromatograms of father and mother, respectively.

Patient 3DS7, 3 years old female suspected to have DS, had myclonus and febrile seizures. No mutation was found in coding regions of the SCN1A gene. The genotype of the patient is shown in Table 5.9. However, a point mutation was detected in exon 26 (c.5418 A>G) in heterozygous condition (Figure 5.17b). This transversion did not change the corresponding amino acid (Glu1806Glu); hence, was a silent mutation and was not found in the current SNP database. A base deletion was also seen in IVS 9. Forward primers were used for sequencing. The mutation was confirmed by sequencing the region with the reverse primer after re-amplification of the region.

Table 5.9. DNA sequence analysis of SCN1A coding region in patient 3DS7

Exon	Nucleotide (bp)	Nucleotides Read	SNPs	Genotype	Comments
1	(-119) - 329	All	dbSNP566839 dbSNP41265141 dbSNP35595680	T G TC	-
2	14776 - 15131	All	dbSNP10930201 dbSNP61741123	T C	-
3	17034 - 17296	All	-	-	-
4	18725 - 19082	All	-	-	Exon was clearly read, but sequence did not show 3' intronic sequences clearly
5	20627 - 20826	All	-	-	-
6	21609 - 22002	All	-	-	-
7	24613 - 24853	All	dbSNP994399 dbSNP1542484	C/T T/C	-
8	25791 - 26110	All	-	-	-
9	26598 - 26960	All	dbSNP93335574 dbSNP7580482 dbSNP56112036 dbSNP6432861	$(AA)_3/(AA)_5$ G/A C G	-
10	28232 - 28711	All	dbSNP13424709 dbSNP7559148	A C	del. A in IVS9
11a	29491 - 29796	All	dbSNP6753355 dbSNP28934003	T/G A	-
11b	29726 - 30040	All	-	-	-
12	31129 - 31475	All	dbSNP59251406	A	-
13a	32001 - 32292	All	-	-	-
13b	32235 - 32511	All	dbSNP6432860	T/C	-
14	33944 - 34281	All	dbSNP490317 dbSNP2126152	A ?	Exon was clearly read, but sequence did not show 3' intronic sequences clearly
15a	35383 - 35730	All	dbSNP12479442 dbSNP563792 dbSNP12617205	T C G	-

Table 5.9. DNA sequence analysis of SCN1A coding region in patient 3DS7 (continued)

15b	35678 - 35953	All	dbSNP10613159 dbSNP2020318	GG ?	Exon was clearly read, but sequence did not show 3' intronic sequences clearly
16a	36980 - 37366	All	dbSNP35620251 dbSNP7601520	GT C/T	-
16b	37266 - 37618	All	dbSNP2298771	G/A	-
17	57850 - 58081	All	-	-	-
18	59695 - 59928	All	-	-	-
19	61256 - 61573	All	dbSNP35420838 dbSNP36031496	TG T	-
20	63744 - 63959	All	-	-	-
21a	70806 - 71108	All	-	-	-
21b	71022 - 71318	All	-	-	-
22	73713 - 73979	All	-	-	-
23	75399 - 75669	All	-	-	-
24	77485 - 77691	All	dbSNP347077958 dbSNP56775989	TG GG	-
25	79108 - 79584	All	-	-	-
26a	81173 - 81419	All	dbSNP55690783	T	-
26b	81366 - 81653	All	-	-	further analysis required
26c	81609 - 81849	All	-	-	A heterozygous silent mutation c.5418 A>G
26d	81815 - 82088	All	-	-	-
26e	82035 - 82369	All	dbSNP3749029 dbSNP35735053	G T	-
26f	82319 - 82560	All	-	-	-

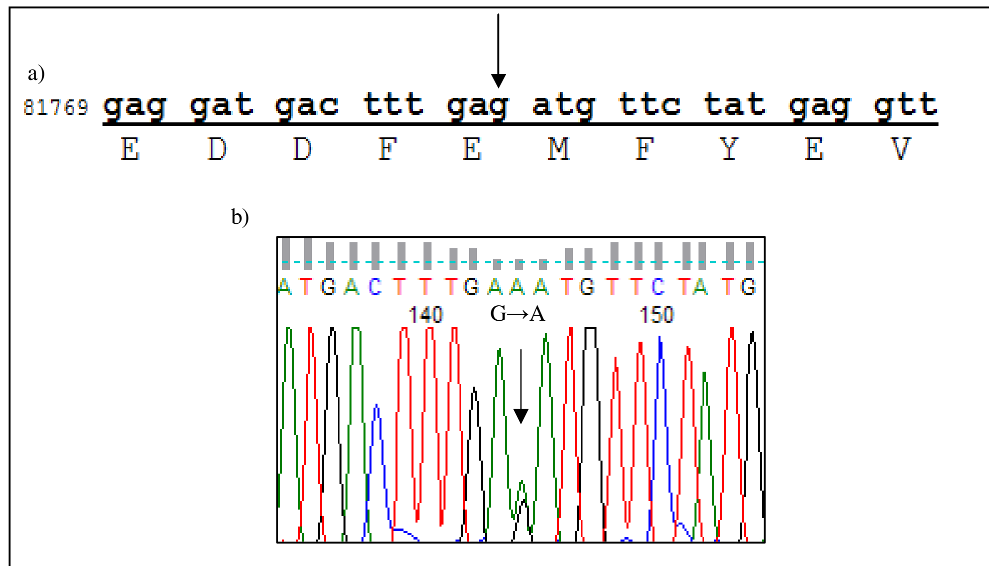

Figure 5.17. DNA sequence analysis of the SCN1A gene showing the silent mutation in patient 3DS7. (a) The partial reference sequence of exon 26. Arrows indicate the position of the nucleotide change. Letters below the codons are the corresponding amino acids. (b) Chromatogram of the relevant region in 3DS7.

Patient 1GEFS1 belonged to a very large family with multiple consanguineous marriages. Several members in the family had different forms of epilepsy, all of which were generally classified under GEFS+ syndrome. Since SCN1A gene is the most relevant gene in IGE syndromes, particularly GEFS+2 phenotype, the index patient was analyzed for mutations in the SCN1A gene to initiate the molecular genetic analysis of epilepsy in the large family. In this patient any sequence alteration was not observed apart from several known SNPs in the SCN1A exons. The SCN1A genotype of the patient was as shown in Table 5.10. This result is acceptable since there is no direct DNA diagnosis of GEFS+ and several other genes may also be involved.

Table 5.10. DNA sequence Analysis of SCN1A Coding Regions in patient 1GEFS1

Exon	Nucleotide (bp)	Nucleotides Read	SNPs	Genotype	Comments
1	(-119) - 329	All	dbSNP566839 dbSNP41265141 dbSNP35595680	T G TC	-

Table 5.10. DNA sequence Analysis of SCN1A Coding Regions in patient 1GEFS1 (continued)

2	14776 - 15131	All	dbSNP10930201 dbSNP61741123	T C	-
3	17034 - 17296	All	-	-	-
4	18725 - 19082	All	dbSNP10930200	T	-
5	20627 - 20826	All	-	-	-
6	21609 - 22002	All	-	-	-
7	24613 - 24853	All	dbSNP994399 dbSNP1542484	T C	-
8	25791 - 26110	All	-	-	-
9	26598 - 26960	All	dbSNP93335574 dbSNP7580482 dbSNP56112036 dbSNP6432861	$(AA)_5$ G C A	-
10	28232 - 28711	All	dbSNP13424709 dbSNP7559148	A C	-
11a	29491 - 29796	All	dbSNP6753355 dbSNP28934003	G A	-
11b	29726 - 30040	All	-	-	-
12	31129 - 31475	All	dbSNP59251406	A	-
13a	32001 - 32292	All	-	-	-
13b	32235 - 32511	All	dbSNP6432860	C	-
14	33944 - 34281	All	dbSNP490317 dbSNP2126152	G C	-
15a	35383 - 35730	All	dbSNP12479442 dbSNP563792 dbSNP12617205	T T G	-
15b	35678 - 35953	All	dbSNP10613159 dbSNP2020318	GG A	-
16a	36980 - 37366	All	dbSNP35620251 dbSNP7601520	GT C	-
17	57850 - 58081	All	-	-	-
18	59695 - 59928	All	-	-	-
19	61256 - 61573	All	dbSNP35420838 dbSNP36031496	TG T	-
20	63744 - 63959	All	-	-	-
21a	70806 - 71108	All	-	-	-
21b	71022 - 71318	All	-	-	-
22	73713 - 73979	All	-	-	-
23	75399 - 75669	All	-	-	-
24	77485 - 77691	All	dbSNP347077958 dbSNP56775989	TG GG	-
25	79108 - 79584	All	-	-	-
26a	81173 - 81419	All	dbSNP55690783	T	-
26b	81366 - 81653	All	-	-	further analysis required
26c	81609 - 81849	All	-	-	-
26d	81815 - 82088	All	-		-
26e	82035 - 82369	All	dbSNP3749029 dbSNP35735053	G T	-
26f	82319 - 82560	All	-	-	-

In all our patients (1GEFS1, 1DS1, 2DS4 and 3DS7) and also in 3 apparently normal individuals several consistent alterations in heterozygous condition were observed in exon 26b (Figure 5.18).

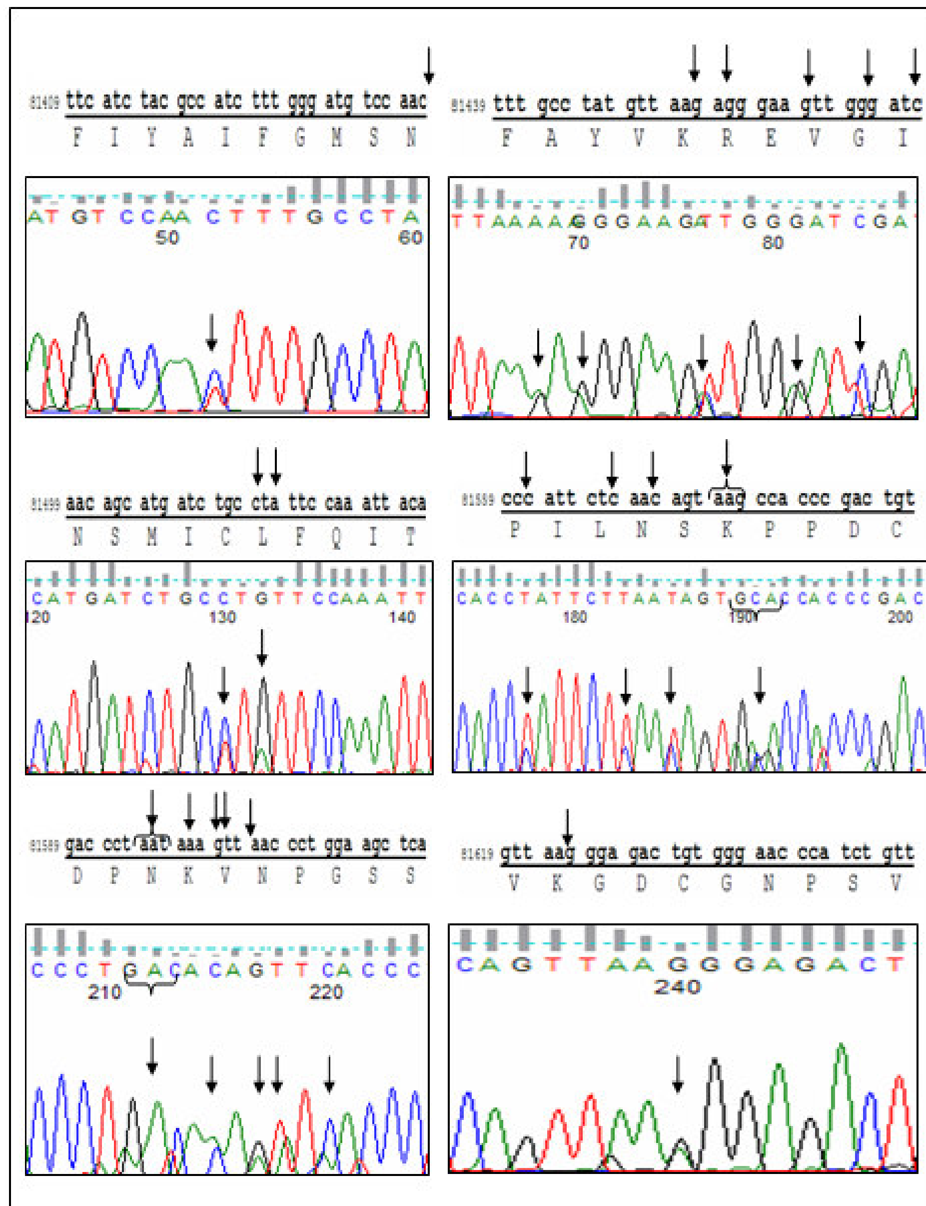

Figure 5.18. Alterations seen in exon 26b of SCN1A gene. The partial sequences of exon 26b in the SCN1A gene are shown above the chromatograms. Arrows indicate the position of nucleotide changes. Letters below the codons are the corresponding amino acids.

Among the heterozygous alterations, only one changed the corresponding amino acid. However, the charge of the amino acid remained the same. Whether this observation was an artifact of DNA sequence analysis or they represent novel polymorphisms in the Turkish population remains to be seen.

The alterations found in patients suspected to have LD, GEFS+ and DS is shown in summary in Table 5.11.

Table 5.11. Alterations found in patients with LD and DS

Patient ID	**Exon**	**Base Change**	**Amino Acid Change**	**Genotype**	**Effect**
3PME5	Exon 1 (EPM2B)	(c.391) G→T	G131X	Homozygous	Nonsense
3PME6	Exon 1 (EPM2B)	(c.391) G→T	G131X	Homozygous	Nonsense
4PME10	Exon 4 (EPM2A)	(c.721) C→T	R241X	Homozygous	Nonsense
1DS1	Exon 5 IVS 9 (SCN1A)	(c.655) A→G (IVS 9) del. C	R219G -	Heterozygous Heterozygous	Missense Not known
1DS2	IVS 9 (SCN1A)	(IVS 9) del. C	-	Heterozygous	Not known
2DS4	Exon 16 (SCN1A)	(c.2365)delAG	-	Heterozygous	Defective splice site
3DS7	Exon 26 (SCN1A)	(c.5418)A→G (IVS 9) del. A	E1806E -	Heterozygous Heterozygous	Silent Not known

6. DISCUSSION

The aim of this study was to establish the methods for mutational analysis of the EPM1, EPM2A, EPM2B and SCN1A genes in patients suspected to have LD and DS, to help discriminative diagnosis for these two diseases and to initiate the study for eliciting the mutation profiles and prevalances of these diseases in Turkey. Amplification conditions of EPM1A, EPM2A, EPM2B and SCN1A genes were optimized and mutational analyses were carried out on eight patients, four were suspected to have LD, three were suspected to have DS and one was suspected to have GEFS+. A novel mutation in EPM2B, a recurrent mutation in EPM2A, two novel mutations and a polymorphism in coding regions of SCN1A and two deletions in intronic regions of SCN1A were found.

6.1. DNA Diagnosis for LD

The hallmark of LD is LBs seen in skin biopsy; however, they may form as a result of the disease and may not be the primary cause of the epileptic phenotype (Singh *et al.*, 2005; Vilchez et al. 2007). Skin biopsy can also give false negative or positive results (Lohi *et al*. 2007). Among four patients suspected to have LD due to clinical manifestations and skin biopsy, the diagnosis was confirmed by DNA analysis in three patients in two families. In one family two sisters were found to carry a novel mutation in homozygous condition. The mutation was also seen in parents and brother in heterozygous condition. Although skin biopsy results were negative in the younger sister, she had some clinical signs of LD and her DNA analysis made the suspicion stronger and showed that her disease may progress later in life. Therefore, it is very important to follow up her prognosis and determine her age of developing LBs. In this family, DNA diagnosis also revealed that the brother who had no signs of LD can be excluded from having LD. A recurrent mutation, the second frequently seen one in the database was found in another patient whose parents were also carriers. Although the complete coding regions of EPM2A and EPM2B genes in the patient could not be read due to sequencing problems, the presence of the highly recurrent mutation confirmed LD diagnosis. In the third family, the patient had inconsistent skin biopsy results and no mutation upon complete sequencing of the coding regions of EPM2A and EPM2B genes. As skin biopsy results may not be reliable and ULD patients show similar clinical features as of

LD patients, coding regions and dodecamer repeat region of EPM1A gene were also analyzed in this patient and neither a point mutation nor a repeat expansion was observed. During a two year follow-up her disease did not progress and her clinical findings improved.

In conclusion, the molecular genetic analysis of four patients suspected to have LD confirmed that DNA analysis is a valuable tool in the diagnosis of LD and determination of the carrier status of individuals in affected families. Some studies suggest that LD patients with EPM2B mutations have milder disease course than patients with EPM2A mutations (Ganesh *et al*., 2006). In this regard, more patients need to be analyzed at the molecular level to confirm the relationship between the severity of the disease and mutation location. At the same time, long-term follow-up of the patients especially who carry a causative mutation but lack LBs in skin biopsy is also very important as it may shed light on the disease mechanism. The follow-up of patients with no mutations in all three genes is also very important since recent studies showed that there may be a third locus involved in the pathogenesis of the LD (Chan *et al*., 2004; Singh *et al*., 2005).

In the framework of this thesis, the molecular genetic analysis of the coding regions of EPM1A, EPM2A and EPM2B genes are well established, however, dodecamer repeat amplification in the EPM1A gene and repeat size determination requires further optimization in the presence of a positive control such as a family with individuals carrying the instable alleles that contain 12-17 repeats and affected individuals with the expanded allele that has more than 30 repeats (Lalioti *et al*., 1998).

6.2. DNA Diagnosis for DS and GEFS+

SCN1A gene is the most relevant gene that may carry mutations leading to DS and SMEI, but there are many patients that do not have a mutation in the SCN1A gene and yet they develop these progressive syndromes. Even in the presence of a SCN1A mutation and its functional analysis showing the loss of function in the sodium channel, SCN1A gene can not conclusively be responsible from the disease phenotype due to the complex inheritance pattern of DS. In addition although there are not any mutations in SCN1A described to cause a different disorder apart from DS or GEFS+2, mutations in several VGICs and LGICs may lead to distinct phenotypes apart from epilepsy syndromes. However, the search for a

mutation in the SCN1A gene in patients suspected to have DS is the first widely accepted step in DNA diagnosis of the syndrome. In the case of GEFS+ the situation is similar and molecular genetic analysis begins with the search of a SCN1A mutation, followed by scanning of the other relevant genes SCN1B, SCN2A, GABRAG2 and GABRAD. Although, GEFS+ have been typed from 1-5 due to the presence of mutations in different genes, this classification is rather superficial since there is no single gene causing GEFS+ due to its complex inheritance pattern. As GEFS+ and DS are syndromes that are relatively recently described there is not enough data showing the relationship between the mutation, modifier factors, the pathogenesis and the prognosis of the disease. One can only argue that if a mutation has a major effect on the phenotype it may be responsible for the monogenic-like inheritance in the family. Since GEFS+ is a mild disease the effect of the mutation on the phenotype is expected to be small. DS on the other hand is expected to be caused by sporadic mutations with a dominant and major effect. Nevertheless, in the light of current knowledge, the molecular genetic analysis of both DS and GEFS+ begins with the search for a mutation in the SCN1A gene.

In the framework of this thesis the molecular analysis of the SCN1A gene was initiated and 3 patients suspected to have DS and one patient with GEFS+ was analyzed. Two novel *de novo* mutations were found in two DS patients. The mutation R219G resided in the evolutionary conserved voltage sensing part of the Na_v 1.1 in the patient and was shown to be non polymorphic in the Turkish population by direct DNA sequence analysis of 60 apparently healthy individuals. It can be concluded that this novel mutation possibly with a major effect is responsible for DS in the patient. However, follow up of the patient is important for confirmation of DS in this patient. In addition, a C deletion was observed in the IVS 9 of the SCN1A gene of the same patient. The deletion did not reside in a location that would affect the splicing and a recent study that searched and evaluated the conserved non-coding sequences of SCN1A to find potential biologically functional sequences such as miRNAs did not reveal such sites in IVS 9 (Martin *et al.*, 2007). Therefore, this change probably does not have an affect on the disease phenotype.

The novel AG deletion at a splice junction found in the second patient probably resulted in defective splicing involving the linker part between the second and the third domains. Studies showed that there were several SMEI and SMEB patients carrying mutations in this

linker part (Claes *et al.*, 2001; Ohmori *et al.* 2002) although there are not any modification site on domain II-III linker. However, the patient's clinical prognosis showed that she began to respond to drug treatment. Although DS is an autosomal dominant disorder, either one copy may be enough to compensate the other one or the use of cryptic splice site may not affect the function of the channel protein in this patient

Two novel changes were found in the third patient suspected to have DS. One was a silent mutation in exon 26 and the other was an A deletion in IVS 9. The A deletion in IVS 9 also did not reside in a location that would affect the splicing. This patient also had good prognosis and she responded to drug treatment.

The mutational analysis of the SCN1A gene through sequence analysis of the coding regions, is not a sufficient strategy for finding causative mutations or mutations that might have a large effect on the phenotype since exon deletions are also known to be common in DS patients. However, the good prognosis observed in the last two patients indicated that they probably did not have a mutation that would have a major effect on the phenotype and that may have GEFS+ instead of DS. The other relevant genes such as SCN1B, SCN2A, GABRAG2 and GABRAD could also be investigated in these patients, along with the patient suspected to have GEFS+ since this patient also did not have any changes in the coding regions of the SCN1A gene upon complete DNA sequence analysis of the coding regions.

The DNA sequence analysis of the SCN1A gene also revealed several consistent alterations in heterozygous condition exon 26b in all 4 of the above patients and 3 apparently normal individuals. Among the heterozygous alterations, only one changed the corresponding amino acid. However, the charge of the amino acid remained the same. None of these alterations were at restriction enzyme cutting sites, and they could not be confirmed. Whether this observation is an artifact of the DNA sequence analysis or they represent novel polymorphisms in the Turkish population remains to be seen.

In conclusion, molecular genetic analysis of the SCN1A gene have also been successfully established by complete DNA sequence analysis of the coding regions; however, by itself it is far from a decisive molecular genetic diagnosis of either DS or GEFS+ due to the complexity of these syndromes. Whole genome studies, twin studies and animal studies are

also necessary to reveal the genotype-phenotype relationships. In addition, as ion channel diseases are a group of paroxysmal disorders (Steinlein, 2001), regulatory gene regions and intronic regions should also be examined.

The SNP genotype data of all patients examined is given along with the identified mutations for a future reference. The genotype data of individuals may be important when other relevant genes are examined for each epilepsy subtype and as gene-gene interactions become available. As a future perspective, the establishment of the molecular genetic analysis of the 4 genes enables the analysis of more patients to reveal the prevalence of LD in the Turkish population and to further explain the molecular mechanisms leading to both LD and DS.

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