PROSPECTIVE CAUSAL GENES FOR GENETIC GENERALIZED EPILEPSY

A thesis submitted for the degree of

Doctor of Philosophy

By

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Dedicated to…

Patients and their families

and

my parents

Declaration

I hereby declare that this thesis, entitled ***"Prospective causal genes for genetic generalized epilepsy",*** *is an authentic record of research work carried out by me under the guidance of Prof. Anuranjan Anand in the Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India and that this work has not been submitted elsewhere for the award of any other degree.*

In keeping the norm of reporting scientific observations, due acknowledgements has been made wherever the work described here has been based on the findings of other investigators. Any omission, which might have occurred by oversight or misjudgment, is regretted.

Manpreet Kaur

Place: Bangalore
Date:

Certificate

This is to certify that the work described in this thesis entitled ***"Prospective causal genes for genetic generalized epilepsy"*** *is the result of the investigations carried out by Ms. Manpreet Kaur in the Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under my guidance, and that the results presented in this thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.*

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Abbreviations

°C	Degree Celsius
µg	micrograms
µl	microlitres
µM	micromolar
bp	Base pairs
CAE	Childhood absence epilepsy
CCDS	Consensus Coding Sequence
cDNA	Complimentary deoxyribonucleic acid
CGH	comparative genomic hybridization
Chr	Chromosome
CNV	Copy number variation
C-terminal	Carboxy-terminal
Da	Dalton
DAPI	6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EEG	Electroencephalography
FBS	Fetal bovine serum
g	Grams
Gb	Gigabases
GGE	Genetic generalized epilepsy
gtf	Gene transfer format
HRP	Horse-Radish Peroxidase
IGE	Idiopathic generalized epilepsy
ILAE	International League Against Epilepsy
Indel	Insertion and deletion
JAE	Juvenile absence epilepsy
JME	Juvenile myoclonic epilepsy
kb	Kilobases
kDa	KiloDaltons
LOD	Logarithm of odds
MAF	Minor allele frequency
Mb	Megabases
mg	Milligrams
ml	Millilitres
mm	Millimetres

mM	Millimolar
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
mV	Millivolts
MW	Molecular weight
ng	Nanograms
NGS	Next-generation sequencing
N-terminal	Amino-terminal
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
pH	Power of hydrogen
pmol	Picomoles
RefSeq	NCBI reference sequence database
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
SNP	Single-nucleotide polymorphism
SNV	Single nucleotide variation
Taq	*Thermus aquaticus*
TSS	Transcription start site
U	Units
UTR	Untranslated region
WES	Whole exome sequencing
WGS	Whole genome sequencing
θ	Recombination fraction

Chapter 1

Introduction

Epilepsy is a group of neurological disorders defined by recurrent and unprovoked epileptic seizures. According to the most recent clinical definition of epilepsy by International League Against Epilepsy (ILAE), an individual may be considered epileptic when any of the following criteria are met: (i) at least two unprovoked or reflex seizures occurring >24 h apart; (ii) one unprovoked or reflex seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures, occurring over the next 10 years; (iii) diagnosis of an epilepsy syndrome (Fisher et al 2014). An epileptic seizure, in turn, is a transient occurrence of symptoms due to abnormal excessive or synchronous neuronal activity in brain, which is usually intermittent, self-limiting and occur with or without loss of consciousness. Unprovoked epileptic seizures may occur unpredictably and are not caused due to factors such as head trauma, stroke, intracranial infections, acute metabolic abnormalities and drug or toxin poisoning.

Present day understanding and concepts of epilepsy and seizure originated from the contributions of Robert Bentley Todd and John Hughlings Jackson in 1850s (York and Steinberg 2011). The work and clinical observations of Hughlings Jackson, a physician, advocated for the understanding of different seizure types and laid the foundation for a scientific approach to the study of epilepsies. The ILAE introduced the classification and terminology for different types of epilepsies and seizures and published it in 1960. These were updated in the classifications of epileptic seizures in 1981 (Commission on Classification and Terminology of the International League Against Epilepsy 1981) and in 1989 (Commission on Classification and Terminology of the International League Against Epilepsy 1989). The most recent revision of terminology and classification of seizure types

and different forms of epilepsies were reported in 2010 (Report of the ILAE Commission on Classification and Terminology 2005–2009, Berg et al 2010).

The widely accepted terminology of epileptic seizures in both basic and clinical research is of two types: focal or partial epilepsy and generalized epilepsy. Focal seizures originate primarily in one hemisphere of the brain, which may be localized to a region or widely distributed. These are further subdivided, according to impairment of consciousness during the seizure episode into simple partial seizures, complex partial seizures and partial seizures evolving to generalized seizures. However, the revised seizure classification suggests description of focal seizures according to specific manifestations such as subjective (auras), motor, autonomic, and dyscognitive features (Berg et al 2010). Generalized epileptic seizures originate from bilateral networks involving both the brain hemispheres and include absences, myoclonus, tonic, atonic, clonic and primary generalized tonic clonic seizures.

The concept of epilepsy syndrome was given in 1989 and is defined as a "cluster of signs and symptoms customarily occurring together; including factors such as type of seizure, etiology, anatomy, precipitating factors, age of onset, severity, chronicity, diurnal and circadian cycling, and sometimes prognosis". The 1989 classification organizes forms of epilepsy syndromes in three classes: idiopathic, symptomatic and cryptogenic depending on their characteristics and the cause of manifestation. Whereas, the revised classifications incorporate additional criteria to categorize epilepsies on the basis of etiology into "genetic" (with presumed underlying genetic defect), "structural/metabolic" (associated with distinct structural or metabolic disease) and "unknown" (underlying cause is not yet known) (Berg et al 2010). Each class can further be organized on the basis of age of onset, specific underlying cause etc.

1.1 Genetic Generalized Epilepsies (GGE)

Genetic generalized epilepsies (GGE) constitute a heterogeneous group of epilepsies with generalized seizures, and are associated with bilaterally

synchronous and symmetrical EEG discharges. GGE syndromes are common and account for 30-40% of all human epilepsies (Sander 1996, Jallon and Latour 2005, Huber et al 2009). They are usually not associated with any brain lesions, mental impairment or neurological abnormalities other than seizures. This group of epilepsies was formerly called "idiopathic epilepsies" and is now often termed as "genetic epilepsies" (Berg and Scheffer 2011) due to absence of any apparent etiology other than a strong possibility of genetic predisposition. GGE represent electro-clinical syndromes, in which a combination of clinical features such as seizure types, age of onset and electroencephalogram (EEG) features (generalized spike wave discharges) are required for diagnosis.

The classical GGEs are divided based on the age of onset and the type of seizure, into four common sub-syndromes: childhood absence epilepsy (CAE), juvenile absence epilepsy (JAE), juvenile myoclonic epilepsy (JME) and epilepsy with generalized tonic-clonic seizures (EGTCS). In addition, there are several rare GGE sub-syndromes that include benign myoclonic epilepsy of infancy (BMEI), early onset absence epilepsy, myoclonic astatic epilepsy (MAE), epilepsy with myoclonic absences, eyelid myoclonia with absences and absence status epilepsy (Huber et al 2009). The frequent seizure types associated with GGE are absence seizures, myoclonic seizures and generalized tonic-clonic seizures (Mattson 2003). The EEG trait of generalized spike-waves is the defining factor in the diagnosis of GGE, indicating bilateral synchronous discharges in a normal background.

Genetic determinants are known to play a major role in pathophysiology of GGE. Several twin and family segregation studies have provided evidence for its genetic basis and indicated towards its complex inheritance pattern. Determining the genetic causes of GGE has been challenging due to its complex genetic architecture comprising of multiple genetic factors (polygenic) with or without their interaction with environmental factors. JME is the most common heritable GGE sub-syndrome and has been emphasis of my research interest for this thesis. Therefore, the study presented here focuses largely on this GGE subtype.

1.2 Juvenile Myoclonic Epilepsy (JME)

JME was first 'discovered' in Switzerland and France in the 19th century. The initial cases were reported by Theodore Herpin (Herpin 1867); and Rabot described the characteristics of myoclonic jerks (Rabot 1899). A comprehensive description of JME as a separate clinically defined syndrome was first provided by Janz and Christian, in a report of 47 cases from Germany and the name "impulsive petit mal" was proposed (Janz and Matthes 1955). Subsequently, the term "juvenile myoclonic epilepsy" was coined (Lund 1975) (review by Genton and Gelisse 2013). In 1989, ILAE suggested "juvenile myoclonic epilepsy" and "impulsive petit mal" as equivalent terms to define this clinical entity (ILAE 1989). In the last three decades or so, JME cases have been intensively studied and reported worldwide from different populations (Delgado-Escueta and Bascal 1984, Asconapé and Penry 1984, Murthy et al 1998, Nicoletti et al 1999).

1.2.1 Prevalence and incidence

JME is a relatively common subtype of genetic generalized epilepsy and accounts for 4-11% of all epilepsies (Hauser 1993, Montalenti et al 2001). Based on hospital and clinical records, it has a prevalence of 12%, whereas its population-based prevalence accounts for 3%. The persistent clinical symptoms of JME and its early age-of-onset result in a high prevalence rate in epilepsy speciality clinics than in referral centres. The prevalence rates reported in various studies are 4.3% in Germany (Janz 1969), 2.8% in Brazil (Figueredo et al 1999), 5.5% in Malaysia (Manonmani et al 1999) and 10.7% in Saudi Arabia (Obeid and Panayiotopoulos 1988). In different parts of India, the reported prevalence rates of JME are 9% (Jain et al 2003) in north India, 7.7% (Murthy et al 1998) and 5.9% (Mani and Rangan 1995) in south India. JME apparently has an equal sex distribution but several reports in literature support a slight female preponderance (Janz and Durner 1997, Panagariya et al 2001). A few studies suggest male preponderance as well (Delgado-Escueta 1984, Mehndiratta and Aggarwal 2002, Vijai et al 2003a).

1.2.2 Age of onset

The onset of JME is age-related, generally from age 12 to 18 years, but may vary from 8 years to 26 years. The mean age of seizure onset in patients with JME is 14 ± 2 years, slightly earlier in females than in males (Marini et al 2004) and about 18 years in the affected family members (Cvetkovska et al 2014). In rare cases with the onset occurring outside the range of 8-26 years, diagnosis should be done with caution. In studies from India, the mean age at onset for JME has been reported as 13.4 ± 4.9 years (± SD) (Murthy et al 1998) and 14.1 ± 3.1 years (± SD) (Mehndiratta and Aggarwal 2002). The seizure types for JME comprise myoclonic jerks (MJ), generalized tonic-clonic seizures (GTCS) and absence seizures (Ab). The mean age of onset for absence seizures is 10.5 ± 3.4 years; for myoclonic seizures is 15 ± 3.5 years and for GTCS is 16 ± 3.5 years (Panayiotopoulos et al 1994).

1.2.3 Clinical and neurological characteristics

The characteristic clinical symptom of JME is myoclonic jerks, which are usually accompanied by generalized tonic-clonic and absence seizures. Myoclonic jerks often occur within one hour of awakening and are associated with sleep-awake cycle (Janz and Durner 1997). The seizures can be precipitated by factors such as sleep deprivation, disturbed sleep-awake cycle, fatigue, alcohol intake and photic stimulation (Janz 1985, Pederson and Peterson 1998, Dhanuka et al 2001, da Silva Sousa et al 2005, Welty 2006).

Myoclonic seizures are the leading symptoms of JME and in about 5% of the patients it is the only seizures present (Greenberg et al 1988, Jain et al 1998). The MJ are characterized as short, bilateral mostly symmetric synchronous muscle contractions predominantly involving the extensor muscles of arms and shoulders. A myoclonic jerk is brief and can be single or repetitive, but usually arrhythmic. Consciousness is maintained, but may be impaired during the jerks. Myoclonic jerks generally precede generalized tonic-clonic seizures (GTCS) (clonic-tonic-clonic sequence) in most of the patients, by an average of about two years. GTCS are observed in most (> 90%) of the JME patients (Greenberg et al

1988, Montalenti et al 2001). In studies involving JME patients from India, history of GTCS has been reported in 75.7% (Mehndiratta and Aggarwal 2002) and 92.9% (Vijai et al 2003) of the cases. In a GTCS seizure, whole body is involved and episodes are usually longer and intense. The tonic phase causing stiffening of muscles is followed by clonic phase causing rhythmic and rapid jerks in arms and legs. The tonic-clonic seizure generally lasts 1 to 3 minutes and occurs mainly after awakening, but may also be nocturnal or random. Absence seizures are subtle in revelation and often go unrecognized in JME patients. The absences are clinically represented as transient phases of severe impairment/loss of consciousness reported as flashes of blackouts, momentary lack of concentration or state of non-responsiveness. They may occur several times a day without circadian variation. The frequency and severity of absence seizures is age-dependent, with those of late-onset (>10 years) being less severe. About 10-30% of JME patients manifest absence seizures, beginning at the mean age of 10.5-11.5 years (Janz 1969, Delgado-Escueta 1984, Panayiotopoulos et al 1994). Among studies from India, JME cases reported with absence seizures varied from 8-40% (Jain et al 2003, Murthy et al 1998, Mehndiratta and Aggarwal 2002, Vijai et al 2003a).

General clinical examination of JME patients is usually normal and is not associated with conditions such as head injury, brain tumor, or encephalitis (Janz and Durner 1997). A few structural and functional imaging studies have highlighted the existence of underlying anatomical abnormalities associated with JME (Meencke and Janz 1984, Meencke and Janz 1985). Specialized staining techniques on a few JME patients have revealed grey and white matter abnormalities, collectively known as microdysgenesis (Meencke and Janz 1984, Meencke and Janz 1985). However, pathological significance of these observations remains unknown. At neurotransmission level, impairment in both serotonin (Meschaks et al 2005) and dopamine (Ciumas et al 2008) systems may be involved in its pathophysiology. Advances in the development of highly sensitive neuroimaging techniques and neuroimaging analysis methods have allowed detailed investigation of the underlying structural alterations in brain anatomy of JME patients. Quantitative MRI and molecular imaging studies report

changes in medial prefrontal cortex (Woermann et al 1999, O'Muircheartaigh et al 2011), thalamus (Kim et al 2007, Pulsipher et al 2009), dorsolateral prefrontal cortex (Koepp et al 1997) and increased structural connections between prefrontal cortex and motor cortex (Vollmar et al 2012). These studies suggest alterations and dysfunction in thalamo-frontocortical network as a possible cause of functional impairment in JME (O'Muircheartaigh et al 2012, Wandschneider et al 2012, Koepp et al 2013). Overall, these findings have generated several hypotheses for origin of epileptic discharges such as cortical onset, thalamic onset or corticoreticular onset and indicate that interplay between thalamus and cortex is necessary for generation and maintenance of generalized discharges.

1.2.4 Electroencephalogram (EEG) characteristics

Electroencephalography (EEG) plays an important role in diagnosis and assessment of patients with JME. The EEG pattern for JME shows normal background activity along with typical interictal generalized spike-wave (GSW) and polyspike-wave (PSW) discharges of frequency of 4-6 Hz (Asconape and Penry 1984), with a frontocentral predominance (Rodin et al 1987). The generalized spike-wave discharges (GSWD) may last from less than 3 seconds to more than 30 seconds, and are mostly observed at sleep onset or awakenings. Classical 3 Hz spike-wave complexes or 3 Hz polyspike-wave complexes, characteristic of typical absence seizures, occur in approximately 17% of EEGs. Existence of focal features in generalized discharges is well known and is reported among one-third of GGE/JME patients (Panayiotopoulos et al 1994, Aliberti et al 1994, Usui et al 2005, Jayalakshmi et al 2010, Seneviratne et al 2014). The ictal EEG is characterized by 10- to 16-Hz medium-high amplitude spikes followed by irregular slow waves. The number of spikes ranges from 5 to 20 per episode and correlates with the intensity rather than the duration of each seizure (Janz and Christian 1957). Provocative measures, such as photic stimulation and sleep deprivation, can help elicit the characteristic EEG abnormalities; and conducting the EEG recording during the process of awakening may be necessary for the diagnosis (Janz 1985). Atypical EEG findings in some patients also contribute to the misdiagnosis of JME (Lancman et al 1994, Montalenti et al 2001). The absence of abnormal EEG readings in JME probands is not unusual (Montalenti et al 2001,

Murthy et al 1998, Jain et al 1998). The combination of EEG with functional magnetic resonance imaging (fMRI) and recent advancements in its data analyses offer a high spatial resolution and detailed *in vivo* electrophysiology than routine scalp EEG, giving an insight to redefine pathophysiology of generalized epilepsies (Gotman et al 2006, Gotman 2008, Kay and Szaflarski 2014).

1.2.5 Photoparoxysmal response (PPR)

Photosensitivity or photoparoxysmal response (PPR) is an abnormal electroencephalographic response to intermittent photic stimulation (IPS), indicated as diffuse paroxysmal discharge. It can be described as altered visual contrast gain control or heightened sensitivity to specific wavelengths. Photosensitivity is a frequently observed feature in GGE with a robust association with JME (Kasteleijn-Nolst Trenite 2001, Guerrini and Genton 2004, Seneviratne et al 2012, Koeleman et al 2013). Photosensitivity is reported in 13-18% of idiopathic absence epilepsies and in 30-35% of patients with JME (Wolf and Goosses 1986, Covanis 2005, Kasteleijn-Nolst Trenit 2006). The photic stimulation response can be influenced by several factors such as age, gender, ethnicity, anti-epileptic drug (AED) treatment, genetics, sleep deprivation and the stimulation technique. Moreover, PPR can also be provoked in 90% of patients with JME depending on mode and intensity of IPS (Appleton et al 2000). The prevalence rates of photosensitivity reported in various studies are as follows: 33% in patients from USA (Asconapé and Penry, 1984), 27.3% in patients from Saudi Arabia (Panayiotopoulos et al 1994), 17.5% in patients from Japan (Shiraishi et al 2001) and 54% in 13 families from Macedonia (Cvetkovska et al 2014). The prevalence rates of photosensitivity reported from JME cases in India are 9% (Jain et al 1998) and 11.6% (Mehndiratta and Aggarwal 2002) from north India, 13% (Murthy et al 1998) and 9.9% (Vijai et al 2003a) from south India. PPR is more often seen in the younger age group of patients and in females (Wolf and Goosses 1986, Kasteleijn-Nolst Trenit 1989, Harding and Jeavons 1994). The EEG-fMRI performed during photic stimulation indicates that the photoparoxysmal response in JME patients may be due to pathological network alterations in the striato-thalamocortical system of the brain (Bartolini et al 2014).

1.3 Genetics of juvenile myoclonic epilepsy

Juvenile myoclonic epilepsy is a heritable subtype of genetic generalized epilepsy. Its etiology is primarily genetic in origin and follows both Mendelian and complex modes of inheritance. In JME families, the clinical and EEG traits are largely inherited in an autosomal dominant inheritance pattern. However, the inherited mutant alleles often show incomplete penetrance and lead to phenotypic heterogeneity within families. Several studies indicate complex polygenic genetic architecture of JME suggesting the implicated genes may be modified by additional genes or possibly by environmental factors. To date, a few genes with disease-segregating mutations or susceptibility-conferring alleles have been identified in a rather small number of JME patients, indicating that its genetic determinants are largely undiscovered.

1.3.1 Clinical genetics

A high genetic predisposition associated with genetic generalized epilepsies has been reported by several clinical genetic studies (Janz et al 1989, Vijai et al 2003a, Cvetkovska et al 2014). The clinical genetic data obtained by twin studies and family aggregation studies suggests the genetic contribution of GGE (Helbig et al 2008, Koepp et al 2014). Prevalence of epilepsy in first- and second-degree relatives of the proband and concordance amongst twins indicate a shared genetic factor associated with the disease. The positive family history has been reported in 17-50% of the JME patients with first- or second-degree relatives affected with the same or different subtype of generalized epilepsy (Janz 1985, Mehndiratta and Aggarwal 2002, Vijai et al 2003a, Marini et al 2004). Twin studies compare concordances (presence of disease) in identical (monozygotic) and non-identical (dizygotic) twin pairs. High concordance in monozygotic twins than dizygotic twins has been reported in studies with large number of twin pairs having genetic generalized epilepsy (Berkovic et al 1998, Kjeldsen et al 2003, Vadlamudi et al 2004, Vadlamudi et al 2014), indicative of a strong genetic basis. The studies on multiplex families with several of its members affected with GGE, report heterogeneity in the clinical phenotypes and syndrome subtype as well, possibly due to variable expressivity of the causative mutation (Kapoor et al 2008). So far,

such multi-generational families have been extremely helpful to discover the disease-linked sub-genomic regions and genes in subtypes of generalized epilepsies.

1.3.2 Molecular genetics

Over the past two decades, JME has been a subject of genetic analysis, which has led to identification of several loci and genes (Delgado-Escueta et al 2013, Koepp et al 2014). These studies further support and establish the view of JME as a complex trait which is associated with high genetic and phenotypic heterogeneity. Genetic linkage- and association studies have unravelled genes, loci as well as several susceptibility alleles for JME, reflecting it's monogenic, oligogenic and polygenic genetic architecture.

1.3.2.1 Linkage studies and mutational analysis of candidate genes

JME, a GGE sub-syndrome manifests a complex mode of inheritance and is also inherited in a Mendelian or near-Mendelian manner in multi-generational families. Though, the underlying genetic factor can be a mutation of a large effect responsible for the disease or a rare allele with moderate effect along with modifier alleles resulting in phenotypic and clinical heterogeneity among the affected relatives. So far, linkage studies on such relatively uncommon large multiplex JME families have identified a few loci and genes with disease-segregating mutations in affected family members. However, the mutations in these genes do not account for majority of the JME patients from across different populations. This indicates a complex genetic and polygenic basis of JME, wherein only a small fraction of causative determinants have been deciphered and validated. The family-based linkage studies help by focusing the search to JME-linked specific chromosome loci and filtering the candidate genes to be screened, excluding major part of the exome or genome for involvement in JME.

Screening of candidate genes for mutations is based on a prior hypothesis, and aims at identifying more rare variants in epilepsy patients as compared to control subjects. These genes are normally chosen due to their association with an

epilepsy sub-syndrome or seizure phenotype in either humans or animal models or both (Escayg et al 2000, Arsov et al 2012, Tao et al 2011). So far, both these approaches have led to identification of genetic determinants for JME in a small number of families, and risk alleles remain undefined in about 90% of the patients. Here, I discuss findings regarding a few JME genes where mutations have been discovered in multiplex families using linkage-based studies and mutational analysis of candidate genes. Besides these, findings from a few genome-wide association studies identifying susceptibility loci for GGE/JME are discussed.

1.3.2.1a GABRA1 at EJM5 (5q34)

In a study by Cossette and colleagues, genetic linkage analysis on a French Canadian family with JME mapped the disease-linked region to 5q34 (Cossette et al 2002). A heterozygous mutation leading to Ala322Asp in *GABRA1*, α1 subunit of $GABA_A$ was found in the affected members of the family. $GABA_A$ receptor with the mutant α1 subunit exhibited loss of function phenotype resulting in reduced amplitude of the GABA-evoked currents (Cossette et al 2002) and reduced ligand affinity (Krampfl et al 2005). This was the first report of a gene mutation segregating with JME phenotype in a Mendelian fashion. A cohort of unrelated 35 JME patients from south India showed absence of *GABRA1*, Ala322Asp, suggestive of it being an uncommon cause of JME in south Indian population (Kapoor et al 2003). Additional mutations in the α1 subunit of $GABA_A$ receptor were found in two unrelated French Canadian GGE families (Lachance-Touchette et al 2011). The Lys353delins18X and Asp219Asn mutations in *GABRA1* altered the plasma membrane expression of $GABA_A$ receptor thus affecting its cellular functioning. Additionally, a frameshift mutation in *GABRA1*, Ser326fs328X has been reported in a patient with childhood absence epilepsy (Maljevic et al 2006). The loss of α1 subunit in heterozygous *Gabra1*+/− mice leads to increased spike-wave discharges and absence-like seizures (Arain 2012).

$GABA_A$ receptors are ligand-gated (ionotropic) chloride channels and are the major inhibitory neurotransmitter receptors in mammalian brain. They mediate fast inhibitory synaptic transmission in the central nervous system. The GABA receptor is a pentameric multi-subunit complex assembled from subunits (α_{1-6}, β_{1-}

$_4$, γ_{1-3}, δ, ε, π, θ), with α1β2γ2 receptor isoform being most abundant in the brain (Macdonald and Olsen 1994, Sigel and Steinmann 2012). Mutations in α1 subunit of $GABA_A$ receptor affect its normal cellular expression and thus, disturb the inhibitory synaptic transmission. This impairment can disrupt the fine-tuned balance between excitatory and inhibitory input in neurons, leading to seizure induction by uncontrolled hyperexcitability.

1.3.2.1b EFHC1 at EJM1 (6p12-p11)

The JME locus at 6p21.2-p11 was identified by genetic analysis of a four-generation family from Los Angeles-Belize and seven multiplex families with JME (Liu et al 1995). The critical region was mapped and refined to the 6p12-p11 location (EJM1) by genotyping and recombination analysis of members of additional Mexican families (Liu et al 1996, Bai et al 2002). Mutational analyses of 18 genes in this interval excluded all except Myoclonin1/*EFHC1* (Suzuki et al 2002, Suzuki et al 2006). Five missense mutations in *EFHC1* were identified to co-segregate with epilepsy in six unrelated families. *EFHC1* encodes a 640-amino acid protein, EF-hand domain-containing protein 1, harbouring three DM10 domains and an EF-hand calcium-binding motif. EFHC1 enhances the calcium influx through $Ca_v2.3$ channel and regulates the cellular apoptosis. Mutations in this gene affect the EFHC1-induced calcium influx through the R-type calcium channel and reduce its effect on the programmed cellular death mechanism (Suzuki et al 2004). Additional *EFHC1* mutations have been reported in patients with JME (Medina et al 2008, Jara-Prado et al 2012) and JAE (Stogmann 2006) across different populations.

While the autosomal dominantly inherited single copy mutations in this gene were reported in JME patients, its homozygous mutation is associated with a severe form of epilepsy syndrome, severe intractable epilepsy of infancy, resulting in death within 18-36 months (Berger et al 2012). Although linkage analysis data from 45 mostly Mexican families mapped EJM1 to 6p12-p11, no disease-segregating *EFHC1* mutations were found in 39 of these, indicating that exonic mutations in *EFHC1* account for only a small fraction of JME cases. Additionally, three mutations reported by Suzuki et al (2004) were found relatively common in

non-Mexican populations (Greenberg and Stewart 2014). However, the occurrence of frequent and spontaneous myoclonias in heterozygous and homozygous Efhc1-null adult mice suggest that decreased function of *EFHC1* may lead to seizure susceptibility and epileptogenicity in JME patients with *EFHC1* mutations (Suzuki et al 2009).

EFHC1 is a microtubule-associated-protein (MAP) involved in cell division and neuronal migration during cortical development (de Nijs et al 2009). It associates with mitotic spindle and the midbody during mitosis and cytokinesis, respectively (de Nijs et al 2006). The *EFHC1* mutations identified in JME patients can apparently cause neuronal migration defects suggesting structural abnormalities during brain development (de Nijs et al 2012). Interestingly, a number of functional and neuroimaging studies have reported occurrence of subtle structural abnormalities in brains of JME patients, involving cortical grey matter, hippocampus, frontal lobe, etc (review by Kay and Szaflarski 2014). These pathological observations and the potential biological role/s of EFHC1 in cortical development disrupted by JME mutations, suggest that JME is a neurodevelopmental disorder and *EFHC1* mutations may cause subtle disruption of the developmental process (de Nijs et al 2013).

1.3.2.1c CACNB4 at EJM6 (2q22-q23)

The spontaneous mutation in *Cacnb4* (beta subunit gene) in *lethargic* mouse causes ataxia and seizures. It results in a 4-nucleotide insertion in a splice donor site leading to exon skipping, translational frameshift, and protein truncation (Burgess et al 1997). Inactivation of Cacnb4 in mouse results in a complex neurological condition including absence seizures and ataxia. Following this observation, the role of this gene in human epilepsies was explored by screening for mutations in *CACNB4* (calcium channel beta-4 subunit) in small families with epilepsy and ataxia, wherein a premature-termination mutation Arg482X in a patient with JME was found (Escayg et al 2000) (EJM6). Additionally, *CACNB4* mutations were detected in a German family with GGE and praxis-induced seizures (EIG9) and in a French Canadian family with episodic ataxia type 5 (EA5)

(Escayg et al 2000). The *CACNB4* gene maps to 2q22-q23 (Escayg et al 1998) and encodes for beta-4 subunit for a voltage-dependent calcium channel (VGCC). The Arg482X mutation leads to truncation of 38 amino acid residues within its C-terminus, disrupting its interaction domain for the α1 subunit. Few studies show that VGCC subunit β4 regulate gene expression via its nuclear targeting and association with regulatory protein complex (Colecraft et al 2002, Hibino et al 2003, Subramanyam et al 2009). The Arg482X mutation apparently causes conformational changes in β4 affecting its nuclear targeting, and thus results in altered gene expression of a few genes (Tadmouri et al 2012). In contrast, normal nuclear targeting of the truncated protein ($\beta_{1\text{-}481}$) was observed in different cell types such as tsA-201 cells, skeletal myotubes, and in hippocampal neurons (Etemad et al 2014). Thus, it is conflicting to conclude whether the calcium channel-dependent or nuclear function of *CACNB4* is involved in the etiology of epilepsy in humans and mouse models.

1.3.2.1d GABRD at EJM7 (1p36.33)

A homozygous Arg220His missense mutation in γ-aminobutyric acid A receptor, delta (*GABRD*) gene was identified in a patient with JME (Dibbens et al 2004). The heterozygous variation Arg220His was found in about 8.3% of the patients with genetic generalized epilepsy (EIG10) and in 4.2% of controls. As this heterozygous variation is found in the general population, its homozygosity may have contributed to the pathogenesis of JME. Besides this, the same study reported a heterozygous mutation, Glu177Ala to be associated with generalized epilepsy with febrile seizures plus. The *GABRD* gene encodes δ-subunit of the ligand-gated chloride channel for gamma-aminobutyric acid (GABA). The Arg220His mutation resulted in decreased $GABA_A$ receptor current amplitudes, which may be associated with increased neuronal excitability and predispose for seizure phenotype (Dibbens et al 2004). However, no association was observed between Arg220His variation with GGE or JME among 562 German patients (Lenzen et al 2005), excluding *GABRD* as a common susceptibility gene for JME. The absence of spontaneous seizures in *GABRD* null mice (Mihalek et al 1999) indicates that *GABRD* itself is not sufficient and may act in conjunction with other genes or environment to produce epilepsy phenotype. These studies suggest that

GABRD variants are likely to be a small part of the complex genetic architecture in contributing towards susceptibility to common epilepsies.

1.3.2.1e SLC2A1 at EIG12 (1p34)

The defective transport of glucose across the blood-brain barrier in patients with GLUT1 deficiency syndrome-1 has been associated with mutations in *SLC2A1* (solute carrier family 2, member 1; *GLUT1*, glucose transporter 1) (De Vivo et al 1991, Seidner et al 1998). Apart from severe neurological defects observed in *GLUT1* deficiency syndrome, *SLC2A1* mutations have also been reported in large and small families with less severe disorders such as paroxysomal exercise-induced dyskinesia (Suls et al 2008) and genetic generalized epilepsies (Suls et al 2009, Mullen et al 2010, Striano et al 2012) (EIG12). However, genetic heterogeneity and phenotypic variability is notable in patients with *GLUT1* mutations, varying from early onset-childhood absence epilepsy (CAE), epilepsy with myoclonic-atonic seizures to CAE evolving to JME. On screening of a cohort of 504 GGE probands from Israel and Australia, *GLUT1* mutations were found in 7 probands, contributing to about 1% of GGE (Arsov et al 2012). The functional effect of these variations varied from marked decrease in glucose transport to no effect on the protein function. These studies implicate that mutations in *GLUT1* may act as causative or susceptibility factors in GGE and other milder forms of epilepsies.

1.3.2.1f CHRNA7 at EJM2 (15q14)

At 20q13, mutations in the *CHRNA4* gene encoding the alpha-4 subunit of the neuronal nicotinic acetylcholine receptor (nAChR) were identified in rare Mendelian idiopathic epilepsy, autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) (Steinlein et al 1995). Subsequently, markers around all nAChR genes were tested in 34 European JME families and significant evidence of linkage was obtained on chromosome 15q13–q14 close to the *CHRNA*7 gene (Elmslie et al 1997).

However, a few independent reports provided evidence against linkage of JME to the 15q14 locus. Sander et al (1999) found no evidence in favour of linkage to 15q14 in 27 JME and 30 IAE families analyzed together. Durner et al (2000) analyzed 53 families ascertained through a JME proband, but no significant evidence of linkage to chromosome 15q14 was obtained assuming genetic homogeneity and autosomal recessive mode of inheritance with 50% penetrance value. Another study supported the linkage at 15q13–14 for JME only and not with a broadly defined phenotype of GGE (Taske et al 2002) but, it failed to identify any disease-causing variant in *CHRNA7* in the 34 JME families examined earlier. Additionally, 15q13.3 microdeletions encompassing *CHRNA7* have been identified in 12 of 1,223 individuals with GGE (Helbig et al 2009), of which, six had JME.

1.3.2.1g PRICKLE1 and PRICKLE2

The role of prickle genes in epilepsy was first noted when a homozygous mutation was found in three families with progressive myoclonus epilepsy-ataxia syndrome (Bassuk et al 2008). Later, two heterozygous missense mutations each in the *PRICKLE1* and *PRICKLE2* genes were discovered in GGE/JME patients with myoclonic seizures and generalized EEG patterns (Tao et al 2011). PRICKLE proteins characterized by their PET and LIM domains, are core components to establish cell polarity during embryonic development functioning via the noncanonical WNT signaling pathway (Liu et al 2013). Prickle1 mutant mice and *Drosophila* prickle mutants exhibit a decreased seizure threshold along with generalized disorganization of the peripheral nervous system in heterozygous mutant flies. Prickle2 mutations affect the intracellular calcium release in zebrafish; and *Prickle2*$^{-/-}$ and *Prickle2*$^{+/-}$ mice show increased epileptiform discharges (Tao et al 2011). This study concludes that deregulation of the *PRICKLE* signaling pathway plays a role in causation of seizures. Its disruption may be affecting the cell polarity and positioning of neurons during development or altering the prickle-mediated calcium signaling in nervous system.

1.3.2.1h Additional whole-genome wide linkage and meta-analysis studies

Besides the studies described above, genome-wide linkage analysis on large number of multiplex families has led to identification of susceptibility loci for GGE with significant or suggestive evidence for linkage. In a collaborative study of European Consortium on the genetics of genetic generalized epilepsy, linkage analysis was conducted in 130 multiplex GGE families from the UK, France, Germany, Italy and Netherlands, with probands with CAE (n=53), JAE (n=23) and JME (n=54). Non-parametric multipoint linkage analysis provided significant evidence of a novel GGE susceptibility locus on 3q26 (Z_{NPL} = 4.19 at D3S3725) and suggestive evidence for two additional GGE loci on chromosomes 14q23 (Z_{NPL} = 3.28 at D14S63) and 2q36 (Z_{NPL} = 2.98 at D2S1371) (Sander et al 2000). Durner et al (2001) performed linkage studies on 91 multiplex GGE families and reported strong evidence for a locus common to most GGE subtypes on chromosome 18q21 (lod score 4.4 and 5.2 in multipoint and two-point analysis respectively), assuming autosomal recessive mode of inheritance. Both JME and non-JME families gave positive evidence for linkage at the 18q21 locus. Linkage was observed for the HLA region on chromosome 6 (lod score 2.5 and 4.2 in multipoint and two-point respectively) in the JME families. However, non-JME families did not show linkage to the HLA region and a suggestive evidence of linkage was detected at 5p15 and 5q14-q22 for absence epilepsies. These results supported the oligogenic model for GGE, with interactions of different combination of susceptibility loci leading to heterogeneous clinical presentations in GGE subtypes (Durner et al 2001). Another genetic linkage study involving 130 multiplex GGE families, failed to support evidence for a major GGE locus at 18q21.1 region (Sander et al 2002).

An additional genetic study was conducted which included the 126 GGE-multiplex families collected by an Australian and six European groups (Hempelmann et al 2006). To dissect out the seizure type-specific susceptibility loci, heterogeneity was decreased by conducting linkage analysis on distinct subgroups comprising 53 JME families and 73 IAE families (non-JME). The genome-wide analysis revealed linkage to two chromosomal regions: 6p12 (Z_{NPL} = 3.05 at D6S2410) and 19q13 (Z_{NPL} = 3.46 near D19S585) and evidence of a suggestive linkage at 5q34

(Z_{NPL} = 2.77 at *GABGR2*- D5S423), conferring susceptibility to myoclonic and GTCS seizures. The subgroup of IAE families (non-JME) revealed susceptibility loci mapping to 11q13 and 13q22-q31 regions, thus conferring risk for absence seizures. This study showed that distinct and shared genetic defects seem to constitute the complex genetic architecture of GGE sub-syndromes (Hempelmann et al 2006).

Most of the linked loci for GGE have not been replicated in independent set of families, reflecting the underlying genetic complexity and heterogeneity of generalized epilepsies. As these individual studies had been performed on a smaller sample size, providing a low power for detection in majority of families, they fail to reveal replicable susceptibility loci. To address this issue, EPICURE consortium performed meta-analysis of three linkage datasets comprising 379 genetic generalized epilepsies (GGE)-multiplex families of European descent (Leu et al 2012, EPICURE Consortium). These families included the families reported earlier by Sander et al (2000) and Hempelmann et al (2006) consisting of 1920 family members of whom 982 were affected by GGE [JAE/CAE (504), JME (258), EGTCS alone (205) and unclassified GGE syndromes (15)]. Under the broad trait model (all GGEs), multipoint non-parametric (NPL) and parametric HLOD analysis revealed suggestive evidence of linkage at 3p14.2 (LOD_{npl} = 2.96 at rs624755, dominant: HLOD = 2.84 at rs782728; recessive: HLOD = 3.21 at rs1374679), 5q34 (LOD_{npl} = 1.95 at rs1432881), 13q12.12 (LOD_{npl} = 2.42 at rs1008812), 19q13.42 (LOD_{npl} = 2.86 at rs9788), 1p36.22 (dominant: HLOD = 2.50 at rs1216213), and at 13q31.3 (recessive: HLOD = 2.67 at D13S1230). However, linkage analysis to dissect out the seizure type-related susceptibility genes was carried under narrow trait model for two family subgroups (235 GAE and 118 JME). The genome-wide parametric and non-parametric analysis revealed suggestive linkage at 5q34 (LODnpl = 2.31 at rs244903, dominant: HLOD = 3.23 at rs357608) and a significant linkage evidence in the chromosomal region 13q31.3 (recessive: HLOD = 5.02 at rs1332470) for absence seizures. Whereas, the parametric and nonparametric analysis for 118 JME families exhibited significant linkage at 2q34 (LOD_{npl} = 3.43 at D2S143, dominant: HLOD = 2.50 at D2S143; recessive: HLOD = 2.59 at D2S143); and a suggestive evidence of linkage at 5q34 (LOD_{npl} = 2.62 at

rs1025482, dominant: HLOD = 2.96 at rs2069347) and at 21q22.3 (dominant: HLOD 2.57 at rs2839377). Notably, some of these loci are supported by previous findings: 13q31.3 has been previously implicated for GGE and photosensitive GGE (Tauer et al 2005, Hempelmann et al 2006), 5q34 wherein a mutation in *GABRA1* causes JME in a large multi-generational family (Cossette et al 2002) and 2q34 by a report of significant linkage to JME to the region 2q33-q36 in a south Indian multi-generational family (Ratnapriya et al 2010). These meta-analysis results indicate that the risk factor at 5q34 predisposes to broad spectrum of GGE sub-syndromes, whereas susceptibility loci at 2q34 and 13q31.3 preferentially confer risk to myoclonic seizures or absence seizures, respectively (Leu et al 2012, EPICURE Consortium).

1.3.2.2 Association studies

Like many other complex phenotypes, GGE/JME has been subjected to genetic association studies in various populations (Tan, Mulley & Berkovic 2004). Many candidate genes, chosen on the basis of their physiological role or their localization in and around JME-linked loci, have been analyzed for association with JME. A few genome-wide association studies (GWAS) for epilepsy patients have been performed and results from these studies suggest that common genetic variations make a modest contribution towards epilepsy susceptibility.

Since the first locus for JME was found to be in the proximity of the HLA region on chromosome 6, initial association studies in JME addressed whether JME-locus is inside or outside the HLA region. Preliminary evidence for association between JME and alleles in the HLA region was reported in German (Durner et al 1992) and Saudi Arabian (Obeid et al 1994) population. Greenberg and colleagues (1996) reported that frequency of HLA-DR13 and HLA-DQB1 alleles were significantly higher in JME patients compared to non-JME patients, indicating that the JME locus probably lies within the HLA region, and that JME is genetically different from certain other forms of GGEs. However, other groups found no significant evidence of association of HLA alleles with JME in various populations (Moen et al 1995, Sander et al 1997, Le Hellard et al 1999).

Table 1.1: Chromosome loci and genes for GGE/JME

Chromosome location	Phenotype; OMIM	Locus/Gene/ Marker	Country/ethnic group/ancestral origin[#]	Type of study	Reference
1p36.33	GGE/JME (EJM7, EIG10)	*GABRD*	72 unrelated patients with GGE	Mutational analysis	Dibbens et al (2004)
1p36.22	GGE	Suggestive linkage	European descent families (235 with GAE and 118 with JME)	Meta-analysis of genome-wide linkage studies	Leu et al 2012 (EPICURE Consortium)
1p34	GGE (EIG12)	*SLC2A1*	Families with GGE	Mutational analysis	Suls et al 2009, Striano et al 2012, Arsov et al 2012
1q43	GGE/JME	Significant association (*CHRM3*)	3020 GGE patients and 3914 controls of European ancestry	Genome-wide association study	Steffens et al 2012 (EPICURE Consortium and EMINet Consortium)
2p16.1	GGE	*VRK2/FANCL*	2606 cases and 18990 controls (European, Asian and admixed African-American)	Meta-analysis of genome-wide association studies	ILAE Consortium on Complex Epilepsies 2014
2p16.1	GGE	Significant association (*VRK2*)	3020 GGE patients and 3914 controls of European ancestry	Genome-wide association study	Steffens et al 2012 (EPICURE Consortium and EMINet Consortium)
2q22-q23	GGE/JME (EIG9, EJM6)	*CACNB4*	90 GGE families of European descent (49 JME families)	Mutational analysis	Escayg et al 2000
2q24.3	GGE	Suggestive association (*SCN1A*)	3020 GGE patients and 3914 controls of European ancestry	Genome-wide association study	Steffens et al 2012 (EPICURE Consortium and EMINet Consortium)
2q34	JME	Significant linkage	European descent 118 families with JME	Meta-analysis of genome-wide linkage studies	Leu et al 2012 (EPICURE Consortium)

2q33–q36	JME (EJM9)	Significant linkage	4-generation family from south India	Linkage analysis	Ratnapriya et al 2010
2q36	GGE	Suggestive linkage	130 GGE multiplex families of European descent (JME, n=54)	Linkage analysis	Sander et al 2000
3p14.2	GGE	Suggestive linkage	European descent families (235 with GAE and 118 with JME)	Meta-analysis of genome-wide linkage studies	Leu et al 2012 (EPICURE Consortium)
3q13.3-q21	GGE (EIG8)	Significant linkage	3-generational south Indian family	Linkage and mutational analysis	Kapoor et al 2008
3q26/3q27.1	GGE/JME (EIG11, EJM8)	Significant linkage	130 GGE multiplex families of European descent (JME, n=54)	Linkage analysis	Sander et al 2000
4p15.1	GGE	Suggestive association	2606 cases and 18990 controls (European, Asian and admixed African-American)	Meta-analysis of genome-wide association studies	ILAE Consortium on Complex Epilepsies 2014
5q12–q14	JME (EJM4)	Significant linkage	Multi-affected three generation family from south India	Linkage analysis	Kapoor et al 2007
5q22.3	GGE	Suggestive association	2606 cases and 18990 controls (European, Asian and admixed African-American)	Meta-analysis of genome-wide association studies	ILAE Consortium on Complex Epilepsies 2014
5q34	JME (EIG13, EJM5)	*GABRA1*	Multi-affected French Canadian family; 2 unrelated French Canadian families with GGE	Linkage and mutational analysis	Cossette et al 2002; Lachance-Touchette et al 2011
5q34	GGE, JME and Absences	Suggestive linkage	European descent families (235 with GAE and 118 with JME)	Meta-analysis of genome-wide linkage studies	Leu et al 2012 (EPICURE Consortium)
5q34	GGE/JME	Suggestive linkage	126 GGE multiplex families of European descent (JME, n=53)	Linkage analysis	Hempelmann et al 2006

6p21.3	GGE/JME	Significant linkage	91 GGE multiplex families (JME, n=53)	Linkage analysis	Durner et al 2001
6p21	JME (EJM3)	*BRD2* (RING3)	JME families	Linkage and association analysis	Greenberg et al 1988, Weissbecker et al 1991, Pal et al 2003
6p12	GGE/JME	Significant linkage	126 GGE multiplex families of European descent (JME, n=53)	Linkage analysis	Hempelmann et al 2006
6p12-p11	JME (EJM1)	*EFHC1*	Hispanics (Los Angeles-Belize, Mexico), 6 Mexican families	Linkage analysis and Mutational analysis	Liu et al 1995, Liu et al 1996, Bai et al 2002, Suzuki et al 2004
8q24	GGE (EIG1)	Significant linkage	10 GGE families	Linkage analysis	Zara et al 1995
8q24	JME	Allelic association of *KCNQ3*	119 cases from south India	Association study	Vijai et al 2003b
9q32-q33	GGE (EIG3)	Suggestive linkage	A consanguineous Turkish family	Linkage analysis	Baykan et al 2004
10p11.22	GGE (EIG5)	Significant linkage	17 French-Canadian families	Linkage analysis	Kinirons et al 2008
10q25-q26	GGE/GTCS (EIG4)	Significant linkage	5 families from north India	Linkage analysis	Puranam et al 2005
11q22.2	GGE (GGE)	Suggestive association	2606 cases and 18990 controls (European, Asian and admixed African-American)	Meta-analysis of genome-wide association studies	ILAE Consortium on Complex Epilepsies 2014
13q12.12	GGE (GGE)	Suggestive linkage	European descent families (235 with GAE and 118 with JME)	Meta-analysis of genome-wide linkage studies	Leu et al 2012 (EPICURE Consortium)

13q31.3	GGE and Absences	Significant linkage	European descent 235 GAE families	Meta-analysis of genome-wide linkage studies	Leu et al 2012 (EPICURE Consortium)
13q31.3	GGE/PPR	Suggestive linkage	25 European descent families GGE and PPR	Linkage analysis	Tauer et al 2005
14q23	GGE (EIG2)	Suggestive linkage	130 GGE multiplex families of European descent (JME, n=54)	Linkage analysis	Sander et al 2000
15q11.2	GGE	Microdeletions	1234 GGE patients and 3022 controls of European ancestry, 399 GGE patients (JME, n=189)	Association study, Whole-genome oligonucleotide array CGH analysis	de Kovel et al 2010, Mefford et al 2010
15q13.3	GGE (EIG7, EJM2)	Microdeletions (*CHRNA7*)	1223 GGE patients from Europe and North America, 539 GGE cases from Australia and Europe (JME, n=183), 399 GGE patients (JME, n=189)	Case-control study/ case-control and familial segregation analysis, Whole-genome oligonucleotide array CGH analysis	Helbig et al 2009, Dibbens et al 2009, Mefford et al 2010
15q14	JME	Allelic association of *CX36*	46 JME families	Association study	Mas et al 2004
15q14	JME (EIG7, EJM2)	*CHRNA7*	34 European families with JME	Linkage analysis	Elmslie et al 1997
16p13.3	GGE/CAE (EIG6, ECA6)	*CACNA1H*	192 GGE patients; 240 epilepsy patients	Mutational analysis	Heron et al 2004; Heron et al 2007
16p13.11	GGE	Microdeletions	1234 GGE patients and 3022 controls of European ancestry, 399 GGE patients (JME, n=189)	Association study, Whole-genome oligonucleotide array CGH analysis	de Kovel et al 2010, Mefford et al 2010
17q21.32	GGE	Significant association (*PNPO*)	3020 GGE patients and 3914 controls of European ancestry	Genome-wide association study	Steffens et al 2012 (EPICURE Consortium and EMINet Consortium)

18q21	GGE/JME	Significant linkage	91 GGE multiplex families (JME, n=53)	Linkage analysis	Durner et al 2001
18q21	GGE	SNP haplotype association of *ME2*	156 GGE patients/108 GGE families	Case-control analysis/ family-based association	Greenberg et al 2005
19q13	GGE (GGE)	Suggestive linkage	European descent families (235 with GAE and 118 with JME)	Meta-analysis of genome-wide linkage studies	Leu et al 2012 (EPICURE Consortium)
19q13	GGE/JME	Significant linkage	126 GGE multiplex families of European descent (JME, n=53)	Linkage analysis	Hempelmann et al 2006
21q22.3	JME	Suggestive linkage	European descent 118 families with JME	Meta-analysis of genome-wide linkage studies	Leu et al 2012 (EPICURE Consortium)

#Patients and families with GGE/JME analysed in respective studies are enlisted. GGE, genetic generalized epilepsy; JME, juvenile myoclonic epilepsy; CAE, childhood absence epilepsy; GGE, genetic generalized epilepsy; PPR, photoparoxysmal response. GGE-associated rare variants in *GREM1, OR10S1*, *PPEF2, CHD1* and *PSME2* genes, identified by whole exome sequencing of 118 GGE patients [JME, n = 93] (Heinzen et al 2012) are not included in this list.

Pal et al (2003) identified highly significant linkage disequilibrium (LD) between JME and a core haplotype of five SNP and microsatellite markers with the LD peaking at *BRD2* at 6p21.3. Two JME-associated SNP variants in the promoter region of *BRD2* were detected; however, no other potentially causative variants were identified in 20 probands from families which had shown positive linkage to the locus. Subsequently, a multi-center study examining the role of *BRD2* as a risk factor for JME replicated the result in the British cohort, found suggestive evidence for its role in Ireland, but showed lack of replication in the German, Australian and Indian populations studied (Cavalleri et al 2007). Similarly, association analysis in a Dutch population identified no significant difference between the allele frequencies of SNP markers located in *BRD2* in cases and controls (de Kovel et al 2007).

In a group of 46 JME patients, including 29 individuals from families used to identify susceptibility locus at 15q14 (Elmslie et al 1997), a significant association between JME and the c.588C>T variant, located in exon 2 of the *CX36* gene was observed (Mas et al 2004). Bioinformatics analysis of the associated SNP suggested that it perturbed the expression of *CX36* by affecting exonic splicing enhancers. For the GGE-linked locus at 18q21, case-control and family-based association methods identified *ME2* (malic enzyme 2) as a gene predisposing to GGE. An increased risk to GGE was found when *ME2*-centered nine-SNP haplotype was present in the homozygous state (Greenberg et al 2005). However, a population-based association study involving 660 GGE patients and 666 controls of German descent did not exhibit association of genetic variations in *ME2* to common GGE syndromes (Lenzen et al 2005).

A two-staged genome-wide association study, including 3020 patients with GGE (1434 GAE and 1134 JME patients) and 3914 controls of European ancestry was carried out by EPICURE Consortium and EMINet Consortium to identify susceptibility alleles for common generalized epilepsies (Steffens et al 2012, EPICURE Consortium and EMINet Consortium). Joint analysis for stage-1 and -2 of the study revealed significant association at 2p16.1 (rs13026414) and at

17q21.32 (rs72823592) for genetic generalized epilepsies. The search for syndrome-related susceptibility genes discovered significant association at 2q22.3 (rs10496964) for GAE (absence epilepsies) and at 1q43 (rs12059546) for JME. The JME-associated region at 1q43 encompasses the gene encoding the M3 muscarinic acetylcholine receptor (*CHRM3*) (rs12059546).

Recently, a meta-analysis of genome-wide association studies from 12 cohorts of epilepsy patients and controls was conducted to detect risk variants for common epilepsies (ILAE Consortium on Complex Epilepsies 2014). The data obtained from published and unpublished genetic cohort studies from EPICURE, EPIGEN, Philadelphia (PA, USA), the Imperial-Liverpool-Melbourne Collaboration, GenEpa, and Hong Kong (China) were used for the meta-analysis. This study included 8696 cases and 26157 controls, belonging to European, Asian or African ancestry. Analysis in all-epilepsy category revealed significant association at 2q24.3 (*SCN1A*) and 4p15.1 (*PCDH7*). However for meta-analysis of genetic generalized epilepsy (GGE), 2606 cases and 18990 controls from eight cohorts were used. The analysis detected the genome-wide significant signal at 2p16.1 (rs2947349), located near the genes encoding vaccinia-related kinase 2 (*VRK2*) and Fanconi anaemia, complementation group L (*FANCL*). The association of 2p16.1 region in GGE cohort had been earlier reported by EPICURE Consortium (Steffens et al 2012, EPICURE Consortium and EMINet Consortium). Additionally, suggestive evidence for association with GGE was found at regions 4p15.1 (*PCDH7*), 5q22.3 (intergenic) and 11q22.2 (*MMP8*).

1.3.2.3 Massive parallel sequencing studies

Given that linkage-based candidate gene analysis and association studies have not had much success in identifying large-effect variants in majority of patients with common generalized epilepsies, comprehensive characterization of variants across the genome by next-generation sequencing (NGS) may help reveal common risk factors for epilepsies. The advancement in next-generation sequencing technologies and the reduced costs have made whole-exome sequencing (WES) or whole-genome sequencing (WGS) of individual epilepsy patients feasible.

The first reported NGS study for GGE had analyzed the exome sequences of 118 GGE patients [juvenile myoclonic epilepsy (n = 93) and absence epilepsy (n = 25)] and 242 controls of European ancestry (Heinzen et al 2012). The study employed two-stage approach involving exome sequencing of 118 GGE-affected individuals and a subsequent large-scale follow-up genotyping of identified candidate variants in a larger cohort of 878 GGE-affected subjects and 1830 controls. In total, 3897 candidate variants were subsequently genotyped in the larger follow-up cohort. No variant reached the statistical significance level of association in the study. However, a number of variants were enriched in patients than controls, notably, in genes *GREM1, OR10S1, PPEF2* and *CHD1*. A total of 1289 variants were reported exclusive to GGE patients and among these, a missense mutation (c.620C>T, p.Ala207Val) in *PSME2* was observed in five unrelated cases. Findings from this study failed to reveal a single variant that could account for more than 1% of the cases, indicating high genetic heterogeneity in generalized epilepsies. It suggests that identification of disease-causing rare variants that are present at very low frequencies needs be carried out through genome-/exome-based sequencing studies in very large sample sizes or co-segregation analyses in multiplex families (Heinzen et al 2012).

Additionally, the massive parallel sequencing technology has provided diagnostic screening method for genetically complex and heterogeneous disorders such as epilepsies. One such study reported use of targeted panels comprising 265 most relevant epilepsy genes or genes related to epilepsy phenotypes (37 GGE associated genes) to identify mutations for epilepsy. Disease causing mutations were detected in 16 out of 33 patients with different epileptic syndromes, belonging to a cohort from Germany and Switzerland (Lemke et al 2012).

1.3.2.4 Copy number variations

Copy number variations are a form of structural variation, which results in alteration of number of copies of one or more sections of DNA. They correspond to large segments of DNA being deleted (fewer than normal number) or duplicated (more than normal number); and may range from a kilobase to several megabases in size. CNVs are found on all chromosomes without any clinical significance;

however, they can be enriched in certain genomic regions known as "hotspots". CNVs are increasingly found in epilepsy patients, when often associated with developmental delay, autism spectrum disorder (ASD), intellectual disability and dysmorphic features (Sharp et al 2008, Shinawi et al 2010, Moreira et al 2014).

Besides these, CNVs have also been reported in epilepsy syndromes associated with normal intellect. Helbig and colleagues (2009) identified microdeletions at 15q13.3 in 12 out of 1,223 individuals with GGE from Europe and North America. This region was earlier implicated in 15q13.3 microdeletion syndrome in of individuals with mental retardation and epilepsy, schizophrenia, autism and other neuropsychiatric features (Sharp et al 2008, Stefansson et al 2008, Miller et al 2008). The deletions in this critical region encompass several genes such as the *CHRNA7* (a7 subunit of the nicotinergic acetylcholine receptor), which is considered a plausible candidate gene for epilepsy (Elmslie et al 1997). In another independent cohort of 539 GGE familial and sporadic GGE cases (JME, n=183) from Australia and Europe, 15q13.3 microdeletions were observed in 7 cases (3 individuals with JME) (Dibbens et al 2009). The 15q13.3 microdeletions were found *de novo* in three probands, two individuals had inherited from the unaffected parent and the transmission could not be determined in the other two probands. Though 15q13.3 microdeletions exhibit strong association with GGE in case-control studies, it does not segregate as a dominant Mendelian trait in multiplex GGE families, indicating it as a susceptibility genetic factor for epilepsies. In a group of 246 cases with 15q13.3 microdeletion syndrome, 28% of the individuals were reported to have epilepsy/seizures (Lowther et al 2014).

de Kovel et al (2010) reported a study aimed to investigate the genomic hotspot regions, previously implicated in various neuropsychiatric disorders, in order to identify the genetic factors conferring risk to GGE syndromes. A group of 1234 GGE patients from North-western Europe and 3022 controls from Germany were assessed using high-density SNP arrays; and recurrent microdeletions were found at 15q11.2 and 16p13.11. In an additional study, by using whole-genome oligonucleotide array CGH to a cohort of 517 patients with GGE or non-lesional epilepsies (JME, n=189), one or more rare CNVs were detected in 8.9% of the

affected individuals. Of these, 2.9% of the probands were found to harbour deletions in regions 15q11.2, 15q13.3, or 16p13.11 (Mefford et al 2010).

Several studies report CNVs associated with epilepsies in the absence of dysmorphic features in the same hotspot regions which were previously identified in patients with the neurocognitive phenotypes, suggesting that these common etiological genetic factors have highly variable phenotypic expressivity (Mefford 2014, Scheffer and Mefford 2014). However, the microdeletions at three genomic hotspots 15q11.2, 15q13.3 and 16p13.11 are notably associated with GGE than any other epilepsy type, though with incomplete penetrance. It indicates that the microdeletion itself is not sufficient to cause epilepsy and may act as a risk factor in the complex genetic architecture known for GGE.

1.4 Perspective

It is evident from the research findings presented in the above sections that genetic generalized epilepsies have substantial genetic component to their etiology with a complex, heterogeneous genetic architecture and phenotype-genotype correlation. These features make the identification of etiological factors for GGE quite challenging.

Over past 10 years or so, advancements in the field of human genetics and clinical diagnosis have provided a better understanding of the genetic basis of various GGE syndromes. Advanced imaging analysis techniques and EEG-triggered fMRI have provided new insights for generalized epilepsies, by enabling detailed *in vivo* studies of electrophysiology and elucidation of network abnormalities in different brain regions. Failure to elicit the history of myoclonic jerks in a clinical examination often results in a delayed diagnosis or misinterpretation of the GGE syndromes. Co-occurrence of JME with other generalized seizures, presence of EEG abnormalities in asymptomatic family members and familial clustering of different GGE syndromes indicates complexity in phenotypic characterization for this disease. Due to considerable overlap in the seizure components of common

GGE sub-syndromes, several studies tend to lump together different subtypes of GGE to find a common defining locus. Other studies advocate splitting different epilepsies on the basis of syndrome classification to identify syndrome-specific locus. However, recent meta-analysis of large datasets of linkage and association studies have employed both broad- and narrow-trait approaches to discover shared and distinct genetic factors, respectively for GGE syndromes. Such an approach has identified susceptibility loci that confer risk to a broad spectrum of GGE syndromes along with ones that preferentially predispose to myoclonic or absence seizures.

Despite using various approaches comprising family-based genetic studies as well as multi-centre linkage and association analyses of large number of GGE patients, so far only a limited number of susceptibility genes and loci have been identified, which account for a small proportion of complete genetic component of complex epilepsies. Limited success in such studies can be attributed to presence of locus- and allele- heterogeneity; and the involvement of population specific risk factors. Next-generation sequencing (NGS) methodologies have enabled whole-exome- and whole-genome- sequencing of individuals at a reasonable cost and thus, providing opportunity to identify patient-specific, disease-associated rare variants. But, such exome-based sequencing of a large number of GGE patients has apparently not yielded variants of large effects, due to high genetic heterogeneity of GGE syndromes (Heinzen et al 2012).

Due to the limitation of any single genetic approach to understand the complex genetic basis of GGE/JME, a combinatorial methodology is suggested along with improved phenotypic classification of affected individuals and their family members. Carefully clinically diagnosed affected individuals shall provide phenotypic homogeneity for seizure-specific analysis in the large datasets, thus providing verifiable common and syndrome-specific genetic signals. With this in view, combining unbiased genome-wide linkage- or association- studies with sequencing of the linked or susceptibility loci by NGS methods, might be successful in identifying the disease-linked variants for genetic generalized epilepsies.

1.5 Objectives of the present study

In this thesis, I present research work aimed towards understanding the molecular genetic basis of genetic generalized epilepsies, mainly JME. A two-step approach was employed to identify the causative genes/mutations in two multi-generational families, NIH2 and GLH5 manifesting GGE/JME, hailing from the southern parts of India. In earlier studies from our laboratory, GGE/JME-linked regions: EIG8 at 3q13.3-q21 (Kapoor et al 2008) and EJM4 at 5q12-q14 (Kapoor et al 2007), have been reported in these two families. In the present work, whole exome-based sequencing methods were used to identify the epilepsy-linked mutations in EIG8 and EJM4. These studies were conducted with the following goals: (i) To identify the epilepsy co-segregating novel/rare alleles in genes at the EIG8-3q13.3-q21 locus in NIH2, using whole-exome based sequencing approaches. And, to functionally evaluate the effect of rare variations identified in the potential epilepsy-causing gene using first line of cell-based assays (Chapter 2). (ii) To identify the epilepsy-causing genetic factor at the EJM4-5q12-q14 locus in GLH5 (Chapter 3).

Chapter 2

Exome sequencing identifies *CASR* as the epilepsy-causing gene at EIG8

The EIG8 (3q13.3-q21) locus for GGE was identified in a three-generation multi-affected epilepsy family, NIH2 and a novel variant, c.2693G>A (p.Arg898Gln) co-segregating with the disorder, was found in the CASR gene (Kapoor et al 2008). In the present study, detailed sequence analysis of a larger haplotype of about 64 Mb (3p14.2-q21) being shared by all affected members of NIH2, has been conducted by whole-exome sequencing (WES). Five novel/rare disease co-segregating variants in the EPHA6, ABI3BP, KIAA1407, IQCB1 and CASR genes were found in this critical interval. Of these, c.2693G>A (p.Arg898Gln) in CASR was predicted to be damaging. It alters an evolutionary conserved residue located in the crucial functional domain of CASR receptor. Thus, analysis of all protein-coding genes in this region allowed me to establish that c.2693G>A (p.Arg898Gln) in CASR is the causative factor in NIH2. CASR encodes an extracellular calcium-sensing receptor belonging to the family C of G-protein coupled receptors. It plays a key role in maintenance of systemic calcium homeostasis and is known to regulate several cellular processes by modulating different intracellular signaling pathways. Further, with an aim to gather additional evidence for involvement of CASR in epilepsy, sequence analysis of CASR was carried out in unrelated 480 JME/GGE patients. Five additional rare nonsynonymous mutations, p.Glu354Ala, p.Asp433His, p.Ser580Asn, p.Ile686Val and p.Ala988Val were found. The amino acid residues, Glu354, Asp433, Ser580, Ile686, Arg898 and Ala988 were found to be conserved across various species and mapped to conserved domains of the receptor, thereby suggesting their functional importance. The effect of these six epilepsy-associated nonsynonymous mutations on the CASR-regulated MAPK and PLC-IP3 pathways was evaluated in transiently transfected HEK293T cells. In these signaling assays, the mutant CASR receptors exhibited a variable leftward shift in the dose-response curves showing an enhanced responsiveness towards extracellular calcium concentrations as compared to the

wild-type receptor, thus suggesting their activating nature. Based on these observations, we propose that gain-of-function effect of these mutations may alter the CASR-regulated functions in the brain.

2.1 Introduction

A previous genetic study involving whole-genome based linkage mapping had identified a novel genetic locus, EIG8 for genetic generalized epilepsy (GGE) in a three-generation family from south India, with several of its members affected with epilepsy (Kapoor et al 2008). EIG8 (MIM: 612899) was localized to chromosome 3q13.3-q21. Genetic boundaries for the critical genomic interval were defined by D3S3675 (centromeric) and D3S1551 (telomeric), respectively. While the distal boundary of EIG8 was defined by a recombination event between D3S1267 and D3S1551 in an affected individual (II:2), its proximal boundary was defined by a recombination event between D3S3675 and D3S1278 in an unaffected individual (II:9). II:9 had no history of seizures or any neurological condition. He was 39 years of age at the time of participation in the study and was apparently well past the age of onset of JME. His *prima facie* unaffected status was considered while defining the proximal boundary of the locus. Based on these observations, Kapoor and colleagues (2008) concluded EIG8, a genomic interval of about 12 megabases (Mb) as the disease-linked locus in NIH2.

The 3q13.3-q21 interval (D3S3675 - D3S1551) harbors 78 annotated protein-coding genes (RefSeq, NCBI, build 37.3). Among these, 13 prioritized candidate genes were analyzed by Sanger sequencing, and a novel, rare heterozygous variant, c.2693G>A (p.Arg898Gln) in the extracellular calcium-sensing receptor gene *(CASR*) was identified. The variation, c.2693G>A was found in all affected members of the Family NIH2 (Figure 2.1) and was absent in 504 chromosomes of control cohort individuals (Kapoor et al 2008).

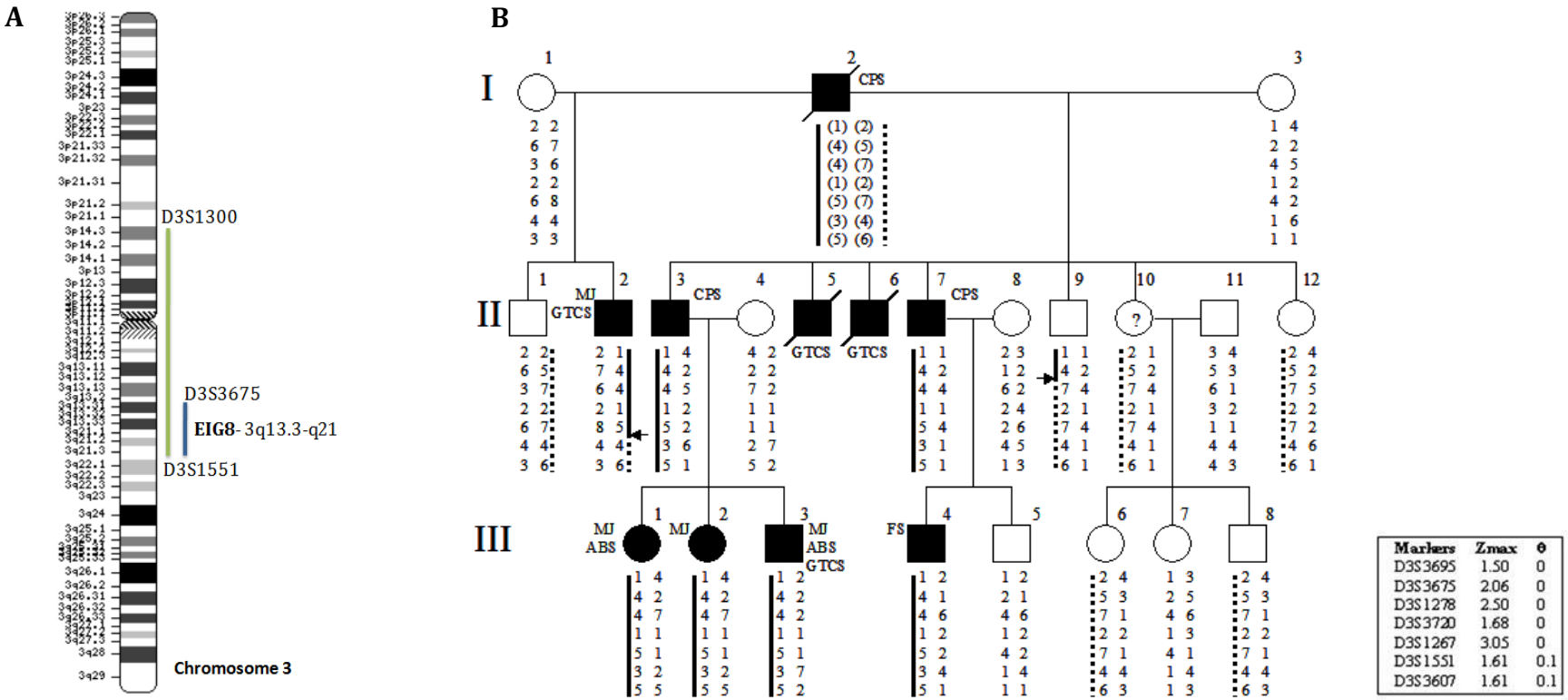


Figure 2.1: GGE-linked genetic interval in NIH2: A. Ideogram of chromosome 3 representing the location of the EIG8 locus (3q13.3-q21) on its q arm along with the extended disease-linked region (3p14.2-q21) defined by markers D3S1300 and D3S1551. **B.** Pedigree of Family NIH2 depicting the disease-linked haplotype (EIG8) shared among the affected members. Males are denoted by squares and females are denoted by circles. Filled symbols represent affected individuals and empty, unaffected ones. Haplotype consisting of 7 markers at 3q13.3-q21 is shown below the symbols. Upper and lower recombination boundaries denoting the critical region are indicated by arrows in individuals II:2 (affected) and II:9 (unaffected). Clinical features of affected subjects are indicated along with the symbols (MJ: Myoclonic jerks, GTCS: Generalized tonic-clonic seizures, CPS: Complex partial seizures, ABS: Absence seizures, FS: Febrile seizures). Adapted from Kapoor et al 2008.

At the time of publication of this report in the year 2008, majority of the genes in the critical genetic interval remained unexamined. I have revisited this study by examining all protein-coding genes (RefSeq) in the locus in two affected members of the family by using the next-generation sequencing (NGS) methods. The NGS approach permitted me to study the genes located not just in the locus delimited by boundaries in the affected-unaffected individuals (II:2 and II:9), but to examine all genes located in a larger interval (D3S1300 - D3S1551, 3p14.2-q21) defined based on recombination events in two affected individuals (II:3 and II:2) (Figure 2.1).

Further, to examine the role of *CASR* in JME/GGE, I examined its complete transcript structure in additional unrelated JME patients. The effects of identified rare nonsynonymous variants were evaluated by conducting cellular assays targeting signal transduction pathways modulated by *CASR*. Results of this study are presented in this chapter.

2.2 Materials and methods

2.2.1 Ascertainment and clinical characterization of patients and controls

The NIH2 family examined in the present study has been reported earlier, with genetic generalized epilepsy (GGE) segregating in an autosomal dominant inheritance mode (Kapoor et al 2008). Affected members of the family manifest myoclonic jerks (MJ), generalized tonic-clonic seizures (GTCS), absence seizures (AbS), complex partial seizures (CPS) and febrile seizures (FS). The family was ascertained at Department of Neurology, National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore, through the proband, III:3 (Figure 2.1). From among 23 members of the family, 20 were available for the study. The family members were clinically evaluated by two neurologists and diagnosed as per the guidelines of International League against Epilepsy (ILAE) for

classification of epilepsy and epilepsy syndromes (Commission of Classification and Terminology of the International League Against Epilepsy 1989).

The proband (III:3), a 17-year old male, exhibited early morning myoclonic jerks appearing since the age of 8. He later manifested absence seizures and generalized tonic-clonic seizures (GTCS) occurring at the age of 9 and 14, respectively. His scalp electroencephalographic (EEG) recordings were abnormal, representing characteristic features of generalized epilepsy comprising generalized bursts of spikes with polyspikes. No apparent brain lesion or abnormality was observed in magnetic resonance imaging (MRI). Additional six affected individuals in the family were available for the study. Individuals III:1 and III:2, sisters of proband, had histories of myoclonic seizures, absence seizures and nocturnal myoclonic seizures. Individual II:3, father of proband and individual II:7, had histories of complex partial seizures with secondary generalization. While the EEG recordings of II:7 appeared normal, brain MRI showed presence of a calcified lesion. A few episodes of febrile seizures along with GTCS were observed in individual III:4. Individual II:2 had a history of myoclonic jerks and GTCS. Diagnosis of deceased individuals and those who could not be examined was arrived at by information provided by other members of the family. Individuals II:5 and II:6 had histories of GTCS and died in accidents during epileptic episodes. Individual I:2 was considered having CPS according to the information provided. Due to unclear clinical profile, individual II:10, was considered of unknown clinical status for genetic analysis. Individual II:12 had delayed developmental milestones with no history of seizures. The remaining members of the family were reported normal and were considered unaffected.

Additionally, about 500 JME patients were ascertained from NIMHANS, Bangalore, SCTIMST (Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum) and a few epilepsy specialty clinics in south India. Clinical diagnoses of these patients were established according to the ILAE guidelines. The control cohort comprised about 250 healthy individuals from south India. These were above 20 years of age and had no apparent history of neurological disorders. The study was approved by the institutional ethics committees and ten milliliters of

peripheral blood was collected from all the subjects after obtaining a written informed consent for participation in the study. Genomic DNA was extracted using the phenol-chloroform method (Sambrook and Russel 2001).

2.2.2 Library preparation, targeted capture and exome sequencing

Whole-exome sequencing was performed on an affected parent-offspring pair (II:3 and III:2) from the family, using Agilent SureSelect All Exon 50 Mb Reagents (Agilent Technologies). Five micrograms of genomic DNA was fragmented by sonication (Bioruptor, Diagenode) to obtain fragment sizes ranging from 100 bp to 400 bp. The resulting fragmented DNA was cleaned up using Agencourt AMPure XP beads (Beckman Coulter) and checked for size distribution on a High-Sensitivity Bioanalyzer Chip (Agilent). Subsequently, genomic DNA libraries were constructed according to SureSelectXT Target Enrichment System (Agilent) for Illumina Paired-End Sequencing. DNA was subjected to a series of enzymatic reactions to repair the frayed ends, phosphorylate the fragments, add a single nucleotide A overhang and for ligation of 60 bp sequence adaptors to fragment ends. After ligation, adaptor-ligated fragments were enriched by polymerase chain reaction (PCR) amplification. The library prepared in this manner was concentrated using a vacuum concentrator (Eppendorf) and then hybridized to SureSelect™ biotinylated RNA baits at 65°C for 24-72 hours. Hybridized library fragments were isolated by magnetic capture using Dynal M-280 streptavidin coated beads (Invitrogen) followed by purification of the captured library-bead solution with AMPure XP beads. The captured library was amplified using PCR and the reaction products were purified with AMPure XP beads. The quality of the sequencing libraries was verified by capillary electrophoresis on an Agilent High Sensitivity Bioanalyzer Chip and the concentration was measured using a spectrophotometer (NanoDrop Technologies). The capture efficiency was evaluated with pre- and post-capture library samples using Brilliant II SYBR® Green QPCR Master Mix (Agilent) on Mx3005P Real-Time PCR System (Stratagene). The sequencing (72 bp paired-end reads) was carried out using an Illumina Genome Analyzer IIx (GAIIx) following the manufacturer's instructions.

Table 2.1: Summary of the next-generation sequencing data

Statistics	II:3	III:2
Data generated	19.9 Gb	20.44 Gb
Read length in bases (paired end)	72	72
Total number of reads [Percentage of high quality reads[a]]	93553724 [93.14]	96125622 [92.8]
Total number of bases [Percentage of high quality bases[b]]	6.73 Gb [94.2]	6.92 Gb [93.9]
Percentage of non-ATGC characters	0.52	0.51
Percentage of reads with non-ATGC characters	0.77	0.75
Total SureSelect probe[#] region length (whole-exome)	51.5 Mb	51.5 Mb
SureSelect probe region covered (whole-exome)	49.8 Mb	48.6 Mb
Total SureSelect probe region length (3p14.2-q21)	575 kb	575 kb
SureSelect probe region covered (3p14.2-q21)	567 kb	564 kb
Average read depth (whole-exome)	66.75	67.36
Average read depth (3p14.2-q21)	80.72	86.58

[a]Reads with ≥70% bases with Phred-like quality score ≥20. [b]Nucleotide bases with Phred-like quality score ≥20. High quality reads are the quality filtered reads from the raw sequence data. [#]Probes used in the Agilent SureSelect Human All Exon 50 Mb kit to capture whole-exome sequence.

Table 2.2: Sequence coverage summary for the SureSelect probe region in the whole-exome sequencing experiment

Percentage of probe region covered*	Whole-exome		Chr 3 region (3p14.2-q21)	
	II:3	III:2	II:3	III:2
Total probe region	96.78	94.45	98.57	97.99
Read depth ≥ 5x	91.25	86.84	97.08	95.8
Read depth ≥ 10x	86.49	80.64	95.45	93.91
Read depth ≥ 15x	82.22	75.57	93.66	91.67
Read depth ≥20x	78.01	71.14	91.34	89.12

*Percentage of SureSelect probe region coverage calculated after sequences were aligned to Human genome reference sequence (hg19/GRCh37). Chr, chromosome.

2.2.3 Variant analysis

The Agilent SureSelect Human All Exon 50 Mb Kit design includes exon sequences from Consensus Coding Sequence (CCDS), NCBI Reference Sequence (RefSeq) and GENCODE annotations. The SureSelect human exome kit is devised to enrich 334,378 target exons of nearly 20,965 genes covering 51 Mb of genome. The raw sequence data (FASTQ) obtained from the sequencer was processed through an in-house analysis pipeline. The unprocessed raw reads were quality checked using SeqQC v2.1. Removal of low quality bases and trimming of adapter sequences from 3' read end was done using a customized script. Quality criteria adapted for filtering was that 70% of the bases in a sequence read should have Phred-like quality score ≥ 20, and a minimum of 50 bp read length was taken further for analysis. The high-quality processed reads were aligned to human genome reference assembly hg19 (GRCh37) using Burrows–Wheeler Aligner v0.6.0 (Li and Durbin 2009). Removal of PCR duplicates (pair of reads with identical outer coordinates) was done by Samtools v0.1.7a (Li et al 2009). Coverage statistics for aligned data were calculated using custom scripts (written in Perl) and hg19 reference gtf (gene transfer format) file (whole-exome and the 3p14.2-q21 interval). Single Nucleotide Variations (SNVs) and short insertions/deletions (Indels) were called using Samtools v0.1.7a and annotated into functional categories of nonsynonymous variants, synonymous variants, and intronic variants with SnpEff v2.0.5 (Cingolani et al 2012). These changes were further segregated as the known and novel ones, based on their presence/absence in the Database of Single Nucleotide Polymorphisms (dbSNP) 131. The shortlisted variations were then checked in the updated release of dbSNP and 1000 Genomes.

The gene variants located in the critical region (3p14.2-q21) were considered for further analysis and in order to avoid missing any potentially 'meaningful' variants, all novel heterozygous variations and indels up to 3x read depth were examined and verified. Additionally, there were about 100 exons in different genes in this 64 Mb region, which were uncovered or had low coverage in the exome sequencing data, due to their absence from the design of the Agilent capture probes. To examine these low coverage exons or gaps, each of them were PCR amplified, Sanger sequenced and examined.

2.2.4 Validation of novel/rare variants (NGS-chr 3 region)

All novel and rare variants identified in the 3p14.2-q21 region by NGS were verified by Sanger sequencing. Primers for the variant-harboring exon/amplicons and missing exons were designed using Primer 3 (Untergrasser et al 2012, Koressaar et al 2007) and Oligo Calc (Kibbe 2007), and obtained from Sigma-Aldrich (Appendix I A2.2). Genomic DNA (100 ng) was amplified by PCR in a reaction volume of 20 µl containing 0.25 µM of each forward and reverse primer, 800 µM of dNTPs (200 µM of each dNTP), 1X reaction buffer (50 mM KCl, 20 mM Tris-HCl pH 8.4), 1.5 mM $MgCl_2$ and 1 U of *Taq* polymerase (Invitrogen, Life Technologies). Amplifications were performed using a GeneAmp® 9700 (Applied Biosystems, Life Technologies) at conditions: initial denaturation at 94°C for 10 minutes, 30-40 cycles of denaturation of 94°C for 30 seconds, annealing at 53-64°C for 30 seconds and extension 72°C for 30 seconds and a final extension at 72°C for 10 minutes. Amplification products were verified by 1.5% agarose/TAE/EtBr gel electrophoresis. These products were purified using the 96-well Multiscreen® Filter plates (Millipore) and eluted in 20 µl of deionized water.

DNA sequencing was performed by cycle sequencing using 1 µl Ready Reaction Mix of the BigDye Terminator Cycle Sequencing kit v3.0 (Applied Biosystems) containing fluorescent-labeled dideoxy-terminators, 2-3 µl (50-100 ng) of purified PCR product and 0.4 µM of primer in a 20 µl reaction volume. The cycle sequencing was performed at following conditions: initial denaturation (96°C, 1 minute) followed by 25 cycles of denaturation (96°C, 10 seconds), annealing (50°C, 5 seconds) and extension (60°C, 4 minutes). After cycle sequencing, the products were purified by ethanol precipitation. The pellet was dissolved in 10 µl of Hi-Di formamide (Applied Biosystems), denatured at 95°C for 5 minutes and analyzed on an automated 48-capillary sequencer (ABI 3730 Genetic analyzer, Applied Biosystems). The sequences obtained were aligned to the respective reference gene sequence (RefSeq database, NCBI) using SeqMan II v5.01 (DNASTAR Inc.). The variants common to the NGS-sequenced samples (II:3 and III:2) were checked

for their presence/absence in other family members of NIH2. The disease-segregating alleles were further examined in the control cohort.

2.2.5 Bioinformatics analysis of novel/rare disease-segregating variants (NGS-chr 3 region)

To predict the plausible effect of the novel missense variations in *CASR* and *KIAA1407*, on the structure and function of their respective proteins, PolyPhen-2 (Adzhubei et al 2010), Sorting Intolerant from Tolerant (SIFT) (Ng et al 2003), Align GVGD (Tavtigian et al 2006), Screening for Non-Acceptable Polymorphisms (SNAP) (Bromberg and Rost 2007) and PMut (Ferrer-Costa et al 2005) were used. The effects of non-coding variants in *EPHA6*, *ABI3BP* and *IQCB1* on the splicing mechanism and translation start process were assessed using *in silico* tools: NetStart 1.0 (Pedersen and Nielsen 1997) NetGene2 (Brunak et al 1991), Human Splicing Finder v2.4.1 (Desmet et al 2009), Alternative Splice Site Predictor (ASSP) (Wang and Marín 2006) and NNSPLICE0.9v (Reese et al 1997). Multiple sequence alignments of DNA or protein sequences from different organisms were carried out by Clustal Omega (Sievers et al 2011). The respective reference nucleotide or protein sequence of each organism was taken from NCBI (GenBank) (Appendix I A2.3).

2.2.6 Mutational analysis of CASR

Complete transcript structure of *CASR* comprising of its exons and their flanking intronic regions (NM_000388.3) were screened by Sanger sequencing in apparently unrelated 480 JME patients and 252 control individuals. Primer pairs for amplification were designed using online software tools, Primer 3 and Oligo Calc (Appendix I A2.2). PCR amplification for individual fragments was performed at their respective standardized conditions on a GeneAmp® 9700. The conditions used were: initial denaturation at 94°C for 5 minutes, followed by 35-40 cycles of denaturation at 94°C for 30 seconds, annealing at 55-60°C for 30 seconds and extension at 72°C for 30 seconds, followed by final extension at 72°C for 10 minutes. PCR amplification was carried out using 100 ng of genomic DNA, 0.25 µM of each forward and reverse primer, 800 µM of dNTPs (200 µM of each dNTP),

1.5 mM $MgCl_2$, 1X reaction buffer (50 mM KCl, 20 mM Tris-HCl pH 8.4) and 1 U of *Taq* DNA polymerase in a reaction volume of 20 µl. The amplified products were verified by gel electrophoresis using 1.5% agarose/TAE/EtBr. These were then purified using 96-well Multiscreen® Filter plates according to the manufacturer's protocol and eluted in 20 µl of deionized water. DNA sequencing was performed as described earlier and products were analyzed on an ABI 3730 Genetic analyzer. The sequences were compared to the *CASR* reference sequence (NM_000388.3), available at the RefSeq database, NCBI and sequence variants were identified using SeqMan II v5.01.

2.2.7 Bioinformatics analysis of CASR mutations

Potential pathogenicity of the rare nonsynonymous variants identified in *CASR*, was evaluated using bioinformatics tools: Sorting Intolerant from Tolerant (SIFT), Polyphen-2, AlignGVGD and Mutation Assessor (Reva et al 2011). The protein sequence of CASR (NP_000379.2) from NCBI was used as the input file for these tools. To observe the conservation status of the altered amino acid residues across various organisms of different genera, alignments of the CASR protein sequences were performed using Clustal Omega. The CASR reference protein sequence of each organism used for sequence alignment was obtained from NCBI (Appendix I A2.3).

2.2.8 Cloning of CASR cDNA and site-directed mutagenesis

CASR cDNA (NM_000388.3, CCDS 3010.1) encoding the 1078 amino acids receptor, was PCR amplified from human adult brain cDNA (Marathon-Ready™ cDNA, Clontech). For amplification, gene-specific primers 5'-TGGAGAGCTAGCG CCACCATGGCATTTTATAGCTGC-3' (forward) and 5'-CTCTCTGCATCTCGAGTAG CCCAGTCTTCTCCTTCC-3' (reverse) were used, harboring the NheI and XhoI restriction enzyme sites incorporated to facilitate cloning. Amplification was performed using 1.5 ng of whole brain cDNA as template with 5 pmol of each primer and 2% DMSO. The amplified product was purified and cloned into the pcDNA3.1 (+) mammalian expression vector (Invitrogen) as a NheI-XhoI fragment (adapted from Kapoor A, PhD thesis 2006). The sequence of the cDNA insert was

verified by Sanger sequencing using cDNA insert primers (Appendix I A2.2). The *CASR* cDNA constructs with mutations incorporated, were generated by site-directed mutagenesis, carried out using QuikChange® II XL mutagenesis reagents (Stratagene). The mutagenic oligonucleotide primer pairs (Appendix I A2.2) were designed to incorporate point mutations in the cDNA and subsequently used for amplification of parental *CASR* pcDNA3.1 vector by Pfu Turbo DNA polymerase. After digestion of the parental DNA construct with DpnI for one hour at 37°C, E.coli XL-10 cells were transformed with 2-5 µl of the reaction containing amplified DNA with incorporated nucleotide substitution. The plasmid DNA was isolated from bacterial culture of insert positive clones using QIAprep® Spin Miniprep Kit (Qiagen). The transformants were screened by sequencing of the mutation-harboring cDNA in the plasmid, using the BigDye Terminator Cycle Sequencing reagents on an ABI3730 Genetic Analyzer. The obtained cDNA insert sequences were compared to *CASR* reference sequence (NM_000388.3, CCDS 3010.1) using Seqman II v5.01.

2.2.9 Cell culture and transient transfections

Human embryonic kidney (HEK293T) cells were grown in Ca^{2+}-free high glucose Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% heat inactivated fetal bovine serum (FBS, Sigma), 2mM L-glutamine (Sigma) and antibiotics (100 U/ml penicillin and 10 mg/ml streptomycin, Sigma) at 37°C in humidified atmosphere of 5% CO_2. For signaling assays, HEK293T cells were sub-cultured in 6-well or 24-well plates (BD biosciences). Next day, the culture medium was replaced with the antibiotic- and serum-free Ca^{2+}-free DMEM for transient transfection with different vector constructs using Lipofactamine® 2000 transfection reagent (Invitrogen).

2.2.10 MAPK (mitogen-activated protein kinase) assay

The effect of missense mutations on functional aspects of CASR regulated MAPK signaling was evaluated by using PathDetect® *in vivo* Signal Transduction Pathway Elk-1 *trans*-Reporting System (Stratagene). This cell-based assay measured the activity of Elk-1, an ETS domain containing transcription factor targeted by MAPK

pathways in response to various extracellular calcium concentrations. HEK293T cells were grown in Ca^{2+}-free DMEM in 6-well plates. At 70% confluence, cells were transiently co-transfected with 1 μg of wild-type (WT)- or mutant- CASR-pcDNA3.1 or empty vector (pcDNA3.1+), 1 μg of pFR-Luc reporter plasmid (Stratagene), 50 ng of pFA2-Elk1 plasmid (Stratagene) and 50 ng of pCMVβ-galactosidase plasmid (transfection control plasmid) (Clontech) using Lipofectamine™ 2000 (Invitrogen) in serum-free DMEM. Six hours post-transfection, the medium was replaced to serum supplemented DMEM. The following day, the cells were serum starved in DMEM with 0.5 mM $CaCl_2$ for 8 hours and then replaced with media containing different concentrations of $CaCl_2$ (0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12 and 15 mM) for 16 hours. The cells were washed with 1X phosphate-buffered saline (PBS) and lysed using 200 μl of 1X Reporter Lysis Buffer (RLB, Promega) on ice. Post lysis, the cells were centrifuged at 15,000 rpm for 2 minutes at 4°C and the supernatant was collected for activity measurements. Luciferase activity was measured on a Luminometer (Berthold) using 2 μl of cell lysate and 20 μl luciferase assay reagent (1:10) (Luciferase Assay System, Promega). β-galactosidase enzyme activity was measured in a 96-well microplate reader (Molecular Devices) using 50 μl of diluted cell lysate (5 μl cell lysate and 45 μl of 1X RLB) and 20 μl of Assay 2X Buffer (Promega) in each well. The assay mixture was incubated at 37°C for 15-20 minutes (appearance of yellow colour) and the activity was stopped using 50 μl of 1 M Na_2CO_3. Then, the absorbance values were measured at 420 nm. Luciferase activity was normalized to the β-galactosidase reading for each cell lysate.

2.2.11 Inositol monophosphate (IP1) assay

The response of cultured HEK293T cells transiently expressing wild-type or mutant CASR to various extracellular Ca^{2+} was assessed by measuring intracellular IP1 (Inositol 1-phosphate) accumulation using an Elisa-based method (IP-One Cell Elisa Assay Kit, CISBIO Bioassays). HEK293T were sub-cultured in Ca^{2+}-free DMEM in 24-well plates. Next day, they are transfected with 0.5 μg of WT- or mutant- CASR-pcDNA3.1 or empty vector (pcDNA3.1+) along with 25 ng of pCMV β-galactosidase plasmid (transfection control plasmid) using Lipofectamine® 2000

(Invitrogen) in serum-free medium. Forty-eight hours post-transfection, DMEM was removed and cells were treated with 200 µl of Stimulation Buffer (10 mM Hepes, 0.5 mM $MgCl_2$, 4.2 mM KCl, 146 mM NaCl, 5.5 mM Glucose, 50 mM LiCl pH=7.4) containing different $CaCl_2$ concentrations (0, 2, 3, 4, 5, 6, 8, 10 mM) for 1 hour at 37°C and 5% CO_2. Following treatment, cells were lysed for 30 minutes at 37°C with 50 µl of 2.5% IP-One ELISA Kit Lysis Reagent and the assay was performed according to the manufacturer's guidelines. The ODs were determined at 450 nm (with optical correction at 620 nm) on Varioskan™ Flash (Thermo Scientific). IP-One ELISA Kit determines the accumulation of intracellular IP1 based on competition between free IP1 and IP1-HRP (Horse-Radish Peroxidase) conjugate, for binding sites on an anti-IP1 monoclonal antibody. The IP1 accumulation for each experiment was calculated as percentage inhibition of IP1-HRP binding = [1 - IP1-HRP binding in stimulated cells/IP1-HRP binding in unstimulated cells] X 100. Therefore, IP1 accumulation in wild-type or mutant CASR transfected cells in response to increasing Ca^{2+} concentration was expressed as percentage inhibition of IP1-HRP binding.

2.2.12 Statistical data analysis

For MAPK assay, the signaling activity represented by relative luciferase units for wild-type and mutant receptors was plotted as mean ± SEM of three independent experiments. In IP-One ELISA based assay, accumulation of IP1 (percentage inhibition of IP1- HRP binding by IP1) for each receptor was represented as mean ± SEM of six independent experiments. The curve fitting for dose-response values were performed using non-linear regression in Prism 5 (GraphPad software, Inc) and EC_{50} (the agonist concentration giving one-half of the maximal response) was calculated using Boltzmann equation in Origin 6.1 (OriginLab Corporation). The mean EC_{50} values for wild type or each mutant CASR receptor in response to increasing concentrations of Ca^{2+} was calculated from the EC_{50} values for all of the individual experiments and is expressed with the standard error of the mean (SEM). The bar graphs to compare signaling response values of mutant receptors to wild-type CASR at three calcium concentrations were plotted using GraphPad Prism 5. Statistical analyses were performed using unpaired Student's t test (GraphPad Prism 5), and P values < 0.05 (two-tailed) were regarded as significant.

2.3 Results

2.3.1 Exome sequencing and variant analysis

Pursuing the previous linkage and candidate gene study regarding the EIG8 locus (Kapoor et al 2008), I utilized the next-generation sequencing methods to identify if there are additional gene variants which may be causative beyond the proposal of c.2693G>A (p.Arg898Gln) in *CASR* for GGE. An extended haplotype of 64 Mb shared by all affected individuals in the family was considered for analysis. And this disease-linked critical interval harbors 214 well annotated protein-coding genes (RefSeq, NCBI, build 37.3).

Whole-exome sequencing (WES) was performed on genomic DNA from an affected parent-offspring pair (II:3 and III:2). Individuals II:3 and III:2 share the GGE-linked haplotype between D3S1300 and D3S1551. Sequencing libraries from both samples were prepared using SureSelect target enrichment system (Agilent SureSelect Human All Exon 50MB kit) and sequenced as 72 bp paired-end reads on Illumina GAIIx platform. A total of 18 Gb of raw sequence data with 93-96 million reads was obtained per sample. After removal of low quality bases and adaptor sequences, about 92% sequence reads were found to be of high-quality (≥70% bases in a read with Phred-like quality score ≥20) (Table 2.1). The sequence reads were aligned to the human genome reference (hg19) using BWA (v0.6.0). The target sequence length of SureSelect probes covers about 51 Mb of exonic region of the human genome. On average, 95% of the SureSelect probe targeted region was covered at least once. For the whole-exome probe region, 10-fold coverage was 80-86%; and 71-78% of the probe region was covered by at least 20 reads (Table 2.2). For further analysis, we focused on variations in genes of the extended GGE-linked interval (64 Mb) on chromosome 3 between the markers D3S1300 and D3S1551 (3p14.2-q21). The SureSelect probes cover about 575 kb of exonic region in this interval. The intended probe region had an average read depth of 83x with nearly 90% of the region covered at least 20 times (Table 2.2).

Table 2.3: Novel/rare variants observed in the 3p14.2-q21 region from both NGS-ed samples (II:3 and III:2)

Position on chr 3[a]	Gene	Location/ type	Sequence variant[b]	Position of amino acid change	Disease co-segregation[c]	Status in databases (MAF)[d]	Status in control cohort (MAF)
66512894	*LRIG1*	Synonymous	ENST00000273261.3:c.258T>C	Ala86=	No	rs372237438, G = 0.0001	
69028848	*C3orf64*	Synonymous	ENST00000295571.5:c.1053C>T	Phe351=	No	_	
75832493	*ZNF717*	Synonymous (Hom)	ENST00000422325.1:c.21C>G	Gly7=	_	_	
88198984	*C3orf38*	5'UTR	ENST00000318887.3:c.-216delG	_	No	_	
93802884	*NSUN3*	Intron	ENST00000314622.4:c.123-67G>A	_	Yes	_	A = 0.01
97194120	***EPHA6***	**Intron**	**ENST00000389672.5:c.1895-76T>C**	**_**	**Yes**	_	**0**
97356988	*EPHA6*	Intron	ENST00000389672.5:c.2784+62G>C	_	No	_	
100039605	*TBC1D23*	Intron	ENST00000394144.4:c.1824-16_1824-15insT	_	No	_	
100472646	*ABI3BP*	Intron	ENST00000284322.5:c.2827+19A>G	_	No	_	
100585640	***ABI3BP***	**Intron**	**ENST00000284322.5:c.1009+83A>G**	**_**	**Yes**	_	**0**
107096831	*CCDC54*	Intron	ENST00000261058.1:c.397A>G	Ile133Val	No	_	
108095484	*HHLA2*	Intron	ENST00000357759.5:c.1224+80T>C	_	No	_	

108372983	*DZIP3*	Intron	ENST00000361582.3:c.2034-9C>T	_	No	rs201141252, T = 0.003	
108549473	*TRAT1*	Intron	ENST00000295756.6:c.8-44G>A	_	Yes	rs371243572, A = 0.003	A = 0.026
108567961	*TRAT1*	Intron	ENST00000295756.6:c.215-52A>G	_	Yes	rs372684266, G = 0.003	G = 0.015
111710781	*ABHD10*	3'UTR	ENST00000273359.3:c.*213_*214insT	_	No	_	
112328944	*CCDC80*	Intron	ENST00000206423.3:c.2322-16T>G	_	No	_	
113005777	*BOC*	3'UTR	ENST00000495514.1:c.*69delAC	_	No	_	
113346706	*SIDT1*	3'UTR	ENST00000264852.4:c.*151G>A	_	No	_	
113753976	***KIAA1407***	**Nonsynonymous**	**ENST00000295878.3:c.614G>A**	**Arg205Lys**	**Yes**	_	**0**
121547865	***IQCB1***	**Intron**	**ENST00000310864.6:c.-12-46T>C**	**_**	**Yes**	_	**0**
122003494	***CASR***	**Nonsynonymous**	**ENST00000490131.1:c.2693G>A**	**Arg898Gln**	**Yes**	rs121909269 (CM083599)	**0**
122474013	*HSPBAP1*	Intron	ENST00000306103.2:c.741+94A>G	_	No	_	
123019154	*ADCY5*	Intron	ENST00000462833.1:c.2725-12C>G	_	Yes	_	G = 0.01

[a]Genomic position of the nucleotide base on chromosome 3 (chr 3) (GRCh37, NCBI). [b]Nomenclature of variants in introns or untranslated regions (5'UTR and 3'UTR) is with respect to first base of the corresponding cDNA. The position of variations is numbered according to cDNA of the longest protein-coding transcript. [c]Variants present in all affected members of NIH2 were considered positive for disease co-segregation. [d]The novel/rare variants were checked in updated release of dbSNP and 1000 Genomes; and the variants present at minor allele frequency (MAF) ≤ 0.005 in the databases were included. The five new variants represented in bold segregated with clinical phenotype in NIH2 and were found absent in the control cohort individuals (n=96 for intronic variations and n=250 for nonsynonymous variations).

The coding exons along with their 100 bp flanking intronic sequence were considered for identification of rare variants segregating with epilepsy phenotype in NIH2. Given the autosomal dominant mode of inheritance of disease in the family, heterozygous variants were selected. We identified 24 novel/rare variants, of which, 9 were found to co-segregate with epilepsy in the family (Table 2.3; Figure 2.3). The allele frequencies of these 9 variations were examined in the control cohort and databases. Five variants: c.1895-76T>C in *EPHA6*, c.1009+83A>G in *ABI3BP*, c.614G>A in *KIAA1407*, c.-12-46T>C in *IQCB1* and c.2693G>A in *CASR* were found to be absent among controls examined (Table 2.3). The common variants or polymorphisms identified in the region are enlisted in Appendix I A2.1.

2.3.2 Predictive effect of novel/rare disease-segregating variants (NGS-chr 3 region)

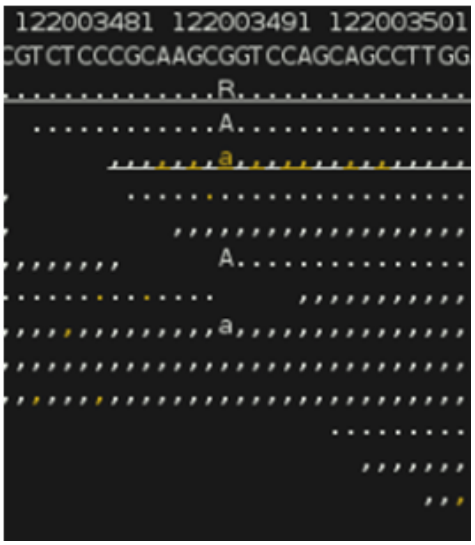
The five new variations were studied for their predictive pathogenicity and evolutionary conservation across various organisms using multiple *in silico* tools. These variants were located in the following genes: *EPHA6* (MIM: 600066), *ABI3BP* (MIM: 606279), *KIAA1407*, *IQCB1* (MIM: 609237) and *CASR* (MIM: 601199). The intronic variants c.1895-76T>C in *EPHA6* and c.1009+83A>G in *ABI3BP* were predicted to have no apparent effect on the splicing regulation of the neighboring exons (Table 2.4). The c.-12-46T>C variation in *IQCB1* is present in the intron between two non-coding exons and lies 46 bases upstream of the translation start site. According to the predictive tools, it neither affects the splicing of non-coding exons nor the translation start site. Two variations that lead to missense substitutions were, c.614G>A (p.Arg205Lys) in *KIAA1407* and c.2693G>A (p.Arg898Gln) in *CASR* (Figure 2.2). The p.Arg205Lys in KIAA1407 alters arginine, a positively charged amino acid residue to a basic amino acid residue, lysine and was therefore predicted to have a neutral effect to the protein. However, p.Arg898Gln substitution in CASR alters a positively charged amino acid to a neutral amino acid and is predicted to have damaging functional consequence to the protein (SIFT score- 0.01; Polyphen-2 score-0.68, Align-GVGD- C35) (Table 2.4).

Table 2.4: Bioinformatics analysis of the five novel disease co-segregating variants (3p14.2-q21)

***In silico* tool**	***EPHA6*, c.1895-76T>C**	***ABI3BP*, c.1009+83A>G**	***IQCB1*, c.-12-46T>C**	**KIAA1407, p.Arg205Lys**	**CASR, p.Arg898Gln**
Predicted effect of the variant					
SIFT	_	_	_	Tolerated (0.31)	Damaging (0.01)
Polyphen-2	_	_	_	Benign (0.005)	Possibly damaging (0.68)
SNAP	_	_	_	Neutral	Non-neutral
PMut	_	_	_	Neutral	Neutral
Align GVGD#	_	_	_	Class C25	Class C35
NetStart 1.0*	_	_	No effect	_	_
NNSPLICE 0.9v	No effect	No effect	No effect	_	_
HSF v2.4.1	No effect	No effect	No effect	_	_
NetGene2.0	No effect	No effect	_	_	_
ASSP	No effect	No effect	_	_	_
Evolutionary conservation of parent nucleotide/residue across organisms					
Clustal Omega§	3 out of 9	4 out of 10	6 out of 9	11 out of 13	25 out of 26

The effect of missense substitutions was predicted using SIFT, Polyphen-2, SNAP, PMut and Align GVGD. Tools such as NNSPLICE, HSF, NetGene and ASSP evaluated the effect of intronic variants on splicing of the flanking exons. *NetStart 1.0 examined the effect of the intronic variant, c.-12-46T>C in *IQCB1*, present between two non-coding exons. §DNA or protein sequence alignment was done using Clustal Omega (found conserved in out of those examined). #In AlignGVGD, Class C65 to C25 ranges from most to least likely to interfere in protein function.

A



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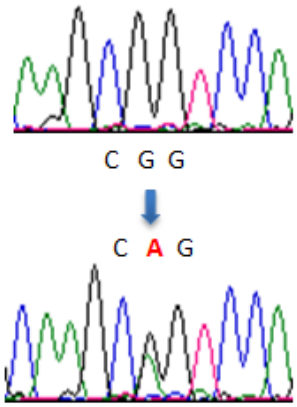


C

Zebrafish	SGFRK**R**SSSVG
Guinea Pig	NVSRKGTAAYG
Mouse	NISRK**R**SSSLG
Rat	NISRK**R**SSSLG
Rhesus Monkey	NVSRK**R**SSSLG
Common Marmoset	NVSRK**R**SSSLG
Orangutan	NVSRK**R**SSSLG
Chimpanzee	NVSRK**R**SSSLG
Human	NVSRK**R**SSSLG
Gorilla	NVSRK**R**SSSLG
Armadillo	NVSRQ**R**SSSLG
Pig	NVSRQ**R**SSSLG
Killer Whale	NVSRQ**R**SSSLG
Cow	NVSRQ**R**SSSLG
Sheep	NVSRQ**R**SSSLG
Horse	NVSRK**R**SSSLG
Rhinoceros	NVSRK**R**SSSLG
Dog	NVSRK**R**SGSLG
Walrus	NVSRK**R**SSSLG
Cat	NVSRK**R**SSSLG
Giant Panda	NVSRK**R**SSSLG
Green Lizard	NVSRK**R**SNSLG
Zebra Finch	NVSRK**R**SNSLG
Wild Duck	NVSRK**R**SNSLG
Chicken	NVSRK**R**SNSLG
Wild Turkey	NVSRK**R**SNSLG

Figure 2.2: The c.2693 G>A (p.Arg898Gln) mutation in *CASR*: **A.** Alignment of the exome sequence reads against human genome reference sequence as viewed in tview of samtools v0.1.7a. The snapshot here shows the reads in forward (caps) and reverse (no caps) strands indicating the heterozygous G>A variation at g.122003494 in *CASR*. The dot and comma represent the matched reference allele and alphabet shows the variant allele. **B.** Electropherograms of sequence with wild-type (G/G) and variant allele (G/A) for the c.2693G>A variation in *CASR*. The arrow indicates the nucleotide showing heterozygous variation shown by presence of two peaks. **C.** Multiple protein sequence alignment of CASR from 26 species by Clustal Omega. The arginine (R) 898 is highlighted in blue.

The conservation of nucleotide residue or amino acid residue was observed by multi-species DNA/protein sequence alignment using Clustal Omega. The nucleotide positions c.1895-76T in *EPHA6*, c.1009+83A in *ABI3BP* and c.-12-46T in *IQCB1* were found to be variable. The amino acid residue p.Arg205 in KIAA1407 was found to be moderately conserved in other organisms. But, the amino acid residue Arg898 in CASR was found to be highly conserved in species as distantly related as humans and zebrafish, indicating its high functional significance (Figure 2.2).

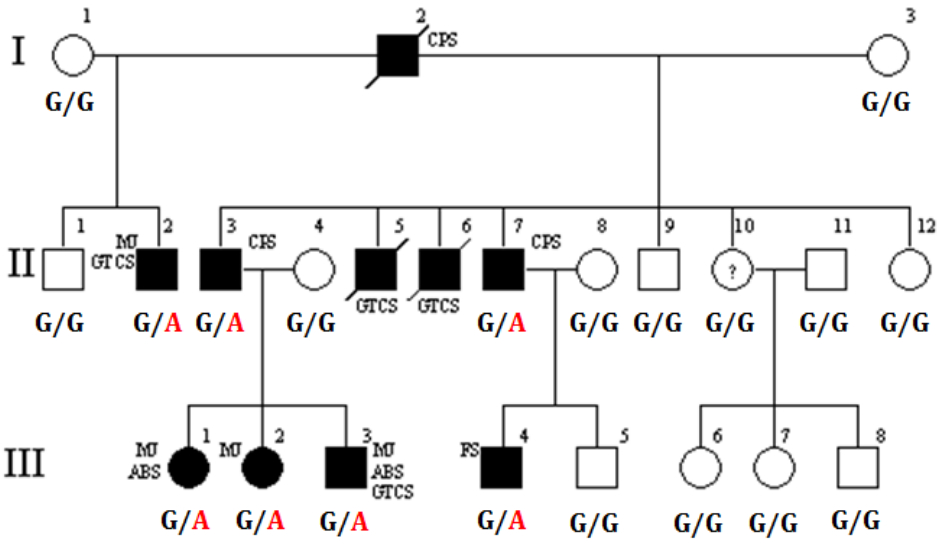


Figure 2.3: Segregation analysis of *CASR* variation, c.2693G>A in NIH2: The genotypes (G/G or G/A) for c.2693 nucleotide position are written below the respective symbols representing family members. The wild-type genotype is G/G and the variant allele (A) present in heterozygous state is shown as G/A (red).

Table 2.5: Novel/rare *CASR* variants observed in 480 JME patients

Position on chr 3[a]	Position of nucleotide change[b]	Position of amino acid change[b]	Location in gene/ protein[c]	Patient counts (n=480)	Status in control cohort (MAF)[d]	Status in databases (MAF)[e]	Earlier report[f]
121902587	c.-315G>T	_	5' UTR	2	T = 0.002	ss1305902404, T = 0.0002	
121902613	c.-289G>A	_	5' UTR	2	_	_	
121902671	c.-243(+12)G>A	_	Intron	1	_	_	
121972900	c.-137C>T		5' UTR	2	_	rs201074178	
121972921	c.-116T>C	_	5' UTR	1	_	_	
121980419	c.537A>G	Gln179=	ECD	9	G = 0.004	rs200129212, G = 0.001	
121980614	c.732C>T	Ser244=	ECD	1	_	_	
121980893	c.1011C>G	Val337=	ECD	1	G = 0.002	rs201820733, G = 0.0002	
121980943	c.1061A>C	Glu354Ala	ECD	1	_	CM083600	Kapoor et al 2008
121981046	c.1164G>A	Ser388=	ECD	1	A = 0.002	rs200898785, A = 0.0008	

121981179	c.1297G>C	Asp433His	ECD	3	C = 0.004	rs199511990 (CM081198), C = 0.0004	Murugaian et al 2008
122001016	c.1665T>C	Ile555=	ECD	2	_	rs201955278, C = 0.001	
122001089	c.1732+6G>A	_	Intron	1	_	_	
122002540	c.1739G>A	Ser580Asn	ECD	1	_	_	
122002628	c.1827G>A	Thr609=	ECD	3	_	rs200868156, A = 0.0001	
122002857	c.2056A>G	Ile686Val	TMD	5	G = 0.002	CM083598	Kapoor et al 2008
122003494	c.2693G>A	Arg898Gln	ICD	1	_	rs121909269 (CM083599)	Kapoor et al 2008
122003702	c.2901C>A	Ile967=	ICD	4	A = 0.004	rs199594582, A = 0.0008	
122003764	c.2963C>T	Ala988Val	ICD	3	_	CM083601	Kapoor et al 2008

[a]Genomic position of the nucleotide base on chromosome 3 (chr 3) (GRCh37, NCBI). [b]The nucleotide and amino acid residue position of the variations in *CASR* is according to its shorter transcript encoding for receptor of 1078 amino acids (NM_000388.3, NP_000379.2). [c]ECD: extracellular domain, TMD: transmembrane domain, ICD: intracellular domain. [d]Minor allele frequencies (MAF) is calculated in 252 control individuals. [e]Variations were checked for their presence in updated release of dbSNP and 1000 Genomes; and variants present at minor allele frequency (MAF) ≤ 0.005 in the databases were included. [f]The CASR mutations p.Glu354Ala, p.Ile686Val, p.Arg898Gln and p.Ala988Val were reported for epilepsy in our earlier study (Kapoor et al 2008). The p.Asp433His has been reported for chronic pancreatitis by another study (Murugaian et al 2008). These reported mutations are present in the Human Genome Mutation Database (HGMD) and are designated by CM_ID.

2.3.3 Rare CASR variants in JME patients

To examine the potential contribution of the *CASR* gene for predisposition to epilepsy, all exons of the gene were sequenced in a set of 480 JME patients and 252 individuals of control cohort. The sequences obtained were compared to the *CASR* reference sequence (NM_000388.3, RefSeq, NCBI). Six rare nonsynonymous variations altering highly conserved residues in the receptor were found. These were: c.1061A>C (p.Glu354Ala), c.1297G>C (p.Asp433His), c.1739G>A (p.Ser580Asn), c.2056A>G (p.Ile686Val), c.2693G>A (p.Arg898Gln) and c.2963C>T (p.Ala988Val) (Table 2.5, Figure 2.4)). These rare alleles were either absent or present with minor allele frequencies (MAF) ≤ 0.005 in 504 control chromosomes examined, dbSNP and 1000 Genomes database. The mutations, p.Glu354Ala, p.Ile686Val, p.Arg898Gln and p.Ala988Val were reported earlier in epilepsy patients (Kapoor et al 2008). The p.Asp433His allele was reported in a patient with tropical chronic pancreatitis (Murugaian et al 2008). These mutations are present in the databases with Human genome Mutation Database (HGMD) ID.

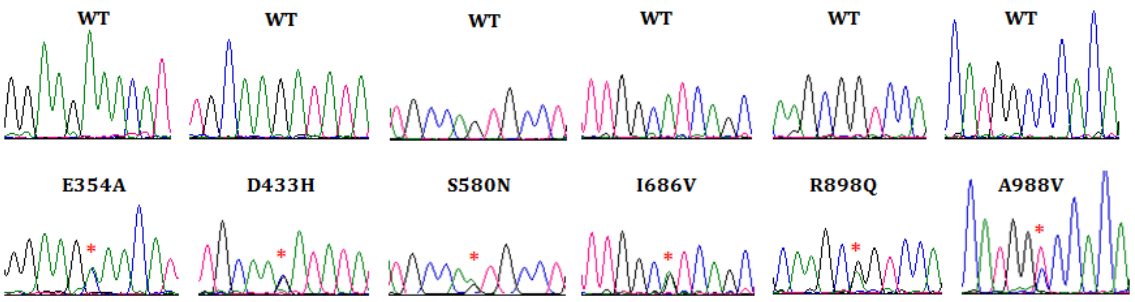
The mutation detected in NIH2, c.2693G>A (p.Arg898Gln) was found in one additional JME patient. The mutations, p.Asp433His, p.Ile686Val and p.Ala988Val variants were observed in more than one unrelated JME affected individuals (Table 2.6). A few synonymous substitutions and rare variants located in the 5'UTR and intronic regions of the gene were also found (Table 2.5) along with certain common polymorphisms (Appendix I A2.4). I have considered the novel/rare nonsynonymous variations for further analysis.

Table 2.6: Clinical characteristics of JME patients harboring CASR mutation

CASR mutation	Gender	Age of onset (years)	Seizure types	EEG	Medication
Glu354Ala	Female	9	MJ, ABS, GTCS	Gen SW	VPA
Asp433His	Female	NA	MJ	NA	NA
Asp433His	Male	8	MJ, ABS, GTCS	SW	VPA
Asp433His	Female	14	MJ, GTCS	Gen SW	VPA
Ser580Asn	Female	NA	MJ	NA	VPA
Ile686Val	Female	18	MJ, GTCS	Gen SW	VPA
Ile686Val	Female	13	MJ	Gen SW	VPA
Ile686Val	Female	14	MJ, GTCS	Gen SW	VPA
Ile686Val	Female	6	MJ, GTCS	Gen SW	VPA
Ile686Val	Male	12	MJ, GTCS	NA	CBZ
Arg898Gln	Male	13	MJ, GTCS	Gen SW	VPA
Ala988Val	Female	12	MJ, GTCS	Gen SW	VPA
Ala988Val	Female	14	MJ	Normal	VPA
Ala988Val	Male	19	MJ	NA	VPA

MJ: myoclonic jerks, GTCS: generalized tonic-clonic seizures, ABS: absence seizures, Gen SW: generalized spike-wave, SW: spike-wave, VPA: Valporic acid, CBZ: Carbamazepine, NA: Not available.

A



B

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CASR_Zebrafish          REFWEET ALQDILT KDASFCV AFGISFV FRKRSSS SMK----
CASR_Guinea Pig         KEFWEET ALQDIYT TDASACD AFGISFV SRKGTAA NATAHRH
CASR_Mouse              KEFWEET ALQDIYT TDASACD AFGISFV SRKRSSS NAMAHRN
CASR_Rat                KEFWEET ALQDIYT TDASACD AFGISFV SRKRSSS NAMAHRN
CASR_Rhesus Monkey      KEFWEET ALQDIYT TDASACN AFGISFV SRKRSSS NAMAHRN
CASR_Common Marmoset    KEFWEET ALQDIYT TDASACN AFGISFV SRKRSSS NAMAHRN
CASR_Orangutan          KEFWEET ALQDIYT TDASACN AFGISFV SRKRSSS NAMAHRN
CASR_Chimpanzee         KEFWEET ALQDIYT TDASACN AFGISFV SRKRSSS NAMAHRN
CASR_Human              KEFWEET ALQDIYT TDASACN AFGISFV SRKRSSS NAMAHRN
CASR_Gorilla            KEFWEET ALQDIYT TDASACN AFGISFV SRKRSSS NAMAHRN
CASR_Armadillo          KEFWEET ALQDIYT TDASACD AFGISFV SRQRSSS NAVAHRN
CASR_Pig                KEFWEET ALQDIYT TDASACD AFGISFV SRQRSSS SATAHRN
CASR_Killer Whale       KEFWEET ALQDIYT TDASACD AFGISFV SRQRSSS SAMAHRN
CASR_Cow                KEFWEET ALQDIYT TDASACD AFGISFV SRQRSSS TAVAHRN
CASR_Sheep              KEFWEET ALQDIYT TDASACD AFGISFV SRQRSSS SAVAHRN
CASR_Horse              KEFWEET ALQDIYT TDASACD AFGISFV SRKRSSS SAMAHRN
CASR_Rhinoceros         KEFWEET ALQDIYT TDASACD AFGISFV SRKRSSS SAMAHRN
CASR_Dog                KEFWEET ALQDIYT TDASACD AFGISFV SRKRSGS SAAAPRN
CASR_Walrus             KEFWEET ALQDIYT TDASACD AFGISFV SRKRSSS SAMAPRN
CASR_Cat                KEFWEET ALQDIYT TDASACD AFGISFV SRKRSSS SAMAHRN
CASR_Giant Panda        KEFWEET ALQDIYT TDASACD AFGISFV SRKRSSS SAMAHRN
CASR_Green Lizard       KEFWEET ALQDIYT TDASACE AFGISFV SRKRSNS NAVANKN
CASR_Zebra Finch        KEFWEET ALQDIYT TDASACD AFGISFV SRKRSNS NAMANRN
CASR_Wild Duck          KEFWEET ALQDIYT TDASACD AFGISFV SRKRSNS NAMANRN
CASR_Chicken            KEFWEET ALQDIYT TDASACD AFGISFV SRKRSNS NAMANRN
CASR_Wild Turkey        KEFWEET ALQDIYT TDASACD AFGISFV SRKRSNS NAMANRN
```

Figure 2.4: *CASR* mutations associated with epilepsy: A. Electropherograms showing DNA sequence of the respective wild-type (WT) (upper panel) and the mutation (lower panel) from the genomic DNA. The presence of two peaks at one nucleotide position indicates the heterozygous variation (marked with red*) for each mutation (p.Glu354Ala, p.Asp433His, p.Ser580Asn, p.Ile686Val, p.Arg898Gln and p.Ala988Val). **B**. Alignment of CASR protein sequence from various organisms by Clustal Omega, showing only the region near the location of mutated amino acid residue. The wild-type amino acid residue for each mutation is highlighted in blue (E354, D433, S580, I686, R898 and A988).

2.3.4 Predictive effect of CASR mutations

According to the *in silico* tools used, the six missense mutations (p.Glu354Ala, p.Asp433His, p.Ser580Asn, p.Ile686Val, p.Arg898Gln, p.Ala988Val) identified in the CASR receptor have benign to damaging functional effect on the protein (Table 2.7). The multiple sequence alignment of human CASR protein with its orthologs by Clustal Omega showed that the amino acid residues Glu354, Asp433, Ser580, Ile686, Arg898, and Ala988 are highly conserved (Figure 2.4). This suggests that these amino acid residues have structural and functional biological importance.

Table 2.7: Bioinformatics analysis of CASR nonsynonymous mutations

CASR mutation	SIFT	Polyphen-2[a]	Align GVGD[b]	Mutation assessor[c]	Evolutionary conservation
Glu354Ala	Tolerated (0.33)	Probably damaging (0.992)	C65 (106.71)	Medium FI (2.305)	26/26
Asp433His	Tolerated (0.1)	Probably damaging (0.967)	C65 (81.24)	Medium FI (2.655)	26/26
Ser580Asn	Tolerated (0.33)	Probably damaging (0.997)	C65 (46.24)	Low FI (1.515)	26/26
Ile686Val	Tolerated (0.18)	Benign (0.012)	C25 (28.68)	Low FI (0.985)	26/26
Arg898Gln	Damaging (0.01)	Possibly damaging (0.555)	C35 (42.81)	Medium FI (2.515)	25/26
Ala988Val	Tolerated (0.14)	Benign (0.005)	C65 (65.28)	Medium FI (1.905)	25/26

[a]Using HumDiv prediction model of Polyphen-2. [b]In Align GVGD, Class C65 to C25 ranges from most to least likely to interfere in protein function, [c]FI: functional impact on protein. [d]Alignment of CASR protein sequence from 26 organisms using Clustal Omega, showing number of organisms with conserved wild-type amino acid residue/organisms examined.

2.3.5 Effect of CASR mutations on MAPK pathway

CASR plays an important role in maintenance of calcium ion homeostasis by sensing its extracellular levels and responding to its changes via regulation of different downstream signaling pathways such as MAPK (mitogen-activated protein kinase), Phospholipase C (PLC) - mediated inositol triphosphate (IP3) formation, etc. (Ward 2004, Conigrave and Ward 2013). The effect of mutations in CASR receptor, towards response to changes in extracellular calcium concentrations, was studied using PathDetect *trans*-reporting system to measure the activity of Elk-1. The Elk-1 belongs to Ets oncogene family of transcription factors containing a highly conserved DNA-binding domain (Ets domain) and is targeted by MAPK pathways (Janknecht et al 1993, Besnard 2011). In this system, the activation domain of Elk-1 is expressed as a fusion *trans*-activator protein and is phosphorylated and activated by MAPK. This activity regulates the expression of luciferase gene, which in turn, reflects the activation status of the signaling events downstream of CASR in response extracellular calcium (Figure 2.5).

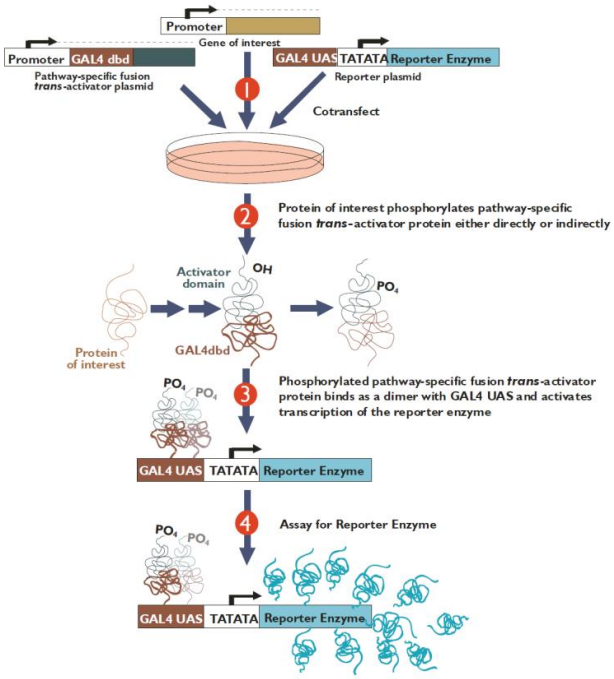
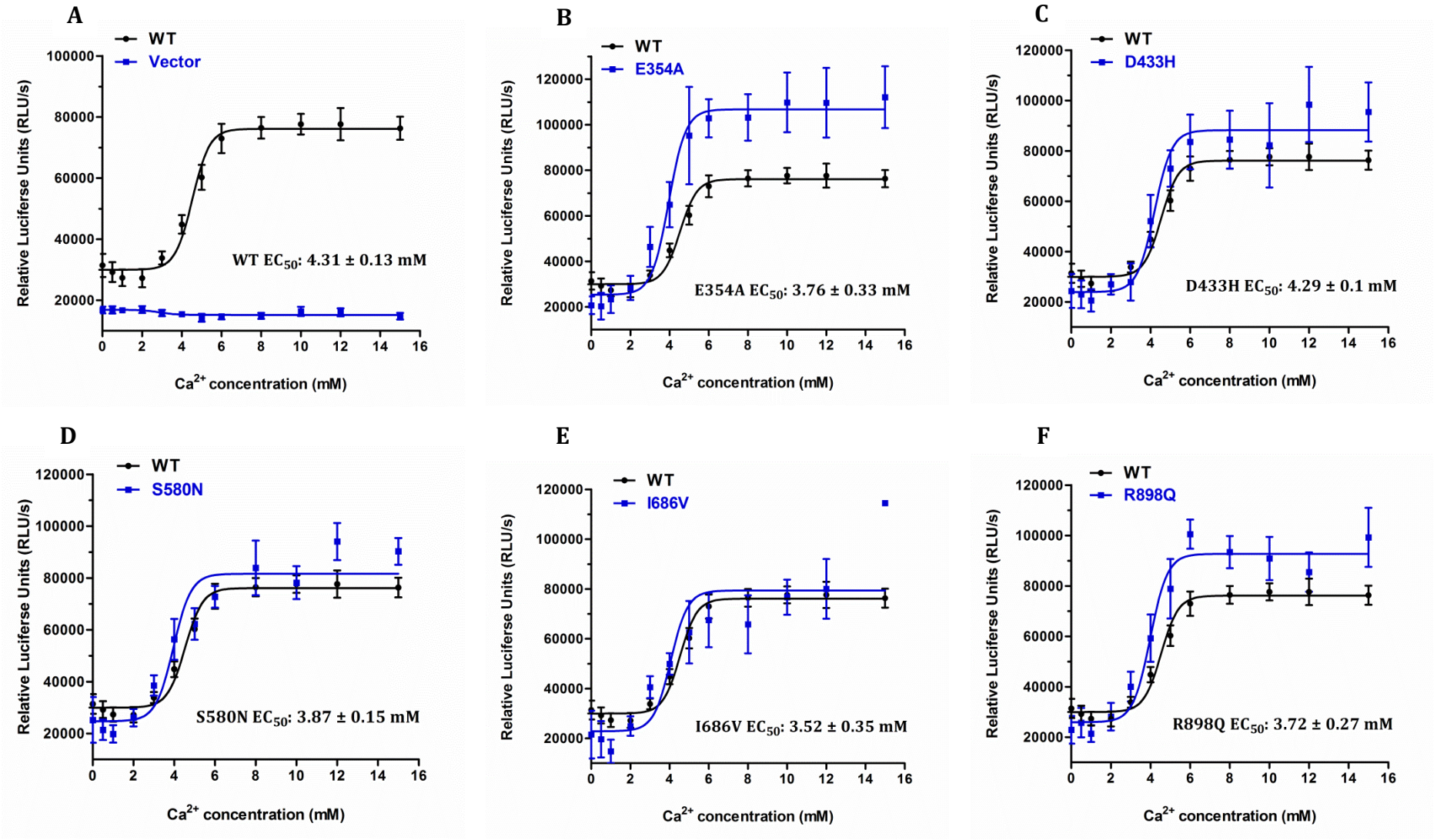


Figure 2.5: The PathDetect *in vivo* Signal Transduction Pathway *trans*-reporting system. (Source: Stratagene)


A
WT
Vector
Relative Luciferse Units (RLU/s)
WT EC50: 4.31 ± 0.13 mM
Ca2+ concentration (mM)
B
WT
E354A
Relative Luciferse Units (RLU/s)
E354A EC50: 3.76 ± 0.33 mM
Ca2+ concentration (mM)
C
WT
D433H
Relative Luciferse Units (RLU/s)
D433H EC50: 4.29 ± 0.1 mM
Ca2+ concentration (mM)
D
WT
S580N
Relative Luciferse Units (RLU/s)
S580N EC50: 3.87 ± 0.15 mM
Ca2+ concentration (mM)
E
WT
I686V
Relative Luciferse Units (RLU/s)
I686V EC50: 3.52 ± 0.35 mM
Ca2+ concentration (mM)
F
WT
R898Q
Relative Luciferse Units (RLU/s)
R898Q EC50: 3.72 ± 0.27 mM
Ca2+ concentration (mM)

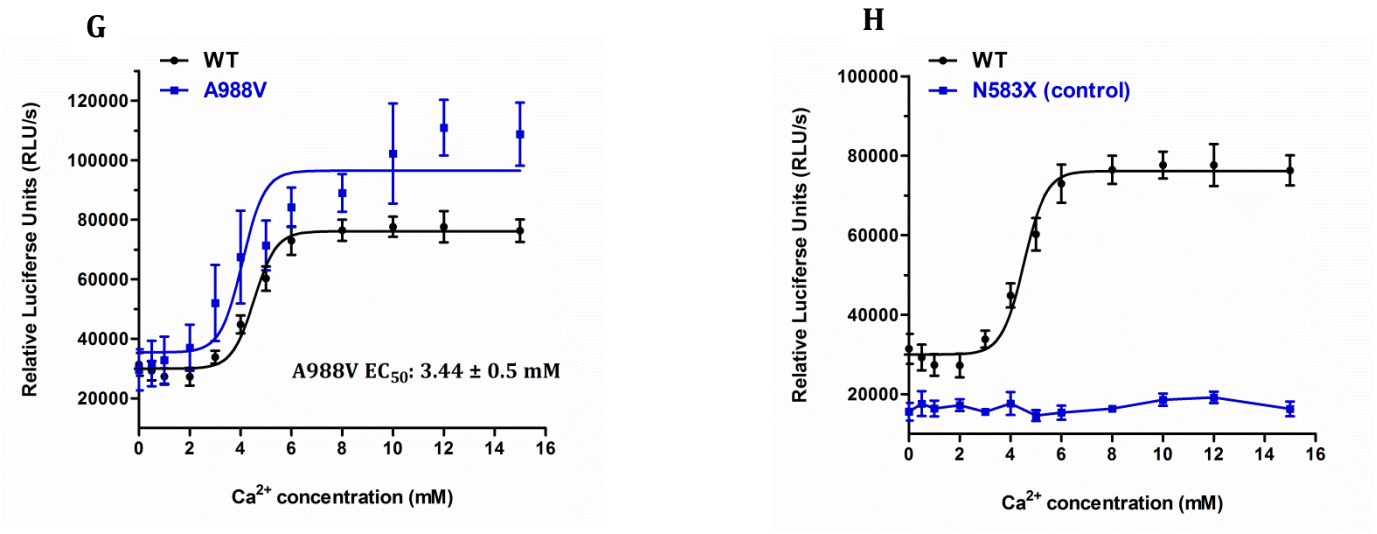


Figure 2.6: Cell signaling (MAPK pathway) of wild-type and mutant CASR receptors in transfected HEK293T cells: Extracellular calcium-evoked increase in MAPK activity in HEK293T cells transiently transfected with control (pcDNA3.1 vector or CASR-N583X), wild-type (WT) or mutation harbouring (E354A, D433H, S580N, I686V, R898Q, A988V) *CASR* cDNA constructs along with vectors of MAPK *trans*-reporting system. In each plot, activity response of WT is presented in black, whereas, of mutant receptor is shown in blue. The basal level signaling activity in cells with control vectors is shown in plots A and H. The dose-response curves of signaling response by mutant receptors is presented in graphs B-G. Values shown here are mean of readings from three independent experiments and are plotted as mean ± SEM.

With exponentially increasing activity in the 3-6 mM range of Ca^{2+} concentration and saturation at higher calcium levels, the signaling activity of CASR across different extracellular Ca^{2+} concentrations (0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12 and 15 mM) generated a sigmoidal dose-response curve. In cultured HEK293T cells, the wild-type (WT) CASR receptor showed a half-maximal response (EC50) at 4.31 ± 0.13 mM (mean ± SEM). The signaling response by p.Glu354Ala, p.Asp433His, p.Ser580Asn, p.Ile686Val, p.Arg898Gln and p.Ala988Val mutant receptors exhibited a variable leftward shift in the dose-response curves (Figure 2.6). The EC50 values of mutant receptors p.Ile686Val: 3.52± 0.35 mM, p.Arg898Gln: 3.72 ± 0.27 mM and p.Ala988Val: 3.44 ± 0.5 mM were significantly lower than that of wild-type ($p < 0.05$). But, the EC50 values for p.Glu354Ala: 3.76 ± 0.33 mM, p.Asp433His: 4.29 ± 0.1 mM, p.Ser580Asn: 3.87 ± 0.15 mM did not differ significantly from that of the wild-type CASR. The vector (pcDNA3.1) and control (CASR-Asn583X pcDNA3.1) transfected cells exhibited basal level activity in response to increasing extracellular Ca^{2+} concentrations.

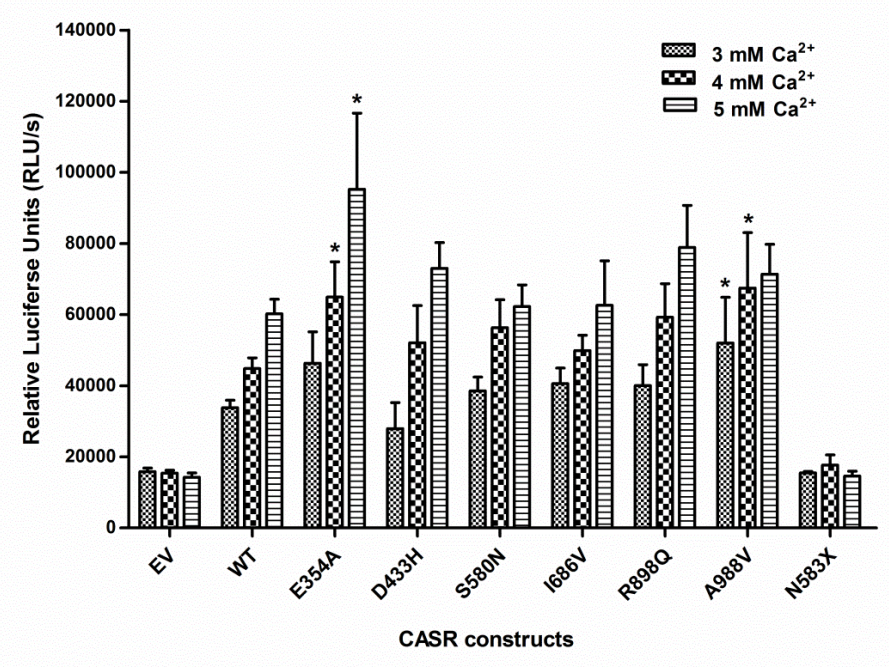


Figure 2.7: Effect of *CASR* mutations on MAPK signaling in response to extracellular calcium: The signaling activity of HEK293T cells transfected with control, WT or mutation-harbouring (E354A, D433H, S580N, I686V, R898Q, A988V) CASR constructs at 3 mM, 4 mM and 5 mM extracellular Ca^{2+} concentrations. The values are plotted as mean ± SEM obtained in three independent experiments. The P values < 0.05 (two-tailed) were regarded significant (unpaired Student's t test, GraphPad Prism 5).

As the wild-type CASR receptor exhibited an exponential increase in signaling activity at 3mM, 4mM and 5mM extracellular Ca^{2+} (Figure 2.6a), the signaling response of mutant CASR receptors was compared to that of wild-type CASR at these three Ca^{2+} concentrations by a bar graph representation (Figure 2.10). The mutation p.Ala988Val, showed significantly higher signaling response than the wild-type CASR at extracellular Ca^{2+} concentrations of 3 mM and 4 mM ($p < 0.05$) (Figure 2.7). The p.Glu354Ala mutant receptor also exhibited an enhanced response than wild-type CASR at 4 mM and 5 mM Ca^{2+} concentrations ($p < 0.05$). The maximum response by p.Glu354Ala and p.Arg898Gln receptors, observed at 6 mM Ca^{2+} was significantly higher than that of the wild-type CASR receptor ($p \leq 0.01$). The reduced EC50 values and enhanced signaling response (at 3 mM, 4 mM, 5 mM and 6 mM Ca^{2+} concentrations) generate a leftward shift in the dose-response curves of p.Glu354Ala, p.Ile686Val, p.Arg898Gln and p.Ala988Val mutant receptors, suggesting a significant gain-of-function effect. The p.Asp433His and p.Ser580Asn mutations caused only slight differences in the dose-response and EC50 values, as compared to wild-type CASR suggesting subtle-effects of these variants on CASR-regulated MAPK pathway. Taken together, it does seem that the CASR mutant receptors exhibit variable gain-of-function effect for the MAPK signaling.

2.3.6 Effect of CASR mutations on intracellular IP1 levels

The effect of CASR wild-type and mutant receptors on Phospholipase C (PLC) coupled Inositol 1,4,5-trisphosphate (IP3) pathway in response to extracellular Ca^{2+} was examined by measuring intracellular IP1 accumulation by IP-One ELISA reagents. As the lifetime of IP3 is less than 30 seconds in the cell, the levels of IP1 were quantified by competitive ELISA in presence of LiCl (prevents conversion of IP1 to Myo-inositol) (Figure 2.8). The cells treated with stimulation buffer without $CaCl_2$ were considered unstimulated for each experiment.

The transiently expressed wild-type CASR, in cultured HEK293T cells exhibited a sigmoidal dose-response curve for increasing extracellular calcium concentrations (0, 2, 4, 6, 8, 10 mM) with an EC50 (agonist concentration at half of the maximal

response) of 4.05 ± 0.13 mM (mean ± SEM). As compared to the wild type CASR, EC50 values of the receptors carrying mutations were as follows: p.Glu354Ala: 3.25 ± 0.18 mM ($p<0.01$), p.Ser580Asn: 3.38 ± 0.16 mM ($p<0.01$), p.Arg898Gln: 3.05 ± 0.18 mM ($p<0.001$), p.Ala988Val: 3.42 ± 0.23 mM ($p<0.05$) suggesting a gain-of-function effect (Figure 2.9). However, the change in EC50 values for the p.Asp433His (3.78 ± 0.16 mM) and p.Ile686Val (3.79 ± 0.2 mM) alleles were not significant. The signaling response curves for p.Glu354Ala, p.Ser580Asn, p.Arg898Gln and p.Ala988Val showed a leftward shift and saturation at a lower Ca^{2+} concentration as compared to wild-type CASR. The cells transfected with control vectors (pcDNA3.1 and CASR-Asn583X pcDNA3.1) had no change in intracellular IP1 levels due to increasing calcium concentration.

Due to an exponential increase in the intracellular IP1 levels in wild-type CASR transfected cells at 3 mM, 4 mM and 5 mM extracellular Ca^{2+}concentrations (Figure 2.9a), IP1 accumulation in cells transfected with the mutant CASR receptors was compared to that of wild-type CASR at these three Ca^{2+} concentrations in a bar graph representation (Figure 2.10).

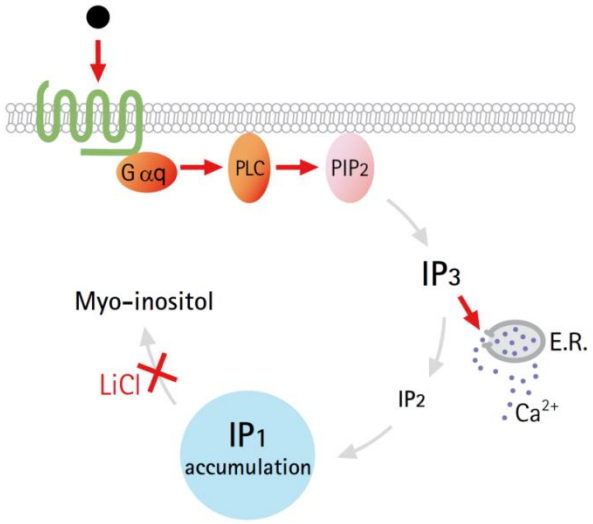
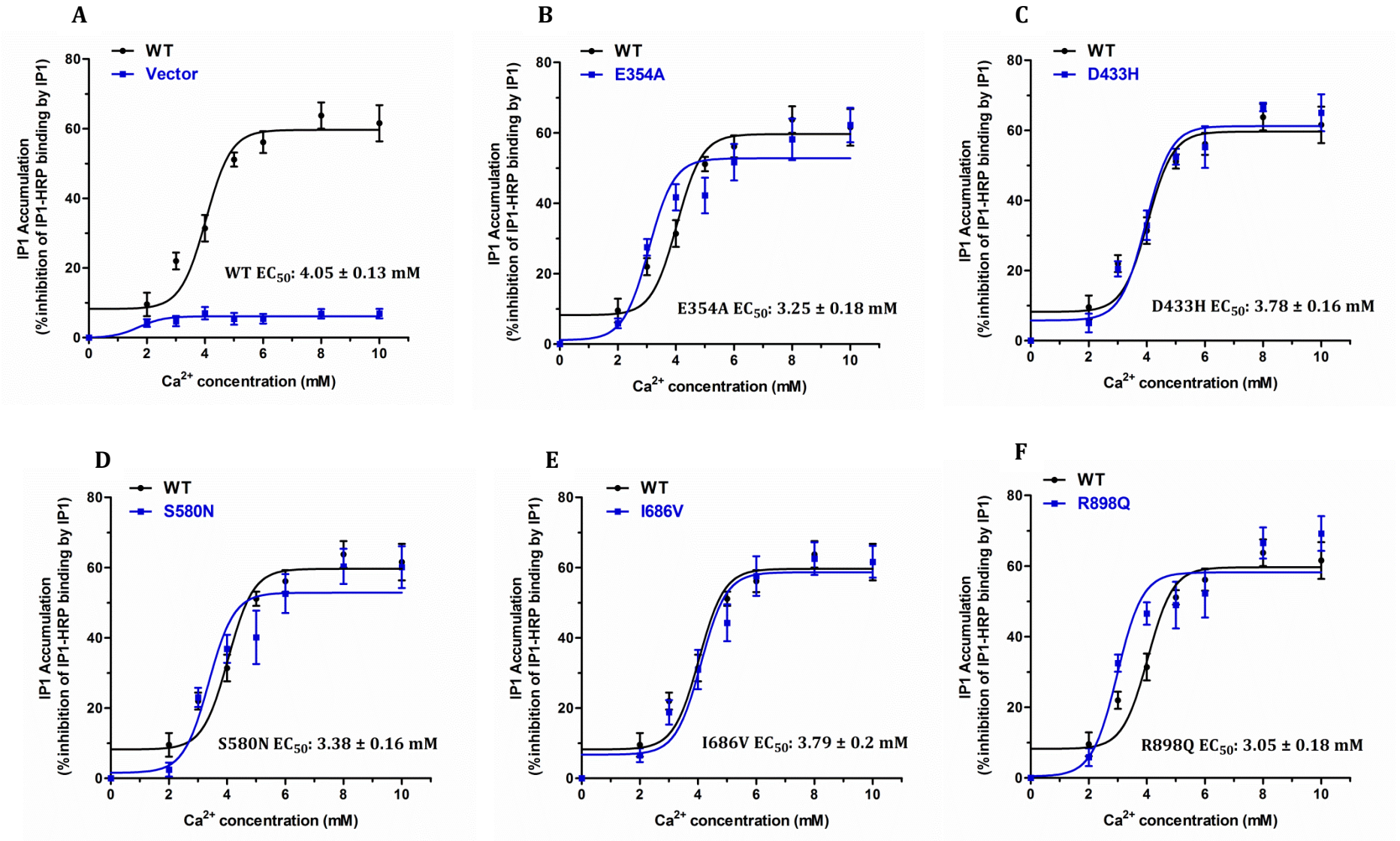


Figure 2.8: Schematic representation of activation of PLC-mediated IP3 signaling pathway by a Gq-coupled GPCR. (Source: Cisbio Bioassays)


A
WT
Vector
WT EC_{50}: 4.05 ± 0.13 mM
B
WT
E354A
E354A EC_{50}: 3.25 ± 0.18 mM
C
WT
D433H
D433H EC_{50}: 3.78 ± 0.16 mM
D
WT
S580N
S580N EC_{50}: 3.38 ± 0.16 mM
E
WT
I686V
I686V EC_{50}: 3.79 ± 0.2 mM
F
WT
R898Q
R898Q EC_{50}: 3.05 ± 0.18 mM
IP1 Accumulation (%inhibition of IP1-HRP binding by IP1)
Ca^{2+} concentration (mM)

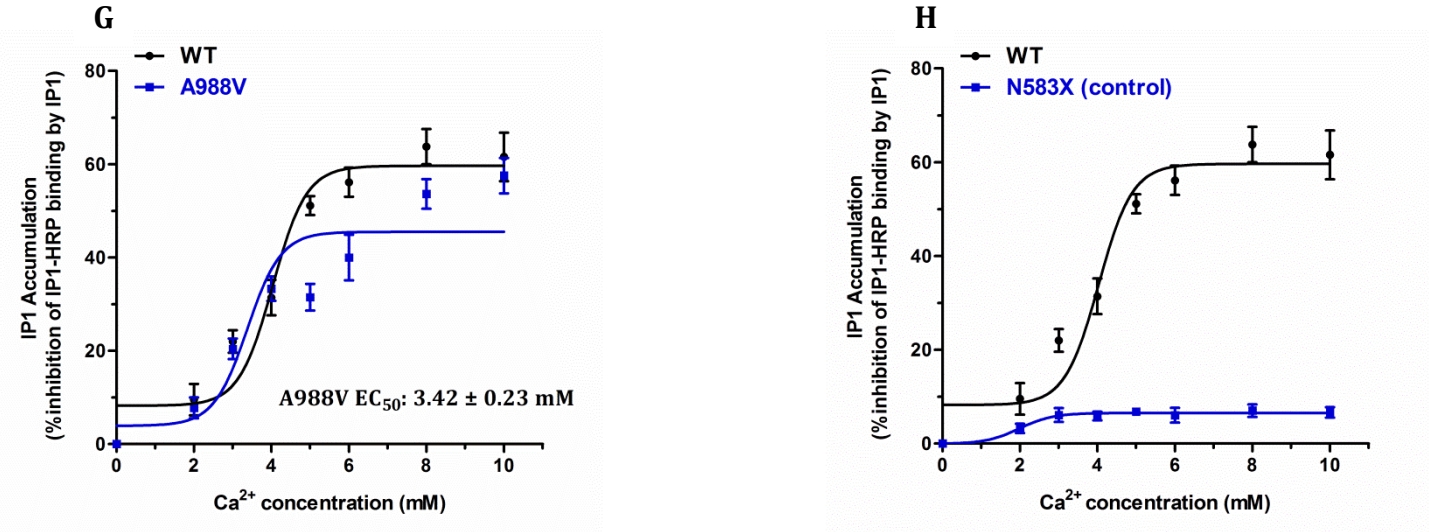


Figure 2.9: Cell signaling (PLC-IP3 pathway) of wild-type and mutant CASR receptors in transfected HEK293T cells: The PLC-mediated IP3 signaling activity is observed by measuring intracellular IP1 accumulation. Changes in IP1 accumulation in response to increasing extracellular Ca^{2+} concentrations were measured in HEK293T cells transiently transfected with control (pcDNA3.1 vector or CASR-N583X), wild-type (WT) or mutation harbouring (E354A, D433H, S580N, I686V, R898Q, A988V) *CASR* cDNA constructs. In each concentration-response curve, activity response of WT is presented in black, whereas, of mutant receptor is shown in blue. The basal level effect in cells with control vectors is shown in plots A and H. The dose-response curves showing changes in intracellular IP1 levels for mutant receptors are presented in graphs B-G. IP1 accumulation was measured using IP-One ELISA Kit and is shown here as the percentage inhibition of IP1-HRP binding (mean ± SEM), obtained from readings of six independent experiments.

The p.Arg898Gln exhibited significantly high levels at 3 mM and 4 mM Ca^{2+} concentrations than the wild-type CASR ($p<0.05$) (Figure 2.10). The HEK293T cells transfected with p.Ala988Val mutant receptor showed significantly reduced intracellular IP1 levels than wild-type CASR at 5 mM ($p<0.001$) and 6 mM ($p<0.05$), exhibiting saturation at lower calcium levels. The significantly leftward shifted dose-response curves of p.Glu354Ala, p.Ser580Asn, p.Arg898Gln and p.Ala988Val mutant receptors indicate an enhanced responsiveness towards calcium and a gain-of-function effect. However, p.Asp433His and p.Ile686Val mutant CASR receptors appear to have a subtle functional effect on the signaling pathway. Yet again, the CASR mutations seem to be of activating nature causing a variable gain-of-function effect on the CASR-regulated IP3-IP1 signaling pathway.

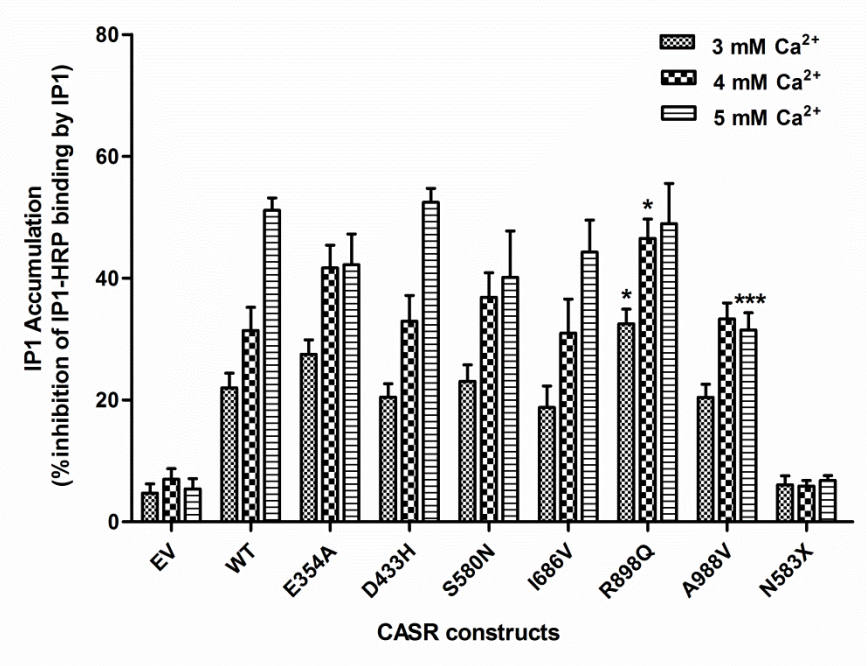


Figure 2.10: Effect of *CASR* mutations on PLC-IP3 signaling in response to extracellular calcium: The intracellular IP1 levels in HEK293T cells transfected with control, WT or mutation-harbouring (E354A, D433H, S580N, I686V, R898Q, A988V) CASR constructs at 3 mM, 4 mM and 5 mM extracellular Ca^{2+} concentrations. The values represented here are of the percentage inhibition of IP1-HRP binding (mean ± SEM), obtained from readings of six independent experiments. The P values < 0.05 (two-tailed) were regarded significant (unpaired Student's t test, GraphPad Prism 5).

2.4 Discussion

Genetic generalized epilepsies are a common form of human epilepsies with substantial genetic basis to their etiology, and exhibit a complex mode of inheritance (Helbig et al 2008, Greenberg and Stewart 2012, Petrovski and Kwan 2013). Although both generalized and focal epilepsies are well defined clinical entities, co-existence of generalized and focal seizures in certain affected individuals in an age-dependent manner as well as presence of both phenotypes in families with multiple affected members, have been reported (Dimova and Daskalov 2002, Caraballo et al 2004, Nicolson et al 2004, Deng et al 2007, Cerminara et al 2012). These findings have suggested an overlapping genetic predisposition for both generalized and focal epilepsies. A few studies have reported presence of febrile convulsions along with GGE in large kindreds (Wallace et al 2001, Kananura et al 2002, Marini et al 2003, Bonanni et al 2004).

Likewise, a three-generation family (NIH2) from south India was reported with its affected members manifesting generalized and focal seizures along with febrile seizures (Kapoor et al 2008). Whole-genome wide linkage analysis identified a critical genetic interval at 3q13.3-q21 (EIG8) with highest LOD score of 3.05. Sanger sequencing of a few candidate genes had led to finding of a rare disease-segregating variation in calcium-sensing receptor gene (*CASR)*, suggesting it as a plausible cause of GGE in NIH2.

In this study, I reanalyzed this disease-linked genomic interval on chromosome 3 by whole-exome sequencing and examining an extended haplotype of 64 Mb (3p14.2–q21) that was being shared by all affected members of NIH2. This analysis identified five rare alleles in the *EPHA6*, *AB3IBP*, *KIAA1407*, *IQCB1* and *CASR* genes, segregating with the clinical phenotype in NIH2. Of these, only c.2693G>A (p.Arg898Gln) in *CASR* fulfilled the criteria of being a causative mutation i.e. absence in databases (dbSNP and 1000 Genomes) and our control cohort, predictive damaging effect on protein due to the substitution and

evolutionary conservation of the parent amino acid residue. These findings strongly suggest a role of CASR in causation of GGE in NIH2 family.

Human *CASR* gene encodes for extracellular calcium-sensing receptor that localizes to plasma membrane of the cell. It is a seven transmembrane protein that belongs to family C of G-protein coupled receptors (GPCR), with other family members being the metabotropic glutamate receptors (mGluR 1-8), γ-aminobutyric acid B receptors (*GABAB*), Retinoic acid-inducible orphan G protein-coupled (RAIG) receptors and taste receptors (T1R). *CASR* plays an essential role in maintenance of systemic calcium homeostasis and regulates several cellular functions via modulation of different intracellular signaling pathways (Brown and MacLeod 2001, Hofer and Brown 2003, Brown 2013). The physiological observations of certain cells being responsive to small changes in extracellular calcium concentrations suggested the presence of an extracellular calcium sensor at the cell surface. The expression cloning in *Xenopus laevis* oocytes using bovine parathyroid poly (A)+ RNA enabled isolation of BoPCaR (bovine parathyroid Ca^{2+}-sensing receptor) (Brown et al 1993). Since then, extracellular calcium-sensing receptor has been identified in several species and in a wide range of tissues such as bone, kidney, intestine and brain (Garrett et al 1995, Brown and Hebert 1997, Zanotti and Charles 1997, Yamaguchi et al 2002, Cheng et al 2002, Chen and Goodman 2004, Loretz 2008, Bystrova et al 2010). *CASR* from brain was first isolated from cDNA library of rat striatal brain (Ruat et al 1995).

The human CASR receptor is a 1078 amino acid polypeptide, comprising of a large extracellular domain (ECD) of about 600 amino acids, seven transmembrane-spanning domains (TMD) and a large ~200-residue carboxyl-terminal intracellular domain (ICD) (Figure 2.12) (Chattopadhyay and Brown 2000). Its large extracellular domain is structurally similar to that of bacterial periplasmic binding proteins (Venus-fly trap domain) and shares sequence identity with the ECD of mGluRs (Bai 2004). The extracellular domain of CASR has been proposed to contain major Ca^{2+}-binding sites and thus, supporting its role in maintaining systemic calcium homeostasis (Huang et al 2007). CASR forms functional homodimers, the active form of receptor on the cell surface (Bai et al 1998a) and

this dimerization is mediated through conserved cysteine residues of ECD (Hu et al 2000). The seven transmembrane domains are involved in the signal transduction mechanism from the ECD, with its intracellular loops providing interaction domains to its respective G proteins. CASR receptor has conserved domain architecture across species in vertebrates (Figure 2.11). Like many other members of family C of GPCRs, CASR has a distinctly large intracellular domain of 216 amino acids, which plays key role in its cell surface expression, and signal transduction via interaction with various intracellular components (Ray et al 1997, Gama and Breitwieser 1998, Awata et al 2001).

CASR senses extracellular calcium (Ca^{2+}_o) and regulates several cellular processes by modulating different intracellular signaling pathways (Brown et al 2001, Ward 2004, Brown 2013) (Figure 2.13). It regulates the secretion of parathyroid hormone and calcitonin in response to Ca^{2+}_o concentration, wherein it acts via activation of phopholipases C, A2 and D (Kifor et al 1997).

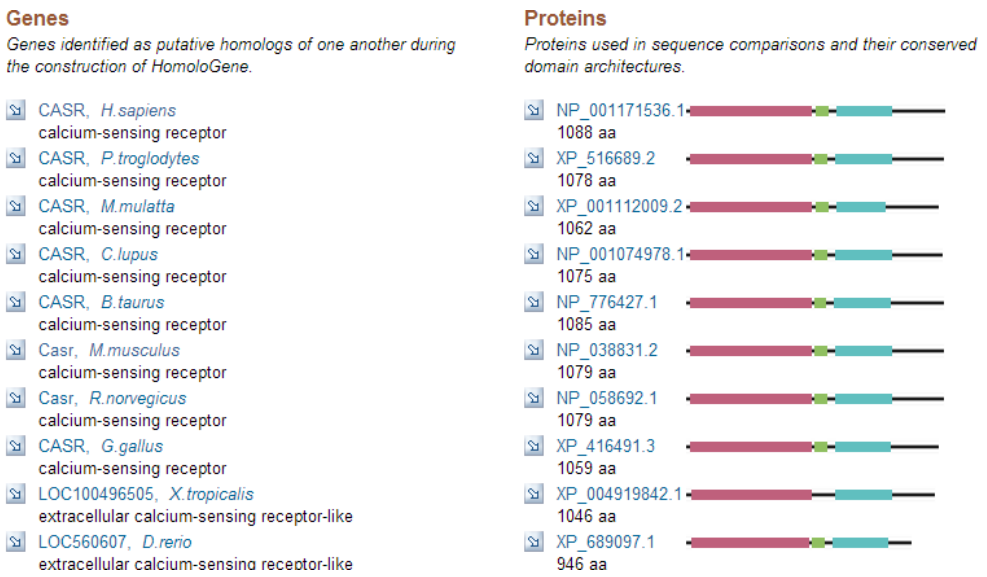


Figure 2.11: Conservation of CASR in Euteleostomi (Homologene, NCBI)

It has also been proposed that *CASR* regulates the fundamental biological processes of cellular proliferation, differentiation and apoptosis in various cell types. *CASR* modulates these functions via interacting with different G proteins, activating PLC mediated IP3 pathway, inhibition of c-AMP, stimulation of the MAPK cascade etc (Darè et al 1998, Kifor et al 2001, Brown et al 2001, Conigrave and Ward 2013). This indicates its role in physiological mechanisms that are apparently quite independent of its role in maintenance of systemic calcium homeostasis.

So far, several studies have shown presence of *CASR* in different brain regions and cell types (Rogers et al 1997, Ferry et al 2000, Kapoor et al 2008, Mudò et al 2009, Chen et al 2010, Ruat and Traiffort 2013), providing a glimpse of its diverse brain-related functions. Expression of *CASR* at transcript and protein level has been observed in different brain regions (Kapoor et al 2008). During development in mice, *CASR* modulates neurite extension in sympathetic neurons and growth of postnatal hippocampal pyramidal dendrites (Vizard et al 2008) and mediates it via ERK1/2 signaling (Vizard et al 2015). A few reports have shown that *CASR* regulates a Ca^{2+}-activated K^{+} channel and a non-selective cation channel (NSCC) in different neuronal cell types and cell lines, thus modulating synaptic plasticity and neurotransmission in neurons (Ye et al 1996, Xiong et al 1997, Chattopadhyay et al 1999a, Chattopadhyay et al 1999b, Chattopadhyay et al 1999c, Phillips et al 2008).

The physiological importance of *CASR* has been established by identifying mutations in this gene in inherited disorders of calcium homeostasis in humans (Egbuna and Brown 2008). Its loss of function mutations are associated with two disorders, familial hypocalciuric hypercalcemia (FHH; MIM: 145980), with one defective copy of gene, and a more severe form of FHH, neonatal severe hyperparathyroidism (NSHPT; MIM: 239200), due to defects in two copies of the gene. The activating mutations that reduce the set point for Ca^{2+} (an enhanced sensitivity to extracellular calcium) are responsible for autosomal dominant hypocalcemia (ADH; MIM: 601198). A few case reports have indicated patients with hypocalcemia manifesting seizures, calcification of basal ganglia or any brain

regions and mental retardation (Pearce et al 1996, Pidasheva et al 2005, Sfar et al 2011, Wong et al 2011), suggesting that derangement in calcium-sensing or homeostasis can have profound effects in normal brain functioning as well.

In this chapter, I present results of whole-exome sequencing analysis of the 64 Mb interval on chromosome 3, confirming the c.2693G>A (p.Arg898Gln) variation in *CASR* to be the epilepsy-causing genetic factor in NIH2. The arginine at 898 amino acid position lies in the intracellular domain of the receptor and is evolutionary highly conserved (Figure 2.12, Figure 2.2c). It is located close to three potential phosphorylation sites for protein kinase C (Thr888, Ser895, Ser915), known to modulate the coupling of *CASR* to intracellular signaling pathways (Bai et al 1998b, Jiang et al 2002). CASR interacts with calmodulin via a calmodulin-binding domain (residues 871-898) in its C-terminus region, and this calcium-dependent association is crucial for its calcium responsiveness and cell surface expression (Huang et al 2010). The ubiquitination and degradation of CASR receptor is mediated by the E3 Ubiquitin Ligase Dorfin, by its region of interaction with dorfin localized to 880-900 residues in intracellular tail (Huang et al 2006). Another report also suggests the gain-of-function effect of p.Arg898Gln and indicates that it disrupts the distal R-K-R motif of the arginine-rich region in ICD leading to abundance of mutant receptor on the plasma membrane (Stepanchick et al 2010). The presence of arginine 898 in the crucial functional domains of the receptor; the calmodulin-binding domain and the dorfin-interacting region, suggests that the alteration of this residue may affect its interactions with other proteins and their mediated functions.

Further, I attempted to gather additional evidence for the role of *CASR* behind causation of epilepsy and was able to identify additional missense mutations in 14 unrelated JME patients (Table 2.6). Among the rare alleles found, effect of six heterozygous missense mutations were evaluated in cell-based functional assays. These mutations occurred at evolutionary conserved residues (p.Glu354Ala, p.Asp433His, p.Ser580Asn, p.Ile686Val, p.Arg898Gln and p.Ala988Val) in CASR (Figure 2.4b). The amino acid residues Glu354, Asp433 and Ser580 are present in the extracellular domain (ECD) of the protein (Figure 2.12).

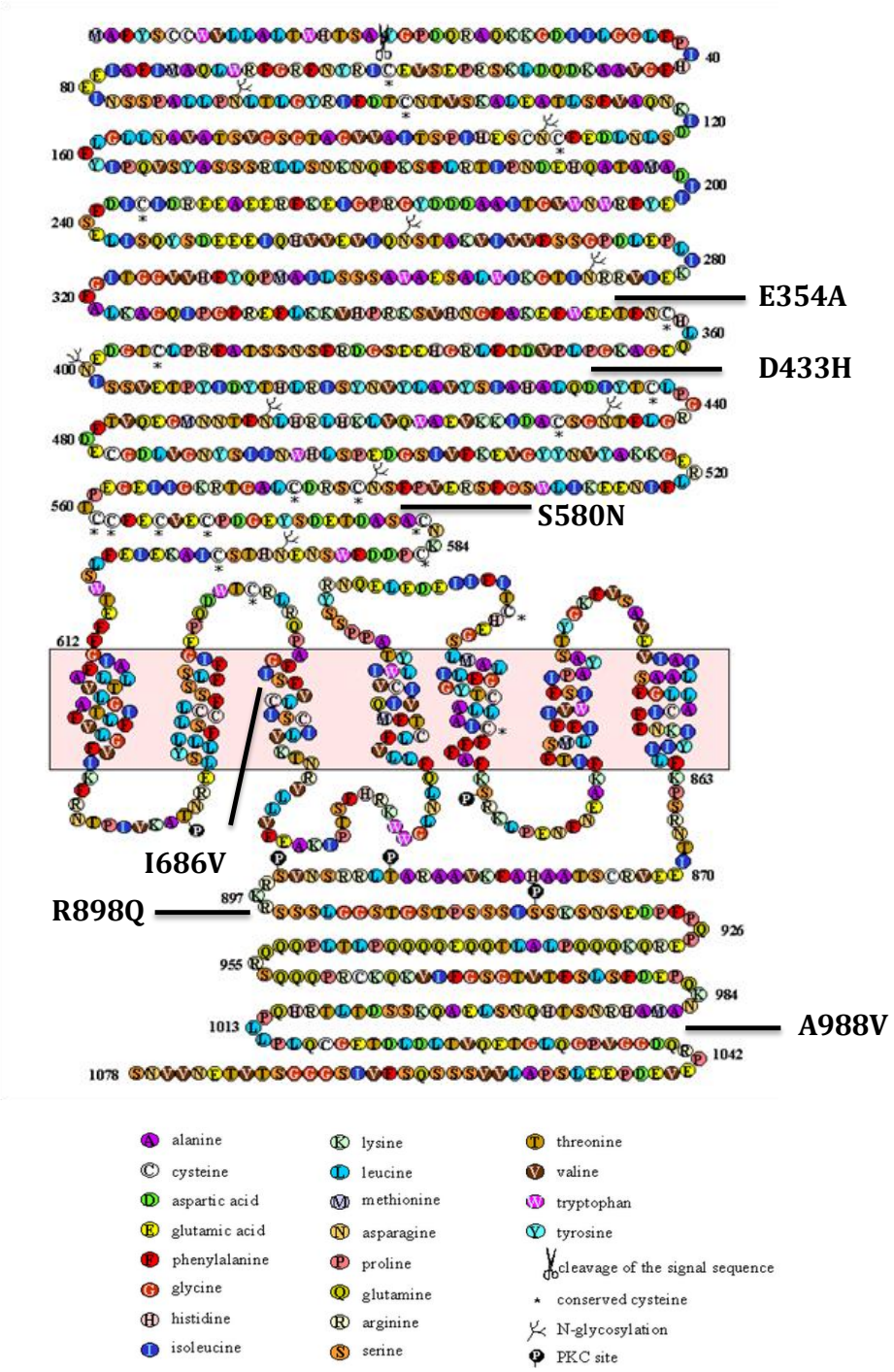


Figure 2.12: Protein sequence and structural topology of CASR: The location of mutations (E354A, D433H, S580N, I686V, R898Q and A988V) identified in JME patients are indicated with a line. CASR comprises of an extracellular domain (1-612 a.a), a seven transmembrane domains (613-862 a.a) and an intracellular domain (863-1078 a.a). The mutations Glu354Ala, Asp433His, Ser580Asn are located in the extracellular domain; Ile686Val is present in the third transmembrane helix; and Arg898Gln and Ala988Val are situated in the intracellular domain of the receptor. (Adapted from www.casrdb.mcgill.ca)

The unusually large extracellular domain of CASR harbors three calcium-binding subdomains, each with two or three predicted Ca^{2+}-binding sites (Huang et al 2009). The Glu354 lies among the crucial amino acid residues in a Ca^{2+}-binding site of the third globular subdomain, suggesting its alteration may affect the conformation or ligand interaction of the calcium-binding site. The Ile686 residue is present in the third transmembrane helix and its substitution to valine may lead to conformational changes affecting the downstream intracellular signaling. The intracellular carboxy-terminal domain (ICD) of CASR has more than 200 amino acid residues and is crucial for its cell surface expression; the ligand-binding modulated downstream signaling and its interaction to proteins such as filamin and calmodulin. Besides p.Arg898Gln, the p.Ala988Val mutation is also located in the intracellular domain of the receptor (Figure 2.12). The Ala988 is present in the filamin-binding domain of ICD, a region essential for CASR-mediated MAPK signaling. CASR interacts with filamin, a scaffolding protein, via a 20-amino acid residue region in ICD and this interaction is known to play a crucial role in the *CASR* mediated-MAPK signaling (Hjälm et al 2001, Awata et al 2001).

Acting via multiple signaling pathways, *CASR* has been proposed to modulate several physiological functions in response to extracellular calcium concentration in a cell- and tissue-specific manner (Figure 2.13). The specific CASR-regulated pathways influencing neuronal excitability in the brain are not yet known. However, CASR is known to regulate Ca^{2+}-activated K^+ channel via p38-MAPK pathway in U87 astrocytoma cells (Ye et al 2004) and the IP3-IP1 signaling regulated-intracellular calcium stores modulate several neuronal functions (Berridge 1998). With this in view, we evaluated the functional effect of the six missense *CASR* mutations on two CASR-regulated signaling pathways: MAPK and IP3-IP1 in relative to wild-type CASR receptor, using transiently transfected HEK293T cells. The signaling activity was measured in the cells transfected with wild-type- or mutant- CASR pcDNA3.1 (+), and cells transfected with pcDNA3.1 (+) vector and the *CASR* cDNA carrying p.Asn583X mutation were considered as controls.

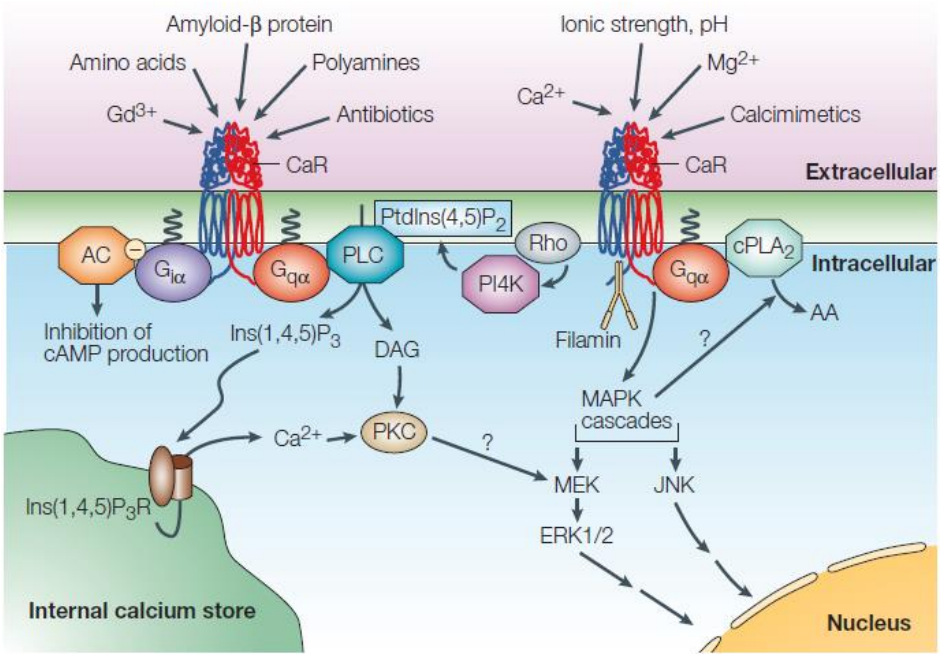


Figure 2.13: CASR-regulated signaling pathways: Several ligands bind to CASR and activate signal transduction cascades, which in turn, regulate various intracellular processes. [AA, arachidonic acid; AC, adenylate cyclase; cAMP, cyclic AMP; cPLA2, cytosolic phospholipase A2; DAG, diacylglycerol; ERK, extracellular-signal-regulated kinase; $G_{i\alpha}$ and $G_{q\alpha}$, α subunits of the i- and q-type heterotrimeric G proteins, respectively; Ins(1,4,5)P3, inositol-1,4,5-trisphosphate; Ins(1,4,5)P3R, inositol-1,4,5-trisphosphate receptor; JNK, Jun amino-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PI4K, phosphatidylinositol 4-kinase; PKC, protein kinase C; PLC, phospholipase C; PtdIns(4,5)P2, phoshatidylinositol-4,5-bisphosphate (from Hofer and Brown 2003)

The wild-type and mutant CASR receptors exhibited a sigmoidal dose-response curve for MAPK and IP3-IP1 signaling on treatment with increasing extracellular Ca^{2+} concentrations. But, the mutant CASR receptors exhibited a variable leftward shift in the dose-response curves of signaling activity. In the MAPK trans-reporting assay, the mutant CASR receptors, p.Glu354Ala, p.Ile686Val, p.Arg898Gln and p.Ala988Val showed significant gain-of-function effect than the wild-type CASR. The leftward shift in the dose-response curves and an enhanced response at crucial Ca^{2+} concentrations (3 mM, 4 mM, 5 mM and 6 mM) suggests these mutations are of activating nature (Figure 2.6 and 2.7). We further assessed the effect of mutations on the signaling activity leading to PLC-mediated 1, 2-diacylglycerol (DAG) formation, thus leading to the IP3/IP1 production, using an

ELISA-based IP-One assay. In IP3-IP1 pathway, the mutant CASR receptors, p.Glu354Ala, p.Ser580Asn, p.Arg898Gln and p.Ala988Val exhibited a significant increased responsiveness towards calcium and saturated at Ca^{2+} concentration lower than that of wild-type CASR (Figure 2.9 and 2.10). Taken together, the CASR mutations, p.Glu354Ala, p.Ser580Asn, p.Ile686Val, p.Arg898Gln and p.Ala988Val apparently have a significant gain-of-function of the CASR-regulated signaling pathways by exhibiting an enhanced responsiveness towards calcium, thus suggesting these to be activating mutations. However, the mild effect of p.Asp433His mutation in altering the signaling activity of CASR receptor suggests, either it is of subtle functional effect and is not solely sufficient to cause functional impairment of the receptor, or it may perturb any other domain-specific CASR function not analyzed here. These findings show that five of six identified epilepsy-associated missense mutations in *CASR* are activating in nature and show a significant gain-of-function effect on the CASR-mediated intracellular signaling.

Similarly, *SLC2A1* mutations in GGE patients are observed to have a spectrum of effects causing marked to mild reduction in the glucose transport by GLUT1 (Arsov et al 2012). Though, no correlation between the severity of seizures in GGE families and functional impairment due to *SLC2A1* mutations was established, the mutations resulting in mild effects on the glucose transport were preferentially observed in families showing variable inheritance pattern. The GGE family with three affected members with JME harbored a mild-effect *SLC2A1* mutation. Such previous reports and our findings indicate that mutations causing relatively moderate effect on the functioning of protein seem sufficient to be causative genetic determinants of GGE/JME.

Our findings indicate a role of CASR in causation of GGE/JME based on the evidence provided at two levels as suggested by McArthur et al 2014. At gene level, all the rare variants in the disease-linked region on chromosome 3, except p.Arg898Gln in CASR, had been excluded based on segregation status in NIH2, allele frequency in databases (dbSNP and 1000 Genomes) and control cohort, evolutionary conservation and predictive pathogenicity using bioinformatics tools. Additionally, we find excess of rare variants in *CASR* in cases than controls and

presence of six rare missense variants in 14 JME patients. The evidence at variant level is provided by their enrichment in cases than controls, strong evolutionary conservation of the variant site, presence in the crucial functional domains of the protein, predictive damaging functional effect and the observed gain-of-function effect of 5 out of 6 variants in the CASR-regulated signaling pathways in HEK293T cells. Additionally, in a large scale study wherein whole exome sequencing was carried out on a group of 118 GGE patients (JME, n=93), a heterozygous rare variation, c.854G>A (p. p.Arg285Gln) in *CASR* was identified in one JME patient (Heinzen et al 2012).

Taken together, we propose that *CASR* is a causative gene for epilepsy with its role predominantly in the GGE/JME phenotype, but also behind seizure types typically not associated with JME, and its potential brain-related functions may underlie its role in susceptibility to epilepsy. Several reports associate activating *CASR* mutations in ADH with hypocalcaemia, muscle cramps, recurrent seizures, basal ganglia calcifications, etc. Seizures are the most frequent symptom associated with ADH, observed in about 56% of the reported pediatric ADH cases (review by Thim et al 2014). And here, we find activating *CASR* mutations linked to a seizure disorder. Thus, it is suggested that gain-of-function effects of *CASR* mutations may perturb the regulation of CASR-activated ion channels or cause an imbalance in calcium homeostasis in the neurons, leading to disruption of synaptic transmission. As the molecular mechanisms how CASR signaling regulates ion channels or other brain-related functions is not yet established, the direct effect of these activating mutations in causing seizure onset is not clear. Thus, it is essential to explore and understand the role of *CASR*-regulated mechanisms in the brain to completely comprehend its role in pathophysiology of epilepsy.

Chapter 3

Exome sequencing reveals a mutation in *TMEM171* at EJM4

EJM4 (5q12-q14) was identified in a three-generation south Indian family (GLH5) with several of its members affected with JME (Kapoor et al 2007). I have carried out whole-exome sequencing (WES) to discover the causative factor in GLH5. Analysis of all WES variants in the 5q12-q14 region led to identification of two novel variants co-segregating with JME. These were c.476G>C in TMEM171 and c.1873-22A>G in COL4A3BP. These variants were found absent in databases and our control cohort. Bioinformatics analysis suggested c.476G>C (p.Gly159Ala) in TMEM171 to be a damaging variant and c.1873-22A>G in COL4A3BP capable of forming an additional splice-site. The c.476G>C variant in TMEM171 converts a highly conserved residue, glycine to alanine (p.Gly159Ala) and is predicted to have a potentially pathogenic effect. A minigene-based splicing assay carried out for the c.1873-22A>G in COL4A3BP in HEK293T and COS-7 cells, showed absence of any alternate spliced product, indicating that this intronic variant is likely to be an uncommon, benign polymorphism. These results suggest that mutation in TMEM171 is the likely cause of JME in GLH5. Further, to identify additional novel/rare epilepsy-associated variants in TMEM171, I examined the gene sequence in a cohort of 480 JME patients. Besides c.476G>C (p.Gly159Ala), two new variations: c.269A>G (p.Gln90Arg) and c.466C>T (p.Arg156Trp) were found. TMEM171 encodes a proline-rich transmembrane protein with an unknown biological function and localizes to plasma membrane. RT-PCR analysis showed expression of TMEM171 in different human brain regions. Though, the epilepsy-associated mutations in TMEM171 did not seem to affect its membrane localization or expression levels, we propose that they may have a damaging effect on its cellular function/s in the brain.

3.1 Introduction

Genetic linkage studies on families with several of their members affected with juvenile myoclonic epilepsy (JME) have helped identify a number of genes for the disorder. An earlier report from this laboratory identified EJM4 (MIM: 611364), a locus at chromosome 5q12-q14 linked to JME in a three-generation south Indian family, Family GLH5 (Kapoor et al 2007) (Figure 3.1).

In the whole-genome based linkage mapping study involving GLH5, the highest two-point LOD (logarithm of odds) score of 3.33 at recombination fraction (θ) = 0.0, was obtained for D5S641 and D5S459. The proximal limit of the disease-linked haplotype was defined by a recombination event between D5S624 and D5S427 in an affected individual (II:7; Figure 3.1), whereas, a recombination event between D5S459 and D5S428 in another affected individual, III:7, defined the distal boundary of the linked region. On the basis of these two recombination events, the centromeric and telomeric boundaries of the critical EJM4 interval were defined by D5S624 and D5S428, respectively. EJM4 encompasses 25 Mb of the genome and harbors 118 well annotated protein coding genes (GRCh37/hg19).

In this chapter, I present the results of a whole-exome sequencing experiment performed on two affected individuals of GLH5, wherein, a novel heterozygous variation, c.476G>C (p.Gly159Ala) in *TMEM171* was identified as the most probable cause of the disease. *TMEM171* is a relatively poorly characterized gene encoding a proline-rich transmembrane protein with an unknown biological function. To gather additional evidence for the role of *TMEM171* in epilepsy, we examined a group of apparently unrelated JME patients for mutations in this gene. Identification of additional novel and rare epilepsy-associated mutations in *TMEM171* is presented here.

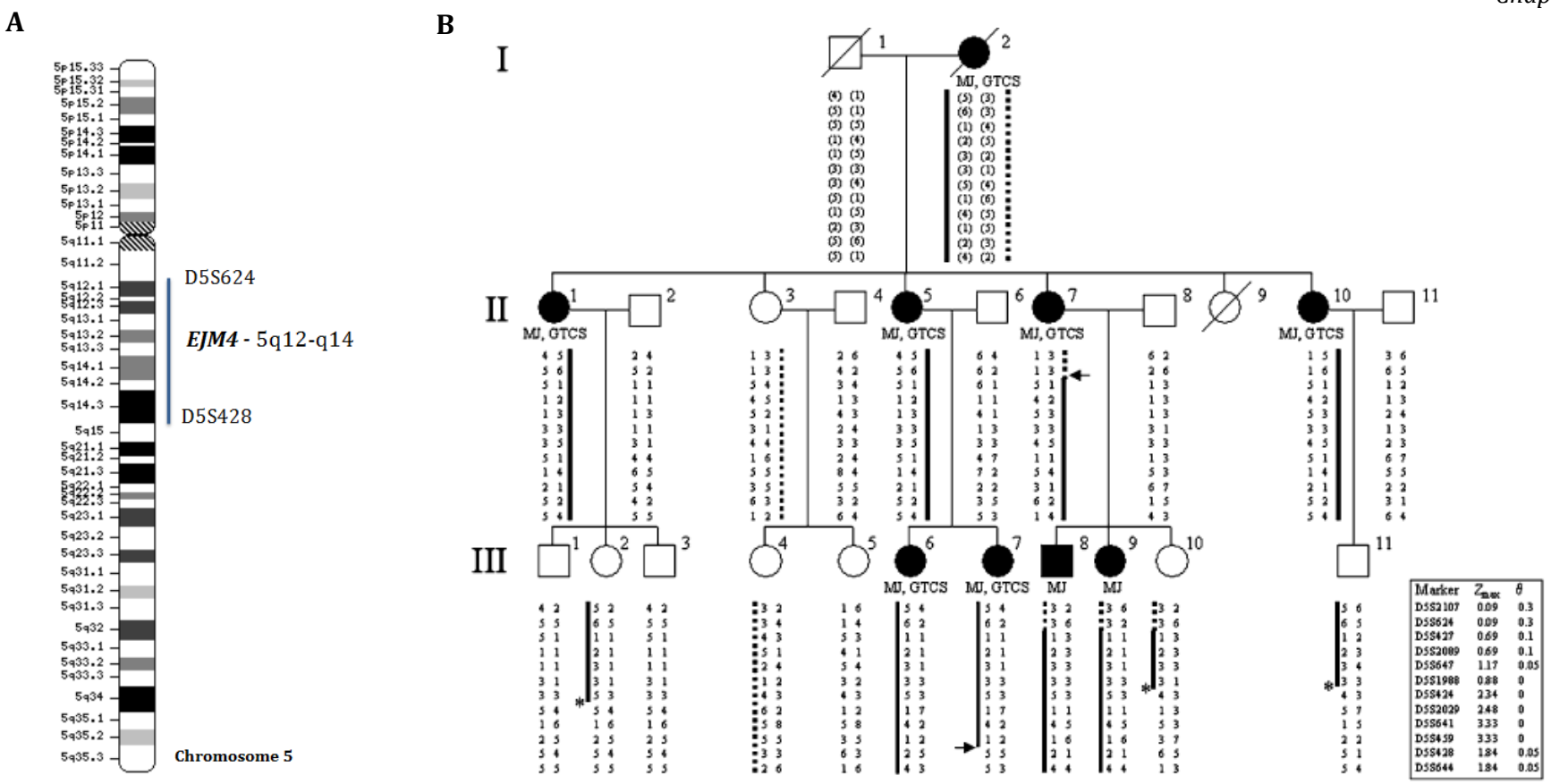


Figure 3.1: The EJM4 locus in Family GLH5: A. Ideogram of chromosome 5 representing the location of the JME locus, EJM4 (5q12-q14). **B.** Pedigree of GLH5 depicting the disease-linked haplotype (EJM4) shared among its affected members and three unaffected members (III:2, III:10 and III:11). Males are denoted by squares and females are denoted by circles. Filled symbols represent affected individuals and empty, unaffected ones. Haplotypes are shown below the symbols. Upper and lower recombination boundaries denoting the critical region are indicated by arrows in individuals II:7 and III:7. Clinical features of affected subjects are indicated along with the symbols (MJ: Myoclonic jerks, GTCS: Generalized tonic-clonic seizures). Adapted from Kapoor et al 2007.

3.2 Materials and methods

3.2.1 Subjects ascertainment and clinical characterization

The three-generation family, GLH5 discussed in this chapter was first reported by Kapoor and colleagues (2007) (Figure 3.1). Of the 24 members in the family, 21 members had consented to participate. They were clinically evaluated by a neurologist according to the guidelines of International League against Epilepsy (ILAE; Commission on Classification and Terminology of the International League Against Epilepsy 1989).

Family GLH5 was ascertained through proband (II:10), who presented distinct characteristics of JME. She had a history of myoclonic jerks (MJ) and generalized tonic-clonic seizures (GTCS) beginning at the age of 14 and 20, respectively. Seizures occurred primarily in the morning after awakening. Her EEG recordings showed generalized spikes, polyspikes and wave discharges (Figure 3.2).

Additional affected members of the family had a history of myoclonic jerks, may be accompanied with GTCS with an age of onset ranging from 12-18 years (detailed description in Table 3.1). At the time of participation in this study, the unaffected subjects reported no history of seizures. For the three deceased members, diagnosis was inferred based on the information provided by other family members. Family GLH5 presents largely homogenous manifestation of JME. For genetic analysis, subjects, I:2, II:1, II:5, II:7, II:10, III:6, III:7, III:8 and III:9 were considered affected and remaining members, unaffected.

A cohort of about 500 apparently unrelated JME patients were ascertained from NIMHANS, SCTIMST and a few epilepsy specialty clinics in south India and were diagnosed according to the guidelines of ILAE. The control cohort comprised of 250 healthy individuals from south India, above 20 years of age and with no apparent family history of neurological disorders. The study was approved by the institutional ethics committees and participating families or individuals provided written informed consent. Ten milliliters of peripheral venous blood was

Table 3.1: Clinical characteristics of the affected individuals of GLH5

Patient ID	Age of onset (years)	Gender	Type of seizures manifested	EEG	Photoparoxysmal response (15Hz)
I:2	_	Female	Myoclonic, GTCS	NA	No
II:1	13	Female	Myoclonic, GTCS	Normal	No
II:5	15	Female	Myoclonic, GTCS	GSW	No
II:7	16	Female	Myoclonic, GTCS	GSW	No
II:10	14	Female	Myoclonic, GTCS	GSW	No
III:6	12	Female	Myoclonic, GTCS	GSW	No
III:7	9	Female	Myoclonic, GTCS	GSW	No
III:8	14	Male	Myoclonic	GSW	Yes
III:9	12	Female	Myoclonic	Normal	No

The affected members of GLH5 exhibited homogenous clinical characteristics of juvenile myoclonic epilepsy. The photoparoxysmal response was observed only in individual III:8. GTCS: generalized tonic-clonic seizures; GSW: generalized spike-wave or polyspike-wave discharges (4-5 Hz); EEG: electroencephalography; NA: not available.

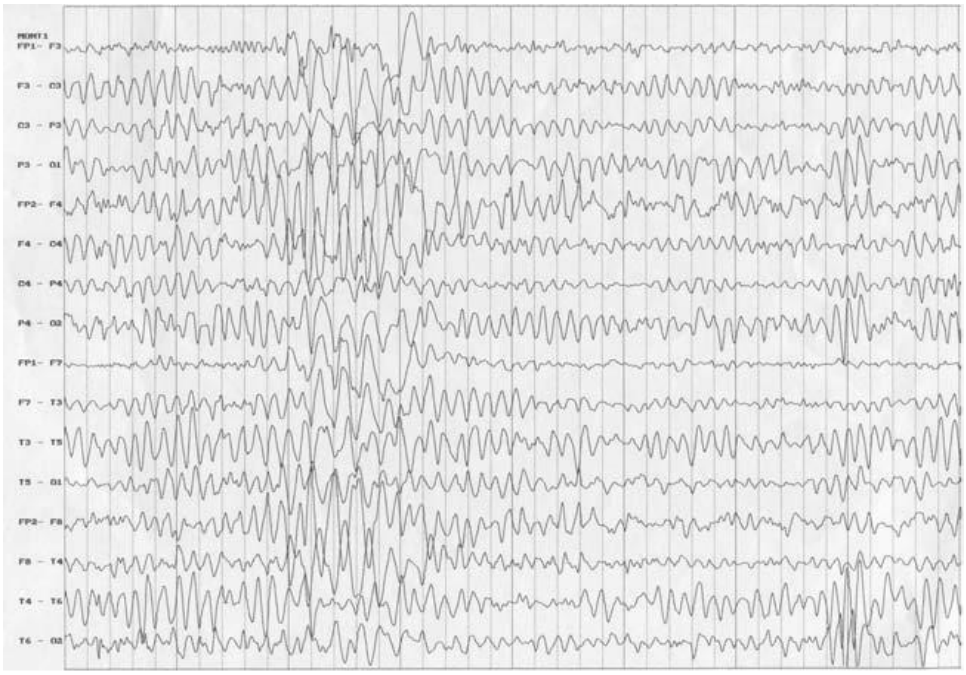


Figure 3.2: Interictal scalp electroencephalogram of proband, II:10 showing generalized polyspike and wave discharges. (Adapted from Kapoor et al 2007)

collected from the participants and genomic DNA was isolated using the phenol-chloroform method (Sambrook and Russell 2001).

3.2.2 Exome sequencing and variant analysis

Genomic DNA from the parent-offspring pair, II:5 and III:6 was used for the next-generation sequencing experiments. Whole-exome sequencing was performed for II:5 and III:6 individuals, using the Agilent SureSelect All Exon 50 Mb Kit. Genomic DNA libraries were constructed from sheared DNA fragments using Agilent's SureSelect XT Target Enrichment System for Illumina Paired-End Sequencing Library. The prepared library was captured using SureSelect™ biotinylated RNA baits and subjected to PCR based amplification (detailed description in chapter 2). The sequencing was then carried out using Genome Analyzer GAIIx (Illumina) obtaining 72 bp paired-end sequence reads.

The raw sequence data (FASTQ) was processed through an in-house analysis pipeline. The quality check was done using SeqQC v2.1 and the low quality bases and 3' adapter sequences were removed. The sequence reads with 70% of its bases having Phred-like quality score ≥ 20 and having a minimum of 50 bp length were considered. The high-quality processed reads were aligned to human genome reference assembly hg19 (GRCh37) using Burrows–Wheeler Aligner (v0.6.0) and PCR duplicates were removed. Coverage statistics for aligned data were calculated using custom scripts (written in Perl) and hg19 reference gtf file format (whole-exome and the 5q12-q14 region interval). Variations and indels were called using Samtools (v0.1.7a) and annotated into functional categories of nonsynonymous variants, synonymous variants, and intronic variants with snpEff (v2.0.5). These changes were further segregated as the known and novel ones, based on their presence/absence in the Database of Single Nucleotide Polymorphisms (dbSNP) 131. The shortlisted variations were then checked in the updated release of dbSNP and 1000 Genomes.

The gene variants located in the epilepsy locus (5q12-q14) were considered for further analysis and the novel/rare heterozygous variations and indels till 3x read

Table 3.2: Summary of the next-generation sequencing data

Statistics	II:5	III:6
Data generated	20.24 Gb	19.48 Gb
Read length in bases (paired end)	72	72
Total number of reads [% of high quality reads[a]]	95194784 [94.12]	91572152 [91.7]
Total number of bases [% of high quality bases[b]]	6.85 Gb [95.01]	6.59 Gb [93.0]
Percentage of non-ATGC characters	0.51	0.52
Percentage of reads with non-ATGC characters	0.75	0.77
Total SureSelect probe[#] region length (whole-exome)	51.5 Mb	51.5 Mb
SureSelect probe region covered (whole-exome)	49.6 Mb	49.4 Mb
Total SureSelect probe region length (5q12-q14)	370 kb	370 kb
SureSelect probe region covered (5q12-q14)	362 kb	362 kb
Average read depth (whole-exome)	70.18	62.95
Average read depth (5q12-q14)	91.53	81.77

II:5 and III:6 are the next-generation sequenced individuals of GLH5. [a]Reads with ≥70% bases with Phred score ≥20. [b]Nucleotide bases with Phred-like quality score ≥20. High quality reads are the quality filtered reads from the raw sequence data. [#]Probes used in the Agilent SureSelect Human All Exon 50 Mb kit to capture 51 Mb of exome sequence.

Table 3.3: Sequence coverage summary for the SureSelect probe region in the whole-exome sequencing experiment

Percentage of probe region covered*	Whole-exome		Chr 5 region (5q12-14)	
	II:5	III:6	II:5	III:6
Total probe region	96.29	96.01	98.07	98.07
Read depth ≥ 5x	90.64	89.93	96.45	96.39
Read depth ≥ 10x	85.89	84.78	95.15	94.94
Read depth ≥ 15x	81.63	80.11	93.49	93.45
Read depth ≥20x	77.53	75.52	91.61	91.18

*Percentage of SureSelect probe region coverage calculated after sequences were aligned to Human genome reference sequence (hg19/GRCh37). Chr, chromosome.

depth were examined and verified. Careful examination of all annotated protein coding genes at 5q12-q14 helped identify a few coding exons with no or poor sequence read coverage in the exome data set. These low coverage exonic regions were PCR amplified, sequenced and examined.

3.2.3 Validation of novel/rare variants (NGS-chr 5 region)

Sanger sequencing was carried out to validate the novel and rare variants identified in the exome data set of individuals II:5 and III:6. Primers were designed spanning the variant-harboring amplicons and low coverage exonic regions, using online tools, Primer3 and Oligocalc (Appendix II A3.2). Amplification by polymerase chain reaction (PCR) was performed using 100 ng of genomic DNA in a reaction volume of 20 µl containing 0.25 µM of each forward and reverse primer, 800 µM of dNTPs (200 µM of each dNTP), 1x reaction buffer (50 mM KCl, 20 mM Tris-HCl pH 8.4), 1.5 mM $MgCl_2$ and 1 U of *Taq* polymerase (Invitrogen). The amplifications were carried using a GeneAmp® 9700 at their respective standardized annealing temperatures. The sequencing was performed using the BigDye Terminator Cycle Sequencing reagents (Applied Biosystems) on an ABI3730 Genetic Analyzer (detailed method in chapter 2). The sequences thus obtained were aligned to the respective reference gene sequences obtained from the RefSeq database (NCBI) using SeqMan II v5.01 (DNASTAR Inc.). A family-based segregation analysis was carried out for the rare and novel heterozygous variations that were common to the next-generation sequenced samples (II:5 and III:6). The gene variants which co-segregated with epilepsy in the Family GLH5 were further examined in the control cohort of 250 individuals.

3.2.4 Bioinformatics analysis of novel/rare disease-segregating variants (NGS-chr 5 region)

Various *in silico* tools were employed to predict the potential pathogenic impact of the novel/rare segregating variants and to observe the evolutionary conservation of the amino acid residue altered due to the nonsynonymous variation in the protein. The effect of nonsynonymous change/s was predicted using SIFT, Polyphen-2, Align-GVGD, SNAP (screening for non-acceptable polymorphisms) and PMut. Whereas, to assess the effect of intronic variant/s, tools such as NetGene2

and NNSPLICE 0.9 (BDGP-Berkeley Drosophila Genome Project) were used. Multiple protein sequence alignment of TMEM171 from different organisms was carried out by Clustal Omega. The respective reference TMEM171 protein sequence of each organism was taken from NCBI (Appendix II A3.3).

3.2.5 Minigene construction

The predicted effect of an intronic variant c.1873-22A>G in *COL4A3BP* on splicing of exons 15 and 16 of the gene, was studied by constructing a minigene from this gene region. The variant in question is positioned 22 bases upstream of exon 16 (NM_001130105.1, ENST00000380494). The minigene comprised exons 15, 16 and the intervening intron (Figure 3.5). The ~2.7 kb (2764 bp) insert of minigene was amplified from the genomic DNA of an affected individual of GLH5 family, carrying c.1873-22A>G variation in the heterozygous state. The primers used for amplification were, forward- 5'-tcttcaaagcttcaactatagaaaactttcatgtggtgg-3' and reverse- 5'-acatgtctcgagaggagcactgtcatgatccacag-3'. PCR was done in a 50 µl volume containing 200µM of each dNTP, 2 mM $MgSO_4$, 0.4 µM of each primer, 100 ng of genomic DNA and 1U Platinum® Taq High Fidelity (Invitrogen). Amplification was performed using a GeneAmp® 9700 at following conditions: initial denaturation at 94°C (1 minute), followed by 18-20 cycles of denaturation at 94°C (30 seconds), annealing at 55°C (30 seconds) and extension at 68°C (3 minutes) followed by final extension at 72°C (7 minutes). The amplified products were electrophoresed and visualized on a 0.8% agarose/TAE/EtBr gel (Figure 3.6a). The amplified minigene-insert was purified using QIAquick® PCR purification reagents (Qiagen) and its identity was confirmed by sequencing using insert-specific primers (Appendix II A3.2). The 2764 bp minigene-insert was cloned into pcDNA3.1 (+) (Invitrogen), mammalian expression vector, using HindIII and XhoI restriction enzyme sites. As the genomic DNA template carried the heterozygous variation, screening of the 'positive' colonies helped identify both wild-type (G) and variation (A) harboring vectors (Figure 3.6b). The wild-type and variant carrying minigene pcDNA3.1 (+) vectors were prepared using QIAprep Spin Miniprep Kit (Qiagen) and used to study the splicing of exons 15 and 16 of *COL4A3BP* in the cultured cells.

3.2.6 Cell culture and transient transfections

HEK293T and COS-7 cells were grown in T-25 flasks (BD Biosciences) and were sub-cultured when at 70-80% confluence. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% heat-inactivated FBS (Sigma), 2 mM L-glutamine (Sigma) and antibiotics (100 U/ml penicillin and 10 mg/ml streptomycin, Sigma) in a humidified atmosphere of 5% CO_2 at 37°C. For transient transfections, culture media of the cells was replaced to antibiotic- and serum- free DMEM. The transfections were performed using Lipofectamine™ 2000 (Invitrogen) following the manufacturer's instructions at a ratio of 1:1 for DNA to transfection reagent (for a 35 mm dish, 2 µg of plasmid DNA: 2 µl of Lipofectamine™ 2000). Six hours post-transfection, the media in culture dishes was changed to serum containing DMEM.

For minigene splicing analysis, HEK293T and COS-7 cells were sub-cultured in 35 mm dishes and used for total RNA isolation after 36 hours of transfection. For cellular localization experiment, HEK293T cells were sub-cultured in 35 mm dishes containing poly-L-lysine (0.1 mg/ml, Sigma) coated coverslips and in 6-well plates for western blot analysis. Immunocytochemistry experiments and whole cell protein extraction for western blot analysis were performed 36-48 hours after transfection.

3.2.7 RT-PCR and splicing analysis of minigene transfected cells

Thirty six hours post-transfection, total RNA was isolated from the minigene-transfected HEK293T cells, using the trizol method. One ml of Trizol reagent (Gibco BRL, Life Technologies) was used per 35 mm culture dish. Precipitated RNA was treated with DNAse I (New England Biolabs) for removal of contaminating genomic DNA if any. The cDNA was synthesized using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) from 2 µg of isolated RNA using random hexamers following the manufacturer's instructions. The control reaction (RT-) for cDNA synthesis from wild-type (WT) and mutant/variant-harboring (MT) minigene transfected cells was done in absence of SuperScript™ III reverse transcriptase. The spliced product from two exons (exon 15 and 16 of *COL4A3BP*) of wild-type and mutant/variant-harboring

minigene was amplified by PCR using T7F and BGHR primers from the synthesized cDNA (Appendix A3.2). Human β-actin gene primers provided along with the cDNA synthesis system (sense primer: 5'-GCTCGTCGTCGACAACGGCTC-3', antisense primer: 5'-CAAACATGATCTGGGTCATCTTCTC-3') were used as control. The amplified products of minigene constructs, control reactions (RT-) and human β-actin control reactions were visualized by 2% agarose/TAE/EtBr gel electrophoresis.

3.2.8 Mutational analysis of TMEM171

The coding exons and intron-exon boundaries of *TMEM171* (NM_173490.7, ENST00000454765) were Sanger sequenced in 480 JME patients and 240 control individuals. Primer pairs for PCR amplification of the exons were designed using online tools, Primer3 and OligoCalc (Appendix II A3.2). PCR amplification of individual fragments was performed at their respective standardized conditions on a GeneAmp® 9700 as described earlier. The amplified products were purified and cycle sequencing was performed. DNA sequencing was carried out using BigDye Terminator Cycle Sequencing kit v3.0 and ABI 3730 Genetic analyzer (detailed protocol in chapter 2). The sequences were compared to *TMEM171* reference gene sequence (NM_173490.7) (RefSeq, NCBI) using SeqMan II v5.01 and the sequence variants were identified.

3.2.9 Bioinformatics analysis

To predict the possible effect of novel/rare nonsynonymous mutations in *TMEM171* identified in JME patients, *in silico* tools, SIFT, Polyphen-2, and Align-GVGD was used. The TMEM171 protein sequence (NP_775761.4) from NCBI was used as the input file for these tools. The evolutionary conservation status of the amino acid residues altered due to the nonsynonymous mutations was evaluated by performing multiple protein sequence alignment of TMEM171 from various organisms using Clustal Omega. The TMEM171 protein sequence of each organism was obtained from NCBI (Appendix II A3.3).

As *TMEM171* encodes for an uncharacterized protein, we used online databases such as NCBI and GeneCards to acquire any available information. The TMEM171

protein was studied using bioinformatics tools, TMHMM and PRED-TMR to evaluate its predictive transmembrane regions. To predict the sub-cellular localization of the TMEM171 protein, LOCTREE 3 tool was employed.

3.2.10 Expression analysis of TMEM171 in the human brain

The presence of the *TMEM171* transcript in different regions of human brain was evaluated by RT-PCR (Reverse transcriptase-PCR) using Marathon-Ready™ cDNAs from hippocampus, cerebral cortex, hypothalamus and cerebellum (Clontech). The source of poly A^+ RNA for these cDNAs is pooled from brains tissues of Caucasian male/female aborted fetuses or adults that died of sudden death. A set of intron-spanning primers 5'-GCCCAGTGCCTTATCTTTGG-3' and 5'TAGTTCCAGGACTCTCAGCG-3' located within exon 1 and exon 2 of *TMEM171*, respectively were used for the amplifications. PCR was performed in a 20 µl reaction volume containing 0.5 ng of Marathon-Ready™ cDNA, 0.25 µM of each primer, 800 µM of dNTPs (200 µM of each dNTP), 1.5 mM $MgCl_2$, 1X reaction buffer (50 mM KCl, 20 mM Tris-HCl pH 8.4) and 1 U of *Taq* DNA polymerase (Invitrogen). PCR amplification was performed on GeneAmp® 9700 at the following conditions: initial denaturation (94°C, 5 minutes), followed by 35 cycles of denaturation (94°C, 30 seconds), annealing (58°C, 30 seconds) and extension (72°C, 30 seconds), and followed by final extension at 72°C for 10 minutes. The amplified products were verified by 1.5% agarose/TAE/EtBr gel electrophoresis. The products were then purified and subjected to bi-directional DNA sequencing on an ABI3730 Genetic analyzer. The amplification of cDNA fragment using the same primer pair from a plasmid containing the *TMEM171* cDNA cloned in pcDNA3.1(+) vector was regarded as a positive control, and no DNA template reaction was considered, negative control.

3.2.11 Generation of TMEM171 cDNA wild-type and mutant constructs

The wild-type cDNA of *TMEM171* transcript (NM_173490.7, CCDS4017.1) cloned in pCMV6-AC-GFP expression vector was obtained from OriGene (Cat. No. RG205538). The identity of the clone was verified by sequencing using overlapping insert-specific primers (Appendix II A3.2). For sub-cloning into the

mammalian expression vector pcDNA3.1 (+), *TMEM171* cDNA of 975 base pairs (bp) encoding a protein of 324 amino acids was amplified from pCMV6-AC-GFP-TMEM171 vector. PCR amplification was performed using a primer pair 5'-GCTGGCTAGCGCCACCATGTCTCCTGCAGCTGCTGCT-3' (forward) and 5'-TAGACTCGAGTTACGGTGGGGAAGGCTCAGA-3' (reverse), harboring sites for restriction enzymes NheI and XhoI, respectively. Amplification was done in a reaction volume of 50 µl using 100 ng of pCMV6-AC-GFP-TMEM171 plasmid, 0.25 µM of each primer, 800 µM of dNTPs (200 µM of each dNTP), 1 mM $MgSO_4$, 1x Pfx amplification reaction buffer (Invitrogen) and 1 U of Platinum Pfx *Taq* DNA polymerase (Invitrogen). The conditions followed for amplification were: initial denaturation (94°C, 5 minutes), followed by 25 cycles of denaturation (94°C, 30 seconds), annealing (55°C, 30 seconds) and extension (72°C, 60 seconds), and followed by final extension at 72°C for 10 minutes. The amplified cDNA was visualized by 0.8 agarose/TAE/EtBr gel electrophoresis and purified using QIAquick® PCR purification kit (Qiagen) (Figure 3.10). The cDNA insert was cloned using Quick T4 DNA ligase (New England Biolabs) into the pcDNA3.1 (+) mammalian expression vector (Invitrogen) as a NheI-XhoI (New England Biolabs) fragment. Plasmid DNA was isolated from positive bacterial clones using QIAprep Spin Miniprep Kit (Qiagen). The identity of the cloned insert was verified by its sequencing using vector-specific primers (T7F and BGHR) and overlapping insert-specific primers (Appendix II A3.2) using BigDye Terminator v3.0 Cycle sequencing Ready Mix on an ABI3730 Genetic Analyzer. The *TMEM171* cDNA insert sequence was compared to its reference sequence (NM_173490.7, CCDS4017.1) (RefSeq, NCBI) using SeqMan II v5.01.

The *TMEM171* cDNA constructs harboring mutations were generated by site-directed mutagenesis using QuikChange® II XL mutagenesis reagents (Stratagene). The mutagenic oligonucleotide primer pairs (Appendix II A3.2) were designed to incorporate point mutations in the cDNA and subsequently used for amplification of wild-type *TMEM171* pcDNA3.1 vector by Pfu Turbo DNA polymerase. After digestion of the parental DNA construct with DpnI for 1 hour at 37°C, E.coli XL-10 cells were transformed with 2-5 µl of reaction containing amplified DNA with incorporated nucleotide substitution. The transformants were screened by

sequencing of the mutated cDNA plasmid using the overlapping insert-specific primers as described earlier.

3.2.12 Cellular localization by fluorescence immunocytochemistry

HEK293T cells grown on poly-L-lysine coated coverslips were transiently transfected with wild-type or mutant *TMEM171* pcDNA3.1 (+) constructs. After 36 hours of transfection, cells were rinsed three times with 1X phosphate buffered saline (PBS) and fixed using 2% paraformaldehyde (Sigma) in PBS for 15 minutes at room temperature. PBS-washed cells were treated with blocking solution (3% BSA with or without 0.1% Triton X-100) for one hour at room temperature. The transfected cells were treated at two conditions separately: non-permeabilized (absence of Triton X-100) and permeabilized (in presence of Triton X-100). Cells were then incubated with polyclonal anti-TMEM171 mouse raised antibody (Sigma) (2 µg/µl) in 1X PBS with 1% BSA for one hour at room temperature. After washing the cells three times with 1X PBS-1% BSA solution, they were incubated for an hour with Alexa Fluor® 568 goat anti-mouse IgG (Molecular Probes) at a dilution of 1:500 in 1X PBS-1% BSA at room temperature. The washed cells were stained with DAPI (0.2 µg/ml) for 10 minutes and then given three washes with PBS to remove excess stain. The stained coverslips were mounted on glass slides using mounting medium (Sigma) and dried overnight. Confocal images of labeled cells were acquired using a LSM 510 Meta (Carl Zeiss) and photomicrographs were taken with a 63x/63x zoom oil immersion objective.

3.2.13 Protein expression analysis by immunoblot analysis

Forty-eight hours post-transfection, cell lysates were prepared from HEK293T cells transiently transfected with wild-type and mutant *TMEM171* pcDNA3.1 (+) constructs. The cells were washed thrice with 1X PBS. The cells were then dislodged in PBS and resuspended in RIPA buffer (150 mM NaCl, 10 mM Tris pH 7.5, 0.1% SDS, 1% Triton X-100, 1% Sodium deoxycholate, 5 mM EDTA) supplemented with protease inhibitor cocktail (1:1000, Sigma), on ice for 30 minutes. The cell lysate was intermittently passed through 26 gauge needle attached to a 1 ml syringe for complete lysis. The lysate was centrifuged at 14000 rpm for 20 minutes at 4°C to remove the cell debris and the cell lysate in the

supernatant was used for western analysis. The total protein content in the whole cell lysates was determined using the bicinchoninic acid assay kit (Sigma). About 20 µg of total protein was subjected to SDS-containing polyacrylamide gel electrophoresis (PAGE) (12%). The proteins on the gel were subsequently electrotransferred (Bio-Rad) to nitrocellulose membrane (GE Healthcare) at 20V for one hour. The membrane was blocked [2% BSA (bovine serum albumin, Sigma) in PBS containing 0.05% Tween-20 (Sigma)] for four hours at 4°C. It was then incubated with polyclonal anti-TMEM171 mouse raised antibody (1 µg/µl, Sigma) in PBS containing 1% BSA and 0.05% Tween-20 for 10-12 hours at 4°C. The membrane was then washed four times with wash buffer (0.05% Tween-20 in PBS), each for 15 minutes at room temperature. It was further incubated with horseradish peroxidase (HRP)-conjugated, rabbit anti-mouse IgG (Bangalore Genei) in PBS containing 1% BSA and 0.05% Tween-20 (1:10000 for 3 hours at 4°C). After washing the membrane with wash buffer (4 X 15 minutes), it was treated with enhanced chemiluminescent (ECL) substrate for HRP (Pierce) for 5 minutes and exposed to film to visualize the ECL signal. The α-Tubulin was used as control, wherein after transfer of cell lysate protein onto the nitrocellulose membrane blot, it was treated separately. The control protein membrane was blocked for 4 hours at 4 °C, incubated with monoclonal anti-α-Tubulin mouse raised antibody (Sigma) (1:10000 dilution for 10-12 hrs, 4°C), followed by treatment with 1:10000 dilution of HRP-conjugated rabbit anti-mouse IgG (Bangalore Genei) for 3 hours at 4°C. The steps of washing and detection remained same as above.

3.3 Results

3.3.1 Analysis of the variants from whole-exome sequencing (5q12-q14)

To identify the genetic cause of JME in Family GLH5, genomic DNA of an affected parent-offspring pair (II:5 and III:6) was subjected to whole-exome sequencing using Agilent SureSelect 50 MB Kit. An average of 19 Gb per sample of raw sequence data was generated (Table 3.2). The SeqQC v2.1 reported an average of 93% reads with high quality (≥ 70% bases in a sequence read with Phred-like

Table 3.4: Novel/rare variants identified at EJM4 (5q12-q14) in II:5 and III:6 by whole-exome sequencing experiment

Position on chr 5[a]	Gene	Sequence variant[b]	Location/ Type	Position of amino acid change	Disease co-segregation[c]	Status in databases (MAF)[d]	Status in control cohort (MAF)
61662229	*KIF2A*	ENST00000381103.2:c.1519-12T>C	Intron	_	No	_	_
61778876	*IPO11*	ENST00000325324.6:c.829-52T>A	Intron	_	No	_	_
63513212	*RNF180*	ENST00000389100.4:c.1216C>T	Synonymous	Leu406=	No	_	_
64850663	*CENPK*	ENST00000396679.1:c.72T>C	Synonymous	Leu24=	No	rs201745679, G = 0.001	_
69338446	*SERF1B*	ENST00000380750.3:c.*879C>T	3'UTR	_	No	_	_
72419676	***TMEM171***	**ENST00000454765.2:c.476G>C**	**Nonsynonymous**	**Gly159Ala**	**Yes**	_	**0**
72980754	*ARHGEF28*	ENST00000545377.1:c.33+45T>A	Intron	_	No	rs370524120, A = 0.001	_
73090249	*ARHGEF28*	ENST00000545377.1:c.933C>T	Synonymous	Ser311=	No	_	_
74677924	***COL4A3BP***	**ENST00000380494.5:c.1873-22A>G**	**Intron**	_	**Yes**	_	**0**
79281499	*MTX3*	ENST00000512560.1:c.557-17C>T	Intron	_	No	_	_

79744232	*ZFYVE16*	ENST00000338008.5:c.3103+9G>T	Intron	_	No	_	_
79815630	*FAM151B*	ENST00000282226.4:c.436G>C	Nonsynonymous	Asp146His	No	_	_
82868208	*VCAN*	ENST00000265077.3:c.9736-27A>G	Intron	_	No	_	_

[a]Genomic position of the nucleotide base on chromosome 5 (chr 5) (GRCh37, NCBI). [b]Nomenclature of variants in introns or UTR is with respect to first base of the corresponding cDNA. The position of the variation is given according to cDNA of the longest protein-coding transcript of the respective gene. [c]Variants present in all affected members of GLH5 were considered positive for disease co-segregation. [d]The novel/rare variations were checked in updated release of dbSNP and 1000 Genomes; and the variants present at minor allele frequency (MAF) ≤ 0.005 in the databases were included. The two new variants represented in bold segregated with JME in GLH5 and were found to be absent in 250 individuals of our control cohort.

quality score ≥20). The high quality processed sequencing reads were mapped to the human genome reference sequence (hg19/GRCh37) using BWA (v0.6.0). The sequence reads covered about 96% of the SureSelect probe region in the whole-exome (51.5 Mb) with mean coverage read depth of 66 (Table 3.3). On average, 90% and 76% of the whole-exome probe region was covered by at least 5 and 20 reads, respectively. As the disease-linked haplotype (EJM4) on chromosome 5 maps to 5q12-q14, we focused our analysis exclusively on this 25 Mb critical region. The SureSelect probes cover about 370 kb of exome region in this 25 Mb interval, of which 98% was covered with sequenced reads (91% coverage at 20x read depth) (Table 3.3).

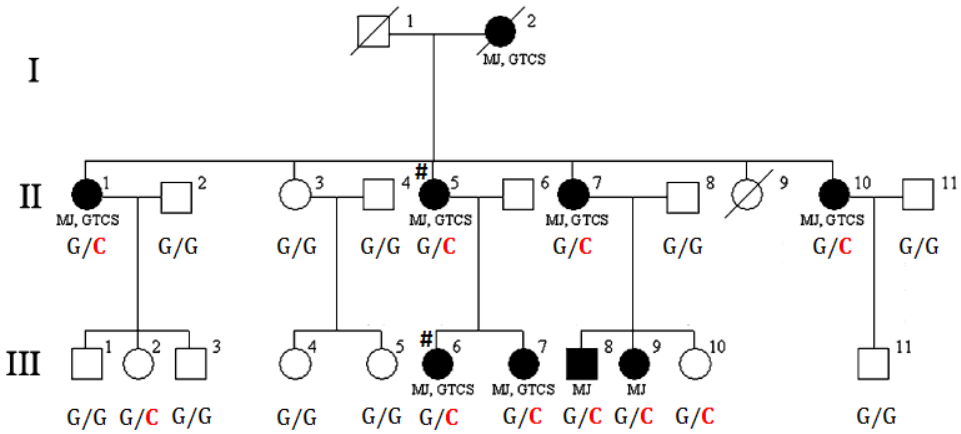
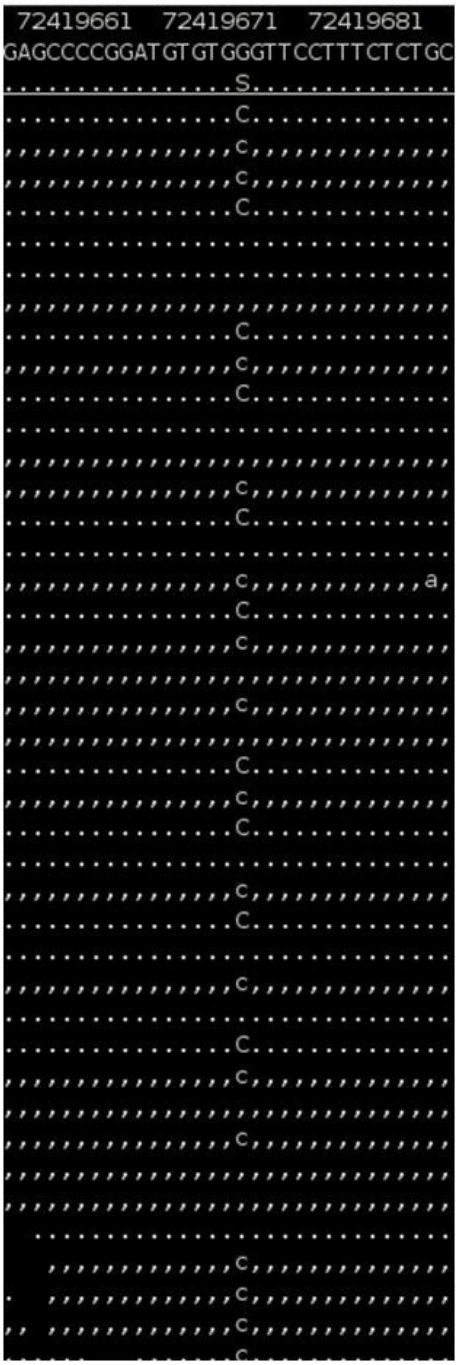


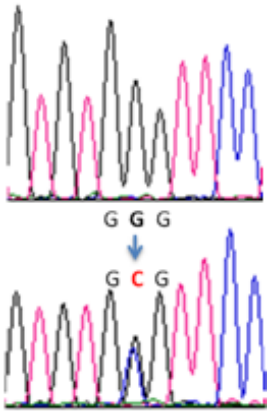
Figure 3.3: Segregation analysis of *TMEM171*, c.476G>C in GLH5: Pedigree of the three-generation GLH5 showing the segregation of c.476G>C mutation in *TMEM171* in all the affected members of the family. The mutation was found to be present in two unaffected individuals (III:2 and III:10) of the family. #Next-generation sequenced individuals (II:5 and III:6).

Small nucleotide variations (SNV) and indels (small insertions and deletions) were called from the aligned reads using Pileup utility of SAMTOOLS. Variants in the coding exons ± 100 bp of intronic sequence of all protein-coding genes in the 5q12-q14 region were only considered for analysis. Assuming autosomal mode of inheritance of JME in GLH5, 13 novel/rare heterozygous variations were identified (Table 3.4). These novel/rare variants were either absent or present at minor

A



B



C

Lizard	ELQDC**G**FHSVQ
Parakeet	DLQGC**G**FLSLQ
Chicken	DLQGC**G**FLSFQ
X.laevis	DFKTC**G**FLSLQ
X.tropicalis	DYKTC**G**FLSLQ
Opossum	EPQAC**G**FLSLQ
Platypus	ESRGC**G**FLSLQ
Guinea Pig	EPQIC**G**LLSMQ
Mouse	EPQIC**G**FLSLQ
Rat	EPQICSFLSLQ
Rabbit	GPPIC**G**FLSLQ
Common Shrew	EPQIC**G**LLSLQ
Horse	EPHIC**G**FLPLQ
Rhinoceros	EPPMC**G**FLSLQ
Dog	EPQMC**G**FLSLQ
Bat	EPQFC**G**FLSLQ
Rhesus macaque	KPRMC**G**FLSLQ
Gibbon	EPRMC**G**FLSLQ
Chimpanzee	EPRMC**G**FLSLQ
Human	EPRMC**G**FLSLQ
Gorilla	EPRMC**G**FLSLQ
Cat	EPQIC**G**FLSLQ
Cow	EPQIC**G**FLSLQ
Wild Boar	ESQIC**G**FLSLQ
Dolphin	EPQIC**G**FLSLQ

Figure 3.4: The c.476G>C (p.Gly159Ala) variation in *TMEM171*: **A.** Alignment of the exome sequence reads against human genome reference sequence as viewed in tview of samtools v0.1.7a. The snapshot here shows the reads in forward (caps) and reverse (no caps) strands indicating the heterozygous G>C variation at g.72419676 in *TMEM171*. The dot and comma represent the matched reference allele and alphabet shows the variant allele. **B.** Electropherograms of sequence with wild-type (G/G) and variant allele (G/C) for the c.476G>C variation. The arrow indicates the nucleotide showing heterozygous variation shown by presence of two peaks. **C.** Multi-species protein sequence alignment of TMEM171 by Clustal Omega. The glycine (G) 159 is highlighted in blue.

allele frequency (MAF) ≤ 0.005 in the public databases. Out of 13 novel/rare variants, only two were found to co-segregate with epilepsy in the family. These are: an intronic variant c.1873-22A>G in *COL4A3BP* (MIM: 604677) and a nonsynonymous variant c.476G>C (p.Gly159Ala) in *TMEM171* (Figure 3.3, Figure 3.4). Both variations were found absent in 250 individuals of control cohort. The common variants or polymorphisms identified in the region are enlisted in Appendix II A3.1.

3.3.2 Bioinformatics and minigene splicing analysis of the COL4A3BP variant

The c.1873-22A>G variation in *COL4A3BP* is present 22 bases upstream of its exon 16, in the intronic region of exon 15 and 16. According to the predictive bioinformatics tools, the variation forms an additional splice acceptor site upstream of exon 16, but with a score lower than wild-type splice site [BDGP: 0.93 (WT) and 0.54 (MT); NetGene2: 0.48 (WT) and 0.14 (MT)]. This predictive effect may lead to incorporation of 21 bases between the exons 15 and 16 due to aberrant splicing. The effect of the c.1873-22A>G variant on the normal splicing process of exon 15 and 16 was examined by constructing a minigene (Figure 3.5). The minigene consisted of ~2.7 kb insert comprising of exons 15 and 16 and their intervening intron, cloned into a mammalian expression vector pcDNA3.1 (+). In wild-type minigene (WT), the c.1873-22 position had 'A', whereas, the variant harboring minigene (MT) had 'G' at c.1873-22 nucleotide position. This minigene utilized the promoter sequence, transcription start and end signals of the vector pcDNA3.1 (+) and the splicing of the two exons was carried out by cellular splicing machinery. Wild-type and variant carrying minigene were transfected into HEK293T and COS-7 cells and cDNA was synthesized from their isolated total RNA. RT-PCR analysis identified the 309 bp spliced product from the wild-type and mutant minigene (Figure 3.6c). Sequencing of both amplified spliced products (exon 15 + exon 16) revealed no additional 21 bases due to the predicted additional splice acceptor site in the mutant minigene. This suggests that the intronic variation c.1873-22A>G neither affects the normal splicing of its flanking exons (15 and 16) in *COL4A3BP* nor forms any alternate transcript with additional

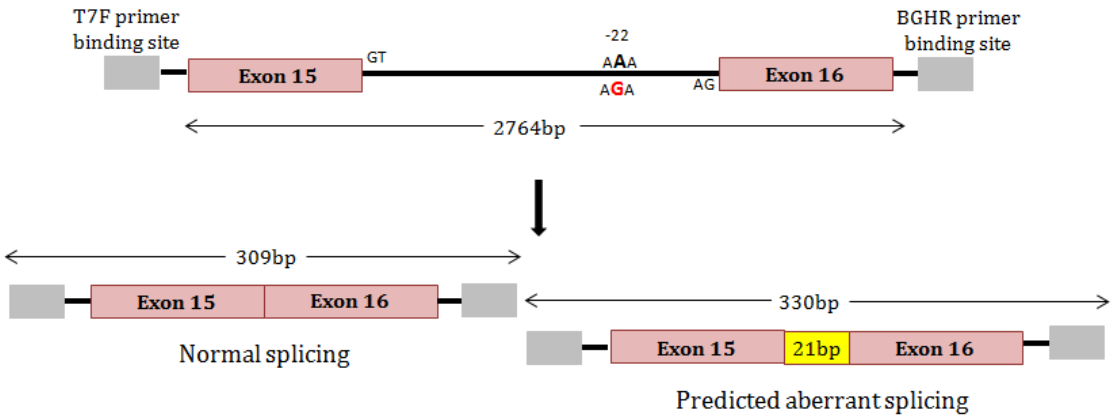


Figure 3.5: Schematic of *COL4A3BP* minigene construct: It represents normal splicing process of exons 15 and 16 of *COL4A3BP* and the predicted alternate splicing due to c.1873-22A>G variation upstream of exon 16. The normal spliced product of the minigene (from T7F to BGHR primer binding sites) is 309 bp, whereas if the variation leads to formation of additional splice acceptor site, the resulting product would have 21 bp inserted between exons 15 and 16 (330 bp).

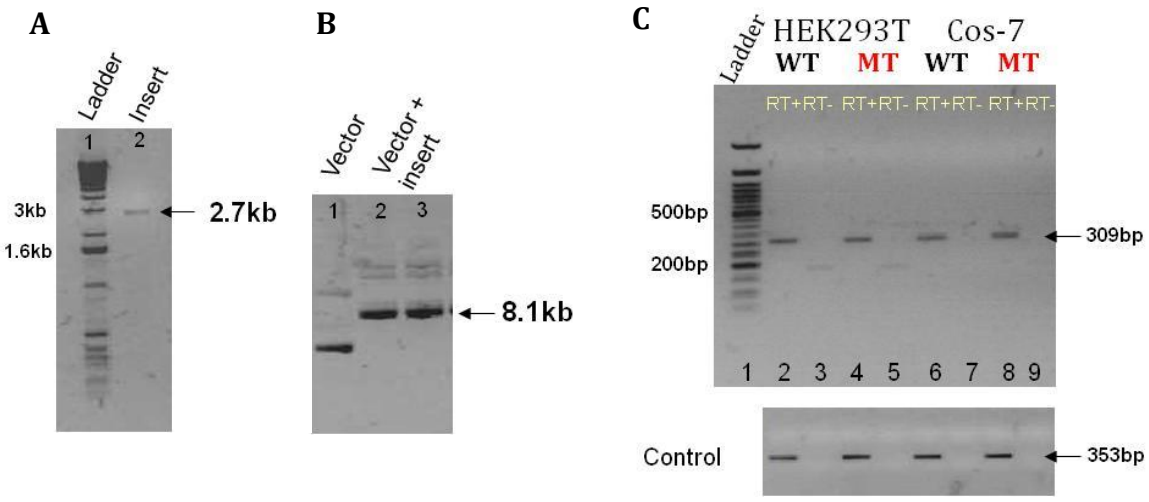


Figure 3.6: Minigene splicing analysis of *COL4A3BP* variant, c.1873-22A>G: **A**. The 2764 bp minigene consisting of exons 15 and 16 of *COL4A3BP* and their intervening intron was amplified from the genomic DNA of an affected individual of GLH5 family. The first lane is 1 kb ladder (Gibco) and in the second lane arrow indicates the ~2.7 kb amplified insert. **B.** Lane 1 shows the band for the pcDNA3.1(+) vector, and bands in lanes 2 and 3 represent the 8.1 kb constructs of minigene cloned in pcDNA3.1(+). **C.** The RT-PCR analysis of cDNA from wild-type (WT) and mutant/variant-harboring (MT) minigene transfected cells indicate presence of the wild-type spliced product of exons 15 and 16 (309 bp) in both. The arrows indicate the 309 bp spliced product of *COL4A3BP* minigene (upper panel) and the 353 bp product of human β-actin gene (control, lower panel). The lanes marked RT+ (2, 4, 6, 8) and RT- (control) (3, 5, 7, 9) indicate the reactions performed in presence and absence of SuperScript™ III reverse transcriptase. Lane 1 shows the 50 bp ladder (NEB).

21 base pairs. It indicates this allele to be apparently neutral and is not the disease-causing variant in GLH5.

3.3.3 Bioinformatics analysis of the TMEM171 variant

The heterozygous c.476G>C variant in *TMEM171* leads to alteration of glycine at 159 amino acid position to alanine (NP_775761.4). Multiple-species protein sequence alignment by Clustal Omega shows glycine at 159 amino acid position to be evolutionary highly conserved, indicating its functional importance (Figure 3.4c). The effect of p.Gly159Ala substitution in TMEM171 was predicted to be damaging by SIFT (score: 0.03) and probably damaging by Polyphen-2 (score: 0.988). SNAP and Pmut analysis predicted the effect of p.Gly159Ala to be non-neutral and pathological, respectively with a high reliability score. Align-GVGD classifies this missense mutation in Class C55, indicative of its high likelihood for interference in the protein function.

3.3.4 Mutational analysis of TMEM171

Sequence analysis of the coding exons and the flanking intronic region of *TMEM171* in JME patients revealed 12 novel/rare variants (Table 3.5). Of these, three variants c.269A>G (p.Gln90Arg), c.466C>T (p.Arg156Trp) and c.709G>A (p.Ala237Thr) result in uncommon missense mutations. The heterozygous variant alleles c.269A>G and c.466C>T were found to be either absent or present at minor allele frequency (MAF) less than 0.005 in the control cohort of 240 individuals and in dbSNP and 1000 Genomes databases. Due to the presence of c.709G>A as a homozygous variation in a control individual, it was not considered for further analysis. Apart from the p.Gly159Ala mutation in TMEM171 identified in GLH5 (Figure 3.4), two additional JME-associated mutations are: p.Gln90Arg and p.Arg156Trp (Figure 3.7). The p.Gln90Arg mutation was found in two unrelated JME patients (Table 3.6). Besides these, a few common variations were also detected in this screen (Appendix II A3.4).

Table 3.5: Novel/rare *TMEM171* variations observed in 480 JME patients

Position on chr 5[a]	Position of nucleotide change[b]	Position of amino acid change[b]	Location/Type	Patient counts (n=480)	Status in control cohort (MAF)[c]	Status in databases (MAF)[d]
72419386	c.186G>A	Ala62=	Synonymous	1	A = 0.004	rs146544114, A = 0.0004
72419469	c.269A>G	Gln90Arg	Nonsynonymous	2	_	rs543295611, G = 0.0004
72419494	c.294C>T	Asp98=	Synonymous	2	_	_
72419666	c.466C>T	Arg156Trp	Nonsynonymous	1	T = 0.004	rs374701617, T = 0.0002
72419853	c.640+13T>C	_	Intron	2	_	rs368542341, C = 0.0002
72424207	c.641-10T>C	_	Intron	1	_	_
72424242	c.666C>T	Pro222=	Synonymous	1	_	_
72424281	c.705G>A	Ala235=	Synonymous	1	_	rs142658267, A = 0.0002
72424285	c.709G>A	Ala237Thr	Nonsynonymous	1	A = 0.006 (1 hom)	rs186159238, A = 0.002
72424317	c.741G>A	Pro247=	Synonymous	1	_	rs150115532, A = 0.0001
72424317	c.741G>A	Pro247=	Synonymous	1	_	rs150115532, A = 0.0001
72427633	c.*76A>T	_	3'UTR	1	_	_

[a]Genomic position of the nucleotide base on chromosome 5 (chr5) (GRCh37, NCBI). [b]The nucleotide and amino acid residue position of the variations in *TMEM171* is according its longer transcript encoding for protein of 324 amino acids (NM_173490.7, NP_775761.4). [c]Minor allele frequencies (MAF) are calculated in 240 control individuals. [d]Variations were checked for their presence in updated release of dbSNP and 1000 Genomes; and variants present at minor allele frequency (MAF) ≤ 0.005 in the databases were included. Hom, homozygous.

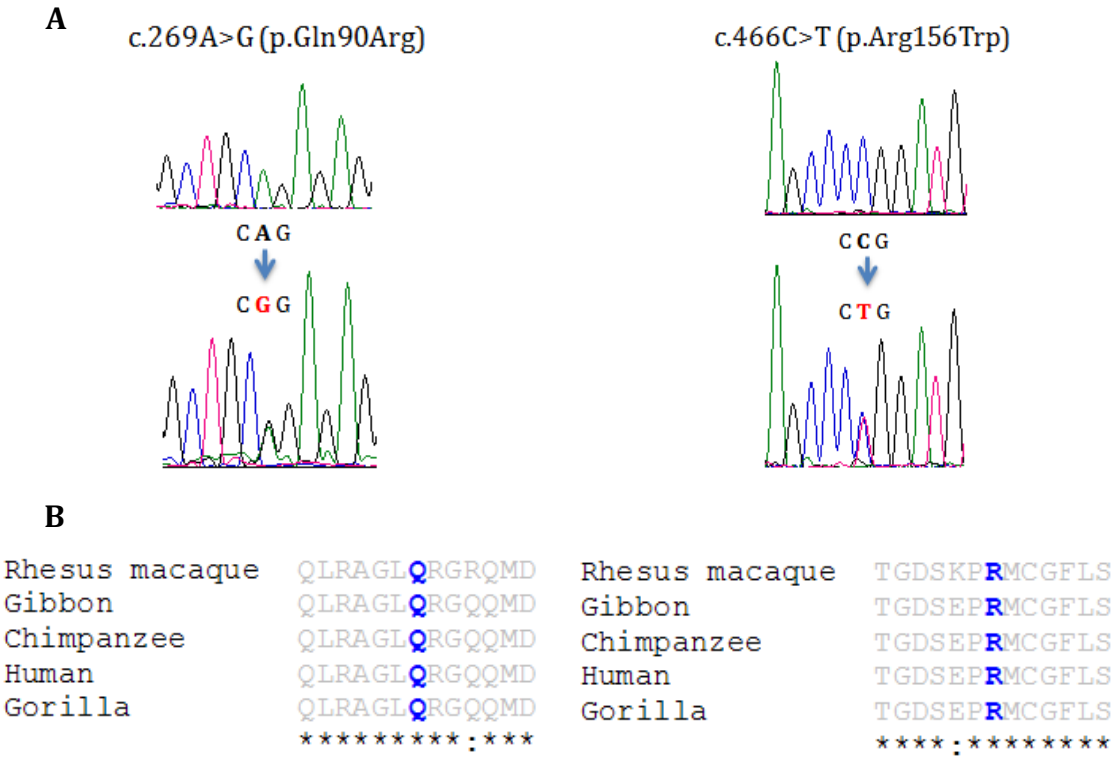


Figure: 3.7: *TMEM171* mutations associated with epilepsy: A. Electropherograms showing DNA sequence of the respective wild-type (upper panel) and the mutation (lower panel) from the genomic DNA. The presence of two peaks at one nucleotide position indicates the heterozygous variation for each mutation (c.269A>G and c.466C>T). The substitution of the reference (black) to variant allele (red) is marked by an arrow. **B.** Alignment of TMEM171 protein sequence from few primates by Clustal Omega, showing only the region near the location of mutated amino acid residue. The wild-type amino acid residue for each mutation is highlighted in blue (Q90 and R156).

Table 3.6: Clinical characteristics of JME patients harboring *TMEM171* mutation

***TMEM171* mutation**	**Gender**	**Age of onset (years)**	**Seizure types**	**EEG**	**Medication**
p.Gln90Arg	Female	15	MJ, ABS, GTCS	Normal	VPA
p.Gln90Arg	Female	11	MJ, GTCS	Gen SW	VPA
p.Arg156Trp	Female	NA	MJ	NA	VPA

MJ: myoclonic jerks, GTCS: generalized tonic-clonic seizures, ABS: absence seizures, Gen SW: generalized spike wave, VPA: Valporic acid, NA: Not available.

3.3.5 Bioinformatics analysis

According to information given in databases, human *TMEM171* gene is located at 5q13.2 and comprises three coding and one non-coding exon. It produces two alternate transcripts (NM_173490.7 and NM_001161342.2) encoding proteins of 324 (NP_775761.4) and 323 (NP_001154814.1) amino acids, respectively. The bioinformatics tools, TMHMM and PRED-TMR suggest that TMEM171 protein has four transmembrane domains (Figure 3.8). Information from LOCTREE3 and GeneCards (COMPARTMENTS) reveals that TMEM171 is likely to be a multi-pass membrane protein localizing to plasma membrane of the cell. The blastp algorithm of NCBI shows TMEM171 to be highly conserved protein across various organisms. Human TMEM171 shares more than 90% similarity with its orthologs in primates and ~60-80% in other mammals, with its proline-rich regions being the most conserved.

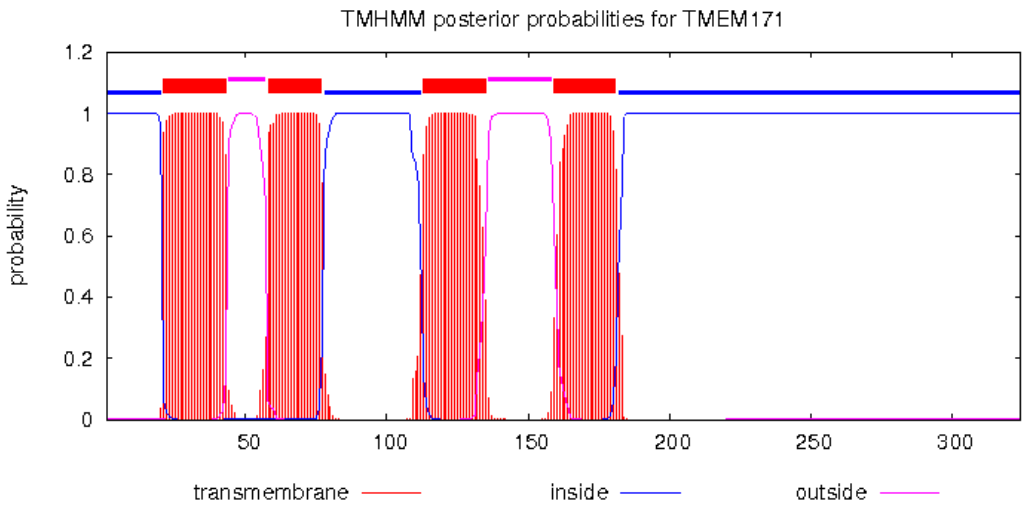


Figure 3.8: TMHMM2.0 prediction of TMEM171 transmembrane domains: The four predicted transmembrane domains of TMEM171 by TMHMM2.0. The probable amino acid positions of four transmembrane helices are 21-43, 58-77, 113-135, and 159-181.

The bioinformatics tools, SIFT, Polyphen-2 and Align-GVGD predict the functional effect of amino acid substitutions in a protein based on the protein sequence homology and biophysical properties of the amino acids. The mutation, p.Gly159Ala identified in GLH5 was predicted to be of damaging nature by these *in silico* tools. SIFT and Polyphen-2 predicts p.Gln90Arg as a benign mutation and p.Arg156Trp to be of damaging nature. Align-GVGD classifies p.Gln90Arg in class C35 indicating that it may affect the protein function and p.Arg156Trp, in class C65 suggesting that it is most likely to interfere with the protein function. The multi-species protein sequence alignment of TMEM171 by Clustal Omega showed glycine at 159 amino acid position to be evolutionary conserved (Figure 3.4c); and p.Gln90 and p.Arg156 to be moderately conserved across various organisms (Figure 3.7b).

3.3.6 Expression of TMEM171 in human brain regions

RT-PCR analysis was performed to detect the expression of *TMEM171* in human brain sub-regions, using Marathon-Ready™ cDNAs (Clontech) from hippocampus, cerebral cortex, hypothalamus and cerebellum. The intron-spanning primer pair specific to *TMEM171* yielded a 400 bp PCR product from the brain sub-regions analyzed and in the positive control [*TMEM171* pcDNA3.1 (+)] (Figure 3.9a). The forward primer was present in first coding exon and reverse primer was in the second coding exon of the gene. Sequencing of the amplified product revealed complete match with the nucleotide sequence of the *TMEM171* transcript (NM_173490.7, CCDS4017.1) in the region spanned by the primers. The amplified product from cDNA of hippocampus, cerebral cortex, hypothalamus and cerebellum indicates the expression of *TMEM171* at the transcript level in these human brain regions.

The expression profile of TMEM171 in various tissues was observed in the online database of GeneCards. According to the RNA expression studies, moderate levels of *TMEM171* expression was observed in brain regions (Figure 3.9b).

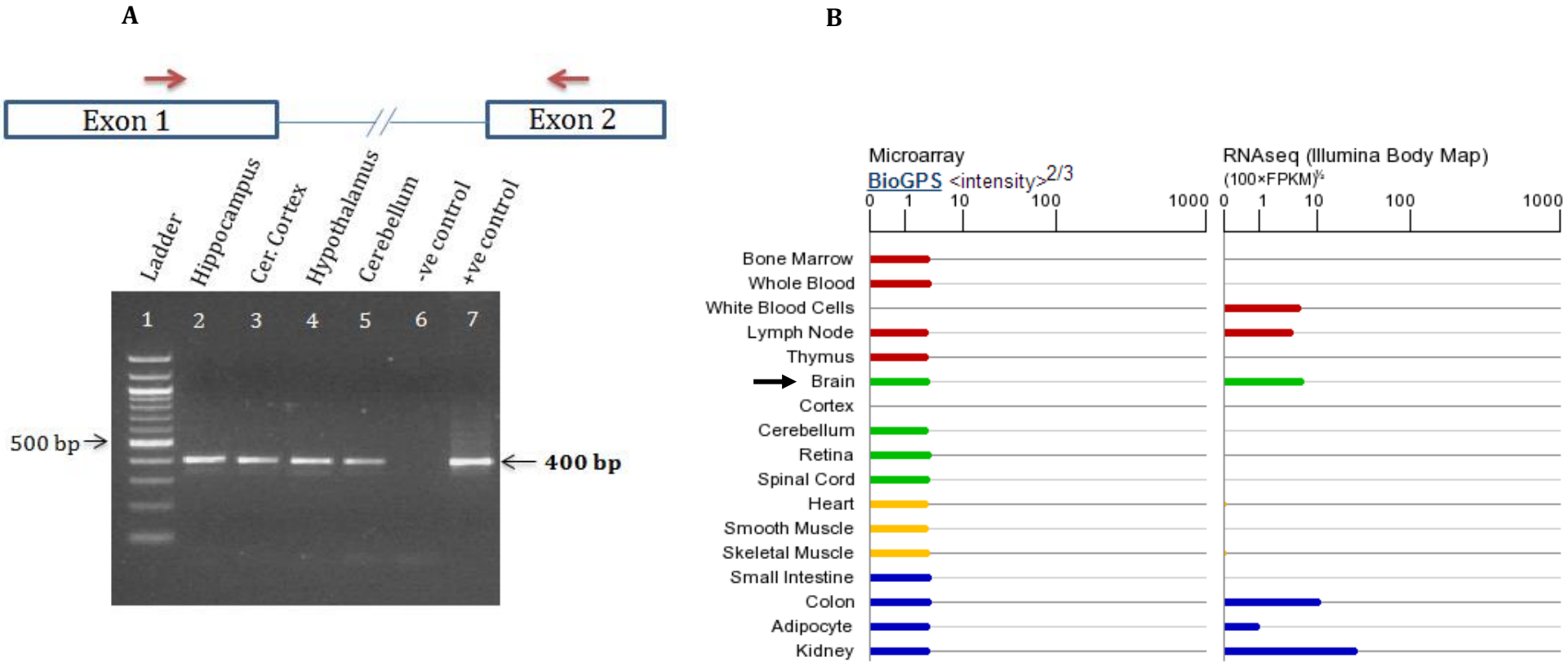


Figure 3.9: Expression of *TMEM171* in the human brain regions: A. RT-PCR analysis of cDNA from different brain regions (hippocampus, cerebral cortex, hypothalamus, and cerebellum). The arrow (lower panel) indicates the 400 bp product amplified by cDNA specific intron-spanning primers located in exon 1 and exon 2 (marked by arrows in upper panel) of *TMEM171*. Lane 6 shows the negative control (no DNA template) and lane 7 indicates the 400 bp product from the positive control [wild-type TMEM171 pcDNA3.1 (+) construct]. Lane 1 is the 100 bp ladder. **B.** Expression of *TMEM171* in various human tissues shown by the RNA expression data summarized in GeneCards. The arrow points to the normalized expression of *TMEM171* in human brain.

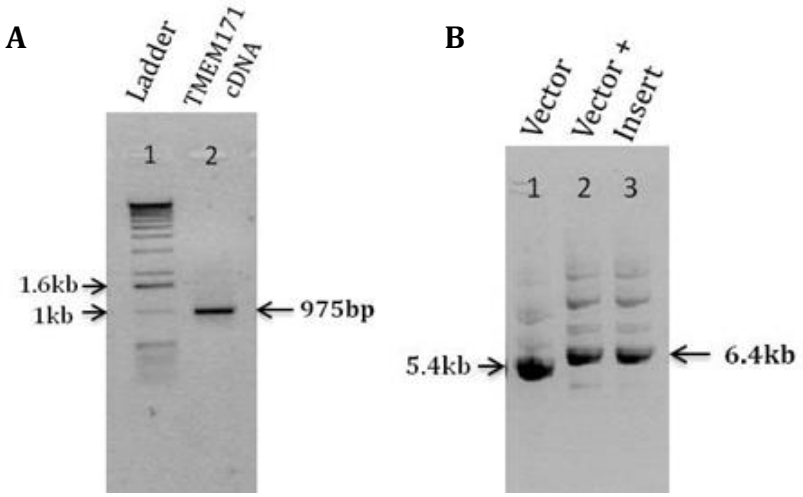


Figure 3.10: *TMEM171* cDNA cloning: A. The coding region of *TMEM171* transcript (NM_173490.7) amplified as a 975 bp product (indicated by arrow) from the TMEM171-AC-GFP (OriGene) construct. Lane 1 is the 1 kb ladder. **B.** The amplified *TMEM171* cDNA insert cloned in mammalian expression vector, pcDNA3.1 (+). Lane 1 indicate the 5.4 kb band of pcDNA3.1 (+), and lane 2 and 3 show the 6.4 kb constructs of wild-type TMEM171-pcDNA3.1 (+).

3.3.7 Cellular localization and expression of wild-type and mutant TMEM171 proteins

To examine the cellular localization of TMEM171 and to test whether the identified mutations (p.Gln90Arg, p.Arg156Trp, p.Gly159Ala) affect its localization, fluorescence immunocytochemistry was performed on HEK293T cells transiently transfected with the wild-type and mutant *TMEM171* pcDNA3.1 (+) constructs. Immunoreactivity was observed using mouse polyclonal anti-TMEM171 antibody (Sigma). TMEM171 protein expression was observed on the plasma membrane of the cell at non-permeabilized conditions and in the cytoplasm when cells were permeabilized (Figure 3.11). Though, the staining in permeabilized cells show TMEM171 expression in cytoplasmic region, its association to a particular sub-cellular organelle remains to be analyzed.

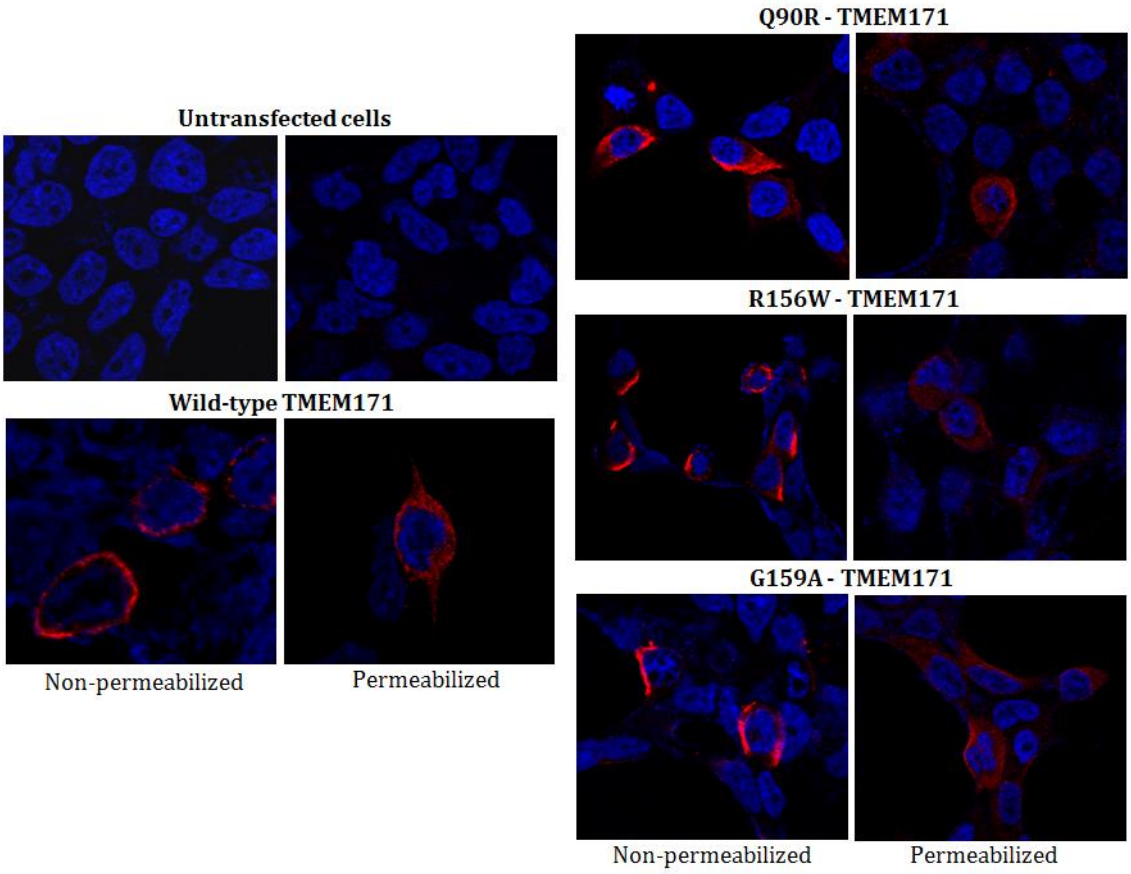


Figure 3.11: Cellular localization of WT and mutant (Q90R, R156W and G159A) TMEM171 proteins in transfected HEK293T cells: Fluorescence immunocytochemistry performed on HEK293T cells transiently transfected with wild-type and mutant (Gln90Arg, Arg156Trp, Gly159Ala) *TMEM171* cDNAs and the untransfected cells. Immunostaining was carried out at non-permeabilized and permeabilized conditions to observe cell surface and intracellular staining of TMEM171, respectively. Localization of TMEM171 was observed with mouse polyclonal anti-TMEM171 antibody (1 µg/µl, Sigma) and detected using Alexa Fluor® 568 goat anti-mouse IgG. The wild-type TMEM171 image shown here is obtained at 63x zoom. The untransfected HEK293T cells do not show any staining with the anti-TMEM171 antibody used.

The HEK293T cells transfected with mutant *TMEM171* constructs harboring mutations for p.Gln90Arg, p.Arg156Trp, p.Gly159Ala exhibited similar expression profile as seen for the wild-type protein at both non-permeabilized and permeabilized conditions (Figure 3.11). This suggests that these missense mutations do not seem to alter the plasma membrane localization of TMEM171. Untransfected HEK293T cells did not show any immunoreactivity with the anti-TMEM171 antibody used.

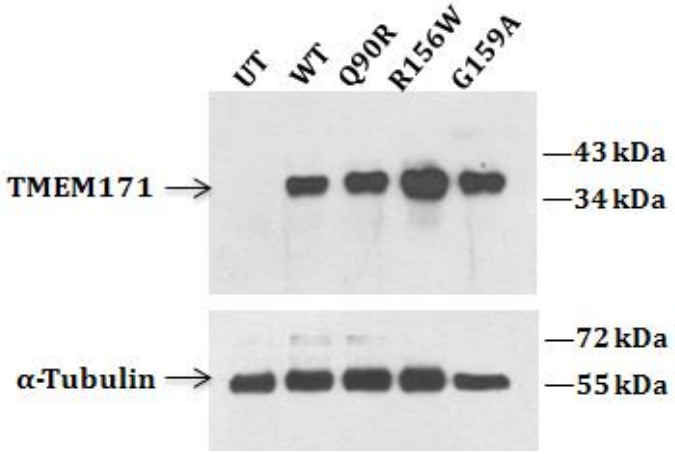


Figure 3.12: Expression of WT and mutant (Q90R, R156W and G159A) TMEM171 proteins in transfected HEK293T cells: Western blot analysis performed on lysates of HEK293T cells transiently transfected with wild-type (WT) and mutant (Gln90Arg, Arg156Trp, Gly159Ala) *TMEM171* pcDNA3.1 (+) and untransfected cells. Immunoreactivity for TMEM171 was observed with mouse polyclonal anti-TMEM171 antibody (1 µg/µl, Sigma). The α-Tubulin used here as a control was stained with the anti-α-Tubulin mouse monoclonal antibody. The HRP-conjugated rabbit anti-mouse IgG was used for detection for both, TMEM171 and α-Tubulin. The lysate from untransfected cells (UT) did not show any reactivity with the anti-TMEM171 antibody used.

Western blot analysis was conducted to examine the expression of TMEM171 in HEK293T cells transiently transfected with wild-type and mutant cDNAs, using a mouse polyclonal anti-TMEM171 antibody raised against the full-length human TMEM171 protein (Sigma, Cat. No. SAB1401999). The whole cell protein lysate from cells transfected with wild-type TMEM171 pcDNA3.1 (+) construct exhibits immunoreactivity with the antibody used, showing a specific band representing a protein of about 35 kDa (TMEM171 calculated mass: 34760 Da) (Figure 3.12). The protein from lysate of untransfected cells does not show any signal with this antibody. The mutant TMEM171 proteins with p.Gln90Arg, p.Arg156Trp or p.Gly159Ala, are observed to express at equivalent levels to that of the wild-type protein (Figure 3.12). Due to the over expression of the wild-type and mutant proteins in the transfected cells, quantifying small changes in the expression levels caused due to the mutations is not plausible.

The epilepsy-associated mutations in TMEM171, p.Gln90Arg, p.Arg156Trp and p.Gly159Ala, do not seem to alter the localization or expression levels of their respective mutant proteins in transiently transfected HEK293T cells.

3.4 Discussion

In this chapter, findings from the whole-exome sequencing experiment, to identify potentially causative genes at EJM4 are presented. The NGS analysis of exome dataset from two affected individuals of Family GLH5, led to identification of novel/rare variants in two genes, *COL4A3BP* and *TMEM171* at 5q12-q14 locus, which appeared suitable for further studies.

The intronic variant in *COL4A3BP* does not seem to affect the splicing and appears to be a rare benign change. But, the missense mutation in *TMEM171* substitutes an evolutionary conserved amino acid residue, glycine at position 159 to alanine; and is predicted to have deleterious effect on the protein structure and function. This heterozygous variant was found in all the affected individuals of GLH5 and was absent in our control cohort and public databases such as dbSNP and 1000 Genomes. However in 1000 Genomes database, a rare variation (MAF: 0.0008) leading to p.Gly159Glu in TMEM171 has been recorded. Due to rarity of this alteration and possibility of multiple mutations at same amino acid residue, the glycine to alanine change at 159 in TMEM171 was examined further. Thus, whole-exome sequencing analysis of the EJM4 locus (5q12-q14) facilitated the discovery of a novel mutation in a plausible epilepsy-causing gene, *TMEM171*.

TMEM171 encodes a 324 amino acid proline-rich transmembrane protein, also known as PRP2 (Proline rich protein 2). The biological structure and function of TMEM171 protein is not known so far. It is predicted to have four transmembrane domains (Figure 3.8) and localizes to the plasma membrane (Figure 3.11). According to available information in databases, *TMEM171* mRNA was detected in spleen, brain, spinal cord, fetal brain, kidney, liver etc (GeneCards) (Figure 3.9b). In nervous system, its mRNA expression is observed in prefrontal cortex, hypothalamus, amygdala, pons, medulla oblongata, dorsal root ganglion (RNA

expression data, GeneCards). As a primary step in examining the role of *TMEM171* in a brain disorder, we identified its expression at transcript level in cDNA library from different human brain sub-regions (Figure 3.9a). So far, no study has either characterized the biological function of TMEM171 or reported its role in any brain-related disorders. However, genome-wide association studies suggest that the genomic region nearby *TMEM171* may be associated with serum urate concentrations and kidney function-related traits (Köttgen et al 2013, Okada et al 2012).

Further, while exploring the role of *TMEM171* as an epilepsy-linked gene, we identified two additional JME-associated novel/rare mutations, p.Gln90Arg and p.Arg156Trp in our cohort of 480 JME patients. Taken together, the three mutations identified in this study alter conserved amino acid residues in TMEM171 and two of the three variations are predicted to have damaging effect on the protein function. Though these missense mutations do not seem to affect the membrane targeting or expression of the protein, it is suggested that they may have an effect on its biological function. We propose that these mutations may either perturb the interaction of TMEM171 with other proteins or may alter its structural-functional properties. We hypothesize based on few studies from the literature, such as mutations in *TBC1D24*, a protein with unknown function, impair its interaction with *ARF6* (regulates dendritic branching and neurite extension) and leads to neuronal hyperexcitability in familial infantile myoclonic epilepsy (Falace et al 2010). Interestingly, mutations in various proteins belonging to the group of uncharacterized transmembrane proteins have been implicated in neurological disorders, such as *PRRT2* gene which encodes for a proline-rich transmembrane domain protein 2 of an unknown function. Mutations in *PRRT2* have been identified for disease phenotypes such as paroxysmal kinesigenic dyskinesia (PKD), Paroxysmal kinesigenic dyskinesia with infantile convulsions (PKD/IC), Benign familial infantile epilepsy (BFIE) and infantile convulsions and choreoathetosis (ICCA) (Chen et al 2011, Lee et al 2012, Heron et al 2012). So far no homology has been found between TMEM171 and other reported TMEM molecules which we have analyzed. However, mutations in other TMEM proteins have also been identified such as in *TMEM216* and *TMEM237* for the Joubert

syndrome related disorders (Edvardson et al 2010, Valente et al 2010, Huang et al 2011); and in *TMEM240* for spinocerebellar ataxia with mental retardation and severe cognitive impairment (Delplanque et al 2014).

Therefore, based on the findings presented in this chapter, a suggestion is made for *TMEM171* as an underlying genetic cause for epilepsy in GLH5 at EJM4 (5q12-q14). We provide evidence at gene- and variant- level for its association to epilepsy (McArthur et al 2014). Gene-level evidence is based on the exclusion of all rare variants except p.Gly159Ala in *TMEM171* in the disease-linked region on chromosome 5, presence/absence in dbSNP and 1000 Genomes databases and control cohort from south India, conservation of the variant residue and predicted damaging functional impact. Two additional rare missense variants were found in three unrelated JME patients. Variant-level evidence is provided by their rare presence (M.A.F<0.005), evolutionary conservation of the altered residue and predictive functional pathogenicity. While the physiological function of *TMEM171* and its role in epilepsy mechanism needs further studies, identification of novel/rare epilepsy-linked mutations in *TMEM171* provides genetic evidence for its involvement in causation of GGE/JME.

Appendix I

A2.1: Known/common variations observed in the 3p14.2-q21 region in the exome sequencing data of individuals II:3 and III:2 of NIH2

Gene	Position on chr3[a]	Variant alleles	Het/ Hom	Location/ type	Position of amino acid change[b]	dbSNP ID
PTPRG	61734539	T/C	Hom	Intron	_	rs3821880
	62189189	G/A	Het	Missense	Gly574Ser	rs2292245
	62189437	G/C	Het	Synonymous	Thr656=	rs17634074
	62229553	C/T	Hom	Synonymous	Ile816=	rs1352882
	62267213	>3bp ins	Het	Intron	_	rs373128198
CADPS	62431402	C/A	Hom	Intron	_	rs73840192
	62431415	T/C	Hom	Intron	_	rs6791296
	62431478	G/A	Het	Intron	_	rs147390947
	62451935	T/C	Het	Intron	_	rs6445273
	62459828	T/-	Het	Intron	_	rs71752520
	62477233	_/AC	Het	Intron	_	rs35911391
	62498378	G/A	Hom	Intron	_	rs1376917
	62556622	A/G	Hom	Intron	_	rs833663
	62631553	G/T	Het	Intron	_	rs4588354
	62636826	C/T	Hom	Intron	_	rs4688302
	62647904	T/C	Het	Intron	_	rs11712073
	62647928	A/G	Hom/Het	Intron	_	rs6770658
	62647942	T/A	Het	Intron	_	rs13093807
	62739092	A/G	Hom/Het	Intron	_	rs478123
SNTN	63640535	A/-	Het	Intron	_	rs5849568
	63645575	T/C	Het	Intron	_	rs34568643
THOC7	63821914	C/G	Hom	Intron	_	rs2637982
ATXN7	63981635	C/T	Het	Synonymous	Leu713=	rs3733125
	63982082	G/A	Het	Missense	Val862Met	rs3774729
PSMD6	63996262	C/T	Het	3'UTR	_	rs1046025
	63999322	G/A	Het	Intron	_	rs3816157
	64008898	C/A	Het	Intron	_	rs139959634
PRICKLE2	64133350	A/G	Hom	Synonymous	Asp272=	rs27673
	64184397	G/T	Het	Intron	_	rs696017
	64184676	G/A	Het	Intron	_	rs695937
	64184685	_/T	Het	Intron	_	rs374502686
	64211102	G/A	Het	5'UTR		rs697287
ADAMTS9	64518994	T/C	Hom	Intron	_	rs1017537

	64526717	G/C	Hom	Intron	_	rs3796384
	64526942	A/C	Hom	Intron	_	rs3796383
	64527321	T/C	Hom	Intron	_	rs1373163
	64527336	C/T	Hom	Intron	_	rs1036919
	64527378	G/A	Hom	Intron	_	rs1036918
	64527465	C/T	Hom	Intron	_	rs1036917
	64554254	T/C	Hom	Intron	_	rs7635677
	64580169	C/T	Het	Intron	_	rs13320542
	64589851	A/G	Hom	Intron	_	rs9985304
	64601550	T/C	Het	Intron	_	rs4589926
	64617042	G/C	Het	Intron	_	rs6768487
	64617070	C/T	Het	Intron	_	rs6445420
	64617699	A/G	Het	Intron	_	rs7640062
	64619048	C/T	Het	Intron	_	rs11717158
	64627448	C/T	Het	Intron	_	rs9832057
	64627752	C/T	Het	Intron	_	rs7632802
	64640207	A/-	Het	Intron	_	rs11326156
	64667059	G/T	Het	Intron	_	rs73124286
MAGI1	65350694	A/G	Het	Intron	_	rs55638583
	65416648	C/A	Hom	Intron	_	rs4688566
	65425588	C/T	Het	Synonymous	Gln412=	rs139785185
	65425591	T/C	Het	Synonymous	Gln411=	rs79701778
	65456199	_/A	Het	Intron	_	rs57455654
	65464522	_/T	Het	Intron	_	rs11409972
	65607637	A/G	Het	Intron	_	rs3749455
	66023592	A/G	Het	Intron	_	rs28696109
SLC25A26	66286930	T/-	Het	Intron	_	TMP_ESP_3_66286930_66286930
	66396768	_/T/TT	Het	Intron	_	TMP_ESP_3_66396768_66396768
	66396845	G/A	Hom/Het	Intron	_	rs332354
	66396898	>6bp ins	Het	Intron	_	rs71616220
	66419883	T/C	Hom/Het	Intron	_	rs2293193
	66419956	C/T	Hom/Het	Missense	Thr120Met	rs13874
LRIG1	66432680	A/G	Hom/Het	Intron	_	rs332371
	66433312	A/G	Hom/Het	Intron	_	rs900172
	66433676	A/G	Hom/Het	Synonymous	Leu741=	rs900171
	66434372	>2bp ins	Het	Intron	_	rs4071605
	66434643	T/C	Hom/Het	Missense	Met615Val	rs2306272

	66444697	A/C	Hom/Het	Intron	_	rs17775391
	66463251	G/A	Hom	Intron	_	rs4856924
	66465259	T/C	Hom	Intron	_	rs4856928
KBTBD8	67048762	T/C	Hom	5'UTR	_	rs4856833
SUCLG2	67411103	G/A	Het	Missense	Val425Ile	rs902320
	67411166	T/C	Het	Missense	Tyr404His	rs902321
	67425983	C/T	Het	Intron	_	rs1065400
	67426125	C/T	Het	Intron	_	rs1065399
	67579412	T/A	Het	Intron	_	rs2634731
	67659817	G/A	Hom/Het	Intron	_	rs2290173
	67660040	G/A	Hom/Het	Intron	_	rs2290174
	67660049	A/G	Hom/Het	Intron	_	rs2290175
FAM19A1	68593852	A/G	Het	3'UTR	_	rs11717918
FAM19A4	68782213	A/G	Hom/Het	3'UTR	_	rs6549143
	68788363	G/T	Het	Intron	_	rs1504301
	68934434	T/G	Het	5'UTR	_	rs4855535
C3orf64	69025792	_/GT	Het	3'UTR	_	rs71618257
	69036707	G/A	Het	Intron	_	rs4855542
	69047141	G/A	Het	Intron	_	rs4855544
	69053444	T/C	Het	Intron	_	rs35171227
	69053447	A/T	Hom/Het	Intron	_	rs9860095
	69054213	A/-	Het	Intron	_	rs11320020
	69054266	_/T	Het	Intron	_	rs11417320
	69054341	T/C	Het	Synonymous	Arg155=	rs6781612
	69059084	C/A	Het	Intron	_	rs55952435
	69059101	C/A	Het	Intron	_	rs73835394
TMF1	69073420	T/-	Het	Intron	_	rs201235194
	69074930	G/A	Het	Intron	_	rs2279936
	69077486	A/G	Het	Intron	_	rs139736121
	69088918	C ins	Het	Intron	_	rs146136734
	69088944	T/C	Het	Intron	_	rs67036817
	69093598	T/C	Hom/Het	Intron	_	rs2271118
	69096614	G/A	Hom/Het	Synonymous	Ser414=	rs2292199
UBA3	69104695	T/A	Hom	Intron	_	rs6549179
	69104752	C/T	Hom	Intron	_	rs6549180
	69112049	C/T	Het	Intron	_	rs2289245
	69113153	T/C	Het	Intron	_	rs13090606
	69127133	T/C	Het	Intron	_	rs3772979
ARL6IP5	69153854	G/T	Hom/Het	3'UTR	_	rs7038

	69153935	A/T	Het	3'UTR	_	rs11128112
FRMD4B	69225588	T/G	Hom	Intron	_	rs11128118
	69225875	T/A	Hom	Intron	_	rs9872199
	69229976	G/C	Hom	Intron	_	rs12054011
	69230026	T/C	Hom	Intron	_	rs9818314
	69230061	G/A	Hom	Missense	Ser947Leu	rs9831516
	69230801	G/A	Hom	Synonymous	His700=	rs9836305
	69242771	A/-	Hom	Intron	_	rs11310698
	69244162	G/A	Hom	Intron	_	rs9853587
	69245454	T/C	Hom	Missense	Ala396Thr	rs9310141
	69245623	A/G	Het	Intron	_	rs73095903
	69246152	A/G	Hom	Synonymous	Leu331=	rs13059488
	69246274	A/G	Hom	Intron	_	rs13059687
	69265371	G/C	Hom	Intron	_	rs62254460
	69267578	A/T	Hom	Intron	_	rs4855301
	69271032	G/A	Het	Synonymous	Leu236=	rs62254461
	69299233	C/G	Het	Missense	Glu173Asp	rs4361282
	69351463	AG/-	Het	Intron	_	rs533192007
	69362706	C/T	Hom/Het	Intron	_	rs1483895
	69362722	C/A	Het	Intron	_	rs2314992
FOXP1	71008342	_/T	Het	5'UTR	_	rs112773801
	71015021	G/T	Hom	Intron	_	rs7638391
	71161817	C/T	Het	Intron	_	rs113050068
	71247257	A/G	Het	Intron	_	rs939845
	71247304	A/G	Hom/Het	Intron	_	rs2037474
	71247572	A/T	Het	Intron	_	rs56850311
	71247576	A/T	Het	Intron	_	rs9855825
EIF4E3	71739070	A/G	Hom/Het	Intron	_	rs949642
PROK2	71823582	C/T	Het	Intron	_	rs3796224
RYBP	72495593	_/A	Het	Intron	_	rs11460842
	72495777	C/G	Hom	Intron	_	rs13066407
GXYLT2	73004157	T/G	Het	Intron	_	rs1532190
	73016635	C/G	Het	Intron	_	rs3732442
	73024350	C/A	Het	3'UTR	_	rs1052278
PPP4R2	73096591	4bp ins	Het	Intron	_	rs71277513
	73110076	G/A	Hom	Intron	_	rs726029
EBLN2	73111368	T/C	Hom	Missense	Ser46Pro	rs2231924
	73111809	A/G	Hom	Missense	Ile193Val	rs2231926
	73111973	A/G	Hom	Synonymous	Pro247=	rs2231928

	73111998	C/T	Hom	Missense	Leu256Phe	rs1060584
PPP4R2	73112919	_/A	Hom	Intron	_	rs3841614
	73112974	A/T	Hom	Intron	_	rs11708712
PDZRN3	73433369	G/A	Het	Missense	A481V	rs3205537
	73433494	T/C	Het	Synonymous	S439S	rs13091636
	73434086	A/G	Hom/Het	Intron	_	rs6787588
	73437112	A/G	Hom	Intron	_	rs6549532
	73437225	_/A	Hom	Intron	_	rs3830251
	73440108	_/A	Hom/Het	Intron	_	rs35427155
	73450198	G/A	Hom/Het	Intron	_	rs3816831
	73453201	C/T	Hom	Intron	_	rs2291464
	73453325	C/T	Hom	Synonymous	Val380=	rs2291463
	73651469	C/T	Het	Intron	_	rs6797804
	73651622	A/-	Het	Intron	_	rs11343439
CNTN3	74315580	C/G	Hom/Het	Intron		rs615824
	74334560	C/T	Het	Missense	Arg867Gln	rs143021649
	74350997	T/C	Het	Intron	_	rs475988
	74413676	T/C	Hom/Het	Synonymous	Gln385=	rs6549590
	74474166	T/C	Hom	Intron	_	rs7619184
FRG2C	75713496	A/G	Het	5'UTR	_	rs73840316
	75713782	G/A	Het	Intron	_	rs62247154
	75714337	T/G	Het	Missense	Ile106Ser	rs73840323
	75714702	A/G	Het	Missense	Asn120Ser	rs62247157
	75714976	C/T	Het	Synonymous	Leu211=	rs62247960
	75715261	C/T	Het	3'UTR	_	rs3966935
	75715319	T/A	Het	3'UTR	_	rs75314439
	75715340	G/A	Het	3'UTR	_	rs75393029
	75715423	A/G	Het	3'UTR	_	rs78262248
	75715640	C/T	Het	3'UTR	_	rs62247967
ZNF717	75786230	C/T	Het	Synonymous	Arg848=	rs149559986
	75786833	G/A	Het	Synonymous	Tyr647=	rs139448820
	75787049	C/A	Hom	Missense	Lys575Asn	rs62250106
	75787057	G/A	Hom	Missense	His573Tyr	rs62250107
	75787540	T/C	Hom	Missense	Ile412Val	rs200986341
	75787794	A/G	Het	Missense	Ile327Thr	rs75467043
	75787813	_/T	Het	Frameshift	Ser321Lysfs	rs143242394
	75790370	A/C	Het	Intron	_	rs75296464
	75790376	T/C	Het	Intron	_	rs78006287
	75790394	C/G	Het	Intron	_	rs151121572

	75790409	T/C	Het	Intron	_	rs73843027
	75790427	C/T	Het	Missense	Ala93Thr	rs199946555
	75790448	T/C	Hom	Missense	Thr86Ala	rs141704469
	75790478	G/T	Hom	Missense	Gln76Lys	rs142265598
	75790536	A/T	Het	Intron	_	rs148282009
	75790554	C/T	Het	Intron	_	rs77885642
	75790583	G/A	Het	Intron	_	rs73843028
	75790668	G/T	Het	Intron	_	rs73843030
	75790797	C/T	Het	Missense	Val50Met	rs147946451
	75790811	G/T	Het	Missense	Thr45Asn	rs199577560
	75790822	A/G	Hom	Synonymous	Asp41=	rs147081315
	75790838	C/T	Het	Nonsense	Trp36Ter	rs113991634
	75790860	C/T	Het	Missense	Val29Met	rs73117241
	75832479	A/T	Het	Missense	Leu12Gln	rs201922232
	75832500	A/G	Het	Missense	Phe5Ser	rs199904132
	75832609	A/G	Het	Intron	_	rs77460238
ROBO2	77089699	T/G	Het	5'UTR	_	rs3923744
	77147112	G/A	Het	Intron	_	rs9874095
	77607070	_/C	Het	Intron	_	rs5850333
	77645984	A/G	Het	Intron	_	rs775718
ROBO1	78663956	G/A	Het	Intron	_	rs1027832
	78664011	G/T	Het	Intron	_	rs3732661
	78666765	T/C	Het	Intron	_	rs9839790
	78676405	T/C	Het	Intron	_	rs7625555
	78676422	G/A	Het	Intron	_	rs7614084
	78676467	A/G	Het	Intron	_	rs7636043
	78688824	G/T	Het	Intron	_	rs78790905
	78700901	G/T	Het	Synonymous	Thr931=	rs6795556
	78706519	C/T	Het	Intron	_	rs9818170
	78708811	4 bp ins	Hom	Intron	_	rs67851307
	78709044	G/A	Het	Intron	_	rs9829907
	78711350	T/C	Het	Intron	_	rs9864412
	78717343	C/T	Het	Synonymous	Ser580=	rs2271151
	78717508	G/A	Het	Intron	_	rs2255164
	78737962	G/A	Het	Intron	_	rs967454
	78796078	C/A	Het	Intron	_	rs2304503
GBE1	81539382	C/T	Het	3'UTR	_	rs846
	81584269	T/-	Het	Intron	_	rs60133692
	81643167	T/C	Hom	Missense	Val334Ile	rs2172397

	81698130	T/C	Het	Missense	Arg190Gly	rs2229519
	81810516	C/A	Het	Intron	_	rs9820490
	81810703	_/G	Hom	5'UTR	_	rs55840733
	81810725	C/T	Het	5'UTR	_	rs2290081
	81810770	G/A	Het	5'UTR	_	rs78982329
CADM2	85984830	T/C	Het	Intron	_	rs36080783
	86010600	G/A	Het	Intron	_	rs9819556
CHMP2B	87295049	T/C	Het	Synonymous	Thr104=	rs11540913
POU1F1	87308905	A/T	Het	3'UTR	_	rs4988463
HTR1F	88041023	A/C	Het	3'UTR	_	rs76428532
ZNF654	88188420	G/A	Hom	5'UTR	_	rs9813894
	88189341	T/C	Hom	Missense	Ile294Thr	rs7653652
	88190768	C/A	Hom	Intron	_	rs7432826
	88190809	C/T	Hom	3'UTR	_	rs7432838
C3orf38	88199179	T/C	Hom	5'UTR	_	rs6551277
	88199185	_/C	Hom	5'UTR	_	rs11374933
	88199298	T/C	Hom	Synonymous	Thr32=	rs6551278
EPHA3	89498619	A/G	Het	Intron	_	rs1028011
PROS1	93593119	T/C	Het	Synonymous	Pro667=	rs6123
ARL13B	93758804	T/C	Het	Missense	Phe257Ser	rs78945131
	93768268	C/G	Het	Missense	Thr348Ser	rs33944211
DHFRL1	93779860	C/T	Het	Missense	Val166Ile	rs17855824
EPHA6	97367231	_/A	Hom	3'UTR	_	rs63703260
	97466432	T/G	Het	Intron	_	rs79617067
	97467362	4 bp del	Het	Intron	_	rs138837886
MINA	97664725	C/T	Het	Missense	Ala386Thr	rs2172257
	97666183	CTC/-	Het	Intron	_	rs2307808
	97673268	A/C	Hom	Synonymous	Ser251=	rs832079
	97686575	T/C	Het	Intron	_	rs699304
GABRR3	97726747	T/A	Hom	Synonymous	Tyr205=	rs832032
	97727727	G/A	Hom	Intron	_	rs832030
	97727753	G/A	Hom	Intron	_	rs832029
	97731434	_/A	Hom	Intron	_	rs11426005
	97744611	A/G	Hom	Intron	_	rs832041
	97753931	T/C	Het	Intron	_	rs832070
CLDND1	98240317	_/A	Het	Intron	_	rs142862428
GPR15	98250986	C/T	Hom/Het	Missense	Pro37Ser	rs2230344
	98252027	G/A	Hom	3' near gene	_	rs1529047
CPOX	98304467	T/C	Het	Synonymous	Glu330=	rs1729995

	98307630	C/T	Het	Missense	Val294Ile	rs2228056
ST3GAL6	98487206	A/G	Het	Intron	_	rs3213958
	98489686	A/G	Het	Intron	_	rs1440153
	98491619	G/T	Het	Intron	_	rs2334230
	98503776	A/G	Het	Intron	_	rs999147
	98503941	G/A	Het	Intron	_	rs62278486
	98512825	T/A	Het	3'UTR	_	rs14310
DCBLD2	98518072	A/G	Het	3'UTR	_	rs17270986
	98518160	_/A	Hom	3'UTR	_	rs62821060
	98531342	A/G	Hom/Het	Intron	_	rs9814819
	98536571	T/C	Hom/Het	Intron	_	rs1440150
	98540982	A/G	Het	Intron	_	rs852701
	98541012	G/A	Hom	Intron	_	rs866873
	98541116	G/A	Het	Synonymous	Ile262=	rs828616
	98541422	G/T	Het	Intron	_	rs828617
	98544124	A/-	Het	Intron	_	rs11341324
FILIP1L	99643176	C/T	Het	Missense	Arg168His	rs793440
TBC1D23	100015146	G/T	Hom	Intron	_	rs3772698
	100015166	C/T	Het	Intron	_	rs3772697
	100016727	_/T	Het	Intron	_	rs3832199
	100029173	A/G	Het	Intron	_	rs4928168
	100030628	_/T	Hom	Intron	_	rs3217495
	100034849	A/T	Hom	Intron	_	rs4928169
NIT2	100057846	C/T	Het	Intron	_	rs1214381
	100058102	G/A	Het	Intron	_	rs166747
	100073725	C/G	Het	Intron	_	rs57703319
TOMM70A	100084432	G/A	Het	Synonymous	Tyr601=	rs277644
	100086861	T/C	Hom/Het	Intron	_	rs277640
	100092526	_/G	Hom/Het	Intron	_	rs11384494
	100092528	_/GCA	Hom/Het	Intron	_	rs3832198
	100093841	T/C	Het	Intron	_	rs60463428
	100119548	T/C	Het	Synonymous	Glu82=	rs7645509
LNP1	100170589	A/G	Het	Synonymous	Ser61=	rs6786064
	100170596	T/C	Het	3'UTR	_	rs75122231
	100170598	C/T	Het	3'UTR	_	rs78633312
	100170600	A/T	Het	3'UTR	_	rs76354691
	100170628	A/G	Het	Synonymous	Gln74=	rs9844083
	100170634	T/C	Het	Synonymous	Phe76=	rs9848109
	100170825	G/A	Het	Intron	_	rs6797397

	100174722	G/A	Het	Synonymous	Glu163=	rs1132022
GPR128	100354683	G/A	Hom	Intron	_	rs1520652
	100354698	T/A	Hom	Intron	_	rs1718281
	100368692	A/G	Hom	Intron	_	rs1144118
	100374740	T/C	Hom	Intron	_	rs9866111
	100387709	A/G	Hom	Intron	_	rs1144110
TFG	100432752	A/T	Hom	Intron	_	rs9824942
	100467018	T/C	Hom	Synonymous	Pro282=	rs11353
	100467595	CT/-	Het	3'UTR	_	rs202022416
ABI3BP	100473505	C/T	Hom	Synonymous	Pro916=	rs10936352
	100493607	T/C	Hom	Intron	_	rs3736534
	100511446	T/A	Hom	Intron	_	rs989795
	100515198	T/-	Hom	Intron	_	rs201011497
	100523771	A/C	Het	Intron	_	rs10511184
	100594327	6 bp del	Het	Intron	_	rs3839090
	100605206	A/G	Het	Intron	_	rs1987384
	100617680	C/T	Het	Synonymous	Ser136=	rs2245370
	100621426	C/A	Hom	Intron	_	rs2576365
IMPG2	100949842	G/A	Hom	Synonymous	Leu1127=	rs348867
	100950161	A/C	Hom/Het			rs348868
	100963154	G/A	Het	Missense	Thr674Ile	rs571391
	100992385	C/T	Het	Intron	_	rs348866
	100994497	C/T	Het	Intron	_	rs533852
	101038339	G/A	Het	Intron	_	rs546090
	101039107	A/C	Het	Intron	_	rs573908
SENP7	101046671	C/A	Het	Intron	_	rs9849770
	101051794	C/T	Het	Intron	_	rs2068494
	101066964	T/C	Het	Intron	_	rs7636229
	101066992	A/T	Hom	Intron	_	rs2553421
	101070420	A/C	Het	Intron	_	rs75017790
	101080694	T/C	Het	Synonymous	Glu496=	rs7616677
	101085208	A/G	Het	Intron	_	rs75513564
	101085413	G/A	Het	Synonymous	Thr393=	rs939443
	101085684	5 bp del	Het	Intron	_	rs58636547
	101136340	C/T	Het	Intron	_	rs9822356
	101136385	C/A	Het	Intron	_	rs4482646
	101177902	A/-	Hom	Intron	_	rs151020849
	101212889	C/T	Het	Intron	_	rs3846088
	101219818	G/A	Het	Intron	_	rs7619540

	101219889	G/C	Het	Intron	_	rs7621830
	101231871	C/T	Het	Intron	_	rs9681493
	101231921	T/C	Het	Intron	_	rs9682313
	101232057	G/A	Het	5'UTR	_	rs9682739
RG9MTD1	101283792	C/G	Het	Missense	Pro56Arg	rs3762735
ZBTB11	101370551	T/C	Het	Intron	_	rs78127229
	101373480	A/G	Het	Intron	_	rs34508490
	101383321	T/C	Het	Intron	_	rs13081846
	101383562	G/A	Het	Intron	_	rs11712748
	101383800	C/T	Het	Intron	_	rs11720934
CEP97	101443461	T/C	Hom	5'UTR	_	rs994573
	101446208	AT/-	Het	Intron	_	rs3070526
	101484335	G/A	Hom	Synonymous	Gln846=	rs2625282
FAM55C	101504217	T/C	Hom	Intron	_	rs115721253
	101504624	A/C	Hom	Intron	_	rs2625289
	101540150	A/-	Hom	Intron	_	rs35027466
NFKBIZ	101571550	A/G	Het	Intron	_	rs587555
	101574503	G/A	Hom/Het	Intron	_	rs677011
	101575882	T/C	Hom	Intron	_	rs595788
	101576029	>6bp ins	Het	Intron	_	rs3217713
	101576175	T/C	Het	Synonymous	Leu659=	rs14134
ZPLD1	102189169	T/C	Het	Intron	_	rs17822656
ALCAM	105238891	T/-	Hom/Het	Intron	_	rs11291806
	105238901	T/C	Hom/Het	Intron	_	rs2291376
	105252377	G/C	Het	Intron	_	rs2171146
	105260518	G/A	Het	Synonymous	Leu300=	rs579565
	105260520	C/T	Het	Missense	Thr301Met	rs1044243
	105260596	T/C	Hom	Synonymous	Ala326=	rs599278
	105264025	C/T	Het	Intron	_	rs72989914
	105266331	A/G	Het	Synonymous	Gln446=	rs9855810
	105266413	T/-	Hom	Intron	_	rs143206743
CBLB	105389153	A/G	Het	Synonymous	Pro871=	rs11713094
	105397492	T/C	Het	Intron	_	rs55847214
	105412286	_/TT	Hom	Intron	_	rs10635036
	105422844	C/T	Hom	Synonymous	Thr527=	rs2305037
	105423088	T/C	Hom	Intron	_	rs1548056
	105438806	G/A	Hom	Intron	_	rs7624400
	105438957	T/G	Het	Synonymous	Leu447=	rs2305036
	105439026	G/A	Het	Synonymous	Asp424=	rs2305035

	105453034	A/G	Hom	Intron	_	rs6768096
	105453069	C/T	Het	Intron	_	rs9657924
	105455955	T/C	Hom	Intron	_	rs2289746
	105495432	G/T	Hom	Intron	_	rs9809911
CCDC54	107096547	G/A	Het	Missense	Arg38Gln	rs709564
	107097346	C/A	Het	Synonymous	Thr304=	rs3811061
BBX	107447857	T/A	Hom	Intron	_	rs3761963
	107451907	C/T	Hom	Intron	_	rs4855764
	107463457	G/-	Het	Intron	_	rs3838316
	107491759	AGA/-	Hom	Inframe	Glu399del	rs34531902
	107519843	T/C	Hom	Intron	_	rs1532325
CD47	107776511	C/T	Hom	Intron	_	rs709436
IFT57	107881152	T/C	Het	3'UTR	_	rs58273586
	107882643	G/A	Hom	Intron	_	rs327168
	107884211	T/C	Hom	Intron	_	rs327167
	107884269	A/G	Het	Intron	_	rs62264150
	107885875	A/G	Het	Intron	_	rs2305551
	107910328	_/T	Het	Intron	_	rs532732144
	107937286	A/G	Hom	Intron	_	rs1289760
	107937360	C/T	Het	Intron	_	rs56273515
	107937408	C/T	Het	Intron	_	rs1135897
	107937544	C/T	Het	Intron	_	rs2305447
	107937571	G/A	Het	Intron	_	rs1289759
	107938240	G/C	Het	Intron	_	rs1289754
HHLA2	108070556	T/C	Hom/Het	Intron	_	rs9846218
	108072243	C/T	Hom/Het	Intron	_	rs6766283
	108072298	T/C	Hom/Het	Missense	Ile30Thr	rs6779254
	108072704	T/C	Hom/Het	Intron	_	rs6779650
	108081217	C/A	Het	Synonymous	Asn344Lys	rs3792332
	108096098	C/G	Hom/Het	Intron	_	rs1463426
	108096227	G/C	Hom	3'UTR	_	rs1463425
MYH15	108102586	T/C	Hom	Intron	_	rs7426463
	108135608	_/T	Het	Intron	_	rs537644034
	108135799	C/G	Hom	Intron	_	rs11927603
	108140148	A/G	Hom	Intron	_	rs4593038
	108147295	C/A	Hom	Intron	_	rs1350235
	108179063	T/C	Hom	Intron	_	rs3957558
	108179220	T/-	Hom	Intron	_	rs11303627
	108189750	C/T	Hom	Intron	_	rs12495639

	108211869	4 bp ins	Hom	Intron	_	rs140450152
	108214537	T/G	Hom	Intron	_	rs2603140
	108224700	_/A	Het	Intron	_	rs55971936
KIAA1524	108272414	_/A	Hom	Intron	_	rs76336584
	108269838	C/T	Hom	3'UTR	_	rs58758610
	108295067	G/A	Hom	Intron	_	rs16854713
DZIP3	108324365	C/T	Hom	Intron	_	rs41267019
	108327050	_/AAC	Hom	Intron	_	rs10684119
	108355424	T/-	Het	Intron	_	rs10711079
	108355576	T/-	Hom	Intron	_	rs10711080
	108366978	A/G	Hom	3'UTR	_	rs2399235
	108372983	C/T	Het	Intron	_	rs201141252
	108394771	G/A	Hom	Intron	_	rs12486790
	108403019	_/G	Het	Intron	_	rs146441770
	108403086	T/C	Hom	3'UTR	_	rs9856097
RETNLB	108474507	T/C	Hom/Het	3'UTR	_	rs1374821
	108474567	G/T	Het	3'UTR	_	rs75657243
	108475974	G/A	Het	Missense	Pro20Leu	rs11708527
	108476205	G/A	Hom/Het	5'UTR	_	rs10933959
TRAT1	108549720	T/C	Het	Intron	_	rs2290055
	108565815	T/C	Hom/Het	Intron	_	rs2593831
	108567923	G/A	Het	Intron	_	rs2715716
GUCA1C	108634973	C/A	Het	Missense	Gly148Val	rs10933973
	108635099	T/C	Het	Intron	_	rs9845098
	108639270	C/T	Het	Intron	_	rs6791441
	108639384	T/C	Het	Missense	Met85Val	rs6804162
	108639423	C/T	Het	Missense	Val72Ile	rs2715687
	108672336	A/-	Het	Intron	_	rs11330501
MORC1	108677821	C/A	Het	Synonymous	Ser982=	rs2197737
	108678002	G/A	Het	Intron	_	rs2197738
	108682218	A/G	Het	Intron	_	rs2715755
	108690221	G/A	Het	Missense	His836Tyr	rs2593943
	108703518	G/T	Het	Intron	_	rs2593958
	108703548	C/T	Het	Intron	_	rs2593959
	108703702	A/T	Het	Intron	_	rs2593960
	108705715	_/T	Hom/Het	Intron	_	rs11433147
	108719470	C/G	Het	Synonymous	Leu707=	rs3762698
	108725849	TTGC/-	Hom	Intron	_	rs5851630
	108725862	T/C	Het	Intron	_	rs2044589

	108725984	G/C	Het	Intron	_	rs2044590
	108746836	T/C	Het	Intron	_	rs17665933
	108754146	G/A	Het	Intron	_	rs9821261
	108754238	A/T	Het	Missense	Phe470Ile	rs4855576
	108754260	A/G	Het	Synonymous	Asp462=	rs3762696
	108754320	_/A	Hom/Het	Intron	_	rs36119926
	108773772	A/G	Het	Intron	_	rs7653625
	108776343	C/T	Het	Intron	_	rs1031252
	108780831	G/A	Het	Intron	_	rs7653651
	108782160	T/C	Het	Intron	_	rs895738
	108788461	T/C	Het	Intron	_	rs2305238
DPPA2	109027157	C/T	Hom	Intron	_	rs7611135
	109027820	G/A	Hom/Het	Intron	_	rs7611871
DPPA4	109046621	G/A	Het	3'UTR	_	rs1183655
	109047928	C/T	Het	Synonymous	Arg229=	rs1163439
	109049720	6 bp ins	Het	Intron	_	rs112463757
	109052935	C/T	Het	Intron	_	rs1351737
CD96	111260975	C/T	Het	5'UTR	_	rs2276873
	111263826	G/A	Het	Intron	_	rs1513325
ZBED2	111312305	T/C	Het	3'UTR	_	rs7612391
CD96	111356083	G/C	Hom	Synonymous	Pro470=	rs1533270
	111368813	C/T	Het	3'UTR	_	rs3733176
PLCXD2	111432805	G/A	Het	Synonymous	Ala232=	rs17423699
	111451383	A/C	Het	Intron	_	rs13085147
	111451603	G/A	Hom/Het	Intron	_	rs3821919
PHLDB2	111602828	G/A	Hom/Het	Intron	_	rs6805359
	111632614	A/G	Het	Intron	_	rs1282947
	111637904	G/A	Hom	Intron	_	rs2399399
	111638132	A/C	Hom	Intron	_	rs951660
	111639353	G/T	Hom	Intron	_	rs937551
	111658290	T/G	Het	Intron	_	rs712509
	111658486	T/G	Hom	Intron	_	rs712510
	111659377	T/C	Hom	Intron	_	rs698358
	111686738	G/A	Hom	Intron	_	rs799570
	111688578	C/T	Het	Synonymous	Asp1119=	rs774854
	111692693	A/G	Het	Intron	_	rs2895388
ABHD10	111697867	T/C	Het	5'UTR	_	rs2289598
	111705275	5 bp ins	Het	Intron	_	rs150902974
	111705992	A/G	Het	Intron	_	rs3804765

	111710140	T/G	Het	Intron	_	rs6795440
	111710202	T/-	Het	Intron	_	rs71773737
	111710682	A/C	Het	3'UTR	_	rs3749308
TAGLN3	111719879	C/T	Het	Intron	_	rs2292582
	111730546	G/A	Hom	Intron	_	rs3749310
	111730591	G/T	Hom	Intron	_	rs3749311
	111730766	G/A	Het	Intron	_	rs3828394
TMPRSS7	111760771	A/G	Hom	5'UTR	_	rs774774
	111763085	C/T	Hom	Intron	_	rs4682350
	111766559	T/G	Hom	Intron	_	rs1688304
	111785208	G/A	Hom	Intron	_	rs11715561
	111793316	G/T	Hom	Intron	_	rs191454
	111794365	A/G	Hom	Intron	_	rs1907639
	111799845	A/T	Hom	Missense	Ser690Cys	rs340151
	111800035	G/T	Het	3'UTR	_	rs148130890
C3orf52	111828384	_/T	Hom/Het	Intron	_	rs5851818
	111828423	G/A	Hom	Missense	Ser144Gly	rs340167
	111828476	A/G	Het	Intron	_	rs2120095
GCET2	111851890	A/G	Het	Intron	_	rs879652
SLC9A10	111860222	A/G	Het	Intron	_	rs1492480
	111873902	_/A	Hom	Intron	_	rs11369523
	111873996	C/T	Het	Intron	_	rs7622733
	111887792	C/A	Het	Missense	Ala1057Ser	rs76007436
	111898387	A/G	Het	Synonymous	Thr970=	rs74367861
	111918215	C/T	Het	Missense	Gly826Ser	rs28516377
	111918216	A/G	Hom	Synonymous	Phe825=	rs13098660
	111921010	A/G	Het	Intron	_	rs9821935
	111921116	C/A	Het	Missense	Ser768Ile	rs9288938
	111921225	G/T	Hom	Missense	Gln732Lys	rs6781844
	111923047	G/A	Hom	Intron	_	rs4422273
	111923123	G/A	Hom	Missense	Thr705Ile	rs4434123
	111936200	C/A	Hom	Intron	_	rs1388857
	111936491	A/G	Hom	Intron	_	rs9288939
	111950367	C/G	Het	Intron	_	rs55735606
	111958679	T/C	Het	Intron	_	rs13065023
	111958862	C/A	Het	Intron	_	rs13089635
	111962774	T/C	Het	Intron	_	rs13089316
	111962851	T/C	Het	Missense	Thr424Ala	rs6768523
	111981697	A/-	Hom/Het	Intron	_	rs5851826

	111981704	C/T	Hom/Het	Intron	_	rs5003791
	111981849	C/T	Hom/Het	Synonymous	Lys373=	rs9860819
	111981878	T/C	Hom/Het	Missense	Ile364Val	rs9809384
	111981924	T/C	Hom/Het	Missense	Ile348Met	rs9809404
	111983197	C/T	Hom/Het	Intron	_	rs6788397
	111985048	G/A	Het	Intron	_	rs4682099
	111985107	T/C	Het	Missense	Ile286Val	rs9872691
	111988992	T/G	Het	Intron	_	rs13069541
	111993692	C/-	Het	Intron	_	rs11324928
	111993716	G/A	Hom	Intron	_	rs6805112
	111993915	A/G	Het	Intron	_	rs4682100
	111996554	T/C	Het	Missense	Ile158Val	rs9828502
	111996726	A/G	Het	Intron	_	rs6791182
	111996730	G/A	Het	Intron	_	rs9809174
	111997510	T/A	Het	Intron	_	rs12632408
	111997791	G/A	Het	Intron	_	rs13314977
	112005691	C/G	Het	5'UTR	_	rs79950192
CD200	112059768	C/G	Hom	Missense	Ser36Cys	rs1131199
	112066360	C/T	Hom/Het	Intron	_	rs7612748
BTLA	112184927	A/G	Hom	3'UTR	_	rs2171513
	112185025	G/A	Het	Missense	Pro267Leu	rs9288952
	112190137	G/T	Hom	Missense	Ser157Arg	rs2931761
ATG3	112253058	_/A	Het	Intron	_	rs35560667
	112253193	_/A	Hom	Intron	_	rs11381118
	112253234	AAG/-	Hom	Intron	_	rs138522909
	112256630	A/G	Het	3'UTR	_	rs2969896
	112267523	G/A	Hom/Het	Intron	_	rs9809247
	112280218	G/A	Hom/Het	Intron	_	rs2279532
SLC35A5	112289320	G/A	Hom/Het	Intron	_	rs9869455
	112289372	G/A	Hom/Het	Intron	_	rs9869579
	112289577	_/T	Hom	Intron	_	rs11411476
	112299732	A/G	Het	Synonymous	Glu256=	rs2292442
	112301458	9 bp ins	Hom	Intron	_	rs5851844
CCDC80	112338027	T/C	Het	Intron	_	rs13084615
CD200R1L	112538572	T/-	Hom/Het	Intron	_	rs35585943
	112545911	T/-	Hom/Het	Frameshift	His203Profs	rs58161637
	112546306	C/A	Het	Intron	_	rs4682119
	112564663	G/C	Het	5'UTR	_	rs4682438
	112564665	C/T	Het	5'UTR	_	rs4682439

	112564801	T/A	Het	Upstream	_	rs6766388
CD200R1	112642568	C/G	Het	Missense	Glu335Gln	rs9865242
	112647613	T/C	Het	Intron	_	rs1466872
	112647832	A/C	Het	Missense	His200Gln	rs9826308
	112647988	C/T	Het	Intron	_	rs9868053
	112648125	G/T	Het	Synonymous	Thr144=	rs6438117
	112648127	T/G	Het	Missense	Thr144Pro	rs4596117
	112648222	T/C	Het	Missense	Lys112Arg	rs2171509
	112666615	A/G	Het	Intron	_	rs72952145
	112693753	A/T	Hom/Het	5'UTR	_	rs62263759
GTPBP8	112709828	C/G	Hom	5'UTR	_	rs2272393
	112710242	T/C	Hom/Het	Intron	_	rs2248029
	112719792	A/-	Het	3'UTR	_	rs200301737
C3orf17	112727184	A/T	Hom	3'UTR	_	rs2306857
	112732310	T/-	Het	Intron	_	rs5851869
BOC	112991196	C/G	Het	Intron	_	rs3930154
	112991312	C/T	Het	Synonymous	Ile241=	rs3814398
	112991842	A/G	Het	Intron	_	rs11717833
	112991959	C/T	Het	Synonymous	Pro335=	rs11710894
	112997554	A/G	Het	Synonymous	Lys579=	rs775228
	112998090	T/C	Het	Intron	_	rs3856720
	112998265	A/G	Het	Synonymous	Pro661=	rs2649878
	112999407	C/T	Het	Synonymous	Tyr735=	rs367753465
	113003595	G/C	Het	Intron	_	rs775222
	113005754	A/T	Het	3'UTR	_	rs5022662
WDR52	113010303	G/A	Het	3'UTR	_	rs17321330
	113010466	T/C	Hom	3'UTR	_	rs2291905
	113015553	A/C	Hom	Intron	_	rs808950
	113015660	G/A	Het	Missense	Thr1717Met	rs2270781
	113022870	T/C	Hom	Missense	His1657Arg	rs4682484
	113022996	G/A	Hom	Intron	_	rs2270782
	113027214	A/C	Hom	Intron	_	rs775231
	113092197	A/G	Het	Intron	_	rs1463640
	113119301	C/T	Het	Intron	_	rs7647220
	113125966	A/G	Het	Intron	_	rs73237123
	113135345	A/C	Het	Intron	_	rs12635786
	113135543	G/A	Het	Intron	_	rs12630518
	113138763	T/G	Het	Intron	_	rs2129434
	113145006	C/T	Het	Synonymous	Leu124=	rs73239107

	113146130	T/C	Het	Missense	Lys53Glu	rs59722850
	113146271	T/C	Het	Intron	_	rs9869318
	113152596	T/A	Het	Intron	_	rs2291413
SPICE1	113164109	A/G	Het	3'UTR	_	rs2054823
	113175945	A/-	Hom	Intron	_	rs5851894
	113176122	G/A	Het	Synonymous	Pro506=	rs7637618
	113207758	G/A	Het	Intron	_	rs7625841
	113211953	C/T	Het	Intron	_	rs3732804
	113211957	T/C	Het	Intron	_	rs3732803
	113212041	G/A	Het	Intron	_	rs1471884
	113218206	C/T	Het	Intron	_	rs6798938
	113222036	G/A	Het	Synonymous	Pro46=	rs11537650
SIDT1	113285186	TG/-	Hom	Intron	_	rs71633326
	113300183	A/T	Het	Intron	_	rs2271494
	113322006	T/G	Het	Intron	_	rs17325765
KIAA2018	113373930	A/G	Hom	Missense	Val2200Ala	rs930818
	113377505	T/G	Hom	Synonymous	Leu1008=	rs16861271
GRAMD1C	113594377	A/G	Het	5'UTR	_	rs13079383
	113595156	C/G	Hom	Intron	_	rs2712350
	113619872	C/T	Hom	Intron	_	rs2632244
	113655207	C/T	Het	Synonymous	Asn517=	rs3765114
	113656833	C/G	Het	Intron	_	rs1963015
	113664184	AG/-	Hom/Het	Intron	_	rs68028538
ZDHHC23	113673125	A/G	Hom	Missense	Lys247Arg	rs11921691
	113679589	C/A	Het	3'UTR	_	rs12629415
	113679725	C/T	Hom	3'UTR	_	rs3732785
KIAA1407	113720624	C/T	Hom	Intron	_	rs6438178
	113753801	C/T	Het	Synonymous	Arg263=	rs61741386
	113755662	A/G	Het	Intron	_	rs12635058
	113755670	T/A	Het	Intron	_	rs368890712
QTRTD1	113784008	_/T	Hom/Het	Intron	_	rs34428541
	113795834	G/A	Het	Intron	_	rs7631864
	113801472	G/A	Het	Intron	_	rs72960764
	113804859	T/C	Het	3'UTR	_	rs3732788
DRD3	113849908	5 bp del	Het	Intron	_	rs149281192
	113850332	A/G	Het	Intron	_	rs73232569
	113858350	C/T	Hom	Synonymous	Gln240=	rs2251177
	113890815	C/T	Het	Missense	Gly9Ser	rs6280
ZNF80	113955056	A/-	Het	3'UTR	_	rs11316836

	113955164	T/G	Hom/Het	Missense	Asp253Ala	rs3732782
	113955265	G/A	Hom/Het	Synonymous	Cys219=	rs6438190
	113955320	C/T	Hom/Het	Missense	Arg201His	rs6438191
TIGIT	114014301	T/C	Het	Intron	_	rs77967063
	114026673	A/G	Het	Intron	_	rs11714612
	114027084	A/G	Het	3'UTR	_	rs370636006
ZBTB20	114099335	_/T	Hom	Intron	_	rs538392137
LSAMP	115529088	C/G	Het	3'UTR	_	rs75235287
	115529092	G/C	Het	3'UTR	_	rs56145932
	115529360	A/G	Het	Intron	_	rs2289269
	116163635	AC/-	Het	Intron	_	rs138580275
IGSF11	118621664	C/A	Het	Missense	Glu333Asp	rs36052974
	118621833	T/C	Het	Intron	_	rs73185803
	118623414	T/C	Hom/Het	Intron	_	rs4687959
	118624631	G/A	Het	Intron	_	rs6782002
	118645118	A/G	Het	Intron	_	rs9848979
	118645201	A/T	Het	Intron	_	rs16829163
	118649060	G/T	Hom	Missense	Pro39Thr	rs2903250
	118753542	G/T	Hom/Het	Intron	_	rs73185835
	118824042	_/TTC	Hom/Het	5'UTR	_	rs143862519
	118864912	G/A	Het	5'UTR	_	rs10049288
C3orf30	118865801	C/T	Het	Synonymous	Ser255=	rs4077931
	118865970	G/A	Hom/Het	Missense	Gly312Ser	rs4077930
	118866515	C/T	Hom	Intron	_	rs9864127
	118867047	C/G	Het	Missense	Asp473Glu	rs9289122
	118870026	_/C	Het	Intron	_	rs68187836
UPK1B	118909159	A/G	Het	Missense	Gln113Arg	rs9840317
	118909962	T/C	Het	Intron	_	rs3796356
	118909987	C/T	Het	Intron	_	rs3796357
	118913337	A/G	Het	Intron	_	rs7628485
B4GALT4	118931389	C/T	Hom	3'UTR	_	rs6784208
	118935285	7 bp del	Hom	Intron	_	rs68075325
ARHGAP31	119013714	C/A	Het	5'UTR	_	rs72960626
	119013928	A/T	Het	Intron	_	rs3732412
	119118104	A/G	Hom	Synonymous	Val355=	rs4688001
	119120577	G/C	Hom/Het	Intron	_	rs10511390
	119128628	A/G	Hom	Intron	_	rs1463139
	119128634	T/C	Hom	Intron	_	rs1463138
	119128712	T/A	Hom	Intron	_	rs12636976

	119133183	G/A	Hom	Missense	Gly803Ser	rs3732413
	119133554	G/A	Het	Synonymous	Ala926=	rs61740281
TMEM39A	119150697	T/C	Het	3'UTR	_	rs79702234
	119155605	G/A	Het	Intron	_	rs116718899
	119171280	C/G	Hom	Intron	_	rs1919586
	119181093	G/A	Het	Intron	_	rs375582328
POGLUT1	119196334	G/T	Hom	Intron	_	rs3732419
	119198873	G/A	Hom	Intron	_	rs4688007
	119199100	T/-	Hom	Intron	_	rs139662431
	119209658	A/G	Het	Intron	_	rs62263444
	119211384	T/G	Het	3'UTR	_	rs11556604
TIMMDC1	119219573	A/G	Het	3'UTR	_	rs11539377
	119235977	G/A	Het	Intron	_	rs4447803
	119236017	G/T	Hom/Het	Intron	_	rs4461452
	119236179	G/A	Het	Intron	_	rs60006470
	119242443	C/T	Het	Intron	_	rs58978800
	119242778	T/C	Het	3'UTR	_	rs13532
CD80	119263680	C/T	Het	Synonymous	Val45=	rs2228017
	119276377	A/G	Het	Intron	_	rs66604554
ADPRH	119301139	C/T	Het	Synonymous	Gly41=	rs20568
	119305379	T/A	Het	Synonymous	Ala182=	rs25676
PLA1A	119325552	A/G	Het	Intron	_	rs530389
	119325907	G/A	Het	Intron	_	rs2251566
	119327605	C/-	Het	Intron	_	rs5852212
	119327653	T/C	Het	Synonymous	Phe104=	rs1723969
	119328450	7 bp del	Hom	Intron	_	rs3832185
	119331892	C/G	Het	Synonymous	Thr181=	rs2272269
	119332042	G/A	Het	Intron	_	rs2272268
	119334986	G/A	Het	Intron	_	rs2247660
POPDC2	119367390	T/C	Hom	Synonymous	Ser242=	rs2688643
	119373257	A/G	Hom	Intron	_	rs2688631
	119373301	G/A	Hom	Intron	_	rs2688630
	119373315	T/C	Hom	Intron	_	rs2688629
C3orf15	119422106	C/A	Het	Intron	_	rs7612954
	119426307	T/C	Het	3'UTR	_	rs9848716
	119434527	G/C	Hom	3'UTR	_	rs6438544
	119459639	A/T	Het	Intron	_	rs34948068
NR1I2	119501580	_/C	Hom	5'UTR	_	rs3841391
	119501780	G/A	Hom	Intron	_	rs2472668

	119501798	T/A	Hom	Intron	_	rs2461831
	119526349	G/A	Het	Intron	_	rs1464603
	119526372	G/A	Het	Intron	_	rs1464602
	119533733	G/A	Het	Intron	_	rs6785049
	119533773	T/C	Hom/Het	Intron	_	rs6797879
	119533910	T/C	Hom	Synonymous	Asn332=	rs4058490
	119534153	C/T	Het	Intron	_	rs2276707
GSK3B	119666266	C/T	Het	Intron	_	rs13312998
GPR156	119886548	G/C	Hom/Het	Synonymous	Leu588=	rs9858566
	119886776	T/A	Hom/Het	Missense	Glu516Asp	rs902790
	119892121	C/G	Het	Intron	_	rs13064815
	119900212	G/C	Het	Intron	_	rs1488765
	119900260	T/C	Hom/Het	Intron	_	rs1488764
	119904125	A/G	Hom/Het	Intron	_	rs902791
	119904315	G/C	Hom/Het	Intron	_	rs902792
	119905641	G/A	Hom/Het	Intron	_	rs2319542
	119912297	C/A	Het	Intron	_	rs902793
	119962415	G/T	Het	Intron	_	rs201566565
LRRC58	120067797	G/A	Hom	Synonymous	Ala98=	rs4676696
FSTL1	120130883	>20bp del	Het	Intron	_	rs67083358
	120134721	_/G	Hom/Het	Intron	_	rs34665997
	120134883	T/C	Het	Intron	_	rs2272515
	120134901	C/A	Hom/Het	Intron	_	rs2272516
HGD	120363375	T/C	Het	Intron	_	rs2075504
	120369575	T/G	Hom/Het	Intron	_	rs3817627
	120371369	G/A	Het	Intron	_	rs1054095
	120371414	G/A	Hom	Intron	_	rs2551607
	120371521	G/A	Het	Intron	_	rs182686850
	120389316	T/A	Het	Missense	Gln80His	rs2255543
RABL3	120413158	G/A	Hom	Intron	_	rs7612975
	120428621	T/C	Hom/Het	Intron	_	rs11720353
GTF2E1	120500446	T/A	Hom/Het	3'UTR	_	rs2229308
STXBP5L	120833994	G/A	Het	Intron	_	rs17195251
	120975997	_/T	Hom	Intron	_	rs150144558
	121100117	T/-	Het	Intron	_	rs35624942
	121100414	A/G	Het	Intron	_	rs17740072
	121132268	T/A	Hom	Intron	_	rs9849118
	121137976	C/T	Hom	3'UTR	_	rs6782025
	121138015	C/T	Hom	3'UTR	_	rs6782033

POLQ	121154974	T/C	Hom	Missense	Gln2513Arg	rs1381057
	121155198	C/T	Hom/Het	Intron	_	rs2030531
	121186543	A/G	Hom/Het	Intron	_	rs1381058
	121192350	G/A	Het	Intron	_	rs3218647
	121195303	AC/-	Het	Intron	_	rs71678533
	121202134	TT/-	Hom	Intron	_	rs397755922
	121207637	G/T	Het	Missense	Pro1381Thr	rs3218642
	121208833	G/C	Hom/Het	Missense	Thr982Arg	rs3218649
	121212278	T/-	Het	Intron	_	rs11351457
	121228400	G/C	Het	Intron	_	rs41544013
	121228833	A/C	Hom/Het	Intron	_	rs3732406
	121238749	G/A	Hom	Synonymous	Gly479=	rs702018
	121238863	C/T	Het	Synonymous	Ala441=	rs35766343
	121239027	G/A	Hom	Intron	_	rs702019
	121241058	C/G	Hom/Het	Intron	_	rs13059229
	121258368	G/A	Het	Synonymous	Phe181=	rs36065146
	121263720	C/A	Hom	Missense	Arg66Ile	rs702017
ARGFX	121295569	CT/-	Het	Intron	_	rs371869945
FBXO40	121339354	T/C	Hom	Intron	_	rs7640859
	121339520	A/C	Hom/Het	Intron	_	rs35295338
	121340536	T/C	Hom/Het	Missense	Val87Ala	rs4676684
	121345874	G/C	Hom/Het	3'UTR	_	rs2840143
	121347408	A/G	Hom	3'UTR	_	rs4676686
HCLS1	121350573	A/G	Het	3'UTR	_	rs1128163
	121350583	C/T	Het	3'UTR	_	rs1128159
	121350966	C/G	Hom	Missense	Leu436Val	rs9869984
	121351315	G/A	Het	Synonymous	Pro368=	rs80289672
	121351480	T/C	Het	Intron	_	rs9815500
	121353254	T/C	Het	Missense	Thr235Ala	rs2070179
	121354583	G/A	Het	Synonymous	Ala230=	rs3772126
GOLGB1	121383297	G/A	Het	3'UTR	_	rs7153
	121400725	G/A	Het	Intron	_	rs1463736
	121413637	G/A	Het	Synonymous	Ser1911=	rs34833153
	121414061	C/T	Het	Missense	Gly1770Asp	rs1127412
	121415720	T/C	Het	Missense	Tyr1217Cys	rs3732410
	121416623	G/C	Het	Missense	Thr916Ser	rs3732407
	121435621	T/C	Het	Synonymous	Gln417=	rs9832267
	121435624	C/T	Het	Synonymous	Glu416=	rs9812411
IQCB1	121489440	A/-	Hom/Het	Intron	_	rs148949254

	121500699	C/T	Hom	Missense	Cys434Tyr	rs17849995
	121508830	A/T	Hom	Intron	_	rs6802815
	121514459	A/-	Het	Intron	_	rs141330650
	121526099	A/G	Hom/Het	Intron	_	rs4582090
	121526204	G/A	Hom/Het	Synonymous	Leu192=	rs4543051
SLC15A2	121613205	G/C	Hom	5'UTR	_	rs4603998
	121630328	A/G	Het	Intron	_	rs6438687
	121634172	_/T	Hom	Intron	_	rs3215370
	121634410	T/A	Het	Intron	_	rs9812515
	121641528	G/A	Het	Intron	_	rs7637569
	121641693	G/A	Het	Synonymous	Ala284=	rs2293616
	121641821	A/G	Het	Intron	_	rs2293615
	121642048	A/T	Het	Intron	_	rs3817601
	121643143	_/T	Hom	Intron	_	rs557681416
	121643170	A/T	Het	Intron	_	rs2257109
	121643303	T/A	Het	Intron	_	rs2257115
	121643699	G/A	Het	Intron	_	rs2257132
	121643804	C/T	Het	Missense	Leu350Phe	rs2257212
	121643893	T/C	Het	Intron	_	rs2257214
	121646641	A/G	Het	Synonymous	Ala387=	rs1143670
	121646759	G/A	Het	Intron	_	rs3762819
	121647286	C/T	Het	Missense	Pro409Ser	rs1143671
	121647467	C/T	Het	Intron	_	rs1316301
	121647782	C/G	Het	Intron	_	rs1316397
	121648168	G/A	Het	Missense	Arg509Lys	rs1143672
	121659197	G/A	Het	Intron	_	rs3817599
ILDR1	121712051	A/C	Hom	Synonymous	Leu515=	rs2877561
	121712805	G/C	Hom/Het	Missense	Pro264Arg	rs3915061
	121712980	C/T	Hom	Intron	_	rs3915060
	121720494	C/T	Het	Intron	_	rs62269207
	121720515	T/-	Het	Intron	_	rs35657414
CD86	121810428	T/-	Hom	Intron	_	rs63114861
	121825197	G/A	Hom	Missense	Glu181=	rs2681417
	121836967	GA/-	Hom	Intron	_	rs10580127
	121838319	G/A	Het	Missense	Ala310Thr	rs1129055
CASR	121976253	G/A	Hom/Het	Intron	_	rs9869985
	122000871	C/T	Het	Intron	_	rs4678174
	122003045	G/C	Hom	Synonymous	Pro758=	rs2036400
	122003832	G/C	Hom/Het	Missense	Gln1021Glu	rs1801726

	122004098	A/T	Hom/Het	3'UTR	_	rs4677948
CSTA	122056432	C/T	Het	Synonymous	Tyr35=	rs17589
	122060274	A/C	Het	Intron	_	rs6762112
CCDC58	122086960	C/T	Hom/Het	Intron	_	rs3749212
FAM162A	122128757	T/C	Het	3'UTR	_	rs16833120
WDR5B	122133830	T/G	Het	Synonymous	Ile182=	rs3749213
KPNA1	122145810	T/A	Hom	3'UTR	_	rs4677950
	122168341	C/A	Het	Intron	_	rs3749209
	122186141	A/G	Het	Intron	_	rs75578364
	122186188	C/T	Hom	Missense	Ser73Asn	rs4678193
PARP9	122247606	A/G	Het	Intron	_	rs7628052
	122259606	T/C	Het	Missense	Tyr528Cys	rs9851180
	122259745	G/T	Het	Intron	_	rs9831600
	122259782	A/G	Hom	Intron	_	rs9868934
	122269380	A/G	Hom	Intron	_	rs1979853
	122274757	G/C	Het	Synonymous	Val122=	rs73192127
DTX3L	122285005	G/A	Het	Intron	_	rs12485294
	122287289	T/C	Hom/Het	Intron	_	rs9839782
	122288210	A/G	Het	Missense	Arg425Lys	rs2332285
	122290579	C/T	Het	Synonymous	Ala736=	rs2036342
PARP15	122345870	C/T	Het	Synonymous	Ser476=	rs17208928
	122354037	A/G	Het	Synonymous	Lys581=	rs1106346
	122354716	C/T	Het	Synonymous	Asp602=	rs61754895
PARP14	122437321	T/C	Hom	Synonymous	Tyr1441=	rs7645033
	122437834	A/G	Het	Intron	_	rs3821688
	122447574	A/C	Het	3'UTR	_	rs3732832
	122447596	C/T	Hom	3'UTR	_	rs7648262
HSPBAP1	122471408	A/-	Hom	Intron	_	rs72280610
	122474305	C/A	Het	Intron	_	rs6794019
	122478032	6 bp ins	Het	Intron	_	rs10657578
	122496747	T/C	Het	Missense	His24Arg	rs141356063
	122512631	G/A	Het	5'UTR	_	rs3806643
DIRC2	122598291	A/G	Het	3'UTR	_	rs2288677
SEMA5B	122629589	A/G	Het	Intron	_	rs7622250
	122630323	T/G	Het	Intron	_	rs2303982
	122630346	T/C	Het	Missense	Asp1082Gly	rs2303983
	122631896	A/T	Hom	Missense	Val894Ala	rs2276782
	122642401	G/A	Het	Intron	_	rs2276780
	122642415	G/A	Het	Intron	_	rs2276779

	122642590	G/A	Het	Synonymous	Ile436=	rs2276778
	122658417	G/T	Het	Intron	_	rs3732827
	122680141	A/G	Het	5'UTR	_	rs13094003
	122680171	A/G	Hom	Intron	_	rs4677985
	122680240	C/A	Het	Intron	_	rs34020905
PDIA5	122807995	G/A	Hom/Het	Intron	_	rs836860
	122821385	T/C	Hom	Intron	_	rs2241961
	122821671	A/G	Hom	Intron	_	rs2241963
	122821672	T/A	Hom	Intron	_	rs61182591
	122835210	A/G	Hom	Intron	_	rs836840
	122835232	T/C	Het	Intron	_	rs2278668
	122843020	T/C	Hom/Het	Intron	_	rs836854
	122843110	G/T	Hom	Intron	_	rs861375
	122843212	C/T	Het	Intron	_	rs2305051
	122864436	G/A	Hom	3'UTR	_	rs8739
	122865136	T/A	Hom	Intron	_	rs2673345
	122873923	G/C	Hom	Intron	_	rs2717225
	122880118	G/A	Hom/Het	Intron	_	rs3828393
	122880191	T/C	Het	3'UTR	_	rs8935
SEC22A	122942358	A/G	Het	Intron	_	rs1546605
	122942402	G/A	Het	Intron	_	rs1546604
	122964900	C/G	Het	Intron	_	rs12493729
	122978486	G/A	Het	Intron	_	rs9289216
	122990291	C/T	Het	Intron	_	rs9860349
	122990734	>6bp ins	Hom	3'UTR	_	rs141585719
ADCY5	123008519	T/G	Hom	Intron	_	rs4677881
	123010276	G/A	Het	Intron	_	rs9809236
	123014877	C/T	Hom/Het	Intron	_	rs9881951
	123014904	C/T	Het	Intron	_	rs56407630
	123015094	G/C	Hom	Intron	_	rs9882534
	123018963	A/G	Hom/Het	Intron	_	rs4482616
	123021838	A/G	Hom	Intron	_	rs9877581
	123021870	G/C	Hom/Het	Intron	_	rs9844212
	123037047	A/C	Hom	Intron	_	rs4327330
	123039496	A/G	Het	Intron	_	rs3935566
	123047478	A/G	Het	Intron	_	rs9855635
	123047666	A/G	Hom	Intron	_	rs9855969
	123049678	C/T	Het	Intron	_	rs4678009
	123049707	G/T	Het	Intron	_	rs9829332

	123049938	A/C	Hom	Intron	_	rs6806529
	123066555	4 bp ins	Het	Intron	_	rs112525497
PTPLB	123301242	C/G	Het	Intron	_	rs820469
	123303821	G/C	Het	Synonymous	Gly18=	rs1271004
	123303824	A/G	Het	Synonymous	Gly17=	rs112371142
	123303960	C/A	Het	5'UTR	_	rs820468
MYLK	123332875	T/-	Het	3'UTR	_	rs35930843
	123337414	C/G	Het	Intron	_	rs860224
	123338956	T/A	Het	Intron	_	rs820457
	123357037	A/G	Het	Synonymous	Asn1614=	rs820463
	123357062	G/C	Het	Intron	_	rs820464
	123368013	A/G	Het	Synonymous	Asp1439=	rs1254392
	123411522	G/C	Het	Intron	_	rs2305631
	123411589	G/A	Het	Synonymous	Thr1186=	rs40305
	123418913	G/A	Hom	Synonymous	Asn1134=	rs865358
	123419117	TTC/-	Het	Missense	Glu1066del	rs75967604
	123419288	C/T	Het	Synonymous	Glu1009=	rs12172926
	123419573	G/T	Het	Missense	Asp914Glu	rs3732487
	123419733	A/G	Het	Missense	Leu861Pro	rs3732486
	123420426	G/A	Het	Intron	_	rs57186134
	123428525	G/A	Het	Intron	_	rs150648437
	123440967	G/A	Hom	Intron	_	rs820355
	123444785	A/T	Het	Intron	_	rs820329
	123451773	G/C	Hom	Missense	Leu496Val	rs9833275
	123452838	G/A	Het	Synonymous	Thr335=	rs4678047
	123454174	G/C	Het	Intron	_	rs58485680
	123456432	C/T	Hom	Intron	_	rs2168439
	123457711	C/A	Hom	Intron	_	rs2198766
	123457893	G/A	Het	Missense	Pro147Ser	rs9840993
	123512627	G/T	Het	Missense	Pro21His	rs28497577
KALRN	123983604	C/T	Het	Intron	_	rs2276739
	123988039	T/C	Hom	Synonymous	His300=	rs2272486
	124044949	C/T	Hom	Synonymous	Phe403=	rs2289778
	124113929	A/T	Hom/Het	Intron	_	rs482285
	124132263	A/T	Hom/Het	Intron	_	rs2289846
	124132568	T/G	Hom	Intron	_	rs6762346
	124210344	C/A	Hom/Het	Intron	_	rs10755077
	124281980	T/C	Het	Intron	_	rs2256831
	124303786	G/C	Het	Intron	_	rs2250757

	124351316	G/A	Het	Synonymous	Val1742=	rs1708303
	124351424	T/C	Het	Synonymous	Leu1778=	rs1660038
	124352868	A/T	Het	Intron	_	rs10428097
	124369640	_/A	Hom	Intron	_	rs11372663
	124369650	T/C	Het	Intron	_	rs2291988
	124374405	A/C	Het	Intron	_	rs4677939
	124376653	G/A	Het	Intron	_	rs106520
	124377196	G/A	Het	Intron	_	rs10804562
	124378386	C/G	Het	Intron	_	rs333297
	124379748	G/C	Het	Intron	_	rs333290
	124379817	T/C	Het	Synonymous	Ile2087=	rs333289
	124380839	G/A	Het	Intron	_	rs73193769
	124385250	G/T	Het	Intron	_	rs76992353
	124385301	T/C	Het	Synonymous	Thr2116=	rs55689161
	124385361	A/G	Het	Synonymous	Ala2136=	rs41264663
	124385556	C/A	Het	Intron	_	rs73193781
	124385814	A/T	Het	Intron	_	rs2289422
	124385823	T/C	Het	Intron	_	rs2289421
	124385942	T/C	Het	Synonymous	Val2204=	rs2289420
	124385960	C/T	Het	Synonymous	Asn2210=	rs2289419
	124390722	G/A	Het	Missense	Gly2306Arg	rs35653635
	124397250	T/-	Hom	Intron	_	rs376122378
	124412634	G/T	Het	Intron	_	rs333283
	124438329	C/T	Het	3'UTR	_	rs4234221
	124438439	T/C	Het	3'UTR	_	rs332512
UMPS	124449252	T/C	Het	5'UTR	_	rs2279199
	124449291	A/G	Het	5'UTR	_	rs1139538
	124454174	A/G	Het	Intron	_	rs3772804
	124456742	G/C	Het	Intron	_	rs1801019
	124458816	C/G	Het	Intron	_	rs694897
	124462710	G/C	Het	Intron	_	rs3772807
	124462808	C/T	Het	3'UTR	_	rs13146
ITGB5	124483190	G/A	Het	Intron	_	rs61761681
	124485235	A/G	Het	Intron	_	rs2291081
	124487849	A/G	Het	Intron	_	rs2291082
	124515308	A/G	Het	Synonymous	Phe540=	rs1803825
	124515509	G/A	Het	Synonymous	Ser473=	rs2291088
	124538764	C/A	Het	Intron	_	rs12490538
MUC13	124629223	A/-	Hom	Intron	_	rs34208404

	124642587	T/-	Hom	Intron	_	rs3841931
	124646594	A/G	Het	Missense	Ile100Thr	rs4679392
	124646705	_/AAG	Hom	Missense	Phe63Ser	rs10630030
HEG1	124692689	C/T	Het	Synonymous	Pro1294=	rs2270778
	124692785	C/T	Het	Intron	_	rs2270779
	124696808	A/-	Hom	Intron	_	rs61603696
	124728472	C/G	Hom	Intron	_	rs6438868
	124728626	A/G	Hom	Missense	Met1039Thr	rs6438869
	124729193	T/G	Hom	Intron	_	rs6784454
	124731485	C/G	Het	Missense	Val980Leu	rs10804567
	124731689	T/A	Het	Missense	Thr912Ser	rs78680419
	124732419	A/G	Het	Synonymous	Ser668=	rs202026679
	124732618	A/G	Het	Missense	Phe602Ser	rs6790837
	124732842	C/A	Het	Intron	_	rs6438871
	124738107	C/T	Het	Synonymous	Ser529=	rs59232004
	124739892	G/A	Het	Synonymous	Ala332=	rs6438874
	124746049	A/G	Het	Missense	Ser305Pro	rs2981546
	124746182	C/A	Het	Synonymous	Pro260=	rs2333041
	124746347	T/C	Het	Synonymous	Ser205=	rs4404487
	124748226	G/A	Het	Synonymous	Gly141=	rs2860440
SLC12A8	124802549	G/A	Hom	3'UTR	_	rs2002242
	124802566	A/-	Het	3'UTR	_	rs200646050
	124802677	A/G	Hom/Het	3'UTR	_	rs1574340
	124802881	A/G	Het	Synonymous	Pro666=	rs2981483
	124802888	C/T	Het	Missense	Arg664Gln	rs2981482
	124837684	T/C	Hom	Missense	Val281Ile	rs621383
	124839470	G/A	Hom	Missense	Leu266Pro	rs863642
	124854425	T/C	Hom	Intron	_	rs702048
	124854636	A/T	Hom/Het	Intron	_	rs2981503

[a]Genomic position of the nucleotide base on chromosome 3 (chr 3) (GRCh37 p.13, NCBI). [b]The position of amino acid is numbered according to one of the protein-coding transcripts for the gene (dbSNP 142, NCBI). Hom, homozygous; Het, Heterozygous

A2.2 Primer pairs used for amplification and sequencing

Primers for validation of novel/rare NGS variations (3p14.2-q21)		
Amplicon	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
KIAA1407-Exon 6	ccaataaacagtggagttctgg	acagagaaaccccacagtgc
CHST13-Exon 2	aatgtgcctagatgggttgg	caggtctgcactgttgctgt
ABI3BP-Exon10	aagttgttgactggctggtg	cagctattgttttcccaaacg
ABI3BP-Exon 32	taagcccccatcatcttcac	aagtggaaaaacgcgaaatc
ADCY5-Exon 15	ggcccttcactttatatcacacc	cacctccctgcaatgtctc
BOC-Exon 12	aaactctggactggggtagaac	agatttcaccaataccttgtcc
C3orf64-Exon 10	aatacttctctcttctccccatc	gcctcttttctttccagattgtc
CCDC80-Exon 6	ctctggtttcctacattggttt	ccaggaggagagtggtatgg
CCDC54-Exon 1	tgctccaagatgttaaaactgc	ttacccctttctccctccag
DZIP3-Exon 18	cagtatgagcactatccaaacttcc	aaagagtcacaggaaaaaccag
EPHA6-Exon 8	gggtttgcttcatacgtgtc	actgtcagagtggattcttatgg
EPHA6-Exon 14	tacacacacgcacacactgc	cccaggacaatgaaagaaag
HHLA2-Exon 7	gggagaattttggtgtttgc	tatcctgcaacccctgctg
HSPBAP1-Exon 5	ccactgatggtgcctatattcc	aagcctacaactggggaagc
IQCB1-Exon 1	tcaaacaaggcaagaaaagc	tgagcttcctaaaggtgtgatg
KIAA1407-Exon 5	tattgaacactcgcagctttg	caaagactgcttcctgttatcc
LRIG1-Exon 2	gatgccaggttgttgattcc	aagaggattttcccaaagagc
NSUN3-Exon 3	gaggcaaaaggaagagtcagg	caggagcagcacagagatcc
SIDT1-Exon 25	ttgatgtggttcggagagac	ccccaagatggtgagttgac
TMEM39A-Exon 1	ttgcctaaagctgaacagagc	cctccaccaaccaaagtctg
TRAT1-Exon 2	gtttcccggtagagactgtg	ctgcttccataggttttccag
TRAT1-Exon 5	cagtttgggcttccttacctc	caggtcccagaacttgacatc
ZBTB20-Exon 4	ttgaccaaatcgagcagttc	ggttgtatttagaggccagtagc
ZDHHC23-Exon 2	aggactggtgtgccaagtg	agtgggaacatcacaacaacc
CADM2-Exon 10	ggctgaatgctggagaaaac	tccttccctaatcctgttacttg
GOLGB1-Exon 11	cctctttgtggaacctctcttc	ctttcagctcctccagctct
PSMD6-Exon 2	aacagaatccggcaagagc	ttccagctcctcatccaaac
PARP14-Exon 1	caggaaacgaaagcgaaaga	cattaagcgcctggaagc
ZNF717-Exon 1	gaggagggcttttccaggta	cgaaggtggtggttcagact
ZNF717-Exon 4	tcattgcacagtccctgaag	gaggaaaaaggggaaacaga
ILDR1-Exon 6	ccttaacccttactcccatgc	cctcccctatcccaaatctc
PRICKLE2-Exon 1	gcctctgacctttcatgctg	cttccaaagcacagcctgag

DIRC2-Exon 5	gattctggtgttccagtttcc	cgatgttttacccagtccttg
ARHGAP31-Exon 1	ggtggatctcaggctctgc	ccgtctctccctcaaacaaa
ARHGAP31-Exon 12	aatcctggtgtgctgctgta	gctcctcctcagattcaatg
MYLK-Exon 2	gggggcgttatgaggatta	cattggtggcttcacaggta
BOC-Exon 3	accagcaccttcccttctct	gtttgggagacagcagcag
BOC-Exon 17	catctgggcttgtgcactg	aagccccacctttgaagact
ADCY5-Exon 21	ccctctccttctgtgtcagg	aggtcatcatctcgcctttg
ROBO1-Exon 28	ccagaccagccaagaaactg	gcttatcagaaaactgcaaggag
SLC12A8-Exon 6	caggggagattgaaaagcag	ttgccatgaggggaacttac
SEMA5B-Exon 14	gggccatgtcctacctctac	gtccacgccccattcctg
HEG1-3'UTR	aagcaggaagagggacagg	aggaaacctcccacccaat
PRICKLE2-3'UTR	gtggcttatgggggagaaa	agtcttgggtcctggctaat
GAP43-Exon 2	agagcagccaagctgaagag	ctgcctgagtcctctctggt
GPR156-5'UTR	ggggaccagttgaaggatgt	atttcaggctccatgtcacc
BOC-3'UTR	cgtaggacaggaacctggaa	tctccaacctcaacgggtag
TBC1D23-Exon 18	acccaacaattgcctagtgatc	gatttctattcctgaagcactgc
STXBP5L-Exon 21	tcagcccaggagttcaagac	gggaactggactgtcattca
ARL13B-Exon 4	tggcgaaacccagtctctac	tgacatcagcttctcctaaagc
ABHD10-3'UTR	caccgaatgagggaaaaagc	gcaattaagagctggcatagc
C3orf38-5'UTR	gctcttttggaggagggact	caacagacaccggaagtgc

Primers for missed/low coverage exons in NGS data set (3p14.2-q21)

Amplicon	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
PTPRG-Exon 12a	gcctcattcccaatctcaat	tcaccccactcttctccttc
PTPRG-Exon 12b	aaagcgagagtgaggatgg	caccttctgctcccaatttt
FEZF2-Exon1b	ggcttgtgcaaaaccaactg	agcttgaaaaaggggcattc
GPR27-Exon1a	gcgcaccattcctgagat	ctctgcatagaagcggtggt
GPR27-Exon 1c	gtctacctccgcctgctctt	gtccgagccaaagggaaag
LRRC58-Exon 1a	ctctgggtgtctgcctcagt	gactcagccctcggttcag
LRRC58-Exon1b	gctgcctcacaaccgtct	cagaactcactaccgcacag
SYNPR-Exon 1,2	ctcccctgttgtatctaccc	ggcttgagggttgaatctcc
PSMD6-Exon 1	gctcccatcagttacaaacg	ggtatccccaaaccaaagc
MAGI1-Exon 1	gctgccaagtttctctctcc	gtatttcaccgccaccacag
SLC25A26-Exon 1	actacccctcacgactcctc	gccagtaacccgaggacag
SLC25A26-Exon 2	gtggccgagagcttattttg	atgggggtaagaccacacac

KBTBD8-Exon 1	ccatccgaatcagccaatag	ccattcatcctccctcttctc
SUCLG2-Exon 1	aggacgataacctggggact	gcggagaccaagagtagcag
SUCLG2-Exon 11	ccaagaagacgatgacgttg	cccatttaaggagccaaagc
FRMD4B-Exon 1	gggcttctgtgtgccagtc	ctgagcgtttgacccaagg
MITF-Exon 1	caggcccagctaccttcc	gggtccttgtcccagagc
GXYLT2-Exon 1	gctgctgcactcatcctg	ctgtctctccagcgcaaag
PPP4R2-Exon 1	gctctgtcggtcttgctctc	aaccatccgccctgaaag
ZNF717-Exon 3	atgtgccattacaggggttg	tcagggactgtgcaatgaag
GBE1-Exon 1	tccgtcccggctataaagg	caaacctgccactacggaag
CADM2-Exon 1	agcaggaggaggaggagaag	gagactgggaggggagagtg
VGLL3- Exon 1	gaggggctgaagatgaaggt	gatggctttctgctcaagga
PROS1-Exon 1	tccaacactagagcccatcc	ctatccacggctgtttccat
ARL13B-Exon 1	ccaggactcttcagccactc	acaccctgggaatgaaagc
EPHA6-Exon 1	cccaaaccacagcccgag	ggagactctgacccctgtac
C3orf26-TRN1-Exon 1	aactgcttctgcctgtcg	caccattttccccgagatg
C3orf26-TRN2-Exon 1	atgacccttctttgacacc	aactcctcccttctatccaacc
TOMM70A-Exon 1	tccctcggtcttccttcg	cctaaacccaagtgttgtgc
PCNP-Exon 1	cttagaccgcccaaccttc	gtccagatccgcccattc
ZBTB11-Exon 1	ctaagggagccgtcgaagag	ctccgactcgtgggtacg
NFKBIZ-Exon 1	ctgcggcccgttaaatac	cggggagtggtaattgatgg
IFT7-Exon 1	ggtccaacacacctcaagc	ctaacccgctctcacgaaac
MORC1-Exon 1	attgtggctccaagaccaag	gagcacctaaagggttcagc
ABHD10-Exon 1	ctaaacgaaagagccacgac	gggaggagcatttttgaacc
C3orf52-Exon 1	actcacagttgcccctcctg	gactcggctccattcagag
CD200-Exon 1	gaaaacggagtgggagaagg	attgtggcaagagccgaag
ATG3-Exon 1	gcagcgaggacattttctg	actgccttcctacaccttgc
BOC-Exon 9	cactttgtcatgccgcttag	ccctggacctacagaagcaa
SIDT1-Exon 1	tatttgatcggcccttcgtc	cgaagtctcccaaggtcaat
IGSF11-T2-Exon 1	aggggtcggctagtcctg	gcccctgagaaagttggact
KTELC1-Exon 1	cacggtggccatctttgt	tccagagcaggagcagagac
PLA1A-Exon 1	ttggctcaaagagggacagt	ggcttttaggggatcttcca
C3orf15-Exon 1	ctcctccctcctccttgc	ggcaagaggtcagtccagag
NR1I2-Exon 2	actcccacctacacccttcc	cgcatctccacacaagcat
NR1I2-Exon 3	cttttgcctaacggcttctg	atccttggggaacctcagtt
NR1I2-Exon 7	tatggccttgctcctcattc	tgggaagtggttaggtagtgc
POLQ-Exon 1	tccttccccacgagtctatg	gggtatgcaacgaagcaagt

ARGFX-Exon 2	acgcctgaccttccaaactc	gtttgagaacagtctggaccac
EAF2-Exon 1	ggagcgccctttgctaata	ggggtactgtgggaacaaaa
ILDR1-Exon 1	ggttccttggagaaggtggt	gctccaggtttctcagttgc
ILDR1-Exon 7a	tgggagggattcatgctaac	gcttaggctgtccctgtctg
ILDR1-Exon 7b	caccgtagctctaggctgaa	accctctgtggtggaatgag
FAM162A-Exon 1	cacactccacaggaccatct	aagtgcgtcatggaatggag
DTX3L-Exon 1	cagggaagcgaaactgaaac	atactttctcccgccctctc
PARP15-Exon 1	acaatcctgaggcagctgtt	ccggaacagggaagtcagt
DIRC2-Exon 1	tgctgctcaggactattctgc	acctcacacaacccctatgc
SEMA5B-Exon 3	ccctgactttcacccttctg	gctgcccaggtgagttcat
SEMA5B-Exon 10	tcccaaagaagaggcatcc	agaccaaggtggccttgaa
SEMA5B-Exon 11	gcaggctaaagacacgctct	aggcacactagtggagactgc
SEMA5B-Exon 15	ctctcgtcctttccggtctt	agaggcagctttagaacccc
SEMA5B-Exon 20	cctacgtctgagcgttgacc	ggagcaggatgtggaaagag
PDIA5-Exon 1	ttctgtgagctgcgtgagat	agagggctggagcagagag
ADCY5-TRN1-Exon 1c	ttccgctccaagaagttccc	cattcgaaagggtgagggtg
ADCY5-TRN2-Exon 1	taagcagcctggtgtcttcc	gaggtgtccaagaaggtgga
PTPLB-Exon 1	cctctcctcgcgtagtcc	ccgggaatgcactgcctg
MYLK-Exon 17	cagagttggggaggagtttg	caggcaagagtgagtgacca
CCDC14-Exon 1	gccgtttattggcttctcc	cgtgttacgacgggaagatt
KALRN-TRN3-Exon 1	gcagtggctcccagtaagtc	acccttccctccctaccac
DCBLD2-Exon 1	agggacgaaggaggagtagg	atttgttcaggggccaagag
CPOX-Exon 1	ctcaatactccgggggtctg	gacccttttccctgtctcc
EIF4E3-Exon 5	ggatttggaggtgtctgtgg	agggcccagttaatgtgaga

CASR gene sequencing primers

Amplicon	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
CASR-Exon 1	aggggctagggacaaggata	ctgatataaggccagagattcac
CASR-Exon 2	gccaccttagttgcagtggt	ttattttgcgtttggtgcag
CASR-Exon 3	ccagctttgccaggtcttta	tttgtgccagagatgggaat
CASR-Exon 4a	aacagcctggaggctcact	gggatggaccttcttcagga
CASR-Exon 4b	gcaatatcacgggcaagat	gagttgcagcccaactctg
CASR-Exon 5	cagggcacagcctacctaat	agtccagtggggaaaccaa
CASR-Exon 6	gcccaaactcctccctctta	cttccatgggcttcactgac
CASR-Exon 7a	ccaccaccacatgtacactca	gcggtgtagagccagatcac
CASR-Exon 7b	tgtcctcctggtgtttgagg	cttgcgggagacgttgct

CASR-Exon 7c	tcattctcttcaagccatcc	cactacaagtgctggggaca
CASR-Exon 7d	tgatgagcctcagaagaacg	cctcagaggaaaggagtctgg
CASR cDNA cloning and insert primers		
Name	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
CASR-cloning	tggagagctagcgccaccatggcattttatagctgc	ctctctgcatctcgagtagcccagtcttctccttcc
CASR-cDNA-Insert-1	tatagctgctgctgggtcc	ggagtctgctggaggaggc
CASR-cDNA-Insert-2	gaaccatccccaatgatgag	ccttggcaaaaccattgtgg
CASR-cDNA-Insert-3	ctccaagaaggtgcaaaagg	tggagaacccactccacagg
CASR-cDNA-Insert-4	ggaccaggaaagggatcatt	tcttggcctcaaacaccagg
CASR-cDNA-Insert-5	ggttttcctctgcaccttca	ccttgaaagcgtgagctgc
CASR-cDNA-Insert-6	cctcctcctccatcagcag	ctgtaacagtgctgcctcc
Site-directed mutagenesis primers for *CASR* mutations		
Name	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
CASR-Glu354Ala	caaggagttttgggaagcaacatttaactgccacc	ggtggcagttaaatgttgcttcccaaaactccttg
CASR-Asp433His	cattgcccacgccttgcaacatatatatacctgcttacc	ggtaagcaggtatatatatgttgcaaggcgtggcaatg
CASR-Ser580Asn	gatgagacagatgccaatgcctgtaacaagtgc	gcacttgttacaggcattggcatctgtctcatc
CASR-Ile686Val	ccagccggcctttggcgtcagcttcgtgctctgc	gcagagcacgaagctgacgccaaaggccggctgg
CASR-Arg898Gln	gcaacgtctcccgcaagcagtccagcagccttggagg	cctccaaggctgctggactgcttgcgggagacgttgc
CASR-Ala988Val	cagaagaacgccatggtccacaggaattctacg	cgtagaattcctgtggaccatggcgttcttctg
CASR-Asn583X	gacagatgccagtgcctgttaacaagtgcccagatgac	gtcatctgggcacttgttaacaggcactggcatctgtc

A2.3 Reference DNA/protein sequence details of genes from different organisms (Gene, NCBI) used for multiple sequence alignment

***ABI3BP* (ABI family, member 3 (NESH) binding protein)**			
Organism	**Assembly**	**Location**	**Gene ID**
Homo sapiens (human)	GRCh37.p13	Chr3, NC_000003.11 (100468179..100712336, complement)	25890
Mus musculus (house mouse)	GRCm38.p2	Chr16, NC_000082.6 (56477801..56690135)	320712
Rattus norvegicus (Norway rat)	Rnor_5.0	Chr11, NC_005110.3 (49392633..49608013, complement)	363767
Bos taurus (cattle)	Bos_taurus_UMD_3.1	Chr1, AC_000158.1 (45521121..45822702, complement)	538604
Canis lupus familiaris (dog)	CanFam3.1	Chr33, NC_006615.3 (7265804..7498964, complement)	701192
Macaca mulatta (Rhesus monkey)	Mmul_051212	Chr2, NC_007859.1 (20931126..21174997, complement)	701192
Felis catus (domestic cat)	Felis_catus-6.2	ChrC2, NC_018731.1 (48273378..48537683, complement)	101099069
Equus caballus (horse)	EquCab2.0	Chr19, NC_009162.2 (54367719..54606937)	100062052
Sus scrofa (pig)	Sscrofa10.2	Chr13, NC_010455.4 (167676709..167833274)	100154641
Pan troglodytes (chimpanzee)	Pan_troglodytes-2.1.4	Chr3, NC_006490.3 (103908355..104005868, complement)	740047
***EPHA6* (EPH receptor A6)**			
Organism	**Assembly**	**Location**	**Gene ID**
Homo sapiens (human)	GRCh37.p13	Chr3, NC_000003.11 (96533425..97467786)	285220

Mus musculus (house mouse)	GRCm38.p2	Chr16, NC_000082.6 (59653061..60605531, complement)	13840
Rattus norvegicus (Norway rat)	Rnor_5.0	Chr11, NC_005110.3 (45066461..46035688)	29202
Macaca mulatta (Rhesus monkey)	Mmul_051212	Chr2, NC_007859.1 (17187421..18116150)	696491
Pan troglodytes (chimpanzee)	Pan_troglodytes-2.1.4	Chr3, NC_006490.3 (99880733..100844873)	460538
Equus caballus (horse)	EquCab2.0	Chr19, NC_009162.2 (57079023..57740095, complement)	100072575
Canis lupus familiaris (dog)	CanFam3.1	Chr33, NC_006615.3 (4049650..4786776)	607935
Felis catus (domestic cat)	Felis_catus-6.2	ChrC2, NC_018731.1 (44887945..45604483)	101087597
Bos taurus (cattle)	Bos_taurus_UMD_3.1	Chr1, AC_000158.1 (40608642..41624566)	100336601
***IQCB1* (IQ motif containing B1)**			
Organism	**Assembly**	**Location**	**Gene ID**
Homo sapiens (human)	GRCh37.p13	Chr3, NC_000003.12 (121769763..121835079, complement)	9657
Mus musculus (house mouse)	GRCm38.p2	Chr16, NC_000082.6 (36828360..36872719)	320299
Rattus norvegicus (Norway rat)	Rnor_5.0	Chr11, NC_005110.3 (69914664..69969813, complement)	303915
Sus scrofa (pig)	Sscrofa10.2	Chr13, NC_010455.4 (148308751..148368283)	100521475
Bos taurus (cattle)	Bos_taurus_UMD_3.1	Chr1, AC_000158.1 (66854937..66900509, complement)	534598
Equus caballus (horse)	EquCab2.0	Chr19, NC_009162.2 (37498764..37544038)	100060805
Macaca mulatta (Rhesus monkey)	Mmul_051212	Chr2, NC_007859.1 (41759490..41833861, complement)	715032
Pan troglodytes (chimpanzee)	Pan_troglodytes-2.1.4	Chr3, NC_006490.3 (125140426..125205577, complement)	745284

Canis lupus familiaris (dog)	CanFam3.1	Chr33, NC_006615.3 (25046985..25113249, complement)	478582

KIAA1407 (coiled-coil domain-containing protein KIAA1407)

Organism	**Assembly**	**Chromsome; Protein reference ID**	**Gene ID**
Homo sapiens (human)	GRCh37.p13	Chr3; NP_065868.1	57577
Rattus norvegicus (Norway rat)	Rnor_5.0	Chr11; XP_006248381.1	288106
Equus caballus (horse)	EquCab2.0	Chr19; XP_001502864.2	100061193
Macaca mulatta (Rhesus monkey)	Mmul_051212	Chr2; XP_001107271.1	710219
Gorilla gorilla (western gorilla)	gorGor3.1	Chr3; XP_004036134.1	101129982
Pan troglodytes (chimpanzee)	Pan_troglodytes-2.1.4	Chr3; XP_516659.2	460594
Cricetulus griseus (Chinese hamster)	CriGri_1.0	Unplaced Scaffold; XP_007617085.1	100773871
Xenopus (Silurana) tropicalis (western clawed frog)	Xtropicalis_v7	Unplaced Scaffold; XP_004912333.1	100493616
Canis lupus familiaris (dog)	CanFam3.1	Chr33; XP_005639564.1	487982
Loxodonta africana (African savanna elephant)	Loxafr3.0	Unplaced Scaffold; XP_003412902.1	100664267
Capra hircus (Goat)	CHIR_1.0	Chr1; XP_005674998.1	102187529
Bos taurus (cattle)	Bos_taurus_UMD_3.1	Chr1; XP_002684801.1	532832
Gallus gallus (chicken)	Gallus_gallus-4.0	Chr1; XP_416572.3	418352

***CASR* (calcium-sensing receptor)**			
Organism	**Assembly**	**Chromsome; Protein reference ID**	**Gene ID**
Homo sapiens (human)	GRCh37.p13	Chr3; NP_000379.2	846
Mus musculus (house mouse)	GRCm38.p2	Chr16; NP_038831.2	12374
Rattus norvegicus (Norway rat)	Rnor_5.0	Chr11; NP_058692.1	24247
Bos taurus (cattle)	Bos_taurus_UMD_3.1	Chr1; NP_776427.1	281038
Danio rerio (zebrafish)	GRCz10	Chr5; XP_689097.1	560607
Equus caballus (horse)	EquCab2.0	Chr19; NP_001157450.1	100034084
Canis lupus familiaris (dog)	CanFam3.1	Chr33; NP_001074978.1	488007
Gallus gallus (chicken)	Gallus_gallus-4.0	Chr1; XP_416491.3	418266
Ovis aries (sheep)	Oar_v3.1	Chr1; XP_004003020.1	101112527
Felis catus (domestic cat)	Felis_catus-6.2	ChrC2; NP_001158126.1	100302732
Macaca mulatta (Rhesus monkey)	Mmul_051212	Chr2; XP_001112009.2	714441
Pan troglodytes (chimpanzee)	Pan_troglodytes-2.1.4	Chr3; XP_516689.2	460628
Gorilla gorilla (western gorilla)	gorGor3.1	Chr3; XP_004036216.1	101140602
Sus scrofa (pig)	Sscrofa10.2	Chr13; NP_001265677.1	100520980
Pongo abelii (Sumatran orangutan)	P_pygmaeus_2.0.2	Chr3; XP_002813271.1	100459602
Ceratotherium simum simum (southern white rhinoceros)	CerSimSim1.0	Unplaced Scaffold; XP_004436566.1	101397437
Cavia porcellus (domestic guinea pig)	Cavpor3.0	Unplaced Scaffold; XP_005008567.1	100718111

Ailuropoda melanoleuca (giant panda)	AilMel_1.0	Unplaced Scaffold; XP_002927270.1	100483630
Meleagris gallopavo (wild turkey)	Turkey_2.01	Chr1; XP_003202678.1	100545558
Callithrix jacchus (white-tufted-ear marmoset)	Callithrix jacchus-3.2	Chr15; XP_002807659.2	100406413
Dasypus novemcinctus (nine-banded armadillo)	Dasnov3.0	Unplaced Scaffold; XP_004467230.1	101412395
Odobenus rosmarus divergens (Pacific walrus)	Oros_1.0	Unplaced Scaffold; XP_004392009.1	101385726
Orcinus orca (killer whale)	Oorc_1.1	Unplaced Scaffold; XP_004278606.1	101289504
Anolis carolinensis (green anole)	AnoCar2.0	Unplaced Scaffold; XP_008113475.1	100561507
Taeniopygia guttata (zebra finch)	Taeniopygia_guttata-3.2.4	Chr1; XP_002193647.1	100223057
Anas platyrhynchos (wild duck)	BGI_duck_1.0	Unplaced Scaffold; XP_005028444.1	101794634

A2.4 Known/common *CASR* variations observed in 480 GGE/JME patients

Position on chr 3[a]	Position of nucleotide change[b]	Location/type	Position of amino acid change[b]	dbSNP ID	Status in databases (MAF)[c]	Status in control cohort (MAF)[d]
121902627	c.-275C>G	5' UTR	_	rs533697004	G = 0.001	G = 0.006
121973114	c.78C>G	Synonymous	Ala26=	rs77852524	G = 0.011	_
121976253	c.492+19G>A	Intron	_	rs9869985	G = 0.078	G = 0.1
121980455	c.573G>A	Synonymous	Glu191=	rs141631116	A = 0.001	A = 0.008
121994941	c.1608+52G>A	Intron	_	rs2279802	A = 0.209	A = 0.25
122001099	c.1732+16T>C	Intron	_	rs2270916	C = 0.179	C = 0.05
122002576	c.1775A>G	Nonsynonymous	Asn592Ser	rs117375173	G = 0.007	_
122003045	c.2244G>C	Synonymous	Pro748=	rs2036400	G = 0.025	G = 0.0
122003757	c.2956G>T	Nonsynonymous	Ala986Ser	rs1801725	T = 0.076	T = 0.19
122003769	c.2968A>G	Nonsynonymous	Arg990Gly	rs1042636	G = 0.207	G = 0.23
122003832	c.3031.G>C	Nonsynonymous	Glu1011Gln	rs1801726	G = 0.078	G = 0.05
122004098	c.*60A>T	3' UTR	_	rs4677948	A = 0.078	A = 0.06

[a]Genomic position of the nucleotide base on chromosome 3 (chr 3) (GRCh37, NCBI). [b]The nucleotide and amino acid residue position of the variations in *CASR* is according its shorter transcript encoding for receptor of 1078 amino acids (NM_000388.3, NP_000379.2). [c]Minor allele frequencies (MAF) for the variation given in the dbSNP or 1000 genomes databases. [d]Minor allele frequencies (MAF) calculated in 252 individuals of control cohort.

Appendix II

A3.1 Known/common variations observed in exome sequencing data of individuals II:5 and III:6 (GLH5) in the 5q12-q14 region

Gene	Position on chr5[a]	Variant alleles	Het/ Hom	Location/ type	Position of amino acid change[b]	dbSNP ID
ZSWIM6	60839907	C/A	Het	Synonymous	Pro1137=	rs16892374
C5orf64	60982841	G/A	Het	Missense	Ala57Thr	rs16893687
	60999768	C/T	Het	Missense	Arg101Trp	rs436696
KIF2A	61642937	T/C	Het	Intron	_	rs3213940
	61643816	G/A	Het	Intron	_	rs3776615
	61648618	G/A	Het	Intron	_	rs55951418
	61654187	G/C	Het	Intron	_	rs35009
	61657228	T/C	Het	Intron	_	rs247265
	61657377	A/T	Hom	Intron	_	rs247266
	61673670	A/G	Het	Intron	_	rs464058
	61676902	G/T	Het	Intron	_	rs247253
	61676911	T/G	Hom	Intron	_	rs247254
DIMT1	61684883	A/-	Het	Intron	_	rs35130331
	61688892	G/A	Het	Intron	_	rs35014
	61689741	T/-	Hom	Intron	_	rs3215345
	61694379	T/C	Hom	Intron	_	rs247264
	61697859	C/T	Hom	Intron	_	rs27089
	61699231	A/C	Het	Intron	_	rs27693
	61699451	C/T	Hom	Intron	_	rs26649
IPO11	61715040	C/T	Het	Intron	_	rs152208
	61745738	T/-	Het	Intron	_	rs11329383
	61785936	T/C	Het	Intron	_	rs26644
	61826663	G/T	Het	Intron	_	rs32179
HTR1A	63257253	C/T	Het	Synonymous	Val98=	rs6294
RNF180	63496825	A/G	Het	Intron	_	rs72769844
	63509349	G/A	Het	Intron	_	rs12519901
	63665448	G/A	Het	Missense	Arg529His	rs76090587
RGS7BP	63905068	A/G	Hom	Missense	Ile255Val	rs889248
FAM159B	63986351	G/C	Hom/Het	5'UTR	_	rs10471637
	63991473	G/C	Hom/Het	3'UTR	_	rs2305962
SREK1IP1	64023924	C/A	Hom	Intron	_	rs10471638

	64023981	C/T	Hom	Synonymous	Lys77=	rs275819
	64064411	G/C	Het	5'UTR	_	rs3756739
CWC27	64064943	G/A	Het	5'UTR	_	rs1363953
	64070617	>6bp ins	Hom	Intron	_	rs140062546
	64084747	T/-	Hom	Intron	_	rs11340181
	64096047	T/-	Hom	Intron	_	rs79929128
	64096170	G/T	Hom/Het	Intron	_	rs6449760
	64097145	C/G	Het	Missense	Pro256Ala	rs7735338
	64267431	A/G	Hom/Het	Intron	_	rs2278352
	64267595	T/C	Hom/Het	Synonymous	Leu370=	rs2278351
	64273018	G/A	Het	Synonymous	Thr403=	rs1309581
ADAMTS6	64447777	C/T	Het	Intron	_	rs17206779
	64747918	_/G	Het	Intron	_	rs3830368
	64766520	TT/-	Het	Intron	_	rs537077807
	64766559	ATT/-	Het	Intron	_	rs34345975
CENPK	64814175	T/G	Het	3'UTR	_	rs11952932
	64814189	7 bp del	Het	3'UTR	_	rs3050538
	64825102	C/T	Hom	Intron	_	rs1017576
	64838584	G/A	Het	Intron	_	rs6891597
	64838758	G/C	Het	Intron	_	rs6891955
	64847543	T/C	Hom	Intron	_	rs6893491
	64848412	C/T	Het	Intron	_	rs3213939
	64848442	T/C	Hom	Intron	_	rs3213938
	64850663	T/A	Het	Synonymous	Leu24=	rs201745679
PPWD1	64863285	A/C	Het	Intron	_	rs3756301
	64863484	_/A	Hom	Intron	_	rs531527135
	64865405	A/G	Hom	Intron	_	rs149208
	64865658	T/C	Het	Intron	_	rs3752673
	64865848	A/G	Hom/Het	Intron	_	rs3752674
	64865921	C/A	Hom/Het	Intron	_	rs13167451
	64881936	A/G	Hom	3'UTR	_	rs27141
TRIM23	64886375	A/-	Hom	3'UTR	_	rs3836739
	64890479	A/C	Hom	Intron	_	rs36135
	64892939	T/G	Hom/Het	Synonymous	Gly416=	rs33945461
	64906617	T/A	Hom	Intron	_	rs37330
	64913841	T/C	Hom/Het	Intron	_	rs11952500
	64919991	G/A	Hom	Intron	_	rs154854

	64920230	T/C	Hom	5'UTR	_	rs71626590
C5orf44	64920803	C/T	Hom	5'UTR	_	rs37254
	64933444	C/G	Hom/Het	Intron	_	rs71626594
	64933505	A/G	Het	Intron	_	rs11741124
	64933653	_/T	Hom/Het	Intron	_	rs200547399
	64942799	A/G	Hom	Intron	_	rs154955
	64942852	_/T	Het	Intron	_	rs369270319
	64954159	A/G	Het	Intron	_	rs28084
	64960311	4bp ins	Hom	Intron	_	rs71789095
SGTB	64965900	T/C	Het	3'UTR	_	rs1549192
	64968171	T/-	Hom	Intron	_	rs5868414
	64976687	C/T	Hom	Intron	_	rs3213798
	65008947	T/-	Hom	Intron	_	rs3833969
	65016452	A/G	Hom	Intron	_	rs9291846
NLN	65073224	C/A	Hom	Intron	_	rs34979
	65083865	G/A	Het	Intron	_	rs6862580
	65105622	G/A	Hom	Intron	_	rs2561196
	65108215	A/G	Hom	Synonymous	Pro659=	rs2254485
ERBB2IP	65288791	A/G	Hom	Intron	_	rs27132
	65308055	C/G	Het	Intron	_	rs706678
	65317181	C/T	Hom	Synonymous	Leu189=	rs706679
MAST4	66195701	C/T	Het	Intron	_	rs458115
	66416764	C/A	Het	Intron	_	rs2254790
	66429329	G/T	Hom	Intron	_	rs2371882
	66459878	G/C	Hom	Missense	Arg1624Pro	rs1705399
CD180	66480004	T/C	Hom	Missense	Ile223Val	rs1697144
PIK3R1	67522722	C/T	Het	Synonymous	Tyr73=	rs706713
	67522851	A/C	Het	Intron	_	rs706714
	67569391	A/C	Het	Intron	_	rs2302974
	67575344	A/G	Het	Intron	_	rs16897620
	67575642	A/G	Het	Intron	_	rs3815701
	67587994	C/T	Het	Intron	_	rs3730087
	67588000	A/G	Het	Intron	_	rs3730088
	67589188	C/T	Het	Synonymous	Phe392=	rs3730090
SLC30A5	68409094	A/C	Het	Intron	_	rs164393
	68411010	C/T	Het	Intron	_	rs240809
	68412048	_/T	Het	Intron	_	rs34215592

	68413043	T/-	Het	Intron	_	rs3840519
	68417643	C/T	Het	Synonymous	His564=	rs164578
	68417755	A/G	Het	Intron	_	rs164577
	68419054	A/T	Het	Synonymous	Thr600=	rs164572
CCNB1	68463014	G/T	Hom/Het	5'UTR	_	rs164390
	68464234	A/G	Hom/Het	Intron	_	rs163443
CENPH	68491714	>6bp ins	Het	Intron	_	rs11283275
	68504250	G/A	Het	Intron	_	rs2972360
MRPS36	68524006	_/T	Hom/Het	Intron	_	rs113292549
	68524133	A/G	Het	Synonymous	Pro71=	rs2972359
	68525027	C/T	Hom/Het	Intron	_	rs2932777
CDK7	68531126	G/A	Het	Intron	_	rs2972389
	68531253	C/T	Hom/Het	Synonymous	Asn33=	rs2972388
	68548351	T/A	Hom/Het	Intron	_	rs2972365
	68564986	A/-	Het	Intron	_	rs59967147
CCDC125	68578428	T/C	Hom/Het	3'UTR	_	rs4421064
	68602746	G/C	Hom/Het	Intron	_	rs6881767
	68609885	G/T	Hom/Het	Intron	_	rs11749723
TAF9	68665736	T/C	Hom	5'UTR		rs3756400
	68665809	T/C	Hom	5'UTR		rs3756399
RAD17	68692375	_/A	Het	Intron	_	rs377737971
	68695940	T/G	Hom/Het	Missense	Leu546Arg	rs1045051
	68696056	T/-	Het	Intron	_	rs368374295
	68696063	_/A	Het	Intron	_	rs371519612
	68706252	G/T	Hom	Intron	_	rs10940223
	68706438	G/A	Hom	Intron	_	rs6871685
MARVELD2	68715310	C/T	Hom	Missense	Thr33Ile	rs1185246
GTF2H2C	68862362	G/T	Het	Intron	_	rs58303754
	68862365	G/T	Het	Intron	_	rs59898528
	68868255	C/T	Hom	Intron	_	rs201020620
	68874621	C/T	Het	Syn	Leu185Leu	rs233198
SERF1B	69321147	G/C	Hom	5'UTR	_	rs77297406
SMN2	69362949	A/G	Hom/Het	Synonymous	Gln154Gln	rs4915
	69366414	C/T	Het	Intron	_	rs62374808
SERF1A	70196565	G/C	Hom	5'UTR	_	rs77297406
SMN1	70238373	A/G	Hom/Het	Synonymous	Gln154=	rs4915
	70241839	C/T	Het	Intron	_	rs62374808

NAIP	70275668	A/C	Het	Intron	_	rs154360
	70279502	G/A	Het	Intron	_	rs33537
	70283607	C/T	Het	Intron	_	rs150363
	70298058	C/T	Het	Intron	_	rs2568349
	70308251	C/T	Hom/Het	Synonymous	Ala164=	rs28409706
GTF2H2	70344903	G/A	Het	Synonymous	Leu185=	rs199999389
	70357186	C/A	Het	Intron	_	rs199839332
	70357189	C/A	Het	Intron	_	rs201185853
BDP1	70751818	T/G	Hom	Missense	Asp38Glu	rs3748043
	70763088	A/G	Hom	Intron	_	rs7442961
	70786727	_/AC	Hom	Intron	_	rs10641531
	70797501	_/A	Het	Intron	_	rs11441119
	70798541	A/G	Het	Missense	Lys722Glu	rs36009281
	70800538	G/A	Hom	Missense	Val778Met	rs3761967
	70805273	C/-	Hom	Intron	_	rs36098798
	70806457	G/A	Hom	3'UTR	_	rs715748
	70806649	T/A	Hom	3'UTR	_	rs1961760
	70806711	C/G	Hom	3'UTR	_	rs715747
	70806958	G/A	Hom	3'UTR	_	rs6886336
	70807991	G/A	Hom	Intron	_	rs418738
	70809169	A/G	Hom	5'UTR	_	rs1698063
	70809270	T/-	Hom/Het	Intron	_	rs34587827
	70811821	A/G	Hom	Intron	_	rs279322
	70834833	G/A	Hom	Intron	_	rs276590
	70837295	A/C	Hom	Missense	Ile2013Leu	rs6453014
	70840233	C/T	Hom	Synonymous	Leu2154=	rs182190
	70840433	A/G	Hom	Intron	_	rs279290
	70849021	G/T	Hom	Missense	Pro2358=	rs469039
	70860493	T/C	Hom	Intron	_	rs277944
MCCC2	70898466	_/C	Hom	Intron	_	rs200382661
	70922616	G/A	Hom	Intron	_	rs12516456
	70927856	C/T	Hom/Het	Intron	_	rs277980
	70928083	G/A	Hom/Het	Intron	_	rs277981
	70945075	A/G	Hom	Synonymous	Ala456=	rs10064079
	70948654	G/A	Hom	Intron	_	rs2242372
	70952506	A/G	Hom	Intron	_	rs7443786
MAP1B	71411718	T/C	Het	Intron	_	rs1531312

	71490962	A/G	Hom	Missense	Ile594Val	rs1866374
	71495041	T/C	Hom	Synonymous	Gly1953=	rs3805452
MRPS27	71522125	A/T	Hom	Intron	_	rs9293821
ZNF366	71739546	C/G	Hom	3'UTR	_	rs4267851
	71739602	G/C	Het	Missense	Ala739Gly	rs13188519
	71742979	T/C	Het	Intron	_	rs72761172
	71743024	G/C	Hom	Intron	_	rs10043295
	71743322	G/A	Het	Intron	_	rs10043368
	71756102	G/A	Het	Synonymous	Leu408=	rs7721922
	71756670	C/T	Het	Synonymous	Glu218=	rs2278600
	71757266	T/C	Het	Missense	Lys20Glu	rs74460472
	71757267	C/T	Het	Synonymous	Val19=	rs78980395
TNPO1	72112421	T/C	Hom	5'UTR	_	rs34648
	72112430	C/G	Hom	5'UTR	_	rs34647
	72182857	A/G	Het	Intron	_	rs4704045
	72184098	C/T	Het	3'UTR	_	rs17606
	72201058	G/A	Het	Intron	_	rs250518
FCHO2	72348272	A/G	Het	Missense	Met371Val	rs185435
	72364673	A/G	Het	Intron	_	rs470926
	72383955	A/C	Het	3'UTR	_	rs201025785
TMEM171	72419041	T/C	Hom/Het	Intron	_	rs7448965
	72419410	A/G	Hom	Synonymous	Gly70=	rs7731777
	72419456	C/G	Het	Missense	Arg86Gly	rs637450
	72419617	C/A	Het	Missense	Asn139Lys	rs636926
BTF3	72798502	TT/-	Hom/Het	Intron	_	rs200370518
	72798724	T/G	Het	Intron	_	rs347233
ANKRA2	72850282	A/G	Hom	Intron	_	rs343104
UTP15	72872766	C/T	Hom	Intron	_	rs1220622
	72873925	G/A	Hom	Intron	_	rs7443182
	72874801	G/A	Het	Intron	_	rs12652195
	72875635	C/T	Hom	Intron	_	rs10942676
ARHGEF28	73069580	C/T	Het	Intron	_	rs79391401
	73069581	A/G	Het	Intron	_	rs4704097
	73072354	T/C	Het	Missense	Trp225Arg	rs7714670
	73076457	G/A	Het	Intron	_	rs11949860
	73076511	C/A	Hom/Het	Missense	Pro284Gln	rs6453022
	73090165	_/GTT	Hom/Het	Intron	_	rs10683146

	73090261	T/C	Hom/Het	Synonymous	Ala315=	rs7716253
	73091126	T/A	Hom/Het	Intron	_	rs2973549
	73091228	C/T	Hom/Het	Intron	_	rs2973548
	73142380	A/G	Het	Intron	_	rs13161779
	73144845	A/G	Het	Synonymous	Ser560=	rs2973568
	73153390	4bp del	Het	Intron	_	rs10543482
	73160730	G/A	Hom/Het	Intron	_	rs1014550
	73163861	C/T	Hom	Intron	_	rs2931422
	73179672	C/T	Het	Synonymous	Tyr1006=	rs3749645
	73190119	T/A	Het	Intron	_	rs2973530
	73190174	A/G	Het	Intron	_	rs34729826
	73197185	A/C	Het	Intron	_	rs434065
	73197200	G/A	Het	Intron	_	rs384927
	73207372	T/A	Het	Missense	His1640Gln	rs1478453
ENC1	73930442	C/T	Hom	Intron	_	rs300240
	73930751	C/T	Hom	Synonymous	Lys520Asn	rs300239
	73931246	T/C	Hom	Synonymous	Ser355=	rs442425
	73932315	T/C	Hom	5'UTR	_	rs9176
HEXB	73981270	T/C	Hom	Missense	Ser62Leu	rs820878
	73992609	G/A	Hom	Intron	_	rs1665894
	73992881	A/G	Het	5'UTR	_	rs10805890
	74009327	A/G	Het	Intron	_	rs17561000
	74017081	TG/-	Het	3'UTR	_	rs35491723
GFM2	74017312	C/T	Het	5'UTR	_	rs79357178
	74028753	>6bp del	Het	Intron	_	rs367655807
FAM169A	74097345	CA/-	Het	Intron	_	rs149496756
	74097381	A/C	Hom/Het	Intron	_	rs73116717
ANKRD31	74364300	G/A	Homo	3'UTR	_	rs10942729
	74376309	A/C	Homo	Intron	_	rs4447963
	74400386	G/C	Homo	Missense	Asp1609Glu	rs961098
	74433839	_/T	Het	Intron	_	rs5868765
	74442410	G/A	Hom	Synonymous	Phe942=	rs1422699
	74442920	A/G	Hom	Synonymous	Thr772=	rs6888707
	74443132	C/T	Hom	Missense	Asp702Asn	rs1422698
	74484275	C/T	Hom	Intron	_	rs10050948
	74491716	TCA/-	Hom	Inframe	Met252del	rs10563854
	74506658	C/T	Hom	Synonymous	Leu107=	rs2219745

	74526490	T/-	Hom	Intron	_	rs11295265
HMGCR	74641560	G/A	Hom/Het	Intron	_	rs10515198
	74646878	C/T	Hom/Het	Intron	_	rs11742194
	74651084	A/G	Hom/Het	Intron	_	rs3846662
	74654512	G/C	Het	Missense	Glu673Gln	rs200606736
	74655726	C/T	Hom/Het	Intron	_	rs3846663
	74656175	G/A	Hom/Het	3'UTR	_	rs5909
COL4A3BP	74712924	T/C	Het	Intron	_	rs190200142
ANKDD1B	74951901	G/A	Hom/Het	Synonymous	Thr315=	rs7717355
	74965122	G/A	Het	Nonsense	Trp480Ter	rs34358
POC5	74984818	A/T	Hom/Het	Intron	_	rs17563686
	74986481	T/C	Hom/Het	Intron	_	rs4704230
	74988369	A/G	Hom/Het	Intron	_	rs888789
	74998334	G/A	Hom/Het	Intron	_	rs17563863
	74998598	G/A	Hom/Het	Synonymous	His115=	rs35130836
	75001432	A/G	Hom/Het	Intron	_	rs4704234
	75003469	T/C	Hom/Het	Intron	_	rs73126731
	75003678	T/C	Hom/Het	Missense	His36Arg	rs2307111
	75008193	T/C	Hom/Het	Intron	_	rs2047059
SV2C	75427518	C/T	Het	5'UTR	_	rs1423099
	75427935	G/A	Het	Synonymous	Arg120=	rs10070440
IQGAP2	75757556	C/T	Het	Intron	_	rs10045155
	75884734	G/A	Het	Synonymous	Leu154=	rs1131232
	75886168	A/G	Het	Intron	_	rs2270907
	75888788	G/A	Het	Intron	_	rs3736394
	75907065	A/G	Het	Intron	_	rs3797390
	75913305	T/C	Het	3'UTR	_	rs2069685
	75923141	G/A	Het	Intron	_	rs2455221
	75923285	C/T	Het	Synonymous	Ala524=	rs2431351
	75923294	T/G	Hom	Missense	Asp527Glu	rs2431352
	75923307	A/G	Hom	Missense	Glu532Lys	rs2909888
	75923393	T/C	Het	Intron	_	rs2431353
	75927595	C/G	Het	Intron	_	rs431160
	75927606	C/T	Het	Intron	_	rs3797409
	75927635	G/A	Het	Intron	_	rs3797410
	75927938	A/T	Het	Intron	_	rs441157
	75932869	C/T	Het	Synonymous	Asp597=	rs2910819

	75932965	G/C	Het	Missense	Leu629Phe	rs2455230
	75933097	C/T	Het	Intron	_	rs2937415
	75936944	G/A	Het	Intron	_	rs457821
	75948650	A/G	Het	Missense	Ile724Val	rs2431363
	75948685	G/A	Het	Intron	_	rs2431362
	75950193	A/G	Het	Intron	_	rs462307
	75950707	G/A	Het	Intron	_	rs465731
	75954537	C/T	Het	Intron	_	rs4704347
	75954577	A/G	Het	Intron	_	rs4704348
	75960825	G/A	Het	Intron	_	rs253092
	75960865	T/C	Hom	Synonymous	His848Gln	rs253093
	75964702	G/A	Het	Intron	_	rs3764935
	75967862	A/G	Het	Intron	_	rs35919076
	75969747	A/G	Het	Intron	_	rs41271836
	75989353	G/A	Het	Intron	_	rs189347715
	75991420	C/T	Het	Missense	Arg1329Trp	rs17681908
	75996813	C/T	Het	Intron	_	rs73127576
	76003254	A/T	Hom/Het	3'UTR	_	rs463188
	76003258	C/T	Hom/Het	3'UTR	_	rs464494
F2R	76028124	A/T	Het	Intron	_	rs168753
F2RL1	76128521	G/A	Hom	Missense	Asn30Ser	rs616235
	76129053	T/C	Hom	Synonymous	Ile207=	rs631465
S100Z	76171063	G/A	Het	Intron	_	rs1320309
	76171252	A/C	Het	Missense	Glu23Ala	rs1320308
	76173727	G/C	Het	Intron	_	rs1320307
CRHBP	76259350	T/C	Het	Intron	_	rs7728378
AGGF1	76326885	G/A	Het	Intron	_	rs2278241
	76330401	G/A	Het	Intron	_	rs1428355
	76343999	T/C	Het	Synonymous	Ile405=	rs13155212
	76359090	_/A	Het	3'UTR	_	rs34239222
PDE8B	76621612	A/T	Hom	Intron	_	rs4133658
	76704849	T/C	Hom	Synonymous	Pro402=	rs186753
	76707438	A/T	Het	Intron	_	rs35864749
	76708155	GA/-	Het	Intron	_	rs111569967
	76708987	A/G	Hom/Het	Synonymous	Glu588=	rs335614
	76717583	A/G	Hom/Het	Intron	_	rs13160107
	76722443	G/A	Hom/Het	3'UTR	_	rs40594

WDR41	76728837	T/C	Het	3'UTR	_	rs335631
	76729193	G/C	Het	Intron	_	rs462581
	76733256	G/A	Het	Intron	_	rs116738892
	76734084	C/T	Hom/Het	Missense	Val329Ile	rs33204
	76734260	A/G	Het	Intron	_	rs33205
	76736621	G/A	Het	Intron	_	rs2560077
	76736907	G/C	Hom/Het	Intron	_	rs2560076
	76747087	T/C	Hom/Het	Intron	_	rs335609
	76760495	T/C	Hom/Het	Intron	_	rs410620
	76760657	_/A	Hom/Het	Intron	_	rs11433376
	76785406	_/G	Het	Intron	_	rs56195720
	76787919	C/G	Hom/Het	Intron	_	rs42764
TBCA	77004031	A/T	Hom/Het	Intron	_	rs9791124
	77004229	A/G	Hom/Het	Intron	_	rs9791128
	77004268	C/G	Hom/Het	Intron	_	rs78984548
AP3B1	77425028	A/T	Het	Missense	Val585Glu	rs6453373
	77524068	_/A	Het	Intron	_	rs5868908
	77524091	A/G	Het	Intron	_	rs4499809
	77563263	G/A	Het	Intron	_	rs10065892
SCAMP1	77755035	T/C	Hom	Intron	_	rs6453391
LHFPL2	77784542	C/T	Het	3'UTR	_	rs11740697
	77784696	G/A	Het	3'UTR	_	rs17194404
	77784738	A/G	Hom	Synonymous	Asn223=	rs2241566
ARSB	78135201	C/T	Het	Synonymous	Pro397=	rs25413
	78135276	T/G	Het	Intron	_	rs25415
	78181423	C/T	Het	Missense	Val376Met	rs1071598
	78181477	C/T	Het	Missense	Val358Leu	rs1065757
	78251347	A/G	Het	Intron	_	rs6870443
	78265041	A/G	Het	Intron	_	rs3733895
	78265092	C/T	Het	Intron	_	rs918581
	78265096	C/T	Het	Intron	_	rs918580
DMGDH	78320085	T/C	Het	Intron	_	rs2303128
	78340062	T/C	Hom	Intron	_	rs248384
	78340257	C/G	Hom	Synonymous	Leu288=	rs248385
	78340286	A/G	Hom	Missense	Ser279Pro	rs532964
	78340411	T/C	Het	Intron	_	rs531982
	78351582	AT/-	Het	Intron	_	rs3215302

	78351607	G/C	Het	Intron	_	rs2272038
	78351636	A/C	Hom	Synonymous	Gly124=	rs2253262
BHMT2	78373431	C/T	Het	Synonymous	Asp54=	rs682985
	78411822	C/G	Het	Intron	_	rs619100
	78416416	C/T	Het	Intron	_	rs567754
	78416974	A/-	Het	Intron	_	rs112263466
	78421780	G/A	Hom	Intron	_	rs694290
	78421959	G/A	Het	Missense	Arg239Gln	rs3733890
	78426729	C/G	Het	Intron	_	rs73769979
JMY	78533584	G/C	Hom/Het	Intron	_	rs72764988
	78573790	A/T	Het	Missense	Met364Leu	rs13182512
	78573970	T/C	Het	Intron	_	rs16876598
	78596044	T/C	Het	Synonymous	Asp532=	rs10514159
	78608355	T/-	Het	Intron	_	rs35852788
HOMER1	78692796	G/A	Het	Intron	_	rs2201400
	78742980	G/A	Het	Intron	_	rs6862358
PAPD4	78952691	G/A	Het	Intron	_	rs67039976
CMYA5	79024722	T/C	Het	Intron	_	rs61465432
	79024734	A/G	Het	Intron	_	rs1541813
	79024779	A/G	Het	Missense	Tyr64Cys	rs16877109
	79025317	A/G	Het	Synonymous	Gln243=	rs1366270
	79025634	G/A	Het	Missense	Gly349Asp	rs1366271
	79026360	G/A	Het	Missense	Gly591Asp	rs16877124
	79026539	A/C	Het	Missense	Ser651Arg	rs57544556
	79027605	T/C	Het	Missense	Val1006Ala	rs6893869
	79027700	T/C	Het	Missense	Phe1038Leu	rs62621915
	79028472	C/T	Het	Missense	Ala1295Val	rs4704585
	79028513	A/G	Het	Missense	Ile1309Val	rs16877133
	79028586	C/T	Het	Missense	Ala1333Val	rs16877135
	79029288	C/A	Het	Missense	Ala1567Glu	rs1428223
	79029383	T/G	Het	Missense	Ser1599Ala	rs1428224
	79029726	T/A	Het	Missense	Ile1713Asn	rs16877141
	79029749	A/G	Het	Missense	Ile1721Val	rs1428225
	79030045	A/G	Het	Synonymous	Val1819=	rs4639193
	79030212	C/T	Het	Missense	Ala1875Val	rs16877147
	79030338	A/G	Het	Missense	Asp1917Gly	rs16877150
	79030346	A/G	Het	Missense	Ser1920Gly	rs16877151

	79031372	G/C	Het	Missense	Val2262Leu	rs6859595
	79032666	C/T	Het	Missense	Thr2693Ile	rs28362541
	79032711	G/A	Het	Missense	Arg2708His	rs28362542
	79033306	A/C	Het	Missense	Lys2906Asn	rs2278239
	79034662	C/G	Het	Missense	His3358Gln	rs3828611
	79048562	C/T	Het	Synonymous	Phe3685=	rs1366272
	79084949	G/C	Het	Intron	_	rs2302979
	79086903	G/A	Het	Intron	_	rs10052867
	79095145	T/G	Het	Intron	_	rs3749683
MTX3	79282798	G/C	Het	Missense	Ser238Arg	rs9293796
THBS4	79351544	G/A	Hom	Intron	_	rs412379
	79351735	C/T	Het	Synonymous	Ser140=	rs423906
	79351852	A/T	Hom	Synonymous	Pro179=	rs438042
	79351859	G/A	Hom	Intron	_	rs405482
	79351860	G/A	Hom	Intron	_	rs447875
	79354147	C/T	Hom	Intron	_	rs368936
	79354647	C/G	Hom	Intron	_	rs432267
	79354702	C/T	Hom	Intron	_	rs411943
	79355192	T/A	Hom	Intron	_	rs2434302
	79355471	C/T	Het	Intron	_	rs401302
	79361265	G/C	Het	Missense	Ala387Pro	rs1866389
	79366080	T/C	Het	Intron	_	rs256439
	79366249	T/G	Hom/Het	Intron	_	rs256438
	79368016	T/C	Hom	Intron	_	rs256437
	79368049	AGA/-	Het	Intron	_	rs3217460
	79369213	G/-	Het	Intron	_	rs67990303
	79373803	C/T	Het	Intron	_	rs2288394
	79375724	G/C	Het	Intron	_	rs2288395
SERINC5	79407163	G/T	Het	3'UTR	_	rs10805931
	79443169	A/G	Hom	Intron	_	rs7712447
	79473059	_/T	Hom	Intron	_	rs11429938
SPZ1	79616083	C/G	Hom/Het	Missense	Val17Leu	rs1862136
	79617357	T/C	Hom/Het	3'UTR	_	rs16876315
ZFYVE16	79733079	T/C	Hom	Missense	Ile192Thr	rs2544600
	79734297	T/C	Hom	Missense	Thr598Ile	rs259028
	79734409	G/A	Hom	Synonymous	Ser635=	rs259029
	79735858	A/G	Hom	Intron	_	rs168939

	79741018	T/A	Hom	Intron	_	rs259034
	79741305	T/-	het	Intron	_	rs34312342
	79743821	T/A	Het	Intron	_	rs2251759
	79745469	A/G	Hom	Missense	Gly1055Ser	rs249038
	79747291	A/G	Hom	Intron	_	rs249039
	79773028	T/G	Hom	Intron	_	rs166062
FAM151B	79809658	A/T	Het	Intron	_	rs249011
	79815658	T/C	Het	Missense	Ile155Thr	rs369998
ANKRD34B	79855372	A/G	Hom	Missense	Leu156Ser	rs32857
	79855876	T/C	Hom	Intron	_	rs187262
MSH3	79966029	G/A	Het	Synonymous	Pro231=	rs1805355
	79974646	T/C	Hom	Intron	_	rs836807
	80021193	C/T	Het	Intron	_	rs245016
	80040532	A/G	Het	Intron	_	rs3816729
	80088462	AG/-	Het	Intron	_	rs3830224
	80149981	A/G	Hom	Missense	Gln949Arg	rs184967
	80168937	G/A	Hom/Het	Missense	Ala1045Thr	rs26279
RASGRF2	80388554	C/T	Het	Intron	_	rs2592094
	80388823	_/A	Het	Intron	_	rs538933586
	80390225	C/T	Het	Intron	_	rs505748
	80408391	T/A	Het	Intron	_	rs10474647
	80409729	T/C	Het	Synonymous	Ser820=	rs10942942
	80502736	C/G	Hom/Het	Intron	_	rs6453542
	80513377	T/-	Hom/Het	Intron	_	rs397771102
ZCCHC9	80600619	C/G	Hom/Het	Missense	Pro15Ala	rs16878594
	80604733	G/A	Hom/Het	Intron	_	rs10514218
SSBP2	80724572	G/A	Het	Intron	_	rs3764985
ATG10	81474225	A/T	Het	Intron	_	rs9293290
	81549216	C/T	Het	Missense	Thr212Met	rs1864183
	81549240	C/A	Het	Missense	Pro220His	rs1864182
	81549280	C/T	Het	Intron	_	rs1864181
RPS23	81571846	G/A	Het	3'UTR	_	rs226201
	81572184	G/C	Het	Intron	_	rs226200
ATP6AP1L	81601075	G/A	Het	5'UTR	_	rs62365440
TMEM167A	82360783	T/C	Hom	Intron	_	rs2306336
XRCC4	82407098	G/A	Hom	Intron	_	rs1478482
	82407105	A/G	Het	Intron	_	rs1478481

	82499307	G/A	Hom	Intron	_	rs2662238
VCAN	82815408	G/A	Het	Missense	Gly428Asp	rs2287926
	82833145	G/A	Het	Synonymous	Gln454=	rs2548541
	82833369	A/G	Het	Missense	Lys529Arg	rs309559
	82833391	A/G	Het	Synonymous	Thr536=	rs16900528
	82834630	T/C	Het	Synonymous	Gly949=	rs309557
	82835545	A/G	Het	Synonymous	Arg1254=	rs160279
	82835724	T/A	Het	Missense	Phe2301Tyr	rs160278
	82843711	_/ATC	Het	Intron	_	rs3842065
	82849120	T/A	Het	Intron	_	rs6873404
	82875800	C/T	Hom	Synonymous	Val3294=	rs308365
HAPLN1	82940273	C/G	Hom	Synonymous	Gly228=	rs2242128
	82940510	T/C	Hom	Intron	_	rs1457081
	82940548	C/T	Hom	Intron	_	rs1457082
	82948216	A/G	Het	Intron	_	rs2045380
EDIL3	83259225	G/T	Het	Intron	_	rs11746605
	83433002	AC/-	Het	Intron	_	rs202163976
	83476194	G/A	Het	Intron	_	rs163715
BTF3	72794249	T/-	Het	5'UTR	_	rs149919824

[a]Genomic position of the nucleotide base on chromosome 5 (chr 5) (GRCh37 p.13, NCBI). [b]The position of amino acid is numbered according to one of the protein-coding transcripts for the gene (dbSNP 142, NCBI). Hom, homozygous; Het, Heterozygous

A3.2 Primer pairs used for amplification and sequencing

Primers for validation of novel/rare NGS variations (5q12-q14)		
Amplicon	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
TMEM171-Exon 2b	tttgcccagtgccttatc	ccagacgagaaccaagagc
CCNB1-Exon 5	ggtgatggtgttgtttgtgg	acagggttttactgcgttgg
C5ORF44-Exon 3	ccagctgatgagagatgatcc	gaattgcttgaacccaggag
RASGRF2-Exon 13	ctgtcctcaggtatttgtcagg	caattacaacccacatgaaagc
IPO11-Exon 9	tgatgtgcctcaagaagagtt	acgccttcaccaacttctgt
CENPK-Exon 1	cacggtaggagggatttttg	tggcttttggaaactagcac
AGGF1-Exon 10	gggaagtgtgtgcatgtgtg	tcaactggatacaagactgatagc
VCAN-Exon 12	ctcattgccaaatagtagacattcg	ctcattgccaaatagtagacattcg
VCAN-Exon 12	ggctgaaaattcgattgagg	taccaggcagcattcctacg
MTX3-Exon 6	gaatccctctcttcgccttt	cccaatcaggccactgtt
ZFYVE16-Exon 6	ttctcaggttccatcagtgg	gggccagatgtgataaatgg
SMN2-Exon 5	ctcttttctccctccaccac	gggattacaggcgtgagcta
SERF1B-3UTR	tggctcatgcctgtaatctg	ccaggctcttcatatcatgc
CRHBP-5UTR	tttgtctccgcctcattctc	gctaacacgcaaggaaggtg
OTP-Exon 1	taagcacctccccgtctctc	caacattccccaactcctttc
AP3B1-5UTR	tacctctaaagcccggagca	gcaacccaaacctttctcct
BHMT2-3UTR	ctcaccatgccctgctatct	ggattgcctttggagtcatt
FAM151B-Exon 4	ttttccctctggtgctatgc	gtagaatgggttttggtctttg
KIF2A-Exon 16	gcttgccctctgtcaaaaag	tgctaggattacaggcgtgag
TRIM23-3UTR	agacctctgtgtgaagcaagtg	aacatttcactccctcattgtg
MCCC2-3UTR	ttctcgaacatgaggctgttac	aagcccgctaaaatatcacc
HOMER1-5UTR	gtttggttgctcgctccac	tcgcagttgcttttccac
C5ORF44-Exon 5	ccacttttacccttccctctg	ggttcctatgcttcatggtg
IQGAP2-Exon 31	ttcaggagccttatagcagacc	gaacatttccgggctttg
HMGCR-Exon 15	gctgttacttttgtgctcagtttc	tactcctttcccccatttt
COL4A3BP-Exon 8	gtattgctcctcctaccatttg	cttaccttatccagtctcttctgc
ADAMTS6-Exon 19	gcaatggctccaaaataccc	tgttctccatcttgccctct
PPWD1-Exon 5	gagggatgcttgcttgttgt	catacgctggttggataagc
MAP1B-Exon 5	tctttgattgtgtggcatcc	gctggcttcagactttctcg
MAP1B-Exon 7	ccagccttcagtttccctta	gtgtggctggccttggtt
MRPS27-Exon 3	tctttctgaaggccgatgtc	gcctttctggctttcttcc
UTP15-Exon 1	aatggttggctaggtgtgag	cccttctccctcattacgtc
UTP15-Exon 3	gccgatactcccaagaacct	gccacagaccccactaaagtt

GFM2-Exon 5F	gatcaggagggagggacatc	aatgacgcagcatctctgtg
NSA2-Exon 3	taccataaacagcgtcatgc	ccgagaaacaagaacctcaa
NAIP-Exon 4	aaattagccgggtgtggtg	gggggacgacaaaaagagat
ARHGEF28-Exon 1	agtttgcttctccctccaca	ccttcccctcaaaaagacac
ARHGEF28-Exon 7	gctcctgggcaactcattta	gacttggggcacatccttt

Primers for missed/low coverage exons in NGS data set (5q12-q14)

Amplicon	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
LRRC70-Exon 1	tgagatttccgttcagcaatac	cacaggtgacaaatgatgtagg
LRRC70-Exon 2a	aaatagtgggttaatggcctga	tgctacaagagaatgaagtcctg
LRRC70-Exon 2b	gtttgtcagctctgcactgg	atccgaagagcaaccatacc
LRRC70-Exon 2c	gaatcgcctcactgtccttg	aacccatccctagtaacattcc
LRRC70-Exon 2d	tgaagcaatacagccctttg	atggatgggggattctgac
LRRC70-Exon 2e	aacttttgggccttcgagac	gcctcattcaacttctcaacttc
LRRC70-Exon 2f	tgaggcttttgacattttgc	tgcttgatacacagaaatgttagc
ZSWIM6-Exon 2	tgctgtcaagtcccacacat	tgaaaaaggatccagagagga
ZSWIM6-Exon3	gcacaaatcaggaaagttctgg	tggtcagctaagaccctgttc
ZSWIM6-Exon4	tccaccacacacacacacac	gcagctctctcttcagcaatac
ZSWIM6-Exon 4	ttgtctcttgtgttgctgtgg	attcccccaaaaccctgcta
FAM159B-Exon 1	aagctctttctggcatcgag	cttgtcgcctccctagcc
C5orf64-Exon1	tctccctcttctctctcttgc	gcagccaggtcagtgtattg
TPNO1-Exon 1	ggaggttatgctggggtaca	ctgcctctcggcttcaaac
RNF180-Exon 4	gctgaaggacaagtggattg	tcaaggacgcacagaaaatg
C5orf44-Exon 8	ctctctgctccaaaatgtgc	ccccacggaatagaaaattg
NLN-Exon 1	aggctgtgctctctctcagg	caccacacggcgaaagac
SREK1-Exon 1	agcaggggagttgtgaaatg	atcaaatatgggcaccaagg
FAM151B-Exon 1	tcaatcggagttccatcctc	acactgaccctgccattctc
MTRNR2L2-Exon 1	gaactcggcaaaccttaccc	tccttccacatcccaaagac
MAST4-Exon 3	gagatgatgttggaggtcagg	gggagaagggtagagatgagg
MAST4-Exon 5	cctccccacaacaaacagtg	aagggcagtgacatccagtg
SLC30A5-Exon 1	gaggaaaggcgagtgttctg	ggagaaagtgcatcctgagc
CENPH-Exon 1	gatgaaagaggagcccgtag	gaaccctagccccttctgagc
CDK7-Exon 1	tgacacagcagccattgaag	cccccaggatgtaaagaatg
BDP1-Exon 1	gggagggcgtagttcctaat	catttgctcagcttccaggt
MAP1B-Exon 1	agcggagacagtaccttcg	atgagaggtgacgaggagagg
FOXD1-Exon 1a	gcggcgcaggagttataaag	ggatcttgacgaagcagtcg

FOXD1-Exon 1b	gctgagcgagatctgtgagtt	cccgatgatgctctcgat
IPO11-TRN1-Exon 1	ctgtgcaggggtttctgtg	cagtcctccaagctccattc
DIMT1-Exon 1	ccctttttctggaggaggag	ctctgccgatcaggtcttg
TAF9-TRN2-Exon 1	agtaccgacccgagttatgc	tctgaagagggcagtgagg
TAF9-TRN3-Exon 1	gctctgcaccctcaatcttc	cctttgctctacagggagga
FCHO2-Exon 14+15	tgctgcctcatttgcttaac	cttctagccacgcagtaggg
BTF3-Exon 1	gagaatagggactcgctgga	cccggatgaaaggctctac
ARHGEF28-Exon 22	tgacccttaccactcaacaac	tccacctccctttcttcttcc
ARHGEF28-Exon 33	gaggtagttccaaccgaaagg	cccacccctgtctttgagag
ANKRD31-Exon 25	ggttgagggaaaaggtcctg	tgttaaaataggcccaccaga
ANKRD31-Exon 24	tttggggaaataaggggaag	attggaaagctatggtgtgc
ANKRD31-Exon 23	cagaaaagaaggttgcaggtg	atagaggtgggtggttgcac
ANKRD31-Exon 22	catgcccttatgaccatttc	tgtgggagaaagtgatgtgttc
ANKRD31-Exon 21a	ggctgcaggtggtcagatta	cattttcggcaaggacactt
ANKRD31-Exon 21b	catcccaacctgttgctttt	cctccattcaggaagctcag
ANKRD31-Exon 20	tagtggggcagcaggattag	gggattcacaaccagaaagg
ANKRD31-Exon 19	tgtcaatggcatatcagagagc	tcagcctttgtgagctttga
ANKRD31-Exon 18	atccttctcatccccatcac	agcgcaccagcatagcac
ANKRD31-Exon 17	atgcttggcagtggtgtttg	ccctttttccagccctactac
ANKRD31-Exon 16	gagtcttttgctttggcact	acccctaacccactgagaaa
ANKRD31-Exon 15	gatcaggaatacacccattgag	gcaggaggtgttgaaatgtg
ANKRD31-Exon 14b	gatcccaacacaaacgtacc	tcctgttctggaagtgtgg
ANKRD31-Exon 14c	gttgtgttctacaggtggcaaa	gcccacatgtcctcattctt
SMN1-Exon 1	agctactggggaggctgag	cggagtgggaaaagacgtag

***COL4A3BP* minigene cloning and insert primers**

Name	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
COL4A3BP-cloning	tcttcaaagcttcaactatagaaaacttt catgtggtgg	acatgtctcgagaggagcactgtcatg atccacag
COL4A3BP-Insert-1	tgtgcaggcaagaatctgag	gcctgggaaatacagcaaga
COL4A3BP-Insert-2	cagagcgtgacttgagaggtt	cgctctgtgatttttctttgc
COL4A3BP-Insert-3	cggtagctgctcagaaatgc	ccttcagcagattgttttcca
COL4A3BP-Insert-4	ccccaaacagaaactccgta	tgggtaaattgtgtggagacag
COL4A3BP-Insert-5	tgacgctgcataacaaggac	aaggtatgcaaggggaaaca
COL4A3BP-Insert-6	tcctcagatgctttccattt	aaatggaaagcatctgagga

RT-PCR primers for *COL4A3BP* minigene

Name	Forward primer (5'-3')	Reverse primer (5'-3')
pcDNA3.1 (+)-specific	taatacgactcactataggg (T7)	tagaaggcacagtcgagg (BGH)
β-actin (control)	gctcgtcgtcgacaacggctc	caaacatgatctgggtcatcttctc

***TMEM171* gene sequencing primers**

Amplicon	Forward primer (5'-3')	Reverse primer (5'-3')
TMEM171-Exon 2a	gcctcattgtggtgtgatttc	cagttggagccacatccag
TMEM171-Exon 2b	tttgcccagtgccttatc	ccagacgagaaccaagagc
TMEM171-Exon 3	gcttccatttattacccacctg	tcacacttgaatgcctcctg
TMEM171-Exon 4	gttttcatcccctccccttc	gaagcactaatggctggaatc

***TMEM171* cDNA cloning and insert primers**

Name	Forward primer (5'-3')	Reverse primer (5'-3')
TMEM171-cloning	gctggctagcgccaccatgtctcctgcagctgctgct	tagactcgagttacggtggggaaggctcaga
TMEM171-cDNA-Insert-1	ggacagacacgtcagcaaac	cggctctctccacagatgaa
TMEM171-cDNA-Insert-2	gcccagtgccttatctttgg	tagttccaggactctcagcg
TMEM171-cDNA-Insert-3	cccctccaccaccttacttt	gtggggaaggctcagaagat

Site-directed mutagenesis primers for *TMEM171* mutations

Name	Forward primer (5'-3')	Reverse primer (5'-3')
TMEM171- Gln90Arg	gctccgtgcagggctgcggagaggtcagcagatgg	ccatctgctgacctctccgcagccctgcacggagc
TMEM171- Arg156Trp	ctggcgactcagagccctggatgtgtggggttcctt	aaggaacccacacatccagggctctgagtcgccag
TMEM171-Gly159Ala	agagccccggatgtgtgcgttccttttctctgcaga	tctgcagagaaaggaacgcacacatccggggctct

RT-PCR primers for *TMEM171*

Name	Forward primer (5'-3')	Reverse primer (5'-3')
TMEM171-RT-PCR	gcccagtgccttatctttgg	tagttccaggactctcagcg

A3.3 Reference protein sequence of TMEM171 from different organisms (Gene, NCBI) used for multiple sequence alignment

***TMEM171* (transmembrane protein 171)**			
Organism	**Assembly**	**Chromsome; Protein reference ID**	**Gene ID**
Homo sapiens (human)	GRCh37.p13	Chr5; NP_775761.4	134285
Mus musculus (house mouse)	GRCm38.p2	Chr13; NP_001020777.1	380863
Rattus norvegicus (Norway rat)	Rnor_5.0	Chr2; NP_001013925.1	293634
Bos taurus (cattle)	Bos_taurus_UMD_3.1	Chr20; NP_001014951.2	538802
Xenopus (Silurana) tropicalis (western clawed frog)	Xtropicalis_v7	Unplaced Scaffold; NP_001004962.1	448384
Pan troglodytes (chimpanzee)	Pan_troglodytes-2.1.4	Chr5; XP_001152753.1	740843
Canis lupus familiaris (dog)	CanFam3.1	Chr2; XP_003638962.1	100855504
Sus scrofa (pig/wild boar)	Sscrofa10.2	Chr2; NP_001231252.1	100514277
Macaca mulatta (Rhesus monkey)	Mmul_051212	Chr6; XP_001099752.2	702863
Ornithorhynchus anatinus (platypus)	Ornithorhynchus_anatinus-5.0.1	Chr1; XP_007656505.1	100077340
Monodelphis domestica (gray short-tailed opossum)	MonDom5	Chr3; XP_007486578.1	100012418
Tursiops truncatus (bottlenosed dolphin)	Ttru_1.4	Unplaced Scaffold; XP_004317133.1	101325851
Gorilla gorilla (western gorilla)	gorGor3.1	Chr17; XP_004058731.1	101128931
Felis catus (domestic cat)	Felis_catus-6.2	ChrA1; XP_003981120.1	101096947
Sorex araneus (European shrew)	SorAra2.0	Unplaced Scaffold; XP_004608567.1	101546374
Pteropus alecto (black flying fox)	ASM32557v1	Unplaced Scaffold; XP_006913194.1	102886656

Ceratotherium simum simum (southern white rhinoceros)	CerSimSim1.0	Unplaced Scaffold; XP_004420520.1	101394185
Equus caballus (horse)	EquCab2.0	Chr14; XP_001504741.2	100065684
Nomascus leucogenys (northern white-cheeked gibbon)	Nleu_3.0	Chr18; XP_003266102.1	100591103
Xenopus laevis (African clawed frog)	_	NP_001086574.1	446409
Oryctolagus cuniculus (rabbit)	OryCun2.0	Unplaced Scaffold; XP_008272757.1	100343682
Cavia porcellus (domestic guinea pig)	Cavpor3.0	Unplaced Scaffold; XP_003461971.1	100735597
Melopsittacus undulatus (budgerigar/parakeet)	Melopsittacus_undulatus_6.3	Unplaced Scaffold; XP_005155084.1	101873540
Anolis carolinensis (green anole/lizard)	AnoCar2.0	Chr2; XP_008101177.1	100551859
Gallus gallus (chicken)	Gallus_gallus-4.0	ChrZ; XP_004937424.1	427220

A3.4 Known/common *TMEM171* variations observed in 480 GGE/JME patients

Position on chr 5[a]	Position of nucleotide change[b]	Location/type	Position of amino acid change[b]	dbSNP ID	Status in databases (MAF)[c]	Status in control cohort (MAF)[d]
72419267	c.67T>C	Nonsynonymous	Phe23Leu	rs638333	C = 0.1689	C = 0.071
72419280	c.80T>C	Nonsynonymous	Val27Ala	rs568311141	C = 0.0010	C = 0.006
72419287	c.87C>T	Synonymous	Gly29=	rs35987724	T = 0.0150	T = 0.006
72419410	c.210A>G	Synonymous	Gly70=	rs7731777	A = 0.0002	A = 0.000
72419456	c.256C>G	Nonsynonymous	Arg86Gly	rs637450	G = 0.4383	G = 0.683
72419488	c.288C>A	Nonsynonymous	Asp96Glu	rs376673995	A = 0.0012	A = 0.010
72419617	c.417C>A	Nonsynonymous	Asn139Lys	rs636926	A = 0.4483	A = 0.416
72419859	c.640+19C>T	Intron	_	rs201735508	T = 0.0012	T = 0.016
72424326	c.750delC	Pro252Leufs	Frameshift	rs555350503	/- = 0.0010	/- = 0.008
72427404	c.819C>T	Synonymous	Asp273=	rs61746311	_	T = 0.023
72427404	c.819C>G	Nonsynonymous	Asp273Glu	rs61746311	G = 0.0313	G = 0.048
72427587	c.*30A>C	3'UTR	_	rs375389928	C = 0.0070	C = 0.041

[a]Genomic position of the nucleotide base on chromosome 5 (chr5) (GRCh37, NCBI). [b]The nucleotide and amino acid residue position of the variations in *TMEM171* is according its longer transcript encoding for protein of 324 amino acids (NM_173490.7, NP_775761.4). [c]Minor allele frequencies (MAF) for the variation given in the dbSNP or 1000 genomes databases. [d]Minor allele frequencies (MAF) calculated in 240 individuals of control cohort.

Appendix III

URLs

Align GVGD: http://agvgd.iarc.fr/agvgd_input.php

ASSP: http://wangcomputing.com/assp/index.html

Blastp: http://blast.ncbi.nlm.nih.gov/Blast.cgi

Clustal Omega: http://www.ebi.ac.uk/Tools/msa/clustalo/

Gene, NCBI: http://www.ncbi.nlm.nih.gov/gene/

GeneCards: http://www.genecards.org/

Homologene, NCBI: http://www.ncbi.nlm.nih.gov/Homologene

Human Splicing Finder v2.4.1: http://www.umd.be/HSF/

LOCTREE 3: https://rostlab.org/services/loctree2/

Mutation Assessor: http://mutationassessor.org/

NCBI: http://www.ncbi.nlm.nih.gov/

NetGene2: http://www.cbs.dtu.dk/services/NetGene2/

NetStart 1.0: http://www.cbs.dtu.dk/services/NetStart/

NNSPLICE0.9v: http://www.fruitfly.org/seq_tools/splice.html

Oligocalc: http://www.basic.northwestern.edu/biotools/oligocalc.html

OMIM, NCBI: http://www.ncbi.nlm.nih.gov/omim

PMut: http://mmb2.pcb.ub.es:8080/PMut/

PolyPhen-2: http://genetics.bwh.harvard.edu/pph2/

PRED-TMR: http://athina.biol.uoa.gr/PRED-TMR/input.html

Primer 3: http://primer3.ut.ee/

SeqQC: http://genotypic.co.in/Products/7/Seq-QC.aspx

SIFT: http://sift.jcvi.org/www/SIFT_enst_submit.html

SNAP: https://rostlab.org/services/snap/

TMHMM: http://www.cbs.dtu.dk/services/TMHMM/

Variation Viewer, NCBI: http://www.ncbi.nlm.nih.gov/variation/view/

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