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Exploring the Genetic Associations Between the Use of Statins and Alzheimer's Disease

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ABSTRACT

Objective: Alzheimer's disease (AD) is the most common cause of dementia. The statins have shown beneficial effects on cognitive functions and reduced the risk of dementia development. However, the exact mechanisms of statin effects in AD are not yet fully understood. In this study, we aimed to explore the underlying mechanisms of statin on AD.

Methods: We downloaded AD blood dataset (GSE63060) and statin-related blood gene expression dataset (GSE86216). Then we performed gene expression analysis of each dataset and compared blood gene expressions between AD patients and statin-treated patients. Then, we downloaded mouse embryonic neural stem cell dataset (GSE111945) and performed gene expression analysis.

Results: From the human blood dataset, we identified upregulated/downregulated genes in AD patients and statin-treated patients. Some of the upregulated genes (*AEN*, *MBTPS1*, *ABCG1*) in the blood of AD patients are downregulated in statin-treated patients. Several downregulated genes (*FGL2*, *HMGCS1*, *PSME2*, *SRSF3*, and *ATG3*) are upregulated in statin-treated patients. Gene set enrichment analysis using mouse stem cell dataset revealed a significant relationship of Kyoto Encyclopedia of Genes and Genomes-defined pathway of AD in statin-treated neural stem cells compared to vehicle-treated neural stem cells (normalized enrichment score: −2.24 in male and −1.6 in female).

Conclusion: These gene expression analyses from human blood and mouse neural stem cell demonstrate the important clues on the molecular mechanisms of impacts of statin on AD disease. Further studies are needed to investigate the exact role of candidate genes and pathways suggested in our AD pathogenesis study.

Keywords: Alzheimer's disease; HMG-COA reductase inhibitor; Statin

INTRODUCTION

Dementia has become a global health problem, and Alzheimer's disease (AD) is the most common cause of dementia.[1] AD affects 5.8 million people in the United States, and 47 million people worldwide live with dementia.[1] Unfortunately, we cannot have effective drugs for treating AD.[2] Recent clinical trials on AD treatment using anti-amyloid antibodies or[3] tau antibodies have also failed.[4-6] Although we cannot cure this disease, we

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can delay its progression and improve its symptoms via lifestyle modification and drug treatment.[2] Currently, we manage AD patients with behavioral therapy and several Food and Drug Administration-approved medications, such as anticholinesterase inhibitors and noncompetitive N-methyl-d-aspartate receptor antagonists.[2]

Statins, also known as hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are the most commonly prescribed lipid-lowering drugs.[7] Statins block the conversion of HMG-CoA to mevalonate by HMG-CoA reductase, a rate-limiting enzyme involved in the *de novo* synthesis of cholesterol.[7] Thus, statin therapy effectively reduces low-density lipoprotein cholesterol and triglyceride levels[8] and shows significant benefits against mortality and morbidity related to cardiovascular diseases.[7] Clinical studies have shown the clinical benefits of statins in various diseases, such as pneumonia,[9] chronic kidney disease,[10] cancer,[11] as well as cerebrovascular diseases.[12] Statins have shown beneficial effects in vascular dementia.[13]

In addition to their clinical benefits in vascular and inflammatory diseases, statins have shown negative associations with AD risk in many clinical studies.[14-17] Statin users showed a lower incidence of AD than non-users (1.1% vs. 2.36%; $p<0.001$) in the United States.[14] Meta-analysis data also showed that statin use was associated with a decrease in both AD and non-AD dementia.[15-17] However, some concerns exist regarding the routine use of statins for AD treatment. Although many studies provide strong evidence regarding the beneficial effects of statins in AD, some studies have reported insignificant results of statins on cognitive functions.[18,19] Besides, the exact mechanisms underlying the effects of statins in AD remain unclear. Elucidating the molecular mechanisms underlying the effects of statins in the brain may help determine scientific indications for statin use in AD. In this study, followed by analyzing publicly available microarray and RNA-sequencing data from patients with AD and statin users, we aimed to determine the mechanisms underlying the effect of statins in AD patients.

MATERIALS AND METHODS

1. Human blood dataset analysis

We downloaded the AD blood dataset GSE63060[20] and the statin-related blood dataset GSE86216.[21] The AD blood dataset consists of blood RNA microarray data from an AD case-control cohort.[20] The statin-related blood dataset comprised blood RNA microarray data from patients who underwent high-dose statin therapy (YELLOW II trial).[21] Since both datasets had already been normalized values for gene expression, we did not conduct normalization. We excluded 30% of the probes with the lowest variance and 30% with the lowest mean gene expression across samples. To compare the gene expression of different platforms, we mapped all probe IDs to the corresponding Entrez IDs and removed those that did not have matching Entrez IDs. If multiple probes were mapped to a gene, we selected the probe with the max-mean value by using the "collapseRows" function in the Weighted Gene Co-Expression Network Analysis package.[22]

We curated differentially expressed genes (DEGs) between cases (patients with AD or statin users) and controls by using the limma package.[23] We selected genes with a false discovery rate (FDR)-adjusted p-value of less than 0.05 as DEGs. Pathway analysis was conducted using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases,[24,25] which were obtained from MSigDB.[26] The degree of enrichment or overlap between genes in the databases and our candidate genes was measured using a hypergeometric test. We selected a pathway with an FDR-adjusted p-value of less than 0.05 as an enriched pathway.

2. Mouse neural stem cell dataset analysis

We obtained publicly available RNA-sequencing data to analyze the DEGs within each group. Four conditions had to be satisfied, i.e., the sexually dimorphic regulation in an environment of pravastatin treatment or the lack of pravastatin treatment (GSE111945).[27] Accordingly, the sample datasets were grouped by 2 based on sex: female (SRR6847750, SRR6847751, SRR6847752, SRR6847753, SRR6847754, and SRR6847755) and male (SRR6847756, SRR6847757, SRR6847758, SRR6847759, SRR6847760, and SRR6847761), paired with treatment condition and control. We processed quality checks of FASTQ by using quality control tools, such as FastQC. Following sequence alignment using STAR, the output for each gene labeled by the Ensembl ID resulted in a BAM format file.[28] MultiQC and HTSeq have reported potential problems.[29,30] The raw RNA count matrix was normalized using the "estimateSizeFactors" function in the DESeq2 package.[31] We developed several statistically significant filters in which 50% of the samples should be over raw count 1 in each gene. Normalized gene counts, which were log2 transformed, were visualized using heat maps for genome-wide gene expression. We determined DEGs by using the "DESeqDataSetFromMatrix" function in the DESeq2 package. The volcano plots were described through the "EnhancedVolcano" package with a significant cutoff, lower p-value than 0.01, and over the absolute value of log2 fold change than 0.5. KEGG and GO pathway accounted for the ranked genelists, performed by the DAVID website.[32] Gene set enrichment analysis (GSEA) was processed using the GSEA program.[33] Except for the KEGG pathway, GO pathway, and GSEA, data processing was performed using R.

3. Ethics

All the data analyzed in this study are publicly available. The consent of participants included in the human blood dataset was obtained by the corresponding studies.[20,21]

RESULTS

1. Overlapping genes from the human blood dataset analysis

The AD blood dataset included 145 patients with AD and 104 healthy controls. The statin-related blood dataset comprised 72 and 72 blood samples collected before and after statin administration, respectively. The AD and statin-related blood datasets included the expression values for 11,342 and 10,937 genes, respectively. The number of intersected genes between the 2 datasets was 9,140. Using the limma package, we curated 3,222 and 85 DEGs in the AD and statin-related blood datasets, respectively. Among the 3,222 DEGs, the numbers of upregulated and downregulated genes (AD-UpGs and AD-DoGs) were 1,742 and 1,480, respectively. In cases of the statin-related blood gene expression dataset, among the 85 statin-DEGs, 62 and 23 genes were upregulated and downregulated (statin-UpGs and statin-DoGs), respectively.

The AD-UpGs were associated with immune-related pathways, such as the myeloid leukocyte activation pathway (**Table 1** and **Fig. 1**). The statin-UpGs were associated with an inflammation-related pathway, namely the interferon activation pathway. The AD-DoGs were associated with neurodegenerative disease-, energy-, and inflammation-related pathways. The statin-DoGs were associated with membrane-related pathways, such as those involved in sterol transporter activity and flippase activity.

Table 1. Pathways enriched by AD- and statin-related genes

Variables	Name
Pathway enriched by up-regulated genes in AD sample	GO_MYELOID_LEUKOCYTE_ACTIVATION
	GO_MYELOID_LEUKOCYTE_MEDIATED_IMMUNITY
Pathway enriched by down-regulated genes in AD sample	KEGG_SPLICEOSOME
	KEGG_RIBOSOME
	KEGG_PROTEASOME
	KEGG_PARKINSONS_DISEASE
	KEGG_OXIDATIVE_PHOSPHORYLATION
	GO_ATP_SYNTHESIS_COUPLED_ELECTRON_TRANSPORT
	GO_ANTIGEN_PROCESSING_AND_PRESENTATION_OF_EXOGENOUS_PEPTIDE_ANTIGEN_VIA_MHC_CLASS_I
	GO_ANAPHASE_PROMOTING_COMPLEX_DEPENDENT_CATABOLIC_PROCESS
Pathway enriched by up-regulated genes in statin use sample	GO_RESPONSE_TO_INTERFERON_GAMMA
Pathway enriched by down-regulated genes in statin use sample	GO_STEROL_TRANSPORTER_ACTIVITY
	GO_PHOSPHATIDYLCHOLINE_FLOPPASE_ACTIVITY
	GO_FLOPPASE_ACTIVITY
Pathway enriched by both DEG$_{AD}$* and DEG$_{STATIN}$†	GO_CELL_ACTIVATION
	GO_INTRACELLULAR_PROTEIN_TRANSPORT
	GO_INTRACELLULAR_TRANSPORT
	GO_HOMEOSTASIS_OF_NUMBER_OF_CELLS
	GO_CELLULAR_MACROMOLECULE_LOCALIZATION

AD, Alzheimer's disease; DEG, differentially expressed gene.
*DEG$_{AD}$ indicates differentially expressed genes between the blood AD and control samples; †DEG$_{STATIN}$ indicates differentially expressed genes between the blood samples before and after administration of statin.
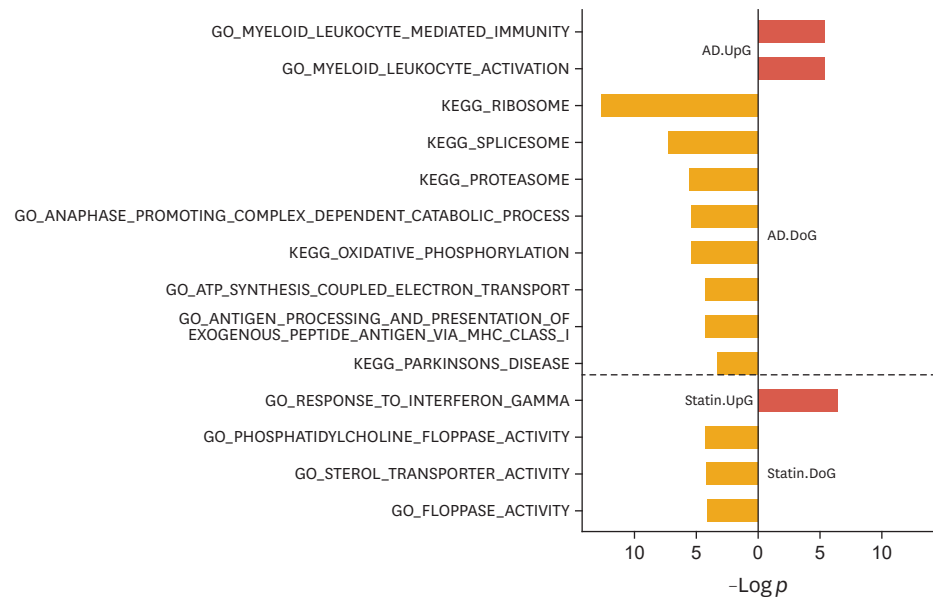
Fig. 1. Pathways enriched by the AD- and statin-related genes in the blood transcriptome (GSE63060 and GSE86216, respectively).
AD, Alzheimer's disease; AD-UpGs, upregulated genes in patients with AD; AD-DoGs, downregulated genes in patients with AD; statin-UpGs, upregulated genes in statin users; statin-DoGs, downregulated genes in statin users.

On comparing the AD-UpGs with the statin-DoGs, we found three common genes, namely, apoptosis enhancing nuclease (*AEN*), membrane-bound transcription factor peptidase 1 (*MBTPS1*), and ATP-binding cassette subfamily G member 1 (*ABCG1*) (**Table 2**). On comparing the AD-DoGs with the statin-UpGs, we found five common genes, namely, fibrinogen like 2 (*FGL2*), 3-hydroxy-3-methylglutaryl-CoA synthase 1 (*HMGCS1*), proteosome activator subunit 2 (*PSME2*), serine/arginine-rich splicing factor 3 (*SRSF3*), and autophagy-related protein 3 (*ATG3*) (**Table 2**).

Table 2. Differentially expressed genes in subjects diagnosed as AD and administered by statin

Variables	Up regulated genes in samples using statin	Down regulated genes in samples using statin
Up regulated genes in AD samples	CEBPB, RRP12, IVD, LDLR, LYN, P2RX7, AGPAT3, C15orf39, STAT1, SLC16A3	AEN, MBTPS1, ABCG1
Down regulated genes in AD samples	FGL2, HMGCS1, PSME2, SRSF3, ATG3	BTG3, SESN1, KIF5C, RPL31, CCNG1

AD, Alzheimer's disease.

## 2 Mouse neural stem cell dataset analysis

To explore how statin treatment affected AD pathogenesis, we performed RNA-sequencing data analysis. For this, we used the mouse neural stem cell dataset GSE111945 composed of RNA-sequencing data from neural stem and progenitor cells of male and female mice.[34] The neural stem and progenitor cells derived from embryonic mouse brains were cultured with pravastatin or vehicle (phosphate-buffered saline) for 24 hours, and RNA from each cell type was extracted and used for generating a transcriptome library.[34]

Fig. 2 shows the results of the RNA-sequencing analysis of male neural stem cells. We identified 116 DEGs (**Fig. 2A and B**). Statin treatment altered the expressions of AD-related genes, such as platelet-derived growth factor receptor-β (*Pdgfrb*), acetyl-CoA synthetase 2 (*Acss2*), and ATP-binding cassette, subfamily A, prune homolog 2 with BCH domain (*Prune2*). Human brain tissue analysis showed that the loss of PDGFRB was related to fibrillar Aβ accumulation in the AD brain.[35] ACSS2 is an important enzyme involved in brain histone acetylation,[36] and ACSS2 dysfunction leads to the loss of protective histone acetylation as well as memory loss in AD.[36] PRUNE2 is related to AD susceptibility in humans.[37,38]

To further investigate the effects of statins in male neural stem cells, we performed a GSEA of DEGs from the neural stem cell dataset. Pathway analysis showed that many of the DEGs were related to the functions of cellular organelles (e.g., nucleosome, ribosome, and mitochondria) and nucleic acids as well as energy metabolism (**Fig. 2C**). The lipid metabolic process and the immune response showed a negative enrichment score (ES), as expected from statin treatment.[39] The DEGs were functionally categorized into three groups: biological process (BP), cellular component (CC), and molecular function (MF) (**Fig. 2D**). The three most enriched terms in the BP were "small molecule transport," "DNA-dependent transcription," and "cell cycle." The three most enriched terms in the CC were "nucleus," "cytoplasm," and "membrane." The three most enriched terms in the MF were "protein binding," "metal ion binding," and "nucleotide binding."

Fig. 3 shows the results of the RNA-sequencing analysis of female neural stem cells. The female dataset showed similar features to the male neural stem cell dataset. *Acss2*, *Prune2*, and *Pdgfrb* were significantly elevated in the statin-treated group (**Fig. 3A and B**). The transmembrane 7 superfamily member 2 (*Tm7sf2*) and HMG-CoA reductase (*Hmgcr*) genes were DEGs in the female dataset but not in the male dataset. These 2 genes are related to cholesterol biosynthesis and are known DEGs in AD-related genetic studies.[40,41]

Fig. 3C and D show the results of the GSEA and DEGs from the female neural stem cell dataset. Pathway analysis showed that many of the DEGs were related to the functions of G-protein-coupled receptor (GPCR) and nucleic acid metabolism (**Fig. 3C**). The lipid metabolic process and oxidation-reduction process showed a negative ES. The three most enriched terms in the BP were "lipid metabolic process," "oxidation-reduction process," and "steroid metabolic process." The three most enriched terms in the CC were "membrane," "internal component of the membrane," and "cytoplasm." The three most enriched terms in the MF were "nucleotide binding," "transferase activity," and "ATP-binding."
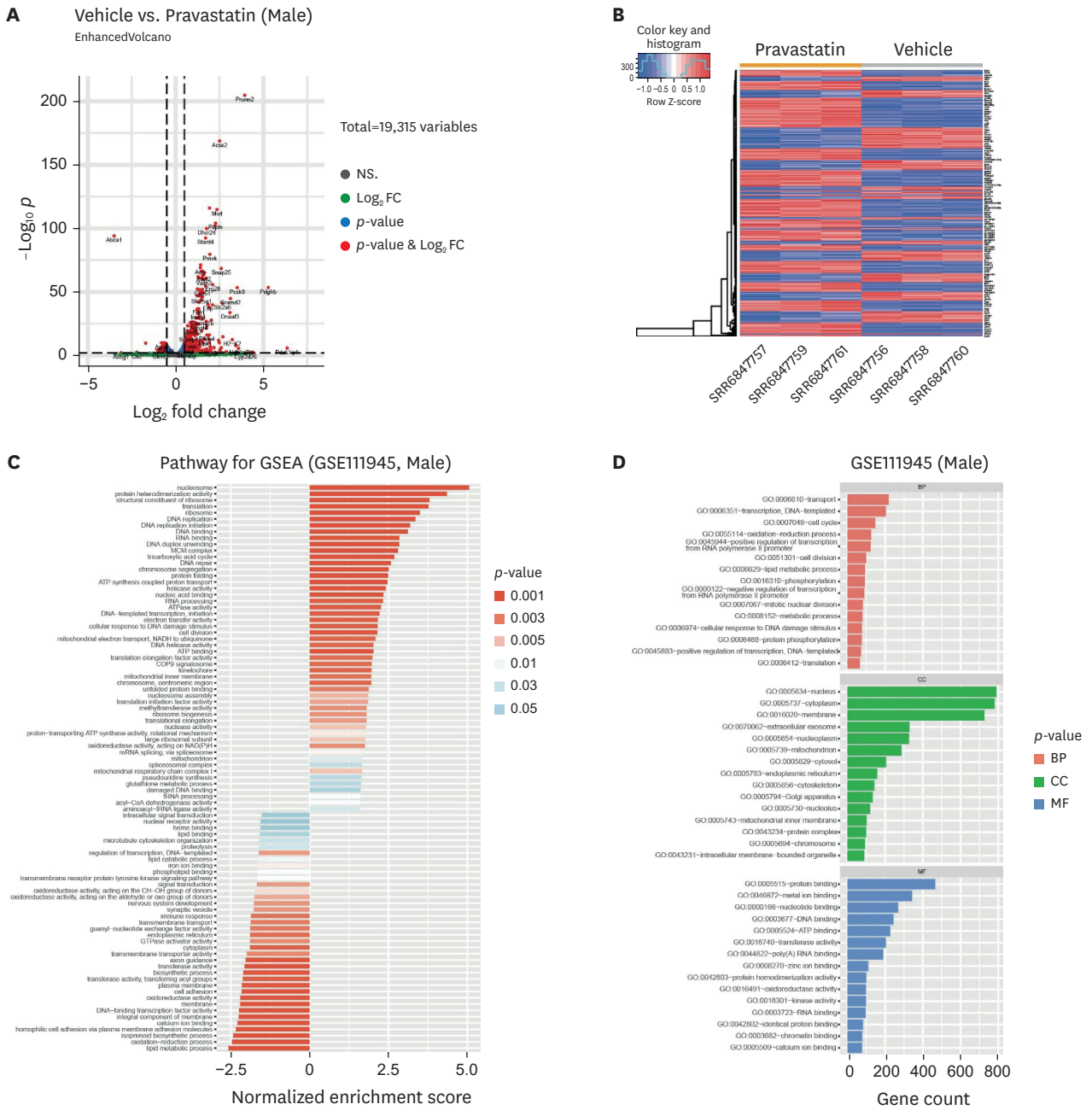
Fig. 2. Gene expression changes in the neural stem cells of a male mouse after 10 μM pravastatin treatment for 24 hours (GSE111945).[34] (A, B) Volcano plot and heatmap of RNA-sequencing data show the DEGs according to statin treatment of the neural stem cells. (C) Enriched pathway analysis of the DEGs from the GSEA. (D) GO enrichment analysis for the DEGs between statin versus vehicle treatment.

DEGs, differentially expressed genes; GSEA, gene set enrichment analysis; GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function.

3. GSEA of AD-related genes from the KEGG database

To further evaluate the role of statins in AD, we performed KEGG pathway analysis. For this, we used gene sets of the KEGG-defined pathway of AD (166 genes). **Fig. 4** shows the GSEA enrichment analysis of KEGG-AD. DEGs associated with statin treatment were enriched in AD-related pathways in both male (ES: −0.47; normalized enrichment score [NES]: −2.24; nominal p-value: 0.0; FDR q-value: 0.0) and female neural stem cells (ES: −0.37; NES: −1.60; nominal p-value: 0.0; FDR q-value: 0.0) (**Fig. 4A and C**). **Fig. 4B** and **4D** are heatmaps of the
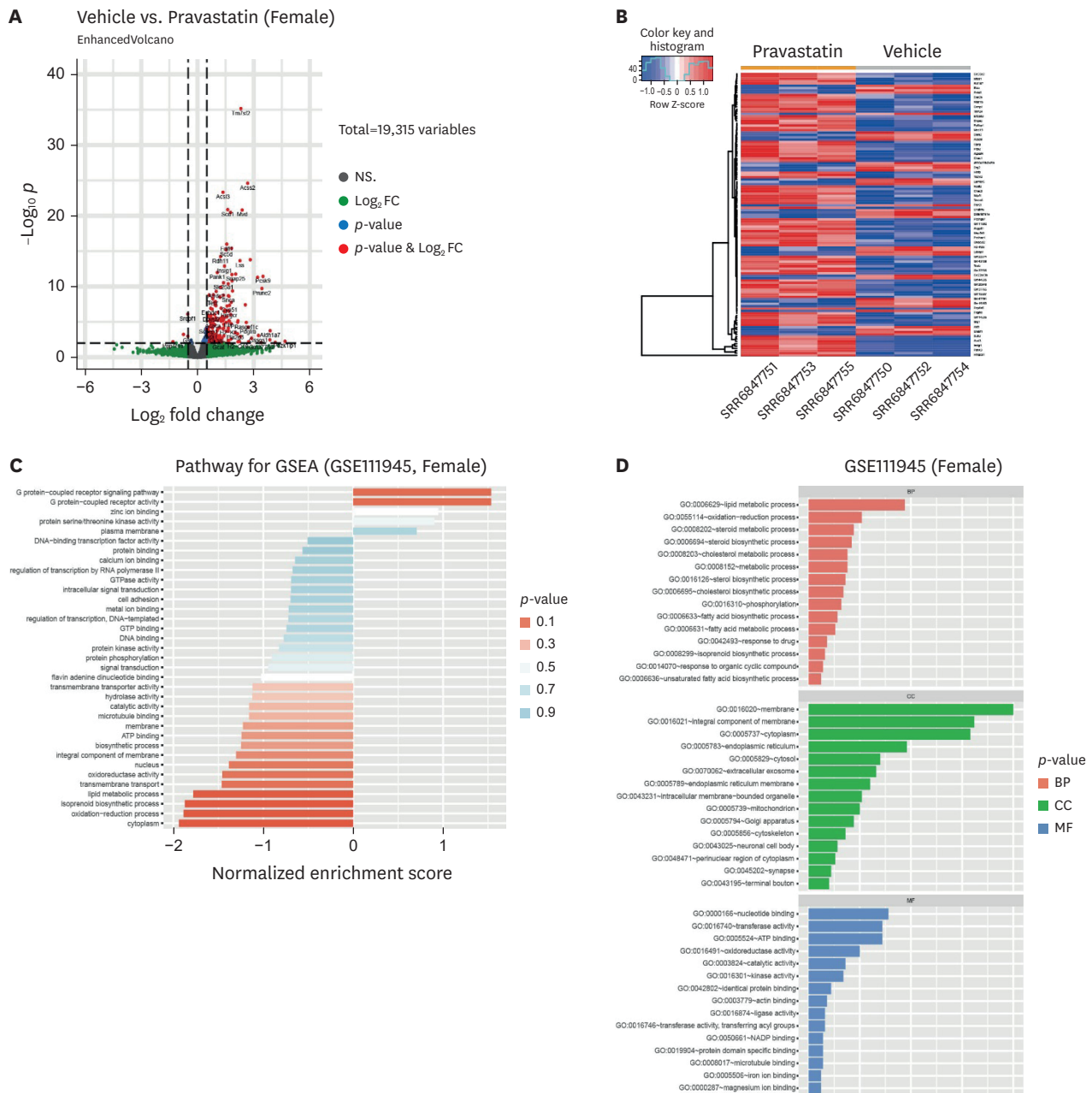
Fig. 3. Gene expression changes in the neural stem cells of a female mouse after 10 μM pravastatin treatment for 24 hours (GSE111945).[34] (A, B) Volcano plot and heatmap of RNA-sequencing data show the DEGs according to statin treatment of the neural stem cells. (C) Enriched pathway analysis of the DEGs from the GSEA. (D) GO enrichment analysis for the DEGs between statin versus vehicle treatment.

DEGs, differentially expressed genes; GSEA, gene set enrichment analysis; GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function.

DEGs in the KEGG-defined pathway of AD. Most DEGs were downregulated in the statin-treated group.

DISCUSSION

A high blood cholesterol level is a well-known risk factor for AD.[42] Moreover, polymorphism in the apolipoprotein E gene (*APOE*) is a major risk factor for AD development.[43] Animal

A Male

B Female

C Male
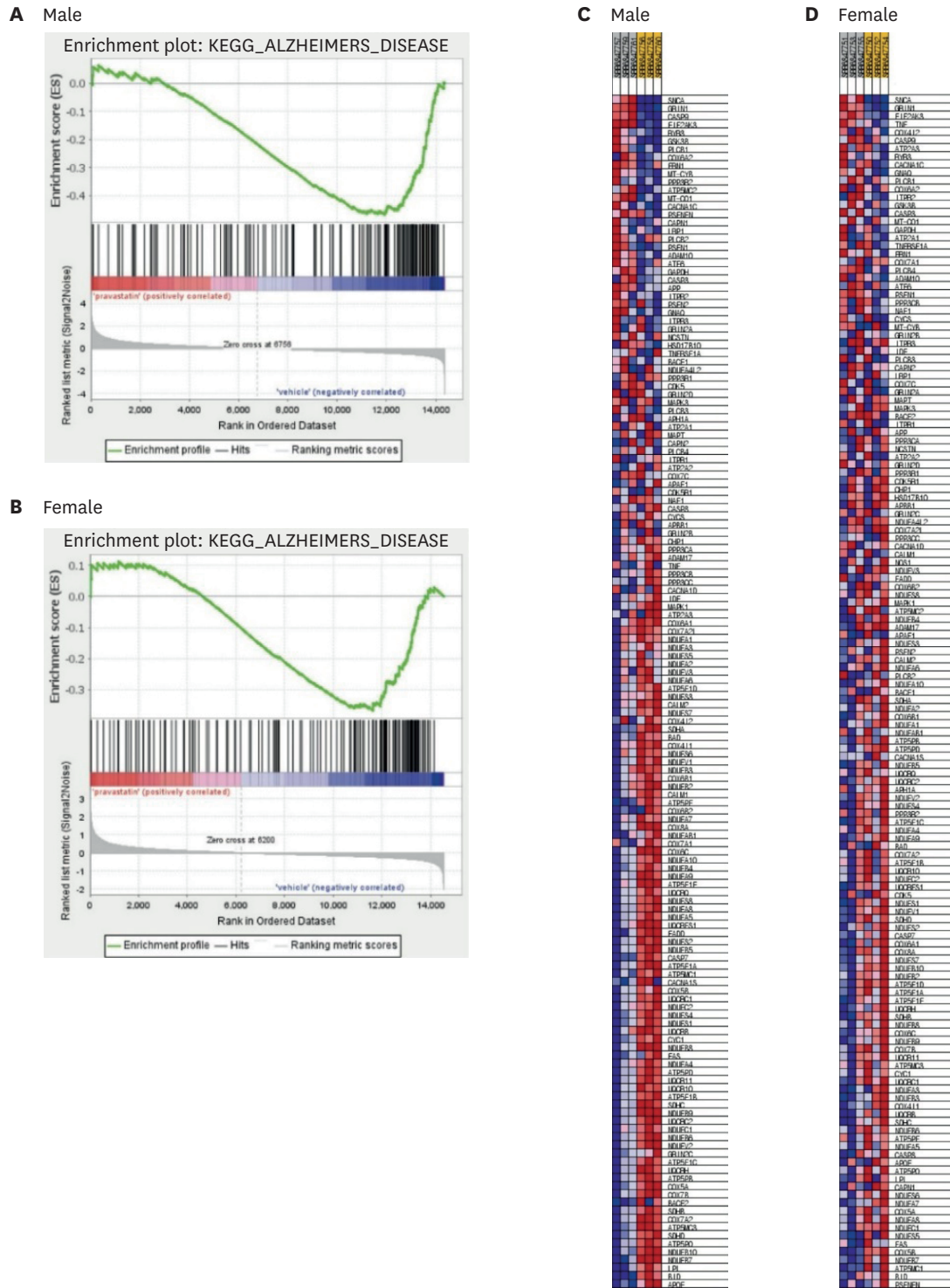
D Female

Fig. 4. Enrichment plots and heatmaps from the GSEA. Enrichment plots generated using the KEGG-defined pathway of AD in male (A, enrichment score: −0.47; NES: −2.24) and female neural stem cells (B, enrichment score: −0.37; NES: −1.60). Heatmaps of genes enriched in the KEGG-defined pathway of AD in male (C, 3 left columns: statin-treated group; 3 right columns: vehicle-treated group) and female neural stem cells (D, 3 left columns: statin-treated group; 3 right columns: vehicle-treated group). Red color indicates a higher expression and blue color indicates a lower expression.
GSEA, gene set enrichment analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; AD, Alzheimer's disease; NES, normalized enrichment score.

studies have shown that high blood cholesterol disrupts autophagy-mediated amyloid β clearance.[44] Cholesterol binds to amyloid β peptides and aids the formation of neurotoxic oligomers.[45] Statins lower cholesterol levels by inhibiting HMG-CoA reductase and improve neurovascular dysfunction by modulating oxidative stress, nitrosative stress, and inflammation.[46,47] Therefore, the major benefits of statins in AD might be attributed to these 2 mechanisms. Our results showed the pleiotropic effects of statins in AD pathology. Based on various mechanisms, these results suggest the beneficial effects of statin for therapy are by lowering lipid levels and reducing oxidative stress.

We performed gene expression analyses in human blood and mouse neural stem cells, and our results provide important clues regarding the molecular mechanisms underlying the effects of statins in AD. To obtain genetic evidence of the effects of statins in patients with AD, we analyzed the whole gene expression patterns of patients with AD and statin users. We then compared the DEGs between the two groups (control versus patients with AD or statin users). This blood transcriptome analysis showed enriched pathways (**Table 1**) and 23 common DEGs (**Table 2**). Among the 23 DEGs, 8 (*AEN*, *MBTPS1*, *ABCG1*, *FGL2*, *HMGCS1*, *PSME2*, *SRSF3*, and *ATG3*) had inverse case-related patterns.

These eight genes showed significant associations with AD pathology. The expression of *AEN*, *MBTPS1*, and *ABCG1* genes increased in the blood transcriptome of patients with AD and decreased in the blood transcriptome of statin users. AEN is a nuclear exonuclease that mediates p53-dependent apoptosis[48]. p53 plays a significant role in neurodegeneration,[49] and AEN is a susceptibility gene for AD in a genome-wide association study.[50] MBTPS1, also called Golgi-resident site-1 protease, is a serine protease that regulates lipid metabolism via the cleavage of substrates and is essential for lysosome biogenesis.[51] The protease cleaves activating transcription factor 6 (ATF6) in the Golgi apparatus for the unfolded protein response-related activation of ATF6, and cleaved ATF6 mediates autophagy functions in neurodegenerative disease.[52] The cholesterol transporter *ABCG1* influences brain cholesterol biosynthesis and increases the secretion of both amyloidogenic and non-amyloidogenic precursor proteins.[53] *FGL2*, *HMGCS1*, *PSME2*, *SRSF3*, and *ATG3* gene expressions decreased in the blood transcriptome of patients with AD and increased in the blood transcriptome of statin users. *FGL2* encodes fibrinogen-like protein 2, and the expression of *FGL2* was significantly decreased (fold change: −6.4) in cultured microglia after 24 hours of amyloid β exposure.[54] HMGCS1 is a mevalonate precursor enzyme that mediates cholesterol biosynthesis in the brain.[55] Yao et al.[56] analyzed the GEO database of patients with AD and healthy controls and suggested that HMGCS1 is a key protein related to AD pathogenesis based on protein-protein interaction analysis. *PSME2* is a major immunoproteasome component related to the type I interferon signaling pathway, which mediates neuroinflammation in AD models.[57] SRSF3 is a splicing factor that regulates RNA splicing.[58] It regulates innate immune gene translation in microglia and is a candidate target for restoring immune homeostasis in AD.[59] ATG3 is an essential protein that mediates autophagosome formation,[60,61] and defective autophagy is the major component of AD pathogenesis.[62]

Analysis of the transcriptome of statin-treated mouse neural stem cells suggests possible molecular mechanisms underlying the effects of statins in AD pathogenesis. Impairment of ribosome function and protein synthesis has already been reported in the brain of patients with AD.[63,64] Our data showed that pathways related to ribosome functions and biogenesis were positively enriched in male neural stem cells, but were decreased in the blood of patients with AD (**Figs. 1** and **2C**). However, in cardiomyocytes, statin treatment reduced protein synthesis.[65] Therefore, further studies are needed to investigate the effects of statins on ribosome function in

the brain. Splicing dysregulation is another possible mechanism underlying AD pathogenesis.[66] Hinrich et al.[67] reported that APOE receptor 2 splicing is impaired in the brain of patients with AD and that restoring normal splicing rescues cognitive function in an AD mouse model. Our data showed that pathways related to the spliceosome were decreased in the blood of patients with AD and increased in statin-treated mouse neural stem cells (**Figs. 1** and **2C**).

Statins block the synthesis of isoprenoid intermediates and cholesterol biosynthesis.[68] Statins also decrease the isoprenylation of signaling molecules such as Ras and Rho GTPase.[68] Ostrowski et al.[69] suggested that the inhibition of protein isoprenylation is the mechanism underlying the beneficial effects of statins on AD. Statin treatment inhibits the membrane localization of Rho and Rab proteins, which are involved in vesicular trafficking, and this inhibition of GTPase resulted in decreased amyloid β secretion in neuroblastoma cell lines.[69] Mouse neural stem cells showed consistent results. Statin treatment showed negatively enriched pathways related to the isoprenoid biosynthetic process and GTPase activity (**Figs. 2C** and **3C**). GSEA of AD-related genes provided more statistical evidence for the beneficial effects of statins in AD. The expressions of most AD-related genes were decreased in statin-treated neural stem cells (**Fig. 4**).

Interestingly, sex differences in neural stem cells resulted in different responses to statin treatment (**Figs. 2** and **3**). For example, pathways related to the spliceosome and ribosome were significantly enriched in male neural stem cells but not in female neural stem cells. In contrast, GPCR signaling pathways were highly enriched in female neural stem cells but not in male neural stem cells. GO term analysis also showed results that differed according to sex. Two-third of patients with AD are women, and sex-related differences such as life expectancy, psychiatric symptoms, pregnancy, and menopause impact AD incidence and disease severity.[70] Statin responses also differ between men and women.[71,72] Statin causes more side effects and fewer cardiovascular benefits in women than in men.[71] This implies that there are sex-related differences in the effects of statins in AD, and we should consider these differences when treating patients with AD.

Our study has several limitations. First, we did not obtain a human brain dataset to compare AD-related and statin-related DEGs. Instead, we used the human blood transcriptome data. Further studies using human brain datasets are warranted to our results. Second, we analyzed the mouse embryonic neural stem cell transcriptome to investigate the mechanism underlying the effects of statin therapy. Although neural stem cells are critical components of the brain and therapeutic targets for AD treatment,[73] embryonic stem cells have features different from adult or aged neural stem cells.[74] Third, it is very difficult to conclude that the AD- and the administration of statin-related biological pathways were shared only using the whole transcriptomic dataset. Therefore, we could not propose the generalized result for the significant common biological mechanisms between AD and statin, but suggested the potential hypothesis. Further studies using neural stem cells or the brain tissue of aged mouse models or humans are needed to confirm these findings.

In conclusion, our study suggests possible mechanisms underlying the effects of statin therapy in AD pathogenesis. Statins inversely regulate AD-related genes and compensate for AD-related dysfunctions by modulating ribosome-related, spliceosome-related, and other signaling pathways.

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