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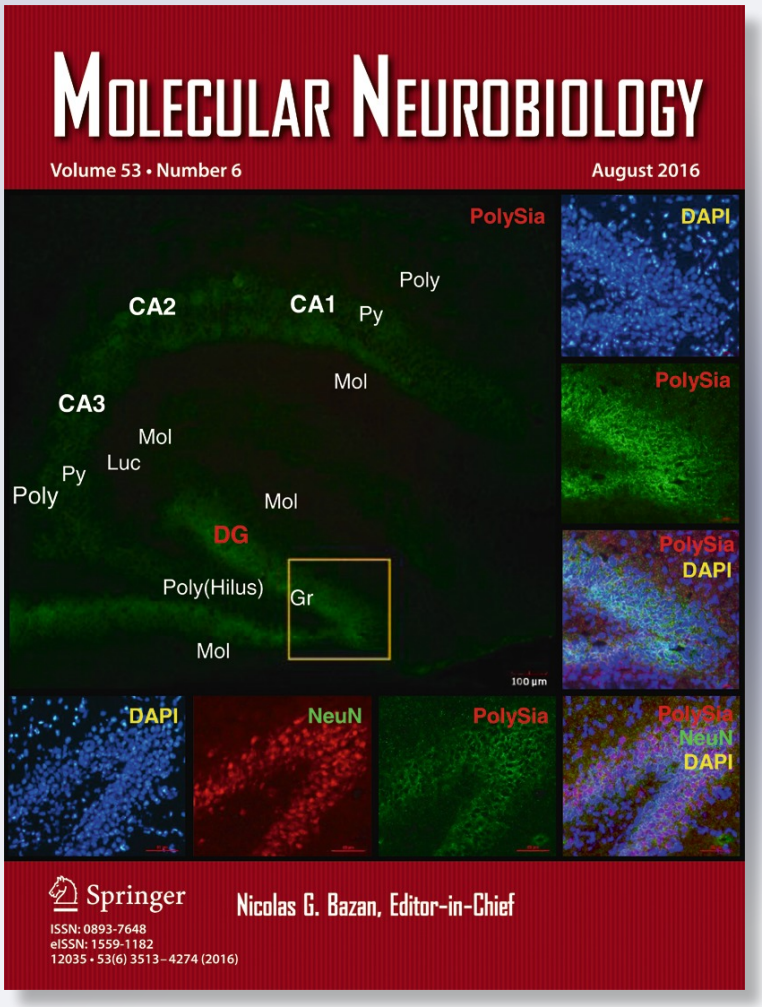

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PLD3 in Alzheimer's Disease: a Modest Effect as Revealed by Updated Association and Expression Analyses

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Abstract Alzheimer's disease (AD) is the most common form of dementia. Numerous genome-wide association studies (GWASs) have found several AD susceptibility common loci but with limited effect size. Recent next-generation sequencing studies of large AD pedigrees had identified phospholipase D3 (*PLD3*) p.V232M as the potentially functional rare variant with causal effect. However, four follow-up replication studies (*Brief Communications Arising* on *Nature*) questioned that *PLD3* V232M might not be so important in AD. In this study, we re-analyzed all public-available genetic (rare and common variants) and expression data of *PLD3*, and screened coding variants within *PLD3* in probands of 18 Han Chinese families with AD, to clarify the exact involvement of *PLD3* in AD. Two closest homologues of PLD3, PLD1 and PLD2, were also analyzed to comprehensively understand the role of phospholipase D members in AD. We found that *PLD3* variant V232M was associated with AD risk in overall sample sets (~40,000 subjects) with a modest to moderate effect size (odds ratio [OR]=1.53). Our results also showed that common variants and mRNA expression alterations of *PLD2* play a role in AD genetic risk and pathology. Although we provided a systematic view of the involvement of *PLD3* in AD at the genetic, mRNA expression, and protein levels, we could not define the exact causal or essential role of *PLD3* rare variants in AD based on currently available data.

Keywords Alzheimer's disease · PLD3 · Meta-analysis · Common variant · Rare variant

Introduction

Alzheimer's disease (AD), the most common form of dementia, is a typical neurodegenerative disease [1]. It is characterized by accumulation of beta-amyloid (Aβ) plaques and tau tangles, neuronal damage, and cognitive impairment [1]. The cause of AD is mostly unclear, except for early-onset familial AD with causal mutations in the amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*) genes [1, 2]. It is believed that late-onset AD is polygenic and develops as a result of interaction of multiple environmental and genetic risk factors [2].

The genetic heritability of AD ranges from 49 to 79 % based on twin and family studies [3]. Numerous genome-wide association studies (GWASs) have found many AD susceptibility genes, such as apolipoprotein E (*APOE*), bridging integrator 1 (*BIN1*), Clusterin (*CLU*), phosphatidylinositol binding clathrin assembly protein (*PICALM*), and *CD33* (cf. http://www.alzgene.org/) [4, 5]. However, these GWAS-identified genes with common variants have a limited effect size (odds ratio [OR]<1.2). The only exception is *APOE*,

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which was recognized as the only unequivocal risk gene of late-onset AD with a large effect (OR>4) [4]. All identified genetic factors contribute only 30 % of the genetic mechanism of the disease [6]. The missing heritability is evident, and there are unknown underlying rare functional variants with larger effect size.

The recent application of next-generation sequencing (NGS) technology offers a good strategy to the discovery of causal rare variants. Among the limited list of whole genome and exome sequencing studies for large AD cohorts or pedigrees, triggering receptor expressed on myeloid cells 2 (*TREM2*, p.R47H, rs75932628) and phospholipase D3 (*PLD3*, p.V232M, rs145999145) are the top two hits [7–9]. The rare variants in these two gene were recognized to have an effect size close to that of one *APOE* ε4 allele (OR>3) [7–9]. The association of *TREM2* p.R47H with AD was well validated in several independent association studies [10–12], and its causal role was also confirmed in animal models [13]. However, the *PLD3* gene, which received as high attention as *TREM2* [14], was mired in conflicts most recently.

In their original report, Cruchaga et al. [9] performed whole exome sequencing in 14 large AD families and found a rare non-synonymous *PLD3* variant V232M (rs145999145) segregated with disease status in two independent families. They also demonstrated the association of *PLD3* V232M with sporadic AD (OR>2) in both case–control and family samples [9]. Further overexpression and knockdown assays showed that PLD3 affects APP processing and Aβ production [9]. This study served as a perfect paradigm for identifying rare causal variants using high throughput techniques and functional assays. However, four recent replication reports (*Brief Communications Arising* on *Nature*) questioned that *PLD3* V232M might not be so important in AD [15–18].

Here, we re-analyzed all public-available genetic (rare and common variants) and expression data of *PLD3*, and screened coding variants within the *PLD3* gene region in probands of 18 Han Chinese families with AD, to further clarify the potential involvement of *PLD3* in AD. In addition, we analyzed two closest homologues of PLD3, PLD1 and PLD2, with an intention to understand the role of phospholipase D members in AD. Our study showed a modest effect of *PLD3* in AD.

Methods and Materials

Evolutionary Conservation Analysis and Protein Structure Modeling

To evaluate the importance of the p.V232M mutation, evolutionary conservation analysis of the PLD3 protein sequence and secondary structure modeling of the protein were performed. We retrieved the PLD3 amino acid sequences of 13 mammalian species, e.g., human (*Homo sapiens*, ENSP00000387050), chimpanzee (*Pan troglodytes*, ENSPTRG00000010994), gorilla (*Gorilla gorilla gorilla*, ENSGGOG00000011018), gibbon (*Nomascus leucogenys*, ENSNLEG00000014094), macaque (*Macaca mulatta*, ENSMMUG00000006071), marmoset (*Callithrix jacchus*, ENSCJAG00000014633), tree shrew (*Tupaia belangeri*, ENSTBEG00000014835), rat (*Rattus norvegicus*, ENSRNOG00000018390), mouse (*Mus musculus*, ENSMUSG00000003363), sheep (*Ovis aries*, ENSOARG00000006607), pig (*Sus scrofa*, ENSSSCG00000025109), cow (*Bos taurus*, ENSBTAG00000019150), and dog (*Canis lupus familiaris*, ENSCAFG00000005362) from Ensembl (http://asia.ensembl.org/index.html). Sequence alignment was performed by ClusterW method using software MEGA 4.0 [19].

For secondary structure modeling of the PLD3 protein, we used three webservers/programs to perform and cross-validate the Protein homology modeling. The RaptorX program (http://raptorx.uchicago.edu/) is a protein structure prediction server for protein sequences without close homologues in the Protein Data Bank (PDB) [20, 21]. Given an input sequence, RaptorX predicts its secondary and tertiary structures. More details are described in the original papers describing this approach [21, 20]. The Protein Homology/analogY Recognition Engine V 2.0 (Phyre, http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index), another top webserver for structure prediction using homology modeling [22], was used to validate the results suggested by RaptorX. The Iterative Threading ASSEmbly Refinement (I-TASSER, (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) program [23–25], which employs a hierarchical method for protein structure and function prediction, was used to predict the structure and function of PLD3 protein. Active sites and effects of mutant p.V232M on the protein activity were shown using I-TASSER.

Meta-Analysis of the Association Between PLD3 Variant V232M and AD

A total of seven case–control cohorts were reported by Cruchaga et al. [9] including 1,106 cases and 928 controls from the National Institute of Ageing Late Onset Alzheimer Disease (NIA-LOAD), 1,114 cases and 913 controls from the Knight Alzheimer's Disease Research Centre (Knight-ADRC), 143 cases and 183 controls from NIA-UK, 255 cases and 2,471 controls from Cache-County, 265 cases and 246 controls from the University of Toronto data set (U. Toronto), 525 cases and 274 controls from the University of Nottingham data set (U. Nottingham), and 1,268 cases and 964 controls from the University of Pittsburgh data set (U. Pittsburgh), resulting in 4,676 cases and 5,979 controls in total. In the four recent studies, van

der Lee et al. [17] analyzed 1,914 cases and 8,021 controls, including 146 cases and 2,383 controls from Age, Gene/Environment Susceptibility-Reykjiavik Study (AGES), 454 cases and 613 controls from Dutch Alzheimer centers, 111 cases and 989 controls from Genetic Research in Isolated Populations (GRIP), 476 cases and 2,412 controls from Rotterdam Study (RS), 499 cases and 293 controls from Alzheimer's Disease Neuroimaging Initiative (ADNI), and 228 cases and 1,331 controls from Framingham Heart Study (FHS). Lambert et al. [18] replicated the association in a French cohort of 2,083 cases and 6,536 controls. Heilmann et al. [16] analyzed three populations including 2,166 cases and 2,754 controls from Fundacio' ACE, 461 cases and 180 controls from St. Pau Hospital, and 941 cases and 933 controls from Germany, resulting totally in 3,568 cases and 3,867 controls. Hooli et al. [15] used the National Institute of Mental Health Alzheimer's Genetics Initiative Study Sample (NIMH, 439 multiplex families comprising 1,440 subjects which were partly included in the Cruchaga et al. study [9]). The NIMH data set reported by Hooli et al. [15] was not included in the current meta-analysis because of the family-based design and the unavailability of detailed genotype data. In addition to the above-reported data, we also included another Germany cohort of 1,089 cases and 1,456 controls, which was previously reported by Schulte et al. [26] but was not considered in the Hooli et al. meta-analysis [15]. In total, 39,189 subjects (13,330 patients and 25,859 controls) from 18 independent populations were included in our meta-analysis. Meta-analysis was performed by software Revman 5.2 (http://tech.cochrane.org/revman/download) under Mantel-Haenszel method. Random effect model was used if there was a heterogeneity (I^2>50 %), and fixed-effect model was used when I^2<50 %. Leave-one-out analyses were conducted to ensure that no single study unduly influenced the estimate.

Expression Alterations of the *PLD* Genes in AD Brain Tissues

We aimed to validate *PLD3* mRNA expression change reported by Cruchaga et al. [9] using four Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) data sets focusing on AD. The first data set (GEO reference series GSE1297 [27]) analyzed expression profiling of brain hippocampus from 9 control postmortem subjects and 22 AD patients at various stages of severity (7 incipient patients, 8 moderate patients, and 7 severe patients) by microarray. The second data set was GSE4757 [28], which reported an expression profiling of entorhinal cortex neurons containing neurofibrillary tangles (NFT) compared to histopathologically normal neurons from the same patients and brain regions from ten mid-stage AD patients. The third data set was GSE36980 [29], which contained postmortem brain tissues including frontal cortex, temporal cortex, and hippocampus from 79 patients. The last data set was GSE29652 [30], which was focusing on analyzing gene expression profile of temporal cortex astrocytes representing different Braak stages (six stage I–II cases, six stage III–IV cases, six stage V–VI cases). Gene expression data of *PLD1*, *PLD2*, *PLD3*, and *APP* were retrieved from these four data sets. Comparisons of mRNA expression level of these four genes between AD patients and healthy controls or between different stages of patients were performed using Student's *t* test by GraphPad Prism (GraphPad Software Inc., La Jolla, CA). Correlation of *PLD3* and *APP* mRNA expression levels was measured by the Pearson correlation coefficient.

Association of *PLD* Common Variants with AD

To test whether there are common variants that affecting AD risk, we retrieved the summary statistics of the International Genomics of Alzheimer's Project (IGAP, http://www.pasteur-lille.fr/en/recherche/u744/igap/igap_download.php), the most recent and largest genetic study of Alzheimer's disease based upon GWAS of individuals of European ancestry [31]. We used meta-analyses data from IGAP stage 1 which were genotyped and imputed 7,055,881 single nucleotide polymorphisms (SNPs) in four previously published GWAS data sets consisting of 17,008 AD cases and 37,154 controls. SNPs within ±10 kb region of the *PLD1*, *PLD2*, and *PLD3* genes were retrieved. Gene-based test was performed using an online tool Versatile Gene-based Association Study (VEGAS, http://gump.qimr.edu.au/VEGAS/) [32], a free program for gene-based association test based on patterns of linkage disequilibrium for each gene.

Expression Quantitative Trait Loci (eQTL) Effects of PLD Common Variants

To test whether these AD-risk variants affect *PLD* expression, we evaluated the eQTL effects of the potential AD risk *PLD* SNPs on *PLD* gene expression alterations based on the available databases. The Genotype-Tissue Expression project (GTEx, http://www.gtexportal.org/home/) [33] provides a comprehensive atlas of gene expression and regulation across multiple human tissues including whole blood and brain tissues. The Brain eQTL Almanac (BRAINEAC, http://caprica.genetics.kcl.ac.uk/BRAINEAC/) [34], a web-based resource to access the UK Brain Expression Consortium (UKBEC) data set, provides the brain eQTL data across ten brain tissues of 134 neurological normal individuals.

PLD3 Coding Variants in Han Chinese AD Patients

Except for the above-mentioned European origin samples, *PLD3* mutation was also detected in a Han Chinese

cohort of 360 patients with sporadic AD and 400 healthy individuals [35]. To further confirm the involvement of *PLD* mutations in familial AD cases, we sequenced whole exons of *PLD1*, *PLD2*, and *PLD3* of 18 AD probands with familial disease history. These patients were diagnosed following the DSM-IV and the NINCDS-ADRDA criteria independently by at least two senior clinicians. Each AD pedigrees contained at least two patients. The whole exons and flanking regions of the 18 probands were sequenced by paired-end reads on the HiSeq 2500 sequencer (Illumina). Clean sequence reads were aligned to the reference genome hg19 by Burrows-Wheeler Aligner (BWA, http://bio-bwa.sourceforge.net/) [36] and Samtools (http://samtools.sourceforge.net/) [37]. SNP calling was performed by Genome Analysis Toolkit (GATK, https://www.broadinstitute.org/gatk/) [38].

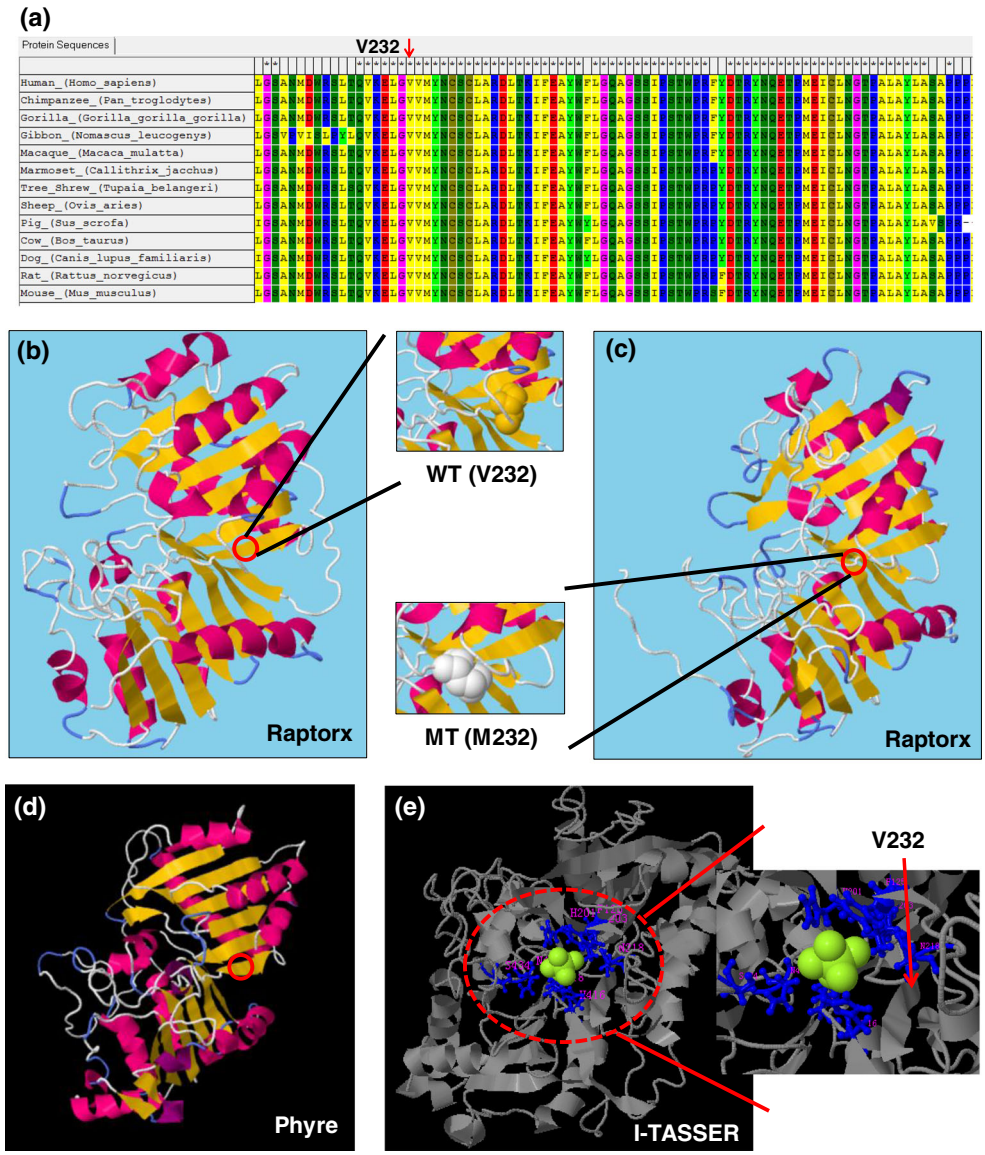

Fig. 1 Sequence conservation (**a**) and secondary structure modeling (**b**–**e**) of the PLD3 protein. PLD3 peptide sequences of human (*Homo sapiens*), chimpanzee (*Pan troglodytes*), gorilla (*Gorilla gorilla gorilla*), gibbon (*Nomascus leucogenys*), macaque (*Macaca mulatta*), marmoset (*Callithrix jacchus*), tree shrew (*Tupaia belangeri*), rat (*Rattus norvegicus*), mouse (*Mus musculus*), sheep (*Ovis aries*), pig (*Sus scrofa*), cow (*Bos taurus*), and dog (*Canis lupus familiaris*) were retrieved from NCBI (www.ncbi.nlm.nih.gov/). Protein sequence alignment was performed by ClusterW method using software MEGA 4.0 [19]. For secondary structure modeling of the PLD3 protein, three software were used to predict and cross-validate the Protein homology modeling. Given an input sequence, RaptorX (http://raptorx.uchicago.edu/) [20, 21] predicts its secondary and tertiary structures. More details are described in their original paper [21, 20]. Protein Homology/analogY Recognition Engine V 2.0 (Phyre, http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) [22] was used to validate the results given by the RaptorX. The Iterative Threading ASSEmbly Refinement (I-TASSER, http://zhanglab.ccmb.med.umich.edu/I-TASSER/) program [23–25], which employs a hierarchical method for protein structure and function prediction, was also used to predict the structure and function of PLD3 protein. Active sites and effects of p.V232M on the protein activity were shown by I-TASSER

Results

Mutant p.V232M Is Located in a Conservative Region and Affects the Secondary Structure of the PLD3 Protein

In general, a rare non-synonymous mutation located in a conservative region would be more likely to be functional or deleterious [39]. We found that amino acid sequence surrounding the 232nd position is conservative in the analyzed mammalian species (Fig. 1). Mutation in this conservative site might be damaging to the structure and/or function of the protein. Indeed, we found that mutant PLD3 (p.M232) protein has a different 3D structure compared to the wild-type protein (p.V232) (Fig. 1). The modeling results were consistent when different programs or algorithms were used (Fig. 1). In addition, the 232nd site is close to the activity center of the protein, indicating a potentially essential role of this residue in the formation of the activity center (Fig. 1). Evidently, PLD3 p.V232 is located in an evolutionarily conservative region, and mutation at this site might affect the structure and function of the protein.

PLD3 V232M Is Significantly Associated with AD, with a Modest Effect Size in the Meta-Analysis

Cruchaga et al. [9] reported an overall meta-analysis OR of 2.10 ($P=2.93\times10^{-5}$) for *PLD3* V232M (rs145999145) in seven case–control cohorts (4,676 cases and 5,979 controls). In the meta-analysis performed by Hooli et al. [15], they combined three other follow-up reports of *Brief Communications Arising* on *Nature* (Heilmann et al. [16], Lambert et al. [18], and van der Lee et al. [17]) and reported a non-significant effect of p.V232M (OR=1.29, $P=0.132$) in 7,565 cases and 18,424 controls. However, when the three follow-up case–control cohorts were combined together with the samples of Cruchaga et al. [9], a significant result (OR=1.62, $P=3.47\times10^{-4}$) was observed in their analysis. In the current meta-analysis, we included another Germany cohort

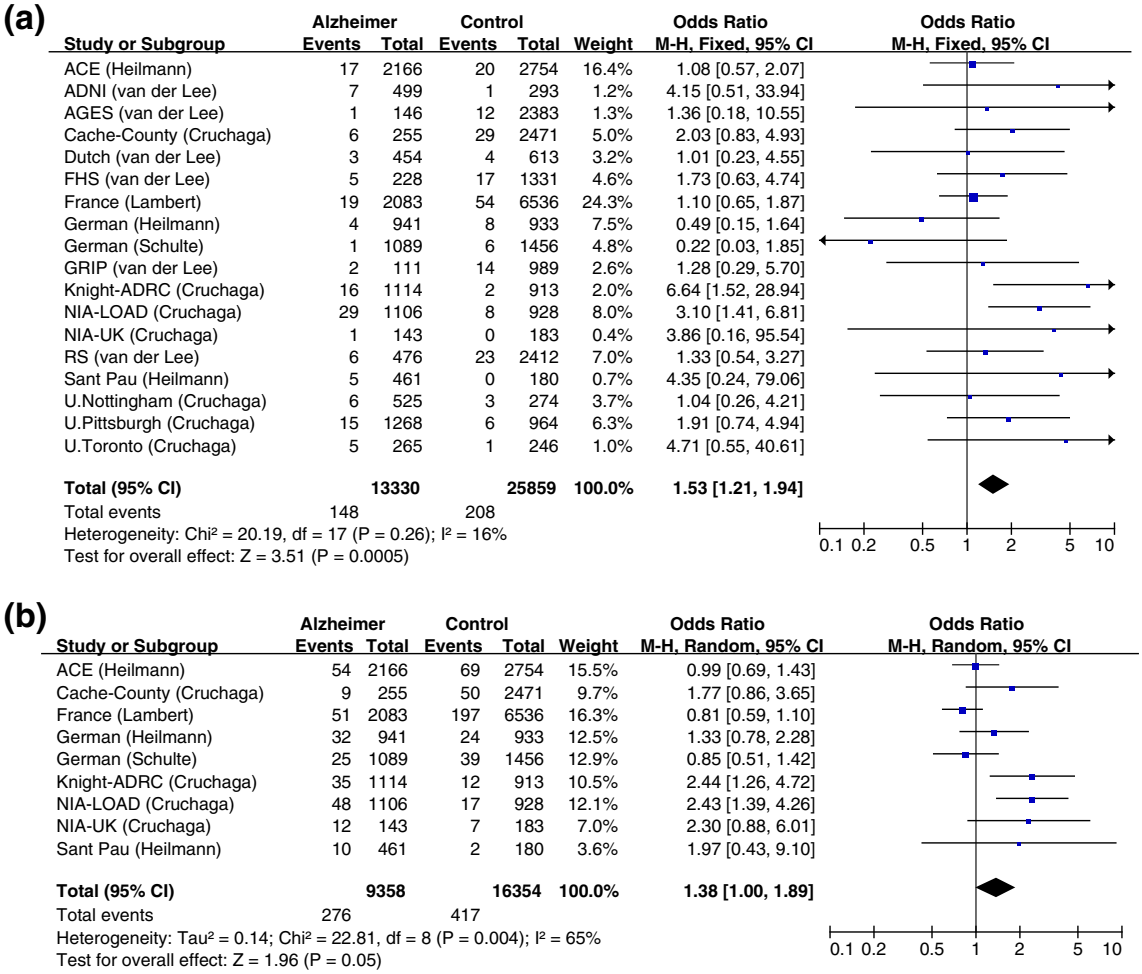

Fig. 2 Meta-analysis for association of *PLD3* variants V232M (**a**) and A442A (**b**) with sporadic AD. Patients (13,330) and controls (25,859) were included in the current meta-analysis. Meta-analysis was performed by software Revman 5.2 (http://tech.cochrane.org/revman/download) under Mantel-Haenszel method

of 1,089 cases and 1,456 controls reported by Schulte et al. [26], which was not considered in the Hooli et al. meta-analysis [15]. As the Cruchaga et al. study [9] contained multiple different data sets, we did not divide all these data sets into two independent "initial data sets" and "validation data sets" groups as Hooli et al. did [15]. In total, 13,330 patients and 25,859 controls from 18 independent populations were combined in our meta-analysis, and this data set covered all the available data so far. An OR of 1.53 ($P=5.0\times10^{-4}$) was observed for V232M (Fig. 2) in our fixed-effect meta-analysis in ~40,000 subjects.

For another *PLD3* variant A442A (rs4819), Cruchaga et al. [9] reported an overall meta-analysis OR of 2.12 ($P=3.78\times10^{-7}$) in four case–control cohorts (2,618 cases and 4,495 controls), while Hooli et al. [15] observed a non-significant effect of p.A442A (OR=0.97, $P=0.752$) in 6,679 cases and 11,911 controls. When these data were combined together, Hooli et al. found a nominally significant result (OR=1.19, $P=0.043$) [15]. In our meta-analysis based on 9,358 patients and 16,354 controls (including the Germany cohort of 1,089 cases and 1,456 controls reported by Schulte et al. [26]), we got a marginal significant effect of p.A442A in these sporadic patients (OR=1.38, $P=0.05$) (Fig. 2). Note that the leave-one-out sensitivity analysis showed that the nominal significance was caused by cohorts reported by Cruchaga et al. [9]. In addition, a significant heterogeneity ($I^2=65$ %, $P=0.004$) was observed for A442A among these data sets. The heterogeneity is mainly introduced by populations from France reported by Lambert et al. [18] (OR=0.81) and from Germany reported by Schulte et al. (OR=0.85) [26]. As the differences of minor allele frequency among these studies were mainly observed in the cases (2~4 % in the Chruchaga et al. study [9], 1.2 % in the Lambert et al. study [18], and 1 % in the Schulte et al. study [26]), rather than in controls (0.7~1.9 % in the Chruchaga et al. study [9], 1.5 % in the Lambert et al. study [18], and 1.3 % in the Schulte et al. study [26]), it would be hard to define whether the heterogeneity was caused by population stratification. Moreover, potential publication bias might also contribute to the heterogeneity as revealed by the funnel plot (Supplementary Fig. S1). Further investigations using larger sample size with correction for potential population stratification will help to clarify this issue.

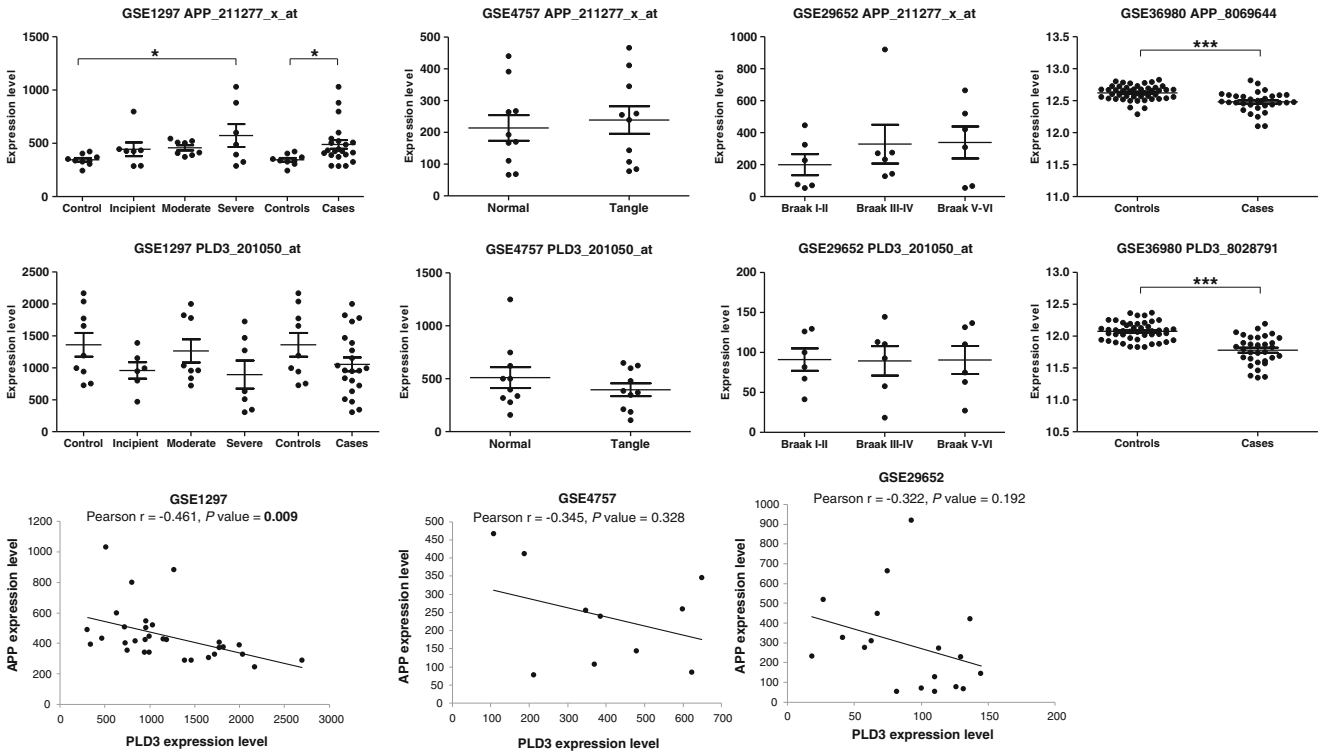

Fig. 3 Alteration of *PLD3* expression level in AD brain tissues and correlation of *PLD3* expression level with *APP* expression level. We re-analyzed expression data from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo). GSE1297 [27] analyzed expression profiling of brain hippocampus from 9 control postmortem subjects and 22 AD patients at various stages of severity by microarray. GSE4757 [28] is expression profiling of entorhinal cortex neurons containing neurofibrillary tangles (*NFT*) compared to histopathologically normal neurons from the same patients and brain region from ten mid-stage AD patients. GSE36980 [29] analyzed postmortem brain tissues including frontal cortex, temporal cortex, and hippocampus from 79 AD subjects. GSE29652 [30] analyzed gene expression profile of temporal cortex astrocytes representing different Braak stages (six stage I–II cases, six stage III–IV cases, six stage V–VI cases). Comparisons of mRNA expression level of these six genes between AD and control or different stage of patients were performed using Student's *t* test by GraphPad Prism (GraphPad Software Inc., La Jolla, CA). Correlation of *PLD3* and *APP* mRNA expression level was measured by Pearson correlation coefficient

PLD3 and *PLD2* Are Differentially Expressed in AD Brain Tissues

In addition to the genetic evidence, Cruchaga et al. [9] showed that expression level of *PLD3* was significantly decreased in AD brains. Moreover, the expression level of *PLD3* mRNA level was reversely correlated with *APP* mRNA level. To validate this observation, we retrieved gene expression data of *PLD1*, *PLD2*, *PLD3*, and *APP* from four data sets involving several AD brain tissues (for details see "Methods and Materials" section). We found that the *PLD3* mRNA level was decreased in AD brain tissues relative to the healthy controls across all four data sets, albeit the decrease did not reach a statistical significance (Fig. 3). Note that *PLD3* mRNA level decreased significantly in brain tissues (including frontal cortex, temporal cortex, and hippocampus) from 79 AD subjects (GSE36980 [29]). The *APP* mRNA level was also significantly decreased in this data set. We excluded this data set for *PLD3*-*APP* correlation analysis. A significant reverse correlation between the mRNA expression levels of *PLD3* and APP was observed in hippocampus from 9 controls and 22 AD patients at various stages of severity (GSE1297 [27]). This trend was confirmed in the other two data sets (GSE4757 [28] and GSE29652 [30]), although the correlation did not reach a statistical significance. Taken together, the observations that *PLD3* mRNA expression level was decreased in AD brains and reversely correlated with *APP* mRNA levels reported by Cruchaga et al. [9] were partially validated in the current analyses, yet our results did not reach statistical significance, partially because of limited number of individuals (Fig. 3). We were unable to perform joint analyses of the expression data currently because the original study design and tissue types of the four data sets are different. It will be good to further define the expression pattern of *PLD3* in AD brains using larger independent sample sets.

We also found an increase of mRNA expression level of *PLD2*, which was reported to be active in AD pathology [40–43], in AD hippocampus tissues, and the increase was positively related to stage of severity. Intriguingly, a significantly and consistently positive correlation between the *PLD3* and *PLD2* mRNA expression levels was observed (Fig. 4).

Common eQTL Variants of *PLD2*, not *PLD3*, Show Associations with AD

Our current meta-analysis showed that *PLD3* V232M confers susceptibility to AD at a moderate effect size (OR=1.53), indicating a susceptibility role rather than a major causal role of PLD3 in AD. There are several other *PLD3* variants, which conferred genetic risk to AD according to Cruchaga et al. [9]. It is thus reasonable to speculate that there might be other *PLD3* variants, in particular common variants affecting *PLD* expression and contributing to AD susceptibility.

We found several positively associated SNPs within *PLD3* in 17,008 AD cases and 37,154 controls from the IGAP project [31] (Table 1). However, none of these significant SNPs could survive after multiple testing corrections. When we performed the gene-based test, no positive association at the gene level was observed. In addition, all these nominally significant

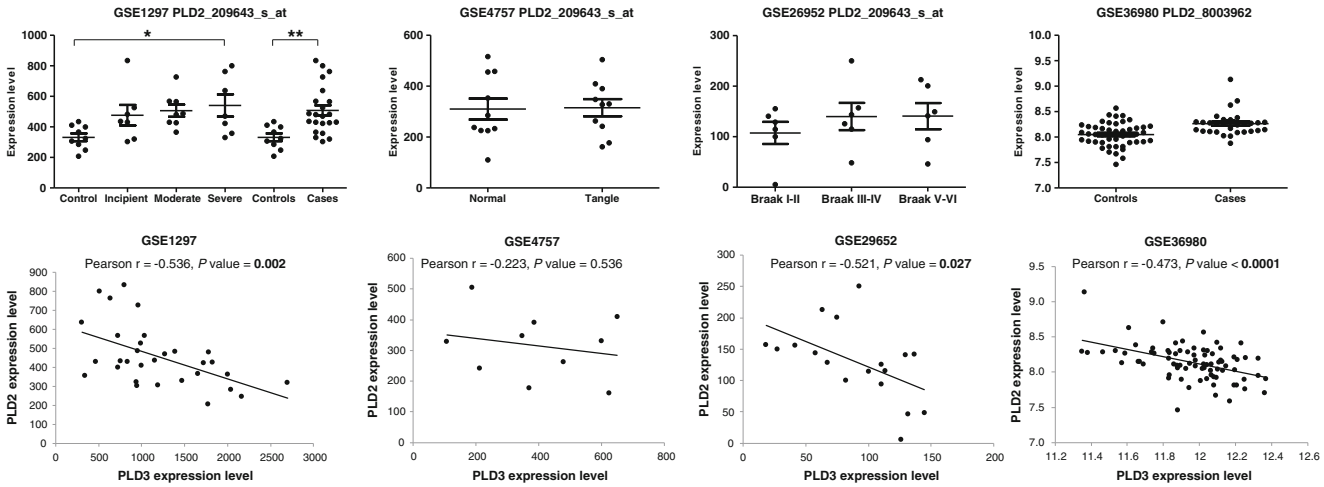

Fig. 4 Alteration of *PLD2* expression level in AD brain tissues and correlation of *PLD2* expression level with *PLD3* expression level. We re-analyzed expression data from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo). GSE1297 [27] analyzed expression profiling of brain hippocampus from 9 control postmortem subjects and 22 AD patients at various stages of severity by microarray. GSE4757 [28] is expression profiling of entorhinal cortex neurons containing NFT compared to histopathologically normal neurons from the same patients and brain region from 10 mid-stage AD patients. GSE36980 [29] analyzed postmortem brain tissues including frontal cortex, temporal cortex, and hippocampus from 79 AD subjects. GSE29652 [30] analyzed gene expression profile of temporal cortex astrocytes representing different Braak stages (six stage I–II cases, six stage III–IV cases, six stage V–VI cases). Comparisons of mRNA expression level of these six genes between AD and control or different stage of patients were performed using Student's *t* test by GraphPad Prism (GraphPad Software Inc., La Jolla, CA). Correlation of *PLD3* and *PLD2* mRNA expression level was measured by Pearson correlation coefficient

Table 1 Top AD-related common variants of the *PLD* genes

Gene	SNP	Chr.	Position	A1	A2	Beta	SE	*P* value
PLD3	rs7249819	19	40853376	C	T	0.08	0.03	0.015
	rs7245751	19	40847233	T	C	0.07	0.03	0.026
	rs11670323	19	40851131	A	G	0.07	0.03	0.026
	rs11667768	19	40854432	T	C	0.07	0.03	0.027
	rs77827612	19	40857900	G	C	0.06	0.03	0.029
	rs76895241	19	40856521	T	C	0.06	0.03	0.029
	rs77209829	19	40846468	C	G	0.07	0.03	0.031
	rs11667774	19	40854454	G	C	0.06	0.03	0.031
	rs11666860	19	40861863	C	T	0.07	0.03	0.033
	rs78044607	19	40854192	T	C	0.06	0.03	0.037
	rs8110888	19	40855255	C	T	−0.03	0.02	0.043
	rs7249146	19	40853492	C	T	−0.03	0.02	0.045
PLD2	rs113124299	17	4727352	A	C	0.12	0.03	2.60E-04
	rs78535545	17	4727727	T	C	0.12	0.03	3.40E-04
	rs74610625	17	4725695	C	G	0.12	0.03	3.70E-04
	rs72835011	17	4729412	C	G	0.11	0.03	0.001
	rs76705156	17	4729684	T	C	0.11	0.03	0.001
	rs74476245	17	4722698	G	C	0.19	0.06	0.002
	rs75364523	17	4723121	A	G	0.1	0.03	0.003
	rs2286670	17	4718027	T	G	0.07	0.02	0.006
	rs17854914	17	4721376	G	A	0.06	0.02	0.015
	rs72833202	17	4706109	C	T	0.06	0.02	0.016
	rs75326412	17	4734271	G	A	0.04	0.02	0.025
	rs2875842	17	4710486	C	G	0.06	0.03	0.027
	rs11654671	17	4732467	T	A	0.04	0.02	0.033
	rs73341629	17	4727684	G	A	0.04	0.02	0.047

The data were retrieved from the summary statistics of the International Genomics of Alzheimer's Project (IGAP, http://www.pasteur-lille.fr/en/recherche/u744/igap/igap_download.php)

A1 effect allele or minor allele, *A2* reference allele, *Beta* overall estimated effect size for the effect allele, *SE* overall standard error for effect size estimate, *P value* meta-analysis *P* value using regression coefficients

SNPs showed no eQTLs signal in both the brain eQTL data set BRAINEAC [34] and the GTEx database [33]. These observations showed that common variants within the *PLD3* gene had no essential effect on *PLD3* mRNA expression and AD risk.

We observed no nominally significant SNPs within the *PLD1* gene region. However, several *PLD2* SNPs showed strong associations with AD (Table 1), although no positive association at the gene level was observed for the *PLD2* gene. Among these significantly associated *PLD2* SNPs, two variants (rs17854914, $P=7.0\times10^{-7}$; rs72833202, $P=2.0\times10^{-7}$) showed strong eQTL effects in whole blood in the GTEx database [33] (Supplementary Fig. S2) but had no eQTL signal in the brain eQTL data set [34]. Intriguingly, three top AD-related *PLD2* SNPs (rs113124299, $P=3.9\times10^{-3}$; rs78535545, $P=4.0\times10^{-3}$; rs74610625, $P=3.8\times10^{-3}$) showed significant exon-specific (exprID 3707228, http://caprica.genetics.kcl.ac.uk/BRAINEAC/) eQTL effect only in hippocampus of the brain eQTL data set (Supplementary Fig. S3). These results suggested that *PLD2* common variants have tissue-specific eQTL effects and may confer genetic susceptibility to AD.

No Enrichment of *PLD* Coding Variants in Han Chinese AD Patients

In the replication study aiming at the rare causal variant *PLD3* V232M in Han Chinese, Jiao et al. [35] detected no p.V232M mutation in 360 patients and 400 control individuals. Similarly, we found no p.V232M in these familial AD probands. No missense SNPs was found in the *PLD3* gene, while three missense SNPs out of five *PLD2* SNPs and four missense SNPs out of sven *PLD1* SNPs were observed in the 18 AD probands. Among these missense SNPs, we found no rare variant (minor allele frequency [MAF]≤0.05) of the *PLD2* gene. Three rare variants (rs567323716 [p.R885H], rs137972331 [p.P514L], rs149506327 [p.R90H]), all had a MAF less than 0.05 in the 1000 genome project (http://www.1000genomes.org/ [44]), were observed in the *PLD1* gene. However, the

three rare missense mutations were observed only once in different individuals out of the 18 AD probands (Table 2). Together, the *PLD* coding variants might be less important in Han Chinese AD patients.

Discussion

Numerous GWASs of AD have identified several common susceptibility loci with modest to moderate effect size [31, 2, 4]. The underlying causal variants, rather than the susceptibility loci, await further characterization. The most famous rare functional variant, *TREM2* R47H, was identified to be a large effect causal variant in AD [7, 8]. *PLD3* rare variant V232M (rs145999145), which was first identified by Cruchaga et al. [9] through whole exome sequencing in large AD families and well-replication in case–control and families cohorts, was questioned by recent follow-up reports (*Brief Communications Arising on Nature*) [16, 15, 18, 17] that failed to replicate the importance of *PLD3* V232M in AD. In brief, in the seven case–control cohorts reported by Cruchaga et al. [9], only two sample sets, NIA-LOAD (1,106 cases and 928 controls, $P=4.0\times10^{-3}$) and Knight-ADRC (1,114 cases and 913 controls, $P=3.4\times10^{-3}$), showed a significant association of p.V232M with AD. The other five sample sets showed the same but not significant effect, whereas the overall meta-analysis effect (OR=2.1) reached a statistical significance ($P=2.93\times10^{-5}$) [9]. This inconsistent trend was also observed in the five follow-up studies: van der Lee et al. [17] obtained a nominal significance (OR=1.94, $P=0.03$) in combined 1,914 cases and 8,021 controls containing five independent non-significant sample sets (AGES, Dutch Alzheimer centers, GRIP, RS, ADNI, and FHS); Lambert et al. [18] failed to replicate the association in a French cohort of 2,083 cases and 6,536 controls (OR=1.17, $P=0.58$); Heilmann et al. [16] found no significant association of p.V232M with AD in three populations of 3,568 cases and 3,867 controls; and Hooli et al. [15] observed a nominally significant signal ($P=0.0212$) for p.V232M with the NIMH subjects (1,440 subjects of 439 families, which were partially included in the Cruchaga et al. study), and they observed an enrichment of p.V232M in unaffected individuals rather than in cases. Notably, this contrast direction was also observed in the Schulte et al. study (MAF=0.20 % in controls vs. MAF=0.05 % in cases) [26]. Considering a MAF of 0.2 % as observed for p.V232M, one needs more than 19,000 samples to achieve a statistical power of 80 % to detect the effect size (OR) of 1.5 under the dominant model. Thus, the inconsistent results of these independent validation studies might be caused by limited sample size. Our meta-analysis involving 13,330 patients and 25,859 controls from all 18 independent populations showed a significant association of V232M with AD susceptibility (OR=1.53, $P=5.0\times10^{-4}$). For another *PLD3* rare variant, p.A442A, negatively or marginally significant associations were consistently observed in the follow-up replications and meta-analyses, except for the Cruchaga et al. report [9]. Taken an overview of those reported top AD genetic risk loci (Fig. 5), we found that the *PLD3* rare variant V232M would increase AD risk with an effect size (OR=1.53), comparable to that of those GWAS-identified common loci (OR≈1.2). Therefore, we suggested that *PLD3* rare

Table 2 Variants within the *PLD* coding regions identified in 18 Han Chinese AD probands

Position	dbSNP ID	Carriers	MAF	Gene	Function
chr3:171319991	rs139035422	1	0.157	PLD1	utr-3
chr3:171320227	rs9822322	8	0.477	PLD1	utr-3
chr3:171330183	rs567323716	9	0.001	PLD1	p.R885H
chr3:171395468	rs2124147	4	0.465	PLD1	Synonymous
chr3:171404478	rs2290480	2	0.166	PLD1	p.A622S
chr3:171405373	rs137972331	1	0.010	PLD1	p.P514L
chr3:171455341	rs149506327	1	0.003	PLD1	p.R90H
chr17:4712395	rs1132446	9	0.316	PLD2	Synonymous
chr17:4712617	rs2286672	11	0.192	PLD2	p.R172C
chr17:4718776	rs1132448	17	0.375	PLD2	Synonymous
chr17:4721376	rs17854914	2	0.076	PLD2	p.E632G
chr17:4722876	rs3764897	2	0.183	PLD2	p.G821S
chr19:40854432	rs11667768	5	0.114	PLD3	utr-5
chr19:40854454	rs11667774	4	0.110	PLD3	utr-5
chr19:40884121	rs201300702	2	0.0002	PLD3	utr-3
chr19:40884160	rs4635	9	0.432	PLD3	utr-3

Global minor allele frequency was retrieved from dbSNP (http://www.ncbi.nlm.nih.gov/snp), using 1000 Genome phase 1 genotype data from 1,094 worldwide individuals

MAF minor allele frequency

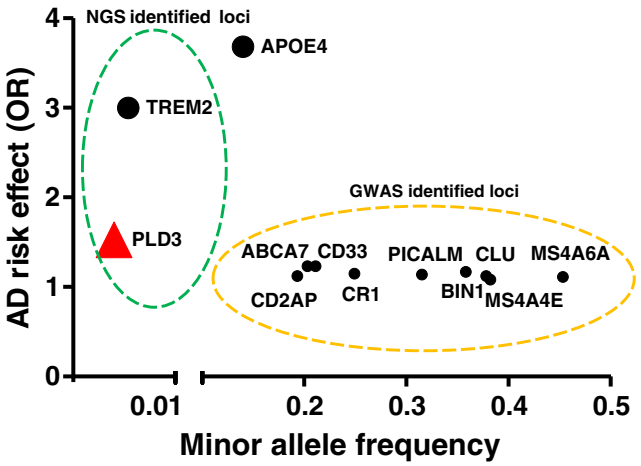

Fig. 5 Effect size of *PLD3* and other top genetic factors in AD. Effect size of the AD-related top genetic risk loci was retrieved from the AlzGene database (http://www.alzgene.org/) [4]. *NGS*, next-generation sequencing technology; *GWAS*, genome-wide association study

variants might contribute to AD risk, with a modest to moderate effect size but not causal or strong effect.

In addition to the genetic association evidence, Cruchaga et al. [9] demonstrated that *PLD3* is down-regulated in AD brain tissues and functions in APP processing. The decreased expression level of *PLD3* and reverse correlation with *APP* level in AD brain tissues could be validated in our analysis, though results of some data sets did not reach a statistical significance. Our result is consistent with another report, which showed a modest reduction of *PLD3* mRNA and protein in AD brains [45]. Expression level of *PLD3* homologues, *PLD1* and *PLD2*, were previously recognized to be regulated in brain tissues of AD patients [46, 47, 43, 48, 41, 42] and affected APP processing and Aβ production. Phospholipase D was said to be a potential therapeutic target in brain disorders [40]. Consistently, we found that *PLD2* is up-regulated and correlated with *PLD3* in AD brain tissues. We further showed that common SNPs affecting *PLD2* expression were associated with AD risk. Taken together, our results indicate a potential synergy effect between *PLD3* and *PLD2* in AD. Phospholipase D family members, especially *PLD2*, might have an essential role in AD pathogenesis.

Another line of evidence for a functional role of p.V232M was suggested by our evolutionary conservation analysis and in silico structure modeling analysis, in which we showed that p.V232M mutation can alter structure and function of the protein, adding more support for the involvement of p.V232M in AD. However, we found no association or occurrence of p.V232M in Han Chinese AD patients. This could be explained by population-specific effect or limited sample size of the Chinese cohorts. It is evident that validation the association of *PLD3* V232M with AD in Han Chinese needs large sample size.

In short, *PLD3* V232M was associated with AD risk according to our meta-analysis of ~40,000 subjects at a modest to moderate effect size. The phospholipase D family members, especially *PLD2*, might play a role in AD development and pathogenesis. Based on the available data, we cannot define the causal or essential role of *PLD3* variants in AD. The exact involvement of *PLD* genes in AD needs further investigation.

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Conflict of Interest The authors declare that they have no competing interests.

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Supplementary Figures

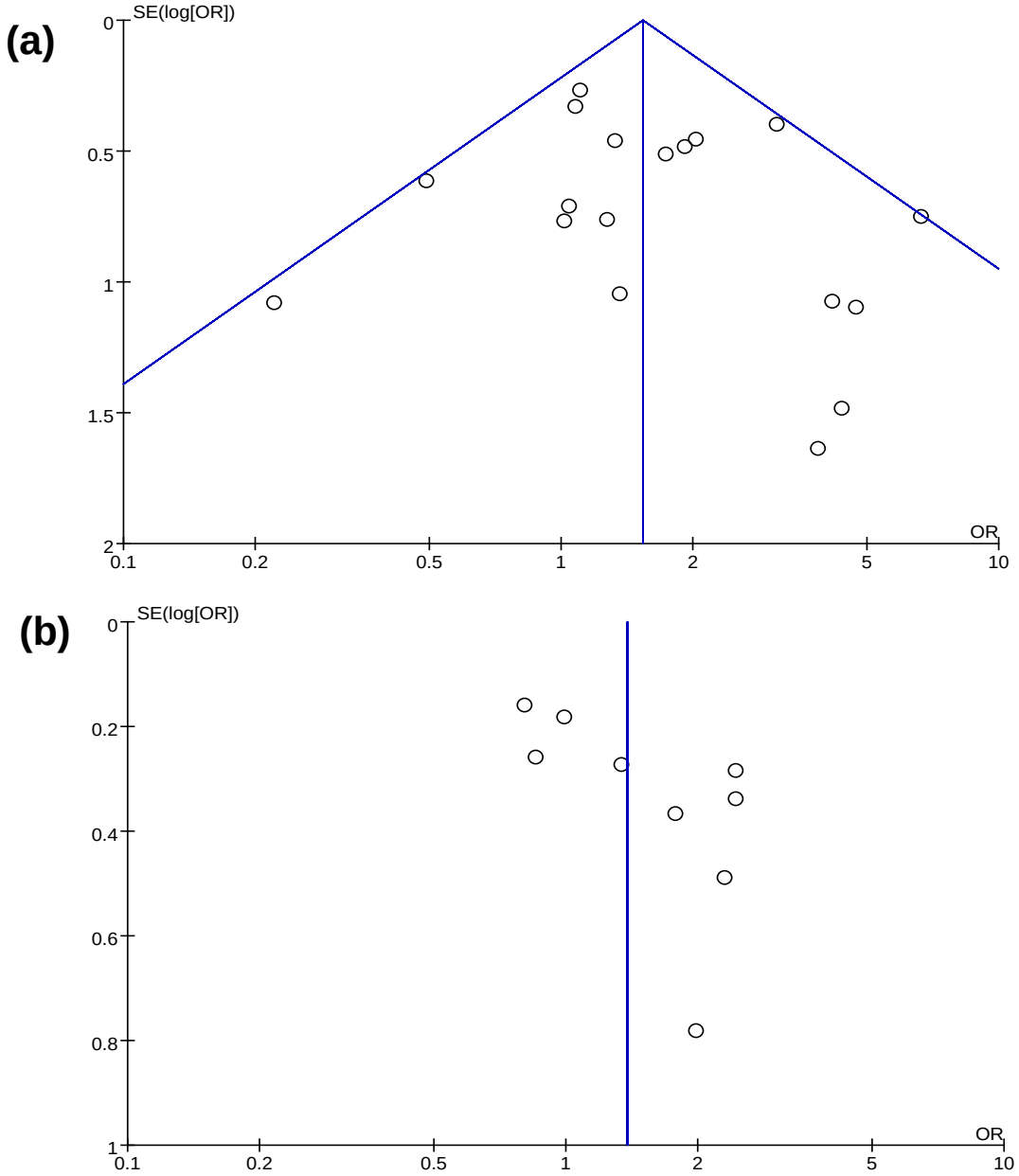

Figure S1. Funnel plot of the meta-analysis for V232M (a) and A42A (b). Meta-analysis was performed by software Revman 5.2 (http://tech.cochrane.org/revman/download) under Mantel-Haenszel method. Random effect model was used if there was heterogeneity ($I^2 > 50\%$), and fixed effect model was applied when $I^2 < 50\%$. Potential bias for A42A was revealed by the funnel plot.

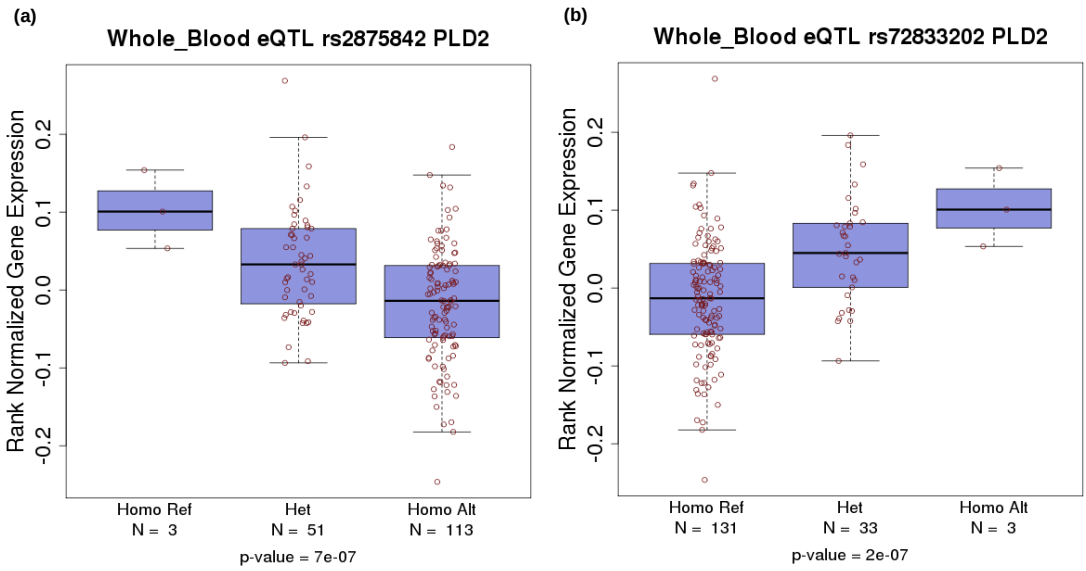

Figure S2. Two AD-related *PLD2* variants rs17854914 (a) and rs72833202 (b) showed strong eQTL effects in whole blood (N = 168) in the GTEx database. The GTEx (Genotype-Tissue Expression project, http://www.gtexportal.org/home/) provides a comprehensive atlas of gene expression and regulation across multiple human tissues.

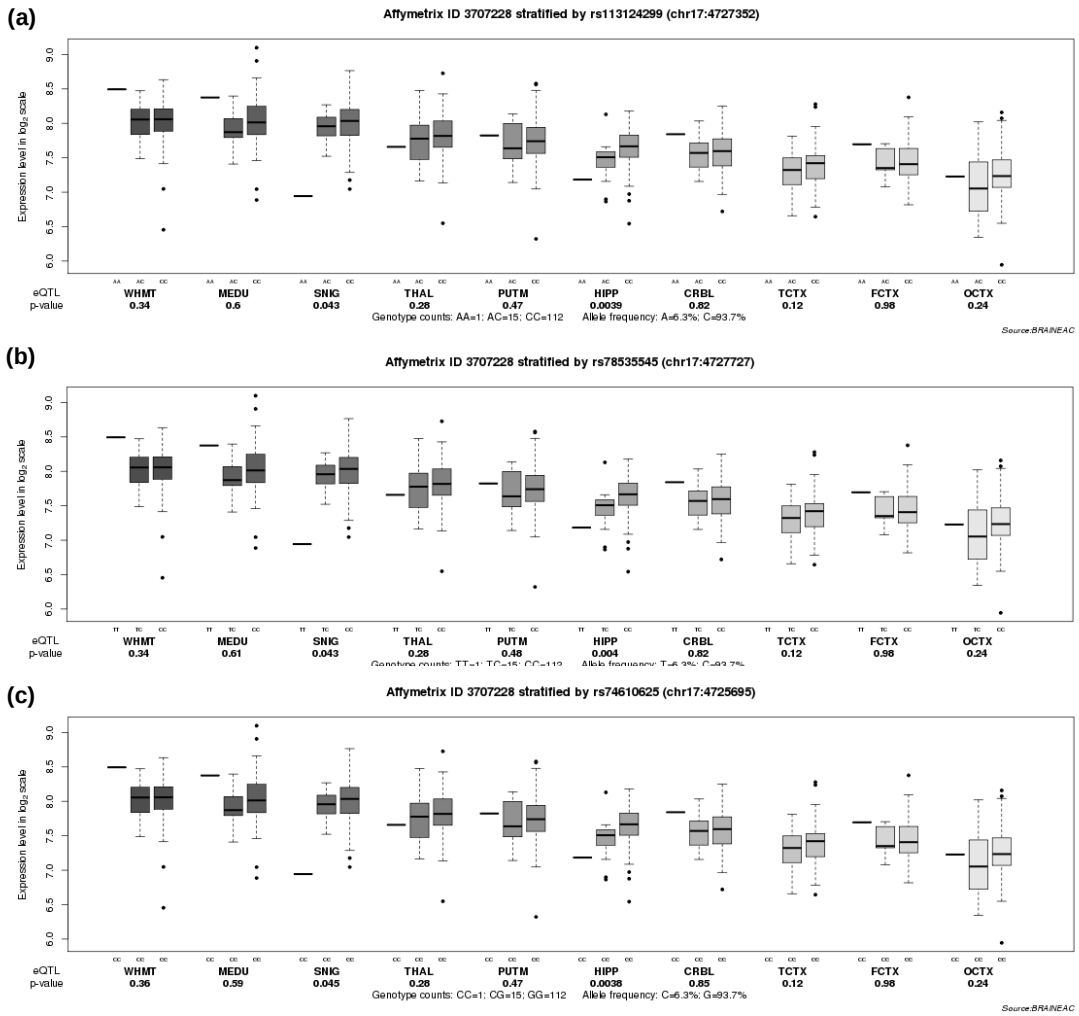

Figure S3. Three top AD-related *PLD2* SNPs rs113124299 (a), rs78535545 (b), and rs74610625 (c) showed significant exon-specific (exprID 3707228) eQTL effect in the hippocampus in the Brain eQTL dataset. The Brain eQTL Almanac (Braineac, http://caprica.genetics.kcl.ac.uk/BRAINEAC/), a web-based resource to access the UK Brain Expression Consortium (UKBEC) dataset, provides the brain eQTL data across ten brain tissues of 134 neurological normal individuals.