Linkage and mutational analysis of *CLCN2* in childhood absence epilepsy.

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Abstract

In order to assess the chloride channel gene *CLCN2* as a candidate susceptibility gene for childhood absence epilepsy, parametric and non-parametric linkage analysis was performed in 65 nuclear pedigrees. This provided suggestive evidence for linkage with heterogeneity: NPL score = 2.3, $p<0.009$; HLOD = 1.5, $\alpha = 0.44$. Mutational analysis of the entire genomic sequence of *CLCN2* was performed in 24 unrelated patients from pedigrees consistent with linkage, identifying 45 sequence variants including the known non-synonymous polymorphism rs2228292 (G2154C, Glu718Asp) and a novel variant IVS4+12G>A. Intra-familial association analysis using the pedigrees and a further 308 parent-child trios showed suggestive evidence for transmission disequilibrium of the G2154C minor allele: AVE-PDT $\chi^2_{(1)} = 5.17$, $p<0.03$. Case-control analysis provided evidence for a protective effect of the IVS4+12G>A minor allele: $\chi^2_{(1)} = 7.27$, $p<0.008$. The 65 nuclear pedigrees were screened for three previously identified mutations shown to segregate with a variety of idiopathic generalised epilepsy phenotypes (597insG, IVS2-14del11, and G2144A) but none were found. We conclude that *CLCN2* may be a susceptibility locus in a subset of cases of childhood absence epilepsy.

Keywords: childhood absence epilepsy; linkage; association; mutation screening; *CLCN2*

1. Introduction

The absence epilepsies are a group of idiopathic generalised epilepsies (IGEs) including childhood absence epilepsy (CAE), juvenile absence epilepsy (JAE), epilepsy with myoclonic absences and eyelid myoclonus with absences (Engel, Jr.,

2001). The main features of CAE are frequent absence seizures (10-40 per day) and bilateral, synchronous, symmetric 3-Hz spike waves on EEG. Age of onset peaks at 6-7 years. Generalized tonic-clonic seizures (GTCS) often develop during adolescence. Otherwise, absence seizures may either remit or persist into adulthood. In contrast, JAE manifests around puberty with absence seizures and GTCS (1989). There is evidence to suggest that CAE and JAE share a similar genetic architecture allowing them to be assessed as a single phenotype in genetic studies (Berkovic et al., 1987;Marini et al., 2004).

Genome-wide linkage analysis of IGE-multiplex families has demonstrated evidence for susceptibility loci on chromosomes 3q26, 14q23 and 2q36 (Sander et al., 2000). Mutations in the chloride channel gene *CLCN2* (chromosome 3q27-q28) have been identified in three of 46 unrelated IGE families demonstrating linkage to 3q26 (Haug et al., 2003). These mutations result in a premature stop codon (M200fsX231), an altered splice site (del74-117) and a single amino-acid substitution (Gly715Glu). Two different mutations (Arg688Gly and Glu718Asp) and an intronic variant (IVS17-3C>T) have been shown to cosegregate with epilepsy in two families with idiopathic epilepsy (D'Agostino et al., 2004).

CLCN2 encodes an 898-residue transmembrane protein ClC-2 which is widely expressed in the brain and other tissues (Thiemann et al., 1992). ClC-2 has been shown to be involved in the control of GABAergic synaptic transmission (Sik et al., 2000) thus providing a plausible biological basis for how loss of *CLCN2* function could affect neuronal excitability.

Available evidence thus suggests that mutations in *CLCN2* may underlie a proportion of IGE phenotypes potentially including CAE and JAE. The aim of this work was to test this hypothesis by linkage analysis using microsatellite loci spanning

CLCN2 in 65 nuclear families each with a proband with CAE and at least one other member affected by CAE or JAE, and to perform direct re-sequencing of the entire genomic sequence in 24 affected unrelated individuals from pedigrees consistent with linkage to determine the presence of any potentially disease-associated variants. The disease-associated mutations previously identified by Haug *et al*. were also screened for in our resource because some of their cases with the mutations had either CAE or JAE.

2. Materials and methods

2.1. Subjects

A resource of 65 nuclear pedigrees each with a proband with CAE and at least one other member affected by CAE or JAE (including 145 cases) and 308 independent parent-child trios with CAE was ascertained. They were all of Caucasian origin from European populations including the UK, France, Germany, Austria, the Netherlands, Denmark, Sweden, Finland, Italy and Greece, and from the USA and Canada. Affected individuals were classified using diagnostic criteria based on the ILAE classification of CAE and JAE (Engel, Jr., 2001). Inclusion criteria for CAE were as follows: age of onset between 3 and 10 years; normal neurological state and development; brief (four to 20 seconds, occasionally longer) and often frequent (tens per day) absence seizures with abrupt and severe impairment of consciousness; automatisms may occur; mild myoclonic jerks of the eyes, eyebrows or eyelids may occur; generalised tonic-clonic seizures may occur; seizures may persist into adulthood; the ictal EEG shows bilateral, synchronous, symmetrical discharges of 2.5-

4Hz spike-wave complexes on a normal background or polyspike-wave complexes; and photosensitivity may be present. Exclusion criteria included: significant developmental delay; persistent or focal neurological deficit; and clear abnormalities on neuroimaging. Inclusion criteria for JAE were as follows: age of onset between 10 and 17 years; normal neurological state and development; brief absence seizures with abrupt and severe impairment of consciousness occurring sporadically; generalised tonic-clonic seizures may occur possibly as the initial seizure type; ictal EEG of symmetrical, generalised spike-wave discharges more prominent in the frontal region at a frequency of 3.5Hz-4.5Hz; photosensitivity may occur. Exclusion criteria include prominent bilateral myoclonic seizures as occur in juvenile myoclonic epilepsy.

Genomic DNA was extracted from whole blood or cheek swab samples according to standard protocols.

Control samples were obtained from the European Collection of Cell Cultures (ECACC) human random control panels 1, 2 and 3 (HRC-1, HRC-2 and HRC-3; 94 samples per plate). The ECACC HRC panels include DNA from blood donors who are all UK Caucasians and are characterised by gender and age at venesection. All donors have given written informed consent for their blood to be used for research purposes. Different numbers of controls were used at different stages of the analysis simply due to availability of these samples to us (Table 1 summarises in which samples each variant was genotyped). Due to variation in quality between assays, the number of successful genotypes varies between SNPs in both cases and controls, thus these final numbers will be quoted in the text. Conformation to Hardy-Weinberg Equilibrium (HWE) was verified in all SNPs typed in controls.

2.2. Linkage Analysis

Linkage analysis was performed using two fluorescently-labelled microsatellite markers, *D3S3609* and *D3S3583*, which flank *CLCN2*. They span a physical distance of 250kb (NCBI sequence map) over a predicted genetic distance of 1.14cM. Only the 65 pedigrees were used for linkage analysis.

Genotyping was performed on the ABI 373 Sequence Analyser using the Genescan® and Genotyper® software. All pedigrees were checked for Mendelian inheritance using the PedCheck program (O'Connell and Weeks, 1998). Any pedigrees which failed this test were re-genotyped. Formal linkage analysis was performed using GeneHunter 2.1 (Kruglyak et al., 1996). Only those individuals with CAE or JAE were considered affected. Parametric analysis was performed under the assumption of autosomal dominant inheritance with a penetrance of 50%. These parameters correspond to the available data on Mendelian segregation in this trait. They are conservative and the low penetrance reduces the risk of false negative results. A disease allele frequency of 0.01 and a phenocopy rate of 0.0001 were assumed. A disease allele frequency of 1% allows the variant to be classifiable as a polymorphism which would fit the common disease/common variant hypothesis. A phenocopy rate of 0.0001 allows for the assumed heterogeneous architecture of CAE. The heterogeneity LOD score (HLOD) and an estimate of α, which represents the proportion of pedigrees consistent with linkage at a specific locus, were calculated. The nonparametric linkage (NPL) statistic (Sall), along with the corresponding degree of significance, was also calculated by GeneHunter. The Sall statistic assesses the degree of allele-sharing in all affected individuals and is valuable as it is a “model-free” form of analysis thus bypassing inherent problems of a parametric analysis such as misspecification of parameters.

2.3. Screening for Known Variants

Primers were designed in-house for IVS2-14del11 (details available on request) and this mutation was screened for in the 65 nuclear pedigrees using the same methodology as for the microsatellite markers. The other two mutations identified by Haug *et al*. (597insG and G2144A) were screened for in the pedigrees by KBiosciences (http://www.kbioscience.co.uk) using the Amplifluor™ chemistry for genotyping. One further known non-synonymous variant rs9820367 (G2003C, Thr668Ser) was screened for by KBiosciences in our entire resource and in 94 DNA samples from HRC-1.

2.4. CLCN2 Sequencing in 24 Families with Evidence for Linkage

Bi-directional direct re-sequencing of ~15kb of genomic DNA (chromosome 3, 185561969-185547096; accession number GI 51511463) including all 24 coding exons, ~1kb of predicted promoter and ~1kb of the 3' region was performed. Twenty-four unrelated cases were chosen from families consistent with linkage to the *CLCN2* locus. This re-sequencing work was performed by Polymorphic DNA Technologies Inc., using standard Sanger dideoxy sequencing protocols (http://www.polymorphicdna.com). Any putative functional variants were then genotyped in the ECACC plates to determine a population minor allele frequency (MAF) and in the entire resource.

Intrafamilial association analysis was performed using the pedigree disequilibrium test (PDT) (Martin et al., 2000;Martin et al., 2001). The PDT produces

two measures of association, the PDT-AVE and the PDT-SUM. The former gives all families equal weight in the analysis whereas the latter gives more weight to more informative families. Case-control analysis was performed with the Pearson chi-squared test or the Fisher Exact Test (when any of the cells in the calculation table contained a value of 5 or less). Control data was derived either from the ECACC plates alone when the tested SNP was novel, or from the combined genotype information from the ECACC plate and the Caucasian data on dbSNP. Case data was derived from the affected offspring from the trios and the probands from the pedigrees yielding a maximum of 373 unrelated affecteds.

Bioinformatic analysis was performed using the prediction program ESEfinder version 2.0 (Cartegni et al., 2003) and the TransFac and Biobase GmbH databases via NSITE (http://www.softberry.com). ESEfinder identifies putative exonic splicing enhancer sites responsive to the human SR proteins SF2/ASF, SC35, SRp40 and SRp55, and predicts whether exonic mutations will disrupt such elements. The NSITE program searches for motifs of known regulatory elements.

3. Results

3.1. Linkage Analysis

Multipoint analysis gave a maximum HLOD of 1.5 (α=0.44) at microsatellite marker *D3S3609*. The NPL statistic at this position was also at its maximum of 2.3 ($p<0.009$) (Figure 1).

3.2. Screening for Known Variants

None of the three mutations reported by Haug *et al*. (IVS2-14del11, 597insG or G2144A) were detected in our resource of 65 pedigrees. The G allele of the non-synonymous SNP rs9820367 (G2003C, Thr668Ser) was detected with a frequency of 43.9% amongst 329 unrelated affecteds. The comparative minor allele frequency (MAF) was 47.6% in 84 ECACC controls (in HWE). See Table 1 for full details of MAFs in the different populations. Case-control analysis demonstrated that this difference was not significant and intra-familial association analysis was also not significant (data not shown).

3.3. CLCN2 Sequencing in 24 Families with Evidence for Linkage

Re-sequencing identified 45 variants including: 39 novel variants and 6 known polymorphisms; one known missense polymorphism; 3 synonymous variants; one 5' UTR and one 3' UTR variant; and 39 intronic SNPs (Figure 2). All cases had at least 3 polymorphisms, with the highest number being 14 (Figure 3). There did not seem to be any discernible pattern to the possession of these variants. Table 1 provides MAF details of those variants which were consequentially typed in a larger resource based on the available data regarding expected MAF and on the results from the bioinformatics analyses. Table 2 lists the variants which on the basis of the bioinformatics analyses may be putative functional variants. Although intrafamilial and case-control analysis was performed on all of the variants genotyped in the larger resource, only two demonstrated statistical significance. Full descriptive details of these two variants are given below.

3.4. Screening for rs2228292

The known missense polymorphism identified through re-sequencing was rs2228292 (G2154C, Glu718Asp). The MAF amongst our 24 sequenced cases was 4.8%. ESEfinder predicted that this SNP would fall within a binding motif for the SF2/ASF protein and that the change from a G to a C would lower the matrix score (from 2.23 to 2.02) and move the site along by two nucleotides (from chromosome position NC_000003.10:18553288-185553282 to NC_000003.10:18553286-185553280). It also predicted that possessing the C allele would result in the creation of a new binding site for SRp40. Consequently, this SNP was genotyped in the ECACC controls and in our entire resource of pedigrees and trios. The MAF was 3.3% in the 277 ECACC samples and 3.8% in our 339 unrelated affecteds. These differences between cases and controls were not statistically significant (data not shown). However, intrafamilial association analysis was also performed using the PDT and this did demonstrate suggestive evidence for overtransmission of the minor allele (AVE-PDT $\chi^2_{(1)}$=2.27, p=0.023; transmitted 30, not transmitted 14).

3.5. Screening for IVS4+12G>A

A novel variant, IVS4+12G>A, was identified with a MAF of 12.9% amongst our unrelated 24 cases. Due to its proximity to the exon-intron boundary this was deemed potentially functional. Furthermore exons 4 and 5 combine to code for one of three ion-binding domains within the Clc protein thus any disruption to their correct translation could have a major effect on the function of the protein. The MAF in our resource of 357 unrelated affecteds was 27.1% whilst in the 186 ECACC controls it

was 35.2% (HWE was confirmed). Case-control analysis established that this difference was significant ($\chi^2_{(1)}$=7.63, p<0.006). However, intrafamilial association analysis did not demonstrate any evidence for overtransmission of either allele (data not shown).

4. Discussion

ClC channels are members of a large family of Cl^- selective ion channels found in a diverse array of organisms. They allow the flow of Cl^- ions across the cell membrane down the electrochemical gradient. The 3D structure of human ClC channels has not been directly characterised but has been inferred from *Escherichia coli* ClC (EcClC) structure. They are composed of two identical subunits each with its own independent pore for Cl^- ions and 3 ion-binding sites: S_{int}; S_{cen}; and S_{ext}. These 3 sites are formed by 4 conserved sequences of amino acid residues (Dutzler et al., 2002). When open, all 3 binding sites are occupied by Cl^- ions, whilst the S_{ext} site is blocked by the side chain of a glutamate residue when closed (Dutzler et al., 2003). There are 9 isoforms in humans involved in an array of physiological functions (Jentsch et al., 2002). ClC-2 has been shown to be involved in the control of GABAergic synaptic transmission (Sik et al., 2000) and is expressed in various epithelia and in the brain (Thiemann et al., 1992). The *CLCN2* gene was located to chromosome 3q26-qter (Cid et al., 1995) and has been shown to be mutated in a small subset of families with IGE (Haug et al., 2003;Sander et al., 2000). The work presented here used 65 nuclear pedigrees for linkage analysis using two microsatellite markers spanning the gene. The maximum HLOD score was 1.5 with an α of 0.44, which is suggestive of linkage in the case of a complex disease, which is expected to

be polygenic and heterogeneous. Thus no single locus would be expected to produce a high maximum HLOD score in this resource. Furthermore the non-parametric analysis produces a maximum NPL score of 2.3 which is statistically significant ($p<0.009$) at the same position, providing further confirmatory supporting evidence for linkage to this locus. However, mutation screening did not detect the mutations reported to co-segregate with IGE by Haug *et al*. (2003). The previously identified missense polymorphism rs9820367 (Thr668Ser) was detected with similarly high MAF in both our unrelated affecteds and the ECACC controls at a little under 50%. Unsurprisingly, there was no statistically significant evidence for disease association with this variant.

Direct re-sequencing in affecteds from pedigrees consistent with linkage identified a further 45 variants. This is a frequency of approximately one every 300 nucleotides which is similar to the predicted frequency of a polymorphism (Kruglyak and Nickerson, 2001). Four of these variants were genotyped in controls and across our resources, either because the MAF in the 24 cases was markedly different from that quoted on dbSNP, or because of a putative functional effect. Of these four, two demonstrated some statistical support for disease association. The first was a known missense SNP rs2228292 (G2154C, Glu718Asp). Although the MAF amongst our 24 cases differed from that among the control ECACC samples (4.8% vs 3.3%), case-control analysis using more than 300 unrelated affecteds from our whole resource was not significant. However, the PDT does provide evidence for transmission disequilibrium with overtransmission of the minor allele. This difference in result may reflect an underlying population stratification which might affect the case-control analysis but not the PDT. This polymorphism was also detected in a previous study in one Polish family in which the proband and his father had familial frontal lobe

epilepsy (FFLE) (D'Agostino et al., 2004). The minor allele was found to segregate with the disease and was not found in any control samples. Although this is a partial rather than a generalised epilepsy such as CAE, it is possible that rs2228292 has an effect on general seizure susceptibility rather than on predisposition to a specific epilepsy. The residue which is altered by this variant (Glu718Asp) is situated close to the Gly715Glu polymorphism identified by Haug *et al*. (2003). They predict that this change will subtly alter voltage-dependent gating, which may cause membrane depolarization and hyperexcitability. However, functional analysis of this polymorphism has not provided consistent support for this postulate (Niemeyer et al., 2004). An alternative suggestion is that the Gly715Glu variant severely affects the ability of ATP to bind to the CBS domains, which is thought necessary for ClC channels to open (Scott et al., 2004;Vanoye and George, Jr., 2002). The same hypothesis could thus be proposed for the Glu718Asp polymorphism. Although the change from a glutamate to an aspartate is a conservative change with a Grantham score of 45 (Grantham, 1974), it has been predicted that 75% of conservative non-synonymous changes will have a functional effect (Zhang, 2000). All mammalian homologues of *CLCN2* contain a glutamate residue at the position corresponding to human Glu718, providing further support for the suggestion that any alteration at this site could have a functional effect. Finally, the substitution of the G with a C is predicted to affect a potential ESE, causing it to have a weaker effect, as well as creating an ESE that does not exist in the wild type. Both these factors could affect splicing providing an alternative way in which this variant may affect the protein.

The second variant which demonstrated statistical disease association was IVS4+12G>A, which has also been detected in families with idiopathic epilepsy (D'Agostino et al., 2004). We found no evidence for preferential transmission of

either allele to affected individuals. However, case-control analysis established that the A allele occurred at approximately 3 times the frequency in controls compared to cases. This indicates that this variant may be more likely to act as a protective allele rather than as a susceptibility allele. Exons 4 and 5 are crucial to the protein function of clc-2 as together they provide the codons which encode residues which form one of the ion-binding sites of the selectivity filter, S_{int}. If the splicing together of these two exons is altered it could have a direct effect on the function of the protein via affecting the efficacy of ion-binding. It is worth noting however that because the PDT results does not support that demonstrated by case-control analysis, the latter may simply reflect unidentified population stratification.

This study has provided further support for a potential role for *CLCN2* in IGE, specifically CAE. Of the variants identified, the most likely to have a putative functional role would be rs2228292 (Glu718Asp), for a number of reasons: it is a missense polymorphism; there is significant evidence for transmission disequilibrium; the comparative residue is conserved across species; it has previously been found to segregate with the disease in another family with idiopathic epilepsy; it alters an amino-acid located 3 residues along from another epilepsy-associated variant, Gly715Glu, which is predicted to have a functional effect via either altering gating properties or changing the ATP-binding ability of the CBS domains. Lastly, bioinformatics analysis predicts that splicing may be affected via the disruption and creation of ESE motifs. Clearly, functional studies will be necessary to demonstrate if these predictions are true.

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Figure Legends.

Figure 1. Chromosome 3q27-28 locus linkage analysis results.

Figure 2. Location of variants found during re-sequencing of ~15kb in 24 patients. Red triangles = Known SNPs. Blue triangles = novel SNPs. The two SNPs investigated further (rs2228292 and IVS4-12G>A) are shown in boxes.

Figure 3. The number of individuals re-sequenced who possessed the indicated total number of polymorphisms.

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