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Abstract

In the present diploma thesis we investigated a consanguineous Egyptian family with an autosomal recessive trait of temporal lobe epilepsy (TLE), myoclonus and tremor. Five of seven siblings in this family are affected. We employed multipoint linkage analysis to map the causative mutation within chromosome 1q31.3-q32.3 achieving a maximal LOD score of 3.6. This region encompasses 12.5 megabases (Mb) including 136 genes. For further investigation of the linked interval we selected two patients for EXOME sequencing. Within the linked region we identified a homozygous single base pair deletion (c.503_503delG) in the 6th exon of the *CNTN2* gene leading to a frameshift within the coding region. As expected for a recessive disease, the mutation occurred in a homozygous state in the affected children and in a heterozygous state in the clinically healthy parents. *CNTN2* is a glycosylphosphatidylinositol (GPI)-anchored neuronal membrane protein located in the juxtaparanode region of the axon and is responsible for the clustering of K⁺ channels in the underlying axonal membrane.

Previous studies report on *CNTN2* knock out mice suffering from spontaneous seizures. Given the severity of the mutation and the proposed function of the gene, we consider this mutation as the most likely cause for the disease in this family. To find out whether mutations in this gene are contributors to clinical similar phenotypes we employed capillary sequencing of all 22 coding exons in 189 Caucasian patients with TLE or TLE associated with myoclonus. We detected seven rare missense variations in eight individuals. However, as all missense variations occurred throughout in a heterozygous state, their pathogenic significance remains questionable. In summary, we have identified a loss of function mutation in the *CNTN2* gene in a family with recessive temporal lobe epilepsy syndrome. Future studies will reveal the significance of this gene in epilepsy in general.

Zusammenfassung

In der vorliegenden Diplomarbeit untersuchten wir eine konsanguine ägyptische Familie, in der fünf von sieben Kindern an einer autosomal rezessiv vererbten Temporallappenepilepsie verbunden mit Myoklonien und Tremor erkrankt sind. In einer Mehrpunkt-Kopplungsanalyse (LOD 3.6) konnte die Kandidatenregion auf einen Bereich zwischen Chromosom 1q31.3-q32.3 eingegrenzt werden. Dieser Bereich umfasst 12.5 Millionen Basenpaare (Mb) und enthält 136 proteinkodierende Gene. Um die ursächliche Mutation zu finden führten wir bei zwei betroffenen Personen eine EXOM Sequenzierung durch.

Innerhalb der Kandidationregion konnten wir eine homozygote Deletion einer Einzelbase (c.503_503delG) im Exon 6 des *CNTN2* Gens identifizieren, welche zu einer Verschiebung des Leserasters führt. Wie erwartet, zeigte sich die Mutation in den betroffenen Familienmitgliedern in homozygoter Form und in den Eltern in heterozygoter Form. *CNTN2* kodiert für ein neuronales Adhäsionsmolekül, das mittels eines Glycosylphosphatidylinositol (GPI)-Ankers mit der Neuronenmembran verbunden ist. Es ist in der juxtaparanodalen Region des Axons lokalisiert und verantwortlich für die Clusterbildung der K⁺ Kanäle in der darunterliegenden Axonmembran.

In früheren Studien konnte gezeigt werden, dass *CNTN2* defiziente Mäuse spontan zu Krampfanfällen neigen. Unter Berücksichtigung der postulierten Funktion des *CNTN2* Proteins einerseits und der schweren Auswirkung der gefundenen Mutation auf dessen Struktur andererseits, erachten wir die Mutation als wahrscheinlich krankheitsverursachend in dieser Familie. Um Herauszufinden, ob Mutationen in diesem Gen auch bei klinisch ähnlichen Epilepsiesyndromen zur Erkrankung beitragen, sequenzierten wir alle 22 proteinkodierende Exone des Gens in 189 kaukasischen Patienten mit entweder nur Temporallappenepilepsie oder Temporallappenepilepsie mit Myoklonien. Wir fanden sieben seltene missense Variationen in acht Patienten. Da jedoch alle Varianten ausschließlich in heterozygoter Form auftraten, muss die Frage nach deren Pathogenität offen bleiben.

Zusammengefasst lässt sich festhalten, dass wir eine homozygote Mutation im *CNTN2* Gen, welche zu einem Funktionsverlust des Proteins führt, in einer ägyptischen Familie mit einem rezessiven Temporallappenepilepsiesyndrom gefunden haben. Welche Bedeutung dieses neue vielversprechende Kandidatengen für Epilepsie im Allgemeinen hat, müssen zukünftige Studien zeigen.

1 Introduction

1.1 Definition and Epidemiology

Epilepsy is one of the most common chronic neurological disorders in humans defined by the state of recurrent, spontaneous epileptic seizures (Blume et al., ILAE 2001). Epileptic seizures, the main symptoms that subjects experience, arise from periodic neuronal cortical hyperexcitability and can be caused by multiple mechanisms. Epilepsy is a persistent epileptogenic abnormality of the cortical brain that is able to spontaneously generate paroxysmal activity (Engel, 2006). An epilepsy syndrome is a complex of signs and symptoms of epileptic conditions and additional clinical features which comprises a cluster of different seizure types (Engel et al., 2001).

In developed countries the incidence is about 50 cases per 100 000 persons per year (Sander and Shorvon, 1996). Incidence rates vary considerably with age. During the first years of life the incidence is very high, decreases in adolescence and increases again in the elderly (Sander et al., 1990). A more recent study of the epidemiology of epilepsy in Europe shows a prevalence between 4.5 -7.0 per 1000 among all age groups estimating that approximately 6 of 1000 Europeans have active epilepsy, considering incomplete case identification (Forsgren et al., 2005). However, generation of precise epidemiological statistics for a disease like epilepsy which is characterized by heterogeneity is difficult.

1.2 Etiology

Generally, epilepsies are classified into three major categories: (i) idiopathic epilepsy also termed as genetic epilepsy has no known cause. For this form hereditary factors are thought to play a dominant role in disease etiology. In case the genetic mutation is known, family members can be diagnosed by genetic testing. (ii) symptomatic (structural/metabolic) epilepsy is caused by a known or suspected associated deficit (e.g. trauma or tumours). The cause is a primary disease which is associated with the development of epilepsy. The third category, cryptogenic (iii) comprises all forms of epilepsy with a yet unknown underlying cause, which may be a genetic component or a yet undetected disease.

Genetic defects may be some of the most important factors contributing to epilepsy. About 30% of all epilepsies account for idiopathic epilepsies. Most forms of epilepsy follow a complex mode of inheritance involving multiple genes and environmental influences. Hence, because the vast majority of idiopathic epilepsies are complex genetic disorders rather than inherited in a simple mendelian manner, causative gene identification is challenging. The contribution of genes and environment is difficult to measure and boundaries are mostly not very clear leading to an evidence for an interaction of genes and environmental factors (Berkovic, 2006). However, epidemiological studies show that about 5% of patients have a first-degree relative with epilepsy (Bianchi et al., 2003, Vestergaard et al., 2007) and family aggregation studies display recurrence risk ratios of around 2.5 in first degree relatives (Annegers et al., 1982, Hemminki et al., 2006).

In addition, twin studies have supported the genetic background of the disease by showing significantly higher concordance rates for monozygotic compared to dizygotic twins for epileptic seizures and epilepsy (Kjeldsen et al. 2003, Berkovic et al., 1998).

The classification of epilepsy and concepts of their genetics is changing fast. So far, many disease causing genes for rare monogenic forms of idiopathic epilepsy are identified.

1.3 Genetics of epilepsy

The vast majority of genes linked to epilepsy encode proteins which are molecular components of the neuronal signalling complex, encoding subunits of voltage-gated and ligand-gated ion channels. Collectively, they are classified as channelopathies. Ion channels embedded in the cell membrane are gated pores that permit the passive flow of ions through the cell membrane against their electrochemical gradient (**Figure1**). They play a major role in nerve and muscle excitation, hormone secretion, cell proliferation, sensory transduction, learning and memory, regulation of blood pressure, salt and water balance, lymphocyte proliferation, fertilization and cell death (Ashcroft, 2000). The hallmark of ion channels is the selective differentiation between ion species, allowing some to pass and others not, and the transition between open and closed (gating).

Mutations have been found in voltage-dependent Na^+ , K^+ , Ca^+ , Cl^- channels, hyperpolarization-activated cyclic nucleotide-gated (HCN) channels and ligand-gated channels such as nicotinic acetylcholine receptors and Gamma-aminobutyric acid (GABA) receptors (**Table1**).

However, a number of non ion channel genes have also been identified in which mutations were found to cause the disease (Reid et al., 2009).

Besides high risk genes in monogenic forms, candidate gene approaches in recent years have identified a number of low risk susceptibility alleles in complex idiopathic epilepsy. These alleles confer only small increments of the individual risk (Odd 1-2) and are thought to interact with environmental factors.

Although, in the last few years genetic discoveries have led to better understanding of the molecular and cellular deficits major causative genes for common epilepsies remains to be explored.

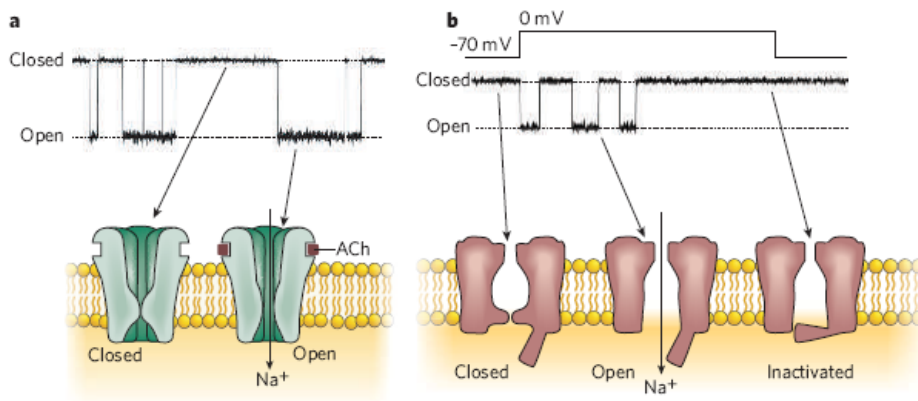


Figure 1: Voltage gated and ligand gated ion channels embedded in the cell membrane. Opening and closing is dependent on ligand binding (ligand gated) (a) or transmembrane voltage (voltage gated) (b). Reprinted from "From molecule to malady." by Ashcroft, F.M., 2006 Nature. 440, p.440

1.4 Neurobiology of seizures and epilepsy

The neuronal signalling system is complex and disruption of one component of the signal pathway can have disastrous consequences. Thus, diverse fundamental components of the central nervous system structure and function serve as possible contributors to seizures.

The nerve cell function and neuronal communication is based on chemical and electrical gradients that provide its ionic microenvironment and electrical activity. In a normal neuronal cell there is a high intracellular concentration of potassium (K^+) and a high extracellular concentration of sodium (Na^+), as well as additional ions which provide a net transmembrane potential of -60mV (Hodgkin and Huxley, 1939; Barnett and Larkman, 2007). This unequal distribution of ions is maintained by ATPase pumps via exchanging internal Na^+ for external K^+ .

By opening the voltage gated ion channels an exchange of intra- and extracellular ions occur, which leads to a transient reversal (1 millisecond) of the polarity of this transmembrane potential termed action potential (Barnett and Larkman, 2007).

The rapid reversal of the action potential is initiated by the transient opening of voltage-gated potassium channels and inactivation of the voltage-gated Na^+ channels. The outward current of K^+ ions restores the resting potential of the cell. The depolarisation of the membrane by the action potential generates local currents in the voltage-gated channels along the neuron and therefore allows the conduction of the impulse from the dendrite to the axon terminal (Barnett and Larkman, 2007). The myelin sheath, formed by the oligodendrocytes, is a multilamellar, wrapped around the axons in segments and separated by the nodes of Ranvier. Therefore the fast saltatory movement of nerve impulses along the neuron can be provided (Hille, 2001). At the synapse, voltage-gated calcium channels allow an inward flow of calcium ions, and synaptic vesicles containing neurotransmitters are released, thus, converting the electrical signal to a chemical signal. The release of neurotransmitters activates ligand-gated receptors in the post-synaptic membrane and a new action potential in the post-synaptic neuron is generated. Considering this, it appears obvious that mutations in components of this signalling way can cause pathological neuronal hyperexcitability, the biological background of epilepsy. So far, various studies investigated how mutations enhance network excitability. Mutations in the subunits of the voltage-dependent sodium and potassium channels alterate ion concentrations. Such an increase or decrease of the ion current into or out of the neuron modifies the exhibition/inhibition balance and can lead to depolarization and resulting action potential discharge (Meisler et al., 2001, Reid et al., 2009). In addition, the hyperexcitability of neurons may be due to either reduction of inhibitory control mechanisms such as GABAergic interneurons, GABA levels and GABA_A receptor sensitivity or due to increase of excitatory mechanisms like astrocytes gap junctions, glutamate level and NMDA receptor sensitivity (Armijo et al., 2002). Blockade of the sodium potassium ATPase in rat hippocampal slice preparations induces seizure activity (Vaillend et al., 2002). Furthermore, seizures themselves provoke an increase in extracellular potassium and therefore an excessive discharge and further depolarization of neurons (Scharfman, 2007).

1.5 Classification of seizures and epilepsy syndromes

In 1981, The International League Against Epilepsy (ILAE) developed an international classification system for epileptic seizures dividing them into two major groups: (i) generalized seizures and (ii) focal seizures. In 1989, ILAE generated a classification for epilepsy syndromes. To date, more than 50 syndromes are described subdivided into clinical syndromes. (ILAE, 1989; Engel, 2001; Engel, 2006) Clinical features of a syndrome include seizure types, their localization, frequency, age of onset, mode of inheritance, physical or mental symptoms and signs, and other clinical, electroencephalographic and imaging features (EEG/MRI). (ILAE, 1989; Engel, 2001; Engel, 2006)

1.5.1 Seizure types

1.5.1.1 Generalized seizures

Generalized seizures are considered as bilaterally synchronous discharges, originating from cortical or subcortical structures or from the whole cortex at once. Although generalized seizures are characterized by the involvement of both hemispheres seizures can emerge localised. EEG shows generalized spike wave activity. However, this location can vary from one seizure to another (Berg et al., 2010). Generalized seizures are sub-categorized into several seizure types (Engel 2006; Blume et al., 2001):

- (i) Tonic-clonic seizures formerly known as “grand mal” start with rigid contraction of muscles (stiffening) followed by rhythmic shaking (jerking) and loss of consciousness. Some individuals can experience the typical tonic-clonic pattern whereas others only undergo the tonic or clonic phase.
- (ii) Myoclonic seizures are brief, rapid contractions of the muscles which result in sudden jerks.
- (iii) Absence seizures are brief periods of staring that last no longer than a few seconds. During the absence awareness and responsiveness are impaired.

(iv) Epileptic spasm is characterized by a sudden flexion extension of predominantly proximal and truncal muscles (epileptic spasm can also be classified as focal seizure).

(v) Atonic seizures are characterized by a sudden loss or diminution of muscle tone lasting 1-2 seconds.

1.5.1.2 Focal seizures

In contrary, focal (partial) seizures are associated with a focal discharge beginning in circumscribed regions of mainly cortical structures limited to one hemisphere. Resulting symptoms are, accordingly to the involved specific cortical structure, sensory sensations or motor signs. Seizures can affect awareness and/or memory. EEG shows localized epileptiform activity. Focal seizures can sometimes turn into generalized seizures, called secondary generalization.

1.5.2 Genetic epilepsy syndromes

1.5.2.1 Age-dependent syndromes

1.5.2.1.1 Newborn to early infancy

The epilepsy syndromes of infancy comprise benign familial neonatal seizures (BFNS), benign familial neonatal-infantile seizures (BFNIS) and benign familial infantile seizures (BFIS). These syndromes differ in the age of onset ranging from the second day of life to a few months (Berkovic et al., 2004). The syndromes, which typically occur in otherwise neurological healthy babies, are characterized by unprovoked seizures which end spontaneously after a period of a few months.

Mutations for these syndromes are found in genes coding for subunits of voltage-dependent K^+ channels (*KCNQ2*, *KCNQ3*), voltage-dependent Na^+ channels (*SCN2A*) and in the *ATP1A2* gene which encodes the alpha subunit of the Na^+/K^+ -ATPase pump.

Febrile seizures (FS) affect 3% of children between 6 months and 6 years of age (Sadleir and Scheffer, 2007). Seizures typically appear in combination with fever. CNS infection or an acute metabolic disturbance is thought as triggering mechanism (Sadleir and Scheffer, 2007). Most FS remit after few years and the child's psychomotor development is unharmed. However, there is an increased risk for later development of an adult form of temporal lobe epilepsy (TLE). FS can occur isolated or in combination with other epilepsy syndromes. Linkage analyses have identified many loci for febrile seizures (Baulac et al., 2004) indicating genetic heterogeneity (Berkovic and Scheffer, 2001). To date, not one high penetrant gene has been found for this form of epilepsy.

Dravet syndrome (DS) is a severe form of myoclonic epilepsy in infancy. DS starts at about the age of 6 months in infants whose development has been normal up to that time. Affected children experience febrile or afebrile generalized tonic-clonic seizures and unilateral seizures that usually occur on alternating sides (Neubauer et al., 2008). DS is associated with developmental regression and prognosis of seizure free life is poor (Dravet et al., 2005). Most of the patients exhibit mutations in the *SCN1A* gene coding for the $\alpha 1$ subunit of voltage-dependent Na^+ channels (Reid et al., 2009). Loss or gain of function mutations result in delayed or impaired inactivation of Na^+ channels, which lead to an ongoing Na^+ current and membrane hyperexcitability. However, in some patients mutations in the *GABRG2* gene coding for the gamma 2 subunit of the GABA_A receptor were identified (Reid et al., 2009).

1.5.2.1.2 Late infancy to adolescence

Generalized epilepsy with febrile seizure plus (GEFS+) links febrile seizures with various other epileptic seizures/syndromes. GEFS+ demonstrates genetic relations between (i) benign and severe and (ii) focal and generalised epileptic disorders (Panayiotopoulos, 2005). It is characterized by phenotypic heterogeneity in affected family members and between different families including classical febrile seizures, febrile seizures plus, mild to severe generalised epilepsy and focal epilepsies (Helbig et al., 2008). GEFS+ is marked by childhood onset of febrile seizures which start

earlier than classical febrile seizures and continue on beyond the age of five years to later childhood or adolescence (Panayiotopoulos, 2005). Remission mostly occurs in adolescence. Mutations for GEFS+ are detected in *SCN1A*, *SCN1B* and *GABRG2* (Reid et al., 2009).

Idiopathic generalized epilepsies (IGE) account for about 30% of all epilepsies (Jallon et al., 2001). IGE's comprise several subsyndromes such as childhood (CAE) and juvenile (JAE) absence epilepsy, juvenile myoclonic epilepsy (JME) and the less frequent but more severe forms myoclonic astatic epilepsy and Lennox-Gastaut syndrome (Reid, 2009).

Idiopathic generalized epilepsy emerges generally in childhood or adolescence with typical absences, myoclonic jerks and generalised tonic clonic seizures. These symptoms can occur alone or in combination with each other and are usually life long. Genes involved in IGE are: *GABRG2*, *GABRA1* and *GABRD* genes coding for subunits of GABA_A receptors, *CLCN2* coding for voltage-dependent Cl⁻ channels gene, *CACNA1H* coding for the Cav3.2 subunit of voltage-activated Ca²⁺ channels and other genes such as *EFHC1*, *ME2*, and *NEDD4L* (Helbig et al., 2008).

1.5.2.2 Age-independent epilepsy syndromes

1.5.2.2.1 Focal epilepsy syndromes

Focal epilepsies account for approximately 50% of all epilepsy patients (Jallon et al., 2001). Traditionally, focal epilepsies have been largely ascribed to acquired/environmental factors such as hippocampal sclerosis, tumours, trauma, and vascular malformations. However, in the past decade, the importance of genetic factors in partial epilepsies was emphasized. Recurrence risk ratios in first-degree relatives of 2-3 (Annegers et al., 1982 Hemminki et al., 2006), the frequent observation of familial clustering and a higher concordance rate in monozygotic (MZ) versus dizygotic (DZ) twins (Berkovic et al., 1998, Kjeldsen et al., 2003) provide clear evidence of a genetic contribution to focal epilepsies.

The first familial focal epilepsy for which the genetic background has been elucidated was the autosomal-dominant nocturnal frontal lobe epilepsy (ADNFLE)(Scheffer et al., 1994).

ADNFLE is characterized by seizures during nonrapid eye movement (NREM) sleep with hypermotor or tonic manifestations and rare tonic clonic seizures. Seizures start in the middle to late childhood and become less frequent during adult life. Three genomic loci have been found to be linked to ADNFLE, chr20q13.2 (Phillips et al., 1995), chr15q24 (Phillips et al., 1998) and chr1q (Gambardella et al., 2000) now referred to as ADNFLE type 1, 2 and 3 (Hirose et al., 2002). For two of the three loci the corresponding gene has been identified. For ADNFLE type 1 mutations have been found in the *CHRNA4* gene coding for the alpha4 subunit of the ligand-gated nicotinic acetylcholine receptors. Mutations in the *CHRNA2* gene coding for the beta 2 subunit of nicotinic acetylcholine receptors respectively were ascribed to ADNFLE type 3.

1.5.2.2.2 Temporal lobe epilepsy (TLE)

TLE is the most common form of epilepsy in adults. Several temporal lobe epilepsy syndromes are acknowledged. They can be either sporadic or familial and categorization in familial or non-familial is not always clear without a profound genetic study and family history investigation.

Familial temporal lobe epilepsies are divided into two major subgroups based on seizure semiology, genetic background and MRI characteristics: (i) Familial mesial temporal lobe epilepsies (FMTLE) and (ii) Familial lateral temporal lobe epilepsies (FLTLE) also termed as autosomal-dominant partial epilepsy with auditory features (ADPEAF). Most of the familial forms of TLE show an autosomal dominant mode of inheritance with incomplete penetrance (Cendes et al., 1998).

FMTLE was first described by Berkovic et al. in 1994. It is characterized by focal seizures which have an onset range between the second and the fifth decade of life. Seizures are typically preceded by an aura with prominent psychic and autonomic

features. Auras are perceptual disturbances manifesting in visual and auditory changes or other sensations such as strange smell, gustatory hallucinations, déjà vu or fear (Shorvon, 2005). Auras are experienced before a seizure occurs. Patients with FMTLE typically show no hippocampal abnormalities (Berkovic et al., 1994). Linkage has been described to 4q13.2-q21.3. However, the corresponding gene has not been found yet (Hedera et al., 2007).

FLTLE or ADPEAF is a benign syndrome with focal seizures and secondary generalization. The focal seizures comprise auditory auras mainly, but complex visual, psychic, autonomic, vertiginous and other sensory symptoms can appear as well (Michelucci et al., 2003). Patients usually have a normal MRI. The age of onset ranges between teenage or early adult life. FLTLE is the first familial epilepsy syndrome where mutations were discovered in a non-ion channel gene. In 1995, linkage to chromosome 10q was established (Ottman et al., 1995) and in 2002 the leucine-rich glioma-inactivated 1 gene (*LG11*), was identified as causative in 5 families with ADPEAF (Kalachikov et al., 2002). LG11 is thought to be a secreted neuronal protein (Fukata et al., 2006) which may play a role for a potassium channel via protein-protein interactions (Schulte et al., 2006). However, not all families with ADPEAF demonstrate LG11 mutations indicating genetic heterogeneity.

Familial Partial Epilepsy with variable foci (FPEVF) is an autosomal dominant syndrome characterized by different types of focal seizures ranging from frontal, temporal, occipital to centroparietal origin (Scheffer et al., 1998). The age of onset is variable but mostly occurs in the first three decades. Linkage for this form has been described to 2q36 (Scheffer et al., 1998) and 22q12 (Xiong et al., 1999). However, the corresponding genes have not been found yet.

1.5.2.2.3 Other rare epilepsy syndromes

Familial cortical myoclonic tremor with epilepsy (FCMTE) comprises a disease spectrum presenting with autosomal dominant inheritance, adult-onset cortical

myoclonus and focal or generalized seizures (van Rootselaar et al., 2005). The wide phenotypic heterogeneity includes syndromes such as: autosomal dominant cortical myoclonus and epilepsy (ADCME), benign adult familial myoclonic epilepsy (BAFME), familial adult myoclonic epilepsy (FAME), familial cortical myoclonic tremor (FCMT), familial cortical tremor with epilepsy (FCTE), familial essential myoclonus and epilepsy (FEME) and familial benign myoclonus epilepsy of adult onset (FMEA). In all syndromes myoclonus is usually the first symptom and characterized by tremulous finger movements and myoclonus of the extremities increased by action and posture (van Rootselaar et al., 2005). Epilepsy is presented by mainly generalized tonic-clonic seizures.

To date, three loci have been mapped but no genes have been identified yet. Linkage was shown to 8q23.3-q24.11 in a Japanese family with BAFME referred to as FCMTE1/FAME1 (Mikami et al., 1999). The second loci mapped on 2p11.1-q12.2 (FCTME2/FAME2) and were established in European pedigrees with ADCME (Guerrini et al., 2001, Laubage et al., 2002, De Falco et al., 2003, Striano et al., 2004). Recently a third locus, FCMTE3, mapped to 5q in a large French family (Depienne et al., 2010).

For summary of epilepsy syndromes see **Table 2**.

Table 1: Epilepsy genes and their associated syndromes (based on Reid et al., 2009)

Channel Receptor			Gene	Epileptic Syndrome
Voltage-gated ion channels				
Na ⁺ channels			SCN1A	GEFS+, SMEI
			SCN2A	BFNIS, GEFS+
			SCN1B	GEFS+
K ⁺ channels			KCNQ2	BFNS
			KCNQ3	BFNS
			KCNA1	partial seizures
			KCNMA1	epilepsy and paroxysmal dyskinesia
			KCNJ11	epilepsy, neonatal diabetes
Ca ⁺ channels			CACNA1H	IGE
			CACNA1A	epilepsy, episodic ataxia
Cl ⁻ channels			CLCN2	IGE
HCN1, HCN2				IGE
Ligand-gated ion channels				
GABA receptors			GABRA1	IGE
			GABRG2	FS/GEFS+
			GABRD	IGE/GEFS+
Neuronal receptors	nicotinic	acetylcholine	CHRNA4	ADNFLE
			CHRNA2	ADNFLE
			CHRNA2	ADNFLE
Non-ion-channel genes				
			LGI1	ADPEAF
			ATP1A2	infantile convulsions, migraine epilepsy syndromes
			ME2	IGE
			EFHC1	IGE
			SLC1A3	epilepsy, migraine, episodic ataxia
			MASS1	FS
			GLUT1	IGE and dyskinesia

Table 2: Classification of electroclinical syndromes and other epilepsies (based on ILAE, A. T. Berg et al., 2010)

Electroclinical syndromes and other epilepsies	
Electroclinical syndromes arranged by age at onset	
<u>Neonatal period</u>	Benign familial neonatal epilepsy (BFNE) Early myoclonic encephalopathy (EME)
<u>Infancy</u>	Epilepsy of infancy with migrating focal seizures West syndrome Myoclonic epilepsy in infancy (MEI) Benign infantile epilepsy Benign familial infantile epilepsy Dravet syndrome Myoclonic encephalopathy in nonprogressive disorders
<u>Childhood</u>	Febrile seizures plus (FS+) (can start in infancy) Panayiotopoulos syndrome Epilepsy with myoclonic atonic (previously astatic) seizures Benign epilepsy with centrotemporal spikes (BECTS) Autosomal-dominant nocturnal frontal lobe epilepsy (ADNFLE) Late onset childhood occipital epilepsy (Gastaut type) Epilepsy with myoclonic absences Lennox-Gastaut syndrome Epileptic encephalopathy with continuous spike-and-wave during sleep (CSWS) Landau-Kleffner syndrome (LKS) Childhood absence epilepsy (CAE)
<u>Adolescence – Adult</u>	Juvenile absence epilepsy (JAE) Juvenile myoclonic epilepsy (JME) Epilepsy with generalized tonic-clonic seizures alone Progressive myoclonus epilepsies (PME) Autosomal dominant epilepsy with auditory features (ADEAF) Other familial temporal lobe epilepsies
Less specific age relationship	Familial focal epilepsy with variable foci (childhood to adult) Reflex epilepsies
Distinctive constellations	Mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE with HS)
Conditions with epileptic seizures that are traditionally not diagnosed as a form of epilepsy per se	Benign neonatal seizures (BNS) Febrile seizures (FS)

1.6 Molecular approaches for identifying genes

The human haploid genome consists of approximately 3 billion DNA bases divided among 20,000-25,000 protein coding genes (International Human Genome Sequencing Consortium, 2004). Genetic changes (variation) contribute to human diseases but many genetic variations have no pathologic effect. At the molecular level, variations mostly occur through (i) structural variants [copy number variants (CNV), insertions/deletions, and block substitutions] and (ii) single nucleotide polymorphisms (SNP's) (Frazer et al., 2009). SNP's are common variants presented by the change in a single base with a minor allele frequency of at least 1% in the population (Kruglyak and Nickerson, 2001). The majority of SNP's are silent and do not alter the function and expression of the gene. Only a small number confer small to moderate effects to the gene. Large biological effects on the phenotype are caused by rare mutations which then provoke monogenetic disorders. Mendelian genetic diseases can either be dominant (only a single copy of a mutated gene cause the disease) or recessive (two mutated alleles of the gene cause the disease) (Mullen et al., 2010). However, the majority of diseases are considered to be complex implying an interaction of genetic and environmental factors. Two models describe the contribution of genetic factors to common diseases. The 'Common Disease, Common Variant' model (CDCV) postulates that the genetic contribution is mainly composed of multiple common variants with small individual risk (relative risk 1.1-1.5) (Mullen et al., 2009). In contrast, the second model 'Common Disease-Multiple Rare Variant' (CDMRV) says that multiple rare variants confer a high disease risk (relative risk 5-10) in a given population (Mullen et al., 2009).

1.6.1 Linkage Analysis and Positional Cloning of Mendelian disease genes

Linkage analysis is used to map genetic loci that predispose to disease. In linkage analysis cosegregation of two or more genes is examined in a pedigree to determine

if they segregate independently or if they do not segregate independently because of their physical proximity (Wiley et al., 1996). In this way the chromosomal region shared by affected members of a large pedigree can be established. Classical linkage is performed in large families with many affected members or multiple families with few affected individuals in which the same mutant gene is responsible for the disease. Naturally occurring crossovers during meiosis limit the candidate region shared by affected individuals. Alleles which are close together on the same chromosome will tend to be inherited together, rather than separated during meiosis (Teare and Barrett, 2005). The pattern of crossovers in the region of the gene among the family members leaves a few hundred predicted genes that might be the disease causing gene (Botstein & Risch, 2003). Subsequently, systematically sequencing of the candidate genes within the linkage region and characterization of their protein products and their associated pathways may identify the putative gene. To prove the causative role of the mutation, it has to be found in the same gene in unrelated families and should show a major impact to the gene function. To infer pathogenicity a lower frequency in controls and a clearly altered function of the gene product is needed.

A requirement for successful linkage is a precise and critical assessment of the phenotypes of the study cohort to ensure exclusion of phenocopies. Phenocopies are defined as family members who have the disease but do not carry the familial mutation. They are thought to suffer from the disease either because of another genetic reason or because of a non-genetic, probably environmental reason. There exists no specific test for any particular disease to distinguish who has the disease gene and who is a phenocopy. Accurate clinical diagnosis does not prevent the occurrence of phenocopies unless some members show certain unusual characteristics of the disease. Correct clinical diagnosis is challenging as clinical heterogeneity with the same mutation is a frequent phenomenon. Misdiagnosis, heterogeneity, complex inheritance and phenocopies can cause failure of linkage analysis (Botstein & Risch, 2003). However, the vast majority of genetic diseases are not transmitted in a Mendelian manner and conventional linkage fails.

1.6.2 Association Studies

Association studies are used to detect differences in allele frequencies of genetic variations between patients and healthy people. Genotypes of large groups of unrelated patients are compared to a control population. An allele is associated with the disease if its frequency differs between cases and controls more than would be predicted by chance (Lander et al., 1994). Two approaches have been widely used in the past few years: (i) Candidate gene-association studies, where a candidate gene already exists due to biological or functional considerations. In these cases only few SNP's within the gene region are tested for association. Within the past 10 years multiple association studies on epilepsy have been performed and more than 85 genes have been claimed to be associated with the disease (<http://geneticassociationdb.nih.gov>). However, most of them could not be confirmed in replication studies. Especially in epilepsies, where heterogeneity is a major variable, findings from association analysis explain little of the disease susceptibility (Helbig et al., 2008). The second approach, (ii) Genome-wide association studies, screen for association of common variants with a disease phenotype across the entire genome (Mullen et al., 2009). In these cases several hundred thousand SNP's across the entire genome are tested for association. The advantage of this approach is that no prior assumption of a functional relationship between a gene and the disease is needed. However, the huge number of tested SNP's makes a correction for multiple testing necessary. Most nominal significant SNP's ($p \leq 0.05$) are likely false positive. To separate the true positive SNP's from false positives, either a very strong p value ($p < 10^{-8}$) is needed or a second round of genotyping with the best significant SNP's in an independent study population has to be performed.

Recently, recurrent microdeletion on chromosome 15q13.3, 16p13.11 and 15q11.2 were identified to constitute a genetic risk factor for common IGE syndromes (Helbig et al., 2009; de Kovel et al., 2009; Dibbens et al., 2009). Each deletion was found in $\sim 1\%$ of affected individuals (Mefford et al., 2010) Notable, the 15q13.3 microdeletion was not identified in the control population, therefore it

can be seen as the major risk factor for IGE, whereas the other two deletions confer a lower genetic risk to IGE (de Kovel et al., 2010).

However, the size of samples needed for a genome- wide association to attain sufficient statistical power is enormous and in epilepsy, the study design is still challenging.

1.6.3 Exome Sequencing

In the past few years sequencing technologies have made great advances. The development of “Next generation sequencing” techniques allows the simultaneous sequencing of genomic regions in the megabase range within a few days. One important application of this technique is the complete analysis of all protein coding exons of the human genome (~ 300.000)(EXOME sequencing). This powerful technique will enhance the identification of familial high risk genes in the near future. For a more detailed description see material and methods.

2 The Aim of the Study

We present a consanguineous Egyptian family with an autosomal recessive inheritance pattern suffering from temporal lobe seizures, myoclonus and tremor. We established linkage to chromosome 1q31.3-q32.2. The aim of the present study was: (i) to identify the causative gene within this family via EXOME next generation sequencing (ii) sequencing of the putative gene in 189 temporal lobe epilepsy patients from Austria and Italy to test whether this gene contributes to TLE phenotypes in general.

3 Material and Methods

3.1 Study population

3.1.1 Egyptian family

We investigated a consanguineous Egyptian family where five of the seven siblings of healthy parents show an autosomal recessive inherited epilepsy syndrome (**Figure 2**). The family exhibits a phenotype with temporal lobe epilepsy, myoclonus and tremor (**Table 3**). Patients were examined by a local neurologist at the University of Ain Shams in Cairo/Egypt.

The initial symptoms in all patients were seizures starting between the ages of 11 to 14 years. The occurring seizures range from complex partial, to secondary generalized tonic clonic and to myoclonic seizures of variable frequency. Three affected members additionally exhibit auras at the onset with hearing childish or illformed voices, olfactory sensations and hallucinations with vivid scenes. All patients responded well to Carbamazepine respectively Lamotrigine. For three patients MRI screening was performed showing a bilateral mesial hippocampus sclerosis in patient IV-7, and was normal in patient IV-3 and IV4. For the other two patients CCT screening demonstrated no abnormalities. EEG showed interictally temporal epileptiform discharges in three of five affected siblings. Altogether, these features suggest a phenotype of temporal lobe epilepsy. Moreover, all five affected patients showed a coarse tremor of the head and upper extremities, which did not respond to

propanolol. Parents of affected siblings gave informed consent and local ethic committee approved the study.

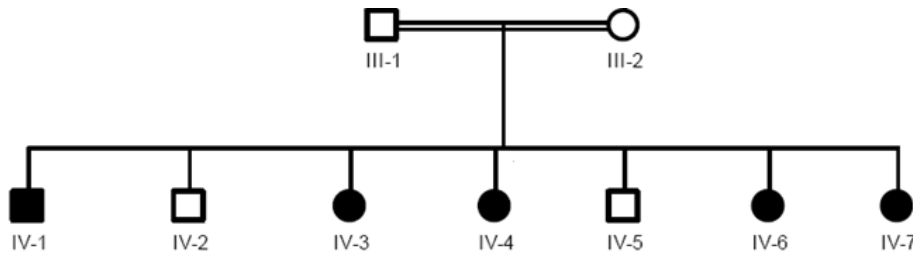


Figure 2: Pedigree of consanguineous Egyptian family with autosomal recessive mode of inheritance. III-1 and III-2: carrier; IV-1, IV-3, IV-4, IV-6 and IV-7: affected;

3.1.2 Temporal lobe epilepsy patients and controls

For the screening for possible mutations of the CNTN2 gene in a larger sample of epilepsy patients we selected 170 Austrian patients and 19 Italian patients with temporal lobe epilepsy (n=189, females=81, males=108). However, none of our patients exhibit exactly the described phenotype. In addition, the pattern of inheritance of our study population varies between sporadic, dominant and likely recessive.

The control individuals for testing, detected putative pathogenic variants consisted of 366 healthy Austrian blood donors and 36 healthy Egyptian volunteers. The Austrian control subjects (n=366, females=145, males=221) were recruited through the Department of Neurology, Medical University of Vienna, as subjects without known history of a neurological disorder, such as non blood-related companions. All patients and controls were Middle European ancestry. The average age of the control group was 40 years. All study participants gave written informed consent and the study was approved by the local ethics committee.

Table 3: Summary of the clinical features of the Egyptian family

Patient	Sex	Age	Onset	Seizure type(s)	Seizure description	Antiepileptic Drugs	EEG	Brain imaging	Associated features
IV-1	M	39	11	GTC	No specific aura	Controlled on CBZ	Abnormal, Not specified	CCT normal	Tremor, head and UE
IV-3	F	37	14	GTC CPS Myoclonus	LOC with aurobuccal automatism	Occasional seizures on CBZ	Interictal: Left posterior temporal epileptiform discharge	MRI normal	Tremor, head and UE
IV-4	F	29	11	Aura GTC CPS Myoclonus	Hearing childish voices, LOC, GTC	Controlled on CBZ	Interictal: No abnormality	MRI normal	Tremor, head and UE
IV-6	F	24	12	Aura GTC CPS Myoclonus	Hallucination with vivid scenes (films), Hearing illformed voices, Bad smell LOC with chewing movements	Difficult to control on CBZ and LTG	Interictal: Left temporo-parietal epileptiform discharge	CCT normal	Tremor, head and UE
IV-7	F	21	11	Aura GTC CPS	Bad smell, Aurobuccal automatism with LOC	Controlled on CBZ	Interictal: Right temporal discharge	MRI: Bilateral mesial temporal sclerosis	Tremor

Generalized tonic clonic (GTC), Complex partial seizures (CPS), Upper Extremities (UE), Carbamazepine (CBZ), Lamotrigine (LTG), Cranial computed tomography (CCT), Magnetic Resonance Imaging (MRI), Loss of Consciousness (LOC);

3.2 Reagents and Instruments

1x TBE (Sigma-Aldrich, St. Louis, US)

10x EDTA Buffer (Applied Biosystems, California US)

40x TaqMan® SNP Genotyping Assay (Applied Biosystems, California US)

7900 HT Fast Real Time PCR System (Applied Biosystems, California US)

Affymetrix GeneChip® Human Mapping10K Xba142 2.0 arrays (Affymetrix, Santa Clara, US)

Agarose (Biozym, Oldendorf Germany)

Sequencing Analysis Software v.5.2 (Applied Biosystems, California US)

Aqua bidestillata (AccuGene®, Lonza, Verviers, Belgium)

BigDye® Terminator v3.1 Sequencing Kit (Applied Biosystems, California US)

BigDye XTerminator® Purification Kit (Applied Biosystems, California US)

DNA Quant-iT™ dsDNA BR Assay Kit (Invitrogen, Eugene, Oregon US)

Ethanol (99.9%) (Sigma-Aldrich, St. Louis, US)

Ethidium bromide (Merck, Darmstadt Germany)

FastRuler™ DNA Ladder, Low Range (Fermentas International, Canada)

Genetic Analyzer 3130 (Applied Biosystems, California US)

IKA® MS 3 Digital vortexer (IKA, Germany)

LiChrosolv water (Merck, Darmstadt Germany)

MultiScreen®PCRµ96 plate (Millipore, Billerica, US)

Pop-7™ (Applied Biosystems, California US)

QIAamp® Blood Midi Kit Spin Protocol (QIAGEN, Hilden Germany)

Qubit™ Fluorometer Reader (Invitrogen, Eugene, Oregon US)

REPLI-g® Mini Kit of Purified Genomic DNA (Qiagen, Hilden Germany)

Sample Loading Buffer (in house made)

Staden Package v. 1.5.

DNA Engine Tetrad® 2 Thermal Cycler (Biorad, California, US)

Taq DNA Polymerase Kit (Qiagen, Hilden, Germany)

Type-it™ Fast SNP Probe PCR Kit (Qiagen, Hilden Germany)

3.3 Techniques

3.3.1 Genomic DNA Isolation

The genomic DNA purification from whole blood was performed according to the QIAamp® Blood Midi Kit Spin Protocol. A 2ml aliquot of human whole blood used for extracting genomic DNA was added to 200µl protease and briefly mixed. After addition of 2.4ml lysis buffer AL and vigorous shaking the homogenous solution was incubated in a water bath at 70°C for 10 minutes. 2ml of 99,9% ethanol was added, thoroughly mixed, half of the solution transferred to the QIAamp Midi column and centrifuged at 1850 x g for 3 minutes. Resulting filtrate was discarded the remainder of the solution transferred in the Midi column and again centrifuged. The bound DNA was washed with 2 different buffers AW1 with following centrifugation at 4500 x g for 1 minute and with AW2 centrifuged at 4500 x g for 15 minutes, respectively. The purified DNA was eluted in two steps with 300µl buffer AE each time incubated for 5 minutes and centrifuged at 4500 x g for 2 minutes.

3.3.2 DNA Quantification

The double-stranded DNA concentration was measured using DNA Quant-iT™ dsDNA BR Assay Kit in conjunction with Qubit™ fluorometer reader.

3µl of each DNA was diluted with buffer and fluorescent reagent up to 200µl and measured with the fluorometer.

Samples were diluted to a working solution of 10ng/µl and stored at -20°C.

3.3.3 Linkage Analysis

Genome-wide linkage analysis using Affymetrix Human Mapping10K Xba142 2.0 arrays was performed. Data for linkage analysis were prepared with a modified version of Alohomora (Ruschendorf, 2005). Multipoint linkage analysis was achieved using Allegro (version1.1d) (Gudbjartsson, 2000). We assumed an autosomal

recessive model. The frequency of the deleterious allele was set to 0.001, and the penetrance to 99% ($q=0.001$; $f_1=0.0$; $f_2=0.0$; $f_3=0.99$).

3.3.4 Whole Genome Amplification

The Whole Genome Amplification based on Multiple Displacement Amplification technology (Dean et al., 2002 and 2001) is an isothermal reaction that provides uniform amplification of whole genomic DNA of limited samples. A 50 μ l MDA-based WGA reaction generates 30-50 μ g of product with consistently >10kb in length regardless of the starting template (Lasken, 2009). Due to its simplicity and reliability MDA is an adequate method for high-throughput applications like DNA sequencing and SNP analysis.

Some of our samples, especially the Italian and Egyptian DNA's, were limited by the amount of DNA template or quality. We performed a whole genome amplification using the REPLI-g® Mini Kit of Purified Genomic DNA according to the manufacturer's specification.

3.3.4.1 Principle and Procedure

Multiple displacement amplification uses the binding of random hexamer primers and phi 29 DNA polymerase, derived from *Bacillus subtilis*, for strand displacement synthesis at a constant temperature (Dean et al., 2002). The phi29 DNA polymerase with its extremely high processivity and strand displacing activity generates high yields of DNA by copying the same template multiple times, extending new primers and displacing the previously extended product whereby the displaced strand functions as a template for replication. (**Figure 3**)

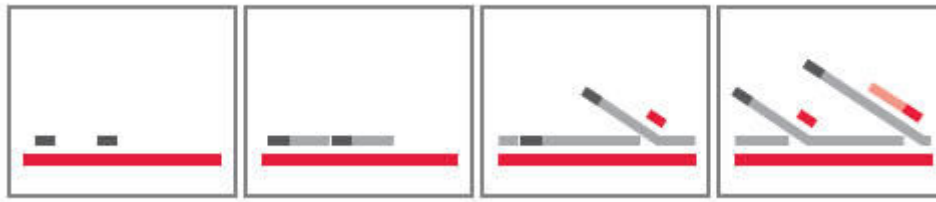


Figure 3: Principle of the whole genome amplification method by multiple displacement amplification. Reprinted from www.qiagen.com, 2010

Phosphorothioate linkage of the 3' end of the random primers prevents them to be degenerated from the 3'-5' exonuclease proofreading activity of the phi 29 DNA polymerase (Dean et al., 2001). Additionally the proofreading activity of the phi 29 DNA polymerase ensures amplification with an error rate of less than 3×10^{-6} (Nelson et al., 2002).

To obtain lysis and denaturation of the DNA 5 μ l buffer D1 was added to 5 μ l purified genomic template DNA vortexed and incubated for 3 minutes at room temperature. For neutralization 10 μ l buffer N1 was added and mixed with the master mix containing 29 μ l reaction buffer and 1 μ l DNA polymerase. To achieve a maximum of DNA yield the samples were incubated in a heating block at 30°C for 16 hours and terminated by heating up to 65°C for 3 minutes. Although typical DNA yields are approximately 10 μ g per 50 μ l reaction we performed accurate quantification with DNA Quant-iT™ dsDNA BR Assay Kit according to the manufacturer's instructions. All samples were diluted to a working solution of 5 ng/ μ l and stored at -20°C.

Various studies (Berthier-Schaad et al. 2007, Lovmar and Syvänen 2006) already used MDA amplified DNA for high throughput DNA arrays and showed excellent concordance of genotype between genomic DNA and amplified DNA as well as good genome coverage and lowest amplification bias.

However, imbalanced amplification can lead to genotyping errors. Due to allelic dropout or low quality genotype data heterozygous genotypes can be mistaken for homozygous.

We gross tested the reliability of our wgaDNA in 20 samples by comparing wgaDNA and gDNA using Taqman Genotyping assay with a randomly chosen SNP from former studies.

3.3.5 TaqMan® Genotyping

3.3.5.1 Principle and Procedure

The Allelic Discrimination assay is a multiplexed assay which detects variants of a single nucleic acid sequence at the end of the PCR process. The TaqMan® SNP Genotyping Assay which consists of two primers and two probes, dual-labelled fluorogenic MGB probes, enables genotyping of the two possible variants at the single-nucleic polymorphism site in a target sequence. One probe matches to the wild type allele and the other probe to the mutated allele allowing biallelic genotyping. (Figure 4) The Allelic Discrimination measures the increase in fluorescence of the reporter dyes associated with the TaqMan® probes (Applied Biosystems, 2010).

The TaqMan® MGB probes contain a fluorescent reporter dye at the 5' end of each probe (VIC®, FAMTM), and a non-fluorescent quencher (NFQ) at the 3' end with a minor groove binder (MGB). The MGB is a modification which enforces shorter probe design resulting in greater differences in T_m values between matched and mismatched probes to secure robust allelic discrimination (Applied Biosystems, 2006).

In the intact probe during the PCR the reporter dye emission is adequately absorbed by the quencher due to their proximity and overlapping absorption spectrum. After hybridization of the probe to the template DNA the DNA polymerase extends the primer bound to the DNA and cleaves the reporter dye from the probe. The separation of the reporter dye from the quencher leads to the characteristic fluorescence signal emitted by the reporter and indicates which alleles are present in the sample. An increase in either VIC® or FAMTM indicates homozygous genotypes

whereas an increase in the fluorescence signal of both reporter dyes signifies a heterozygous genotype.

The increase in the fluorescence signal is measured by the sequence detection software (SDS) which displays the results on a scatter plot of allele X versus allele Y grouped into four genotype clusters: homozygote for allele 1, homozygote for allele 2, heterozygote and non template controls (NTCs).

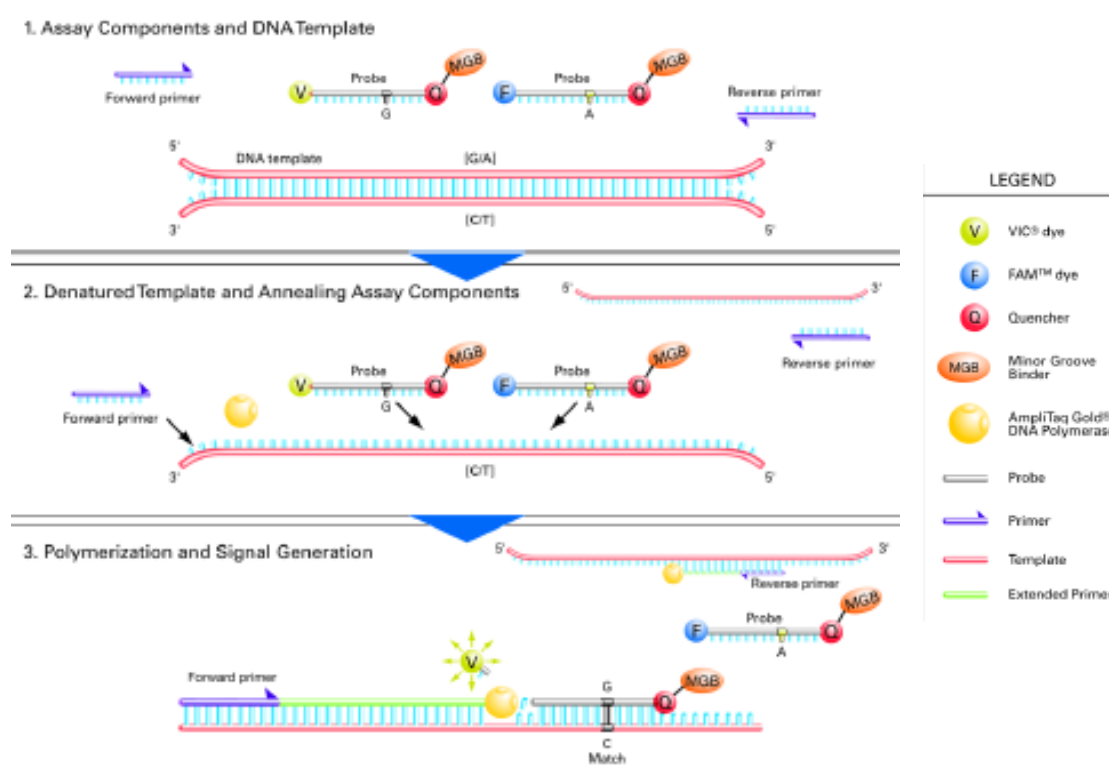


Figure 4: Different steps of the Taqman allelic discrimination assay.
Reprinted from "Allelic Discrimination Getting Started Guide" by Applied Biosystems, 2010

Genotyping was achieved using Type-it™ Fast SNP Probe PCR Kit using standard protocols and reagents.

8µl Genotyping reaction mix, consisting 5µl Type-it Fast SNP PCR Master Mix, 0.25µl 40X TaqMan® SNP Genotyping Assay, 2.75µl Aqua bidestillata was added to 20ng DNA and conducted using optical 96-well reaction plates on a 7900 HT Fast Real

Time PCR System. The TaqMan® SNP Genotyping Assay was for a SNP located on chromosome 10q24.32 in the gene PITX3 which was randomly chosen and had no further significance for us.

Standard cycling conditions were the initial PCR activation step for 5 minutes at 95°C for activation of the HotStarTaq*Plus*DNA Polymerase followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 30 seconds. Before and after the PCR step Allelic Discrimination reads were performed.

In all 20 cases the amplified DNA gave the correct genotype. The results were indistinguishable from the unamplified genomic DNA. (**Figure 5**)

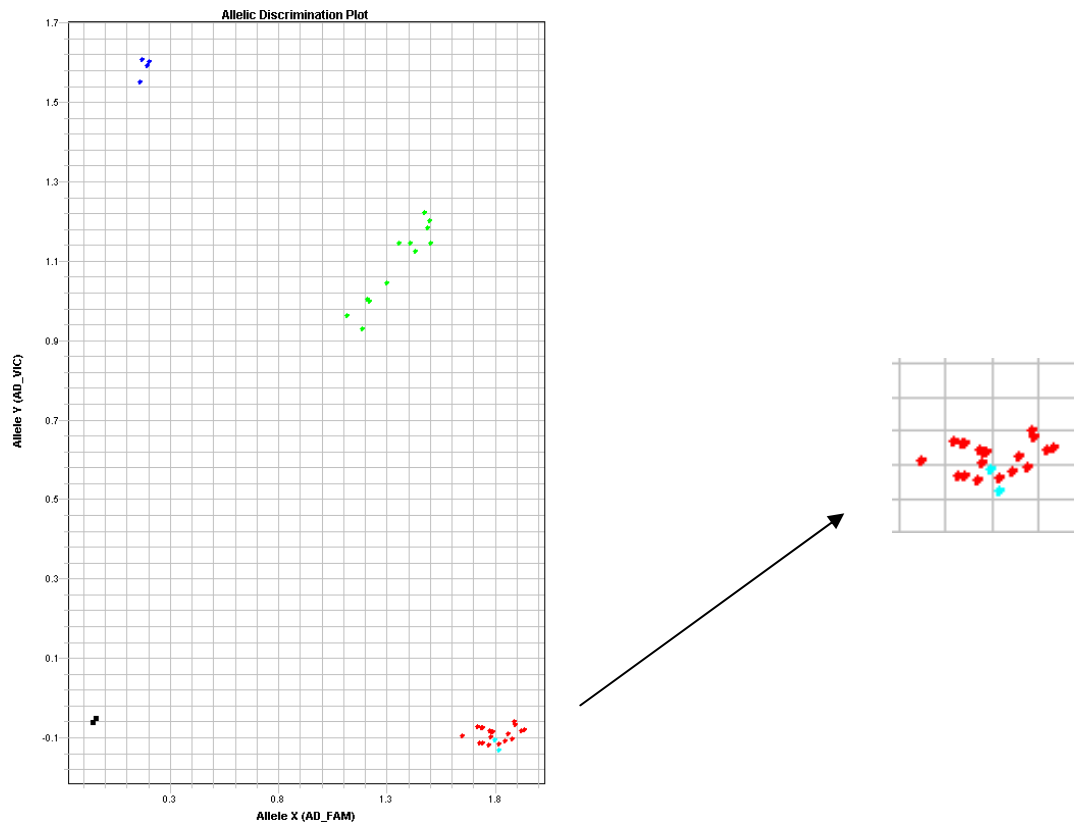


Figure 5: Scatter plot of Taqman allelic discrimination assay for validation of the whole genome amplification.

Demonstrated are results of testing the whole genome amplified DNA versus the genomic DNA, to ensure correct amplification. The vertical axis is the mutated allele, labelled with VIC[®] and the horizontal axis is the wild-type allele, labelled with FAM[™]. Non-template controls are plotted as black squares. Dark blue dots on the upper left side are indicating homozygous mutated samples, green dots in the middle indicating heterozygous samples, red dots on the lower right side indicating homozygous mutated samples. Detail shows two bright blue dots referring once to the amplified DNA and once to the genomic DNA demonstrating no difference between amplified and genomic (non-amplified) DNA.

Taqman allelic discrimination assays were developed to screen the normal control panel for all three variants derived from the Exome sequencing and for all seven novel heterozygous variants resulting from subsequent capillary sequencing using 20ng of DNA as previously described above. All three variants resulting from the Exome sequencing were additionally screened in 189 TLE patients and in the Egyptian family.

3.3.6 Next-Generation Sequencing

3.3.6.1 Principle and Procedure

Next-Generation sequencing enables massively parallel sequencing of many thousands or millions of sequencing reactions rather than 96 as conventional sequencing machines achieve at a time. Traditional sequencing produces read length around 400-800bp whereas next-generation sequencing, depending on the platform, generates far shorter reads which can then be compared to a reference. Additionally, priory template amplification as in conventional sequencing is unnecessary. To date, various next-generation DNA sequencing platforms are available (for review, Shendure & Ji, 2008; Mardis, 2007; Tucker et al., 2009). Although, the different platforms vary in sequencing biochemistry and array generation, their work flows are conceptually similar (Shendure & Ji, 2008).

3.3.6.1.1 Illumina sequencing technology

Illumina sequencing platform is based on the concept of sequencing by synthesis and uses reversible terminator-based sequencing chemistry (Bentley et al., 2008). Libraries can be generated by any method creating adaptor ligated DNA fragments. Fragments are added to the surface of a glass flow cell which has covalently attached oligos complementary to the specific adaptors. Solid-phase bridge amplification (Adessi et al., 2000; Dedurco et al., 2006) occurs by adding unlabeled nucleotides and polymerase and double stranded bridges are built. Complete amplification results in several million dense clusters, each containing ~1.000 copies of the same template (Illumina Sequencing Technology, 2010). Modified sequencing chemistry, consisting of four differentially labelled fluorescent nucleotides and polymerase, only allows single-base incorporation in each cycle. Subsequent laser excitation provokes the fluorescence signal. After sequencing, the reads are aligned to a reference genome and subjected to a quality filtering.

The paired-end module yields two independent reads derived from each end of the template instead a single-end read (Illumina Sequencing Technology, 2010).

3.3.6.2 Whole Exome Sequencing

Whole Exome Sequencing is a second-generation method for targeted sequencing of all protein-coding regions. Although costs of Whole Genome Sequencing are reducing, Whole Exome Sequencing is a good alternative. By exome sequencing, candidate genes for mendelian disorders can be identified within a small number of unrelated, affected individuals. (Ng. et al., 2009).

3.3.6.2.1 Exome Sequencing

Whole Exome Sequencing was performed for two members of the Egyptian family (IV-3, IV-6) by our cooperation partner (Department of Human Genetics, Helmholtz Center, Munich). Libraries for the two exomes were prepared following standard protocols using the SureSelect whole Exome Assay. Sequencing of post-enrichment libraries was carried out on an Illumina Genome Analyzer IIx as 54bp paired-end runs. For each sample two lanes of a flowcell were sequenced. Image analysis and base calling was carried out using the Genome Analyzer Pipeline version 1.5 with default parameters.

3.3.6.2.2 Read Mapping

Alignment of the reads to the hg18 reference sequence was performed with the BWA software (v.0.5.8) using mainly default parameters. Reads were trimmed of low quality bases at the end (-q) using a cutoff quality of 15. A small number (3-4%) of duplicated reads, which were indicated by identical outer coordinates of mapped mate pairs, were removed. The percentage of reads overlapping targeted regions and coverage statistics of targeted regions were calculated using Perl scripts. Mapped reads that directly overlapped the targeted regions were used for variant identification.

About 6.5-7.0 gigabases of mapable sequence data were generated per individual with 34-39% of reads mapping to the target regions. On average, >98% of the

targeted bases were covered at least once, and > 75% of bases were covered 20 fold or more.

3.3.6.2.3 Variant Calling

Initially, 29185 and 29755 variants for the two samples including SNP's and indels using SAMtools (v. 0.1.7) were called respectively. For the variant filter part of SAMtools the default parameters with the exception of setting a maximum read depth of 9999 (parameter-D) was used.

Additional filters were applied to exclude low confidence variants: Median base quality of the variant base of at least 15, a minimum of 15% of reads showing the variant base and that the variant base is indicated by at least 5% of reads coming from different strands. For indels it was required that at least 10% of reads covering this position to indicate the indel.

3.3.6.2.4 Variant Annotation

Variant annotation was performed using custom Perl scripts. For prioritization known SNP's from dbSNPv1.30, from eight sequenced exomes of Hap Map samples (Ng et al., 2009) and from the in-house database were excluded.

Finally, the analysis was confined to the linkage region chr1:195653469-208175909 and all variants that were shared between the two samples were selected.

3.3.7 DNA Capillary Sequencing

For identifying further mutations in the CNTN2 gene we performed directed capillary sequencing. We screened the candidate gene in three family members of the Egyptian family and in 189 TLE patients. The sequencing reaction was carried out using BigDye® Terminator Cycle Sequencing kit v.3. Primer design was achieved

using Primer3 software. For amplification of exon sequences and exon-intron boundaries the following intronic primers were selected:

Table 4: Primer sequences for capillary sequencing of 22 coding exons

Exon	Sequence
exon 2	forward CTCCCCAGGTCCTTCTCAG reverse TCGTCACCCAAATCATAGCA
exon 3-4	forward ACAGCACAGCCCAAGAGG reverse TCTCAGTAATTCTCCTAGCCCC
exon 5	forward GTGAAGGATGAGTCGGGGAG reverse CCTCTGCCAAGCATCTGG
exon 6	forward GAGTGGCCCTGTTAGCCC reverse AAACCCTTTTCCTAATGCCC
exon 7	forward AGTTGGCTCTGAAAGGTGCT reverse CCTGTGAGGGACACCAAGAT
exon 8	forward ACTGGAGGGGTAGGCCC reverse CTTGGGGACAAGGGTGAAG
exon 9	forward GCTGCCCTGATTTTCTGTTT reverse GGAACCAAGTGAGCAGAGGAC
exon 10	forward CCTCCCTGTGGCTCCTG reverse CCACAGGGTATGTGCATGTG
exon 11-12	forward AAAGGGCACGCCAAGTAAC reverse GGGGAGGACAGTACCCTGAG
exon 13	forward CCTGGGCCCATTTCCT reverse ATGGCTAAGGACATCTTGGG
exon 14	forward TGGAGTAGGGGACTCCCAAG reverse CAGCTTTGAGGGAAGGGAG
exon 15	forward TGGCTCAAATTGGAAGCTG reverse TCCACCTCCTACACCCAGTC
exon 16	forward GGTGATTCAGGCCAGTGC reverse ATAAGTGGTGCCAGGGTCTC
exon 17	forward CTCTACAGGCACAGGCTCAG reverse CTGCTCAAGCGGGAGAGG
exon 18	forward CTTGGCAAAGGTTGGGTG reverse ATCCCTGTGGACAAGTGGC

exon 19	forward GCTCTGCAGGACACAGGG reverse CTGTGAGTTGTTCCCACCTG
exon 20	forward GAGAGGAACAGAATGTGGGG reverse AGAGGCACCCACCCTCC
exon 21	forward AAACATCCAGAGAAGGGCTG reverse TGCTGCTGCTTATGTTCACTG
exon 22	forward GGGATGACTCAACGATCAGC reverse GTGGGAATGATCCTCTCAGG
exon 23	forward AAGGGTGGGGCTAGGGTAG reverse ACCTTGGCAGCAGGAAG

3.3.7.1 DNA Template Preparation

Cycle sequencing generates the most reproducible results for sequencing amplified templates. We performed PCR using Taq DNA polymerase kit. The PCR amplification was carried out with primers flanking the region of interest in the target DNA.

3.3.7.2 PCR reaction optimization

To ensure successful amplification of the sequence with primer sets of interest, optimization was performed for all 20 primers. Standard PCR reaction with different annealing time as well as PCR reaction with Q-Solution was set up, respectively.

Primers were synthesized with standard HPLC purification and ordered in lyophilized form at Sigma Aldrich. According to the manufacturer's protocol, primers were dissolved with H₂O to a final concentration of 100pmol/ μ l. The PCR reaction was carried out with a 1:10 dilution of the primer stock.

The standard PCR reaction mix for a 10 μ l approach with 16ng DNA consisted of 1 μ l 10xPCR buffer, 0.2 μ l dNTP's (10mM each), 0.2 μ l primer forward (10pmol/ μ l), 0.2 μ l primer reverse (10pmol/ μ l), 6.33 μ l dd H₂O and 0.07 μ l Taq polymerase (5U/ μ l). For the reaction mix with Q-Solution 1.2 μ l 5xQ-Solution was added and dd H₂O was accordingly reduced. Sequence reaction was carried out using Biorad Tetrad® 2 thermal cycler.

A simplified hot start was performed to avoid mispriming and primer-oligomerization. The PCR program was started and once the thermal cycler reached 95°C the plate was placed in the cycler. Thermal cycler conditions were set to the initial denaturation step at 95°C for 5 min, followed by 35 cycles at 94°C for 30 seconds (denaturation), 50°C-70°C for 30 seconds (annealing) and 72°C for 30 seconds (extension) completed with the final extension at 72°C for 10 minutes. Optimal primer annealing temperatures were determined empirically by a temperature gradient between 50°C and 70°C.

All PCR products were subsequently analyzed on a 2% ethidium bromide-stained agarose gel. The agarose gel, composed of 200µl 1x TBE, 4g agarose and 5µl ethidium bromide (10mg/ml), was loaded with 8µl PCR product mixed with 2 µl loading buffer. Additionally 3µl of low range FastRuler™ DNA Ladder for validating the amplified product size was applied. Gel electrophoresis was carried out at 100 volt for 45 minutes.

3.3.7.3 PCR Reaction

Once the corresponding conditions for each exon were achieved the template generation was performed.

The PCR reaction mix was adjusted for 30µl with 25ng of template DNA.

Table 5: PCR condition for each exon with corresponding mastermix and primer annealing temperature

Mastermix	Exon	Primer annealing temperature
<u>Standard PCR reaction mix:</u> 3 μ l 10xPCR buffer 0.6 μ l dNTP's (10mM each) 0.6 μ l primer forward (10pmol/ μ l) 0.6 μ l primer reverse (10pmol/ μ l) 20 μ l dd H ₂ O and 0.21 μ l Taq polymerase (5U/ μ l)	exon 02	56°C
	exon 03-04	57°C
	exon 05	57°C
	exon 06	56°C
	exon 07	62°C
	exon 08	62°C
	exon 09	58°C
	exon 11-12	59°C
	exon 13	61°C
	exon 14	59°C
	exon 15	59°C
	exon 16	62°C
	exon 18	58°C
	exon 21	58°C
	exon 22	59°C
	exon 23	60°C
<u>PCR reaction mix with Q-Solution:</u> 3 μ l 10xPCR buffer 3.6 μ l Q-Solution 0.6 μ l dNTP's (10mM each) 0.6 μ l primer forward (10pmol/ μ l) 0.6 μ l primer reverse (10pmol/ μ l) 16.4 μ l dd H ₂ O and 0.21 μ l Taq polymerase (5U/ μ l)	exon 10	60°C
	exon 17	58°C
	exon 19	58°C
	exon 20	61°C

Sequence reaction was carried out using Tetrad® 2 Thermal Cycler as described above. DNA quality and approximate quantity was determined by loading 8 μ l of the PCR products on an agarose gel as described above. Purified DNA run as a single band on the gel.

3.3.7.4 Template Purification

Before sequencing PCR product purification is necessary for providing optimal results. Contaminations in the sequencing reaction have a negative effect on polymerase binding, amplification or extension (Applied Biosystems, 2009). To remove contaminating salts, unincorporated dNTP's and non-specific PCR products (primer-dimer artefacts and secondary PCR product) purification with the MultiScreen® PCR μ 96 plate was performed according to the manufacturer's instructions.

The volume of the PCR reactions was adjusted to 100 μ l with dd H₂O and the reactions were transferred to the MultiScreen® PCR μ 96 plate. The plate was placed on a vacuum manifold and applied to vacuum at 20 inches Hg until the wells were empty. DNA was washed with 50 μ l H₂O, mixed several times within each well and vacuum filtration repeated. Purified samples were dissolved in 25 μ l H₂O by shaking on a plate shaker at 1000rpm for 10 minutes.

Samples were retrieved from the purification plate and transferred into a 96-well plate.

3.3.7.5 Cycle Sequencing

3.3.7.5.1 Principle and Procedure

DNA sequencing was first described by Sanger et al. in 1977. Based on the principle of DNA replication sanger dideoxy sequencing, a method where dideoxynucleotides triphosphates (ddNTP's) function as DNA chain terminators, has undergone a process of subsequent improvements. Increased efficiency and accuracy has led to high-throughput semi-automated sequencing technology (Hunkapiller et al., 1991).

Automated cycle sequencing is a process of rounds of denaturation, annealing and extension, which results in linear amplification of extension products. **(Figure 6)** The reaction mix contains DNA template, a sequencing primer, DNA polymerase, nucleotides (dNTP's), reaction buffer and dideoxynucleotides (ddNTP's) which lack the 3'-hydroxyl group essential for the phosphodiester bond formation. Each round of primer extension is terminated by the incorporation of one of the four fluorescent dye-labelled dideoxynucleotide triphosphates.

The sequence is determined by high-resolution capillary electrophoresis using a denaturing flowable polymer. The purified buffered sequence reaction is exposed to high voltage and the negatively charged fragments move into the capillaries toward the positive electrode. The fragments are separated by size based on their total charge. Passing a laser beam each dye emits a unique wavelength and the fluorescent dye on the extension product identifies the 3' terminal dideoxynucleotide as A, G, C, or T (Applied Biosystems, 2009).

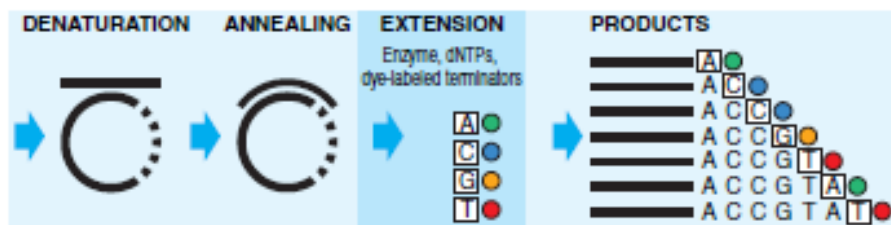


Figure 6: Principle of dye terminator cycle sequencing.
Reprinted from "DNA Sequencing by Capillary Electrophoresis Chemistry Guide." by Applied Biosystems, 2009

We performed cycle sequencing in a 96-well reaction plate using BigDye® Terminator v3.1 Cycle Sequencing Kit. The volume of the 2.5xready reaction premix was reduced to 2µl and accordingly adjusted for a 20µl approach. Therefore, 3µl 5x BigDyeSequencing buffer, 0.5µl primer either forward or reverse (3.2pmol), 5µl template DNA and 9.5µl ddH₂O were added. Template quantity was adapted according to the manufacturer's protocol.

Sequence reaction was carried out using Tetrad® 2 thermal cycler. Cycling conditions were rapid thermal ramp to 96°C and initial denaturation at 96°C for 1 minute followed by 30 cycles at 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. Reaction was completed with a rapid thermal ramp to 4°C.

3.3.7.6 Extension Product Purification

To prevent coinjection of unlabeled and dye-labelled reaction components with the extension products, purification of the product needs to be performed. Purification was carried out with BigDye Xterminator® Purification Kit according to standard protocol. 10µl extension product from the sequencing reaction was transferred in an optical 96-well reaction plate. 10µl XTerminator Solution was mixed with 45µl SAM Solution and directly added to the sequencing reaction products. The plate was vortexed for 30 minutes at 2000rpm to capture and immobilize unwanted components. The centrifugation moved the insoluble fraction and captured reaction components to the bottom of the reaction well, whereas the purified extension products remained in the supernatant.

The plate was either stored at 4°C or immediately placed in the Genetic Analyzer 3130.

3.3.7.7 Capillary Electrophoresis

Capillary electrophoresis was performed on the Genetic Analyzer 3130 using Pop-7™ run module BDx_UltraSeq36_Pop7. For shorter exons run time was reduced. EDTA buffer and LiChrosolv H₂O were used according to standard protocol.

3.3.8 Data Analysis

Primary sequencing analysis was performed with Applied Biosystems Sequencing Analysis Software v.5.2 to process raw data into DNA sequence using KB™ basecaller, which assigns a base to each peak and provides per-base quality value

(QV) predictions. Accurate analysis and alignment was achieved using Staden Package v. 1.5. Each chromatogram was visually inspected for the presence of a putative variant.

3.3.9 In Silico Analysis

Assessments of the cross-species conservation of the CNTN2 amino acid sequence were performed.

Predictions of the effects of amino acid substitution were performed with PolyPhen2.

4 Results

4.1 Clinical data

We identified a consanguineous Egyptian family where five of the seven siblings are affected suffering from autosomal recessive inherited epilepsy syndrome associated with myoclonus and tremor suggesting a phenotype of temporal lobe epilepsy. For details see Material and Methods section.

4.2 Genetic Data

4.2.1 Linkage Analysis of the Egyptian family

We performed Genome-wide linkage analysis in the five affected family members. Under an autosomal recessive model, significant linkage with a LOD score of 3.6 on chromosome 1q31.3-q32.2 between rs927510 and rs724054 was obtained. This region extends 12.5Mb and contains 136 genes (**Figure 9a, b**).

4.2.2 Whole Exome Sequencing

Whole Exome Sequencing was performed for two members (IV-3, IV-6) of the Egyptian family by our cooperation partner (Department of Human Genetics, Helmholtz Center, Munich, HMGU).

Initially, the screening of the two samples showed 29185 and 29755 variants, respectively including SNP's and indels. A number of prioritization steps were applied to reduce the amount of variants. To identify a potentially pathogenic mutation we focused on nonsynonymous variants, splice site acceptor and donor site mutations and coding indels. Variants whose features did not meet quality-control standards were eliminated. We applied the following filters: (i) Removing variants which are present in either the Single Nucleotide Polymorphism Database version 130 or in the eight sequenced exomes of the HapMap samples or in different exomes sequenced at the HMGU and (ii) exclusion of all variants outlying the linkage region. After this filtering, three homozygous variants which were predicted to result in amino acid changes were left (**Table 6**).

(i) *DEAD (Asp-Glu-Ala-Asp) box polypeptide 59, (DDX59)* harboured a single nucleotide variant c.844G>C that introduces a missense mutation at position 282 resulting in an amino acid transition from glutamine acid to glutamine (E282Q). This gene is rarely expressed in the brain and may play a role in ATP binding and helicase activity.

(ii) *Troponin I type 1, (TNNI1)* exhibits a single nucleotide variant c.506C>A that introduces an amino acid change from serine to tyrosine at position 169 (S169Y). *TNNI1* is expressed in cardiac and skeletal muscle only. The encoded protein inhibits calcium-mediated conformational changes in actin-myosin complexes and thus prevents muscle contraction.

(iii) The third variation a homozygous single nucleotide deletion c.503_503delG in *Contactin 2* gene, (*CNTN2*) also termed *TAG-1*, in exon 6 at the amino acid position 168 results in a frameshift onwards of this position. *CNTN2* is a glycosylphosphatidylinositol (GPI)-anchored neuronal membrane protein that functions as a cell adhesion molecule and may play a role in the formation of axon connections in the developing nervous system. The deletion is located in the Ig-like C2-type 2 domain of the protein and therefore yields to a loss of function, making this gene the most likely candidate gene.

Table 6: Exome Sequencing: Variants shared by the two individuals after prioritization.

Gene	Exon	Nucleotide	Amino acid
DDX59	3	c.844G>C	E282Q
TNNI1	7	c.506C>A	S169Y
CNTN2	6	c.503_503delG	Frameshift

4.2.3 Genotyping of DDX59, TNNI1 and CNTN2 variants

We genotyped all three putative pathogenic variants (i) in 8 members of the Egyptian family [5 affected children (IV-1, IV-3, IV-4, IV-6 IV-7), one healthy child (IV-2) and two healthy parents (III-1, III-2)], (ii) in 170 Austrian and 19 Italian TLE patients and (iii) in 366 Austrian and 36 Egyptian healthy controls. All three variations were confirmed to be in a homozygous state in all affected subjects of the family and in a heterozygous state in both healthy parents (**Figure 7**). None of these variants were present in the screened Austrian and Italian TLE patients and Austrian and Egyptian controls.

4.2.4 Capillary Sequencing of the CNTN2 gene

Based on previous published animal studies (Fukamauchi et al., 2001; Poliak, et al., 2003; Traka et al., 2003; Brew et al., 2007), expression data (focusing on expression in the brain) and the impact of the mutation (loss of function mutation), we considered the *CNTN2* gene as the most likely candidate. To test whether *CNTN2* is a more general contributor to TLE we screened all 22 coding exons of this gene in 189 Austrian and Italian patients and in three members (III-1, IV-1 and IV-7) of the Egyptian family using capillary sequencing.

We identified 21 coding sequence variants in our study population (**Table 8**). Twelve of these variants lead to nonsynonymous amino acid substitutions, of which seven were not previously reported in dbSNP 130. Nine variants were synonymous, of which three were already deposited in the SNP database 130.

Each of the seven novel missense variants was genotyped in 366 neurologically normal control samples using the Taqman allelic discrimination assay. To assess the frequency of rare variants (in the CNTN2 gene) in the general population, our cooperation partner in Munich, evaluated Whole Exome Sequencing data from 72 non-epileptic individuals.

In particular, capillary sequencing revealed seven nonsynonymous coding variants (**Table 7, Figure 9e**). We detected a heterozygous missense variation, c.214C>T, in exon 3 leading to an amino acid change from arginine to tryptophan on position 72 (R72W). R72W lies in an evolutionary conserved area and a clearly protein damaging effect is predicted by PolyPhen. It was not present in any of our control individuals.

We found two heterozygous missense variants in exon 8: c.862G>A resulting in a protein change from glutamine acid to lysine at position 288 (E288K). For this variation, found in an Italian patient, the evolutionary conservation is slightly weaker but is still predicted to be possibly damaging and not present in the control population. The second variation in exon 8, c.947C>T induces the amino acid substitution threonine to isoleucine at codon 316 (T316I). T316I lies in an evolutionary conserved region, is predicted to be a benign variation and present in five Austrian control individuals.

In two patients we detected the variation c.1405 C>A in exon 12. The amino acid alteration is a proline to threonine change on position 469 (P469T). One of the patients shows a positive family history. This variant is predicted to be benign and not found in Austrian controls but in one individual of the 72 exome controls.

The fifth variant, c.1460C>T, in exon 12 induces a substitution from the amino acid threonine to isoleucine at codon 487 (T487I). T487I also located in an evolutionary conserved area, is predicted to have a protein damaging effect and was found twice in our control population.

In exon 16 we found the variation c.2057T>C. This variation is marked by an amino acid transition from isoleucine to threonine at the position 686 (I686T). I686T is predicted to be benign and was not present in the control cohort.

In exon 18 we discovered a double nucleotide variant (c.2273-2274GG>AT) provoking a change from tryptophan to tyrosine (W758Y) in an individual with a positive family history. The deceased grandfather was reported to suffer also from a nonspecified epilepsy syndrome. W758Y shows a clear protein damaging effect, considerable evolutionary conservation and absence in both control cohorts.

Evaluation of Whole Exome Sequencing data from 72 non epilepsy patients from the Munich database revealed four rare nonsynonymous coding variants not deposited in the dbSNP130: (**Table 7**)

One variant, P469T, was already found in two of our patients. The second variant detected, c.505C>T in exon 6, provokes an amino acid change from leucine to phenylalanine (L169F). For this variation no protein damaging effect is predicted by PolyPhen. The third identified variation c.1294_1295insG is a single base pair insertion in exon 11. The last variation, c.2701C>T, was found in exon 20 inducing an amino acid substitution from proline to serine (P901S). PolyPhen predicts a protein damaging effect for P901S.

In total, capillary sequencing of patients and evaluation of exome data from control individuals revealed 35 distinct variations in the *CNTN2* gene (**Table 8**).

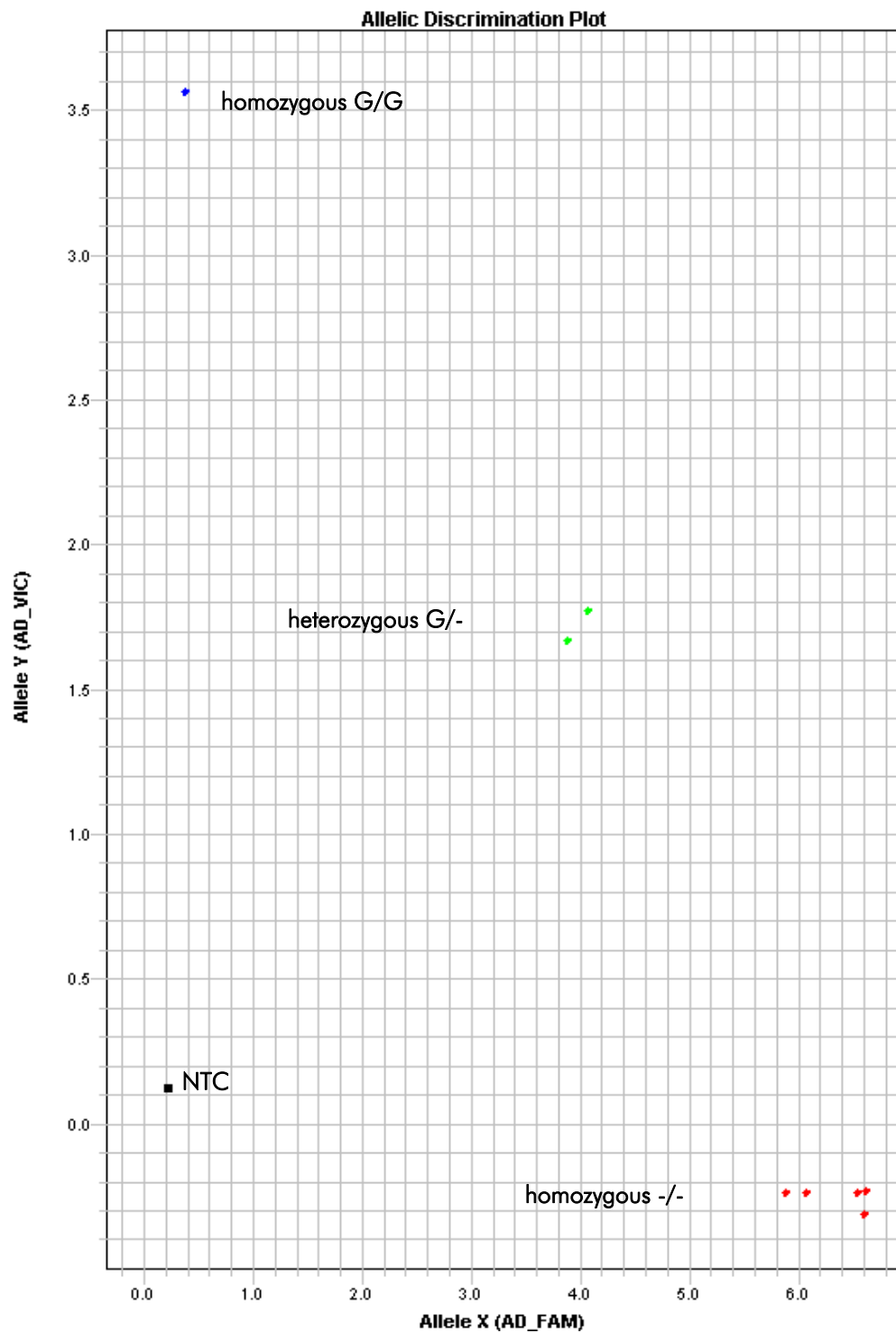


Figure 7: Scatter plot of Taqman allelic discrimination assay of the genotyped c.503-503delG mutation in the Egyptian family. Plotting wt allele (G) versus mutated allele (delG). The vertical axis is the G allele, labelled with VIC® and the horizontal axis is the mutated allele with the deletion, labelled with FAM™. Non-template controls are plotted as black squares. Heterozygous parents (green dots), homozygous affected siblings (red dots) and homozygote wild type GG in the healthy sibling (blue dot) are genotyped accordingly.

Table 7: Rare CNTN2 variants in cases and controls

Cases counts	Exome Controls n=72 counts	Exon	Genomic position	Nucleotide change	Protein change	PolyPhen Prediction	Austrian controls n= 366		
							1/1	1/2	2/2
1		3	205027192	c.214C>T	R72W	probably d.			366
	1	6	205028229	c. 505C>T	L169F	benign			
1		8	205030437	c.862G>A	E288K	possible d.			366
1		8	205030522	c.947C>T	T316I	benign		5	361
	1	11	205033504	c.1294_1295insG					
2/ [1]	1	12	205033764	c.1405C>A	P469T	benign			366
1		12	205033819	c.1460C>T	T487I	probably d.		2	364
1		16	205036310	c.2057T>C	I686T	benign			366
[1]		18	205039031	c.2273_2274GG>AT	W758Y	probably d.			366
	1	20	205041231	2701C->T	P901S	possible d.			

cDNA numbering is based on the reference gene uc001hbr.1 for CNTN2 where +1 corresponds to the A of ATG start translation codon. Familial cases are given in square brackets. Possibly d.: possibly damaging; probably d.: probably damaging. The variant allele was denoted as '1', the reference allele as '2'.

Figure 8: Schematic representation of evolutionary conserved areas in different vertebrates of the seven rare nonsynonymous coding variants detected by capillary sequencing.

	R72W	E288K	T316I
Homo sapiens	PPATY R WKMN G	TTA--EPT L QIP	SKGRDT V QGRI
Pan troglodytes	PPATY R WKMN G	TTA--EPT L QIP	SKGRDT V QGRI
Mus musculus	PPATY R WKMN G	GTA--EPT L QIP	SKGRDT V QGRI
Rattus norvegicus	PPATY R WKMN G	ATA--EPT L QIP	SKGRDT V QGRI
Gallus gallus	PPATY R WKMN G	LSS--E P LLHIQ	IKGRDT Y QGRI
Danio rerio	PAAVY R WRV N G	GTSVDGPILI P	SEGRETHQGR V

	P469T	T487I	I686T	W758Y
Homo sapiens	RVTV T PDG T LI	DEGKY T CFAEN	YEF R VLAS N IL	QG S THWQTAR V
Pan troglodytes	RVTV T PDG T LI	DEGKY T CFAEN	YEF R VLAS N IL	QG S THWQTAR V
Mus musculus	RVTV T LDG T LI	DEGKY T CFAEN	YEF R VSAS N IL	QG S SSWQTAR V
Rattus norvegicus	RVTV T SDG T LI	DEGKY T CFAEN	YEF R VSAS N IL	QG S SSWQTAR V
Gallus gallus	RVT I TADG T LI	DEGKY T CFAEN	YEF R VLAS N IL	KGTQ G WLTAR V
Danio rerio	RITV T PDGIL W	DEGKY T CFAEN	YEFQ V ILAS N IL	QGI P T W TVVK V

Homo sapiens (human), Pan troglodytes (chimpanzee), Mus musculus (mouse), Rattus norvegicus (rat), Gallus gallus (chicken) and Danio rerio (zebrafish). Corresponding amino acid is indicated in bold.

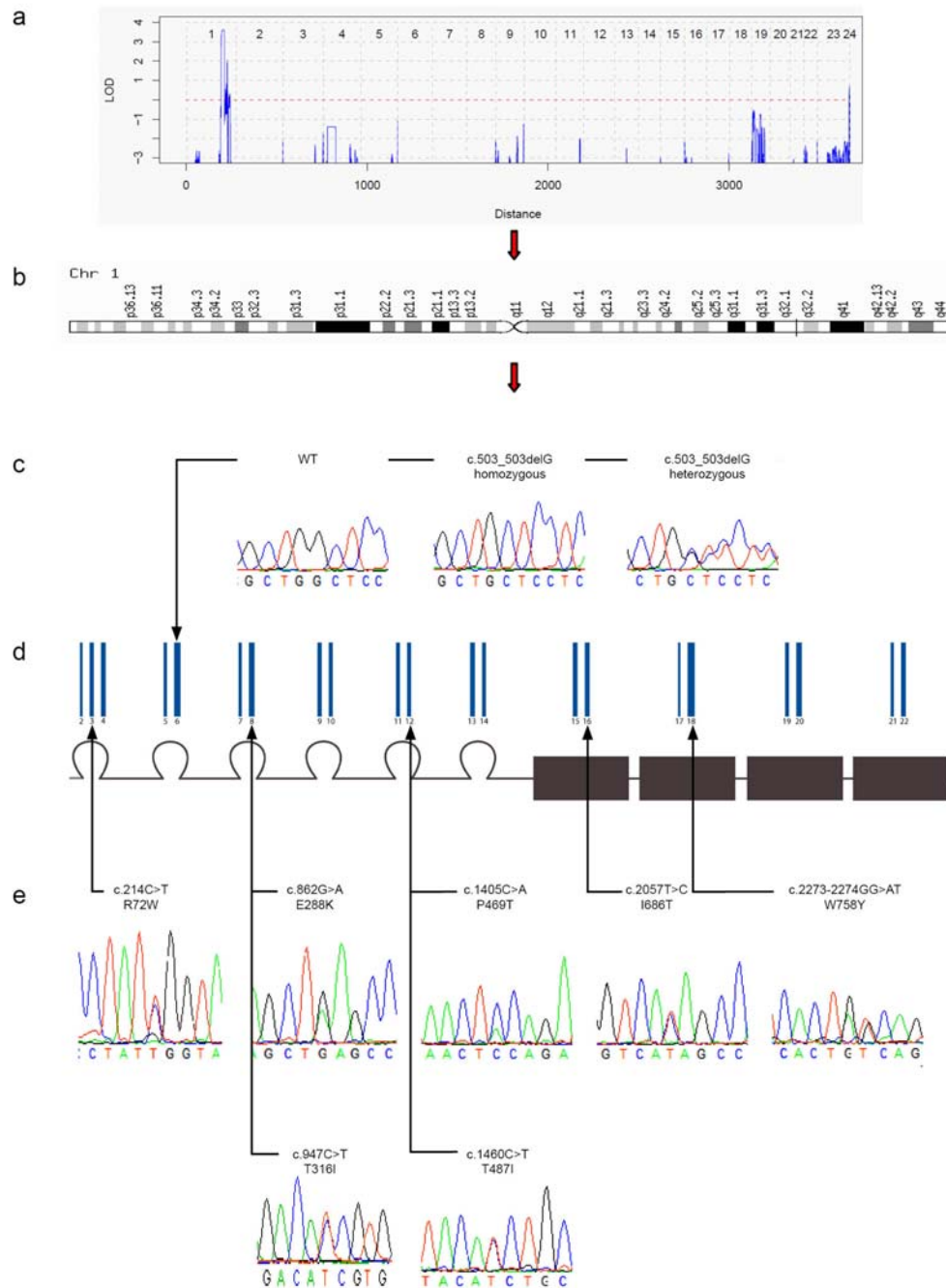


Figure 9: Linkage mapping and subsequent sequencing identifies a CNTN2 mutation in an Egyptian family and heterozygous missense variations in patients with TLE.

(a, b) Genome-wide linkage analysis yields significant positive LOD score on Chr1q31.3-q32.2 defining a possible new locus between rs927510 chr1:195653470 and rs724054 chr1:208175909. (c) Sequence traces of a control sample (WT) and of two members of the Egyptian family. Individual IV-6 carries a homozygous deletion (c.503_503delG) in exon 6 of the CNTN2 gene and the parents demonstrate the c.503_503delG mutation in a heterozygous state, respectively. (d) Exon structure of human CNTN2 transcript and the corresponding protein domain structure consisting of 6 Ig-like and 4 Fibronectin type III domains. The CNTN2 gene extends over 1040aa and contains 22 coding exons (vertical hatches). Arrows indicate positions of detected variations relative to exons and protein domains. (e) Sequence traces of the seven rare heterozygous CNTN2 variations detected in 189 TLE patients. Variation and predicted translational changes are indicated. None of the variations were present in controls except T316I, T487I and P469T.

Table 8: All CNTN2 variations found in 189 TLE patients and 3 Egyptian family members and 72 exome controls

Genomic position on chromosome 1	Exon/Intron	Nucleotide	Aminoacid	Comment	Cases n= 192	Controls Exome n= 72
Exon nonsynonymous						
205027192	3	c.214C>T	R72W		1/182	
205027737	5	c.433G>A	A145T	rs2275697	50/185	20/72
205028229	6	c. 505C>T	L169F			1/72
205030437	8	c.862G>A	E288K		1/192	
205030522	8	c.947C>T	T316I		1/192	
205031116	9	c.1097C>T	P366L	rs2229866	99/190	39/72
205033504	11	c.1294_1295insG				1/72
205033518	11	c.1309C>G	V437L	rs79431021	6/185	5/72
205033764	12	c.1405C>A	P469T		2/185	1/72
205033819	12	c.1460C>T	T487I		1/185	
205035727	15	c.1974A>G	N659D	rs41264871	2/187	2/72
205036310	16	c.2057T>C	I686T		1/188	
205039031	18	c.2273-2274GG>AT	W758Y		1/188	
205041231	20	2701C->T	P901S			1/72
205042840	23	c.3070G>A	V1024I	rs17416074	12/186	10/72
Exon synonymous						
205027083	3	c.105C>T	F35F		1/182	
205027390	4	c.297C>T	N99N	rs9787172	10/182	8/72
205027790	5	c.486A>C	P162P	rs114050151	11/185	3/72
205030397	8	c.822C>T	R274R		1/192	
205030406	8	c.831C>T	D277D		1/192	
205034390	13	c.1695G>A	V565V		1/186	
205039627	19	c.2447C>T	P816P		1/188	
205041158	20	c.2628C>T	S876S	rs2229868	77/190	29/72
205041179	20	c.2649G>A	K883K		1/190	
Intron non-coding						
205027496	4	IVS4+12G>A		rs16855012		1/72
205028438	6	IVS6+18G>T		rs872379	10/178	
205028827	7	IVS7+17insG		rs5780262	40/189	
205028884	7	IVS7+74-75delAG		rs35182846	77/189	
205030587	8	IVS8+39C>T		rs41310859	11/192	
205031744	10	IVS10+47G>A		rs2242001	56/191	
205031769	10	IVS10+72G>A		rs2242000	56/191	
205033650	11	IVS11+50A>G		rs11804498	1/185	
205034434	13	IVS13+44G>T		rs3901740	38/186	
205034853	13	/GACCACCGGCCCTGAC CAGTGCAGCAGGCCA		rs3217591	137/186	
205038731	17	IVS17+42C>T		rs2305275	64/186	

5 Discussion

In the present study we investigated a consanguineous Egyptian family with an autosomal recessive inherited epilepsy syndrome. Five of seven siblings are affected and present a phenotype with temporal lobe epilepsy, myoclonus and tremor. Via linkage analysis and exome sequencing we report a one basepair deletion in exon 6 of the *CNTN2* gene (c.503_503delG) in a homozygous state in all affected siblings and in a heterozygous state in the two healthy parents. The result of this deletion is a frameshift which consequently leads to an impairment or loss of enzyme function, due to change in structure of the protein and therefore may be considered to be a loss of function mutation.

CNTN2, alternatively named TAG-1 (Transient Axonal Glycoprotein-1), is a neuronal cell adhesion molecule (CAM) which belongs to the contactin family of the immunoglobulin superfamily (IgSF). *CNTN2* comprises six N-terminal Immunoglobulin (Ig)-like and four Fibronectin type III domains and is attached to the cell membrane by a glycosylphosphatidylinositol anchor (Furley, et al., 1990) (**Figure10**). The protein is transiently expressed in axons during development of the central and peripheral nervous system and in myelinating glia cells as an anchored form as well as a released form (Karagogeos, 2003; Poliak and Peles, 2003). It is expressed in several types of neurons inclusively in adult neurons of the dorsal root ganglia and their projections and in spinal motor neurons (Dodd et al., 1988; Traka et al., 2002 and 2003). *CNTN2* interacts with a number of different binding partners and may play an important role in the initial stage of axon outgrowth and in

interaction between developing neurons. Further, CNTN2 is implicated in axon-glia interactions as it takes part in the organization of axonal subdomains at the node of Ranvier of myelinating fibers. The myelin sheath is structured in segments separated by the nodes of Ranvier, a myelin free region. The nodes are characterized by a high concentration of Na⁺ channels which are essential for the generation of the action potential during saltatory conduction (Waxman and Ritchie, 1993). Next to the node sits the paranodal junction formed by terminal cytoplasmic loops of the myelin onto the axolemma. The region adjacent to the paranodes is designated as the juxtaparanodes (**Figure 11a**). The juxtaparanode is enriched with shaker-type Kv1 channels. These channels build a complex with the Caspr2 protein which subsequently forms a complex with CNTN2. CNTN2 is required for the juxtaparanodal clustering of Caspr2 and K⁺ channels (Poliak et al., 2003; Traka et al., 2003). In CNTN2 deficient mice (95% homology between human and rat homologues at the amino acid sequence level) axonal Caspr2 did not accumulate at juxtaparanodes and the normal enrichment of shaker-type K⁺ channels in these regions was severely disrupted (Traka et al., 2003). A model for this molecular interaction at the juxtaparanodal region suggests a complex consisting of a glial CNTN2 molecule and the axonal Caspr2 forming a heterodimer through the trans binding of CNTN2 with itself. This suggests that axon-glia interactions mediated by these proteins allow myelinating glia cells to organize ion channels such as Kv1.1 and Kv1.2 in the underlying axonal membrane (Poliak et al., 2003; Traka et al., 2003) (**Figure 11b**). So far, the role of the juxtaparanodal K⁺ channels is not completely understood. It is hypothesized that K⁺ channels act as an active damper of re-entrant excitation (Vabenick et al., 1999) which requires a high spatial clustering of K⁺ channels near the node and they maintain the resting potential for the entire nerve (Chiu and Ritchie, 1984). Although the K⁺ channel clustering was abolished in CNTN2 and Caspr2 deficient mice, the total content of K⁺ channels remained constant and the myelin-concealed K⁺ channels maintained the internodal resting potential (Poliak et al., 2003; Traka et al., 2003). According to these two studies, no gross morphological abnormalities of the central nervous system of

CNTN2 and Caspr2 deficient mice compared with the wild type were reported and no change in the excitability of myelinated nerves occurred. Nevertheless, in a previous study, mice lacking Kv1.1 and Kv1.2 exhibited increased seizure susceptibility and hyperexcitability in axons and Kv1.2 knock-out mice additionally showed myoclonus and tremor (Brew et al., 2007). Moreover, Fukamauchi et al., reported spontaneous epileptic seizures in CNTN2 deficient mice (Fukamauchi et al., 2001). In 2006 Strauss et al. described a recessive symptomatic focal epilepsy in Older Amish People caused by a single base deletion in the CASPR2 gene. Brain samples of these patients showed an altered expression of Kv1.1 and Nav1.2 channels (Strauss et al., 2006).

Considering these findings, *CNTN2* ranks highly among candidate genes for epilepsy. Thus, we subsequently performed capillary sequencing in TLE patients to investigate the contribution of the *CNTN2* gene to the temporal lobe epilepsy phenotype in general. Overall, we did not find any homozygous mutation. The screening of all coding exons revealed heterozygous variants only. Assuming a recessive trait and considering the fact that the parents, heterozygous for the c.503_503delG mutation, presented no clinical symptoms of epilepsy at the time of examination, we cannot prove pathogenicity of these variants in our TLE patients. Thus, there is no indication that heterozygous variations influence the disease susceptibility.

The ‘gold standard’ in proving the pathogenicity of a given high risk variant is segregation in the family. However, as family history is often not available, this proof cannot be applied. In silico predictions of pathogenicity, such as calculated by PolyPhen, have to be considered with caution. Past studies have frequently shown that genetically proven mutations were predicted to be benign by PolyPhen and variations, irrelevant for the disease, were predicted to be probably damaging. The predictions are based on evolutionary conservation and theoretical assumptions of the 3 dimensional structure of a protein. To find out the relevance of a candidate gene in a given disease population it has become standard to screen in a first step

many patients for the whole gene (all exons), and in a second step, to genotype the found variants in a matched control population. Lack of occurrence of a variant in the control group is usually taken as a supportive argument for pathogenicity. The genome of any individual carries many thousands of private or very rare missense variants which do not obviously influence the phenotype of this person. It is therefore a matter of statistical chance that such variants are found in a given gene when screening hundreds of samples. Thus, a more reliable approach is to screen the whole gene in the same number of patients and control individuals. If the frequency of variants is then found to be higher in the patient group, it might be more likely to take this as a supportive argument for pathogenicity. We have evaluated the frequency of variants in the *CNTN2* gene from the exome sequencing data of 72 non epileptic patients. We have found four rare nonsynonymous coding variants in 72 exome data, and seven in our 189 TLE patients. The identical frequency of rare variants in the patients and the control group indicates that *CNTN2* is not likely to be a major contributor for the TLE phenotype in general.

Nevertheless, we cannot preclude compound heterozygous mutations completely (the condition of having two heterogeneous recessive alleles causing the disease) as we did not screen the intronic and regulatory regions of the *CNTN2* genes. Digenic inheritance (the disease is caused by an interaction of two gene-defects) cannot be excluded too. We did not screen any other genes, which may contain a second variant contributing in combination with the heterozygous *CNTN2* variation to the TLE phenotype. According to the parents' heterozygous state, regarding the c.503_503delG mutation, we know that in this case haploinsufficiency (a single copy of the normal gene is not capable of providing sufficient protein product as to assure normal function) of the *CNTN2* protein does not underlie epilepsy. However, we cannot exclude the possibility of a dominant negative effect (heterozygous mutation that results in an altered gene product that acts antagonistically to the wild-type allele) or loss of function (mutation that results in an impaired or abolished function

of the protein) in some of the other found heterozygous variations and thus, being possibly pathogenic.

The remarkable phenotype of the Egyptian family and the limited comparability with our tested sample of TLE patients is further constricting our results. The phenotype of the Egyptian family consisting of an adolescent onset epilepsy syndrome with obvious temporal lobe features, myoclonus and tremor could most probably be arranged with a rare syndrome called familial cortical myoclonic tremor with epilepsy (FCMTE, BAFME, FAME) (van Rootselaar et al., 2005). However, these patients start with generalized tonic clonic seizures in adulthood, exhibit also myoclonus and tremor, but there are no definite references for temporal lobe epilepsy as in our case. Furthermore, contrary to our family, most of these families show an autosomal dominant trait. Three loci on chromosome 2p, 8q and 5q are described for this syndrome to date, but a causing gene has not yet been identified (Mikami et al., 1999, Guerrini et al., 2001, Laubage et al., 2002, De Falco et al., 2003, Striano et al., 2004, Depienne et al., 2010).

It cannot be ruled out that the c.503-503del G mutation, obviously disease causing in the described Egyptian family, could be unique to this family. Therefore it remains to be seen whether mutations affecting *CNTN2* will be identified as causes of temporal lobe epilepsy in other populations.

Nevertheless, the crucial role of *CNTN2* in neuronal excitability makes this gene a perfect candidate for epilepsy. Future studies will show the significance of this new identified gene.



Figure 10: Schematic representation of CNTN2 protein.
The protein comprises 6 N-terminal Ig-like and 4 Fibronectin type III domains.

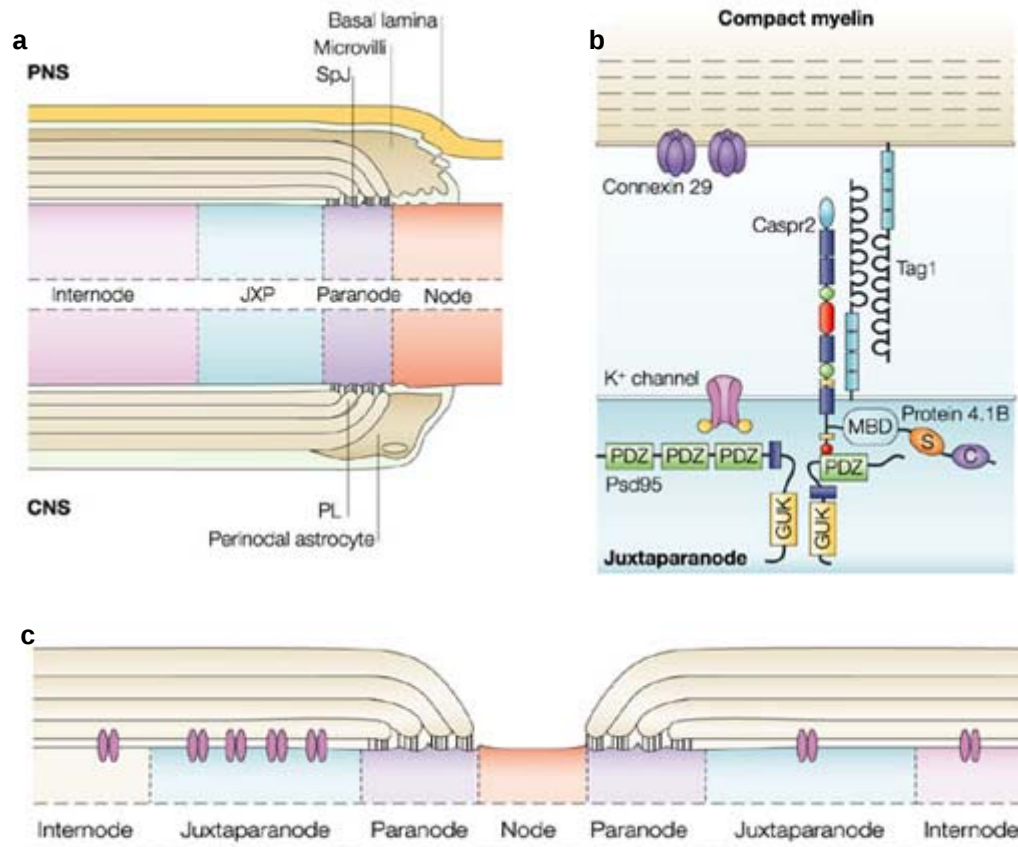


Figure 11: Localization of CNTN2 under the myelin sheath.

(a) Differentiation of the axonal membrane into nodes, paranodal junction, juxtaparanode and internodal region. (b) Axonal CNTN2 (TAG-1) forms a complex with Caspr2 and undergoes a homophilic binding with a glial CNTN2 (TAG-1) molecule at the juxtaparanodal region. Caspr2 in turns interacts with voltage gated K⁺ channels embedded in the membrane. (c) In CNTN2-deficient mice (CNTN2^{-/-}) clustering of K⁺ channels at the juxtaparanode is altered and channels dissipated into the internode.

Reprinted from "The local differentiation of myelinated axons at the Nodes of Ranvier." by Poliak, S. and Peles, E. 2003. Nature Review. 4, p968

6 Appendix

Figure 12: Nucleotide and amino acid sequence of the CNTN2 gene uc001hbr.1.
Rare nonsynonymous coding variations identified by capillary sequencing are indicated in yellow. The c.503_503delG mutation of the Egyptian family is indicated in red.

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      10      20      30      40      50      60
  ----:----|----:----|----:----|----:----|----:----|
1 ATGGGGACAGCCACCAGGAGGAAGCCACACCTGCTGCTGGTAGCTGCTGTGGCCCTTGTC 60
1 M G T A T R R K P H L L L V A A V A L V 20

      70      80      90     100     110     120
  ----:----|----:----|----:----|----:----|----:----|
61 TCCTCTTCAGCTTGGAGTTCAGCCCTGGGATCCCAAACCACCTTCGGGCCTGTCTTTGAA 120
21 S S S A W S S A L G S Q T T F G P V F E 40
   ||
   exon2/exon3

     130     140     150     160     170     180
  ----:----|----:----|----:----|----:----|----:----|
121 GACCAGCCCCTCAGTGTGCTATTCCAGAGGAGTCCACGGAGGAGCAGGTGTTGCTGNCA 180
41 D Q P L S V L F P E E S T E E Q V L L X 60

     190     200     210     220     230     240
  ----:----|----:----|----:----|----:----|----:----|
181 TGCCGCGCCCGGGCCAGCCCTCCAGCCACCTATCGGTGGAAGATGAATGGTACCGAGATG 240
61 C R A R A S P P A T Y R W K M N G T E M 80
   ||
   exon3/exon4

     250     260     270     280     290     300
  ----:----|----:----|----:----|----:----|----:----|
241 AAGCTGGAGCCAGGTTCCTGTCACCACTGGTGGGGGGCAACCTGGTCATCATGAANCCC 300
81 K L E P G S R H Q L V G G N L V I M X P 100

     310     320     330     340     350     360
  ----:----|----:----|----:----|----:----|----:----|
301 ACCAAGGCACAGGATGCCGGGGTCTACCACTGCCTGGCCTCCAACCCAGTGGGCACCGTT 360
101 T K A Q D A G V Y Q C L A S N P V G T V 120

     370     380     390     400     410     420
  ----:----|----:----|----:----|----:----|----:----|
361 GTCAGCAGGGAGGCCATCCTCCGCTTCGGCTTTCTGCAGGAATTCTCCAAGGAGGAGCGA 420
121 V S R E A I L R F G F L Q E F S K E E R 140
   ||
   exon4/exon5

     430     440     450     460     470     480

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-----|-----|-----|-----|-----|-----|
421 GACCCAGTGAAANCTCATGAAGGCTGGGGGGTGATGTTGCCCTGTAACCCACCTGCCCCAC 480
141 D P V K X H E G W G V M L P C N P P A H 160

          490          500          510          520          530          540
-----|-----|-----|-----|-----|-----|
481 TACCCAGGCTTGTCTACCGCTGGCTCCTCAACGAGTTCCCCAACTTCATCCCGACGGAC 540
161 Y P G L S Y R W L L N E F P N F I P T D 180
      ||
      exon5/exon6

          550          560          570          580          590          600
-----|-----|-----|-----|-----|-----|
541 GGGCGTCACTTCGTGTCCAGACCACAGGGAACCTGTACATTGCCCGAACCAATGCCTCA 600
181 G R H F V S Q T T G N L Y I A R T N A S 200

          610          620          630          640          650          660
-----|-----|-----|-----|-----|-----|
601 GACCTGGGCAACTACTCCTGTTTGGCCACCAGCCACATGGACTTCTCCACCAAGAGCGTC 660
201 D L G N Y S C L A T S H M D F S T K S V 220

          670          680          690          700          710          720
-----|-----|-----|-----|-----|-----|
661 TTCAGCAAGTTTGCTCAGCTCAACCTGGCTGCTGAAGATACCCNGCTCTTTGCACCCAGC 720
221 F S K F A Q L N L A A E D T X L F A P S 240
      ||
      exon6/exon7

          730          740          750          760          770          780
-----|-----|-----|-----|-----|-----|
721 ATCAAGGCCCGGTTCCAGCAGAGACCTATGCACTGGTGGGGCAGCAGGTCACCCTGGAG 780
241 I K A R F P A E T Y A L V G Q Q V T L E 260

          790          800          810          820          830          840
-----|-----|-----|-----|-----|-----|
781 TGCTTCGCCTTTGGGAACCCTGTCCCCCGGATCAAGTGGCGCAAAGTGGACGGCTCCCTG 840
261 C F A F G N P V P R I K W R K V D G S L 280
      ||
      exon7/exon8

          850          860          870          880          890          900
-----|-----|-----|-----|-----|-----|
841 TCCCCGCAGTGGACCACAGCTGAGCCACCCCTGCAGATCCCCAGCGTCAGCTTNGAGGAT 900
281 S P Q W T T A E P T L Q I P S V S X E D 300

          910          920          930          940          950          960
-----|-----|-----|-----|-----|-----|
901 GAGGGCACCTACGAGTGTGAGGCGGAGAACTCCAAGGGCCGAGACACCGTGCAGGGCCGC 960
301 E G T Y E C E A E N S K G R D T V Q G R 320

          970          980          990          1000          1010          1020
-----|-----|-----|-----|-----|-----|
961 ATCATCGTGCAGGCTCAGCCTGAGTGGCTAAAAGTGATCTCGGACACAGAGGCTGACATT 1020
321 I I V Q A Q P E W L K V I S D T E A D I 340
      ||
      exon8/exon9

          1030          1040          1050          1060          1070          1080
-----|-----|-----|-----|-----|-----|
1021 GGCTCCAACCTGCGTTGGGGCTGTGCAGCCGCCGGAAGCCCCGGCCTACAGTGCCTGG 1080
341 G S N L R W G C A A A G K P R P T V R W 360

          1090          1100          1110          1120          1130          1140
-----|-----|-----|-----|-----|-----|

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1081 CTGCGGAACGGGGAGCNTCTGGCCTCCCAGAACCAGGTGGAGGTGTTGGCTGGGGACCTG 1140
 361 L R N G E X L A S Q N R V E V L A G D L 380
 ||
 exon9/exon10

1150 1160 1170 1180 1190 1200
 ----:----|----:----|----:----|----:----|----:----|
 1141 CGGTTCTCCAAGCTGAGCCTGGAAGACTCGGGCATGTACCAGTGTGTGGCAGAGAATAAG 1200
 381 R F S K L S L E D S G M Y Q C V A E N K 400

1210 1220 1230 1240 1250 1260
 ----:----|----:----|----:----|----:----|----:----|
 1201 CACGGTACCATCTACGCCAGCGCCGAGCTAGCCGTGCAAGCACTCGCCCCTGACTTCAGG 1260
 401 H G T I Y A S A E L A V Q A L A P D F R 420
 ||
 exon10/exon11

1270 1280 1290 1300 1310 1320
 ----:----|----:----|----:----|----:----|----:----|
 1261 CTGAATCCCGTGAGGCGTCTGATCCCGCGGCCGCGGGGGAGAGATCCTTATCCCCTGC 1320
 421 L N P V R R L I P A A R G G E I L I P C 440

1330 1340 1350 1360 1370 1380
 ----:----|----:----|----:----|----:----|----:----|
 1321 CAGCCCGGGGAGCTCCAAAGGCCGTGGTGCTCTGGAGCAAAGGCACGGAGATTTTGGTC 1380
 441 Q P R A A P K A V V L W S K G T E I L V 460

1390 1400 1410 1420 1430 1440
 ----:----|----:----|----:----|----:----|----:----|
 1381 AACAGCAGCAGAGTGACTGTAACCTCAGATGGCACCTTGATCATAAGAAACATCAGCCGG 1440
 461 N S S R V T V T P D G T L I I R N I S R 480
 ||
 exon11/exon12

1450 1460 1470 1480 1490 1500
 ----:----|----:----|----:----|----:----|----:----|
 1441 TCAGATGAAGGCAAATACACCTGCTTTGCTGAGAACTTCATGGGCAAAGCCAACAGCACT 1500
 481 S D E G K Y T C F A E N F M G K A N S T 500

1510 1520 1530 1540 1550 1560
 ----:----|----:----|----:----|----:----|----:----|
 1501 GGAATCCTATCTGTGCGAGATGCAACCAAAATCACTCTAGCCCCCTCAAGTGCCGACATC 1560
 501 G I L S V R D A T K I T L A P S S A D I 520
 ||
 exon12/exon13

1570 1580 1590 1600 1610 1620
 ----:----|----:----|----:----|----:----|----:----|
 1561 AACTTGGGTGACAACCTGACCCTACAGTGCCATGCCTCCACGACCCACCATGGACCTC 1620
 521 N L G D N L T L Q C H A S H D P T M D L 540

1630 1640 1650 1660 1670 1680
 ----:----|----:----|----:----|----:----|----:----|
 1621 ACCTTCACCTGGACCCTGGACGACTTCCCACATCGACTTTGATAAGCCTGGAGGGCACTAC 1680
 541 T F T W T L D D F P I D F D K P G G H Y 560

1690 1700 1710 1720 1730 1740
 ----:----|----:----|----:----|----:----|----:----|
 1681 CGGAGAACTAATGTGAAGGAGACCATTGGGGATCTGACCATCCTGAACGCCAGCTGCGC 1740
 561 R R T N V K E T I G D L T I L N A Q L R 580
 ||
 exon13/exon14

1750 1760 1770 1780 1790 1800

```

-----|-----|-----|-----|-----|-----|-----|
1741 CATGGGGGGAAGTACACGTGCATGGCCCAGACGGTGGTGGACAGCGCTCCAAGGAGGCC 1800
581 H G G K Y T C M A Q T V V D S A S K E A 600

          1810          1820          1830          1840          1850          1860
-----|-----|-----|-----|-----|-----|-----|
1801 ACAGTCCTGGTCCGAGGTCCGCCANGTCCCCAGGAGGTGTGGTGGTGAGGGACATTGGC 1860
601 T V L V R G P P X P P G G V V V R D I G 620
      ||
      exon14/exon15

          1870          1880          1890          1900          1910          1920
-----|-----|-----|-----|-----|-----|-----|
1861 GACACCACCATCCAGCTCAGCTGGAGCCGTGGCTTCGACAACCACAGCCCCATCGCTAAG 1920
621 D T T I Q L S W S R G F D N H S P I A K 640

          1930          1940          1950          1960          1970          1980
-----|-----|-----|-----|-----|-----|-----|
1921 TACACCCTGCAAGCTCGCACTCCACCTGCAGGGAAGTGGAAGCAGGTTNGGACCNATCCT 1980
641 Y T L Q A R T P P A G K W K Q V X T X P 660
      ||
      exon15/exon16

          1990          2000          2010          2020          2030          2040
-----|-----|-----|-----|-----|-----|-----|
1981 GCAAACATCGAGGGCAATGCCGAGACTGCACAGGTGCTGGGCCTCACCCCTGGATGGAC 2040
661 A N I E G N A E T A Q V L G L T P W M D 680

          2050          2060          2070          2080          2090          2100
-----|-----|-----|-----|-----|-----|-----|
2041 TATGAGTTCCGGGTCATAGCCAGCAACATTCTGGGCACTGGGGAGCCTAGTGGGCCCTCC 2100
681 Y E F R V I A S N I L G T G E P S G P S 700

          2110          2120          2130          2140          2150          2160
-----|-----|-----|-----|-----|-----|-----|
2101 AGCAAAATCCGGACCAGGGAAGCAGCCCCCTCGGTGGCACCCCTCAGGACTCAGCGGAGGA 2160
701 S K I R T R E A A P S V A P S G L S G G 720
      ||
      exon16/exon17

          2170          2180          2190          2200          2210          2220
-----|-----|-----|-----|-----|-----|-----|
2161 GGTGGAGCCCCCGAGAGCTCATCGTCAACTGGACGCCCATGTCACGGGAGTACCAGAAC 2220
721 G G A P G E L I V N W T P M S R E Y Q N 740
      ||
      exon17/exon18

          2230          2240          2250          2260          2270          2280
-----|-----|-----|-----|-----|-----|-----|
2221 GGAGACGGCTTCGGCTACCTGCTGTCCTTCGCGAGGCAGGGCAGCACTCACTGGCAGACC 2280
741 G D G F G Y L L S F R R Q G S T H W Q T 760

          2290          2300          2310          2320          2330          2340
-----|-----|-----|-----|-----|-----|-----|
2281 GCCCGGGTGCCTGGCGCCGATGCCAGTACTTTGTCTACAGCAACGAGAGCGTCCGGCCC 2340
761 A R V P G A D A Q Y F V Y S N E S V R P 780

          2350          2360          2370          2380          2390          2400
-----|-----|-----|-----|-----|-----|-----|
2341 TACACGCCCTTTGAGGTCAAGATCCGCAGCTACAACCGCCGCGGGGATGGGCCCCGAGAGC 2400
781 Y T P F E V K I R S Y N R R G D G P E S 800

          2410          2420          2430          2440          2450          2460
-----|-----|-----|-----|-----|-----|-----|

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2401 CTCACTGCACTCGTGTACTCAGCTGAGGAAGAGCCCAGGGTGGCCCCTACCAAGGTGTGG 2460
 801 L T A L V Y S A E E E P R V A P T K V W 820
 ||
 exon18/exon19

2470 2480 2490 2500 2510 2520
 ----:----|----:----|----:----|----:----|----:----|
 2461 GCCAAAGGGTCTCATCCTCAGAGATGAACGTGACCTGGGAACCCGTGCAGCAGGACATG 2520
 821 A K G V S S S E M N V T W E P V Q Q D M 840

2530 2540 2550 2560 2570 2580
 ----:----|----:----|----:----|----:----|----:----|
 2521 AATGGTATCCTCCTGGGGTATGAGATCCGCTACTGGAAGCTNGGGACAAAGAAGCAGCT 2580
 841 N G I L L G Y E I R Y W K A X D K E A A 860
 ||
 exon19/exon20

2590 2600 2610 2620 2630 2640
 ----:----|----:----|----:----|----:----|----:----|
 2581 GCGGACCGAGTGAGGACAGCAGGGCTGGACACCANTGCCCGAGTCAGNGGCCTGCATCCC 2640
 861 A D R V R T A G L D T X A R V X G L H P 880

2650 2660 2670 2680 2690 2700
 ----:----|----:----|----:----|----:----|----:----|
 2641 AACACCAAGTACCATGTGACCGTGAGGGCCTACAACCGGGCTGGCACTGGGCCTGCCAGC 2700
 881 N T K Y H V T V R A Y N R A G T G P A S 900

2710 2720 2730 2740 2750 2760
 ----:----|----:----|----:----|----:----|----:----|
 2701 CCTTCTGCCAACGCCACGACCATGAAGCCCCCTCCGCGGCGACCTCCTGGCAACATCTCC 2760
 901 P S A N A T T M K P P P R R P P G N I S 920
 ||
 exon20/exon21

2770 2780 2790 2800 2810 2820
 ----:----|----:----|----:----|----:----|----:----|
 2761 TGGACTTTCTCAAGCTCTAGTCTTAGCATTAAAGTGGGACCCTGTGGTCCCTTTCCGAAAT 2820
 921 W T F S S S S L S I K W D P V V P F R N 940

2830 2840 2850 2860 2870 2880
 ----:----|----:----|----:----|----:----|----:----|
 2821 GAGTCTGCAGTCACCGGCTATAAGATGCTGTACCAGAATGACTTACACCTGACTCCACG 2880
 941 E S A V T G Y K M L Y Q N D L H L T P T 960
 ||
 exon21/exon22

2890 2900 2910 2920 2930 2940
 ----:----|----:----|----:----|----:----|----:----|
 2881 CTCCANCTCACCGGCAAGAACTGGATAGAAATCCCAGTGCCTGAAGACATTGGCCATGCC 2940
 961 L X L T G K N W I E I P V P E D I G H A 980

2950 2960 2970 2980 2990 3000
 ----:----|----:----|----:----|----:----|----:----|
 2941 CTGGTACAAATTCGGACCACAGGGCCCGAGGGGATGGGATCCCTGCAGAAGTCCACATC 3000
 981 L V Q I R T T G P G G D G I P A E V H I 1000

3010 3020 3030 3040 3050 3060
 ----:----|----:----|----:----|----:----|----:----|
 3001 GTGAGGAATGGAGGCACAAGCATGATGGTGGAGAACATGGCAGTCCGCCCAGCACCACAC 3060
 1001 V R N G G T S M M V E N M A V R P A P H 1020
 ||
 exon22/exon23

3070 3080 3090 3100 3110 3120

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-----:-----|-----:-----|-----:-----|-----:-----|-----:-----|-----:-----|
3061 CCTGGCACCNATCATTTCCCACTCCGTGGCGATGCTGATCCTCATAGGCTCCCTGGAGCTC 3120
1021 P G T X I S H S V A M L I L I G S L E L 1040

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8 Curriculum vitae

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Besondere Erfahrungen und Kenntnisse

MOLEKULARBIOLOGISCHE UND GENETISCHE TECHNIKEN

DNA Isolierung, PCR, Real Time PCR, Genotypisierung und Expressionsanalysen mittels TaqMan, Kapillarsequenzierung mittels ABI-Genetic Analyzer 3130, Gene Chip Analyse mittels Genome-Wide-Human SNP Array 6.0 (Affymetrix)

COMPUTERPROGRAMME

Sehr gute Kenntnisse in Microsoft Office (Word, PowerPoint, Excel), Adobe Illustrator
Gute Kenntnisse in Umgang mit genetischen Datenbanken (UCSC Genome Browser, NCBI, ENSEMBL) und Sequenzanalyseprogramm (Staden Package)

SPRACHKENNTNISSE

Sehr gute Englisch-Kenntnisse in Wort und Schrift auf wissenschaftlichem Niveau
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AUSLANDERFAHRUNGEN

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Wissenschaftliche Publikationen

1: *Association of transcription factor polymorphisms PITX3 and EN1 with Parkinson's disease.*
Haubenberger D, Reinthaler E, Mueller JC, Pirker W, Katzenschlager R, Froehlich R, Bruecke T, Daniel G, Auff E, Zimprich A. Neurobiol Aging. 2009 Apr 2.

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