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FULL-LENGTH ORIGINAL RESEARCH

An association analysis at 2q36 reveals a new candidate susceptibility gene for juvenile absence epilepsy and/or absence seizures associated with generalized tonic–clonic seizures

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Summary

Purpose: To further evaluate the previously shown linkage of absence epilepsy (AE) to 2q36, both in human and WAG/Rij absence rat models, a 160-kb region at 2q36 containing eight genes with expressions in the brain was targeted in a case–control association study involving 205 Turkish patients with AE and 219 controls.
Methods: Haplotype block and case–control association analysis was carried out using HAPLOVIEW 4.0 and inhibin alpha subunit (*INHA)* gene analysis by DNA sequencing.
Key Findings: An association was found between the G allele of rs7588807 located in the *INHA* gene and juvenile absence epilepsy (JAE) syndrome and patients having generalized tonic–clonic seizures (GTCS) with p-values of 0.003 and 0.0002, respectively (uncorrected for multiple comparisons). DNA sequence analysis of the *INHA* gene in 110 JAE/GTCS patients revealed three point mutations with possible damaging effects on inhibin function in three patients and the presence of a common ACTC haplotype (H1) with a possible dominant protective role conferred by the T allele of rs7588807 with respective p-values of 0.0005 and 0.0014.
Significance: The preceding findings suggest that *INHA* could be a novel candidate susceptibility gene involved in the pathogenesis of JAE or AE associated with GTCS.
KEY WORDS: Absence epilepsy, Association study, Haplotype blocks, *INHA* gene.

Idiopathic generalized epilepsies (IGEs) are complex seizure disorders accounting for up to 30% of all epilepsies, which are mainly caused by genetic factors (Moulard et al., 2001). Childhood absence epilepsy (CAE) and juvenile absence epilepsy (JAE) constitute the common and clinically well-characterized subtypes of IGE. Ion channel dysfunctions are considered to be the most prevalent cause of monogenic forms of epilepsy. Using candidate gene approach studies, mutations have been identified in the genes encoding the γ2, α1, and β3 subunits of γ-aminobutyric acid $(GABA)_A$ receptors (Wallace et al., 2001; Kananura et al., 2002; Maljevic et al., 2006; Tanaka et al., 2008) and in the α1H-subunit of low threshold T-type Ca channels in patients with CAE (Chen et al., 2003). Association studies also confirm that certain single nucleotide polymorphisms (SNPs) in ion channel genes confer susceptibility to AEs but not necessarily in all populations (Feucht et al., 1999; Lu et al., 2004; Liang, 2006; Urak et al., 2006; Everett et al., 2007).

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Epilepsy is possibly not only caused by alterations in the function or structure of the ion channels but also by an imbalance of neurotransmission in the synaptic cleft (Noebels, 2003; Kapur, 2008). Ion channels, receptors, or transporters may be involved directly in affecting membrane excitability or neurotransmitter release, but also the subunits that change the function of the channels and receptors, transcription factors, and hormones that alter the structure of the brain, reducing the threshold of the epileptic seizures, are part of the pathogenesis of epilepsy. Recent studies reveal novel genes other than ion channel genes associated with IGE, such as malic enzyme 2 (*ME2*) and glutamate dehydrogenase (*GDH*), which carry a causative mutation in a photosensitive myoclonic AE family (Greenberg et al., 2005; Bahi-Buisson et al., 2008).

To understand the complex genetic nature of IGEs, three whole-genome linkage studies have been performed (Sander et al., 2000; Durner et al., 2001; Chioza et al., 2009). One of these studies includes patients with juvenile myoclonic epilepsy (JME), and IGE with only generalized tonic–clonic seizure (GTCS) besides JAE, and the results support the presence of a strong disease locus on chromosome 18 and susceptibility loci on chromosome 6 for JME, on chromosome 8 for non-JME, and two loci on chromosome 5 for absence seizures that may affect the seizure phenotypes (Durner et al., 2001). The other genome-wide linkage study includes patients with absence seizures (CAE and JAE) and bilateral myoclonic seizures on awakening and reveals a strong susceptibility locus on 3q26 and possible suggestive loci on 14q23 and 2q36 (Sander et al., 2000). Finally in a genome-wide SNP-based linkage analysis in 41 nuclear families with at least one affected member with CAE syndrome, a susceptibility locus was identified on chromosome 3p23-p14 (Chioza et al., 2009).

A whole-genome linkage analysis in AE model WAG/Rij rats shows that the syntenic 2q33–37 region contains a susceptibility locus that influences the quantitative trait of spike wave discharges (SWDs) (Gauguier et al., 2004). Interestingly, this region overlaps with the candidate region for absence epilepsy in the previous human linkage study (Sander et al., 2000). Screening of the *SLC4A3* gene coding for an anion exchanger residing in 2q36 for possible mutations and susceptibility alleles reveals only a slight contribution of a missense variation to the IGE phenotype (Sander et al., 2002; Vilas et al., 2009). In a recent study, the 2q36 region is also found to confer susceptibility to IGE phenotype in a large family (Klein et al., 2008). However, an attempt to scan the positional candidate potassium channel gene (*KNJ13*) does not reveal any causative mutations in the coding, promoter, and regulatory regions of this gene.

A 160-kb region with high gene density at 2q36 includes eight genes, two of which are ion channels [amiloride-sensitive cation channel (*ACCN4*)and anion exchanger carrier (*SLC4A3*)] that are possible candidates for epileptic seizures, and also others like a transmembrane protein with unknown function (*TMEM198*), GDP-mannose phosphorylase A (*GMPPA*), obscruin-like 1 gene (*OBSL-1*), chondroitin polymerizing factor (*CHPF*), inhibin alpha subunit precursor (*INHA*), and serine/threonine kinase 11 interacting protein (*STK11IP*), all being expressed in the brain (Shmueli et al., 2003).

The present study aims to further assess the association of 2q36 with absence epilepsy, focusing on the 160-kb region in a case–control association analysis, and finds a significant association between rs7588807 in the *INHA* gene and JAE and/or absence with GTCS.

Materials and Methods

Samples

Case–control association study

According to the inclusion criteria of patients with typical absence seizures specified by the epileptologists of The Genetic Commission of Turkish League Against Epilepsy (TLAE), patients had (1) typical absence seizures as defined by the International League Against Epilepsy (ILAE) criteria (Commision on Classification and Terminology of the International League Against Epilepsy, 1981); (2) 3–6 Hz generalized SWDs on their electroencephalography (EEG); (3) normal neurologic status and normal IQ level; and (4) been diagnosed as IGE, including JME (Commision on Classification and Terminology of the International League Against Epilepsy, 1989). The case–control study comprised 205 patients affected with AE, 81 of which were in the form of parent–offspring trios and 219 unrelated control individuals from the general population who did not have a personal or family history of epilepsy. All patients and control individuals were of Turkish origin and were from various regions of the country. The syndromic breakdown of the patient population was such that there were 100 CAE, 72 JAE, 19 JME, 12 eyelid myoclonia with absence epilepsy (EMA), and two recurrent absence status epilepticus (RASE) patients. Two hundred five patients were also subgrouped according to the associated features besides absence seizures. According to this grouping—GTCS, myoclonic, and febrile seizures were observed in 81, 36, and 38 patients, respectively. Photosensitivity was present in 64 patients.

Haplotype block analysis

Parent–offspring trios of 38 controls were used to constitute the haplotype block structure of the 160-kb region at 2q36.

The study was approved by the ethics committee of Boğaziçi University and informed consent were obtained from all subjects included in the study.

DNA analysis

DNA was extracted from 10 ml peripheral blood of patients and their family members by the NaCl method (Miller et al., 1988) and from saliva samples of control individuals by the ORAGENE saliva kit (DNA Genotek, Kanata, ON, Canada).

Genotyping for the identification of the haplotype block structure

Twenty-four biallelic SNPs were genotyped by restriction enzyme analyses and one SNP by hybprobe analysis in the Light Cycler 480 (LC480, Roche Diagnostics, Mannheim, Germany). HAPLOVIEW version 4.0 (Broad Institute, Cambridge, MA, U.S.A.) was used for the analysis of linkage disequilibrium (LD) and haplotype block structure (Barrett et al., 2005). A haplotype block was defined where the first and the last markers were in strong LD with all intermediate markers but that intermediate markers were not necessarily in LD with each other (solid spine analysis).

Genotyping for the case–control association study

Genotyping of a total of 10 SNPs in 205 absence patients and 219 healthy individuals was carried out using hybridization probes designed by TIB Mol Bio (Berlin, Germany) on an LC480 platform based on melting curve analysis.

DNA sequence analysis

The promoter and the coding regions consisting of two exons of the *INHA* gene were amplified in eight polymerase chain reaction (PCR) studies that contained 50 ng genomic DNA, 0.2 pmol of each primer, 0.2 mM of each dNTP, 1× reaction buffer with 1.5 mM [Mg^{2+}], 5% dimethyl sulfoxide (DMSO), and 1.25 U Qiagen taq polymerase (Qiagen, Valencia, CA, U.S.A.) in 25 μl. The PCR products of the three promoter regions and exon 1 were sequenced directly (Macrogen, Seoul, Korea), whereas the four regions of exon 2 were analyzed by high resolution melting analysis (HRMA) on the LC480 platform. The HRMA mixture was prepared in 20 μl volume containing 1× master mix with faststart taq DNA polymerase, reaction buffer, dNTP mix and high-resolution melting dye, 0.2–0.5 mM [Mg^{2+}], 0.2–0.5 pmol of each primer pairs, and 20–40 ng of genomic DNA (See Supporting Information Table S1 for the primer sequences for each PCR region).

Risk analysis of the novel variations found in the *INHA* gene were carried out by "Polyphen" for amino acid substitutions, "ESEfinder" for exonic enhancer sequences, and "Splice Site Predictor" and "Alternative Splice Site Predictor (ASSP) for possible cryptic sites (Reese et al., 1997; Ramenski et al.,2001; Cartegni et al., 2003; Wang & Marín, 2006).

Statistical analysis

Power analysis was carried out by assuming a disease prevalence of 0.0015 in the general population and a type I error rate of 0.05. The power was >90% for a recessive genotype effect with a relative risk (RR) of two for rs7588807. The power for a dominant trait of rs7588807 with a RR2 was 74%. All power calculations were carried out by QUANTO (Gauderman, 2002; Gauderman & Morrison, 2006).

Case–control association analysis was carried out by HAPLOVIEW 4.0 that calculated the chi-square statistics of SNP alleles between two groups at a 0.05 significance level (Barrett et al., 2005). Transmission disequilibrium test (TDT) using patient-mother-father trios and haplotype association analysis was also carried out by the HAPLOVIEW 4.0 program. TDT calculated whether one of the alleles of heterozygous parents was preferentially transmitted to the affected child, and compared the transmitted and nontransmitted alleles. The haplotype association test was carried out by summing the fractional likelihoods of each individual to have a certain haplotype. Bonferroni correction was applied for multiple testing to adjust the p-values in the case–control study. Several correlated tests have been performed for subsets of the case–control studies, but multiple testing corrections were not reported because of their unknown interdependency. The odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using an online calculator for confidence intervals of odds ratios in unmatched case–control study developed by Bland and Altman (2000). The measure of LD between SNP pairs was calculated by using Lewontin's D́.

Results

Haplotype block structure of the 160-kb region and selection of representative SNPs

At the time of the initiation of the study, approximately 60 SNPs were available for the 160-kb region in the HapMap data. The 25 SNPs that had HapMap minor allele frequency (MAF) >0.1 and that were >1 kb apart were selected, and they were genotyped on 38 unrelated control trios to construct the haplotype block structure of the region (Fig. 1). The average distance between each SNP was 6 kb, and all were in Hardy Weinberg equilibrium (HWE). The MAF of the SNPs in the Turkish population varied from 0.017 to 0.454.

Block analysis revealed seven haplotype blocks with high LD between the first and last SNP but not necessarily between the intermediate SNPs. In tagger analysis, 20 of the SNPs in the blocks were tagged indicating a highly heterogeneous haplotype structure. Therefore, not all "tag" SNPs but the most informative SNPs in the Turkish population were chosen to represent the blocks for the following association study to reduce possible false-positive results. Eight

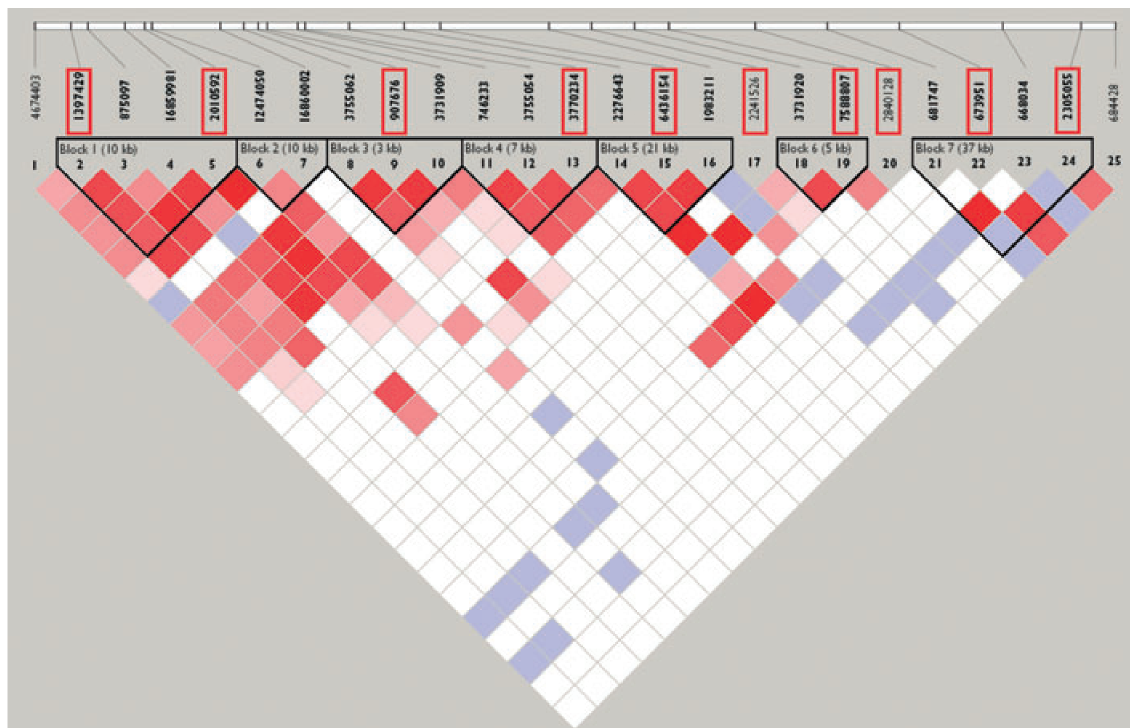

Figure 1.
Haplotype block structure of the 160 kb at 2q36. The red boxes indicate the SNPs chosen for the association study.
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SNPs in the blocks that had MAF >29% were chosen as representative SNPs and two more SNPs (rs2241526 and rs2840128) were also selected to represent the regions between blocks, adding it up to 10 SNPs to be used in the case–control association study.

Association study

A case–control association analysis carried out using the 10 representative SNPs on 205 absence patients and 219 healthy controls showed an association of the G-allele of rs7588807 to the patient sample with a Bonferroni corrected p-value of 0.235. All SNPs were in HWE in both the case and control samples. To clarify whether the locus has an impact on the syndrome or seizure, the patients were subgrouped according to both their syndrome and seizure types. The case–control association analysis on patient subgroups revealed significant associations of the two SNPs in 70% LD, rs7588807 (in block 6) and rs2840128 (between blocks 6 and 7) to JAE, and to patients having GTCS besides absence seizures (Table 1). rs7588807 resides in the intron 1 of inhibin alpha subunit gene (*INHA*), whereas rs2840128 does not fall into any gene. The genotypic frequencies of rs7588807 in both patient groups were also significantly different than the control group (Table 2). TDT is commonly used to see whether the associated allele is overtransmitted to the affected child. Of the total 205 AE cases, parental samples were available for only 81 patients. After the breakdown of the patient population according to the syndrome and associated feature types, only 26 JAE cases and 34 patients with GTCS were in the form of mother-father-patient trios. When a TDT analysis was carried out for rs7588807, the G-allele (p-value = 0.0094) in trios with GTCS were found to be overtransmitted (Table 2).

The pattern of inheritance of rs758807 indicated a similar and reduced risk of GT and TT genotypes in patients with GTCS in the crude genetic model (Table 3). In the model where G allele was recessive, GG genotype carried a risk of 2.7-fold compared to those with GT or TT genotypes, indicating that the G-allele was the susceptibility allele. On the other hand, considering T-allele has a dominant inheritance, GT or TT haplotypes reduced the disease risk by more than half (OR 0.36). Therefore, the T allele could be considered to have a dominant protective effect. Genetic model testing on patients with JAE revealed similar results.

Gene analysis

The highly significant association was found to JAE/GTCS patient subgroups but not to the total 205 AE cases. Therefore, we aimed to resequence all JAE patients and patients with GTCS. There were 72 JAE patients and 81 patients with GTCS among the total 205 cases. The overlap

Table 1. Chi-square and p-values for case–control allelic association analysis for syndrome subgroups and associated features

		Syndrome subgroups				Associated features							
		CAE (N = 100)		JAE (N = 72)		GTCS (N = 81)		Myoclonus (N = 36)		Febrile seizure (N = 38)		Photosensitivity (N = 64)	
SNP name	Associated allele	χ^2	p-value[a]	χ^2	p-value[a]	χ^2	p-value[a]	χ^2	p-value[a]	χ^2	p-value[a]	χ^2	p-value[a]
rs1397429	C	0.4	n/s	0.1	n/s	0.5	n/s	0.3	n/s	0.03	n/s	0.2	n/s
rs2010592	T	2.6	n/s	0.04	n/s	1.4	n/s	3.6	n/s	0.04	n/s	0.06	n/s
rs907676	G	2.6	n/s	0.6	n/s	4.05	n/s	5.3	0.021	0.07	n/s	0.2	n/s
rs3770234	T	1.7	n/s	1.4	n/s	1.5	n/s	0.6	n/s	0.8	n/s	0.06	n/s
rs6436164	A	0.003	n/s	0.9	n/s	0.3	n/s	0.7	n/s	0.2	n/s	0.2	n/s
rs2241526	C	0.3	n/s	1.06	n/s	1.04	n/s	0.3	n/s	0.002	n/s	0.9	n/s
rs7588807	G	0.02	n/s	8.8	0.0030	13.9	0.0002	3.2	n/s	0.8	n/s	3.05	n/s
rs2840128	T	0.5	n/s	4.8	0.0275	6.8	0.0092	0.6	n/s	0.7	n/s	0.6	n/s
rs673951	T	2.6	n/s	0.3	n/s	0.6	n/s	0.8	n/s	0.1	n/s	0.003	n/s
rs2305055	C	1.6	n/s	0.09	n/s	1.5	n/s	0.6	n/s	0.2	n/s	0.08	n/s

n/s, not significant.
[a]Uncorrected values.

Table 2. Allele/genotype counts, frequencies, and TDT analysis for rs7588807

	Allele counts (%)		Genotype counts (%)						
rs7588807	G	T	GG	GT	TT	Overtransmitted allele (transmitted/untransmitted ratio)	Total[a]	χ^2	p-value[b]
JAE patients	93 (73)	45 (27)	34 (48.5)	25 (38)	10 (14)	–	70	10.5	0.0053
AE patients with GTCS	111 (70)	47 (30)	41 (51)	29 (36)	9 (11)	–	81	14.9	0.0005
All cases	248 (61)	158 (39)	79 (38.5)	90 (44)	34 (16.5)	–	205	5.8	n/s
Controls	229 (53)	203 (47)	61 (28)	107 (49)	48 (22)		219		
JAE trios (N = 26)	–	–	–	–	–	G (1.69)	–	2.3	n/s
AE trios with GTCS (N = 34)	–	–	–	–	–	G (2.33)	–	6.4	0.0094

n/s, not significant.
[a]There are missing alleles.
[b]Uncorrected values.

Table 3. Test of association between rs7588807 genotypes and GTCS

	Genotypes					
Genetic model	GG (95% CIE)	GT (95% CIE)	TT (95% CIE)	d.f.	χ^2	p-value
Crude OR (vs. GG)	1	0.40 (0.228–0.713)	0.28 (0.123–0.63)	2	14.9	0.0005 0.0053[a]
Dominant G-allele OR (vs. GG + GT)	1	1	0.45 (0.209–0.0968)	1	4.3	0.03 0.165[a]
Recessive G-allele OR (vs. GT + TT)	2.7 (1.611–4.665)	1	1	1	14.3	0.0001 0.0012[a]
Dominant T-allele OR (vs. GG)	1	0.36 (0.214–0.620)	0.36 (0.214–0.620)	1	14.3	0.0001 0.0012[a]
Allele T versus G	1 (G)	0.47 (T) (0.323–0.705)		1	14	0.0001 0.003[a]

OR, odds ratio, CIE, confidence interval estimates, d.f., degrees of freedom.
[a]p-value in JAE patients.

between JAE and GTCS was such that 43 JAE patients also had GTCS. Seventy-two JAE patients and 38 IA patients with GTCS (81−43 = 38) were resequenced, making up a total of 110 patients. The *INHA* gene with 2 exons comprises an approximately 1,424-bp coding region. DNA sequencing analysis of the 2 exons and the promoter region of a total of 110 patients revealed eight novel nucleotide changes in eight patients (Table 4). R124C and H175Q substitutions seemed to have highly damaging effects on protein function, whereas L249L carried a medium risk for

Table 4. ***INHA* gene novel variations identified in absence epilepsy patients**

Patients (P1–P8) (syndrome/seizure)	Position of the SNP	Nucleotide change /position	Amino acid	Risk analysis (Polyphen, ESEfinder, ASSP, Splice Site Predictor)	Analysis in control samples
P1 (JAE)	Promoter	n.–560G → A	–	Conserved sequence change	Absent in 49 controls
P2 (JAE/GCTS)	Promoter	n.–658A → T	–	Conserved sequence change	Absent in 49 controls
P3 (JAE)	Exon1	n.–106G → C	–	Non-conserved sequence change	Absent in 49 controls
P4 (JME/GCTS)	Exon 2	n.315G → C	E105D	Missense conservative change/benign	Absent in 49 controls
P5 (JAE)	Exon 2	n.370C → T	R124C	Missense nonconservative change/highly damaging	Absent in 249 controls
P6 (JAE)	Exon 2	n.487G → A	V163M	Missense nonconservative change/benign effect	Present in 1/49 control sample
P7 (JAE/GTCS)	Exon 2	n.525C → G	H175Q	Missense conservative change/damaging	Absent in 249 controls
P8 (JAE/GCTS)	Exon 2	n.747G → A	L249L	Splicing regulation/medium risk	Absent in 249 controls

Table 5. Estimated frequencies of three major INHA haplotypes

	H1 (ACTC)			H2 (GCGC)			H3 (GTGT)		
	Frequency in cases and control (ratio)	χ^2	p-value[a]	Frequency in cases and control (ratio)	χ^2	p-value[a]	Frequency in cases and control (ratio)	χ^2	p-value[a]
JAE	0.244:0.480 (0.5)	12.1	0.0005	0.310:0.197 (1.57)	3.6	0.0577	0.266:0.163 (1.63)	3.3	0.0675
GTCS	0.268:0.480 (0.55)	10.2	0.0014	0.333:0.197 (1.69)	5.2	0.0224	0.234:0.163 (1.44)	1.7	0.1865

Haplotypes with frequency lower than 0.01 were not considered.
[a]Uncorrected values.

a splicing defect. None of the three nucleotide changes were detected in 249 controls. The parental samples were available only for patient P5, and R124C substitution was present in the unaffected mother. The two conserved nucleotide changes at the promoter region were not detected in 49 controls but did not coincide with the TF binding sites and, therefore, they were not considered significant.

The sequencing analysis of the *INHA* gene in 110 patients revealed the genotypes of three other known SNPs that were then genotyped in 49 controls. SNPs 1 and 2 (rs11893842, rs35118453) were located in the 5′UTR, and SNPs 3 and 4 (rs7588807 and rs12720063) in intron 1 and exon 2 of the gene, respectively. LD between the four SNPs was 0.82, and by tagger analysis three SNPs (rs11893842, rs35118453, and rs7588807) were tagged. Haplotype estimates pointed to the presence of three major haplotypes: ACTC (H1), GCGC (H2), and GTGT (H3). H1 with the T-allele of rs758807 was observed with a higher frequency in controls with p-values of 0.0005 in analysis with JAE patients and 0.0014 in patients with GTCS (Table 5). On the other hand, the frequency of H2 with the associated G-allele of rs7588807 was significantly higher in patients with GTCS with a p-value of 0.0224.

Discussion

The aim was to identify a possible susceptibility locus for absence seizures at 2q36 found to be associated with IGE phenotypes in previous studies focusing on a 160 kb region that contained eight genes with expressions in the brain through a case–control association study rather than targeting individual candidate genes. An initial study was carried out in 38 Turkish trios in the 160 kb region to identify the block structure and the actual frequencies of the SNPs in the Turkish population, as such an analysis was not done previously for the Turkish population and there was no evidence of whether the HapMap data for this region could be imported for a case–control association analysis in the Turkish population. The haplotype block analysis enabled the selection of the most informative SNPs, reducing the time and cost of the association study. The population stratification among the case and control groups were minimized, since all individuals were of Turkish origin and the heterogeneity in genetic contributions of the patient sample was minimized by selecting them on the basis of a carefully set clinical criteria and restricting the cases to those having an IAE phenotype.

The resulting association of the G allele of rs7588807 with >90% power for a recessive genotype effect indicated the possible involvement of a non–ion channel gene, *INHA* in the pathogenesis of JAE or GTCS. The analysis for the inheritance pattern revealed that carrying GG genotype for rs7588807 meant a 2.7 times greater probability to have the disease. On the other hand, individuals carrying GT or TT had the risk less than by half (OR = 0.36) compared to GG haplotype. Therefore, the G allele was considered as a recessive susceptibility allele and T allele as a dominant protective allele.

A further significant association was found in haplotype analysis that covered the whole *INHA* gene between a certain haplotype (GCGC), including the G allele of rs7588807 in the third position and the case group. The presence of a

common protective haplotype (ACTC) with the T allele of rs7588807 in the control group at a significantly higher frequency also supported the role of *INHA* in JAE/GTCS pathogenesis. The G allele of rs7588807 present in both GCGC and GTGT was also shown to be overtransmitted in trios with GTCS. Apparently, the association of the G allele with JAE/GTCS was due to the underrepresentation of ACTC among cases. These four SNPs reside in the 5′UTR intron and exon 2 of the gene covering the whole gene and there were no other common SNPs with higher heterozygosity in the *INHA* gene. In addition, although less significantly rs2840128, 3′ to the *INHA* gene was also found to be associated with JAE/GTCS in the initial association study, indicating that the region covering the *INHA* gene is associated with JAE/GTCS cases (Table 1).

All association studies target SNPs that may not necessarily be true variants in disease associations. In this study, allelic association suggests that a true variant in LD with the associated SNP (rs7588807) may be responsible for the disease association, either in the form of mutations in the coding regions of the gene or by controlling gene expression. The common associated SNP could be in LD, with the true variant residing in the intronic or promoter regions not examined. Whether the common haplotypes (H2 and H3) that include associated allele (G) have a direct effect on *INHA* gene expression could be evaluated with further experimental work using appropriate constructs. A change in the level of the expression compared to the H1 haplotype might raise the susceptibility to epileptic seizures. The *INHA* gene can also be analyzed at both the DNA and RNA levels in absence animal model WAG/Rij rats. Although the probable true variant is not evident from this study, it actually may not be found in 100% of cases, since a complex inheritance is indicated for the patient group analyzed.

The finding of three rare variants in three JAE/GTCS patients that probably have damaging effects on protein function in the patient group and their absence in 249 control samples are further supporting the involvement of INHA in the disease phenotype, as it is the case with many epilepsy-associated genes, where only a few mutations have been identified in a small number of cases for many candidate genes. It should be noted, however, that because these events are extremely rare, >1,000 controls would be necessary to show that the absence of these variants in nonepileptics is not a ‘‘chance’’ occurrence.

The possible pathogenic effect of the three point mutations can be evaluated as follows on the basis of the knowledge of inhibin protein structure and function using bioinformatic tools. JAE patient P5 with pure absence seizures had the amino acid substitution (R124C) in INHA, where arginine (R), a polar amino acid, is replaced with a nonpolar amino acid cysteine (C). R124 is not conserved in the homologous genes of other species but was always replaced with a polar amino acid like histidine. Cysteine residues are essential in the formation of the three-dimensional (3D) structure of the protein through sulfur bonds. An extra cysteine residue may cause additional and improper sulfur bonds within the protein that may interfere with the dimerization of alpha subunits with βA or βB subunits to form inhibin A or B proteins (Antenos et al., 2007). If the alpha–beta dimerization is not possible, then βA subunits dimerize as monomers to form activin proteins, which are normally inhibited by inhibin activity. The mutation carried by the healthy mother may have contributed to the disease phenotype in the context of the patient’s genotype, but not the mother’s that is through the quantitative pattern of inheritance of the trait. The H175Q substitution at a conserved position in JAE patient P7 replaces basic polar histidine with polar glutamine with a neutral side chain. JAE patient P8 who had both GTCS and absence seizures, carried a G to A transition (n.747G $\rightarrow$ A) that may have resulted in the loss of a consensus motif for SRp55, an exonic splicing enhancer. However, the presence or absence of a putative splicing enhancer and whether the missense mutations have an effect on protein function should further be evaluated experimentally.

There could be some possible mechanisms through which inhibin alpha protein could lead to increased seizure susceptibility in absence epilepsy patients. INHA codes for the alpha subunit of the inhibin protein, which is known to be a gonadal glycoprotein that inhibits the secretion of follicle-stimulating hormone that induces the production of progesterone and estradiol. Progesterone was shown to enhance the SWDs through allopregnanolone, a positive modulator of $GABA_A$ receptors (Van Luijtelaar et al., 2001). In the case of mutant inhibin alpha subunit, higher progesterone may be involved in enhanced SWDs. As a remarkable point, inhibin and activin levels in serum are not detectable until adolescence at approximately 10 years of age, which corresponds to the age of onset of JAE (Vadakkadath & Atwood, 2005).

Inhibin alpha subunit and activin expression and protein localization in different parts of the brain were shown in previous studies (Roberts et al., 1996; Fujimura et al., 1999). However, the exact function of this protein in the brain is unknown. Transgenic mice with dominant negative activin type I receptor shows decreased excitatory glutamatergic current and enhanced GABA release and $GABA_B$ receptor activation (Müller et al., 2006; Zheng et al., 2009). Therefore, decreased levels of inhibin alpha subunit by binding less to type II receptor would increase the activity of activin proteins and enhance the excitability effect of activin in the brain. It may be speculated that a decreased level of inhibin protein due to GCGC or GTGT haplotypes in patients may explain the high level of activin and increased excitability of the brain.

In conclusion, while functional studies, which are beyond the scope of this manuscript, will ultimately determine the nature of our findings; these findings nevertheless support the previously found association of absence seizures with

2q36 and point out that *INHA* could be a novel gene contributing to the pathogenesis of JAE or absence seizures associated with GTCS. They also point out that the involvement of INHA in disease pathogenesis may be exerted by two different mechanisms: some potentially "damaging" mutations that interrupt gene function severely and are seen in only a few patients, and other variations that are more common that might merely raise risk for epilepsy. Obviously, to establish a more concrete association of the *INHA* gene to JAE syndrome and/or to AE with GTCS, evidence from other independent study groups and/or populations are needed besides mutational analysis of more AE patients.

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Disclosure

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

References

Antenos M, Stemler M, Boime I, Woodruff TK. (2007) N-linked oligosaccharides direct the differential assembly and secretion of inhibin alpha- and betaA-subunit dimers. *Mol Endocrinol* 21:1670–1684.

Bahi-Buisson N, El Sabbagh S, Soufflet C, Escande F, Boddaert N, Valayannopoulos V, Bellané-Chantelot C, Lascelles K, Dulac O, Plouin P, de Lonlay PN. (2008) Myoclonic absence epilepsy with photosensitivty and a gain of function mutation in glutamate dehyrogenase. *Seizure* 17:658–664.

Barrett JC, Fry B, Maller J, Daly MJ. (2005) Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 21:263–265.

Bland JM, Altman DG. (2000) Statistics notes: the odds ratio. *BMJ* 320:1468.

Cartegni L, Wang J, Zhu Z, Zhang MQ, Krainer AR. (2003) ESEfinder: a web resource to identify exonic splicing enhancers. *Nucleic Acid Res* 31:3568–3571.

Chen Y, Lu J, Pan H, Zhang Y, Wu H, Xu K, Liu X, Jiang Y, Bao X, Yao Z, Ding K, Lo WH, Qiang B, Chan P, Shen Y, Wu X. (2003) Association between genetic variation of CACNA1H and childhood absence epilepsy. *Ann Neurol* 54:239–243.

Chioza BA, Aicardi J, Aschauer H, Brouwer O, Callenbach P, Covanis A, Dooley JM, Dulac O, Durner M, Eeg-Olofsson O, Feucht M, Friis ML, Guerrini R, Kjeldsen MJ, Nabbout R, Nashef L, Sander T, Sirén A, Wirrell E, McKeigue P, Robinson R, Gardiner RM, Everett KV. (2009) Genome wide high density SNP-based linkage analysis of childhood absence epilepsy identifies a susceptibility locus on chromosome 3p23-p14. *Epilepsy Res* 87:247–255.

Commision on Classification and Terminology of the International League Against Epilepsy. (1981) Proposal for revised clinical and electroencephalographic classification of epileptic seizures. *Epilepsia* 22:489–501.

Commision on Classification and Terminology of the International League Against Epilepsy. (1989) Proposal for revised classification of epilepsies and epileptic syndromes. *Epilepsia* 30:389–399.

Durner M, Keddache MA, Tomasini L, Shinnar S, Resor SR, Cohen J, Harden C, Moshe SL, Rosenbaum D, Kang H, Ballaban-Gil K, Hertz S, Labar DR, Luciano D, Wallace S, Yohai D, Klotz I, Dicker E, Greenberg DA. (2001) Genome scan of idiopathic generalized epilepsy: evidence for major susceptibility gene and modifying genes influencing the seizure type. *Ann Neurol* 49:328–335.

Everett K, Chioza B, Aicardi J, Aschauer H, Brouwer O, Callenbach P, Covanis A, Dooley J, Dulac O, Durner M, Eeg-Olofsson O, Feucht M, Friis M, Guerrini R, Heils A, Kjeldsen M, Nabbout R, Sander T, Wirrell E, McKeigue P, Robinson R, Taske N, Gardiner M. (2007) Linkage and association analysis of CACNG3 in childhood absence epilepsy. *Eur J Hum Genet* 15:463–472.

Feucht M, Fuchs K, Pichlbauer E, Hornik K, Scharfetter J, Goessler R, Füreder T, Cvetkovic N, Sieghart W, Kasper S, Aschauer H. (1999) Possible association between childhood absence epilepsy and the gene encoding GABRB3. *Biol Psychiatry* 46:997–1002.

Fujimura H, Ohsawa K, Funaba M, Murata T, Murata E, Takahashi M, Abe M, Torii K. (1999) Immunological localization and ontogenetic development of inhibin alpha subunit in rat brain. *J Neuroendocrinol* 11:157–163.

Gauderman WJ. (2002) Sample size requirements for matched case-control studies of gene -environment interaction. *Stat Med* 21:35–50.

Gauderman WJ, Morrison JM. (2006) QUANTO 1.1: a computer program for power and sample size calculations for genetic-epidemiology studies, http://hydra.usc.edu/gxe.

Gauguier D, van Luijtelaar G, Bihoreau MT, Wilder SP, Godfrey RF, Vossen J, Coenen A, Cox RD. (2004) Chromosomal mapping of genetic loci controlling absence epilepsy phenotypes in Wag/Rij rats. *Epilepsia* 45:908–915.

Greenberg DA, Cayanis E, Strug L, Marathe S, Durner M, Pal DK, Alvin GB, Klotz I, Dicker E, Shinnar S, Bromfield EB, Resor S, Cohen J, Moshe SL, Harden C, Kang H. (2005) Malic enyzme 2 may underlie susceptibility to adolescent-onset idiopathic generalized epilepsies. *Am J Hum Genet* 76:139–146.

Kananura C, Haug K, Sander T, Runge U, Gu W, Hallmann K, Rebstock J, Heils A, Steinlein OK. (2002) A splice-site mutation in GABRG2 associated with childhood absence epilepsy and febrile convulsions. *Arch Neurol* 59:1137–1141.

Kapur J. (2008) Is epilepsy disease of synaptic transmission? *Epilepsy Curr* 8:139–141.

Klein KM, Preisig-Müller R, Knake S, Hamer HM, Oertel WH, Neubauer BA, Daut J, Rosenow F. (2008) Evaluation of susceptibility loci in an extended pedigree with idiopathic generalized epilepsy. *Epileptic Disord* 10:13–18.

Liang J. (2006) Common polymorphisms in the CACNA1H gene associated with childhood absence epilepsy in the Chinese Han Population. *Ann Hum Genet* 71:325–335.

Lu JJ, Zhang YH, Pan H, Chen YC, Liu XY, Jiang YW, Bao XH, Shen Y, Wu HS, Xu KM, Wu XR. (2004) Case-control and transmission/disequilibrium tests of the genes encoding GABRA5 and GABRB3 in a Chinese Population affected by childhood absence epilepsy. *Chin Med J* 117:1497–1506.

Maljevic S, Krampfl K, Cobilanschi J, Tilgen N, Beyer S, Weber YG, Schlesinger F, Ursu D, Melzer W, Cossette P, Bufler J, Lerche H, Heils A. (2006) A mutation in the GABA(A) receptor alpha(1)-subunit is associated with absence epilepsy. *Ann Neurol* 59:983–987.

Miller SA, Dykes DD, Polesky HF. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 16:1215.

Moulard B, Picard F, Hellard S, Agulhon C, Weiland S, Favre I, Bertrand S, Malafosse A, Bertrand D. (2001) Ion channel variation causes epilepsy. *Brain Res Rev* 36:275–284.

Müller MR, Zheng F, Werner S, Alzheimer C. (2006) Transgenic mice expressing dominant-negative activin receptor IB in forebrain neurons reveal novel functions of activin at glutamatergic synapses. *J Biol Chem* 281:29076–29084.

Noebels JL. (2003) Exploring new gene discoveries in idiopathic generalized epilepsies. *Epilepsia* 44:16–21.

Ramenski V, Bork P, Sunyaev S. (2001) Human non-synonymous SNPs: server and survey. *Nucleic Acid Res* 30:3894–3900.

Reese MG, Eeckman FH, Kulp D, Haussler D. (1997) Improved splice site prediction in gene. *J Comput Biol* 4:311–323.

Roberts VJ, Barth SL, Meunier H, Vale W. (1996) Hybridization histochemical and immunohistochemical localization of inhibin/activin subunits and messenger ribonucleic acids in the rat brain. *J Comp Neurol* 364:473–493.

Sander T, Schulz H, Saar K, Gennaro E, Riggio MC, Bianchi A, Zara F, Luna D, Bulteau C, Kaminska A, Ville D, Cieuta C, Picard F, Prud'homme JF, Bate L, Sundquist A, Gardiner RM, Janssen GA, de Haan GJ, Kasteleijn-Nolst-Trenité DG, Bader A, Lindhout D, Riess O, Wienker TF, Janz V, Reis A. (2000) Genome search for susceptibility loci of common idiopathic generalized epilepsies. *Hum Mol Genet* 9:1465–1472.

Sander T, Toliat MR, Heils A, Leschik G, Becker C, Rüschendorf F, Rohde K, Mundlos S, Nürnberg P. (2002) Association of the 867Asp variant of the human anion exchanger 3 gene with common subtypes of idiopathic generalized epilepsy. *Epilepsy Res* 51:249–255.

Shmueli O, Horn-Saban S, Chalifa-Caspi V, Shmoish M, Ophir R, Benjamin-Rodrig H, Safran M, Domany E, Lancet D. (2003) GeneNote: whole genome expression profiles in normal human tissues. *Comptes Rendus Biologies* 326:1067–1072.

Tanaka M, Olsen RW, Medina MT, Schwartz E, Alonso ME, Duron RM, Castro-Ortega R, Martinez-Juarez IE, Pascual-Castroviejo I, Machado-Salas J, Silva R, Bailey JN, Bai D, Ochoa A, Jara-Prado A, Pineda G, Macdonald RL, Delgado-Escueta AV. (2008) Hyperglycosylation and reduced GABA currents of mutated GABRB3 polypeptide in remitting childhood absence epilepsy. *Am J Hum Genet* 82:1249–1261.

Urak L, Feucht M, Fathi N, Hornik K, Fuchs K. (2006) A GABRB3 promoter haplotype associated with childhood absence epilepsy impairs transcriptioanl activity. *Hum Mol Genet* 15:2533–2541.

Vadakkadath MS, Atwood CS. (2005) The role of hypothalamic-pituitary-gonadal hormones in the normal structure and functioning of the brain. *Cell Mol Life Sci* 62:257–270.

Van Luijtelaar G, Budziszewska B, Jaworska-Feil L, Ellis J, Coenen A, Lasoń W. (2001) The ovarian hormones and absence epilepsy: a long-term EEG study and pharmacological effects in a genetic absence epilepsy model. *Epilepsy Res* 46:225–239.

Vilas GL, Johnson DE, Freund P, Casey JR. (2009) Characterization of an epilepsy-associated variant of the human Cl-/HCO3(−) exchanger AE3. *Am J Physiol Cell Physiol* 297:3.

Wallace RH, Marini C, Petrou S, Harkin LA, Bowser DN, Panchal RG, Williams DA, Sutherland GR, Mulley JC, Scheffer IE, Berkovic SF. (2001) Mutant Gaba (A) receptor gamma2-subunit in childhood absence epilepsy and febrile seizures. *Nat Genet* 28:49–52.

Wang M, Marín A. (2006) Characterization and prediction of alternative splice sites. *Gene* 366:219–227.

Zheng F, Adelsberger H, Müller MR, Fritschy JM, Werner S, Alzheimer C. (2009) Activin tunes GABAergic neurotransmission and modulates anxiety-like behavior. *Mol Psychiatry* 14:332–346.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primer sequences for *INHA* gene.

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