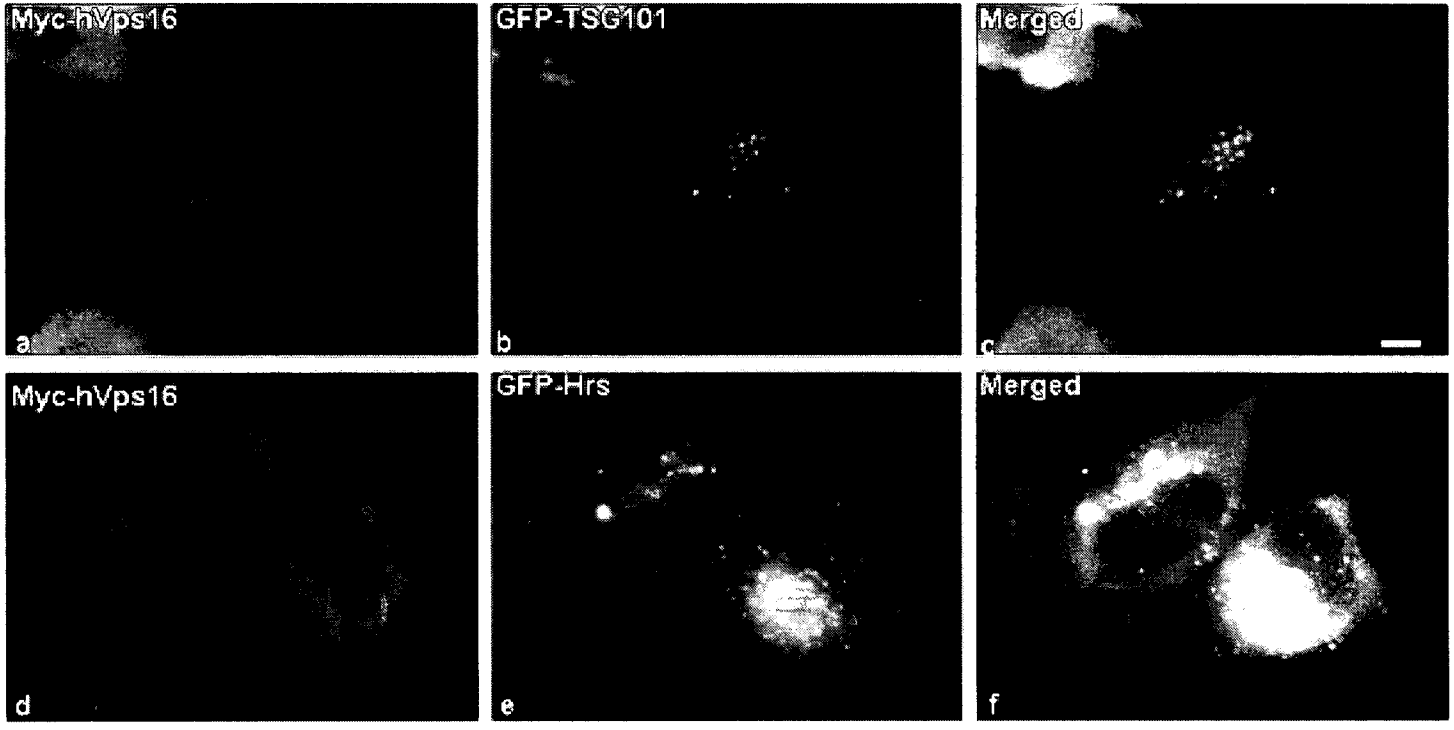
Supplemental Fig. 1, Kim et al



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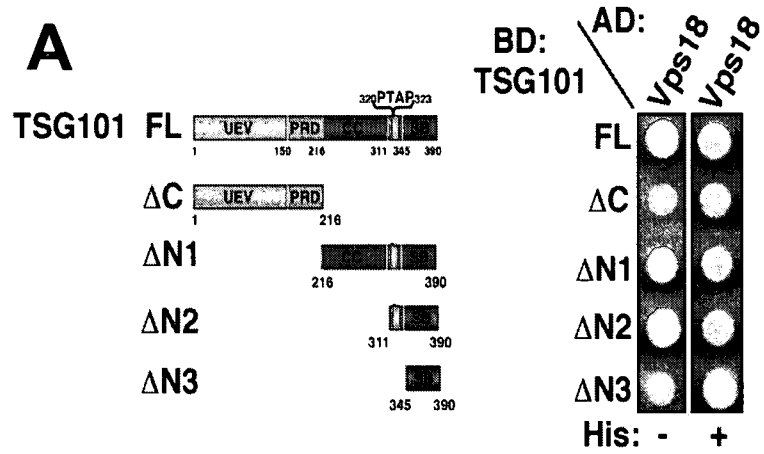

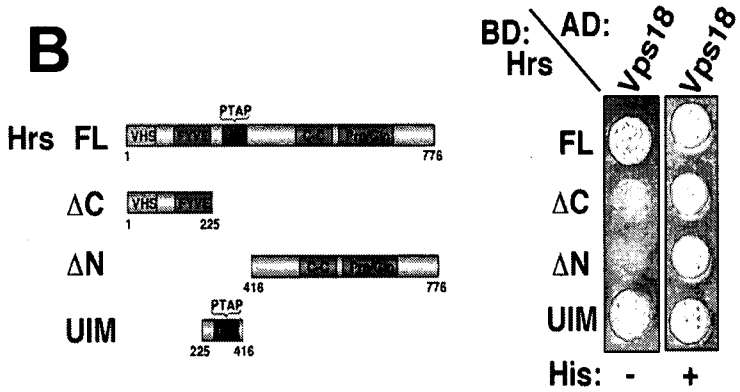

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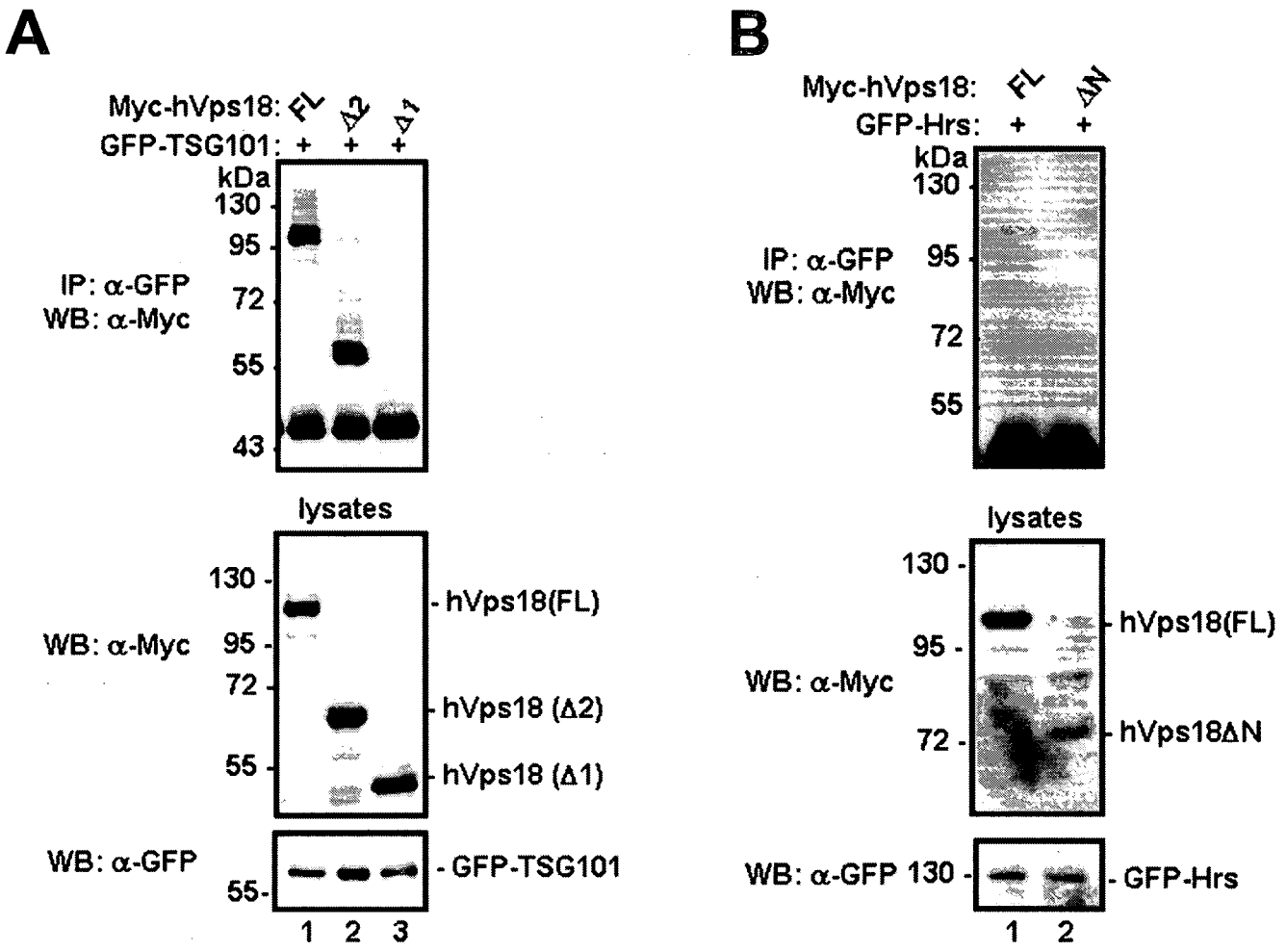
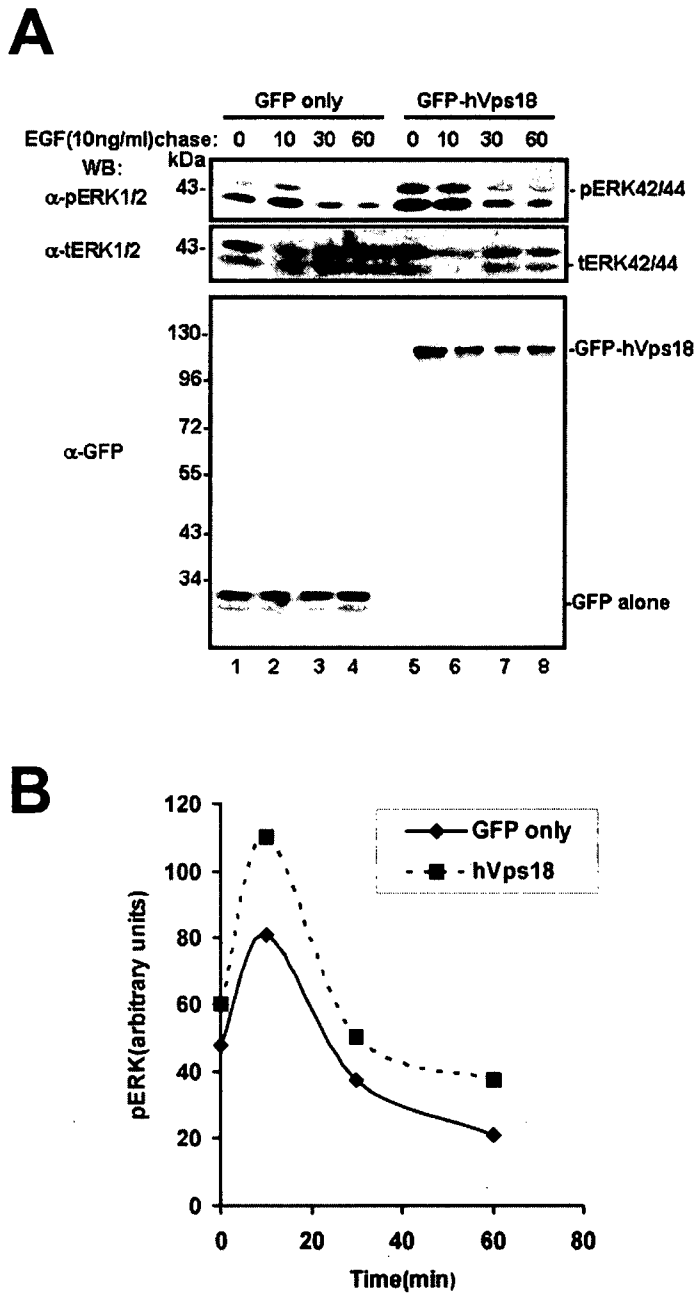


Supplemental fig.4 A, B, Kim et al



Supplemental Figures.

Supplemental Figure 1. Immunofluorescence microscopy analyses of HeLa cells transfected with Myc-Vps16 and GFP-TSG101 (a-c), or GFP-Hrs (d-f). Transfected cells were fixed, permeabilized, and immunostained with a monoclonal antibody to Myc followed by Texas-Red conjugated donkey anti-mouse IgG.. GFP-TSG101 and Hrs were directly visualized by GFP fluorescence (*green,* a and c) and myc-Vps18 (*red*, b and e). Merged images are represented in the third panel of each row (c and f). *Scale bar* represents 10 μm.

Supplemental Figure 2. Identification of TSG101 or Hrs binding sites by deletion analyses. (A, B) Schematic representation of TSG101, and Hrs deletion constructs used in this study and yeast two-hybrid analyses of the interaction with hVps18. Following domains of TSG101 are indicated: UEV, ubiquitin E2 variant; PRD, proline rich domain; SB, steadness box. The VHS, Vps27/Hrs/ Stam domain; PRD, proline rich region; UIM, ubiquitin interacting motif; Pro/Gln, proline/glutamine rich region of Hrs are indicated. Yeast transformants bearing the combination of constructs indicated were spotted onto media with histidine (His +) or without histidine (His -), repectively.

Supplemental Figure 3. The NH_2-terminal regions of hVps18 are critical for the interaction with TSG101 and Hrs *in vivo* (A, B) COS-7 cells were cotransfected Myc-tagged hVps18 wild type and deletion mutants with GFP-tagged TSG101 or Hrs as indicated, and subsequently immunoprecipitations were performed using anti-GFP antibodies. Immunoprecipitates were then subjected to Western blotting using anti-Myc antibodies. Western blot analysis with anti-GFP and anti-Myc antibodies on the soluble cell lysates prior to immunoprecipitation are also included.

Supplemental Figure 4. (A) GFP-vector alone and GFP-hVps18 transfected COS-7 cells were treated with 10 ng/ml EGF for 5 min followed by an acidic wash to remove cell surface EGF. Cells were then chased in serum-free medium for the indicated times and analyzed by immunoblotting with anti-phospho-ERK42/44 (top), anti-ERK42/44 (middle), and anti-GFP antibodies. (B) The level of phospho-ERK42/44 was quantified and normalized against the level of total ERK42/44 and plotted as a percentage of the relative level of phospho-ERL42/44 at chase time. Relative intensities of the bands were quantified using NIH Image (version 1.63).

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Patients with a sodium channel alpha 1 gene mutation show wide phenotypic variation

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Summary We investigated the roles of mutations in voltage-gated sodium channel alpha 1 subunit gene (*SCN1A*) in epilepsies and psychiatric disorders. The *SCN1A* gene was screened for mutations in three unrelated Japanese families with generalized epilepsy with febrile seizure plus (GEFS+), febrile seizure with myoclonic seizures, or intractable childhood epilepsy with generalized tonic–clonic seizures (ICEGTC). In the family with GEFS+, one individual was affected with panic disorder and seizures, and another individual was diagnosed with Asperger syndrome and seizures. The novel mutation V1366I was found in all probands and patients with psychiatric disorders of the three families. These results suggest that *SCN1A* mutations may confer susceptibility to psychiatric disorders in addition to variable epileptic seizures. Unidentified modifiers may play critical roles in determining the ultimate phenotype of patients with sodium channel mutations.

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Introduction

Voltage-gated sodium channels (SCN) are the primary molecules responsible for generating action potentials in nervous system, skeletal and cardiac muscle, and mutations in the genes encoding those expressed in neurons including Nav1.1 encoded by *SCN1A* have been reported to cause epilepsies (Goldin, 2001).

Generalized epilepsy with febrile seizures plus (GEFS+) (OMIM604233) is dominantly inherited epilepsy characterized by febrile seizures in childhood progressing to afebrile generalized epilepsy in adults (Scheffer and Berkovic, 1997; Wallace et al., 2002). More than ten *SCN1A* missense mutations have been reported in GEFS+ families, approximately 10% of cases examined (Abou-Khalil et al., 2001; Annesi et al., 2003; Bonanni et al., 2004; Ceulemans et al., 2004; Escayg et al., 2001; Gerard et al., 2002; Ito et al., 2002; Lerche et al., 2001; Spampanato et al., 2003; Sugawara et al., 2001; Wallace et al., 2001). The penetrance is less than 80% (Scheffer and Berkovic, 1997; Singh et al., 1999; Wallace et al., 2002). In GEFS+, patients in the same pedigree express variable phenotype, febrile seizures often extending age of 6 years and afebrile generalized tonic–clonic seizures, absence, myoclonic and focal seizures (Ito et al., 2002; Scheffer and Berkovic, 1997; Singh et al., 1999).

Mutations of *SCN1A* were also identified in another epileptic syndrome; severe myoclonic epilepsy of infancy (SMEI). This disorder is characterized by early onset refractory generalized tonic–clonic or unilateral convulsions usually within the first 6 months of life, followed by additional myoclonic seizures often accompanied by mental deterioration (OMIM 182389). Some patients experience additional complex partial seizures or absence seizures. More than 150 mutations have been identified in children with this disorder, 50–80% of SMEI patients tested. Similar to GEFS+, the SMEI patients are heterozygous for the mutant alleles. Among 75 cases in which both parents have been tested, in 69 cases, or 90%, the mutations arise as *de novo* in the affected children (Meisler and Kearney, 2005; Yamakawa, 2005). Some group of patients manifest very frequent intractable GTC, usually begins in infancy, develop subsequent mental decline, as well as ataxia. They, devoid of myoclonic seizures, are proposed as intractable childhood epilepsy with generalized tonic–clonic seizures (ICEGTC). This condition has also been proven to be caused by *SCN1A* mutations (Fujiwara et al., 2003). Thus, it has been shown that the SCN mutation cause wide spectrum of febrile sensitive seizure syndromes. SMEI is the most severe phenotype within the spectrum.

In this paper, we present a novel missense mutation found in independent three families GEFS+, FS and myoclonic seizures, and ICEGTC. Members with this mutation in one of those families also showed panic disorder and Asperger syndrome. Our data suggests that the mutations of *SCN1A* provide susceptibility for psychiatric illnesses as well as fever sensitive epileptic syndromes.

Methods

Epilepsy classification

Criteria for diagnosis of SMEI were done according to the International League Against Epilepsy guidelines (1989). Patients who fulfill these criteria other than the absence of myolconic seizure were diagnosed as ICEGTC. We used the Febrile Seizures (FS) to describe with the typical febrile convulsion syndrome where all seizures occurred with fever between 3 months and 6 years. The term FS+ denotes individuals with FS and/or afebrile generalized tonic–clonic seizures (GTCS) extending beyond the age of 6 years.

Genetic analysis

We studied independent three families of GEFS+, FS and myoclonic seizures, and ICEGTC. Written informed consent was obtained from the parents or responsible adults as well as healthy control individuals where necessary and was approved by the Ethical Committees of Kanagawa Children's Medical Center, Shizuoka Medical Institute of Neurological Disorders, and the RIKEN Institute. Methods for mutational analysis of *SCN1A* were described in our previous articles (Fujiwara et al., 2003). Briefly, genomic DNA was extracted from heparin treated blood samples of affected and unaffected individuals using the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany) and from hair samples using the ISOHAIR Kit (Nippon Gene, Toyama, Japan). Genomic DNA was amplified by PCR with Pyrobest (TaKaRa Shuzo Co. Ltd., Tokyo, Japan) and Blend Taq Plus (Toyobo, Osaka, Japan) DNA polymerases according to the manufacturer's instructions. For mutation analysis on *SCN1A*, PCR primers were designed to amplify all 26 coding exons of *SCN1A* defined by comparison of cDNA (GenBank accession no. AY043484) and genome sequences (nos. AC010127 and AC021673). For haplotype analysis, PCR primers were designed to amplify several regions containing 8 single nucleotide polymorphism (SNP) sites in *SCN1A* (NCBI Reference SNP ID nos. rs1020852, rs2169312, rs8191987, rs994399, rs12614431, rs10497276, rs10497275 and rs2217195). PCR products were directly sequenced with a dideoxy terminator kit and analyzed by an automated sequencer (model ABI3730, Applied Biosystems, Foster City, CA).

Patients, family members, and control individuals

Family A

Patient 1 (proband, III-6; see Fig. 1A). A 9-year-old girl exhibited febrile generalized tonic–clonic convulsion at 2 years and 6 months when carbamazepine was initiated. At 3 years of age, she exhibited 1–2 times afebrile tonic convulsion. Her electroencephalograph, taken at 6 years of age, discloses normal except 4–5 Hz diffuse irregular spikes and wave complex at a drowsy state. Then valproic acid was initiated and she experienced no seizures since then. She is now the third grade of normal elementary school.

Patient 2 (III-5). This 7 years old boy, younger brother of III-6, was diagnosed as VATER association from vertebral anomaly, rectal atresia, hypoplasia of right trachea and hydrokidney. He experienced febrile generalized tonic–clonic seizure at 1 year and 2 months. Prevention for seizures by rectal diazepam was initiated since

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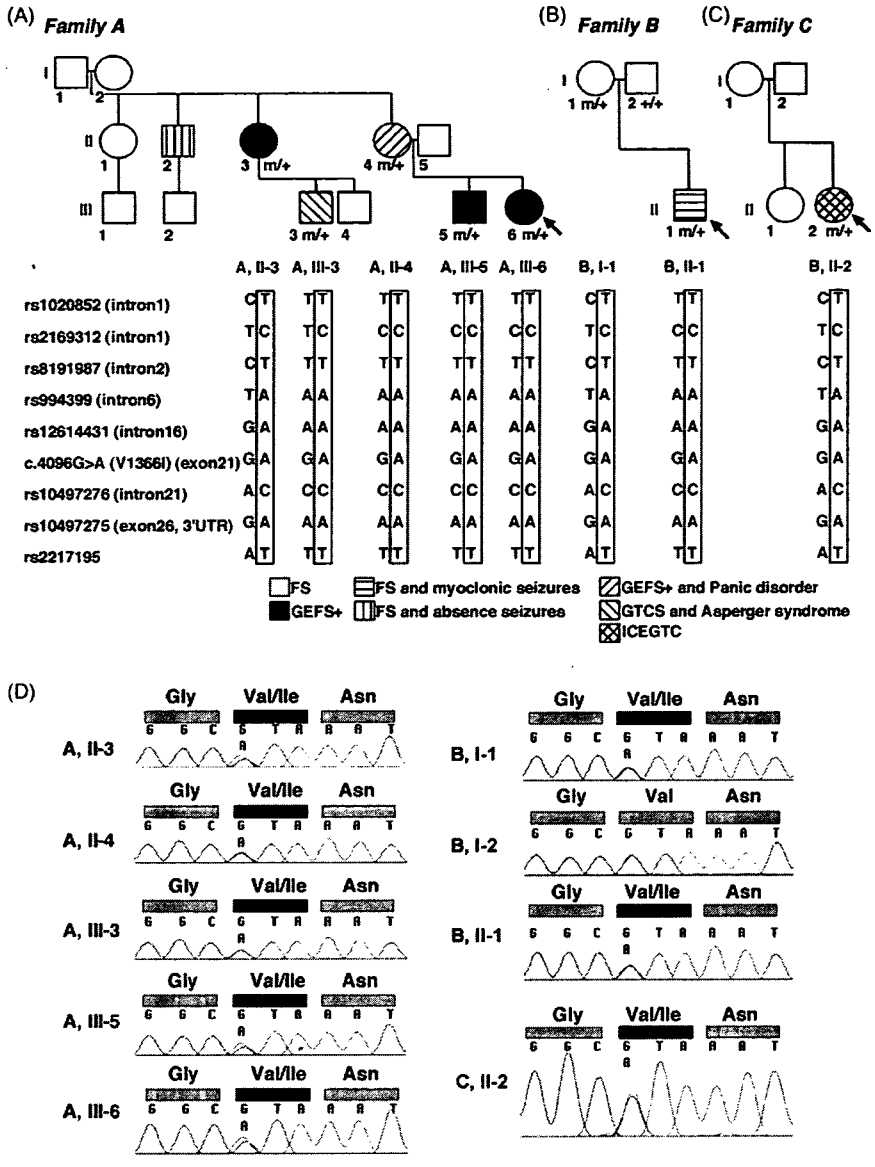

Figure 1 A c.4096G>A (V1366I) mutation of *SCN1A* in Japanese families with epilepsy. (A–C) Pedigree trees of the families A, B, and C. (m) c.4096G>A (V1366I) mutation of *SCN1A*. (+) Wild-type allele. The putative disease-associated haplotype is boxed. Genomic DNAs of members without genotypes were not available for the analysis. Circles, females; squares, males; arrows, probands. Note that members II-4 and III-3 in family A also show panic disorder and Asperger syndrome. (D) Electropherograms obtained by direct sequencings on the PCR products of *SCN1A* coding-exon 21 amplified from indicated members.

then. He exhibited afebrile absence seizures and generalized tonic–clonic seizures and valproic acid was initiated. Although Zonisamide was added for febrile seizure, he continued febrile GTC several times per year. He started to take potassium bromide at 5 years old and experienced no seizures since then. His intelligent quotient is 107 and in the second grade of normal elementary school.

Patient 3 (II-4). She is the 40-years mother of patients 1 and 2. She has febrile seizures several times until 6 yeas of ages. At her 30s, she experienced palpitation, sweating, shivering, and shortness of breathing. She was frightened the idea that she lose control of herself, make traffic accident when she drives. These symptoms worsened when she was in the crowds. She was diagnosed as having panic disorder by diagnostic criteria of DSM IV. Diazepam as anti-anxiety and paxil as anti-depressant relieve her symptoms partially and she is continuing her occupation. Her older sister (II-1) and brother (II-2) both experienced febrile seizures several times before 6 years of ages. In addition, brother (II-2) exhibited absences after 6 years of age and had valproic acid until 20 years of ages.

Patient 4 (III-3). An 8-year-old boy is maternal cousin of patients 1 and 2. He gained meaningful words at 12 months and walked alone at 16 months. He exhibited afebrile GTC at 5 years old. He had hypersensitivity to noise and explained too many anxieties. He had difficulty in social interaction

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and was reluctant to go to kindergarten. At 6 years of ages, he was diagnosed as Asperger's disorder with the diagnostic criteria of DSM-IV-TR. Pimozide relieved his violence to other students and his repetitive behavior.

Family B

Proband (II-1; see Fig. 1B). He exhibited several times of febrile GTC at 11 months. One of them lasted 25 min. EEG revealed no abnormalities. At 13 months, he experienced frequent GTC and myoclonus. Carbamazepin was initiated that was not effective for both seizures. Seizures continue more then 5 times per day. He was admitted our hospital at 21 months old. Valproate and Sodium bromide ceased seizures in 2 weeks. He is now 3 years of age. Besides one FS, he shows no seizures since then. He shows no developmental delay and neurological abnormalities. His mother (I-1) has no seizures and his father (I-2) showed one brief febrile seizure at 3 years of age.

Family C

Proband (II-2; see Fig. 1C). A 30-year-old woman had her first fever-induced GTC at age 7 months. The febrile seizure repeated monthly and from 5 years of age on she began to have afebrile GTC in addition. She once experienced convulsive status epilepticus lasting 40 min at age 5 years. The seizure was also induced by taking bath and exercise. The seizure later tended to occur in clusters a dozen to several times a day during sleep. Although her seizures had been very intractable with various medications, the seizure gradually reduced its frequency and stopped at age 20 years with the medication of valproate, phenobarbital and acetazolamide. She developed almost normally until 7 years of age. Her IQ was 50 at age 10 years and 52 at age 20 years. She showed no neurological abnormalities. She is currently visiting a sheltered workshop. The EEG showed diffuse bilateral spike-waves only when valproate was reduced, otherwise no paroxysmal discharges were recorded. MRI showed no abnormal findings. The clinical course of this patient including early onset fever-sensitive and non-sensitive refractory GTCs, paroxysms-scanty EEG and mental retardation indicates a phenotype of ICEGTC, although the seizure disappearance after 20 years of age is exceptional.

Her mother (I-1) had two febrile convulsions between the ages of 1 and 2 years, and her father (I-2) has no seizures.

Control individuals

DNAs from 304 Japanese healthy individuals were analyzed as controls in this study.

Result

We sequenced *SCN1A* gene on genomic DNAs of probands and members of families A, B, and C (Fig. 1). We identified a novel missense mutation that altered guanine at the nucleotide number at 4096 to adenine (c.4096G>A) resulting in the alteration of Valine residue at the position of 1366 in Nav1.1 into Isoleucine (V1366I) in the probands (III-6 in family A, II-1 in family B, and II-2 in family C). While this mutation was also found in other symptomatic family members (II-3, II-4, III-3, and III-5 in Family A), it was also found in an asymptomatic member (I-1 in Family B) and not found in a symptomatic member who had one febrile seizure (I-2 in Family B). This mutation was not observed in 608 alleles of Japanese healthy control individuals. Genomic DNAs of other family members were not available for the analysis, because we could not get their consent or we could not reach to the members.

We found that the V1366I mutation carriers in families A and B share a common putative SNP haplotype in the 100 kb region corresponding *SCN1A* by using 8 markers (Fig. 1A–C). Although the exact haplotype of the proband in family C were not determined because DNAs of her parents were not available for the study, she is very likely to share the same haplotype with the mutation carriers in families A and B according to the results of genotyping. Therefore, a founder effect is expected in the V1366I mutation in these three families.

Discussion

We here presented a novel *SCN1A* missense mutation, Val 1366 Ile, in three independent families of variable epilepsies including GEFS+, FS and myoclonic seizures, and ICEGTC. Putative position of aa 1366 is transmembrane domain (D3S5) of the Nav1.1 channel. This mutation is a conservative amino acid change and gives a slight bulkiness to the residue. However, the D3S5 domain forms the pore of the channel and the V1366 residue is highly conserved among vertebrate and invertebrate sodium channel alpha subunits and human calcium channel alpha subunits (Fig. 2). Therefore it is still possible that the mutation may critically affect the channel function. Apparently, functional studies are necessary to confirm the causative role of this channel mutation.

The mother (I-1) of the proband in family B is unaffected even if she had the mutation indicating its incomplete penetrance. The father in family B, who had a FS but not the mutation, would be a phenocopy. We recently reported a case of mosaicism in a family of SMEI and proposed that mosaicism may play roles in diverse phenotypes of members harboring identical mutations in some familial SMEI cases (Morimoto et al., 2006). It is also possible that mosaicism plays a role for the phenotype of mother (I-1) in family B. However, mosaicism may not be a player in determining the ultimate phenotype for the proband (II-1) in the family B because his mother has the mutation. Also in the family A, the V1366I mutation has been transmitted through members, and therefore mosaicism is not expected in patients II-3, II-4, III-3, III-5, and III-6. These results suggest that unidentified modifier(s) such as polymorphisms in other genes may be critical for ultimate phenotypes of the patients with the V1366I mutations in these families.

In addition to epileptic syndromes, two members from the family A showed psychiatric conditions that are panic disorder and Asperger syndrome. The DSM-IV (American Psychiatric Association, 1994) defines panic disorder as the spontaneous, unexpected occurrence of panic attacks followed by persistent concern, worry, and anxiety. Asperger

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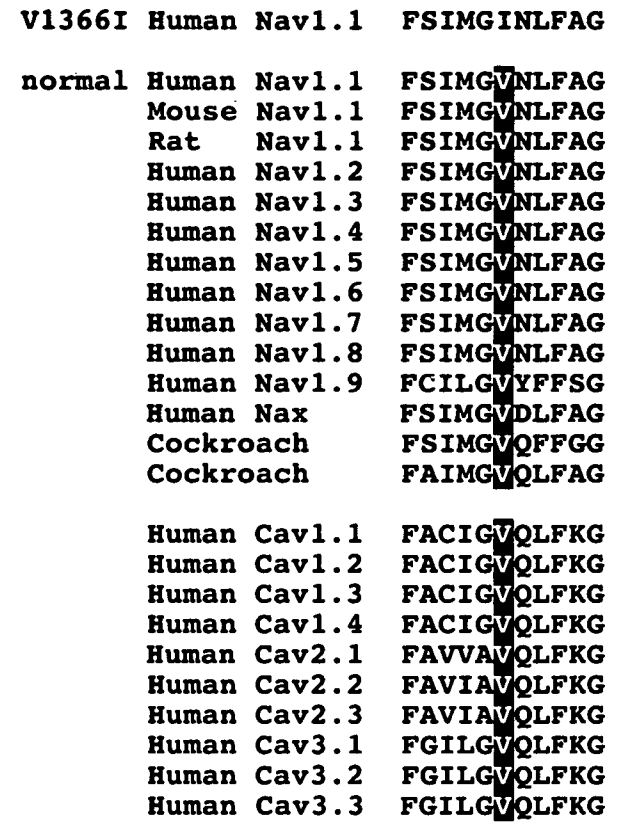

Figure 2 The valine residue for the c.4096G>A (V1366I) mutation is conserved among vertebrate and invertebrate sodium channel alpha subunits and human calcium channel alpha subunits. The valine residue corresponding to V1366 of human Nav1.1 is highlighted in black. Sources of sequences are as follows (notations refer to accession numbers): Human Nav1.1, NP_008851; mouse Nav1.1, NP_061203; rat Nav1.1, NP_110502; human Nav1.2, NP_001035232; human Nav1.3, NP_008853; human Nav1.4, NP_000325; human Nav1.5, NP_932173; human Nav1.6, NP_055006; human Nav1.7, NP_002968; human Nav1.8, NP_006505; human Nav1.9, NP_054858; human Nax, NP_002967; cockroach sodium channels, AAC47483 and AAK01090; human Cav1.1, NP_000060; human Cav1.2, NP_000719; human Cav1.3, NP_000711; human Cav1.4, NP_005174; human Cav2.1, NP_000059; human Cav2.2, NP_000709; human Cav2.3, NP_000712; human Cav3.1, NP_061496; human Cav3.2, NP_066921; human Cav3.3, NP_066919.

syndrome is one condition in the pervasive developmental disorders (autistic spectrum) and shares the common physiological problem with autism in an impairment of social interaction, but it is primarily distinguished from autism by the higher cognitive abilities and a more normal and timely development of language and communicative phrases. Twin and adoption studies showed strong genetic contribution on both panic disorder and autism spectrum (Piven and Palmer, 1999). Anxiety in autism patient is effectively treated with serotonin transporter inhibitors that are used for panic disorders (Kolevzon et al., 2006). This implies that these two distinct conditions may partially overlap in biological abnormality.

Considering the essential role of sodium channels in the CNS and the sensitivity of neuronal firing patterns to subtle mutations in these channels, SCN mutations may affect emotional and cognitive functions (Meisler et al., 2002). However, no autistic spectrum and other psychiatric illnesses had not been reported in families with FC plus; ICEGTC or SMEI. Weiss et al. (2003) sequenced *SCN1A* autism patients from Autism Genetic Research Exchange Families. Among 117 families tested, 5 missence mutations, R542Q, I1034T, F1038L, R1902C, were found in 6 patients. These mutations were not found in control tested. R542Q and F1038L mutations were shared by siblings. They hypothesized that, even though *SCN1A* mutations may not be major determinants of genetic abnormalities in autism, the combined effect of these mutations may predispose to these psychiatric diseases (Meisler and Kearney, 2005; Weiss et al., 2003).

The results described in this study may further support their proposal. Additional examination using model mouse, functional tests and population studies may contribute to the elucidations of molecular pathology of *SCN1A* mutations on epilepsy and psychiatric disorders.

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Spinocerebellar ataxia with axonal neuropathy: consequence of a Tdp1 recessive neomorphic mutation?

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Tyrosyl-DNA phosphodiesterase 1 (Tdp1) cleaves the phosphodiester bond between a covalently stalled topoisomerase I (Topo I) and the 3′ end of DNA. Stalling of Topo I at DNA strand breaks is induced by endogenous DNA damage and the Topo I-specific anticancer drug camptothecin (CPT). The H493R mutation of Tdp1 causes the neurodegenerative disorder spinocerebellar ataxia with axonal neuropathy (SCAN1). Contrary to the hypothesis that SCAN1 arises from catalytically inactive Tdp1, $Tdp1^{-/-}$ mice are indistinguishable from wild-type mice, physically, histologically, behaviorally, and electrophysiologically. However, compared to wild-type mice, $Tdp1^{-/-}$ mice are hypersensitive to CPT and bleomycin but not to etoposide. Consistent with earlier *in vitro* studies, we show that the H493R Tdp1 mutant protein retains residual activity and becomes covalently trapped on the DNA after CPT treatment of SCAN1 cells. This result provides a direct demonstration that Tdp1 repairs Topo I covalent lesions *in vivo* and suggests that SCAN1 arises from the recessive neomorphic mutation H493R. This is a novel mechanism for disease since neomorphic mutations are generally dominant.

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Introduction

DNA topoisomerases, glycosylases, methyltransferases, and recombinases act via formation of a transient covalent intermediate with DNA. When these DNA-processing enzymes become covalently trapped on the DNA, they cause a particularly harmful kind of DNA damage. The repair pathways for these types of lesions are of great interest because they influence the effectiveness of widely used antibacterial and antitumor drugs that act by stabilizing such covalent complexes (Connelly and Leach, 2004).

Inherited defects of DNA repair are associated with a predisposition to cancer and neurological abnormalities (Friedberg *et al*, 2006). To our knowledge, spinocerebellar ataxia with axonal neuropathy (SCAN1) is the first example of a human genetic disorder that results from a failure to repair DNA–protein covalent complexes. More importantly, the mutant protein responsible for the disease becomes itself covalently trapped on the DNA.

SCAN1 is an autosomal recessive disorder characterized by ataxia, cerebellar atrophy, and peripheral neuropathy (Takashima *et al*, 2002). The patients are usually wheelchair bound by early adulthood but retain normal cognitive function suggesting that the disease arises from degeneration or impairment of specific neurons (Takashima *et al*, 2002). SCAN1 has been associated with the *TDP1 1478A>G* mutation, which encodes the missense change H493R that disrupts the active site of tyrosyl-DNA phosphodiesterase 1 (Tdp1) (Interthal *et al*, 2001, 2005b; Takashima *et al*, 2002).

Tdp1 catalyzes the hydrolysis of the phosphodiester bond between a DNA 3′ end and a tyrosine residue, a linkage specific to the enzyme–DNA covalent complex formed when a type IB DNA topoisomerase cleaves DNA (Yang *et al*, 1996). Topoisomerase I (Topo I) becomes covalently trapped in a dead-end complex on the DNA when it fails to religate the DNA after cleavage near endogenous lesions (nicks, gaps, or abasic sites) (Pommier, 2004). Tdp1 participates in the repair of Topo I–DNA complexes as a member of the mammalian DNA single-strand break repair complex (Pouliot *et al*, 2001; El-Khamisy *et al*, 2005; Interthal *et al*, 2005b).

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