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Report

Malic Enzyme 2 May Underlie Susceptibility to Adolescent-Onset Idiopathic Generalized Epilepsy

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Idiopathic generalized epilepsy (IGE) is a class of genetically determined, phenotypically related epilepsy syndromes. Linkage analysis identified a chromosome 18 locus predisposing to a number of adolescent-onset IGEs. We report a single-nucleotide polymorphism (SNP) association analysis of the region around the marker locus with the high LOD score. This analysis, which used both case-control and family-based association methods, yielded strong evidence that malic enzyme 2 (*ME2*) is the gene predisposing to IGE. We also observed association among subgroups of IGE syndromes. An *ME2*-centered nine-SNP haplotype, when present homozygously, increases the risk for IGE (odds ratio 6.1; 95% confidence interval 2.9–12.7) compared with any other genotype. Both the linkage analysis and the association analysis support recessive inheritance for the locus, which is compatible with the fact that ME2 is an enzyme. ME2 is a genome-coded mitochondrial enzyme that converts malate to pyruvate and is involved in neuronal synthesis of the neurotransmitter γ-aminobutyric acid (GABA). The results suggest that GABA synthesis disruption predisposes to common IGE and that clinical seizures are triggered when mutations at other genes, or perhaps other insults, are present.

Idiopathic generalized epilepsy (IGE [MIM 600699]) is one of the most common forms of epilepsy, representing ~30% of all epilepsies (Annegers 1994). Diagnostically, the classification of different forms of IGE is dependent on the specifics of seizure characteristics, including type(s) of seizures, age at onset, timing of seizures, factors precipitating seizures, etc. (International League Against Epilepsy 1989). The most important aspect of syndrome classification in IGE is the type of seizures seen, of which there are usually three forms: myoclonic seizures (bilateral brief jerks of the upper and sometimes lower limbs), absence seizures (brief staring spells with loss of consciousness), and generalized tonic-clonic seizures, which involve loss of consciousness and generalized convulsions. The IGEs usually begin in childhood and adolescence and are primarily, if not exclusively, genetic (Greenberg et al. 1992). The problem for genetic studies is that not only do the syndrome characteristics overlap, thus making the phenotype definition for genetic studies unclear, but different IGE syndromes frequently occur within the same family (Beck-Mannagetta and Janz 1991). This mixed familiality further complicates phenotype definition. The mode of inheritance of the IGEs is complex and likely involves an oligogenic model in which several genes must interact to produce a phenotype (Greenberg et al. 1988*a*, 1992; Durner et al. 2001). There appear to be several loci, some of which are specific for certain seizure types—for example, myoclonic seizures may be determined in part by a gene on chromosome 6, *BRD2* (Greenberg et al. 2000; Pal et al. 2003), whereas absence seizures may be influenced by

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a gene or genes on chromosome 5 (Durner et al. 2001). However, we have identified one locus on chromosome 18 that appears to be common to all the adolescent-onset IGE syndromes we studied: juvenile myoclonic epilepsy (JME [MIM 606904]), juvenile absence epilepsy (JAE [MIM 607631]), and epilepsy with generalized tonic-clonic seizures (EGTCS). Here, we report the identification of that locus and of a specific SNP haplotype that conveys a sixfold increase in the odds of disease for these IGEs.

Our previous linkage analysis results showed a maximum two-point LOD score of 5.2 at *D18S474*, under a recessive model, with evidence of heterogeneity (Durner et al. 2001). The data consisted of many small nuclear families. Unambiguous recombinants, which would have allowed us to limit the candidate region, could not be identified. Because simulation studies of linkage suggested that, at LOD scores >5, the sought-for locus was likely to lie within 3 cM of the peak (Greenberg and Abreu 2001), we started the search for association at the point of maximum evidence for linkage and typed SNPs on either side of *D18S474* (dbSNP Home Page).

Probands had received diagnoses of one of the following IGE syndromes: JME, JAE, or EGTCS. Study-eligibility criteria have been described elsewhere (Greenberg et al. 1995). All subjects signed informed consent forms and were interviewed in person to confirm the diagnosis, to confirm that study entry criteria were met, and to collect blood for DNA extraction. Subjects for the case-control analysis included 156 patients with IGE (88 with JME; 68 with JAE + EGTCS) and 126 randomly chosen controls of European origin. For family-based association, we included 108 families (59 with JME; 49 with JAE + EGTCS) and used the program TRANSMIT (D. Clayton's Web site) to evaluate excess transmission. All family members were included, so that maximum information was obtained even from families missing a parent, but only the proband was used to test for transmission distortion. We used the program SNPHAP to infer haplotypes (Clayton and Jones 1999). We SNP-typed 35 SNPs in the region of *D18S474*. The SNP names and positions are listed in table 1. We also used the program GOLD (Abecasis and Cookson 2000) to examine the marker-marker linkage disequilibrium (LD) in the region.

SNPs centromeric to *D18S474* showed strong and consistent evidence of allelic (as opposed to genotypic [see below]) association in both case-control (fig. 1) and family-based (fig. 2) association studies. For the informative loci between rs674351 and rs654136, the SNPs in the region of malic enzyme 2 (*ME2*) and its putative promoter, the *P* values from the case-control association analysis ranged from .010 to .0001.

One of the most important questions in the genetics of IGE involves phenotype definition. Results of our previous linkage analysis (Durner et al. 2001) suggested that most families with the forms of adolescent-onset IGE we studied showed linkage to *D18S474*. However, whereas JME is genetically linked to chromosome 6, other forms of IGE are not (Greenberg et al. 1995; Durner et al. 1999). We thus performed separate association analyses for JME versus non-JME (non-JME = combined JAE + EGTCS). Both subsets of patients showed the same pattern of association as we saw in the total IGE results, with strong consistent associations with the SNPs in the

Table 1

List of SNPs Used in the Analysis and Their Chromosomal Positions

SNP Number[a]	SNP Name	SNP Position on Chromosome 18 (kb)
1	rs3752087	46442.427
2	rs3892158	46452.5
3	rs1370484	46481.329
4	rs4940007	46510.479
5	rs1470325	46525.278
6	rs1954882	46542.655
7	rs2276186	46579.802
8	rs2849233	46583.54
9	rs2255672	46585.354
10	rs2586775	46588.141
11	rs2218369	46592.104
12	rs2586770	46595.637
13	rs2849239	46596.829
14	rs2586760	46615.12
15	rs2586761	46618.445
16	rs1822459	46626.114
17	rs674351	46639.943
18	rs584087	46644.537
19	rs585344	46650.854
20	rs608781	46659.313
21	rs642698	46674.972
22	rs674210	46698.94
23	rs645088	46708.89
24	rs649224	46710.649
25	rs654136	46716.191
26	rs685373	46732.828
a	rs674965	46744.48
27	rs2027735	46757.401
28	rs620898	46761.135
29	rs2156010	46789.476
b	rs4390682	46804.44
c	rs10502913	46820.258
30	rs3764465	46823.359
31	rs3764466	46823.474
d	rs2276163	46827.376
32	rs2298617	46855.39
33	rs2282543	46945.582
34	rs1893379	46970.523
35	rs1893378	46970.847

NOTE.—See table A1 of appendix A (online only) and appendix B (online only) for further information regarding SNP primers and laboratory conditions.

[a] SNPs a–d were added for analysis of the LD region; see text for details.

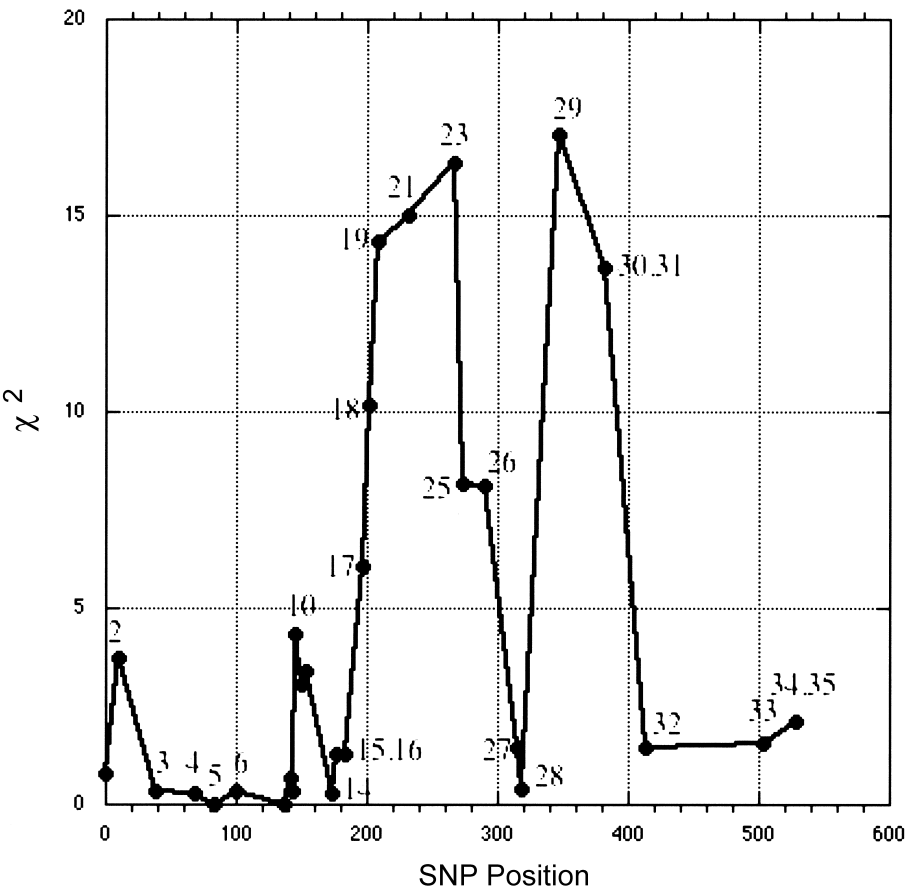

Figure 1 Evidence for association of adolescent-onset IGE on chromosome 18. The graph shows the χ^2 based on the case-control analysis for each SNP tested in this region, which is just centromeric to marker *D18S474*. There appear to be two peaks, the first of which occurs directly over the promoter and locus for ME2. The second occurs in a region of no known genes that exhibits evidence of strong marker-marker disequilibrium. *ME2* is located between SNP locations 200 and 300. The origin of the *X*-axis in figures 1–3 is the first SNP (rs3752087), which is located at position 46442.427 kb. SNP numbers are given next to each data point.

ME2 region, as well as with SNPs just telomeric to *ME2* (fig. 3).

We then asked whether there was a specific haplotype in the *ME2* region that was more prevalent in probands with IGE than in controls. Including only individuals whose haplotype probability was >0.9, we inferred haplotypes for probands and controls in the IGE data set for a nine-SNP-long haplotype covering a 76-kb region encompassing *ME2* and the three SNPs just centromeric to it (table 2). (These latter three SNPs lie in or near the putative promoter region of *ME2,* in a region where there are no known genes and few ESTs [AI024301, BX115263, and AW182546]). Because the original linkage analysis supported recessive inheritance (Greenberg and Berger 1994; Durner et al. 2001), we examined the frequency of *homozygotes* carrying the nine-SNP haplotype versus other genotypes. (We note that, unlike haplotyping heterozygous haplotype alleles by use of case-control data, haplotyping homozygotes is without ambiguity, because there is only one type of base at each locus of the haplotype.) We identified homozygotes for this haplotype and compared the odds of disease in homozygotes with that for any other genotype. We found that 35% of cases were homozygous for the nine-SNP haplotype, compared with 8% of controls. The χ^2 for the case-control association was 30 (1 df; $P < .0001$). The odds of disease, given homozygosity, was 6.1 times the odds for any other genotype (95% CI 2.9–12.7).

Telomeric to the maximum indication of association at the *ME2* locus is a second peak suggesting allelic association. We investigated the marker-marker LD in this region (fig. 4) and found an ~200-kb region of strong LD that ends just centromeric to the start of the *ME2* locus. We also investigated the homozygous haplotype association evidence of this peak and compared the results with those in the *ME2* region. To make this analysis comparable to the *ME2* region analysis, we typed four more SNPs in this region (SNPs a–d in table

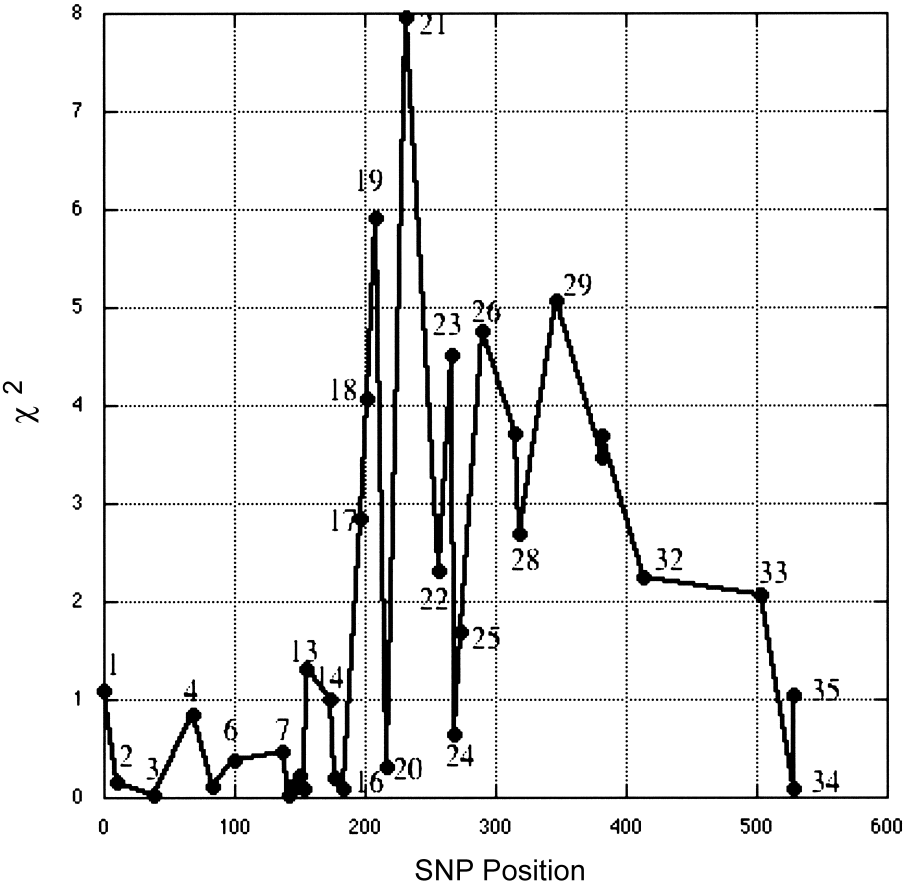

Figure 2 Evidence for association in IGE, from family-based association analysis rather than case-control analysis. SNP numbers are given next to each data point.

1, to bring the total to seven). The region spanned by this seven-SNP haplotype is 10 kb shorter than that spanned by the nine-SNP haplotype over *ME2*. Thus, the *ME2* region has a larger SNP haplotype and has less marker-marker LD, circumstances that, compared with the LD region, should decrease the frequency of homozygotes. In fact, we observed that, in the LD region, 16% of patients were homozygous for the seven-SNP haplotype, versus 3% of controls—a difference of 13%, compared with a 27% difference between homozygotes among cases and controls over the *ME2* region. The odds of disease for a carrier of two copies of this seven-SNP haplotype is 5.96 (95% CI 2.03–17.5; $P = .001$); this CI is wider than that for *ME2* and represents more variability in the estimate of the odds ratio.

The homozygote haplotype analyses support the conclusion that the *ME2* region, and not the nearby LD region, contains the IGE-related gene. The meaning of this second peak, therefore, remains unclear. The second peak could be the result of the block of LD extending into the *ME2* region in a subset of patients, creating a "shadow" peak. There are two known genes in that region telomeric to *ME2: MADH4* (MIM 600993), thought to be a transcription factor related to cancer, and *ELAC1* (MIM 608079), possibly a tumor-suppressor locus. Neither appears related to brain structure or function.

It is also significant that the results of both the haplotype analysis and the original linkage analysis supported recessive inheritance. Enzyme-related diseases tend to show recessive inheritance. Since ME2 is an enzyme, the observation of recessiveness lends added support to the idea that *ME2* is the IGE locus in this region.

A previous genetic study of the IGE syndrome JME suggested, in part, recessive inheritance for an IGE gene (Greenberg et al. 1988*a*). The results of a segregation analysis involving JME (Greenberg et al. 1988*a*) supported an oligogenic inheritance model, suggesting that at least two epistatically interacting loci were responsible for JME susceptibility. The best-fitting model was one in which one locus was dominantly inherited and one recessively inherited. In an oligogenic model, the gene

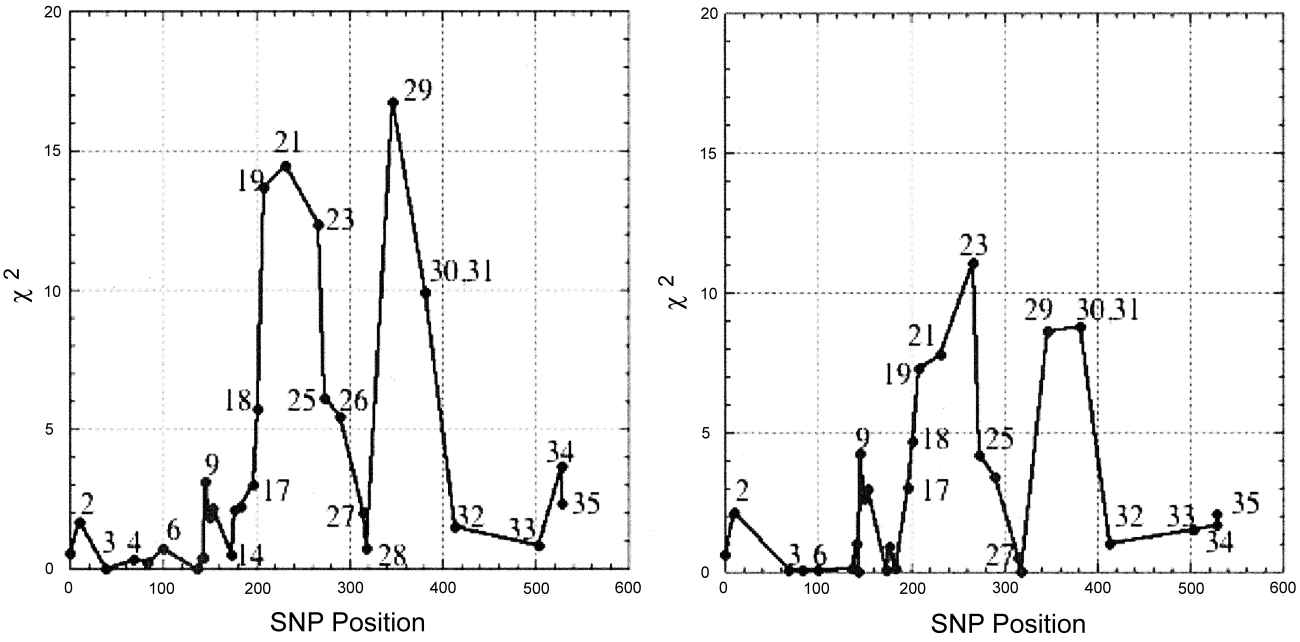

Figure 3 Evidence for association in two separate diagnostic groups with adolescent-onset IGE: JME (*left*) and non-JME (*right*) IGE. Non-JME IGE consists of the two groups: JAE and EGTCS. There were 88 JME cases and 68 JAE + EGTCS cases. The results shown are for case-control analysis. SNP numbers are given next to each data point. Results (not shown) for family-based association were similar for JME, but the sample size for non-JME was not sufficiently large to reach significance.

frequencies of the disease alleles must be high if the population prevalence is at all appreciable, because disease-related alleles must be present at several loci; hence, the proposed high frequency (60%) of the nine-SNP haplotype is predictable. We previously reported evidence that the *BRD2* locus on chromosome 6 is *EJM1,* the gene that predisposes to a common form of JME (Pal et al. 2003), and the linkage analysis and analysis of SNPs and haplotypes of *BRD2* supported dominant inheritance (Greenberg et al. 1988*b,* 2000; Durner et al. 1991; Weissbecker et al. 1991). Thus, the results of both linkage analysis and allelic and genotypic association studies of families with JME and other IGE syndromes have identified two loci that follow the prediction of the segregation analysis.

How do these results help explain the observations of the phenotypic overlap and co-occurrence of different IGE syndromes within families? According to the hypothesis of Durner et al. (2001), the variety of seizure types seen in families with IGE is explained by genes that are specific for different *seizure* types (rather than IGE syndromes), genes that may interact (either directly or indirectly) with a more general epilepsy susceptibility locus. Supporting this hypothesis is (1) suggestive evidence of linkage to chromosome 8 for families identified through patients with absence and/or tonic-clonic seizures (but without myoclonus) (Durner et al. 1999), (2) evidence of a locus on chromosome 5 that conveys susceptibility to absence seizures (Durner et al. 2001), (3) the chromosome 6 locus predisposing to myoclonic seizures (but not to JAE or EGTCS) (Greenberg et al. 1995), and (4) the identification of *ME2* as the likely locus that conveys overall IGE susceptibility.

ME2 is a nuclear genome-coded enzyme that localizes in the mitochondria and is expressed in the brain. Its

Table 2

Bases of the Inferred Nine-SNP Haplotype Located in the Promoter Region and within the *ME2* Locus That Had the Highest Probability in Patients versus Controls

SNP Name	Base
rs674351	A
rs584087	A
rs585344	C
rs608781	A
rs642698	G
rs674210	A
rs645088	C
rs649224	C
rs654136	A

NOTE.—The haplotype was found in 81% of patients and 60% of controls. When present homozygously, it was found in 35% of patients and 8% of controls.

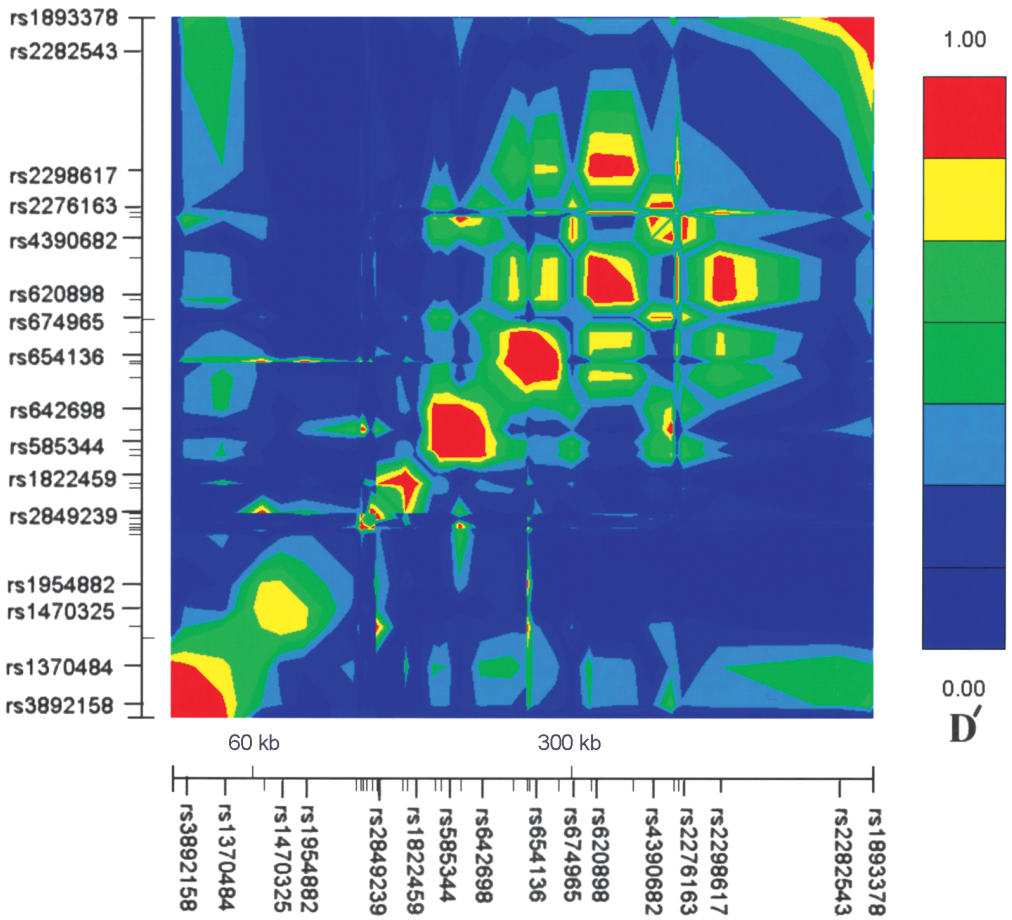

Figure 4 Graph of marker-marker LD in the studied region. The colors correspond to the LD measure *D′*, with red showing the strongest evidence for LD and blue the weakest. The promoter region of *ME2* begins just after SNP rs1822459; *ME2* ends at about SNP rs654136. Note that LD appears to become most pronounced starting just after the *ME2* locus. We suggest that the second peak in association evidence seen in figures 1–3 is due to the LD with *ME2* and not to an independent disease association. The plot uses all SNPs listed in table 1.

primary function appears to be reversibly catalyzing NADP- and NADPH (nicotinamide adenine dinucleotide phosphate)–dependent oxidative decarboxylation of malate to pyruvate and carbon dioxide. However, it also is indirectly involved in the synthesis of γ-aminobutyric acid (GABA) in neurons, in that it supplies a perhaps critical pool of pyruvate for GABA synthesis (Hassel 2001). The connection of GABA to epilepsy susceptibility is compelling. GABA is the main inhibitory neurotransmitter in the CNS, and many antiepilepsy drugs are known to modulate the GABA neurotransmitter system (Meldrum 1982). Mutations in GABA receptors play a role in susceptibility to some forms of epilepsy (Baulac et al. 2001), and GABA is involved in modulation of spike-wave activity (Lang et al. 1996). ME2 activity is reported to be highly enriched in mitochondria from cortical synaptic terminals, compared with mitochondria from cerebellar or cultured cortical neurons (McKenna et al. 2000), and it may also be involved in the glutamate cycle in neurons (Hassel and Brathe 2000). The finding that ME2, an enzyme related to GABA synthesis, is strongly linked to and associated with IGE suggests that disruptions in GABA synthesis in the brain may change the overall threshold of cortical excitability, allowing other mechanisms to trigger specific types of seizures, types determined by malfunctions in other genes. This would explain the observed variety of IGE syndromes, with their overlapping symptoms and multiple seizure types, as well as the occurrence of different IGE syndromes and seizure types within the same family.

The *BRD2* locus has been suggested as playing a strong role in a common form of JME. Both the *BRD2* locus and *ME2* have been shown to be linked and associated with JME, but their functions suggest no obvious interaction. BRD2 is thought to interact directly with H4 histones during DNA transcription (Kanno et al. 2004). The nature of both of these proteins is so fundamental to cell functioning that it suggests that the

mechanisms leading to IGE may be quite subtle and may operate during brain development.

Although there have been a number of reports of loci linked to the phenotype of IGE (Charlier et al. 1998; Escayg et al. 2000; Cossette et al. 2002; Kananura et al. 2002; Haug et al. 2003), they are almost exclusively linkage analyses of single or a few large pedigrees with autosomal dominant inheritance; with multiple affected members, sometimes with phenotypes not typical of IGE; and with forms of epilepsy variant to the classically described syndromes. Such families are atypical of the inheritance patterns observed in families of the common IGEs seen in clinics. The loci and the putative mutations identified at those loci have not been shown to influence the common IGEs.

This report presents analysis of an IGE locus based on data from families collected specifically for common forms of IGE, in a genomic region previously identified through linkage analysis. The linkage and association evidence suggests that *ME2* is a major susceptibility locus for IGE. If confirmed, it may suggest that a general susceptibility to adolescent-onset IGE could be due to a disruption in GABA synthesis, which sets the stage for a second insult to trigger seizures.

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Electronic-Database Information

The URLs for data presented herein are as follows:

dbSNP Home Page, http://www.ncbi.nlm.nih.gov/SNP/

D. Clayton's Web site, http://www-gene.cimr.cam.ac.uk/clayton/software/ (for TRANSMIT)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for IGE, JME, JAE, *MADH4,* and *ELAC1*)

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