LETTER TO THE EDITOR

USP25 in genetic generalized epilepsy: a gene under scrutiny

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Fan et al. [1] recently reported the potential role of *USP25* in Genetic Generalized Epilepsy (GGE). They conducted trio-based whole-exome sequencing on a cohort of 319 unrelated families of East Asian ancestry and identified five heterozygous *USP25* variants. These include one nonsense variant, an in-frame deletion, and three missense variants (**Supplementary Table 1**).[1]

We have concerns regarding the criteria on which the rare variant aggregated association test is based. Their methodology only screened the patients for disease-causing variants categorized as damaging variants with a minor allele frequency (AF) < 0.005 in the Genome Aggregation Database (gnomAD) (v2.1) (**Supplementary Table 1**).[1,2] The authors used five different frameworks, including epilepsy-associated gene, *de novo*, autosomal recessive, X-linked, and co-segregation analysis.[1] The authors performed a rare variant association analysis comparing the aggregated frequency of *USP25* qualifying variants in their cohort to the aggregated frequency of those same five variants in gnomAD and healthy Chinese volunteers.[1] However, this study did not apply the same steps to identify the qualifying variants in (i) the East Asian population in gnomAD,[2] (ii) all populations in gnomAD,[2] and (iii) the 296 healthy Chinese volunteers.[1] This could introduce bias and may not accurately represent the true association of rare variants related

to *USP25*. For example, by querying the gnomAD v2.1 database,[2] we found 27 damaging and highly conserved variants in *USP25* (**Supplementary Table 2**). These 27 variants are present in the East Asian population with AF < 0.005 (**Supplementary Table 2**).[2] Thus, we suspect that deleterious variants may be enriched in East Asians.

Further, utilizing public control databases in rare variant association analyses instead of a matched control group may compare patients from a homogeneous population to a more heterogeneous population in terms of ancestry/population stratification, as well as technical factors related to genetic data processing, or sequencing, and thereby introduce bias.[3] In this respect, methods such as Rare Variant Exome CALIBration using External Repositories (RV-EXCALIBER) have been introduced to leverage the extensive sample size of gnomAD as a control group in rare variant association analyses, accounting for the population heterogeneity and technical obstacles.[3]

In a burden test involving 3,064 GGE cases and 3,962 ancestry-matched controls, we analyzed the effect of damaging missense ultra-rare variants, as defined in the previous study done by Koko et al. (**Supplementary Table 1**),[4] while adjusting for covariates such as sex, top ten principal components, exome-wide variant count, and exome-wide singleton count. Our findings showed no significant difference in the number of *USP25*-related variants between cases and controls in individuals of European descent (4/3,064 GGE cases vs. 0/3,962 controls, OR: 8.69×10^4, $P = 0.94$). This aligns with the recent hypothesis-free analyses of larger cohorts within the Epi25 Collaborative, incorporating 5,499 GGEs and 33,444 controls, which found no exome-wide significant gene associations.[5] Only a single *USP25* truncating variant was found in cases versus 15 in controls (OR: 0.55, $P = 0.45$).[5]

GGE is a complex neurological disorder influenced by the cumulative effect of variants in multiple genes,[5] with rare families segregating rare variants with large effects.[6] Moreover, *USP25* is not under strong selection for loss-of-function (LoF) variants according to gnomAD v2.1 with LoF observed/expected upper bound fraction (LOEUF) score of 0.33 compared to

other known epilepsy-related genes such as *SCN1A* (LOEUF: 0.07).[2] Additionally, there are only two reported *de novo* variants of *USP25* in the Deciphering Developmental Disorders (DDD) study, and it has not been classified among the genes associated with developmental disorders.[7] Therefore, we believe that there is currently not sufficient evidence to categorize *USP25* as a risk factor for GGE. Consequently, stating that "*USP25* as a novel causative gene for generalized epilepsy", as mentioned by the authors in the introduction,[1] may mislead or oversimplify.

Furthermore, *USP25* was curated for gene validity according to ClinGen criteria, which assess gene-disease associations based on standardized criteria,[8] which have been adapted to the epilepsy field.[9] The gene-disease relationship of *USP25* with epilepsy was found to be limited, given the population frequency of the variants,[1,2] and because the functional impact provided by the authors did not meet the threshold for supporting gene-level evidence.[1,9] In particular, the authors assessed *USP25*$^{-/-}$ mice,[1] which do not reflect the human heterozygous condition. The knockout mice did not develop spontaneous seizures,[1] which makes them unsuitable models with regard to epilepsy. In addition, *USP25* has a probability of loss-of-function intolerance (pLI) of 0,[10] making it unlikely that loss-of-function variants are a cause of human disease.

In summary, we have concerns about the validity of *USP25* as a new epilepsy gene, since, in our opinion – as the data are presented and as we have outlined above – the authors' conclusions are not based on best practices in the field. Accordingly, the authors' conclusion should be further validated in a larger cohort, with careful consideration of study design for the analysis of rare variants and their functional validation.

Data availability

The whole-exome sequencing data used for the rare variant association test were obtained from the Epi25 dataset during the first two years of recruitment (http://epi-25.org/). Detailed information on controlled-access exome data is included in the previous publication.[4] In addition, variant-level and gene-based summary statistics, along with data sharing details

regarding the recent exome-wide burden analysis of individuals with epilepsy conducted by the Epi25 Collaborative, are provided in the latest publication.[5]

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

Appendix 1

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