Identification of susceptibility variants to benign childhood epilepsy with centro-temporal spikes (BECTS) in Chinese Han population

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Abbreviations: BECTS, benign childhood epilepsy with centro-temporal spikes; SNPs, single nucleotide polymorphisms; GWAS, genome-wide association analysis; MAF, minor allele frequency; PCS, principal components; SMR, summary-data-based Mendelian randomization; QC, quality control; H-W, Hardy-Weinberg; EEG, electroencephalogram; ADNFLE, autosomal dominant nocturnal frontal lobe epilepsy; nAChRs, nicotinic acetylcholine receptors

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1. Introduction

Benign Childhood Epilepsy with Centro-temporal Spikes (BECTS) is the most common form of idiopathic epilepsy in children, accounting for 8 to 23% of epilepsy in children less than 16 years of age [1, 2]. The typical age of onset is 3 to 14 years, and the condition typically resolves by the early teenage years. Whilst affected children are usually neurodevelopmentally normal, BECTS has been associated with varying degrees of neuropsychological damage, and can be associated with sociological and behavioral problems in adulthood [3]. Increased familiality of BECTS has raised the hypothesis that the condition has genetic underpinnings (reviewed in [4]). However, familial recurrence may occur because of shared environmental or genetic factors, and to date formal heritability studies in twins have not supported a major genetic contribution to susceptibility [5]. To better characterize the genetic architecture of BECTS, a large two-stage case-control GWAS was conducted in the Han Chinese population of 1800 BECTS cases and 7090 healthy ethnically matched controls. A significant common variant heritability of the disease was demonstrated, and several loci identified with suggestive association with BECTS risk.

2. Materials and methods

2.1. Subjects

Patients with BECTS were recruited from outpatient clinics at 30 hospitals in Beijing, Shanghai, and 26 other Chinese provincial capital cities. Diagnosis was determined according to the International League Against Epilepsy (Commission on Classification and Terminology of the International League Against Epilepsy, 1989)[6] definition for BECTS by pediatric neurologists. A patient was diagnosed with epilepsy if he/she had at least two unprovoked epileptic seizures. Healthy, unrelated adult blood donors, from the Beijing and Shanghai were included as controls. Only self-reported Han Chinese ethnicity cases and controls were recruited, and additional ethnicity checks were performed as described below. Written informed consent was obtained from all the parents or guardians, or directly from adult participants, and the study was approved by the relevant ethics committees of the hospitals and institutions involved.

2.2. Genotyping and quality control

DNA was isolated from venous blood samples of study participants. Genome-wide genotyping was performed with Illumina OmniZhongHua-8 version1.0 BeadChips (Stage 1) and Illumina
Human CoreExome-24 version 1.0 BeadChips (Stage 2) on an Illumina iScan array scanner at the Laboratory of Department of Rheumatology and Immunology, Changzheng Hospital (Shanghai, China). Genotype calls were made using the Illumina BeadStudio software; all SNPs with quality scores <0.15 were excluded. The cluster plots of the top-associated single nucleotide polymorphisms (SNPs) were inspected manually.

Genome-wide association analysis was performed using PLINK. We excluded individuals with call rates below 98% and heterozygosity rates >3 standard deviations from the mean. Duplicate subjects or probable relatives were identified by identify-by-descent (IBD) analysis (PL_HAT>0.1875) and excluded.

SNP markers were excluded if they had a minor allele frequency (MAF) below 0.01, a genotype distribution out of Hardy-Weinberg equilibrium (P<10^{-5}), or had a high rate of missing genotype calls (missing genotype call rate>0.02). Outliers on heterozygosity vs misssingness plots were also excluded (Supplementary Figure 1). Sex chromosomes were excluded from the analysis; only autosomal SNPs were analyzed. After these quality control steps, to detect and correct for population stratification we used the Shellfish software (http://www.stats.ox.ac.uk/~davison/software/shellfish/shellfish.php), having first excluded regions of long range LD. To confirm ethnicity, we performed a continental PCA on the Han Chinese dataset, merged with available data from 51 available populations genotyped by Illumina 650Y from the Human Genome Diversity Panel (HGDP-CEPH) [7]. Continental PCA indicated that all the samples came from subjects of Han Chinese descent (East Asian) (Supplementary Figure 2). Cases or controls lying more than 6 standard deviations from the population mean on principal components (PCs) 1–10 were then excluded (Supplementary Figure 3).

2.3. Data analysis

Imputation was performed separately according to SNP microarray used, using the 1000 Genome reference through the Sanger Imputation Service (imputation.sanger.ac.uk/). BECTS associations of all markers with a MAF of 0.01 and imputation quality INFO>0.8 (6,563,936 SNPs in the OmniZhonghua set and 5,742,369 SNPs in the CoreExome set) were analyzed by using logistic regression (PLINK) with dosage output, adding the top PCs as covariates (4 PCs for the CoreExome set) were analyzed by using logistic regression (PLINK) (6,563,936 SNPs in the OmniZhonghua set and 5,742,369 SNPs in the CoreExome set). The strongest associations observed were multiple SNPs in regions on chromosome 3, 15 and 10, located in or nearby the genes KALRN, CHRNA4, and CHRNA5. The second associated signal rs1948 was encompassed by several genes that encode nicotinic cholinergic receptor subunits (CHRNA4/CHRNA5/CHRN2/CHRNA6/Fig. 1B). The third locus on chromosome 10p12.1 (peak SNP rs139905806, Supplementary Figure 6A) is a region which has been previously associated with lamotrigine-induced skin rash in a previous GWAS study in Korean patients with epilepsy [13].

3. Results

3.1. Genome-wide association analysis

The first stage of GWAS included individuals of 997 BECTS cases and 3115 healthy controls. 972 cases and 2916 controls genotyped using the Illumina OmniZhonghua SNP microarray for 805,150 SNPs were retained after a series of stringent quality control (QC) procedures. Minimal residual genomic inflation was observed (genomic inflation factor after correcting top 4 PCs=1.033) (Supplementary Figure 5A). The second stage GWAS was performed with an independent cohort of 803 BECTS cases and 3975 controls. 777 cases and 3768 controls were genotyped using the Illumina Core-Exome SNP microarray for 257,007 SNPs and left/removed for association analysis after QC procedures. Again, minimal residual genomic inflation was observed (genomic inflation factor=1.034) (Supplementary Figure 5B) after correcting for the top 3 principal components (PC). SNP imputation was performed on each stage (imputed lambda of OmniZhonghua and CoreExome cohorts is 1.042 and 1.039 respectively) and imputed genotype data was combined by meta-analysis (final analysis 5,352,724 SNPs in 1738 cases and 6592 controls). Whilst no locus in this analysis achieved genome-wide significance (P<5 x 10^{-8}), 12 independent loci reached suggestive significance (5 x 10^{-6}<P<10^{-8}, Table 1, Supplementary Figure 6), the direction of association being consistent between datasets for each locus (P=2.44 x 10^{-5}).

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3.2. Summary-data-based mendelian randomization analysis

The first stage of GWAS included individuals of 997 BECTS cases and 3115 healthy controls. 972 cases and 2916 controls genotyped using the Illumina OmniZhonghua SNP microarray for 805,150 SNPs were retained after a series of stringent quality control (QC) procedures. Minimal residual genomic inflation was observed (genomic inflation factor after correcting top 4 PCs=1.033) (Supplementary Figure 5A). The second stage GWAS was performed with an independent cohort of 803 BECTS cases and 3975 controls. 777 cases and 3768 controls were genotyped using the Illumina Core-Exome SNP microarray for 257,007 SNPs and left/removed for association analysis after QC procedures. Again, minimal residual genomic inflation was observed (genomic inflation factor=1.034) (Supplementary Figure 5B) after correcting for the top 3 principal components (PC). SNP imputation was performed on each stage (imputed lambda of OmniZhonghua and CoreExome cohorts is 1.042 and 1.039 respectively) and imputed genotype data was combined by meta-analysis (final analysis 5,352,724 SNPs in 1738 cases and 6592 controls). Whilst no locus in this analysis achieved genome-wide significance (P<5 x 10^{-8}), 12 independent loci reached suggestive significance (5 x 10^{-6}<P<10^{-8}, Table 1, Supplementary Figure 6), the direction of association being consistent between datasets for each locus (P=2.44 x 10^{-5}).

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3.2. Summary-data-based mendelian randomization analysis

eQTL analysis of the probes of the 12 most strongly-associated loci demonstrated that 9 were associated with transcriptional levels of the most proximal gene (P_{qqt}<0.05). To investigate whether the observed genetic associations operated through these transcriptional effects, a Summary-data-based Mendelian Randomization (SMR) analysis was performed, using brain tissue gene-expression data. Significant association was observed at CHRNA5 locus, tagged by rs76712448 (P_{smr}=0.028, Fig. 2). A HEIDI test supported this SNP being directly associated with BECTS and CHRNA5 expression. The most strongly associated GWAS hit, rs1948, shows significant association at the CHRNA5 locus in the SMR analysis (P_{smr}=7.9 x 10^{-5}). The association suggests that more than one SNP on the haplotype tagged by rs1948 is associated with BECTS susceptibility through effects on central nervous system (CNS) CHRNA5 expression.
Table 1. Significant SNPs achieving suggestive association (10⁻⁵ < \textit{P} < 10⁻³) in the meta-analysis of imputed GWAS data. Findings are given for the most strongly associated SNP at each locus.

| SNP    | Chr | Gene       | SNP frequency | Allele 1 | Allele 2 | Frequency | Direction | Odds Ratio | P-value |
|--------|-----|------------|---------------|----------|----------|-----------|-----------|------------|---------|
| rs73141536 | 3   | CADM2      | T/G           | 0.19     | 0.19     | 1.26      | ++        | 1.13       | 0.075   |
| rs1561578   | 3   | CADM2      | A/C           | 0.11     | 0.11     | 0.71      | +         | 0.74       | 0.82    |
| rs2175709   | 12  | TMEM5      | A/T           | 0.35     | 0.35     | 0.82      | +         | 0.89       | 0.19    |
| rs60419110  | 18  | C14orf144  | T/C           | 0.017    | 0.017    | 2.51      | +         | 1.36       | 0.19    |

Chr = chromosome, BP = base pair position, hg18. Gene = gene closest to the most strongly associated SNP. Left, Right gene = left- and right-most genes in the strongest association window of the SNP. P-meta = \textit{P}-value from meta-analysis. Direction of association = direction of odds ratio in the individual dataset.

* Heterogeneity analysis showed that the associations of this SNP in each set were significantly different (Heterogeneity \textit{P} value < 0.05). The separate LocusZoom plots in each dataset of this locus were attached in the supplementary file.
Fig. 1. A) The top SNP, rs1561578, is shown in purple, and the remaining SNPs are colored according to their linkage disequilibrium r^2 value with rs1561578. Genotyped SNPs in OmniZhonghua set are shown as dots and imputed SNPs shown as squares. B) The top SNP, rs1948, is shown in purple, and the remaining SNPs are colored according to their linkage disequilibrium r^2 value with rs1948. Genotyped SNPs in OmniZhonghua set are shown as dots and imputed SNPs shown as squares.
these genes (MAF > 1%) do not have major influences on the risk of BECTS.

A combination of suggestive genetic association and strong transcriptomic effect supports the involvement of the gene CHRNA5, encoding the cholinergic receptor nicotinic alpha 5 subunit, in BECTS aetiopathogenesis. While the most strongly associated SNP at this locus (rs1948 at chromosome 15q24) is located within the gene CHRN4B, SMR analysis suggests that the associated gene at this locus is the neighbouring gene CHRNA5. The rs1948 is strongly associated with expression of the t3603436 transcript of the CHRNA5 genes \( P_{\text{eQTL}}=2.10 \times 10^{-12}, P_{\text{smR}}=7.9 \times 10^{-9} \) which encodes a subunit of cholinergic receptors. Acetylcholine is an important excitatory CNS neurotransmitter. Association has previously been reported between multiple SNPs in the CHRNA5-CHRNS3-CHRNB4 gene cluster and cigarette smoking, nicotine dependence, and smoking associated lung diseases [24-26], as well as with cognitive measures [27,28]. There is suggestive evidence that smoking increases the risk of epilepsy overall [29], but whether smoking influences the risk of BECTS specifically is unknown. It was reported that BECTS risk allele rs1948-A is also associated with higher Fagerström Test for Nicotine Dependence (FTND) score in the Chinese Han population [30,31]. Although it is unlikely the patients themselves were smoking at their age of onset, they could have been exposed to secondhand smoke. If the offspring is a carrier of the risk allele, then at least one of the parents must be a carrier too, which means they are more likely to develop tobacco addiction or become a heavier smoker. Studies have shown that prenatal maternal cigarette smoking is associated with febrile seizure [32,33]. It is possible that CHRNA5 influences the onset of BECTS by another mechanism independently of smoking (including maternal or paternal perinatal, or postnatal passive, smoking). The GSRR

**Fig. 2.** Prioritizing genes at a GWAS locus using SMR analysis. The findings for chromosome 15p24 locus for BECTS are displayed. In the top plot, gray dots represent the P values for SNPs from the GWAS meta-analysis for BECTS, and diamonds represent the P values for probes from the SMR test. The middle plot presents the eQTL P values of SNPs from the BRAINEAC study for the transcripts tagging CHRNA5 and CHRNA3. The top and middle plots include all the SNPs available in the region in the GWAS and eQTL summary data, respectively, rather than only the SNPs common to both data sets.

**Fig. 3.** GSMR analysis to test for the effect of maternal smoking around birth on BECTS. The plot shows the relationship between the estimated effects of SNPs \( z \) associated with maternal smoking around birth \( x \) on BECTS \( y \) and the estimated effect of \( z \) on \( x \) \( x \) axis, \( bzx \). The slope of the dashed lines represent the ratio between the \( bzy \) and \( bzx \) as the estimate of the mediation effect of \( x \) on \( y \) \( bzy = bzx / bzx \). Error bars represent the standard errors.
analysis reported here though demonstrates using data from the UK Biobank that maternal smoking around birth is associated with 3.9x increased risk of BECTS. Unfortunately data is not available in UK Biobank about other forms of perinatal or antenatal smoking exposure, and therefore we are unable for example to determine the effect of paternal or other sources of passive smoking on BECTS risk.

Linkage of chromosome 15 (15q24), at which CHRNA3, CHRNA5, and CHRNB4 are encoded, has also been reported in autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) [34], but this finding has not been confirmed in other families [35,36], and the mutations identified to date in CHRN genes in ADNFLE have been in other receptor subunits (reviewed in [37]). Of interest, as with ADNFLE, in BECT seizures occur more commonly at night and during sleep, suggesting overlapping pathogenesis. Studies in rodents have described the anatomical localization and function of the nicotinic acetylcholine receptors (nAChRs) formed by the subunits encoded by this gene cluster. Animal experiments also have shown that microinjection of acetylcholine into the brain can cause seizures in animals, suggesting that acetylcholine, as a neurotransmitter, may play an important role in the development of epilepsy [38,39]. While not definitive, this data supports a role for this locus and a possible link between nicotinic/cholinergic neurostimulation and BECTS, raising the hypothesis that anticholinergic therapies may be effective in BECTS. Further research will be required to test this.

KALRN, associated with Heschl’s gyrus (temporal lobe) morphology in GWAS, induces various signaling mechanisms that regulate neuronal shape, growth, and plasticity, through their effects on the actin cytoskeleton [40]. This locus has not previously been associated with epilepsy but its known association with brain morphology is consistent with a role in a CNS disease like this. Another eight loci achieved suggestive association, and further research will be required such as expanding the GWAS dataset, and/or functional genomic analyses, to determine if they have a role in BECTS.

In conclusion, this study shows that significant common variant heritability contributes to the development of BECTS, and provides evidence that the cholinergic receptor subunit gene CHRNA5, is involved in its pathogenesis. This raises the hypothesis that anticholinergic therapies may be effective in BECTS; further research will be required to test this. Importantly, the study demonstrates through Mendelian randomization approaches that maternal smoking around birth is associated with increased risk of BECTS, thereby identifying to our knowledge the first environmental risk factor for the disease.
Author contributions

Huji Xu, Li-Ping Zou, Perry Bartlett, David Reutens and Matthew A Brown made substantial contributions to conception and design of the study. Geng Wang, Zhiyui Li, Paul Leo, Gabriel Cuellart Partida, Mischa Lundberg and Matthew A Brown contributed to statistical analysis and interpretation of data. Huji Xu, Li-Ping Zou, Xiuyu Shi, Zhisheng Liu, Gefe Wu, Hongmin Zhu, Yuqin Zhang, Dong Li, Li Gao, Liu Yang, Wei Wang, Jianxiang Liao, Jiwen Wang, Shuizhen Zhou, Hua Wang, Xiaojing Li, Jingyun Gao, Li Zhang, Xiaomei Shu, Dan Li, Yan Li, Chunhong Chen, and Xiyou Zhang were responsible for case diagnosis, subject recruitment and the collection of blood samples. David Reutens are responsible for reviewing the diagnosis of the patients. Geng Wang, Matthew Brown, and Huji Xu were primarily responsible for drafting the manuscript and revising it. All authors approved the final version of the manuscript.

Declaration of Competing Interest

The authors have no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2020.102840.

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