

Genome-wide association analysis of genetic generalized epilepsies implicates susceptibility loci at 1q43, 2p16.1, 2q22.3 and 17q21.32

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Genetic generalized epilepsies (GGEs) have a lifetime prevalence of 0.3% and account for 20–30% of all epilepsies. Despite their high heritability of 80%, the genetic factors predisposing to GGEs remain elusive. To identify susceptibility variants shared across common GGE syndromes, we carried out a two-stage genome-wide association study (GWAS) including 3020 patients with GGEs and 3954 controls of European ancestry. To dissect out syndrome-related variants, we also explored two distinct GGE subgroups comprising 1434 patients with genetic absence epilepsies (GAEs) and 1134 patients with juvenile myoclonic epilepsy (JME). Joint Stage-1 and 2 analyses revealed genome-wide significant associations for GGEs at 2p16.1 (rs13026414, $P_{\text{meta}} = 2.5 \times 10^{-9}$, OR[T] = 0.81) and 17q21.32 (rs72823592, $P_{\text{meta}} = 9.3 \times 10^{-9}$, OR[A] = 0.77). The search for syndrome-related susceptibility alleles identified significant associations for GAEs at 2q22.3 (rs10496964, $P_{\text{meta}} = 9.1 \times 10^{-9}$, OR[T] = 0.68) and at 1q43 for JME (rs12059546, $P_{\text{meta}} = 4.1 \times 10^{-8}$, OR[G] = 1.42). Suggestive evidence for an association with GGEs was found in the region 2q24.3 (rs11890028, $P_{\text{meta}} = 4.0 \times 10^{-6}$) nearby the *SCN1A* gene, which is currently the gene with the largest number of known epilepsy-related mutations. The associated regions harbor high-ranking candidate

genes: *CHRM3* at 1q43, *VRK2* at 2p16.1, *ZEB2* at 2q22.3, *SCN1A* at 2q24.3 and *PNPO* at 17q21.32. Further replication efforts are necessary to elucidate whether these positional candidate genes contribute to the heritability of the common GGE syndromes.

INTRODUCTION

Epilepsy is one of the most common neurological disorders characterized by recurrent unprovoked seizures due to neuronal hyperexcitability and abnormal synchronization. Approximately 3% of the general population is affected by epilepsy (1), which has a major impact on an individual's quality of life and carries significant public health consequences. Despite advances in epilepsy research, the heterogeneous and complex molecular mechanisms involved in epileptogenesis remain elusive. Genetic factors play a predominant role in ~40% of epilepsies (for review, see 2). The genetic generalized epilepsies (GGEs, formerly called the idiopathic generalized epilepsies) represent the most common group of genetically determined epilepsies accounting for 20–30% of all epilepsies (3). The GGE syndromes are characterized by age-related recurrent unprovoked generalized seizures in the absence of detectable brain lesions or metabolic abnormalities (4,5). The common classical GGE syndromes include childhood absence epilepsy (CAE), juvenile absence epilepsy (JAE), juvenile myoclonic epilepsy (JME) and epilepsy with generalized tonic–clonic seizures alone (EGTCS) (6). The typical seizure types of the common GGE syndromes are absence seizures (CAE and JAE), bilateral myoclonic seizures on awakening (JME) and generalized tonic–clonic seizures (EGTCS). The electroencephalographic signature is generalized spike-wave discharges, which reflect a synchronized hyperexcitable state of thalamocortical circuits (7).

Concordance rates of 70–80% for GGE in monozygotic twin pairs (8) compared with the rapidly declining recurrence risk of GGEs in siblings ranging from 4 to 10% and the lifetime prevalence of 0.3% in the general population implicate a nearly exclusively genetic etiology of GGEs but also provide compelling evidence for a complex genetic predisposition. The genetic architecture of common GGE syndromes is likely to represent a biological spectrum, in which a small fraction (1–2%) follows monogenic inheritance, whereas the majority of GGE patients presumably display an oligo-/polygenic predisposition. Twin and family studies provide evidence for genetic determinants shared across common GGE syndromes, but also suggest that different genetic configurations specify the phenotypic expression of absence and myoclonic seizures (8–11).

Most of the currently known genes for rare monogenic forms of genetic epilepsies encode voltage-gated or ligand-gated ion channels (e.g. *SCN1A*, *GABRA1*, *KCNQ2*, *CHRNA4*) (12,13). So far, none of these epilepsy genes seems to play a substantial role in the genetic predisposition of common GGE syndromes. In contrast to the positional gene mapping strategies applied in rare monogenic epilepsies, numerous small-scale linkage and candidate gene association studies failed to identify replicable susceptibility genes for common GGE syndromes (14–19). Recently, success in

identifying susceptibility variants has been achieved for the first time through an international research collaboration, which studied more than thousand subjects with common GGE syndromes. Large-scale copy number variation analysis revealed a predisposing role for recurrent genomic microdeletions at 15q11.2, 15q13.3 and 16p13.11, which, albeit individually rare, collectively represent important genetic risk factors in 3% of GGE patients (20–22). These concerted research efforts greatly improve the prospects of disentangling the complex genetic basis of the common epilepsies.

Genome-wide association studies (GWAS) have attracted considerable attention as a powerful and effective approach for the identification of susceptibility genes in complex human diseases (23,24). The high heritability and characteristic clinical features of the common GGEs highlight these as the most promising group of epilepsies for GWAS (25). Here, we report the results of the first GWAS in GGEs including 3020 GGE patients and 3954 population controls of European ancestry. The aims of the present GWAS were to identify susceptibility alleles shared across a wide range of common GGE syndromes and to dissect out syndrome-related variants conferring the risk for either genetic absence epilepsies (GAEs) or JME. Our GWAS results implicate susceptibility loci at 2p16.1 and 17q21.32 shared by common GGE syndromes, and a syndrome-related locus at 2q22.3 for GAEs and at 1q43 for JME, respectively.

RESULTS

GWAS results for GGE

The GWAS Stage-1 discovery cohort comprised 1527 GGE patients of North-Western European descent and 2461 German population controls, matched for genetic ancestry. After single nucleotide polymorphism (SNP) imputation and stringent SNP quality controls, 4.56 million SNPs with an average genotyping call rate (CR) of >99.9% were chosen for association analysis using a linear mixed-model statistic and complementary logistic regression analysis for an additive genetic model to estimate odds ratios (ORs). The plot of the genome-wide $-\log_{10} P$ -values obtained for the linear mixed-model analysis (P_{LMM}) is shown in Figure 1A.

In the discovery stage, none of the Stage-1 SNPs achieved genome-wide significance ($P_{LMM} < 5.0 \times 10^{-8}$). In total, 40 SNPs located at 14 chromosomal loci showed associations with GGE exceeding the Stage-1 screening threshold of $P_{LMM} < 1.0 \times 10^{-5}$ (Table 1; Supplementary Material, Table S1). Four chromosomal regions with strong linkage disequilibrium (LD) structure contained at least four SNPs with $P_{LMM} < 1.0 \times 10^{-5}$: (i) 2p24.3 (SNP rs388556, chr2:13309991; $P_{LMM} = 1.1 \times 10^{-6}$, OR[G] = 0.76, 95%-CI: 0.68–0.84) (Supplementary Material, Fig. S3B), (ii) 2p16.1 near the gene encoding the vaccine-related serine/

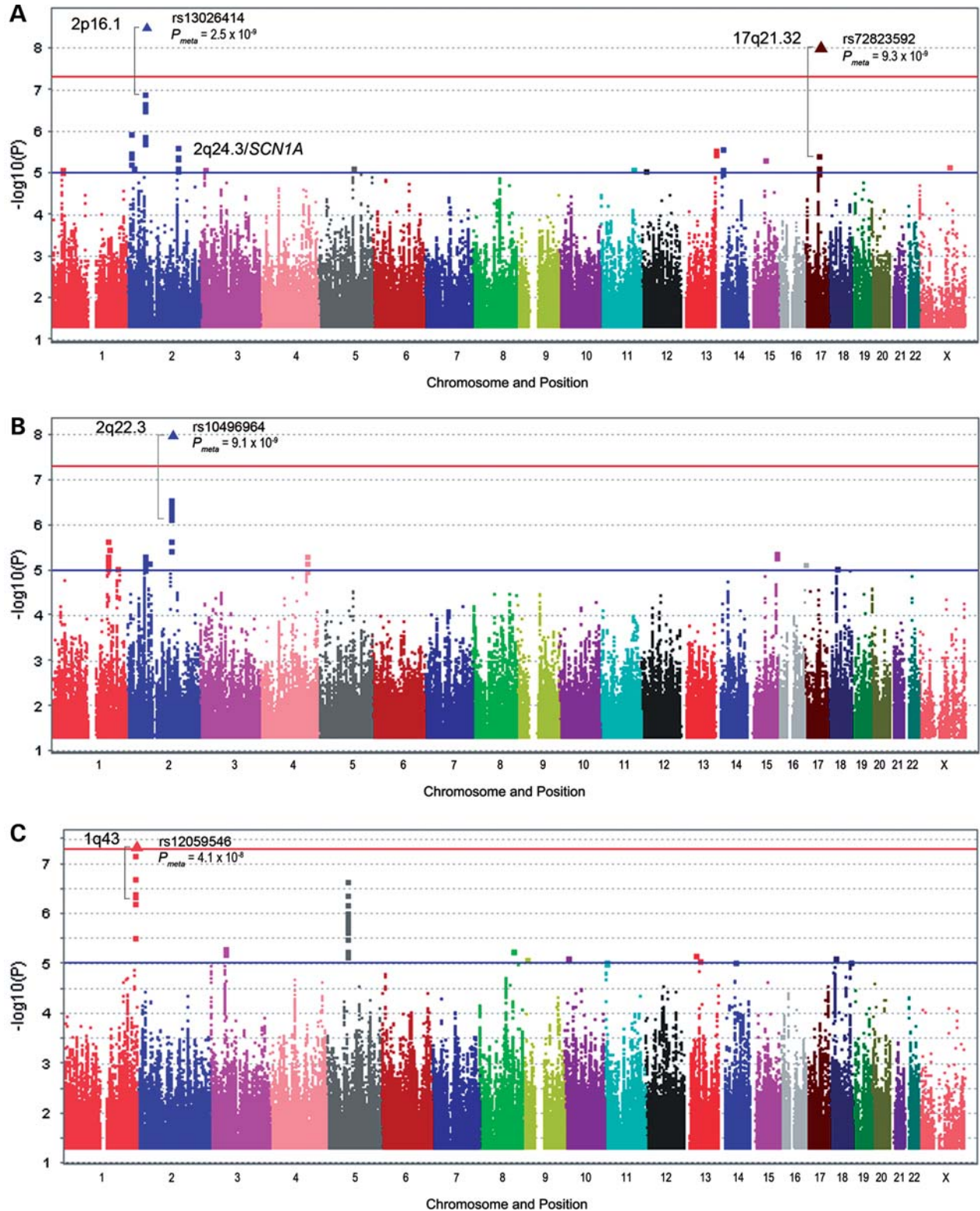


Figure 1. Genome-wide $-\log_{10} P_{LMM}$ -values of the linear mixed-model association analysis. Manhattan plots for genome-wide association analysis of 1527 GGE patients and 2461 controls (A), 702 GAE patients and 2461 controls (B) and 586 JME patients and 2461 controls (C). $-\log_{10} P_{LMM}$ -values of Stage-1 SNPs (~ 4.5 millions) were obtained by a linear mixed-model analysis and plotted chromosome-wise against the physical position of each SNP. The triangle refers to the P_{meta} -value of the combined Stage-1 and 2 association analysis for the significantly associated SNP. The bracket connects the P -values of Stage-1 (lower dot) and combined Stage-1 and 2 (triangle) association analyses. The blue horizontal line indicates the Stage-1 screening threshold at $P_{LMM} = 10^{-5}$ and the red line the threshold for genome-wide significance at $P_{LMM} = 5.0 \times 10^{-8}$. P_{LMM} -values for X-chromosomal SNPs shown in Figure 1A–C refer to females only (947 GGE/438 GAE/372 JME versus 1179 controls). Only P_{LMM} -values < 0.05 were presented.

Table 1. GWAS Stage-1 SNP associations with $P < 10^{-5}$ based on linear mixed-model analysis

Trait	Locus	dbSNP ID	Chr	Position	Minor allele	MAF Ca/Co	P_{LMM}	P_{LRgc}	LR OR 95%-CI	Nearest gene
GGE	1p34.3	rs1170543	1	34516971	G	0.202/0.244	7.93E-06	8.15E-05	0.79 (0.71–0.88)	<i>C1orf94</i> (59.7k)
GGE	1p34.3	rs771390	1	34523523	T	0.202/0.243	8.99E-06	8.63E-05	0.79 (0.71–0.88)	<i>C1orf94</i> (66.2k)
GGE	2p24.3	rs388556	2	13309991	G	0.254/0.310	1.09E-06	5.10E-07	0.76 (0.68–0.84)	
GGE	2p23.3	rs75866363	2	23899930	A	0.099/0.067	7.48E-06	3.24E-06	1.52 (1.29–1.79)	# <i>ATAD2B</i> #
GGE	2p16.1	rs13026414	2	57787559	T	0.365/0.424	1.24E-07	1.61E-08	0.78 (0.71–0.86)	
GGE	2q24.3	rs11890028	2	166651523	G	0.260/0.312	2.37E-06	5.18E-07	0.77 (0.70–0.85)	<i>SCN1A</i> (13.1k)
GGE	2q24.3	rs4667876	2	166703242	G	0.457/0.515	8.40E-07	8.06E-07	0.79 (0.72–0.87)	<i>SCN1A</i> (64.8k)
GGE	3p25.2	rs17832686	3	13108902	T	0.069/0.099	7.68E-06	1.70E-05	0.68 (0.57–0.80)	
GGE	5q22.3	rs158362	5	114121602	A	0.515/0.461	2.27E-06	7.13E-07	1.24 (1.13–1.36)	
GGE	5q22.3	rs289034	5	114150064	T	0.432/0.378	7.57E-06	1.21E-06	1.25 (1.14–1.37)	
GGE	11q22.3	rs11212190	11	106745947	T	0.405/0.457	8.21E-06	1.25E-05	0.81 (0.74–0.89)	# <i>CWF19L2</i> #
GGE	12p13.1	rs11837180	12	13384648	G	0.195/0.237	8.49E-06	6.57E-05	0.78 (0.70–0.87)	<i>C12orf36</i> (30.6k)
GGE	13q34	rs2479965	13	110788841	T	0.400/0.453	3.33E-06	1.03E-05	0.81 (0.74–0.88)	# <i>C13orf16</i> #
GGE	14q11.2	rs2268330	14	22865816	C	0.261/0.214	2.62E-06	1.27E-06	1.30 (1.17–1.44)	<i>PABPNI</i> (0.6k)
GGE	17q21.32	rs12951323	17	43382564	A	0.171/0.217	9.75E-06	1.38E-06	0.75 (0.66–0.84)	<i>PNPO</i> (0.9k)
GGE	17q21.32	rs72823592	17	43478003	A	0.196/0.247	3.74E-06	2.03E-07	0.74 (0.66–0.83)	<i>NFE2L1</i> (2.7k)
GAE	1q31.1	rs72709849	1	185133218	T	0.334/0.272	2.22E-06	5.73E-06	1.34 (1.18–1.53)	# <i>PLA2G4A</i> #
GAE	1q31.1	rs12720541	1	185136695	T	0.324/0.264	6.00E-06	1.55E-05	1.33 (1.17–1.52)	# <i>PLA2G4A</i> #
GAE	2p16.1	rs2717068	2	57948377	T	0.482/0.412	4.62E-06	8.90E-07	1.33 (1.18–1.50)	
GAE	2p13.2	rs114271652	2	72990468	A	0.409/0.348	6.86E-06	8.83E-06	1.29 (1.15–1.46)	<i>EMX1</i> (7.6k)
GAE	2q22.3	rs10496964	2	145076379	T	0.105/0.156	3.64E-07	1.39E-06	0.63 (0.52–0.76)	<i>ZEB2</i> (82.0k)
GAE	2q22.3	rs75917352	2	145087448	T	0.116/0.169	2.77E-07	7.95E-07	0.65 (0.54–0.77)	<i>ZEB2</i> (93.1k)
GAE	4q31.23	rs116127935	4	150920832	T	0.056/0.030	4.81E-06	1.34E-06	1.91 (1.44–2.52)	
GAE	4q31.23	rs10030601	4	150944662	C	0.061/0.035	6.80E-06	8.15E-06	1.78 (1.36–2.32)	
GAE	15q26.2	rs12904369	15	95136678	C	0.295/0.233	4.22E-06	6.12E-06	1.38 (1.21–1.57)	<i>SPATA8</i> (6.8k)
GAE	16q24.2	rs8061677	16	86355067	C	0.486/0.420	7.17E-06	8.19E-06	1.31 (1.16–1.47)	# <i>KLHDC4</i> #
GAE	18q12.1	rs10853686	18	24124522	T	0.364/0.432	8.87E-06	3.96E-06	0.75 (0.67–0.85)	
JME	1q43	rs12059546	1	238036720	G	0.241/0.172	1.97E-07	1.40E-07	1.53 (1.32–1.79)	# <i>CHRM3</i> #
JME	1q43	rs1110615	1	238040793	A	0.236/0.168	4.14E-07	2.32E-07	1.53 (1.31–1.78)	# <i>CHRM3</i> #
JME	3q21.31	rs12635454	3	48738660	A	0.107/0.070	4.82E-06	1.77E-05	1.59 (1.28–1.97)	<i>IHPK2</i> (8.9k)
JME	3q21.31	rs62261251	3	49612053	G	0.097/0.062	6.45E-06	2.43E-05	1.62 (1.30–2.04)	# <i>BSN</i> #
JME	5q12.3	rs39861	5	66188014	C	0.302/0.235	2.25E-07	1.05E-05	1.41 (1.22–1.62)	# <i>MAST4</i> #
JME	8q23.1	rs3019359	8	109824117	T	0.369/0.442	5.40E-06	4.91E-07	0.74 (0.65–0.84)	<i>TMEM74</i> (40.4k)
JME	11p15.4	rs10836956	11	4902623	A	0.079/0.046	9.10E-06	2.81E-05	1.76 (1.37–2.26)	<i>OR51G1</i> (0.5k)
JME	13q13.2	rs17669194	13	33165584	T	0.180/0.129	6.76E-06	2.73E-05	1.48 (1.25–1.76)	
JME	18q11.2	rs6507226	18	18445289	G	0.375/0.439	7.69E-06	1.37E-04	0.77 (0.67–0.87)	
JME	18q22.3	rs4892037	18	68574532	C	0.264/0.199	9.26E-06	6.03E-06	1.45 (1.25–1.68)	# <i>NETO1</i> #

GGEs, genetic generalized epilepsies; GAEs, genetic absence epilepsies; JME, juvenile myoclonic epilepsy; dbSNP, annotation of a single nucleotide polymorphism according to NCBI dbSNP Build 36.3; Chr, chromosome; Position, physical chromosomal position in bps; Minor allele, SNP allele with a frequency <50% in the entire study sample; MAF, minor allele frequency; Ca, cases; Co, control subjects; P_{LMM} , type-1 error rate of the linear mixed-model statistic; P_{LRgc} , P -values of logistic regression (LR) analysis adjusted for the observed genomic inflation factor; OR, odds ratio, 95%-CI: 95%-confidence interval.

threonine kinase 2 (*VRK2*) (SNP rs13026414, chr2:57787559; $P_{LMM} = 1.2 \times 10^{-7}$, OR[T] = 0.78, 95%-CI 0.71–0.86) (Fig. 2A; Supplementary Material, Fig. S3A), (iii) 14q11.2 in the 5'-terminal region of the *PABPNI* gene (rs2268330, chr14:22865816; $P_{LMM} = 2.6 \times 10^{-6}$, OR[C] = 1.30, 95%-CI 1.17–1.44) and (iv) 17q21.32 (rs72823592, chr17:43478003; $P_{LMM} = 3.7 \times 10^{-6}$, OR[A] = 0.74, 95%-CI 0.66–0.83) (Fig. 2C; Supplementary Material, Fig. S3E). Of interest, suggestive evidence for association was obtained in the chromosomal region 2q24.3 encompassing the *SCN1A* gene (rs11890028, chr2:166651523; $P_{LMM} = 2.4 \times 10^{-6}$, OR[G] = 0.77, 95%-CI 0.70–0.85) (Fig. 2B; Supplementary Material, Fig. S3C). The *SCN1A* gene encodes the neuronal sodium channel $\alpha 1$ subunit ($Na_v1.1$) and is currently the gene with the largest number (>700) of known epilepsy-related mutations (26).

Based on the significance of the association signals and the regional LD structure, we selected nine top-ranked SNPs for

Stage-2 replication analysis in two independent cohorts comprising 604 European parent-offspring trios of children with GGE and a European case-control sample consisting of 889 unrelated GGE patients and 889 ethnically matched population controls (Table 2). Joint Stage-1 and 2 analysis revealed genome-wide significant associations at 2p16.1 (SNP rs13026414, chr2:57787559; $P_{meta} = 2.5 \times 10^{-9}$, OR[T] = 0.81, 95%-CI 0.76–0.87) and also 17q21.32 (rs72823592, chr17:43478003; $P_{meta} = 9.3 \times 10^{-9}$, OR[A] = 0.77, 95%-CI 0.71–0.83).

Although Stage-2 replication analysis did not strengthen evidence for an association of GGE with SNP rs11890028 in the 5'-terminal *SCN1A* region, the combined Stage-1 and 2 P -values (top-ranked SNP: rs11890028, chr2:166651523; $P_{meta} = 4.0 \times 10^{-6}$, OR[G] = 0.85, 95%-CI 0.79–0.92) emphasize *SCN1A* as potential susceptibility gene for common GGE syndromes. In line with this finding, supportive evidence for an association of the SNP rs11890028 with focal epilepsies

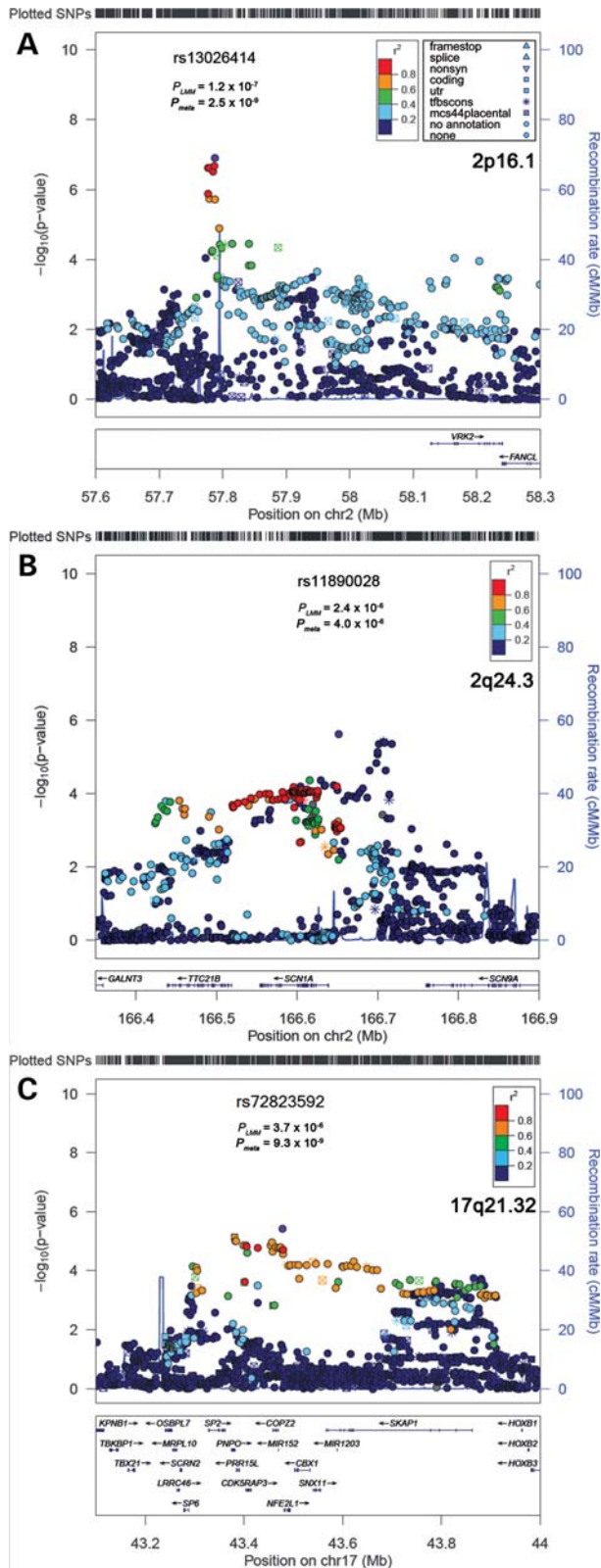


Figure 2. Regional P_{LMM} -values of Stage-1 SNP associations with GGE. Association results of GGE to 2p16.1 (A), 2q24.3 (B) and 17q21.32 (C). P_{LMM} -values of regional Stage-1 SNPs obtained by the linear mixed-model statistic are plotted against their physical chromosomal positions (NCBI build 36.3, hg18). The top-ranked SNP at each locus is shown in purple. The color

has been published in a recent GWAS of entirely independent cohorts, comprising 3445 European patients with various kinds of focal epilepsies and 6935 controls of European ancestry (16). This GWAS reported an association of $P = 4.8 \times 10^{-4}$ for SNP rs11890028 using logistic regression analysis for an additive genetic model. Joint association analysis of both GWAS datasets revealed genome-wide significance for the same risk allele of SNP rs11890028 ($P_{meta} = 4.6 \times 10^{-8}$; Stouffer's z trend statistic).

GWAS results for GAEs

To search for susceptibility genes conferring the risk for GAEs, a genome-wide Stage-1 association scan was carried out in 702 patients with GAEs of North-Western European origin and 2461 ethnically matched controls. The genome-wide plot of the Stage-1 $-\log_{10} P_{LMM}$ -values per chromosome is shown in Figure 1B. None of the observed P_{LMM} -values met genome-wide significance at the discovery stage. In total, 71 SNPs exceeded the Stage-1 screening threshold of $P_{LMM} < 1.0 \times 10^{-5}$ (Table 1; Supplementary Material, Table S1). Four chromosome regions of strong LD contained at least four SNPs with $P_{LMM} < 1.0 \times 10^{-5}$: (i) at 1q31.1 within the *PLA2G4A* gene encoding the cytosolic phospholipase A2 group 4A (rs72709849, chr1:185133218; $P_{LMM} = 2.2 \times 10^{-6}$, OR[T] = 1.34, 95%-CI: 1.18–1.53) (Supplementary Material, Fig. S4F), (ii) at 2q22.3 (rs10496964, chr2:145076379, $P_{LMM} = 3.6 \times 10^{-7}$, OR[T] = 0.63, 95%-CI: 0.52–0.76) (Fig. 3A; Supplementary Material, Fig. S3H) and (iii) in the region 4q31.23 (rs10030601, chr4:150944662; $P_{LMM} = 6.8 \times 10^{-6}$, OR[C] = 1.78, 95%-CI: 1.36–2.32). Like in the GGE GWAS, we observed strong association signals at 2p16.1 (rs2717068, chr2:57948377; $P_{LMM} = 4.6 \times 10^{-6}$, OR[T] = 1.33, 95%-CI: 1.18–1.50).

Stage-2 replication analysis was carried out for seven top-ranked Stage-1 SNPs with $P_{LMM} < 1.0 \times 10^{-5}$ in two independent replication cohorts comprising 347 parent–offspring trios of children with GAEs and a case–control sample consisting of 385 European GAE patients and 385 ethnically matched controls. The association results of the Stage-2 replication and joint Stage-1 and 2 analyses are shown in Table 2. Joint Stage-1 and 2 analysis revealed a significant association at 2q22.3 (top SNP: rs10496964, chr2:145076379, $P_{meta} = 9.1 \times 10^{-9}$, OR[T] = 0.68, 95%-CI: 0.60–0.78). Similar to the GWAS results for GGEs, the combined Stage-1 and 2 results support an association of GAEs with the 2p16.1 locus (rs2717068, chr2:57948377; $P_{meta} = 3.6 \times 10^{-7}$, OR[T] = 1.27; 95%-CI: 1.16–1.40).

GWAS results for JME

To identify susceptibility genes conferring the risk for JME, a genome-wide Stage-1 association scan was carried out in 586

scheme of the SNP dots reflects the pairwise LD between the top-ranked SNP and a neighboring SNP (pairwise r^2 values are obtained from the 1000 Genomes Project HapMap CEU data (June 2010 release)). Estimated recombination rates are plotted in blue. Gene and microRNA annotations are from the UCSC genome browser (hg18). GGEs: genetic generalized epilepsies.

Table 2. GWAS Stage-2 replication and combined association analysis of 21 top-ranked Stage-1 candidate SNPs

Stage-1 case-control cohorts				Stage-2 case-control cohorts				Stage-2 parent-offspring trios			Stage-1 and 2 joint analysis		Nearest gene			
Trait	dbSNP ID	Chr	Position	A1/A2	MAF Ca/Co	P_{LMM}	LR OR 95%-CI	MAF Ca/Co	P_{CMH}	OR 95%-CI	T/U	P_{TDT}		OR 95%-CI	P_{meta}	OR 95%-CI
GGE	rs771390	1	34523523	T/C	0.202/0.243	8.99E-06	0.79 (0.71-0.88)	0.197/0.226	3.40E-02	0.84 (0.71-0.99)	211/231	3.42E-01	0.91 (0.76-1.10)	5.90E-07	0.82 (0.76-0.89)	<i>Clorf94</i> (66.2k)
GGE	rs388556	2	13309991	G/C	0.254/0.310	1.09E-06	0.76 (0.68-0.84)	0.313/0.309	7.75E-01	0.98 (0.85-1.13)	262/241	3.49E-01	0.92 (0.77-1.10)	8.47E-05	0.88 (0.82-0.95)	
GGE	rs13026414	2	57787559	T/C	0.365/0.424	1.24E-07	0.78 (0.71-0.86)	0.373/0.403	7.19E-02	0.88 (0.77-1.01)	248/301	2.37E-02	0.82 (0.70-0.97)	2.47E-09	0.81 (0.76-0.87)	
GGE	rs11890028	2	166651523	G/T	0.260/0.312	2.37E-06	0.77 (0.70-0.85)	0.300/0.317	2.58E-01	0.92 (0.80-1.06)	270/271	9.66E-01	1.00 (0.84-1.18)	4.02E-06	0.85 (0.79-0.92)	<i>SCN1A</i> (13.1k)
GGE	rs289034	5	114150064	T/C	0.432/0.378	7.57E-06	1.25 (1.14-1.37)	0.386/0.395	5.02E-01	0.95 (0.83-1.10)	296/272	3.14E-01	1.09 (0.92-1.28)	7.93E-05	1.14 (1.06-1.22)	
GGE	rs2479965	13	110788841	T/C	0.400/0.453	3.33E-06	0.81 (0.74-0.88)	0.449/0.452	8.58E-01	0.99 (0.87-1.13)	288/281	7.69E-01	1.03 (0.87-1.21)	4.39E-05	0.89 (0.83-0.95)	<i>#C13orf16#</i>
GGE	rs2268330	14	22865816	C/G	0.261/0.214	2.62E-06	1.30 (1.17-1.44)	0.266/0.250	3.01E-01	1.08 (0.93-1.26)	209/250	5.57E-02	0.84 (0.70-1.01)	5.51E-05	1.14 (1.06-1.23)	<i>PABPN1</i> (0.6k)
GGE	rs12951323	17	43382564	A/C	0.171/0.217	9.75E-06	0.75 (0.66-0.84)	0.179/0.209	2.18E-02	0.82 (0.70-0.97)	162/184	2.37E-01	0.88 (0.71-1.09)	3.25E-07	0.79 (0.72-0.86)	<i>PNPO</i> (0.9k)
GGE	rs72823592	17	43478003	A/G	0.196/0.247	3.74E-06	0.74 (0.66-0.83)	0.190/0.237	4.97E-04	0.75 (0.64-0.88)	175/197	2.54E-01	0.89 (0.72-1.09)	9.35E-09	0.77 (0.71-0.83)	<i>NFE2L1</i> (2.7k)
GAE	rs12720541	1	185136695	T/G	0.324/0.264	6.00E-06	1.33 (1.17-1.52)	0.277/0.252	2.72E-01	1.14 (0.91-1.43)	120/128	6.12E-01	0.94 (0.73-1.20)	8.59E-06	1.21 (1.10-1.35)	<i>#PLA2G4A#</i>
GAE	rs1520965	2	57917252	G/T	0.368/0.299	8.47E-06	1.36 (1.20-1.54)	0.367/0.304	8.69E-03	1.33 (1.07-1.64)	150/139	5.18E-01	1.08 (0.86-1.36)	6.59E-07	1.30 (1.18-1.43)	
GAE	rs2717068	2	57948377	T/G	0.482/0.412	4.62E-06	1.33 (1.18-1.50)	0.469/0.395	3.46E-03	1.35 (1.10-1.65)	167/162	7.83E-01	1.03 (0.83-1.28)	3.64E-07	1.27 (1.16-1.40)	
GAE	rs10496964	2	145076379	T/C	0.105/0.156	3.64E-07	0.63 (0.52-0.76)	0.138/0.181	2.13E-02	0.72 (0.55-0.95)	88/114	6.74E-02	0.77 (0.58-1.02)	9.09E-09	0.68 (0.60-0.78)	<i>ZEB2</i> (82.0k)
GAE	rs10030601	4	150944662	C/T	0.061/0.035	6.80E-06	1.78 (1.36-2.32)	0.064/0.058	6.70E-01	1.10 (0.72-1.67)	45/26	2.41E-02	1.73 (1.07-2.81)	1.27E-06	1.58 (1.29-1.93)	
GAE	rs12904369	15	95136678	C/A	0.295/0.233	4.22E-06	1.38 (1.21-1.57)	0.257/0.253	8.83E-01	1.02 (0.81-1.28)	120/135	3.48E-01	0.89 (0.70-1.14)	2.54E-05	1.20 (1.08-1.33)	<i>SPATA8</i> (6.8k)
GAE	rs10853686	18	24124522	T/C	0.364/0.432	8.87E-06	0.75 (0.67-0.85)	0.383/0.392	7.14E-01	0.96 (0.78-1.18)	158/167	6.18E-01	0.95 (0.76-1.18)	1.03E-05	0.83 (0.75-0.91)	
JME	rs12059546	1	238036720	G/A	0.241/0.172	1.97E-07	1.53 (1.32-1.79)	0.232/0.213	3.74E-01	1.12 (0.88-1.42)	62/38	1.64E-02	1.63 (1.09-2.44)	4.14E-08	1.42 (1.26-1.61)	<i>#CHRM3#</i>
JME	rs1110615	1	238040793	A/G	0.236/0.168	4.14E-07	1.53 (1.31-1.78)	0.229/0.205	2.72E-01	1.15 (0.90-1.46)	61/37	1.53E-02	1.65 (1.10-2.48)	6.56E-08	1.43 (1.26-1.62)	<i>#CHRM3#</i>
JME	rs62261251	3	49612053	G/C	0.097/0.062	6.45E-06	1.62 (1.30-2.04)	0.062/0.075	3.09E-01	0.81 (0.54-1.21)	21/29	2.58E-01	0.72 (0.41-1.27)	1.00E-04	1.28 (1.06-1.54)	<i>#BSN#</i>
JME	rs39861	5	66188014	C/T	0.302/0.235	2.25E-07	1.41 (1.22-1.62)	0.272/0.258	5.24E-01	1.08 (0.86-1.35)	50/56	5.60E-01	0.89 (0.61-1.31)	3.25E-07	1.26 (1.13-1.41)	<i>#MAST4#</i>
JME	rs17669194	13	33165584	T/C	0.180/0.129	6.76E-06	1.48 (1.25-1.76)	0.141/0.159	3.19E-01	0.87 (0.65-1.15)	40/40	1.00E+00	1.00 (0.65-1.55)	4.10E-05	1.25 (1.09-1.44)	

GGEs, genetic generalized epilepsies; GAEs, genetic absence epilepsies; JME, juvenile myoclonic epilepsy; dbSNP, SNP, annotation of a single nucleotide polymorphism according to NCBI dbSNP Build 36.3; Chr, chromosome; Position, physical chromosomal position in bps; A1/A2 SNP alleles in the entire study cohort; MAF, minor allele (A1) frequency; Ca, cases; Co, controls; P_{LMM} , type-1 error rate of the linear mixed-model statistic; P_{CMH} , P -value of the Cochran-Mantel-Haenszel test for $2 \times 2 \times 6$ groups; P_{TDT} , P -value of the transmission disequilibrium test; P_{meta} , meta-analysis of Stage-1 and 2 P -values; OR, odds ratio, 95%-CI, 95%-confidence interval. The SNPs exceeding the significance threshold of $P_{meta} < 5.0 \times 10^{-8}$ in the combined Stage-1 and 2 association analysis are marked by bold letters.

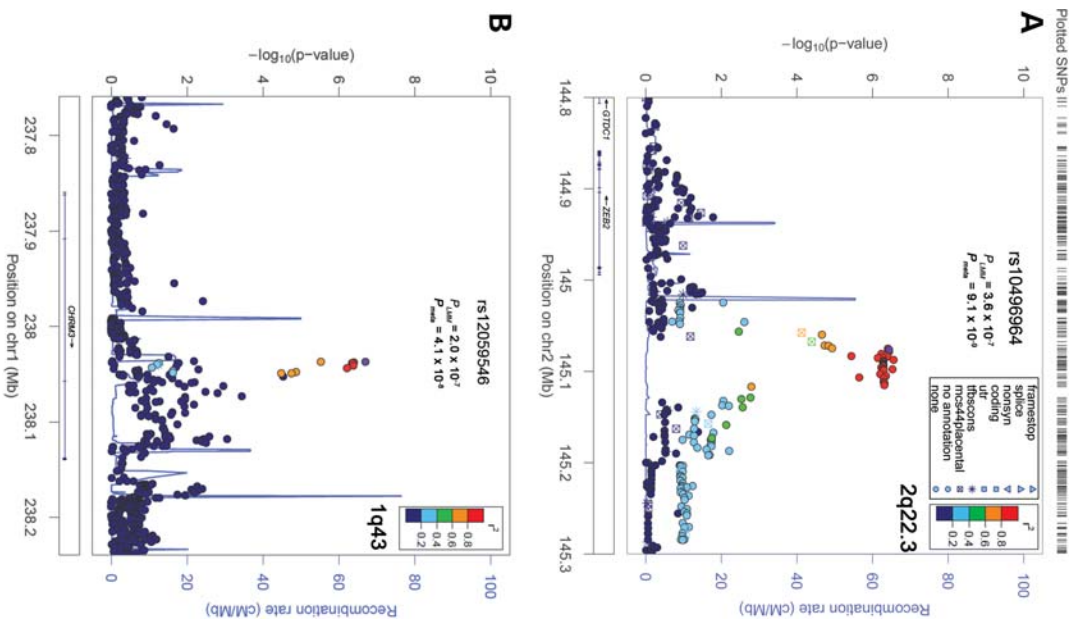


Figure 3. Regional P_{LMM} -values of Stage-1 SNP associations with GAEs and JME. Association of GAEs to 2q22.3 (A) and JME to 1q43 (B). Regional Stage-1 SNP P -values of the linear mixed-model statistic are plotted against their physical chromosomal positions (NCBI build 36.3, hg18). The top-associated SNP at each locus is shown in purple. The color scheme of the SNP dots reflects the pairwise LD between the top-ranked reference SNP and the neighboring SNPs (pairwise r^2 values are obtained from the 1000 Genomes Project HapMap CEU data (June 2010 release)). Estimated recombination rates are plotted in blue. GAEs, genetic absence epilepsies; JME, juvenile myoclonic epilepsy.

JME patients of North-Western European origin and 2461 ethnically matched population controls. The genome-wide plot of the Stage-1 $-\log_{10} P_{LMM}$ -values is shown in Figure 1C. None of the observed Stage-1 P_{LMM} -values reached genome-wide significance. In total, 54 SNPs exceeded the Stage-1 screening threshold of $P_{LMM} < 1.0 \times 10^{-5}$ (Table 1, Supplementary Material, Table S1). The most prominent association signals were obtained in the chromosomal region 1q43 within the gene encoding the M3 muscarinic acetylcholine receptor (*CHRM3*) (rs12059546, chr1:238036720; $P_{LMM} = 2.0 \times 10^{-7}$, OR[G] = 1.53, 95%-CI: 1.32-1.79) (Fig. 3B; Supplementary

Material, Fig. S3I), and on chromosome 5q12.3 within the *MAST4* gene encoding the microtubule-associated serine/threonine kinase 4 (rs39861, chr5:66188014; $P_{LMM} = 2.3 \times 10^{-7}$, OR[C] = 1.41, 95%-CI: 1.22–1.62) (Supplementary Material, Fig. S3J). Furthermore, two adjacent SNPs at chromosome 3q21.31 were located in a region of strong LD harboring the gene encoding the presynaptic scaffolding protein Bassoon (*BSN*) (rs62261251, chr3:49612053, $P_{LMM} = 6.5 \times 10^{-6}$, OR[G] = 1.62, 95%-CI: 1.30–2.04).

Stage-2 replication analysis was carried out for five top-ranked Stage-1 SNPs with $P_{LMM} < 1.0 \times 10^{-5}$ in two independent replication samples comprising 166 parent–offspring trios of children with JME and a case–control sample consisting of 382 European JME patients and 382 ethnically matched controls. The association results of the Stage-2 replication and joint Stage-1 and 2 analyses are shown in Table 2. The joint Stage-1 and 2 P -value of SNP rs12059546, which is located in the gene encoding the M3 muscarinic acetylcholine receptor (*CHRM3*), reached genome-wide significance (rs12059546, chr1:238036720, $P_{meta} = 4.1 \times 10^{-8}$, OR[G] = 1.42, 95%-CI: 1.26–1.61).

DISCUSSION

GGE syndromes are considered the most promising of the common epilepsies for molecular genetic studies, because of their high heritability, diagnostic reliability and prevalence accounting for 20–30% of all epilepsies (2,3,13,25). Despite their predominant genetic etiology, previous linkage and association studies have failed to identify replicable susceptibility genes for common GGEs (14–19). This failure likely reflects the underestimated degree of genetic complexity and heterogeneity as well as the speculative, hypothesis-driven approach of candidate gene studies (17,18,27). Here, we report the first GWAS for common GGEs, including 3020 GGE patients and 3954 controls all of European ancestry. This comprehensive and hypothesis-free approach offers a powerful tool to dissect the unknown genetic architecture of the common GGEs.

The present GWAS revealed significant associations at 2p16.1 and 17q21.32 for GGEs, 2q22.3 for GAEs and at 1q43 for JME (Fig. 1A–C). To exclude technical artifacts, stringent array and SNP quality filters were applied to ensure a high accuracy of SNP genotyping. In addition, we used a novel linear mixed-model statistic that provides a powerful and efficient tool for accounting for all sources of stratification in a GWAS using a case–control design (28). Thereby, we minimized the risk of spurious associations due to confounding by stratification effects or cryptic relatedness. Of note, GGE was selected as target phenotype in the present GWAS but we also explored two GGE subtypes to evaluate the hypothesis that susceptibility alleles differentially predispose to either GAEs or JME. Therefore, the interpretation of the present association results should take into account multiple testing of two additional phenotype models in an exploratory approach. Considering that our GWAS will have primarily detected those susceptibility variants enriched by chance ('winner's curse') (29), further replication efforts are necessary to validate these novel association findings.

Specifically, further validation is required for the JME-related association signal at 1q43 ($P_{meta} = 4.1 \times 10^{-8}$), which is close to the threshold for genome-wide significance ($P < 5.0 \times 10^{-8}$). Of interest, an empirical evaluation of borderline genetic associations (P -value between 10^{-7} – 5.0×10^{-8}) demonstrated that ~70% of the investigated borderline associations reached the significance threshold of $P < 5.0 \times 10^{-8}$ when additional data from subsequent GWASs were considered (30). These empirical data may argue against concerns that the traditional significance threshold of $P < 5.0 \times 10^{-8}$ might be too lenient. Otherwise, it is likely that we have missed common genetic factors of low risk (ORs < 1.3) due to the modest power of the sample sizes investigated in the present GWAS (Supplementary Material, Fig. S1).

The present GWAS in common GGEs identified two genome-wide significant associations in the chromosomal regions 2p16.1 and 17q21.32. The associated genomic segments harbor interesting candidate genes, which warrant further attention. The association for GGEs/GAEs in the chromosomal region 2p16.1 is located in an intergenic region close to a recombination hot spot (Fig. 2A). The associated chromosomal segment consists of ~900 kb encompassing the genes encoding the vaccine-related serine/threonine kinase 2 (*VRK2*) and the Fanconi-anemia-complementation L polypeptide (*FANCL*) distally (Supplementary Material, Fig. S3A). Interestingly, a recent GWAS meta-analysis of schizophrenia revealed a genome-wide association near *VRK2* (rs2312147, chr2:58020264; $P = 1.9 \times 10^{-9}$, OR[C] = 1.09) (31). Notably, the SNP rs2312147 is in modest to strong LD (r^2 : 0.25–0.87, $D' > 0.8$) with the SNPs at 2p16.1 that display the strongest associations with GGE and GAE. These overlapping association signals suggest that schizophrenia and GGEs may share a genetic risk factor at the 2p16.1 locus. An exploration of the phenotype-deletion relationship of the 2p15-p16.1 microdeletion syndrome characterized by intellectual disability, microcephaly and intractable seizures supports the hypothesis that haploinsufficiency of the *VRK2* gene may impair cortical development (32–34). Thereby, *VRK2*-associated neurodevelopmental alterations could increase the risk of GAE/GGE.

The 17q21.32 locus encompasses a chromosomal segment of ~650 kb harboring 10 genes (Fig. 2C; Supplementary Material, Fig. S3E), of which the gene encoding pyridoxine-5'-phosphate oxidase (*PNPO*) represents a plausible candidate gene. *PNPO* catalyzes the oxidation of pyridoxine-5'-phosphate to pyridoxal-5'-phosphate, the active co-factor form of vitamin B₆, which is involved in neurotransmitter metabolism. Recessively inherited *PNPO* mutations lead to a severe deficiency of pyridoxal-5'-phosphate levels in the brain and cause neonatal and infantile seizures typically occurring as status epilepticus during febrile episodes (35,36). In contrast to a severe *PNPO* deficiency caused by highly penetrant recessively inherited *PNPO* mutations, *PNPO* sequence variants with modest effects on pyridoxal-5'-phosphate levels could result in an impaired neurotransmitter homeostasis leading to an increase of seizure susceptibility. With respect to the therapeutic implications of pyridoxal-5'-phosphate deficiency, *PNPO* represents a high-priority metabolic candidate gene with potential clinical relevance (37).

Two other potential candidates at 17q21.32 are the gene encoding the heterochromatin protein-1 (*CBX1*) and the gene encoding the CDK5 regulatory subunit-associated protein 3 (*CDK5RAP3*); both are involved in the control of neuronal differentiation and neuron migration during cortical development (38,39). These neurodevelopmental alterations correspond well with the observation of microdysgenesis of mesiofrontal cortical structures in JME and other GGEs (40–42).

Interestingly, we found suggestive evidence for association of GGE in the chromosomal region 2q24.3 that encompasses the *SCN1A* gene (Fig. 2B; Supplementary Material, Fig. S3C). *SCN1A* is a highly conserved gene encoding the α subunit of the neuronal voltage-gated sodium channel that plays a central role in the generation and propagation of action potentials in both neuronal and glial cells (26). *SCN1A* is currently the gene with the largest number ($n > 700$) of epilepsy-related mutations (for review, see 26). Mutations in the *SCN1A* gene were identified in $\sim 10\%$ of patients with generalized epilepsy with febrile seizures plus (GEFS+) and 95% of children with severe myoclonic epilepsy in infancy (SMEI) (26,43,44). The key role of *SCN1A* mutations in the expression of predominantly generalized seizures in GEFS+ families implicates an involvement of *SCN1A* also in the pathogenesis of common GGE syndromes. Consistently, we obtained association signals achieving intermediate significance for several SNPs at the *SCN1A* locus (top-ranked SNP: rs11890028, $P_{\text{meta}} = 4.0 \times 10^{-6}$). Of interest, supportive evidence for an association of the *SCN1A* SNP rs11890028 with focal epilepsies has also been reported in a recent GWAS of focal epilepsies (16). Joint association analysis reaches genome-wide significance for the SNP rs11890028 ($P_{\text{meta}} = 4.6 \times 10^{-8}$) emphasizing an intriguing role of *SCN1A* as a genetic risk factor for a wide spectrum of common epilepsy syndromes. Taking into account that the top-ranked association signals are located in the intergenic region of 135 kb between the *SCN1A* and *SCN9A* genes (Supplementary Material, Fig. S3C), it is also possible that susceptibility alleles at the *SCN9A* locus may contribute to the association findings in the 2q24.3 region (45).

In an exploratory approach, we also addressed the question whether susceptibility variants differentially predispose to either GAEs or JME. Twin and family studies suggest that heterogeneous configurations of oligo-/polygenic factors, consisting of both shared and specifying susceptibility alleles, differentially determine the expression of either typical absence seizures (CAE and JAE) or JME-related myoclonic seizures (8–11). Despite the relatively low numeric power of the GAE- and JME subgroups, we presumed that a thorough delineation of more homogeneous GGE phenotypes/endophenotypes and narrowly defined inclusion criteria of GGE subgroups may result in an enrichment of GGE subtype-related risk factors that facilitate the dissection of the complex and heterogeneous genetic basis of the common GGE syndromes (17,18). In support of this assumption, we found a genome-wide significant association for GAEs in the chromosomal region 2q22.3 and for JME at chromosome 1q43. Overall, the top-associated loci, achieving only intermediate significance at the discovery stage, considerably differed between both GGE subgroups, although the

same control cohort was used for the case–control association analyses. However, given the limited power of both GGE subgroups, the top-ranked association signals do not reach significance levels that allow clarifying the hypothesis whether heterogeneous sets of genetic risk factors differentially confer susceptibility to either GAEs or JME (see Fig. 1B and C; Supplementary Material, Table S2). Nonetheless, the current results should encourage further studies on larger GGE cohorts to search for syndrome-related susceptibility genes.

The significant association for GAEs in the chromosomal region 2q22.3 is located in an intergenic region (Fig. 2A). The nearest gene encodes the zinc finger E-box-binding homeobox 2 protein (*ZEB2*) which is located ~ 80 kb upstream of the top-ranked association signal (rs10496964, $P_{\text{meta}} = 9.1 \times 10^{-9}$, OR[T] = 0.68). Mutations in the *ZEB2* gene cause the Mowat–Wilson syndrome that is characterized by intellectual disability, distinct facial features and congenital anomalies such as Hirschsprung disease, congenital heart defects, corpus callosum agenesis and urinary tract anomalies (46). About 74% of patients with Mowat–Wilson syndrome are also affected by epilepsy (46). This intriguing comorbidity emphasizes common pathogenic mechanisms that operate in both the conditions.

The association with JME at 1q43 encompasses a chromosomal segment of ~ 100 kb flanked by two recombination hot spots. It covers the distal part of the *CHRM3* gene (Fig. 3B; Supplementary Material, Fig. S3I). Although it is well established that mutations of genes encoding neuronal nicotinic acetylcholine receptor subunits (*CHRNA4*, *CHRN2*) cause autosomal dominant nocturnal frontal lobe epilepsy (for review, see 47), it remains to be determined whether genes encoding muscarinic acetylcholine receptors also play a role in epileptogenesis. Muscarinic effects on striatal cholinergic interneurons modulate thalamic gating of corticostriatal signaling (48). More specifically, the hippocampal CA3 transcriptome signature in surgically removed sclerotic hippocampi of patients with refractory mesial temporal lobe epilepsy revealed a cell-type specific expression of the M3 muscarinic acetylcholine receptor in distinct subtypes of hippocampal interneurons, providing a molecular mechanism for a differential cholinergic modulation of hippocampal circuitry (49,50) which may influence synchronization and excitability of thalamocortical circuits and thereby seizure susceptibility (7,50). However, muscarinic M3-receptor knockout mice do not show increased pilocarpine-induced seizure activity (51).

In summary, we present the first GWAS of GGE syndromes, the most common form of all inherited epilepsies, and report allelic associations at 1q43, 2p16.1, 2q22.3, 2q24.3 and 17q21.3. The associated regions harbor high-ranking candidate genes: *CHRM3* at 1q43, *VRK2* at 2p16.1, *ZEB2* at 2q22.3 and *PNPO* at 17q21.32. In context with the causative role of *SCN1A* mutations in GEFS+ and SMEI (43,44), our results provide emerging evidence that susceptibility variants of the *SCN1A* gene may also increase the risk of common GGE syndromes. Further replication studies are necessary to validate these novel association findings and to delineate their phenotype–genotype relationships.

MATERIALS AND METHODS

Study participants

Epilepsy patients of European ancestry with common GGE syndromes (CAE, JAE, JME and EGTCS alone with age-at-onset <26 years) were recruited as a concerted effort of national and international epilepsy genetics programs integrated in the European EPICURE Project (<http://www.epicureproject.eu>). Phenotyping and diagnostic classification of GGE syndromes were carried out according to standardized protocols (available at: <http://portal.ccg.uni-koeln.de/ccg/research/epilepsy-genetics/sampling-procedure>) (4–6) and were reviewed by experienced epileptologists. Individuals with a history of severe major psychiatric disorders (autism spectrum disorder, schizophrenia, affective disorder: recurrent episodes requiring pharmacotherapy or treatment in a hospital), or severe intellectual disability (no basic education, permanently requiring professional support in their daily life) were excluded. The study protocol was approved by the local institutional review boards and all study participants gave informed consent.

The GWAS followed a two-stage process (52): Stage-1, high-density SNP association scan in a case–control discovery sample; followed by Stage-2 replication and joint (Stage-1 and 2) association analysis of the Stage-1 association signals exceeding $P_{LMM} < 1.0 \times 10^{-5}$ in two independent replication samples of European ancestry.

Stage-1 GGE discovery sample

After quality control (QC) of the individual array data, 1527 unrelated patients with GGE (947 females/580 males) and 2461 German population controls (1179 females/1282 males) were included in the genome-wide high-density SNP scan. The GGE patients had the following syndromes: CAE $n = 480$, JAE $n = 215$, unspecified GAE $n = 7$, JME $n = 586$, EGTCS on awakening $n = 93$ and EGTCS alone $n = 146$. The origins of these subjects, by country, were: Austria $n = 231$, Belgium $n = 41$, Denmark $n = 93$, Germany $n = 885$ and The Netherlands $n = 277$. Array SNP data of 2461 German control subjects were obtained from the PopGen Biobank and the KORA (Cooperative Health Research in the Region of Augsburg) research platform representing epidemiologically recruited cohorts from the Northern (Schleswig, PopGen, $n = 1080$) and Southern (Augsburg, KORA, $n = 1381$) regions of Germany. Although the control subjects were not screened for epilepsy, the loss of power is likely to be minimal as the prevalence of GGEs is only 0.3% (50).

Stage-1 GGE discovery subgroups

To dissect out susceptibility alleles differentially conferring the risk for either GAE or JME, we formed two distinct GGE subgroups consisting of: (i) 702 patients with GAEs (CAE/JAE/unspecified GAE; 438 females/264 males) without JME; and (ii) 586 patients with JME (372 females/214 males) irrespective of whether absence seizures occurred. For the association analyses, each group of GGE patients was compared with the entire sample of 2461 controls.

Stage-2 replication cohorts

The Stage-2 replication cohorts comprised 604 European parent–offspring trios of children with GGEs (378 females/226 males; syndrome classification: CAE/JAE $n = 347$, JME $n = 166$, EGTCS alone $n = 91$; origins by country: Australia/UK $n = 135$, Bosnia/Croatia $n = 4$, Bulgaria $n = 4$, Denmark $n = 31$, Finland $n = 10$, France $n = 18$, Germany $n = 12$, Greece $n = 1$, Italy $n = 260$, Spain $n = 5$, Sweden $n = 1$, The Netherlands $n = 5$, Turkey $n = 118$), and a case–control sample of European descent consisting of 889 patients with GGEs (571 females/318 males; syndrome classification: CAE/JAE $n = 385$, JME $n = 382$, EGTCS alone $n = 122$; origins by country: Australia/UK $n = 264$, Denmark $n = 44$, Finland $n = 6$, France $n = 114$, Germany $n = 130$, Italy $n = 79$, Spain $n = 53$, The Netherlands $n = 39$, Turkey $n = 160$) and 889 ethnically matched control subjects (398 females/491 males). The Stage-2 GAE subgroup included 347 trios and 385 GAE patients and 385 ethnically matched controls. The Stage-2 JME subgroup consisted of 166 trios and 382 JME singletons and 382 ethnically matched controls.

Array quality control and population stratification analysis

Initially, the Stage-1 case–control sample consisted of 1595 GGE patients of North-Western European descent and 2518 unselected German population controls. DNA was extracted from blood samples and lymphoblastoid cell lines using standard procedures. All Stage-1 DNA samples were genotyped by the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix SNP 6.0 array; Affymetrix, Santa Clara, CA, USA) at either the Affymetrix Genotyping Service (Santa Clara) or ATLAS Biolabs (Berlin, Germany). We removed 125 subjects (68 cases and 57 controls) from the Stage-1 case–control sample according to four QC criteria: (i) discordant gender information ($n = 1$); (ii) overall SNP CR <95% or excessive heterozygosity rate of autosomal SNPs >29.5% ($n = 1$); (iii) duplicated or related individuals exceeding an identity-by-state (IBS) allele sharing >1.55 ($n = 12$); (iv) ancestry outliers identified by IBS outlier detection diagnostics (proportion of significantly different other samples >25%; $n = 82$) and subsequently by multidimensional scaling (MDS) analysis of IBS genetic distances ($n = 29$) using PLINK (53). After quality control, the GWAS Stage-1 discovery sample included 1527 GGE patients and 2461 genetically matched controls.

SNP genotype quality control and SNP imputation

Stage-1 SNP 6.0 array genotyping

Genome-wide genotyping of >906 600 SNPs was performed using the Birdseed v2 algorithm implemented in the Affymetrix Genotyping Console 4.0 (genotype confidence score: <0.1, annotation file: GenomeWideSNP_6.na30, NCBI build 36.3) (54). SNP genotyping was carried out separately for 10 batches, each representing array assemblies processed at different time points or locations. SNPs with high genotyping accuracy were selected for SNP imputation according to the following QC criteria: (i) minor allele frequency (MAF) <5% in cases or controls, (ii) CR <98% for SNPs with MAF >10% and CR <99% for SNPs with MAF <10% in either cases or controls, (ii) difference of missing data >1%

between the cases and controls, (iv) deviation from the Hardy–Weinberg equilibrium (HWE) with $P < 1.0 \times 10^{-4}$ in the controls and (v) differences in the allele frequencies with $P < 1.0 \times 10^{-4}$ across three batches of cases and between two batches of controls (KORA and PopGen). Finally, the intensity cluster plots of the top-ranked associated SNPs ($P < 1.0 \times 10^{-5}$) of a provisional association analysis were checked manually for clustering errors. After QC, 572 071 autosomal SNPs with an overall genotyping CR of 99.74% were used for SNP imputation to maximize genomic coverage.

SNP imputation

SNP imputation was performed using a Markov chain Monte Carlo algorithm implemented in the software program IMPUTE version 2 (55). SNP imputation was based on filtered reference haplotypes from the HapMap III CEU samples (release #2, February 2009) and the 1000 Genomes low-coverage CEU pilot haplotypes (NCBI Build 36.3, released June 2010). Imputed SNPs were excluded according to the following QC filters: (a) IMPUTE info quality score ≤ 0.8 , (b) overall CR $< 99\%$, (c) MAF $< 3\%$ in cases or controls and (d) significant HWE deviation with $P < 10^{-6}$ in the cases and $P < 10^{-4}$ in the controls. After QC filtering, the expanded GWAS Stage-1 SNP dataset included 4.56 million SNPs.

Stage-2 SNP selection and genotyping

For Stage-2 replication analysis, we selected 22 Stage-1 SNPs (12 SNPs genotyped by the SNP 6.0 array, 10 untyped SNPs with imputed genotypes) based on their statistical significance ($P_{\text{LMM}} < 1.0 \times 10^{-5}$), the regional LD structure and the imputed information quality score (cut-off: ≥ 0.9). Genotyping of the Stage-2 SNPs was performed by the Sequenom iPLEX Gold system (Sequenom, Inc., San Diego, CA, USA) using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (56) at the genotyping platform at the Institute of Clinical Molecular Biology (University Medical Center Schleswig-Holstein, Kiel, Germany). Two SNPs (SNP ID: rs1110615, assay-ID: C__7539479_10; rs17669194, C__34702952_10) were genotyped by TaqMan[®] SNP genotyping assays according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA).

To assess the accuracy of Stage-1 and 2 SNP genotyping, 112 Stage-1 sample replicates and 68 Stage-2 sample duplicates were genotyped in the Stage-2 replication analysis. For the imputed Stage-1 SNP genotypes, the individual genotype with the highest posterior probability was selected using a cut-off threshold of at least 0.4. The comparison of Stage-1 and Stage-2 SNP genotypes revealed a critical concordance rate of 96% for SNP rs4667876. Therefore, this SNP was removed from Stage-2 analysis. The remaining 21 Stage-2 SNPs showed an allelic replication rate of $> 99.5\%$ with an average concordance rate of 99.8%. For the 68 Stage-2 sample duplicates, the SNP genotype concordance rate was 100%. Overall, we observed one Mendelian error in 604 parent–offspring trios. Altogether, these quality checks demonstrate a high genotyping accuracy of the SNPs selected for Stage-2 replication analysis.

Association analyses and controlling for population structure

Genome-wide Stage-1 association analysis of the imputed SNPs was performed for genotype dosages using SNPTESTv2 (www.stats.ox.ac.uk/~marchini/software/gwas/snpstest.html), which incorporates information about uncertainties of genotype callings (57). To correct for potential confounding effects of population stratification, we applied logistic regression analysis, assuming an additive model adjusted for gender and the top four dimensions of PLINK MDS analysis as covariates. Despite this attempt to correct for structure using MDS components, the quantile–quantile (QQ) plots of the P -values obtained by logistic regression analysis still showed a substantial overall inflation of the test statistic resulting in a genomic inflation factor of $\lambda_{\text{GC}} = 1.11$ ($\lambda_{\text{GC}1000} = 1.06$) for the Stage-1 GGE-, $\lambda_{\text{GC}} = 1.05$ ($\lambda_{\text{GC}1000} = 1.05$) for the GAE- and $\lambda_{\text{GC}} = 1.06$ ($\lambda_{\text{GC}1000} = 1.06$) for the JME case–control samples. To correct for the residual inflation of the test statistic, the observed λ_{GC} was used to adjust the type-I error rates of logistic regression analysis (P_{gc}) (52). Although genomic inflation is expected to some extent in the presence of polygenic inheritance (58), some unexplained inflation could not be removed by logistic regression analysis. To remove any residual inflation of the test statistic, we applied a novel factored spectrally transformed linear mixed-model (FaST-LMM; <http://mscomp.bio.codeplex.com>) that explicitly captures all sources of structure based on estimates of the genetic relatedness of individuals (28). Therefore, a covariance matrix R was generated by calculating for every pair of individuals the genome-wide averaged correlation of their relatedness based on 238 K high-quality SNPs. Thereby, the genomic inflation was almost eliminated, resulting in $\lambda_{\text{GC}} = 1.006$ for the Stage-1 GGE-, $\lambda_{\text{GC}} = 0.988$ for the GAE- and $\lambda_{\text{GC}} = 0.999$ for the JME case–control samples (Supplementary Material, Fig. S2A–C). Association analysis of genotyped X-chromosomal SNPs was restricted to female subjects only. To adjust for multiple testing of ~ 4.56 million correlated SNPs, we assessed the effective number of independent tests using the software tool ‘Genetic type-1 error calculator’ (59). In total, we carried out 764 825 independent tests per phenotype, corresponding to a nominal significance threshold of $P \sim 6.5 \times 10^{-8}$ to achieve a genome-wide type-1 error rate of $P = 0.05$. Accordingly, the threshold for genome-wide significance was set to a nominal P -value of $< 5.0 \times 10^{-8}$. Regional visualization of GWAS results was produced by the program LocusZoom version 1.1 (60). GWAS Manhattan plots were generated using Haploview 4.2 (61). SNP and gene annotations refer to NCBI build 36.3/UCSC hg18.

Power calculations

Power calculations were performed using a CaTS power calculator (62). Joint analysis of the GWAS Stage-1 and 2 GGE samples has a power of 80% to detect a variant with an OR of ≥ 1.31 at a type-I error rate of $P = 5.0 \times 10^{-8}$, assuming a disease prevalence of 0.3%, an additive genetic model, a strong LD ($r^2 = 1$) between the causal variant and the SNP allele, and a frequency of the disease-associated allele of at least 20% in controls. Likewise, 80% power was obtained for OR > 1.44 in GAE and for OR > 1.50 in JME.

More comprehensive power simulations are provided in the Supplementary Material, Figure S1.

Stage-2 replication and joint Stage-1 and 2 association analyses

PLINK was utilized for single marker association analysis using the transmission disequilibrium test (TDT) for the parent–offspring trios and the Cochran–Mantel–Haenszel test for $2 \times 2 \times 6$ stratified case–control subsamples deriving from six different European regions. The Breslow–Day test was applied to test differences in the ORs across the stratified subsamples. The weighted Stouffer’s z trend method was used for joint analysis of Stage-1 and 2 P -values using the MetaP software (<http://compute1.lsrc.duke.edu/software/etap.php>) (63). The inverse variance method was applied for combining ORs and 95%-CIs utilizing the statistical package ‘meta’ in R version 2.13.2. With respect to the relative low power of the Stage-2 replication cohorts, association analysis was based on joint Stage-1 and 2 analysis, which almost always has a superior power compared with Stage-2 analysis alone as replication study for Stage-1 association (62).

SUPPLEMENTARY MATERIAL

Supplementary material is available at *HMG* online.

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