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ELECTRONIC LETTER

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Epilepsy is one of the most common and serious neurological disorders, with up to 60 million people affected worldwide.[1] Juvenile myoclonic epilepsy (JME) is a common familial form that accounts for 5–10% of all epilepsy cases.[2] This form belongs to the idiopathic epilepsy group, due to the absence of detectable structural or metabolic abnormalities. Clinically, JME is mainly characterised by isolated myoclonic jerks on awakening that usually begin during adolescence. It is also highly drug-dependent, since a 90% recurrence is reported after interruption of pharmacological treatment.[3]

Studies on the incidence of epilepsy in relatives of probands with JME, as well as on twins, have provided strong evidence for a genetic contribution.[4 5] Autosomal dominant, autosomal recessive, two locus, monogenic, and polygenic models of inheritance have been suggested.[6] So far, three genes that are mutated in different forms of JME have been identified, namely *CACNB4*,[7] *GABRA1*,[8] and *CLCN2*.[9] In addition, two different susceptibility loci have been identified by linkage analysis. The first locus, termed *EJM1* (OMIM 254770), is on the human leukocyte antigen region of chromosome 6p.[10] Although no trait-causing mutation has yet been identified at this locus, association with a haplotype of the *BRD2* gene has been recently reported.[11] The second locus, termed *EJM2* (OMIM 604827), is in the region of chromosome 15q that contains the gene coding for the α7-nicotinic acetylcholine receptor subunit. Genetic mapping of the *EJM2* locus defined a 15.1 cM candidate region on chromosome 15q14, flanked by the D15S165 and D15S971 loci.[6] Interestingly, this region includes the *CX36* gene, which codes for the first connexin identified in neurons.[12]

Connexins are integral membrane proteins, encoded by a family of at least 20 genes in humans, which form the subunits of gap junction channels.[13] Gap junctions permit the cell-to-cell passage of ions, second messengers, and small metabolites and, in the nervous system, provide the structural basis of electrical synapses.[14] Several studies indicate the relevance of gap junction-mediated coupling in maintaining the synchronous activity of neuronal populations, and the gamma frequency oscillations which are thought to underlie a range of cognitive processes.[15 16] Thus, recordings between fast-spiking cells of neocortex has revealed a high occurrence of electrical coupling,[17] suggesting that electrical and synaptic coupling act synergistically to improve neuronal synchronisation.[18] Accordingly, deletion of *CX36* in mice results in loss of electrical synapses, preventing the synchronous inhibitory activities which underlie gamma oscillations in the cerebral cortex.[19 20] Further experiments have revealed that expression of *Cx36* is markedly reduced in the hippocampus of kindled and kainate-treated rats, an animal model of human temporal epilepsy.[21]

These observations, together with the location of the *CX36* gene on human chromosome 15q14, make this gene a strong candidate for JME. To test this hypothesis, we searched for mutations in the coding regions and intron–exon junctions of *CX36* in most of the European cases of 15q14-linked and in randomly selected (RS) JME patients. We found that these patients had no mutation in the coding regions of *CX36*. However, several single nucleotide polymorphisms (SNPs)

Key points

- Juvenile myoclonic epilepsy (JME) is a generalised form of epilepsy with onset in early adolescence. Genetic factors are likely to play a role in the etiology of JME, and significant evidence supports a major susceptibility locus located on chromosome 15q14, where the gene connexin36 (*CX36*) is also mapped. Since electrotonic communication between neurons connected by gap junctions is likely to be implicated in the generation and maintenance of neuronal synchrony, mutations in the *CX36* gene may be associated with JME.
- Mutation analysis of the human *CX36* gene was undertaken in 29 probands from JME families previously linked to the 15q14 locus, as well as in 17 randomly selected (RS) JME patients. Sequencing identified five single nucleotide polymorphisms (SNPs), c.-127A>T, c.333T>A, c.369C>T, c.588C>T, and c.888G>A, none of which resulted in an amino acid substitution.
- A case control study performed on a sample of 29 15q14-linked JME patients, 140 RS JME patients, and 123 controls, demonstrated a significant association between JME and the c.588C>T polymorphism within exon 2, with significant differences in both allele ($p=0.03$) and genotype ($p=0.017$) frequencies. Subjects with the T/T genotype at position 588 had a significantly increased risk of JME (odds ratio 4.3; 95% CI 1.49 to 12.3), compared with those with a C/C genotype. In addition, HAP2, a haplotype containing c.588C>T, was found to be significantly associated with JME ($p=0.03$).
- Further analysis suggested that the c.588C>T polymorphism may influence Cx36 gene expression by affecting exonic splicing enhancers. This defect may contribute to the pathogenesis of JME.

Abbreviations: CEPH, Centre d'Etude du Polymorphisme Humain; ESE, exonic splicing enhancer; HW, Hardy-Weinberg; ILAE, International League Against Epilepsy; JME, juvenile myoclonic epilepsy; LD, linkage disequilibrium; RS, randomly selected; SNP, single nucleotide polymorphism

were identified, one of which had allelic frequencies statistically different from those of controls. Moreover, a haplotype of *CX36* appeared to be over represented in JME patients. The results provide the first evidence for an association between *CX36* and the 15q14-linked JME families.

METHODS

Cases and control sample

A total of 169 patients with JME were investigated: 29 of these were unrelated individuals from the families used to identify the 15q14 susceptibility locus,[6] whereas the other 140 patients were RS from the neurology departments of five hospitals, irrespective of whether an epileptic syndrome affected first degree relatives or not.[22] The majority of the 15q14-linked patients (2/3) were clinically ascertained from within the UK, the others originating from five other European countries (Denmark, France, Greece, Portugal, and Sweden). Diagnostic evaluation was made according to the classification of the International League Against Epilepsy (ILAE). The control group included unrelated individuals who were randomly selected from families of the Centre d'Etude du Polymorphisme Humain (CEPH) (n = 44), and of the Swiss population (n = 79). SNP c.333T>A was further genotyped on 94 additional Swiss controls. Since allele frequency and distribution of both genotypes and haplotypes were similar in CEPH and Swiss controls, these two sub-populations were combined into a single control group, comprising 123 individuals (50% women). Informed consent was obtained from all participating individuals, and the study was approved by the ethic committees of all participating institutions (National Hospital for Neurology and Neurosurgery, London; Paris Hospital Ethics Committee; and Ethical Committee of the Geneva University Hospital).

Mutation analysis and identification of SNPs

Detection of mutation was performed by genomic PCR amplification and direct sequencing. We designed primers using the human genomic sequences (GenBank AC012271) which encompass *CX36*. Primer pairs were selected to amplify fragments covering the whole coding region of this gene, all intron–exon junctions, and 572 bp of the 5′ UTR. Primers sequences were 5′TAAAAGGAAAGGGGGATTCG3′ and 5′CTCAGTCCAGGTGTGAGAAGG3′ for exon 1; 5′CAGCTCCCCAGTCAAAAGAC3′ and 5′GGTCACATAAATGAGGGTGGA3′ for exon 2; 5′ACGCAGGCGGAGACTACTTA3′ and 5′CCCGATCATAGTGGAGTGCT3′ for the 5′UTR. Amplification reaction was performed with 100 ng total genomic DNA. The 25 µl PCR mixture contained 250 µM deoxynucleotide triphosphates, 200 nM each primer, 2.5 µl 10× PCR buffer (Finnzymes), and 1.25 U Dynazime *Taq* polymerase (Finnzymes, Espoo, Finland). All samples were amplified in a T Gradient Thermocycler 96 (Biometra, Germany), under the following conditions: initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 40 s, and extension at 72°C for 1 min. After purification, the PCR products were sequenced using standard protocols, an ABI 377 automated sequencer, and an additional primer for exon 2 (5′CACCAGTCCGCCAAGCAGCGAG3′). Chromatographs of amplicons from affected individuals were compared to the genomic sequence of the *CX36* gene using the gap4 editor, which is available at the UK HGMP Resource Centre (http://www.hgmp.mrc.ac.uk). Five SNPs were characterised and named with reference to the *CX36* cDNA sequence (GenBank NM_020660).

SNP genotyping

Selected regions encompassing each identified SNP were amplified from genomic *CX36* DNA and analysed by pyrosequencing. The following PCR primer pairs were employed (b indicates biotin): 5′GGTCGGGACGCGCTTGGATTT3′ and 5′bCCCCCTGACCGCCGGCTT3′ for c.–127A>T (expected amplicon 258 bp); 5′CCTACTCTGTGCACCAGTCCG3′ and 5′bCCCCATTCACAATAGCATTT3′ for c.333T>A and c.369C>T (expected amplicon 174 bp); 5′bACTCCACACCCATCAGGTCTA3′ and 5′CGGAACACCACTTGGATAAT3′ for c.588C>T (expected amplicon 95 bp); 5′bCTCAACCTGGCTGAACTCAAC3′ and 5′CCCAGACCTTCATGAAACCTG3′ for c.888G>A (expected amplicon 194 bp). The corresponding sequencing primers were: 5′GATTCGAGGATTTTTT3′, 5′GAACGCCGCTACTCT3′, 5′AGACCCCCCTGAGTC3′, 5′GGCAGGAAGGCATCTC3′, and 5′CAGGTCCTTGTTACGAAT3′, respectively.

All amplification reactions were carried out in a 96 well microtitre plate thermal cycler (Biometra, Germany), under the same conditions used for the mutation analysis. Preparation of single-strand DNA and annealing to the sequencing oligonucleotide were as reported.[23] Briefly, attachment of the biotinylated DNA amplicon to streptavidin-modified paramagnetic beads was followed by a high salt washing step, a 10 min NaOH (0.15 M) denaturation step, and a final annealing step (95°C, 15 s). Purified fragments annealed with the cognate sequencing primers were then processed for sequencing. DNA mini-sequencing was performed using a PSQ96 instrument, enzymes, and dNTPS (all from Pyrosequencing AB), as previously described.[23] All the resulting genotypes, which were automatically assigned to the samples by the SNP analysis software, were checked by two independent investigators.

Statistical analysis

The extent of linkage disequilibrium (LD) of each SNP combination was assessed in all 123 control individuals, by calculating r^2 using a DNA sequence polymorphism program.[24] A two-tailed Fisher's exact test and the Bonferroni procedure were computed to determine the significance of association between polymorphic sites. Five-SNP-locus haplotypes were inferred from genotypes of the control group, using the Bayesian method,[25] as provided by PHASE software. The algorithm deals with missing genotype data, so that a total of 246 haplotypes were inferred in the control group. Similarly, haplotypes of both control and JME groups were inferred for the four SNPs which were selected after LD analysis, for subsequent case control analysis. *CX36* haplotype genealogies were constructed using the reduced-median-network approach, available in Network 3.1 software (Fluxus-engineering; www.fluxus-engineering.com). Hardy-Weinberg (HW) equilibrium was tested for each SNP using the HW exact test, as implemented in Genepop software (http://wbiomed.curtin.edu.au/genepop). Logistic regression models were used to calculate odds ratios (OR, 95% confidential interval). The Fisher's exact test was used for contingency table inference. To correct for multiple comparisons, we used a modified Bonferroni test as described in Sankoh *et al*,[26] which accounts for the level of correlation between linked markers. In this case, p values $\leqslant 0.019$ were taken to indicate a significant difference for $\alpha = 0.05$. For haplotype associations no correction is required and p values $\leqslant 0.05$ were considered to indicate statistically significant difference.

RESULTS

CX36 mutation screening

A total of 46 JME patients, including 29 individuals whose pedigrees had previously been used to determine the 15q14 susceptibility locus, and 17 RS JME patients, were screened for mutations of *CX36*, as compared to four unaffected controls. Screening of the whole coding region,

the intron–exons junctions, 48 bp of the splice donor, 70 bp of the intron splice-acceptor sites, and 70 bp of the 3′-untranslated region revealed no mutation of the *CX36* gene in JME patients which could result in an amino acid substitution or affect the splicing and branching sites of RNA.

Polymorphisms

Four SNPs were detected in *CX36* exon 2 (table 1). One was a T>A transversion (c.333T>A), and the three others included a G>A transition (c.888G>A) and two C>T transitions (c.369C>T; c.588C>T), respectively. An additional SNP was identified in the 5′-untranslated region (5′UTR) of the *CX36* and involved an A>T transversion located 127 bp upstream of the translation initiation codon (c.-127A>T).

Characterisation of SNP genotypes and haplotypes

The allele frequencies of the five *CX36* SNPs were determined in a control group of 123 individuals (table 1). Genotype frequencies for all SNPs were in HW equilibrium (not shown). Haplotypic combinations of the five SNPs are shown in fig 1. A total of eight out of the 32 possible haplotypes were observed. The most common HAP1 haplotype, which represents >30% of all haplotypes, was found to be conserved in non-human primates (fig 1), suggesting that it most likely represents the ancestral haplotype. To explore the evolution of the *CX36* gene, sequence genealogies were constructed, using a method that equally weighted all nucleotide positions. The skeleton network revealed two major branches in the northern European *CX36* genealogy (HAP4 and HAP5 accounting for >30% of all haplotypes, and HAP2 and HAP6 accounting for >25%), which had evolved from a unique ancestral haplotype (HAP1), via two successive mutations events (fig 1).

Linkage disequilibrium analysis

Assessing the strength of pairwise linkage disequilibrium (LD) between the five SNPs of the control population, we found that SNP369, SNP588, and SNP888 were not in significant LD ($r^2 = 0.2–0.01$) with any other *CX36* SNPs (table 2). In contrast, SNP-127 and SNP333 demonstrated a highly significant LD with each other ($r^2 = 0.81$, $p<0.001$) (table 2). These results indicate, first, that *CX36* SNPs do not reside within the same LD block, and, second, that SNP-127 and 333 may be grouped to facilitate association studies, without loss of statistical power.

Case control study

To determine whether SNP333, 369, 588, and 888 were associated with JME, we tested 29 unrelated JME patients that had been shown to have a 15q14 linkage, and 140 RS JME patients. These two JME groups were analysed both separately and as a single population. The 15q14-linked JME sample represents the largest available European set of patients whose linkage to the 15q14 locus has been unambiguously established.[6 27] Using Fisher's exact test, we found that the allele frequency of SNP588 was significantly ($p = 0.03$) different between the 15q14-linked JME group and the control group (table 1). We also found a trend towards a difference ($p = 0.057$) in the allele frequency of SNP588 between the RS JME group and the control group. When all the JME patients were pooled and compared to the control group, the allele frequency of SNP588 was also significantly different ($p = 0.03$) as judged by the Fisher test, but did not reach statistical difference when a conservative correction for multiple testing was applied. This finding suggests that a small difference in the allele frequency may become masked because of the rarity of the disease. The risk of JME increased in the presence of a T allele, in both the

Table 1 Allele frequency of *CX36* SNPs in JME cases and controls

SNP*	Alleles, n (%)		p Value†	Function	Protein residue	Amino acid position	dbSNP ID
SNP-129	A	T		–	–	–	rs2277558
15q14 JME	ND	ND	–				
RS JME	ND	ND	–				
Combined	ND	ND	–				
Control	150 (70.7)	62 (29.3)					
SNP333	T	A		Synonymous	Thr	111	rs651724
15q14 JME	44 (75.9)	14 (24.1)	0.29				
RS JME	160 (70.2)	68 (29.8)	0.58				
Combined	204 (71.3)	82 (28.7)	0.33				
Control	292 (67.9)	138 (32.1)					
SNP369	C	T		Synonymous	Ser	123	–
15q14 JME	56 (96.50)	2 (3.5)	0.37				
RS JME	170 (89.5)	20 (10.5)	0.3				
Combined	226 (91.1)	22 (8.9)	0.61				
Control	211 (92.5)	17 (7.5)					
SNP588	C	T		Synonymous	Ser	196	rs3743123
15q14 JME	34 (58.6)	24 (41.4)	0.033‡				
RS JME	181 (65.1)	97 (34.9)	0.057				
Combined	215 (64)	121 (36)	0.026§				
Control	179 (72.8)	67 (27.2)					
SNP888	G	A		Synonymous	Glu	296	–
15q14 JME	49 (84.5)	9 (15.5)	0.5				
RS JME	229 (88.8)	29 (11.2)	0.88				
Combined	278 (88)	38 (12)	1				
Control	205 (88.4)	27 (11.6)					

ND, Not determined. dbSNP, public database reference (http://www.ncbi.nlm.nih.gov/SNP).
*SNP offset were calculated taking the A of the CX36 start codon as position 1, and using the GenBank accession no. NM_020660; †p Values computed using Fisher's exact test, give the significance value of the comparison of SNPs allele frequencies between JME and control cases. Statistically significant associations are underlined; ‡OR = 1.89; 95% CI 1.03 to 3.45 (odds ratios and their associated 95% CI were calculated for results with $p<0.05$); §OR = 1.5; 95% CI 1.04 to 2.17.

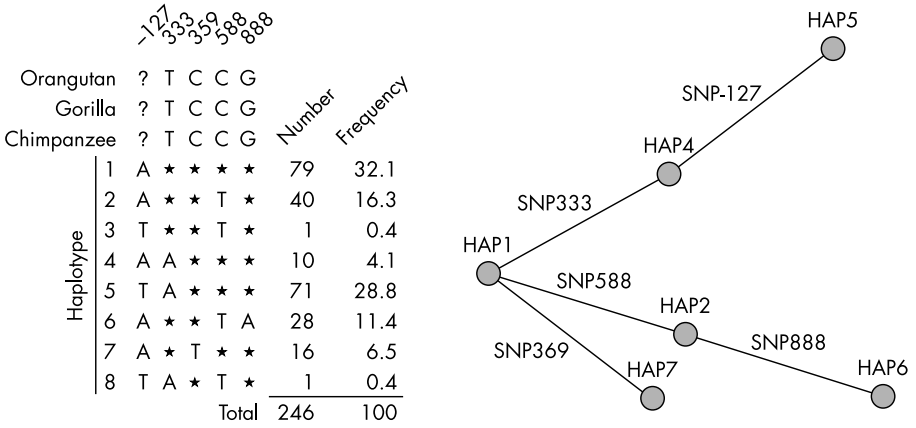

Figure 1 The haplotypes of *Cx36* SNPs are highly conserved in primates and derived from a common ancestor. European haplotype combinations of the *CX36* gene (HAP1 to HAP8) were inferred by PHASE software in a group of 123 Caucasian control individuals. The corresponding sequences of the closely related non-human primates are also indicated. Asterisks (*) indicate identity of the human and ape bases. The most common *CX36* haplotype (HAP1) is identical in humans and apes and, thus, likely represents the ancestral haplotype. The phylogenetic tree generated by reduced-median-network analysis, confirmed that all other haplotypes were derived from this ancestral HAP1.

Table 2 Pairwise linkage disequilibrium (r^2) between *CX36* SNPs (unrelated controls)

SNP	333	369	588	888
−127	0.81***	0.03**	0.14***	0.05***
333		0.03**	0.18***	0.06***
369			0.03**	0.01
588				0.32***

Significant pairwise linkage disequilibrium ($r^2>0.5$) was found between SNP-127 and 333 (underlined value), but not between the three other SNPs.
*0.01<p<0.05; **0.001<p<0.01; ***p<0.001.

15q14-linked cases and the entire JME population (OR = 1.89 and 1.5, respectively; table 1).

We then examined the distribution of SNP588 genotypes (table 3) and found that subjects with a T/T genotype at position 588 were significantly more numerous in the 15q14-linked JME group than in the control population (OR = 4.3; 95% CI 1.49 to 12.3, p = 0.017) as attested by both uncorrected and corrected tests. In addition the c.588C>T transition was not in HW equilibrium in the 29 linked cases' subgroup, due to an over-representation of the T/T genotype (data not shown).

Haplotype association analysis

To assess whether a combination of multiple SNPs increases the risk of JME, the frequencies of haplotypes were compared between cases and controls (table 4). The distribution of Cx36 haplotypes was significantly different (p = 0.02) between the 15q14-linked JME, and the control group (table 4). The haplotype TCTG, accounting for >97% of the Cx36 HAP2 haplotype, was more frequent in the 15q14-linked JME patients than in controls (0.28 *v* 0.17), although the difference failed to reach statistical significance. We found that homozygous carriers of the HAP2 haplotype had a significantly increased risk of JME (p = 0.03) than either heterozygous carriers of the HAP2 haplotype (HAP2/X) or non-carriers (X/X) (table 5). The same increased prevalence of the HAP2/HAP2 combination was also observed when the pooled JME group was compared to controls (p = 0.04).

Folding of *CX36* mRNAs containing synonymous SNPs

To evaluate the effect of the 588T variation, we used the MFOLD program[28] to predict the structure of the mRNA coding for CX36. We observed that the predicted structure of the transcript was similar when the molecule was comprised of nucleotides 588C, 333A/588C, 369T/588C (fig 2), or -127T/588C (not shown), which correspond to haplotypes HAP1, HAP4, HAP7, and HAP5, respectively (fig 1). In contrast, we found that the presence of allelic variant 588T caused an obvious change in the predicted structure of the *CX36* mRNA, as illustrated for HAP2 (fig 2). The folding structure of the three other haplotypes containing the 588T variant (HAP3, HAP6, and HAP8) were also affected, in a similar way to that of HAP2 (not shown).

Alteration of potential exonic splicing enhancers by the 588T nucleotide

To further investigate whether the 588T variation may affect exonic splicing enhancers (ESEs), we used sequence motif-scoring matrices predicting consensus functional ESE sites,[29] to analyse exon 2 of wild type (HAP1) and variant (HAP2) *CX36*. Multiple high-score binding sites, recognised by the essential splicing factors SF2/ASF, SC35, SRp40, and SRp55,

Table 3 Genotype frequency of SNP588 in JME cases and controls

	Genotype at SNP588, n (%)					
Group	CC	CT	TT	Total	OR (95% CI)*	p Value†
15q14 JME	13 (44.8)	8 (27.6)	8 (27.6)	29	4.3 (1.5 to 12.3)	0.017
RS JME	57 (41)	67 (48.2)	15 (10.8)	139	1.37 (0.58 to 3.22)	0.11
Combined	70 (41.7)	75 (44.6)	23 (13.7)	168	1.79 (0.81 to 3.98)	0.09
Control	66 (53.7)	47 (38.2)	10 (8.1)	123		

*Odds ratio (OR) compares the T/T genotype to the pool of all other genotypes; †Given by Fisher's exact test. Statistically significant associations after Bonferroni correction are underlined.

Table 4 Cx36 haplotype frequency in JME cases and controls

Haplotype*	15q14 JME†, n (%)	RS JME, n (%)	Combined, n (%)	Control, n (%)
ACCG	13 (22.4)	81 (28.9)	94 (27.8)	81 (32.9)
TCCG	18 (31.1)	82 (29.3)	100 (29.6)	79 (32.2)
TCTG	16 (27.6)	65 (23.2)	81 (24)	41 (16.7)
TCTA	7 (12.1)	32 (11.4)	39 (11.5)	28 (11.4)
TTCG	1 (1.7)	20 (7.2)	21 (6.2)	16 (6.5)
ACTG	1 (1.7)	0	1 (0.3)	1 (0.3)
TTCA	1 (1.7)	0	1 (0.3)	0
TCCA	1 (1.7)	0	1 (0.3)	0

*Haplotype inferred using PHASE for the four SNPs 333, 369, 588, and 888, respectively; p = 0.02 (p value computed using Fisher's exact test, gives the significance value of the comparison of haplotype frequencies between JME and control cases).

Table 5 Association between HAP2 haplotype combinations and JME

Group	Haplotype HAP2, n (%)*			Total	OR (95% CI)†	p Value‡
	HAP2/HAP2	HAP2/X	X/X			
15q14 JME	3 (10.5)	10 (34.5)	16 (55)	29	14.8 (1.3 to 147.5)	0.026
RS JME	6 (4)	53 (38)	81 (58)	140	5.46 (0.6 to 48)	0.102
Combined	9 (5.5)	63 (37.5)	96 (57)	168	6.91 (0.8 to 57.6)	0.04
Control	1 (0.8)	39 (31.7)	83 (67.5)	123		

*X denotes any haplotype other than HAP2; †OR compare the HAP2/HAP2 combination to all other combinations; ‡Given by Fisher's exact test.
Statistically significant associations are underlined.

were distributed throughout this exon. We found that the 588T synonymous nucleotide specifically disrupted the SF2/ASF motif (CTC**C**CGC) and the SC35 motif (ATCTC**C**G), reducing the cognate score from 2.266 to 0.801 and from 3.176 to 1.441, respectively. The resulting values fell below the calculated default thresholds for the native motifs (1.95 and 2.38, respectively), indicating that the 588T variation rendered the SF2/ASF and SC35 ESEs inactive.

DISCUSSION

Juvenile myoclonic epilepsy is a common form of idiopathic, generalised epilepsy that shows a complex pattern of inheritance. Two major susceptibility loci, *EJM1* on chromosome 6p21 and *EJM2* on chromosome 15q14, have been identified by linkage analysis. The finding of a significant linkage disequilibrium between JME and a haplotype of the *BRD2* gene, which is located on chromosome 6p21, suggested

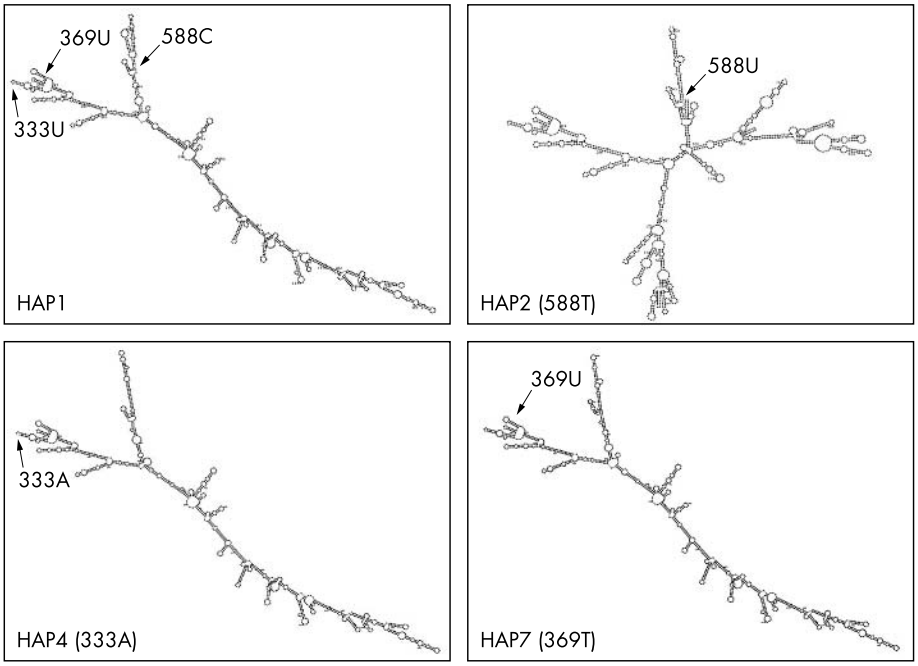

Figure 2 The predicted folding structure of Cx36 mRNA is altered by the 588T SNP. A sequence of 1147 bp, covering the complete coding sequence of Cx36 mRNA and including various synonymous SNPs, was tested with MFOLD software to predict the secondary structure of the transcript. The folding pattern of HAP1, the most common *CX36* haplotype, was not affected by the SNPs we studied (illustrated by HAP4 and HAP7). In contrast, this structure was markedly altered in haplotypes carrying the 588T SNP, for example in HAP2.

that BRD2 is *EJM1*.[11] In contrast, the genes involved at the *EJM2* locus are still unknown. In the present study, we screened the *CX36* gene for SNPs and found that the c.588C>T variant, located in exon 2, is associated with JME. In addition, we found that the homozygous combination of HAP2, one of the *CX36* haplotypes containing SNP588, was significantly associated with JME. These findings suggest that *CX36* is a potential susceptibility gene for juvenile myoclonic epilepsy at the *EJM2* locus.

The mechanisms through which SNP588C>T predisposes to JME remain to be elucidated. First, it is possible that the c.588C>T transition may be a functional variant of the *CX36* gene, conferring susceptibility to JME. This hypothesis is supported by the finding of a C at position 588 in the sequence of the ancestral human *CX36* haplotype (HAP1), as well as in the *CX36* sequence of non-human primates and phylogenetically more distant species, such as the house mouse (GenBank NM_010290.1) and the cow (GenBank NM_174683.1), indicating an evolutionary conservation of this base, which suggests a likely importance for the function of the *CX36* gene. In turn, the c.588C>T variant could alter this function, for example by affecting RNA stability. While most of the elements which control the stability of mRNAs are located in the 5′- and 3′-UTRs of genes, compelling evidence indicates the presence of other stabilising elements also in the coding region of many genes.[30 31] Because individual exons comprise multiple positive and negative cis elements that affect splicing, some exonic SNPs may influence splicing efficiency and, therefore, the levels of gene expression.[32] The predicted changes, both in the secondary structure of Cx36 mRNA and in two potential ESEs, we observed for the synonymous SNP588C>T are consistent with this hypothesis. Further studies, examining the relation between mRNA stability, exon skipping, translation efficiency, and SNP588 genotype, should determine whether this variant is indeed responsible for decreased CX36 levels. Second, it is conceivable that SNP588 may be in linkage disequilibrium with other variants of the same haplotype that confer susceptibility to JME. The existence of such variants is suggested by the association of HAP2, one of the *CX36* haplotypes containing SNP588, with JME. The analysis of haplotype combination showed an over-representation of this homozygous risk haplotype in the 15q14-linked patients, consistent with the LOD score maximisation previously reported at *EJM2*, using a recessive model.[27] Increasing evidence indicates that the interaction of multiple SNPs within a haplotype can lead to functional changes.[31 33] Since SNPs occur approximately every 500–1000 bp of DNA in human chromosomes,[34] several other variants are anticipated in the 5′-, 3′-UTRs and intron regions of the *CX36* gene. The presence of causative mutations in these regulatory regions should be further investigated. Third, our data do not exclude the possibility that the *CX36* SNP associated with JME might be in linkage disequilibrium with variants of another gene, such as the *ACTC* gene or another gene located close to *CX36*. However, no such gene was identified in the proximity of *CX36*, using the UCSC genome browser[35] (not shown).

Recent studies indicate that *CX36* is a likely gene for JME. This gene encodes a protein making channels at electrical synapses between neurons[12] and at gap junctions between the electrically excitable insulin-producing β-cells of pancreas.[36] In a previous study, we have shown that Cx36-made channels control the synchronisation of Ca^{2+} oscillations in a transformed cell line.[37] Although no such control has yet been investigated in neuronal cells, it is consistent with a possible involvement of Cx36 in the regulation of neuronal excitability. Moreover, it has been shown that the generation of myoclonic seizures is closely associated with an abnormal synchronisation of neurons, particularly in the inferior olive,[38] a brain region where Cx36 is highly expressed.[39] In view of the recent findings that deletion of *Cx36* in mice leads to altered oscillations of interneurons in both hippocampus and neocortex,[19 20 40] a change in the levels of CX36 could conceivably be involved in the generation of epileptic seizures. The lack of such epilepsy events in the *Cx36* knock-out model[19 20 40] does not contradict this involvement, inasmuch as these mice may have developed several morphological and electrophysiological changes compensating for the loss of Cx36.[41] These negative findings rather indicate that the existing murine models may not be relevant for investigating the participation of Cx36 in complex diseases, such as human JME. At any rate, this is the first study which associates a genetic variation in the connexin36 gene with JME.

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