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**Failure to replicate** No evidence for a *BRD2* promoter hypermethylation in blood leucocytes of Europeans with juvenile myoclonic epilepsy

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## Summary

Juvenile myoclonic epilepsy (JME) is a common syndrome of genetic generalized epilepsies (GGEs). Linkage and association studies suggest that the gene encoding the bromodomain-containing protein 2 (*BRD2*) may increase risk of JME. The present methylation and association study followed up a recent report highlighting that the *BRD2* promoter CpG island (CpG76) is differentially hypermethylated in lymphoblastoid cells from Caucasian patients with JME compared to patients with other GGE subtypes and unaffected relatives. In contrast, we found a uniform low average percentage of methylation (< 4.5%) for 13 CpG76-CpGs in whole blood cells from 782 unrelated European Caucasians, including 116 JME patients, 196 patients with genetic absence epilepsies and 470 control subjects. We also failed to confirm an allelic association of the *BRD2* promoter SNP rs3918149 with JME (Armitage Trend Test;  $P = 0.98$ ), and we did not detect a substantial impact of SNP rs3918149 on CpG76 methylation in either 116 JME patients (meQTL- $P = 0.29$ ) or 470 German control subjects (meQTL- $P = 0.55$ ). Our results do not support the previous observation that a high DNA methylation level of the *BRD2* promoter CpG76 island is a prevalent epigenetic motif associated with JME in Caucasians.

## KEYWORDS

Juvenile myoclonic epilepsy, genetic generalized epilepsy, *BRD2*, DNA methylation, association analysis

## 1 | INTRODUCTION

Juvenile myoclonic epilepsy (JME) is a common syndrome of genetic generalized epilepsies (GGEs) accounting for 5% to 10% of all epilepsies.<sup>1</sup> Genetic factors play a predominant role in the etiology of JME but the vast majority of the underlying susceptibility genes remains elusive, probably due to an extensive genetic heterogeneity and complex inheritance.<sup>2-4</sup>

Linkage to the chromosomal region 6p21.3 and association studies of positional candidate genes suggest that the gene encoding the bromodomain-containing protein 2 (gene symbol: *BRD2*) may increase risk of JME in Caucasians.<sup>4,5</sup> The reported allelic association of JME with the *BRD2* promoter SNP rs3918149 and the lack of potentially causative coding mutations

suggested that susceptibility of JME could result from a regulatory effect of the promoter SNP on *BRD2* expression.<sup>5</sup> Recently, Pathak and co-workers reported that hypermethylation of a CpG island (CpG76) within the *BRD2* promoter was associated with JME in Caucasians.<sup>6</sup> *BRD2* CpG76 hypermethylation in B-lymphoblastoid cells was found in 12 out of 23 Caucasian JME patients, predominantly in 11 out of 14 JME patients retrieved from families with positive linkage evidence to the *BRD2* locus. In contrast, the *BRD2* CpG76 methylation state was low in 23 unaffected family members and 16 patients with other (non-JME) GGE syndromes. With regard to the relatively small sample size of the original study and the potential clinical relevance of this prevalent epigenetic biomarker for JME, our present methylation and association analyses explored the validity of a JME-associated DNA hypermethylation of the *BRD2* promoter CpG76 island in Caucasians.

## 2 | MATERIALS AND METHODS

### 2.1 | Study participants

Our multicenter study cohort comprised 782 unrelated European Caucasians (411 females, 371 males) (Suppl. Table S1, Suppl. Figures S1A&B), including 116 JME patients (72 females, 44 males), 196 patients with genetic absence epilepsies (GAEs; 120 females, 76 males; 142 childhood and 53 juvenile absence epilepsies, one unspecified genetic absence epilepsy) and 470 German population controls (219 females, 251 males).<sup>7</sup> Twenty-one JME patients were derived from GGE-multiplex families.<sup>8</sup> Diagnostic classification of GGE syndromes was carried out according to the ILAE guidelines, as described elsewhere.<sup>7</sup> The German control subjects were recruited from the Heinz Nixdorf Recall Study (HNR) cohort. The study protocol was approved by the local institutional review boards and all study participants gave informed consent.

### 2.2 | Methylation analysis and data processing

Individual genomic DNA samples were extracted from whole blood cells. Methylation profiling of sodium bisulfite converted DNA (500 ng) was carried out using the Infinium HumanMethylation450 BeadChip (HM450k array; Illumina, San Diego, CA, USA). The signal intensities were extracted using GenomeStudio version 2011.1 based on the HumanMethylation450 manifest version 1. The R-package *meffil 1.0.0* was applied for quality control, functional normalization and surrogate variable analysis (SVA).<sup>9</sup> The CpG methylation

level was assessed as methylation percentage ( $\beta$ -values). After stringent quality filtering,<sup>9,10</sup> 13 CpGs covering the *BRD2* CpG76 island (chr6:32,935,896-32,936,792, hg19) were chosen for methylation analysis (Suppl. Table S2). Two CpG76-CpGs (cg16801540, cg07223713) overlap with the original set of 10 CpG76-CpGs (Dr. Pathak, personal communication).<sup>6</sup> The average CpG76 methylation percentage was determined to infer the methylation state of the *BRD2* promoter CpG76 island.<sup>11</sup> Individual genotypes of the *BRD2* promoter SNP rs3918149 (chr6:32,936,373G>A, hg19) were retrieved from the imputed array-based SNP dataset of the study participants generated for our previous GWAS of common GGE syndromes and genome-wide methylation quantitative trait loci (meQTL) analyses of hippocampal biopsies, respectively.<sup>7,12</sup> The individual sample identity of the SNP and CpG methylation data sets was confirmed based on the genotype concordance of control SNPs assessed by the HM450k and SNP arrays.<sup>7,12</sup>

### 2.3 | Statistical methods

meQTL analysis and differential methylation analysis was carried out by SVA implemented in *meffil*.<sup>9</sup> The covariates, gender and age at sampling, were included in the regression model. The Armitage Trend Test was applied to compare the SNP allele frequencies in case-control cohorts.

## 3 | RESULTS

### 3.1 | ~~Replication analysis~~ Exploration of an JME-associated *BRD2* promoter hypermethylation

Our methylation analysis revealed a low individual percentage of methylation for each single CpG76-CpG among all 782 European study participants, including 116 JME patients, 196 patients with genetic absence epilepsies and 470 German population controls (Figure 1, Suppl. Table S1). The individual CpG methylation states differed marginally across the investigated 13 CpG76-CpGs (Suppl. Table 1, Suppl. Figure S2). Overall, the average methylation percentages of 13 CpG76-CpGs did not exceed 4.5% in the entire study cohort and displayed a unimodal distribution with little interindividual variation (Figure 1). Consistently, the two overlapping CpG76-CpGs displayed uniform individual average methylation percentages below 7% among the 782 study participants (Suppl. Figure S4, Suppl. Table 1).

### 3.2 | Association and meQTL analyses of the *BRD2* SNP rs3918149 for JME

Our association analysis of JME with the *BRD2* promoter SNP rs3918149 obtained similar allele frequencies in 116 European JME patients ( $f_{(A)} = 0.125$ , genotype counts: 1 A/A, 27 A/G, 88 G/G) and 470 German population controls ( $f_{(A)} = 0.126$ , genotype counts: 9 A/A, 100 A/G, 361 G/G; HWE- $P = 0.50$ ; Armitage Trend Test;  $P = 0.983$ , two-sided). Likewise, we found no evidence for an association of the SNP rs3918149 with JME in our previous GWAS including 817 European JME patients ( $f_{(A)} = 0.123$ ; 11 A/A, 177 A/G, 623 G/G) and 2,419 German control subjects ( $f_{(A)} = 0.113$ ; 33 A/A, 478 A/G, 1,892 G/G; HWE- $P = 0.65$ ; Armitage Trend Test;  $P = 0.302$ , two-sided; Suppl. Figure S3).<sup>7</sup> Furthermore, our meQTL analysis provides no evidence for a regulatory *cis*-acting effect of the SNP rs3918149 on the average percentage of CpG76 methylation in 116 JME patients (*cis*-meQTL- $P = 0.285$ ) as well as 470 German control subjects (*cis*-meQTL- $P = 0.547$ ) (Figure 2). Notably, our previous meQTL analysis of 110 hippocampus biopsies revealed no *cis*-meQTL of SNP rs3918149 and CpG76-CpG methylation.<sup>12</sup> Our SVA did not detect significant differences in the average methylation percentage of the 13 *BRD2* CpG76-CpGs from 116 JME patients compared to 196 GAE patients ( $P = 0.637$ ).

## 4 | DISCUSSION

In contrast to the previous report,<sup>6</sup> we did not detect *BRD2* CpG76 hypermethylation in whole blood cells from 116 unrelated European Caucasian JME patients, as well as 196 European GAE patients and 470 German control subjects. We found a unimodal distribution of average CpG76 methylation percentage ranging between 2.4-4.5% with little interindividual variation. These conflicting results could reflect three critical factors: i) genetic heterogeneity, ii) cell-type specific differential methylation, and iii) technical variation of the methylation assays.

Despite of the predominant genetic etiology of JME, extensive genetic heterogeneity and complex inheritance hinder the molecular genetic dissection of the underlying genetic risk factors.<sup>2,3</sup> Considering that we have applied the same diagnostic criteria for JME and recruitment scheme,<sup>6,7</sup> the genetic architecture underlying JME should be similar in the original and the

present study. Given the high prevalence (> 10%) of *BRD2* CpG76 hypermethylation in Caucasian JME patients reported in the original study,<sup>6</sup> it is unlikely ( $P < 10^{-5}$ ) to miss this common epigenetic motif in 116 unrelated European Caucasian JME patients.

Technical variation may be a source for spurious methylation signals. Using accurate experimental procedures, it is unlikely that the technology itself account for the controversial/contradictory methylation results. Likewise, the different only partially overlapping sets of CpG76-CpGs investigated in the original and the present study should not play a critical role, given a uniform methylation pattern of CpGs within promoter CpG islands.<sup>11</sup> Remarkably/Notably, Pathak et al. reported a differential bimodal distribution of the average CpG76 methylation percentages among JME patients (hypermethylated state: 80-90%, hypomethylated state: 5-20%).<sup>6</sup> These distinct CpG76 methylation patterns indicate uniform methylation states across the individual CpG76-CpGs. Consequently, the two overlapping CpG76-CpGs should have detected *BRD2* promoter CpG76 hypermethylation in the present study.

Cell type-specific methylation might explain differential methylation of *BRD2* CpG76 in B-lymphoblastoid cells and whole blood leucocytes.<sup>13,14</sup> B-lymphoblastoid cell lines (LCLs) represent Epstein-Barr virus-immortalized CD19+ B-lymphocytes. B-lymphocytes account for a small proportion of 4-10% of whole blood leucocytes. Therefore, B-lymphocyte-specific hypermethylation may be undetectable in whole blood leucocytes. However, a previous study investigating methylation profiles of flow-sorted blood cell types revealed uniformly low methylation levels of the CpG76-CpGs with marginal variation across the major blood cell types (Suppl. Table S2).<sup>15</sup> With regard to the clonality of LCLs<sup>16</sup> and the widespread random clonal-related monoallelic expression/methylation of human autosomal genes,<sup>14,17</sup> differential methylation/expression patterns obtained from virtually monoclonal LCLs should be interpreted with caution.<sup>16</sup> Moreover, multiple random and directed changes in DNA methylation during cell culturing may lead to aberrant methylation signals.<sup>13,14,18</sup>

Given that the association of JME with the *BRD2* promoter SNP rs3918149 and the CpG76 hypermethylation state have been found in the same JME families,<sup>4-6</sup> we examined the hypothesis that the JME-associated SNP rs3918149 exerts a regulatory effect on CpG76

methylation. Unlike the original association, we fail to confirm an allelic association of SNP rs3918149 with JME. Likewise, our methylation analysis does not provide evidence that the SNP rs3918149 affects CpG76 methylation in whole blood leucocytes and in human hippocampus biopsies.<sup>12</sup> Taken together, our present results do not support previous studies<sup>4-6</sup> highlighting the *BRD2* gene as susceptibility gene for JME.

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## DISCLOSURE OF CONFLICT OF INTEREST

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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### **REFERENCES**

1. Camfield CS, Striano P, Camfield PR. Epidemiology of juvenile myoclonic epilepsy. *Epilepsy Behav.* 2013;28(suppl 1):S15-7.
2. Delgado-Escueta AV, Koeleman BP, Bailey JN, et al. The quest for juvenile myoclonic epilepsy genes. *Epilepsy Behav.* 2013;28(suppl 1):S52-7.
3. Mullen SA, Berkovic SF. Genetic generalized epilepsies. *Epilepsia* 2018;59:1148-53.

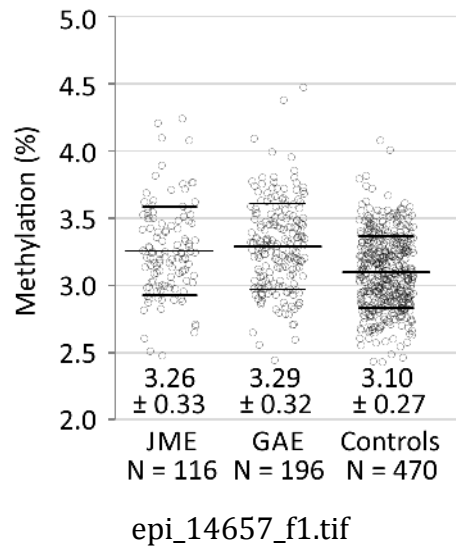
4. Greenberg DA, Durner M, Keddache M, et al. Reproducibility and complications in gene searches: linkage on chromosome 6, heterogeneity, association, and maternal inheritance in juvenile myoclonic epilepsy. *Am J Hum Genet.* 2000;66:508-16.
5. Pal DK, Evgrafov OV, Tabares P, et al. BRD2 (RING3) is a probable major susceptibility gene for common juvenile myoclonic epilepsy. *Am J Hum Genet.* 2003;73:261-70.
6. Pathak S, Miller J, Morris EC, et al. DNA methylation of the BRD2 promoter is associated with juvenile myoclonic epilepsy in Caucasians. *Epilepsia* 2018;59:1011-9.
7. EPICURE Consortium, EMINET Consortium, Steffens M, et al. Genome-wide association analysis of genetic generalized epilepsies implicates susceptibility loci at 1q43, 2p16.1, 2q22.3 and 17q21.32. *Hum Mol Genet.* 2012;21:5359-72.
8. EPICURE Consortium, Leu C, de Kovel CG, et al. Genome-wide linkage meta-analysis identifies susceptibility loci at 2q34 and 13q31.3 for genetic generalized epilepsies. *Epilepsia* 2012;53:308-18.
9. Min JL, Hemani G, Davey Smith G, et al. Meffil: efficient normalization and analysis of very large DNA methylation datasets. *Bioinformatics* 2018;34:3983-9.
10. Zhou W, Laird PW, Shen H. Comprehensive characterization, annotation and innovative use of Infinium DNA methylation BeadChip probes. *Nucleic Acids Res.* 2017;45:e22.
11. Long HK, King HW, Patient RK, et al. Protection of CpG islands from DNA methylation is DNA-encoded and evolutionarily conserved. *Nucleic Acids Res.* 2016;44:6693-706.
12. Schulz H, Ruppert AK, Herms S, et al. Genome-wide mapping of genetic determinants influencing DNA methylation and gene expression in human hippocampus. *Nat Commun.* 2017;8:1511.
13. Thompson TM, Sharfi D, Lee M, et al. Comparison of whole-genome DNA methylation patterns in whole blood, saliva, and lymphoblastoid cell lines. *Behav Genet.* 2013;43:168-76.

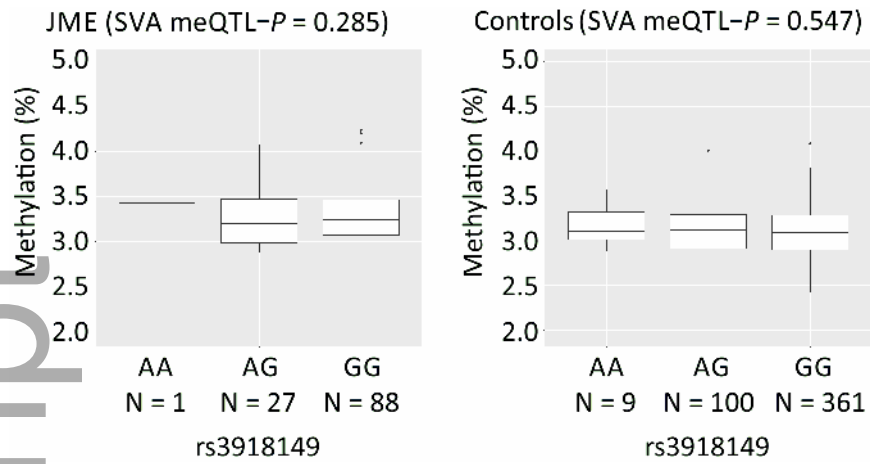
14. Onuchic V, Lurie E, Carrero I, et al. Allele-specific epigenome maps reveal sequence-dependent stochastic switching at regulatory loci. *Science* 2018;361.
15. Jaffe AE, Irizarry RA. Accounting for cellular heterogeneity is critical in epigenome-wide association studies. *Genome Biol.* 2014;15:R31.
16. Plagnol V, Uz E, Wallace C, et al. Extreme clonality in lymphoblastoid cell lines with implications for allele specific expression analyses. *PLoS ONE* 2008;3:e2966.
17. Gimelbrant A, Hutchinson JN, Thompson BR, Chess A. Widespread monoallelic expression on human autosomes. *Science* 2007;318:1136-40.
18. Saferali A, Grundberg E, Berlivet S, et al. Cell culture-induced aberrant methylation of the imprinted IG DMR in human lymphoblastoid cell lines. *Epigenetics* 2010;5:50-60.

### Figure legends

**FIGURE 1** Average percentage of methylation of 13 *BRD2* promoter CpG76-CpGs in whole blood cells from 116 European Caucasian patients with juvenile myoclonic epilepsy (JME), 196 European patients with genetic absence epilepsy (GAE) and 470 German population controls. The mean methylation percentage  $\pm$  standard deviation is given for each study group.

**FIGURE 2** *Cis*-meQTL results of SNP rs3918149 genotypes versus average percentage of methylation of 13 *BRD2* CpG76-CpGs in whole blood cells from a) 116 European JME patients, and b) 470 German control subjects.





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