Failure to replicate previously reported genetic associations with sporadic temporal lobe epilepsy: where to from here?

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Temporal lobe epilepsy (TLE), traditionally thought to develop largely due to environmental factors, has recently become the focus of association studies in an effort to determine genetic risk factors. Here we examine all previous claims of association of genetic polymorphisms with TLE by attempting replication in a cohort of 339 TLE patients of European origin. We also examine if these variants contribute to other types of epilepsy by examination in a larger cohort of 752 patients representing a range of different epilepsies. We fail to clearly replicate any of the previously reported associations and also fail to show a role for these variants in the development of other forms of epilepsy. Although our results cannot definitively rule out a role for these genes, they do suggest that most and perhaps all of the previous associations are false positives. As has been the experience with other diseases, these results highlight the importance of larger sample sizes and replication. In TLE, it appears that collaboration before publication is the best option to increase sample size sufficiently in the short term. These general principles are applicable to other studies undertaken for common complex diseases.

Keywords: epilepsy; TLE; epilepsy genetics; association; replication

Abbreviations: FS = febrile seizures; HS = hippocampal sclerosis; IGE = idiopathic generalized epilepsy; TLE = temporal lobe epilepsy


Introduction

Although major advances have been made in the field of Mendelian genetics, with the discovery of numerous genes underlying monogenic diseases, only a limited number of genetic susceptibility factors underlying common diseases have been convincingly identified (Ioannidis et al., 2004). This is mainly because common diseases arise from interaction of several genes, with additional environmental influences, and are therefore much more complex. Moreover, the best strategy to study such complex traits is a matter of ongoing debate (Botstein and Risch, 2003).

The study of genetic contributions to the aetiology of epilepsy exemplifies these issues. Epilepsy is a serious, common and heterogeneous condition. A growing number of single gene defects are being identified in rare Mendelian forms of epilepsy, but for the more common non-Mendelian forms of the condition, the nature of the genetic aetiology remains uncertain. Temporal lobe epilepsy (TLE) is the most common form of partial epilepsy. According to the focus of seizure origin, TLE can be subclassified further into mesial TLE and lateral or neocortical TLE. Therefore, TLE is itself a heterogeneous condition with a wide range of possible underlying aetiologies. Although traditionally it was considered an acquired disorder, it is now becoming clear that genes play a role in at least a subset of cases. Evidence for this comes from both human and animal studies.

TLE rarely occurs in families as a monogenic trait. So far, the only known gene associated with a monogenic type of TLE is LGII, mutations in which are responsible for some cases of familial lateral TLE manifesting as autosomal dominant partial epilepsy with auditory features (Kalachikov et al., 2002). Several families with autosomal dominant mesial TLE have also been reported, and linkage has been established in some of
them (Berkovic et al., 1996; Baulac et al., 2001; Claes et al., 2004). In addition, individuals with a TLE phenotype have been reported in other multiplex familial epilepsies, such as generalized epilepsy with febrile seizure plus (Abou-Khalil et al., 2001), familial partial epilepsy with variable foci (Scheffer et al., 1998; Xiong et al., 1999) and partial epilepsy with pericentral spikes (Kinton et al., 2002). Finally, genes have been identified in mice, mutations in which cause a phenotype reminiscent of TLE. Examples include Scn2a in the Q54 mouse (Kearney et al., 2001), Jh8 in the jerky mouse (Liu et al., 2002) and Plc-β1 (Bohm et al., 2002). These observations illustrate that there is good evidence for a genetic contribution to TLE.

Hippocampal sclerosis (HS) is the most common histopathology underlying sporadic forms of mesial TLE. In most cases, the cause of HS is not known. A long-standing but controversial hypothesis links childhood febrile seizures (FS) with subsequent HS (Falconer, 1971); patients with TLE are more likely than patients with other focal epilepsies to have a history of FS (Baulac et al., 2004). The heritability of FS appears to be high (Tsuboi, 1987; Tsuboi and Endo, 1991). Taken together, these data imply a genetic component to HS and thus to many cases of mesial TLE.

Over the last 4 years, several studies have reported associations between common variants in specific genes and sporadic TLE including mesial TLE. Significant association with TLE has been reported in four genes: IL-1β (Kanemoto et al., 2000), PDYN (Stogmann et al., 2002), GABBR1 (Gambardella et al., 2003a) and PRNP (Walz et al., 2003). One study has reported an association of APOE with age of onset of TLE (Briellmann et al., 2000). In addition, variants in CHRNA4 (Chou et al., 2003a) and GABRG2 (Chou et al., 2003b) have been reported as risk factors for childhood FS. These associations, rightly or wrongly, have contributed to the growing view that TLE is at least partially genetic (Tan et al., 2004b).

The well-accepted prevalence of false-positive results in genetic association studies stresses the importance of replication to confirm or reject novel association results (Cardon and Bell, 2001). In fact, where follow-up studies have been reported [IL-1β (Heils et al., 2000; Buono et al., 2001; Peltola et al., 2001; Tilgen et al., 2002; Jin et al., 2003); PDYN (Gambardella et al., 2003b; Tilgen et al., 2003); APOE (Blumcke et al., 1997; Gambardella et al., 1999); and CHRNA4 (Mulley et al., 2004)], results have been conflicting, making it currently unclear whether any of the genes have important effects on the development of TLE or FS.

Here we have examined all seven claims of positive association (for TLE and FS) in the literature by attempting to replicate them in our independent cohort of adult patients, which included subcohorts of 339 patients with a syndromic diagnosis of TLE and a partially overlapping subcohort of 107 patients with epilepsy of any type who had a definite previous history of FS.

Further, we added, to make in total a cohort of 752 epilepsy patients, an additional 371 patients with forms of epilepsy other than TLE and examined if these variants contribute to the development of any common type of epilepsy.

Material and methods

Ethical permission was obtained from the Joint Research Ethics Committee of the National Hospital for Neurology and Neurosurgery and Institute of Neurology. All patients provided written informed consent.

The primary aim of this study was to re-test previous association studies involving various TLE phenotypes. We thus classified the 539 patients diagnosed with TLE into relevant subcategories as defined by the original studies reporting each association (see Table 1). In this way, we were able to re-test directly each of the claims for association. We note that these categories are not mutually exclusive, which is why the sum of the subcategories is greater than the total number of patients with TLE examined. For each gene, we make clear which group of patients was used for the association.

In order to examine any effect of these variants on other classifications of epilepsy we have divided our overall cohort of 752 epilepsy patients as having idiopathic generalized epilepsy (IGE, n = 96), cryptogenic (n = 233) or symptomatic epilepsy (n = 330). This scheme follows presumed gradations in the level of genetic contribution to etiology and, for our patients, corresponds approximately, though not exactly, to the classification of epilepsies and epileptic syndromes according to the Commission on Classification and Terminology of the International League Against Epilepsy (1989). Ninety-three individuals were unclassifiable according to these criteria. These patients therefore were only included in the association tests involving ‘all epilepsy’.

All patients and controls were of self-identified European ancestry. We used unrelated individuals from a twin registry as controls (Andrew et al., 2001).

DNA was extracted using standard methods and genotyping was carried out using a variety of techniques outlined below. All arrays were validated on 16 samples previously genotyped using direct sequencing. The variants IL-1β −511 (rs16944), GABBR1 G1465A (rs1805057), PRNP Asn171Ser (no rs number available) and GABRG2 Asn196Asn (rs211037) were genotyped using Applied Biosystems Taqman technology (probe details available on request). The 68 bp variable number tandem repeat in the promoter of the PDYN gene and the CHRNA4 variant (rs1044396) were genotyped as detailed previously (Zimprich et al., 2000; Chou et al., 2003a). To allow comparison with previous studies, we grouped PDYN alleles

<table>
<thead>
<tr>
<th>TLE classification</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippocampal sclerosis*</td>
<td>141</td>
</tr>
<tr>
<td>Familial non-lesional†</td>
<td>50</td>
</tr>
<tr>
<td>Non-lesional‡</td>
<td>245</td>
</tr>
<tr>
<td>Refractory with hippocampal sclerosis¶</td>
<td>121</td>
</tr>
<tr>
<td>Symptomatic refractory¶</td>
<td>181</td>
</tr>
</tbody>
</table>

*The categories of TLE defined here overlap; thus their sum is greater than the total number of TLE patients; †as used by Kanemoto et al. (2000); HS was diagnosed using high-resolution magnetic resonance imaging; ‡as used by Stogmann et al. (2002); non-lesional TLE patients (HS allowed) with first or second degree relatives having a history of seizures; ¶as used by Gambardella et al. (2003a); non-lesional TLE patients (HS allowed); §as used by Wallz et al. (2003); we used criteria outlined in Siddiqui et al. (2003) to define drug resistance. As used by Briellmann et al. (2000); patients with symptomatic, drug-resistant (as defined in Siddiqui et al., 2003) TLE; includes patients with HS.
as low expression 'L' (i.e. those with one or two repeats) and high expression 'H' (i.e. those with three or four repeats). Direct sequencing of a fragment of the APOE gene was applied to genotype the variants rs429358 and rs7412 (used to define the £2, £3 and £4 haplotypes). Primers and reaction conditions are available on request.

We assessed the significance of genotypic and allelic contingency tables using the χ² distribution. For tables with insufficient cell counts, we used an exact probability test as implemented in the program RxC (available at http://bioweb.usu.edu/mpmbio/rxc.asp). Haplotypes were inferred for the APOE gene by applying the EM algorithm as implemented in the program PEM (available at http://www.people.fas.harvard.edu/~junliu/plem/click.html). Haplotypic association was assessed by calculating log-likelihoods of estimated haplotype frequencies (£2, £3 and £4) for each of the cases, controls, and cases and controls. The test statistic 2(ln Lcase + ln Lcontrol – ln Lcase/control) is then applied, giving a χ² value with n – 1 degrees of freedom (where n = number haplotypes). This method was implemented using the EH and PM programs (available at: http://www.iop.kcl.ac.uk/LoP/Departments/PsychMed/GEpibSt/software.shtml).

In order to illustrate the expected power of replication in our cohort, we estimated power of detection using software available at http://statgen.iop.kcl.ac.uk/gpc/. To calculate power of detection at a 0.05 type I error rate level, we used as parameter values: relative risk values, a disease prevalence of 0.1%, risk allele frequencies observed in our control population and the relevant case–control ratio. Direct positive replications are better estimators of genetic effect than original reports (Lohmueller et al., 2003). However, as direct positive replications were not available, we used data from original reports to estimate relative risk and odds ratio (OR) values. We defined the risk allele as that conferring risk in the original report. We emphasize that we used the estimated relative risks, as opposed to the lower bounds on these relative risks. This means of course that we cannot rule out smaller effects for the variants even when our estimated power is high. Although TLE is the most common partial epilepsy, precise prevalence figures are not known. We view the use of a disease prevalence of 0.1% as a conservative estimate for all types of TLE tested here.

We report significance levels for attempted replications for the TLE or FS subjects with uncorrected P-values. However, when testing for association with the four other phenotype groupings, we accept as significant only tests that exceed a corrected value. We corrected for both the types of epilepsy (all patients, symptomatic epilepsy, cryptogenic epilepsy and IGEs), as well as the five polymorphisms. We thus corrected (by the Bonferroni method) for 20 tests resulting in a significance level threshold set at P = 0.0025 (0.05 out of 20), for an experiment-wide type 1 error rate of 0.05. All associations are reported as uncorrected P values to allow evaluation of trends.

We assessed the significance of the relationship between age at onset of habitual seizures with the presence or absence of the APOE £4 allele using the Mann–Whitney U test. All genotypic counts are in Hardy–Weinberg equilibrium after correction for multiple testing.

Results

IL-1β

The IL-1β –511 variant has been reported previously to associate with TLE and HS (Kanemoto et al., 2000), FS (Virta et al., 2002; Kanemoto et al., 2003) and refractory partial epilepsy (Peltola et al., 2001). The first of these reports (Kanemoto et al., 2000) showed an association between the IL-1β –511 genotype and development of TLE accompanied by HS in Japanese patients [P = 0.0085; OR 3.29 confidence interval (CI) 1.28–8.47]. Our results failed to replicate, or show a trend, in support of this association in our European cohort (see Table 2). We estimated our cohort to have 68% power of detection for this association.

We also examined the reported association with FS in Finnish children (Virta et al., 2002) and in Japanese patients with HS (Kanemoto et al., 2003). We were unable to replicate the association with childhood FS in our cohort of 107 patients with all forms of epilepsy and antecedent FS (P = 0.557 and 0.408 for genotype and allele).

Restricting our analysis to HS patients with a history of FS (n = 50) similarly failed to show significance (P = 0.886 and 0.865 for genotype and allele). We estimated our cohort to have 90% power of detection for the FS association. We were unable to calculate power of detection for FS with HS as genotypic counts for these subgroups were not detailed in the original report.

Similarly, we found no association (P = 0.780 and P = 0.672 for genotype and allele) with refractory partial epilepsy in the 372 patients in our overall cohort who matched the phenotype reported in the original positive association study (Peltola et al., 2001). We estimated our cohort to have almost complete power of detection for this association.

Examination in other forms of epilepsy failed to show any contribution of this allele to development of other types of epilepsy. Results are shown in Table 2.

Table 2  IL-1β –511 genotype counts and analysis results

<table>
<thead>
<tr>
<th>Genotype</th>
<th>TLE + HS® (n = 141)</th>
<th>All patients (n = 752)</th>
<th>Symptomatic (n = 330)</th>
<th>Cryptogenic (n = 233)</th>
<th>IGE (n = 96)</th>
<th>Controls (n = 384)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G/G</td>
<td>59 (0.45)</td>
<td>309 (0.45)</td>
<td>133 (0.44)</td>
<td>98 (0.46)</td>
<td>37 (0.42)</td>
<td>161 (0.44)</td>
</tr>
<tr>
<td>G/A</td>
<td>57 (0.44)</td>
<td>306 (0.44)</td>
<td>137 (0.45)</td>
<td>94 (0.44)</td>
<td>41 (0.47)</td>
<td>162 (0.45)</td>
</tr>
<tr>
<td>A/A</td>
<td>15 (0.11)</td>
<td>74 (0.11)</td>
<td>34 (0.11)</td>
<td>21 (0.10)</td>
<td>10 (0.11)</td>
<td>41 (0.11)</td>
</tr>
<tr>
<td>MAF†</td>
<td>0.33</td>
<td>0.33</td>
<td>0.34</td>
<td>0.32</td>
<td>0.35</td>
<td>0.34</td>
</tr>
<tr>
<td>Genotype P‡</td>
<td>0.9808</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Allele P‡</td>
<td>0.9272</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Genotype counts are those for successful typings only. *TLE with hippocampal sclerosis as used by Kanemoto et al. (2000); † minor allele frequency; ‡ P values are uncorrected and calculated from χ² distribution generated from contingency tables; NS = non-significant.
PDYN

Stogmann et al. (2002) reported an effect of a functional PDYN promoter variation on development of non-lesional TLE in patients of middle-European descent whose first and/or second degree relatives also had a history of seizures. The authors reported an over-representation of the low expression allele (‘L’) in the patient cohort \([ P = 0.0025; \text{OR} = 5.33 \ (CI \ 1.94–14.65)]\). We failed to replicate this association in the 50 patients in our overall cohort matching this phenotype (see Table 3). Our cohort is predicted to have 89% power of detection for this association.

The same authors also reported that patients with TLE carrying the low expression PDYN allele had a significantly higher risk of developing frequent secondarily generalized tonic–clonic seizures and status epilepticus. We were unable to replicate these associations as our patient numbers were too low in the case with frequent secondarily generalized seizures and we lacked reliable information that could confirm the absence of a history of status. However, of the 14 patients we identified with frequent secondarily generalized seizures (as defined in the original association), only one carried the low expression allele.

Analysis of the broader cohort suggests the possibility that PDYN may act as a general risk factor for epilepsy \([ P = 0.043]\) and for IGE \((P = 0.041)\). Following the previous observation (Stogmann et al., 2002) that PDYN genotype effect was limited to familial cases of epilepsy, we examined the variant in familial cases of IGE and found the association to strengthen \((P = 0.002)\). In light of the many tests we have conducted here, these results can be considered only as a modest trend. To clarify the role of PDYN in epilepsy, further analysis is required in larger patient cohorts.

GABBR1

Gambardella et al. (2003a) reported the GABBR1 G1465A variant to have a major effect on the development of non-lesional forms of TLE in Italian patients \([ P = <0.0001 \ \text{OR} = 37.95 \ (CI \ 8.84–162.98)]\). In our cohort of patients with non-lesional TLE, however, we see no association (see Table 4). We have 81% power of detection for this association. However, this is a conservative estimate as we used the same relative risk value calculated for the heterozygote as an estimate for the homozygote risk genotype. This was required as it is impossible to calculate a relative risk value for the homozygote risk genotype as that genotype was not observed in the original control cohort.

We saw no effect of this variant on the development of other forms of epilepsy as classified here (see Table 4).

PRNP

The non-synonymous PRNP variant Asn171Ser was reported by Walz et al. (2003) to contribute to the development of refractory TLE with HS in a Brazilian patient cohort which included patients of both European and African descent. The authors observed the variant in 23% of patients but were unable to detect it in controls, suggesting that the variant contributes greatly to disease development \((P = <0.0001)\). We failed to replicate this association in our cohort of 121 patients.

Table 3 PDYN genotype counts and analysis results

<table>
<thead>
<tr>
<th>Genotype</th>
<th>TLE + FH* (n = 50)</th>
<th>All patients (n = 752)</th>
<th>Symptomatic (n = 330)</th>
<th>Cryptogenic (n = 233)</th>
<th>IGE (n = 96)</th>
<th>IGE + FH* (n = 32)</th>
<th>Controls (n = 384)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H/H</td>
<td>17 (0.36)</td>
<td>336 (0.48)</td>
<td>152 (0.49)</td>
<td>106 (0.50)</td>
<td>33 (0.36)</td>
<td>8 (0.25)</td>
<td>175 (0.48)</td>
</tr>
<tr>
<td>L/H</td>
<td>22 (0.47)</td>
<td>270 (0.39)</td>
<td>120 (0.39)</td>
<td>80 (0.38)</td>
<td>46 (0.49)</td>
<td>16 (0.50)</td>
<td>160 (0.44)</td>
</tr>
<tr>
<td>L/L</td>
<td>8 (0.17)</td>
<td>92 (0.13)</td>
<td>37 (0.12)</td>
<td>27 (0.13)</td>
<td>14 (0.15)</td>
<td>8 (0.25)</td>
<td>30 (0.08)</td>
</tr>
<tr>
<td>MAF†</td>
<td>0.4</td>
<td>0.33</td>
<td>0.31</td>
<td>0.31</td>
<td>0.40</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Genotype P‡</td>
<td>0.0898</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.0021</td>
</tr>
<tr>
<td>Allele P‡</td>
<td>0.0429</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.0011</td>
</tr>
</tbody>
</table>

Genotype counts are those for successful typings only. *Familial non-lesional TLE (history of seizures in first and/or second degree relatives) as used by Stogmann et al. (2002); †familial IGE (history of seizures in first and/or second degree relatives); ‡minor allele frequency; †P values are uncorrected and calculated from \(\chi^2\) distribution generated from contingency tables; NS = non-significant.

Table 4 GABBR1 G1465A genotype counts and analysis results

<table>
<thead>
<tr>
<th>Genotype</th>
<th>nl-TLE* (n = 245)</th>
<th>All patients (n = 752)</th>
<th>Symptomatic (n = 330)</th>
<th>Cryptogenic (n = 233)</th>
<th>IGE (n = 96)</th>
<th>Controls (n = 1089)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G/G</td>
<td>218 (0.99)</td>
<td>683 (0.99)</td>
<td>302 (0.99)</td>
<td>210</td>
<td>89 (0.99)</td>
<td>1062 (0.99)</td>
</tr>
<tr>
<td>G/A</td>
<td>2 (0.01)</td>
<td>6 (0.01)</td>
<td>4 (0.01)</td>
<td>0</td>
<td>1 (0.01)</td>
<td>8 (0.01)</td>
</tr>
<tr>
<td>A/A</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MAF†</td>
<td>0.002</td>
<td>0.004</td>
<td>0.006</td>
<td>0.006</td>
<td>0.006</td>
<td>0.004</td>
</tr>
<tr>
<td>Genotype P‡</td>
<td>1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Allele P‡</td>
<td>0.6823</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Genotype counts are those for successful typings only. *Non-lesional TLE patients; includes patients with HS; as used by Gambardella et al. (2003a); †minor allele frequency; †P values are uncorrected and generated using an exact probability test; NS = non-significant.
with refractory TLE and HS (see Table 5). In fact, we were unable to detect this variant in any of our refractory TLE patients. We observed the variant only twice, once in a patient with symptomatic epilepsy secondary to cerebrovascular disease and once in a control individual. As we could not estimate the genotypic relative risk of the variant in the original study (the variant was not observed in the control population), it was not possible to calculate power for this association.

### APOE

Briellmann et al. (2000) reported a significant association in Australian patients between the APOE ε4 allele and age of onset of refractory TLE. Results indicated that the presence of the ε4 allele correlated with a mean age of onset of 5 ± 5 years, whilst in the absence of the allele, mean age of onset was 15 ± 10 years (Mann–Whitney U test $P = 0.004$). Our results, from 181 refractory TLE patients, do not support this association (Mann–Whitney U test $P = 0.1808$). Mean age of seizure onset in patients carrying at least one copy of the ε4 allele (30 of 181 patients) was 13.7 ± 10 years. Patients not carrying a copy of the ε4 allele had a mean age of seizure onset of 16.7 ± 11 years.

We further examined the distribution of the APOE haplotypes across refractory TLE and other subgroups of epilepsy (see Table 6). We note a modest trend in haplotype distribution for the IGE ($P = 0.031$) group and ε4 allele frequency for refractory TLE. In both cases, the trend seems to be generated by an under-representation of the hypothesized risk allele (ε4). We feel that this association is likely to be the result of multiple testing and thus would view it as tentative at best. As such, confirmatory testing in an independent cohort is required.

### CHRNA4

Chou et al. (2003a) reported, in a cohort of Taiwanese children, association of a synonymous SNP variant Ser543Ser with childhood FS ($P = 0.001$; OR = 2.84 [CI 0.87–9.28]). Our results, from the 107 epilepsy patients with a history of FS in our cohort, failed to replicate this association. However, we do note that our study is estimated to have only 17% power of detection for this association.

Analysis across different subgroups of epilepsy failed to support a role for this variant in disease predisposition for the subtypes of epilepsy tested here (see Table 7).

### GABRG2

Chou et al. (2003b) reported a synonymous variant in the GABRG2 gene (rs211037) to associate with FS in a cohort of 104 Taiwanese children [$P = 0.017$, OR = 2.56 (CI 1.01–6.50)]. Our results in a cohort of 107 patients with a history of FS failed to support this association. Again, we note a lack of power, with our cohort estimated to have only 8% power of detection for this association.

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**Table 5** PRNP Asn171Ser genotype counts and analysis results

<table>
<thead>
<tr>
<th></th>
<th>Refractory HS†</th>
<th>All patients (n = 752)</th>
<th>Symptomatic (n = 330)</th>
<th>Cryptogenic (n = 233)</th>
<th>IGE (n = 96)</th>
<th>Controls (n = 384)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asn/Asn</td>
<td>109</td>
<td>718 (0.99)</td>
<td>320 (0.99)</td>
<td>219</td>
<td>93 (0.99)</td>
<td>360 (0.99)</td>
</tr>
<tr>
<td>Asn/Ser</td>
<td>0</td>
<td>1 (0.01)</td>
<td>1 (0.01)</td>
<td>0</td>
<td>0 (0.01)</td>
<td>1 (0.002)</td>
</tr>
<tr>
<td>Ser/Ser</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MAF‡</td>
<td>0</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>Genotype P†</td>
<td>1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Allele P†</td>
<td>1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Genotype counts are those for successful typings only. †refractory TLE with hippocampal sclerosis as used by Walz et al. (2003); ‡uncorrected significance value for 2×2 χ² distribution between the presence and absence of the ε4 allele; †frequency of the ε4 allele only; ‡frequency of the ε4 allele; §uncorrected significance value for 2×2 χ² distribution between the presence and absence of the ε4 allele; NS = non-significant.

**Table 6** APOE genotype counts and analysis results

<table>
<thead>
<tr>
<th></th>
<th>Refractory TLE‡</th>
<th>All patients (n = 752)</th>
<th>Symptomatic (n = 330)</th>
<th>Cryptogenic (n = 233)</th>
<th>IGE (n = 96)</th>
<th>Controls (n = 384)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ε3*</td>
<td>230 (0.80)</td>
<td>926 (0.79)</td>
<td>406 (0.78)</td>
<td>285 (0.8)</td>
<td>125 (0.83)</td>
<td>469 (0.74)</td>
</tr>
<tr>
<td>ε4*</td>
<td>36 (0.13)</td>
<td>156 (0.13)</td>
<td>72 (0.14)</td>
<td>46 (0.13)</td>
<td>14 (0.09)</td>
<td>108 (0.17)</td>
</tr>
<tr>
<td>ε2*</td>
<td>20 (0.07)</td>
<td>92 (0.08)</td>
<td>44 (0.08)</td>
<td>24 (0.07)</td>
<td>11 (0.07)</td>
<td>57 (0.09)</td>
</tr>
<tr>
<td>ε4 frequency‡</td>
<td>0.13</td>
<td>0.13</td>
<td>0.14</td>
<td>0.13</td>
<td>0.09</td>
<td>0.17</td>
</tr>
<tr>
<td>Haplotype P‡</td>
<td>0.205</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>ε4 P³</td>
<td>0.078</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

‡Symptomatic refractory TLE patients as used by Briellmann et al. (2000); †haplotype counts are those inferred from successful genotyping at both loci only; ‡frequency of the ε4 allele; †uncorrected significance value for haplotype distribution as detailed in Materials and methods; §uncorrected significance value for 2×2 χ² distribution between the presence and absence of the ε4 allele; NS = non-significant.
Analysis of the GABRG2 variant in the broader epilepsy cohort fails to support a role for this variant in the development of other forms of epilepsy tested here (see Table 8).

Discussion

This study has examined all positive genetic associations reported thus far on sporadic forms of TLE, and two associations on FS, in a single patient cohort of greater size than used in any of the original reports. We also tested whether the variants might predispose to the development of other forms of epilepsy.

Replication studies have previously been published on four of the seven variants examined here. In the case of APOE, data from other European populations published prior to the positive association (Blumcke et al., 1997; Gambardella et al., 1999) strongly suggest the reported effect on age of onset (Briellmann et al., 2000) to be a false-positive association. The IL1β−511 association with HS was similarly not replicable in European populations, although sample sizes were small (Heils et al., 2000; Buono et al., 2001). It is possible that this association is due to a population-specific effect in terms of functionality or patterning of linkage disequilibrium, but this would appear unlikely since there was also a failed replication in a Chinese population (Jin et al., 2003). Previous re-testing of the PDYN polymorphism in a population of European origin failed to replicate the original result clearly (Tilgen et al., 2003; Gambardella et al., 2003b).

Examination of the CHRNA4 association in an Australian population failed to replicate the original association (Mulley et al., 2004). However, both the Australian and our study lack power of detection for the CHRNA4 association. Similarly, we lacked power to replicate the GABRG2 association. Further research for both of these associations is required, particularly in Asian populations.

Our own results are consistent with these failed replication efforts. In particular, APOE results fail to show an effect in the same direction as the original report either in our study or in the other replication efforts. The results for PDYN are more ambiguous. We do not see association with ‘familial’ non-lesional TLE, as originally reported. However, our results suggest a possible role for PDYN as a risk factor for epilepsy. In particular, we note a trend of association with IGE, which is enhanced in ‘familial’ IGE patients (following the definition of familial as used in the original report). We encourage examination of this variant across large, well-phenotyped cohorts in order to examine this putative role further.

For GABBR1 G1465A and PRNP Asn171Ser, where there were no previous attempts at replication, our results suggest the original associations are likely to be false positives. In contrast to the original reports, we see no evidence for even a marginal effect of these variants in the European population tested here. We cannot, however, rule out the possibility that these variants may be disease-causing or in linkage disequilibrium with disease-causing variants that are specific to one or more population groups. We thus encourage replication attempts in the respective populations. However, in the case of the PRNP work in particular, a more likely explanation is that the original association is due, at least in part, to population stratification; the cases and controls were drawn from a
The results presented here suggest that most or all of the reported associations for TLE are not reproducible in the European population. When these results are considered with previous replication attempts, the fact remains that not one of these associations has been replicated accurately in a population of similar or other ancestry. On the weight of current evidence, it appears likely that most or all of the reported associations for TLE were false positives. Our data certainly do not rule out the presence of risk factors in any of the genes studied. However, given that our sample sizes were always at least as big as the previous reports and usually bigger, it seems fair to conclude that while we have not ruled out a role for these genes, the current association data considered together does not appear to have reliably identified any risk factors for TLE or FS. It would therefore appear that at present we know little about the genetic basis of sporadic TLE. Even worse than our ignorance, however, is the fact that false associations can lead to a waste of effort in trying to understand the biological bases of the involvement of genes which are not in fact involved in the disease, in this case sporadic TLE. How can this situation be improved?

In agreement with recent recommendations (Tan et al., 2004b), we believe methodological weaknesses in study design contribute to the variable results. All seven of the variants tested here have been selected using a sequence-based approach, focusing on functional, or putatively functional, variation. Whilst this approach has obvious advantages, future studies should seek to merge a sequence-based approach with methods that systematically represent variation in candidate genes. Map-based approaches such as the tagging SNP (single nucleotide polymorphism) method address this issue by taking advantage of linkage disequilibrium to allow examination of most variation by typing only a small number of tagging SNPs (Botstein and Risch, 2003; Neale and Sham, 2004). The publicly available HapMap resource, in conjunction with rapidly developing tagging SNP selection techniques (Goldstein et al., 2003), makes this approach feasible even with only modest laboratory resources. Researchers should check for population stratification, especially in ethnically mixed settings. In addition, for an association to be considered replicated, it should be replicated exactly. A follow-up study that shows association with another polymorphism in a gene or a somewhat different phenotype may be of interest, but should not be considered a replication. These sorts of ‘replications’ must be treated with particular care because of the possibility of exploring populations, polymorphisms and phenotypes until something is found in a given gene that can be called a replication. Neale and Sham (2004) have proposed a gene-based definition of replication that overcomes some of these issues.

These methodological issues deserve more serious attention than they have sometimes received, but we believe the central difficulty is more innate, and less easy to rectify.

The most fundamental problem with current efforts is the relatively low sample size in most studies, resulting in limited power to either detect, or to rule out definitively, association. Few investigators working on epilepsy genetics have large cohorts for any particular type of epilepsy, including our own current and previous efforts. It does not appear to us that in the near future individual investigators will be able to increase their own sample sizes to levels sufficient to carry out highly powered association studies within the various forms of epilepsy. For example, our own collection comes from one of the biggest referral centres in Europe and, although our own total phenotyped collection is sizeable, the numbers of patients with any given subtype of epilepsy is still small. Our previous study of refractory epilepsy, for example, included only 200 refractory patients, reporting a modest association with a putatively functional variant in the ABCB1 (MDRI) gene (Siddiqui et al., 2003). This association was not replicated in an exact replication attempt (Tan et al., 2004a), but was reported as ‘replicated’ for a similar but not identical definition of pharmacoresistance (Zimprich et al., 2004).

We therefore see two central lessons from our failure to replicate the TLE associations. First, the field must concentrate more seriously on efforts to determine which polymorphisms have real effects, as opposed to always racing to publish a new association. False positives are clearly exacerbated by the publication of multiple small studies and the practice of data exploration to identify subgroups that show associations. This fact must be recognized and addressed. Secondly, and most fundamentally, we believe it is critical for different research groups to increase substantially the size of their patient cohorts. Given that this will take time, we feel that in the short term, groups should combine their epilepsy samples, and attempt to replicate one another’s results in a population of similar ethnicity before publication. Although collaboration to such a level could be viewed as controversial from a scientific point of view, we feel that the trade-off here is justified. It is only with such steps that the epilepsy community, like other disease areas, will be able to arrive at a reasonable false to true discovery ratio in reported associations. The association between GABBR1 G1465A and non-lesional TLE was recently examined in a North American population of Caucasian descent. The association did not replicate (Ma et al., 2005).

Note added in proof
The association between GABBR1 G1465A and non-lesional TLE was recently examined in a North American population of Caucasian descent. The association did not replicate (Ma et al., 2005).

Acknowledgements
We wish to thank all the individuals who donated DNA and participated in this study. We would also like to thank Dr. Tim Spector for providing control DNA. G.L.C. is supported by the
Annals of Human Genetics Studentship in Human Population Genetics. D.B.G. is a Royal Society/Wolfson research Merit Award holder. C.D. is supported by the National Society for Epilepsy. J.M.L. is supported by a research contract with UCB Pharma. This study was funded by the Royal Society and by Medical Research Council grant G0400126.

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