**TOMM40 genetic variants cause neuroinflammation in Alzheimer's disease**

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**Abstract**

It suggests that mitochondrial dysfunction is linked to neuroinflammation. Translocase of outer mitochondrial membrane 40 (TOMM40) is located in the outer membrane of mitochondria. TOMM40 genetic variants may be involved in mitochondrial function and increase the risk of Alzheimer’s disease (AD). However, t the role of TOMM40 in neuroinflammation of AD remains unclear. In this study, four exonic variants within *TOMM40-APOE* region (rs772262361, rs157581, rs11556505, rs440446) were identified from 80 AD patients by using next-generation sequencing. Four functional variants were further evaluated in 213 normal control, 393 AD patients and 1025 controls from Taiwan biobank. Two TOMM40 missense variant rs157581 (c.339T>C, p.Phe113Leu) and rs11556505 (c.393C>T, p.Phe131Leu) were linked to increased AD susceptibility. Our results demonstrated that TOMM40 genetic variants, but not wild-type (WT) TOMM40, cause microglial activation. TOMM40 missense variants activate NF-κB cascade and NLRP3 inflammasome activation in BV2 microglial cells. Moreover, TOMM40 genetic variant upregulated pro-inflammatory cytokines in microglia cells expressing (F113L) and (F131L) TOMM40, leading to loss of hippocampal neurons. The plasma levels of inflammatory cytokines, including IL-6, IL-18, IL-33 and COX-2, were significantly upregulated in AD patients carrying TOMM40 variants. AD patients carrying TOMM40 genetic variants displayed the upregulated level of inflammatory cytokines.

**Keywords:** Alzheimer's disease, TOMM40, SNP, neuroinflammation, NLRP3, NF-κB, microglia, hippocampal neurons.

**TOMM40 genetic variations and mitochondrial dysfunction and inflammation in Alzheimer's disease**

1. **Introduction**

Alzheimer’s disease (AD), characterized by selective neuronal loss in brain regions involved in emotional and cognitive function, is the most common cause of dementia among older people [1]. Genetic variants increases the risk of developing AD [2-4]. *APOE*, located at 19q13.32, is the strongest risk factor for AD, accounting for approximate 50% of total risk contribution [5-10]. However, not all patients carrying *APOE* variants develop AD. Genome-wide association studies reveal that genetic variants neighboring *APOE* loci also increase the AD risk [11, 12]. *APOE* surrounding genes, such as *TOMM40*, *PVRL2*, and *APOC1*, display strong linkage disequilibrium (LD) in *APOE* region and may also have direct pathogenesis toward AD [13-16].

Translocase of outer mitochondrial membrane 40 (TOMM40) is one of the *APOE* surrounding genes which located adjacent to 5′-upstream of APOE. TOMM40 is the channel-forming subunit of the translocase of the mitochondrial TOMM complex that is essential for protein import into mitochondria [17]. Many studies suggest that TOMM40 gene may contribute to the risk of AD [18, 19]. Single nucleotide polymorphisms (SNPs) within TOMM40 are associated with amyloid deposition and influence the metabolism of amyloid beta (Aβ) [20-22]. One of the possible mechanisms for TOMM40 toward AD pathology is the toxic effect involving mitochondria autophagy for Aβ42. *TOMM40* poly-T repeats polymorphism within intron 6 (rs10524523) has been shown to affect the AD age of onset and contribute to AD susceptibility by modulating the expression of *TOMM40* and *APOE* transcription [23-25]. Differential transcription of *TOMM40* RNA in the brain has been shown to be an indicator of mitochondrial dysfunction in AD [18]. The majority of the genetic variants of *TOMM40* that were associated with AD susceptibility often resided in noncoding regions with unclear functions [26]. In AD, the related quantitative traits of functional genetic variants of *TOMM40* are still unclear. Genetic architecture of AD is still unknown. The pathogenic mechanism of TOMM40 genetic variant remains unclear in the pathophysiology of AD.

Mitochondrial dysfunction is involved in the pathogenesis of AD [27]. Progressive accumulation of mitochondrial Aβ directly lead to neurotoxicity [28]. Cellular trafficking systemis involved in importing Aβ into mitochondria [29], including translocases of the mitochondrial membrane complex and the endoplasmic reticulum-mitochondria transfer [30]. Mitochondrial malfunction and impaired protein transport of mitochondria result in the accumulation of Aβ o phosphorylated tau [31]. The major pathway for mitochondrial import Aβ involves the translocase of the outer membrane (TOMM) and the translocase of the inner membrane (TIMM)[32]. Aβ can directly affect mitochondrial respiratory enzyme activity and change mitochondrial membrane permeability. With Aβ42 accumulated in mitochondria, activities of cytochrome c oxidase (COX) were inhibited, resulting in free radical production and neuronal apoptosis [28, 33]. Mitochondria are the major sources of intracellular reactive oxygen species (ROS) and are vulnerable to oxidative stress at the same time [34]. Damaged mitochondrial DNA leads to dysfunction of respiratory chain, which consequently leads to decline of brain hypometabolism.

Microglial-mediated neuroinflammation, the inflammatory response of central nervous system (CNS), is involved in the etiopathogenesiso of AD [35, 36]. In physiological status, microglia, which act as resident macrophages of CNS, mediate the development of CNS and regulate immune responses in the CNS [37]. In pathological or inflammatory stimuli, microglia alter from resting state to activated state and secret pro-inflammatory cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), which subsequently cause degeneration of CNS neurons [35, 36, 38]. Under pathological or inflammatory conditions, crucial step for pathogen associated molecular pattern (PAMP)- or damage-associated molecular pattern (DAMP)-induced microglial activation is the oligomerization and activation of microglial NLRP3 inflammasome complex, which is composed of nucleotide-binding oligomerization domain and leucine-rich-repeat-and pyrin-domain-containing 3 (NLRP3), apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase-1 [39-42]. Dysregulated overactivity of microglial NLRP3 inflammasome resulting from microglial activation is believed to be involved in the pathogenesis of AD [43-45]. Accumulation of Aβ and tau lead to microglial activation and NLRP3 priming, leading to neurodegeneration [46]. Aβ overloaded microglia display differentially genetic alternation and triggered several AD-related genes, such as *TREM2* and *ApoE* [47, 48]. However, the pathogenic mechanism of TOMM40 in AD remain unclear.

In this study, we identified genetic variants within *TOMM40-APOE* region and determined the significance of variants in AD patients. Missense variants within *TOMM40* gene, rs157581 (c.339T>C, p.Phe113Leu) and rs11556505 (c.393C>T, p.Phe131Leu), were associated with increased risk of AD. In this study, it was hypothesized that TOMM40 genetic variants cause loss of hippocampal neurons by triggering NLRP3 inflammasome and upregulating IL-1β, IL-6 or TNF-α. Our results demonstrated that TOMM40 variants lead to microglial activation. Moreover, TOMM40 genetic variants activate NF-κB cascade and NLRP3 inflammasome activation in microglial cells. The plasma levels of inflammatory cytokines, including IL-6, IL-18, IL-33 and COX-2, were significantly upregulated in AD patients carrying TOMM40 variants.

1. **Results**

**AD patients exhibit genetic variants within *TOMM40* gene**

High-density whole-genome association study has shown strong associations of SNPs neighboring *APOE* loci with AD risk [11]. Although *APOE* is the strong risk factor for AD, it is believed that additional factors with *APOE* locus contribute to pathogenesis of AD [19, 49, 50]. TOMM40 is within APOE surrounding regions and locates proximity to *APOE* gene [19, 50]. DNA samples from 80 AD patients were examined by using targeted panel of whole-genome sequencing. Genetic variants within *TOMM40-APOE* region were identified in AD patients (Table 1). Exonic variants were included rs772262361 (a synonymous variant within *TOMM40*, c.198A>G, p.Ser66=), rs157581 (a missense variant in *TOMM40*, c.339T>C, p.Phe113Leu), rs11556505 (a missense variant within *TOMM40*, c.393C>T, p.Phe131Leu), rs440446 (a missense variant in *APOE*, p.Asn14Lys) (Table 1). SNP rs772262361 was located in a CpG rich loci (Supplementary Figure 1). Up to date, the correlation between rs772262361 and AD remain unclear (Supplementary Figure 1). Three exonic variants were statistically significantly associated with AD (Table 1). We were further examined the significance of genetic variants within *TOMM40-APOE* region in AD patients.

**Exonic SNP of TOMM40 are linked to increased AD susceptibility**

The frequency of rs772262361 in Taiwan was further examined in the normal controls (NC) of Taiwan Biobank (controls). All of four functional variants (rs772262361, rs157581, rs11556505, and rs440446) were evaluated in 213 NC, 393 AD patients and 1025 controls. Genotype and allele frequency ofTOMM40 between normal controls (NC), normal controls from Taiwan Biobank (controls) and AD patients were displayed in Table 2. SNP rs772262361 was in Hardy-Weinberg equilibrium at a significance level of 0.05. When adjusted for age, sex, hypertension, and diabetes, the two SNPs, rs157581 and rs11556505, were significantly associated with AD significantly in additive model (OR=0.17, 95% CI 0.03~0.86, *p*=0.02). We further studied the pathogenic effect of TOMM40 variants, rs157581 (p.Phe113Leu; F113L) and rs11556505 (p.Phe131Leu, F131L) in *in vitro* model of AD.

**Expression of TOMM40 genetic variants, but not wild-type TOMM40, cause microglial activation.**

TOMM40 is located in the outer membrane of mitochondria. TOMM40 genetic variants may be involved in mitochondrial function and increase the risk of AD [18]. It suggests that mitochondrial dysfunction is linked to neuroinflammation [51]. In this study, it was hypothesized that TOMM40 genetic variant caused mitochondrial dysfunction drives NLRP3 inflammasome and upregulates pro-inflammatory cytokines in microglia, which subsequently causes cell death of hippocampal neurons. In vitro study using BV2 microglial cells and HT22 hippocampal neurons was performed to this hypothesis. FLAG-tagged wild-type (WT) TOMM40, (F113L) TOMM40 or (F131L) TOMM40 were into BV2 microglial cells. Immunoblotting using anti-FLAG antibody showed that WT, (F113L) TOMM40 and (F131L) TOMM40 were expressed in BV2 microglial cells (Fig. 1A), but not in vector plasmid transfected BV2 microglial cells (control). Increased ionized calcium-binding adaptor molecule 1 (Iba-1) is an indicator of microglial activation. Compared to control or WT TOMM40-transfected BV2 cells, expression of (F113L) TOMM40 and (F131L) TOMM40 led to the increase of Iba-1 protein (Figure 1B).

**TOMM40 genetic variants activate NF-κB cascade and NLRP3** **inflammasome activation in microglial cells.**

Mitochondrial dysfunction triggers activation of NF-κB pathway and NLRP inflammasome [52]. Moreover, mitochondrial reactive oxygen species (ROS) lead to NLPR3 inflammasome activation [53]. Western blot showed that expression of (F113L) or (F131L) TOMM40 in microglial cells increased upregulating protein expression of phospho-IKKα/βSer176/180 and active phospho-NF-κB p65 (Figure 2A). Compared to control or WT TOMM40, TOMM40 genetic variants increased inflammasome activity of BV2 microglial cells by upregulating protein levels of NLRP3, absent in melanoma 2 (AIM2), ASC and cleaved active caspase-1 (Figure 2B).

**TOMM40 genetic variant upregulates pro-inflammatory cytokines in microglia cells, leading to loss of hippocampal neurons.**

NLPR3 inflammasome activation in microglial cells results in overproduction of pro-inflammatory cytokines, leading to neuronal death [28]. TOMM40 genetic variants caused microglial activation. ELISA analysis showed that the protein expression of pro-inflammatory cytokines IL-1β, IL-6 or TNF-α was significantly increased in culture medium (CM) from BV2 microglial cells expressing (F113L) TOMM40 and (F131L) TOMM40 compared with control or WT TOMM40 (Figure 3A). To demonstrate that pro-inflammatory cytokines from BV2 cells expressing TOMM40 genetic variants lead to neuronal death, CM of HT22 hippocampal neuronal cells were replaced with CM of BV microglia transfected with WT, (F113L) or (F131L) TOMM40. As shown in Figure 3B, CM of BV2 cells transfected with (F113L) or (F131L) TOMM40 decreased cell viability of HT22 neuronal cells compared with control or WT TOMM40.

**TOMM40 genetic variants result in the upregulation of pro-inflammatory cytokines in AD patients.**

To determine whether TOMM40 genetic variants increase plasma level of inflammatory cytokines, ELISA was performed to evaluate plasma protein level of IL-1β, IL-6, IL-18, IL-23, IL-33, TNF-α and COX-2. Compared to NC, AD patients carrying TOMM40 genetic variants had a significantly higher plasma level of IL-6, IL-18, IL-33 and COX-2 (Figure 4 and Table 3).

1. **Discussion**

*APOE*, located with chromosome 19q13.32, is a strong risk gene for AD, accounting for approximate 50% of AD cases [9, 16]. *APOE ε2/ε3/ε4* alleles are haplotypes constructed by two missense variants, rs7412 and rs429358. For AD pathology, APOE is found co-localized with cholesterol and fibrillary Aβ in neuritic plaques and neurofibrillary tangles [54]. Compared with AD patients in western countries, the frequency of *APOE ε4* allele is lower in Asian population [55]. There may be other genetic factors contributed to the pathogenesis of AD in Asian patients [50, 56]. *TOMM40* is adjacent to APOE gene. Increasing studies show that TOMM40-APOE region may contribute for AD susceptibility [19, 50, 57, 58]. TOMM40 processes APOE-dependent effect in contributing the etiology of AD [50, 59]. In Chinese AD population, APOE and TOMM40 variants are increased the risk of AD [50].

TOMM40 poly-T repeats polymorphism within intron 6 (rs10524523) has been shown to affect the AD age of onset and contribute to AD susceptibility by modulating the expression of TOMM40 and APOE transcription [24]. Several functional SNPs located at chromosome 19 within *TOMM40-APOE* region were found in the prior assessment of the genetic variances of late onset AD [23, 60].

Mitochondrial dysfunction and neuroinflammation are involved in neurodegeneration [51]. It suggests that mitochondrial dysfunction is linked to neuroinflammation [51]. Under pathogenic condition, pathogen- and danger-associated molecular patterns (PAMP, DAMP, respectively), bind to receptor on microglia and aggravate neuroinflammation [51]. Moreover, mitochondrial impairment triggers the activation of NF-kB pathway and NLRP3 inflammasome [52].

Neuroinflammation, characterized by the activation of microglia, is inflammatory process in central nerve system and is linked to neurodegeneration of AD [35, 42]. Activated microglia release ROS and pro-inflammatory cytokines, leading to neurotoxicity [35, 38, 61]. TNF-α, IL-1β and IL-6 cytokines cause upregulation of ROS [62]. Overproduction of ROS and ROS-induced lipid peroxidation leads to neuronal death and apoptosis [63].

1. **Materials and Methods**
   1. **Patients and control subjects**

Institutional Review Board of Chang Gung Memorial Hospital governed this investigation (IRB No.201700444B0C602, 201802324B0 and 202002551B0). The ethical approval for the study was granted by the IRB of the Taiwan Biobank before the study was conducted (approval number: 201506095RINC and TWBR10801-01). All participants submitted informed consent. Probable AD patients and age-matched control participants were recruited from the Department of Neurology, Chang Gung Memorial Hospital, Linkou Medical Center. AD was diagnosed according to the criteria of the recommendations from the National Institute on Aging- Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease [64]. Patients who had Modified Hachinski ischemic score of >4 or met the NINDS-AIREN criteria for vascular dementia were excluded [65]. The Taiwan Biobank has officially developed data from community volunteers; https://www.twbiobank.org.tw/new\_web\_en/index.php). The available data will be included in this study, including basic demographic information, mini-mental state examination, and experimental data from whole-genome sequencing (WGS).

* 1. **DNA extraction, WGS and data processing**

Genomic DNA was isolated from blood samples with Gentra Puregene Blood Kit (Qiagen). After library amplification, DNA was analyzed for deep targeted sequencing on Ion Torrent PGM system. The panel covering *PVRL2* (GRCh38: 19:44,846,135-44,889,227), *TOMM40* (GRCh38: 19:44,891,219-44,903,688, and *APOE* (GRCh38: 19:44,905,748-44,909,394) was used to examine the genetic variants in 80 AD patients. Sequencing data were aligned to hg38 human reference genome and analyzed analyzed using Torrent Suite Software.Variants were filtered by Bam-Utils v1.0.2. Filtered variants were annotated using SnpEff v4.2. Additionally, Integrative Genome Viewer (IGV) software (http://software.broadinstitute.org/igv/) was used for mutation analysis. The joint variant calling file (VCF) was annotated with refGene gene regions, single-nucleotide polymorphism (SNP) effects, and functional effect prediction tools, as well as Exome Variant Server (EVS) and 1000 Genomes minor allele frequencies (MAFs) using Annovar (http://www.openbioinformatics.org/annovar/). For all mutations, the variants were interpreted using the Human Gene Mutation Database (HGMD, www.hgmd.cf.ac.uk), AD&FTD (www.molgen.ua.ac.be/admutations/), and AlzForum (alzforum.org/mutations) databases and by literature searches.

* 1. **Sequencing and genotyping of genetic variants**

Genetic variants and SNPs indented from the cohort were further confirmed by performing TaqMan analysis or Sanger DNA sequencing (catalog #4317596, Applied Biosystems). SNP rs157581, rs11556505 and rs440446 were examined using TaqMan genotyping probes (C\_3084827\_10/rs157581, C\_2769404\_10/rs11556505, C\_905012\_20/rs440446 ,Thermo Fisher Scientific).

* 1. **Cell culture**

HT22 mouse hippocampal neuronal cells and BV2 mouse microglia were purchased from Elabscience (Cat. EP-ML-0697 and EP-CL-0493) and cultured in 10 % FBS-containing DMEM medium. Cells were grown at 37°C in a humidified air with 5% CO2 and then sub-cultured into different culture plates.

Culture medium of BV2 microglia cells treated with lipopolysaccharide (Cat. L2630, Sigma-Aldrich) was collected and then added to HT22 cells. Following incubation for 24 hours, HT22 hippocampal neuronal cells were used for various experiments mentioned below. Passage numbers of cells used in this study were 3-18.

* 1. **Transfection of TOMM40 genetic variants**

The cDNAs of wild-type (WT) TOMM40 (GeneID:10452), (F113L) or (F131L) TOMM40 mutant were constructed and subcloned into a mammalian pcDNA3 expression vector (Invitrogen) containing FLAG (DYKDDDDK)-tag sequences. WT or mutant TOMM40 plasmid was transfected into cells using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific). After transfection for 2 or 3 days, transfected cells were utilized for the experiments described below.

* 1. **Immunofluorescence staining**

BV2 microglia cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Fixed cells were interacted with anti-Iba-1 primary antibody () for 2 hours. Cells were incubated with Alexa Fluor 488-conjugated secondary antibody (Invitrogen). Images were visualized on LionHeart FX automatic microscope (BioTek) and then analyzed by using Gen5 software (BioTek).

* 1. **Determination of cell viability**

Cell viability was assessed by using CCK-8 assay (Cat 96992, Sigma-Aldrich). Briefly, HT22 cells (1×104 cells/well) were seeded into the 96-well plate. WST-8 was applied into wells for 1 hour, and optical density (OD) at 450 nm was evaluated with a spectrophotometer.

* 1. **Immunoblotting assays**

Proteins were extracted from microglia-like cells, SH-SY5Y dopaminergic cells, zebrafish brain tissue or mouse SN tissue by using RIPA lysis buffer. Proteins were separated using SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Membranes were interacted overnight at 4°C with diluted primary antibodies (Supplemental Table 1). After washing, membranes were interacted with HRP-conjugated anti-rabbit or anti-mouse secondary antibody. Subsequently, immunoreactive proteins were detected using ECL kit. Relative protein level was quantified by using ImageJ software and normalized to β-actin.

* 1. **Measurement of pro-inflammatory cytokines**

The plasma level of IL-1β, IL-6, IL18, IL-23, IL-33, TNF-α or COX-2 was measured using ELISA kit (Cat. ab46052, ab178013, ab215539, ab221837, ab119547, ab267646, ab181421, Abcam). Briefly, 10 μl of plasma samples was added into 96-well pales coated with primary antibody at 25°C for 150 min. Then, biotinylated antibody was loaded in to wells. Following 1 hour-incubation, HRP-streptavidin reagent was added into 96-wells, and OD450 was detected on microplate reader (TECAN).

* 1. **Statistics**

All results were analyzed by using GraphPad Prism Program and SASsoftware version 9.1.3. Data were presented as mean ± SD value. Statistical significance was evaluated by one-way ANOVA with Tukey’s post-hoc test (multiple groups) or unpaired two-tailed Student’s t-test (two groups). Statistically difference was considered as significant at P value < 0.05. Demographic data and the frequencies of genotypes between AD patients and control subjects were compared by χ2-test or t-test. Multivariable logistic regression adjusted for age, sex, hypertension, and DM were performed for association analyses..

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**Author Contributions**

YCC, YSL, HLW and CCC conceptualized the study and designed the experiments. LSW and KSW performed the experiments and analyzed the data. SCC, WMH, YHH, YYW and YCC conceived and designed the experiments. YCC, HLW and CCC supervised the project and wrote the manuscript. All authors read and approved the final manuscript.

**Conflict of Interest**

The authors declare no conflict of interest.

**Informed Consent Statement**

Informed consent was obtained from all subjects involved in the study.

**Institutional Review Board Statement**

Institutional Review Board of Chang Gung Memorial Hospital governed this investigation (IRB No.201700444B0C602, 201802324B0 and 202002551B0). The ethical approval for the study was granted by the IRB of the Taiwan Biobank before the study was conducted (approval number: 201506095RINC and TWBR10801-01).

**Data Availability Statement:**

All data generated or analyzed during this study are included in this published article.

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**Figure Legends**

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**Figure 1. TOMM40 genetic variants lead to microglial activation.** (A) BV2 microglial cells were transfected with empty pcDNA3 vector (control), cDNA of FLAG-tagged wild-type (WT), (F113L) or (F131L) TOMM40. Immunoblot assay showed that TOMM40 expressed in mitochondrial fraction of BV2 cells. Cytochrome c oxidase subunit IV (COX-IV) was used as an internal control for mitochondrial fraction. (B) immunofluorescence staining showed that expression of (F113L) or (F131L) TOMM40 leads to activation of BV2 microglial cells by upregulating protein level of Iba-1. Each bar represents mean ± SD of four experiments. \*\**P*<0.01 compared to BV2 microglial cells expressing WT TOMM40.



**Figure 2. TOMM40 genetic variants induce activation of NF-κB signaling and NLRP3 inflammasome.** (A) Immunoblot assay showed that (F113L) and (F131L) TOMM40 induced activation of NF-κB cascade by increasing protein expression of phospho-IKKα/βSer176/180 and phospho-NF-κB p65Ser536 in BV2 microglial cells. (B) Compared with control or WT TOMM40, (F113L) and (F131L) TOMM40 caused activation of NLRP3 or AIM2 inflammasome and upregulation of pro-inflammatory cytokines by increasing protein expression of NLRP3, ASC and cleaved caspase-1, in BV2 cells. Each bar shows mean ± SD of four experiments. \**P*<0.05, \*\**P*<0.01, \*\**P*<0.001 compared to BV2 microglial cells transfected with WT TOMM40.

**Figure 3. TOMM40 genetic variants induce the secretion of pro-inflammatory cytokines in microglial cells, leading to cell death of hippocampal neurons.** (A) Compared to control or WT TOMM40, BV2 microglial cells expressing (F113L) or (F131L) TOMM40 resulted in the increase of pro-inflammatory cytokine IL-1β, IL-6 and TNF-α in culture medium (CM). (B) CM of HT22 hippocampal neurons were replaced with CM from BV2 microglial cells transfected WT, (F113L) or (F131L) TOMM40. One day after replacement, CM of BV2 microglia cells expressing TOMM40 genetic variants reduced cell viability of HT22 hippocampal neurons. Each bar represents mean ± SD of four experiments. \**P*< 0.05 or \*\**P*<0.01 compared to control BV2 microglial cells.



**Figure 4. TOMM40 genetic variants result in the upregulation of pro-inflammatory cytokines in AD patients.** (A-G) ELISA assay showed that the expression of IL-6, IL-18, IL-33 and COX-2 was significantly increased in AD patients carrying TOMM40 genetic variants (AD with TOMM40) compared to NC or AD patients. \*\**P* < 0.01 compared with NC.

**Supplement Figure 1. SNPs in TOMM40 in genomic DNA of AD patients.** (A) Physical loci of the associated SNPs within the TOMM40-APOE region. (B) Sanger sequencing confirmed the sequence of rs772262361 from AD patient.

**Table 1. Genetic variants within *TOMM40-APOE* region associated with risk of AD.**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Gene | SNP | Position | MAF (cases/NC) | MAF\*  dbSNP | OR (95% CI) | *P* value |
| *PVRL2*: Intron variant | rs394221 | 45368424 | 0.51/0.38 | 0.45 | 1.7 (1.2~2.4) | 0.001 |
| *TOMM40*:Synonymous, p.Ser66= | **rs772262361**\* | 45394870 | 0.013/0.0 | 0.00004 | - | - |
| *TOMM40*: Intron | rs184017 | 45394969 | 0.34/0.16 | 0.20 | 2.8 (2.0~4.0) | 4.2E-08 |
| *TOMM40*: Intron | rs2075650 | 45395619 | 0.25/0.07 | 0.13 | 4.2 (2.8~6.1) | 1.1E-10 |
| *TOMM40*: Missense, p.Phe113Leu | **rs157581**\* | 45395714 | 0.38/0.23 | 0.23 | 2.1 (1.5~2.9) | 4.4E-05 |
| *TOMM40* : Missense, p.Phe131Leu | **rs11556505**\* | 45396144 | 0.26/0.10 | 0.11 | 3.3 (2.2~4.8) | 2.5E-08 |
| *TOMM40*: Intron | rs157582 | 45396219 | 0.34/0.18 | 0.22 | 2.4 (1.7~3.3) | 3.3E-06 |
| *APOE*: Missense, p.Asn14Lys | **rs440446**\* | 45409167 | 0.56/0.33 | 0.38 | 2.6 (1.9~3.6) | 1.4E-08 |
| *APOE*: Intron | rs769449 | 45412079 | 0.25/0.08 | 0.11 | 3.6 (2.4~5.3) | 2.7E-09 |

\*SNPs marked in bold were missense variants. **rs772262361**was a point mutation.

**Table 2. Demographics of NC, AD patients, Controls from Taiwan Biobank.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **NC** | **AD Patients** | **Controls** | ***P* value1** | ***P* value2** |
| Number | 213 | 393 | 1025 |  |  |
| Age (years) | 67.4 ± 10.3 | 74.0 ± 8.7 | 58.7 ± 5.4 | < 0.0001 | < 0.0001 |
| Men, N (%) | 111 (52.1) | 142 (36.1) | 524 (51.1) | 0.0001 | < 0.0001 |
| Education (years) | 8.1 ± 4.5 | 6.2 ± 4.7 | 5.3 ± 1.1 | < 0.0001 | < 0.0001 |
| Hypertension, N (%) | 99 (46.5) | 191 (48.6) | 207 (20.2) | 0.62 | < 0.0001 |
| Diabetes, N (%) | 153 (71.8) | 271 (69.0) | 81 (7.9) | 0.46 | < 0.0001 |
| Global cognition, MMSE | 24.3 ± 5.4 | 16.5 ± 6.5 | 27.1 ± 2.6 | < 0.0001 | < 0.0001 |
| *APOE* ε4 carrier, N (%) | 12 (5.63) | 201 (51.2) | 166 (16.2) | < 0.0001 | < 0.0001 |
| *TOMM40* |  |  |  |  |  |
| rs772262361, p.Ser66= AA/AG (%) | 100/0 | 99.5/0.5 | 100/0 | - | - |
| rs157581, p.Phe113Leu TT/TC/CC (%) | 67.3/30.3/2.4 | 40/49.9/10.1 | 59.9/35/5.1 | < 0.0001 | < 0.0001 |
| rs11556505, p.Phe131Leu CC/CT/TT (%) | 92.6/7.4/0 | 54.4/40/5.6 | 81.9/17.3/0.9 | < 0.0001 | < 0.0001 |

Data are expressed as percentages or mean ± SD. P value1: NC versus AD; P value2: Control from Taiwan Biobank versus AD.

**Table 3. The plasma level of pro-inflammation cytokines between NC and AD.**

|  |  |  |  |
| --- | --- | --- | --- |
|  | NC  (n =45 ) | AD  (n =37) | *P* value |
| Age (years) | 72.1 ± 9.0 | 72.9 ± 6.0 | 0.6740 |
| Men/Female | 21/24 | 14/23 | 0.4275 |
| APOE ε4 carrier (%) | 0 | 32.4 | <0.0001 |
| IL-1β (pg/ml) | 21.4 ± 8.4 | 23.1 ± 10.8 | 0.3884 |
| IL-6 (pg/ml) | 0.8 ± 1.6 | 1.6 ± 1.5 | 0.0237 |
| IL-18 (pg/ml) | 64.4 ± 53.0 | 193.5 ± 200.6 | <0.0001 |
| IL-23 (pg/ml) | 9.7 ± 3.2 | 10.5 ± 8.9 | 0.5867 |
| IL-33 (pg/ml) | 4.4 ± 0.8 | 4.9 ± 0.3 | 0.0005 |
| TNF-α (pg/ml) | 13.8 ± 8.1 | 14.5 ± 18.9 | 0.9342 |
| COX-2 (ng/ml) | 0.5 ± 0.06 | 0.7 ± 0.7 | 0.0453 |

Data are expressed as percentages or mean ± SD.