Mol Cell Toxicol (2019) 15:345-352
DOI 10.1007/s13273-019-0038-4

ORIGINAL PAPER

Novel *GRN* mutations in Koreans with Alzheimer's disease

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Received: 30 July 2018 / Accepted: 15 January 2019

Abstract

Backgrounds: Alzheimer's disease (AD) and frontotemporal dementia (FTD) are the two most common neurodegenerative diseases leading to early onset dementia (< 65 years). Mutations in the amyloid precursor protein, presenilin, and presenilin 2 genes are involved in some cases of familial early-onset AD (EOAD), while the microtubule-associated protein tau (*MAPT*) and progranulin (*GRN*) mutations have been mainly identified in FTD patients. Clinically, FTD was often misdiagnosed and confused with AD or psychiatric disorders, which could be a challenge in disease diagnosis.
Methods: We performed mutation analysis of *GRN* in 89 Korean patients with clinically diagnosed EOAD. *In silico* predictions were also performed for the variants to estimate their role in different disorders.
Results: No pathological mutations in *MAPT* was identified, but we identified two novel genetic variations in the *GRN* gene: p.Leu585Phe (c.1767G > T) and c.IVS8 + 23_ + 26delTGGG, which occurred independently in two EOAD patients (frequenct of 2/89, 2.2%). Using a combination of clinical and association studies, *in silico* prediction, and 3-D modeling software, we suggest that both mutations are probably pathogenic and involved in FTD.
Conclusion: Our data suggest that it would be important to re-examine EOAD patients who had been diagnosed when the FTD spectrum was not well described and the causative FTD genes had not yet been identified. In addition, we propose initially analyzing genes associated with the first form of suspected dementia and, if the results are negative, studying genes implicated in the other form of dementia.

Keywords: *GRN*, EOAD, AD, Mutation, Korean

Introduction

Alzheimer's disease (AD) and frontotemporal dementia (FTD) are common neurodegenerative diseases, and could under 65 years of age. FTD was initially described as Pick's disease, and until recently was often misdiagnosed as AD or other psychiatric disorders[1]. Approximately, 10-23% cases of FTD occur as an autosomal-dominant form, and 30-50% of FTD patients have family history of dementia[2,3]. To date, more than ten different genes associated with AD and FTD have been identified: amyloid precursor protein (*APP*), presenilin-1 (*PSEN1*), presenilin-2 (*PSEN2*) progranulin (*GRN*), microtubule-associated protein *tau* (*MAPT*), chromosome 9 open reading frame 72 (*C9orf72*), TAR DNA binding protein-43 (*TDP43* or *TARDBP*), fused in sarcoma (*FUS*), valosin-containing protein (*VCP*) and charged multivesicular body protein 2B (*CHMP2B*), and triggering receptor expressed on myeloid cells 2 (*TREM2*). *GRN* and *MAPT* were identified as the main risk factor genes for FTD, and occur with a frequency of 50%[4,5]. Mutations in the other genes were rarely reported.

Although the integrated use of advanced techniques (neuroimaging, biomarkers, and genetics) is gaining popularity, the existing guidelines to aid in the diagno-

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sis of the major forms of dementia remained unclear[6]. From a clinical perspective, FTD and AD may be difficult to distinguish either at onset or during disease progression[7]. Non-cognitive manifestations of FTD such as mood changes, psychosis and variable social conduct can be characterized as initial phases of AD[8]. From a genetic perspective, many of the gene mutations linked to AD, such as *PSEN1* and *PSEN2*, and apolipoprotein E ε4, have also been linked to FTD[9,10]. In patients aged <65 years, the incidence and prevalence of FTD is similar to that of AD[11]. In addition, the *MAPT* gene has been extensively investigated in FTD[12]. and has been recently implicated in AD[13], suggesting that tau pathology might jointly contribute to FTD and AD. On the other hand, some mutations in AD genes have been associated with the early onset familial form of dementia and typical FTD clinical presentations[14,15]. More recently, many patients who were initially diagnosed with AD/EOAD are now recognized as carriers of pathogenic mutations in FTD genes[16,17], especially in the *GRN* gene[18,19].

To date, very few studies on these two genes have been conducted in Asia, especially in Korea. The aim of the present study was to analyze the *MAPT* and *GRN* genes in DNA samples from Korean patients belonging to families clinically classified as probable EOAD, who did not carry mutations in the three main genes linked to AD, namely *PSEN1*, *PSEN2*, and *APP*.

Materials & Methods

Subjects

Genetic screening was performed on 89 probands diagnosed with EOAD under 65 years of age. All samples were obtained from seven hospitals in Korea, from 2010-2014. A diagnosis of EOAD was made based on the clinical criteria of the National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA) criteria[6]. This study was approved by the Ethics Committees of the Seoul National University Bundang Hospital, Chung-Ang University Hospital, Dong-A University Hospital, Inha University Hospital, Myong Ji Hospital, Gil Hospital and Soon Chun Hyang University Hospital.

Genetic analyses

Total DNA was isolated from the buffy coat obtained by density gradient centrifugation of whole blood using the Geneall® Exgene™ Blood SV kit and following the standard procedure (Songpa-gu, Seoul, Korea). All the coding exons and the intron/exon boundaries of *MAPT* and *GRN* were PCR amplified with primers as previously reported[16,17]. Direct sequencing was performed with BigDye Terminator Cyclic sequencing Ready Reaction Kit (company, city, country) on an ABI Prism 3730*xl* DNA analyzer manufactured by BIONEER CORPORATION (DAJEON, Korea). Data alignment was performed using NCBI Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the sequencing data was screened by the DNA BASER (http://www.dnabaser.com) tool. The disease association of the novel mutations we identified was analyzed by screening against 622 unaffected individuals in the Korean Reference Genome Database (KRGDB, http://152.99.75.168/KRGDB/menuPages/intro.jsp). Mutations were also checked in international reference databases, namelythe Exome Aggregation Consortium (ExAC, http://exac.broadinstitute.org/), 1000 Genomes (http://www.1000genomes.org/) databases and UniProt database (http://www.uniprot.org/).

Bioinformatic analysis

Splice site prediction was performed using Human Splicing Finder 3 (HSF3; http://www.umd.be/HSF3/). In order to determine whether the mutations were benign or potentially pathogenic they were screened using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), Sorting Intolerant From Tolerant algorithm (SIFT; http://sift.jcvi.org/), and PROVEAN (http://provean.jcvi.org/index.php) software. Three-dimensional (3D) protein structure was determined using an online Raptor X software (http://raptorx.uchicago.edu/), and structure of the *GRN* variant was compared to the X-ray structure of the normal protein. Superimposed images of variant and normal proteins were processed using Discovery Studio 3.5 Visualizer software[20].

Results

In our study, 89 EOAD paqtients under 65 years of age were screened. They were negative for in *APP*, *PSEN1*, and *PSEN2* genes. While no pathological mutations in *MAPT* were identified, two patients out of the 89 studied (frequency of 3/153, 2.0%) carried two novel different *GRN* mutations: p.Leu585Phe (c.1767G>T), and c.IVS8+23_+26delTGGG (Table 1). Both mutations were missing in the AD and FTD mutation database (http://www.molgen.ua.ac.be/ADMutations/), or in the Alzheimer's Research Forum (http://www.alzforum.org/mutations). These mutations were also checked against the database of Korean Reference Genome Database ([KRGDB]; http://152.99.75.168/KRGDB/menuPages/intro.jsp) and the Exome Aggregation Consortium ([ExAC]; http://exac.broadinstitute.

Table 1. Neuropsychological testing of patients with novel *GRN* mutations.

Patient	Case 1	Case 2
Gender	Female	Female
Age onset	54	58
Diagnosis (final)	AD with logopenic aphavsia	EOAD
Diagnosis Date (MM/DD/YY)	7/6/2010	6/7/2012
Education duration (yrs)	1	12
MMSE (MAX:30)	6	26/30 (1^{st}); 22/30 (2^{nd})
CDR	2	0.5 (1^{st}); 0.5 (2^{nd})
SOB	12	3 (1^{st}); 3.5 (2^{nd})
GDS	6	4 (1^{st}); 4 (2^{nd})
S-IADL (MAX:45)	NA	8/45 (1^{st}); 14/45 (2^{nd})
KNPI (MAX:144)	NA	1/144 (1^{st}); 16/144 (2^{nd})
GDp	22	7 (1^{st}); 4 (2^{nd})
APOE genotype	ε4/ε4	ε3/ε4
Novel mutation	p.L585F (c.1767G>T)	c.IVS8+23_+26delTGGG

GRN: progranulin; MMSE: mini-mental state examination; CDR: Clinical Dementia Rating; SOB: Sum of Box; GDS: Global Deteriorating Scale; S-IADL: Seoul Instrumental Activities of Daily Living; KNPI: Korean Version of Neuropsychiatric Inventory; GDP: Geriatric Depression Scale; F: female; M: male; EOAD: early-onset Alzheimer's disease; AD: Alzheimer's disease; IVS: intron; EX: exon; yrs: years; MAX: maximum; ApoE: apolipoprotein E; del: deletion; 1st: first test; 2nd : second test; NA: not available.

org/about) dataset. *GRN* p.Leu585Phe (c.1767G>T), and c.IVS8+23_+26delTGGG were absent in both of these databases, suggesting that these two mutations could be rare variants.

Upon sequencing, a heterozygous G>T exchange was identified in *GRN* exon 12, resulting in a TTG (Leu)>TTT (Phe) exchange at codon 585 (Figure 1a). p.Leu585 is located at the C-terminal end of the *GRN* protein. *In silico* analysis with PolyPhen2 and 3-D structure prediction suggested that p.Leu585Phe might be a damaging mutation. PolyPhen2 awarded a score of 0.998 for this mutation using the HumDiv data and a score of 0.878 using the HumVar data. SIFT also predicted p.Leu585Phe as a damaging variant, with a score of 0.01. Analysis with PROVEAN suggested that *GRN* p.Leu585Phe is a non-neutral (probably pathogenic) variant. Figure 1b shows a 3-D model of *GRN* with Leu585Phe, compared to the normal *GRN* (#P28799_*GRN*_HUMAN Granulins) for comparison. Differences between the normal and mutant protein were highlighted in the black circle. Leucine was labeled in green and phenylalanine was labeled in red. Significant changes could be seen in the structure of mutant *GRN* due to the charge differences between leucine and phenylalanine. Both leucine and phenylalanine are hydrophobic molecules. Phenylalanine is one of the largest amino acids, and contains a benzene ring as functional group. The benzene ring on phenylalanine may result in extra stress of the C-terminal of granulin. In addition, hydrophobic interactions are induced inside the transmembrane structure. Extra helices could be seen in the loop region due to the phenylalanine, which may result in disturbances of the helical structure by counter electrostatic interactions in the structure of the C-terminal.

GRN c.IVS8+23_+26delTGGG is a 4-base deletion located proximal to the 3' splice junction of exon 8 (Figure 2a). *In silico* analysis of the effect of this variant on splicing was performed using Human Splicing Finder 3 software with several integrated splice site prediction tools (MAXENT, NNSplice, and Genesplicer). These tools predicted that the variant c.IVS8+23_+26delTGGG could decrease the splicing of exon 8, leading to disturbances in the splicing of the GRN transcript, which in turn may cause a frameshift mutation resulting in a premature stop codon and subsequent non-sense-mediated degradation of the gene product (Figure 2b). Below, detailed case reports for the two unknown genetic variants: p.Leu585Phe (c.1767G>T), and c.IVS8+23_+26delTGGG will be presented.

Case report on patient with *GRN* p.Leu585Phe

The p.Leu585Phe (c.1767G>T) is a novel missense variation identified in a female patient, who developed disease at 54 years of age, diagnosed with probable AD in 2009. She had no history of hypertension (HTN), diabetes mellitus (DM) and/or hyperlipidemia. For eight years prior to her visit, she experienced progressive anxiety and memory impairment. Specifically, she had difficulty in sleeping without anxiety, and difficulty in focusing during conversation. In addition, she seemed to have obsessive compulsive disorder (OCD), especially with respect to food. In her daily life, she was able to perform for simple daily tasks such as cooking food, but had difficulties in using kitchen utensils. In addition, she showed some symptoms of language dysfunction. When she was young, she had received only one year of education. Initially, she was suspected to be an AD pa-

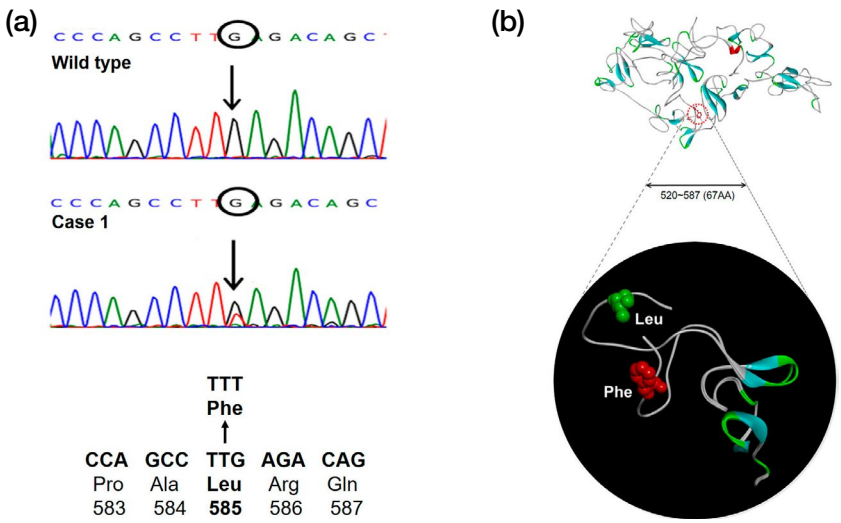

Figure 1. Identification of a novel missense mutation in the *GRN* gene. (a) Sequencing data of the patient with *GRN* p.L585F (c.1767G>T); (b) *In silico* 3-D modeling for *GRN* p.L585F, comparing to the normal *GRN* (#P28799_*GRN*_HUMAN Granulins).

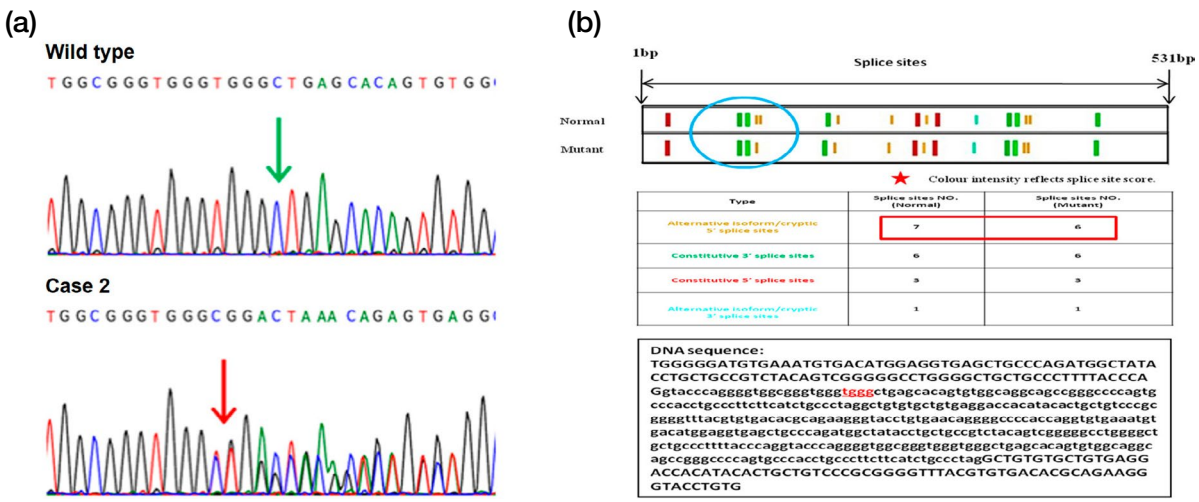

Figure 2. Identification of a novel deletion mutation in the *GRN* gene. (a) Chromatograms of a deletion novel mutation c.IVS8+23_+26delTGGG; (b) Splice sites prediction result showed the novel deletion mutation may cause disturbances in the splicing of *GRN* transcript since it is located near a splice site (shown in the red rectangle and in the blue circle). The four bases that are underlined and highlighted in red indicate the location of the deletion mutation (c.IVS8+23_+26delTGGG) in DNA sequence.

tient. Magnetic resonance imaging (MRI) scan revealed she had a mild degree of ischemic lesions (small vessel disease) in her cerebral white matter, bilaterally. Brain atrophy was also observed. At the time of the neuropsychological examination her Mini-Mental State Examination (MMSE) score was 6/30, and Clinical Dementia Rating (CDR) and Global Deterioration Scale (GDS) scores were 2 and 6, respectively. Fluorodeoxyglucose (FDG)-positron emission tomography (PET) images showed hypermetabolism bilaterally in her frontal, parietal, and temporal cortex, and in the posterior cingulate gyrus (left>right). Pittsburgh compound B (PibPET) showed positive results. She was clinically diagnosed with AD with logopenic aphasia. The patient had a homozygous APOE ε4/ε4.

Case report on patient c.IVS8+23_+26delTGGG

The c.IVS8+23_+26delTGGG is a novel indel identified in a female patient with an age at onset of 58 years diagnosed with probable AD with no family history of dementia. For three years before her first visit to the ho-

spital, she was reported having memory impairment. She received a prescription for donepezil (10 mg) upon initially visiting a doctor owing to complaints of discomfort. She then came to the hospital for her second visit because of her progressively worsening memory. Testing revealed a decline in her ability to compute, and an impaired sense of direction, although she did not get lost at that time. In terms of her daily routine she was not in charge of financial matters for her family, and she was unable to drive in the same year. She was self-employed. When she was young, she had received a 12th grade education. She received a neuropsychological examination twice a year. During the first neuropsychological examination her MMSE was 26/30; CDR and GDS scores were 0.5 and 4, respectively. At the second neuropsychological examination, her MMSE was 22/30; CDR and GDS scores were 0.5 and 4, respectively. She showed impaired color discrimination, and impaired associative ability in these two examinations. An MRI scan showed parietal lobe focal atrophy. Single photon emission computed tomography (SPECT) showed moderate hypoperfusion in both parietal lobes, and in the limbic and right temporal lobes. Neurological examination failed to detect any focal neurological abnormalities. She received a prescription for aricept (10 mg) and wellbutrin (150 mg) after the examination. Since then, the hospital has failed to maintain contact with patient. She was clinically diagnosed with EOAD and had accompanying ApoE ε3/ε4 alleles.

Discussion

One of the challenges facing clinicians who evaluate patients with dementia is determining which clinical diagnosis best fits with the patient's presentation, along with the most likely underlying molecular pathology. Although clinical symptoms often help with this prediction, there may still be variability between diagnosis and pathology. In the past, FTD was recognized as a neurodegenerative dementia that was difficult to clinically distinguish from AD[21], which paved the way for an intense debate about testing the capacity of the current NINCDS-ADRDA criteria[6] to arrive at a clinically differential diagnosis. Today the integration of clinical information including blood biomarkers, genetic analysis, and brain imaging techniques such as Amyloid PET and the FDG-PET facilitates the discrimination of the two different disorders. However, the fact that the clinical symptomatology between AD and FTD is overlapping has been documented[22], leading to the recognition that differential diagnosis of these forms of dementia is difficult in spite of these technologies. In addition, in the early 1990s, the first approach to study patients with familial dementia was genetic analysis for familiar AD. The FTD genes had not yet been discovered as *MAPT* and *GRN*, they were described in 1998 and 2006, respectively[23-25]. In addition, several *GRN* mutations were identified in some patients who had received an initial diagnosis of AD[16,26]. This is an important aspect in mind regarding the differential diagnosis of patients presenting with dementia. Hence, in this study we decided to screen cases that were diagnosed with EOAD, but did not carry pathogenic mutations in AD genes.

Using this approach, two different novel *GRN* variations were identifies from two EOAD Korean patients: p.Leu585Phe (c.1767G>T), and c.IVS8+23_+26delTGGG. The first mutation (p.Leu585Phe) may be a pathogenic mutation, while the second c.IVS8+23_+26delTGGG variant was a single nucleotide polymorphism with unclear pathogenicity. Both patients were characterized by gradual onset, progression, and memory disorder accompanied by deficits in one or more cognitive areas, such as problems with language, motor skills or perception. In addition, at least one abnormal biomarker, observed by structural neuroimaging using MRI or molecular neuroimaging using positron emission tomography (PET), presented in these patients with a clinical presentation consistent with AD[27]. Both patients showed parietal lobe focal atrophy and/or had a positive amyloid PET scan demonstrating fibrillary amyloid pathology suggestive of AD. Notably, each patient carried a *GRN* variant which would be predicted by *in silico* studies to underlie AD pathology rather than FTD. Previous studies have also noted that 9-17% of *GRN* mutations carriers may present with an AD phenotype[26,28]. Another interesting observation is that many *GRN* mutations have been associated with AD clinical diagnoses, followed by poor verbal memory, impairment in working memory and executive functions, language impairment, and corticobasal syndrome (Table 2).

Many studies have demonstrated that polymorphisms in *GRN* modify the risk of developing AD[29-31]. In this report, patient 2 showed a 4-nucleotide deletion in intron 8, near the 3' splice site of exon 7, *GRN* c.IVS8+23_+26delTGGG. The *in silico* tools we used predicted that this variant could decrease the splicing of exon 7, causing disturbances in the splicing of *GRN* transcript. Of note, the nearby mutation *GRN* c.708+6_+9delTGAG has been previously confirmed as pathogenic; thus, altered splicing of exon 7 is a known pathogenic mechanism[32]. Previously, eight intronic polymorphisms (IVS0+3A>T, IVS1+5G>C, IVS2+1G>A, IVS3+2T>C, IVS7+1G>A, IVS7-3C>G, IVS8-1G>C, IVS11-15_EX12+177del;Δ12) have been reported; all of these pathogenic variants are expected to result in loss of a functional *GRN* transcript and consequent haploinsufficiency[17,28,33-37]. Thus, the presence of the novel intronic polymorphism variant we

Table 2. GRN mutations associated with Alzheimer's disease.

Mutation	APOE genotype	Age of onset	Gender	Family history	Neuropsychological testing	Neuroimaging data	Clinical phenotype	Reference
c.1263_1264insGAAGCGAG	ε3/ε4	65	Male	Yes	Poor verbal and visual memory, confrontational naming, and executive function with relative preservation of visuospatial skills	MRI: indicated atrophy of medial and lateral temporal and parietal lobes, right greater than left FDG-PET: revealed hypometabolism in bilateral temporoparietal cortex, demonstrating fibrillary amyloid pathology suggestive of AD	AD	18
c.1A>T, p.M1?	ε3/ε4	54	Female	Yes	Displayed poor verbal memory (that benefitted from recognition), impairment in working memory and executive functions, and language impairment	MRI: There was asymmetric atrophy involving the left temporal lobe, left medial and inferior frontal regions, and left inferior parietal lobe PET: neuritic amyloid plaques, tau-positive neurofibrillary tangles in medial temporal and neocortical regions, revealing autopsy confirmation of AD pathology	AD	
c.1767G>T, p.L585F	ε4/ε4	54	Female	No	She experienced progressive anxiety and memory impairment	MRI: revealed she had a mild degree of ischemic lesions (small vessel disease) in cerebral white matter bilaterally PET: showed hypermetabolism in bilateral frontal, parietal, temporal cortex, and in the posterior cingulate gyrus	EOAD	This study
c.IVS8+23_+26delTGGG	ε3/ε4	58	Female	No	She showed impaired color discrimination, and impaired associative ability in two examinations	MRI scan showed parietal lobe focal atrophy PET: showed moderate hypoperfusion in both parietal lobes, and in the limbic and right temporal lobes.	EOAD	
p.T272SfsX10	64	NA	Female	Yes	Revealed a prominent initial episodic memory impairment, depression and apathies that were the principal cause of compromised daily living activities	NA	AD	19
p.R110X	63	NA	Female	Yes	Being already advanced stage of the disease	NA	AD	
p.C149LfsX10	62	NA	Female	Yes	Characterized by absence of spontaneous speech and severe comprehension impairment	NA	AD	19
p.W304C	65	NA	Female	Yes (two affected brothers)	Disclosed a dysexecutive syndrome with impaired working memory and verbal fluency.	MRI: highlighted diffuse cortical atrophy, more evident in anterior lobes	AD	

GRN: progranulin; APOE: Apolipoprotein E; EOAD: early-onset Alzheimer's disease; AD: Alzheimer's disease; MRI: magnetic resonance imaging; PiB-PET: Positron emission tomography with the beta-amyloid tracer Pittsburgh Compound B; FDG-PET: PET with fluorodeoxyglucose; NA: not available.

identified, c.IVS8+23_+26delTGGG, would support a hypothesis of its pathogenic role. In p.Leu585Phe, the leucine to phenylalanine change at codon 585 is located in granulin D, but this residue is not conserved between motifs; the phenylalanine is found at the homologous residue in granulin motifs A and G. *In silico* models showed that the structure of this variant may be changed in the coil structure at the C-terminus of *GRN*.

On the other hand, the influence of APOE status on the clinical phenotype seen in *GRN* carriers has been previously unstated. Two other studies showed no clear modulatory effect on clinical symptoms[35,38], while one study showed early memory problems in E4 allele carriers[26]. The second patient in this report was heterozygous for the APOE4 allele, while the first patient had homozygous alleles. It could be suggested that the AD pathology seen in these patients could be due to APOE4 status, as the prevalence of amyloid deposition is ~10% in 45-59-year-old cognitively normal E4-carriers, and 37% in 60-69-year-old carriers[39]. On the other hand, the AD-like clinical symptoms and atrophy pattern could be apparent due to the early age of onset of symptoms, as well as the advanced neurofibrillary pathology present in these cases, which may be the main cause of dementia. Future functional studies[40-42] are needed to elucidate the underlying mechanisms by which the mutations contribute to AD pathogenesis. In addition, we propose initially analyzing genes associated with the first form of suspected dementia[1,13,16,43-46] and, if the results are negative, studying genes implicated in the other form of dementia.

In conclusion, we demonstrate that *GRN* p.Leu585Phe (c.1767G>T) and c.IVS8+23_+26delTGGG could be novel pathogenic mutations involved in EOAD. We conducted *in silico* prediction and 3D modeling to arrive at this conclusion, which is in line with previous studies demonstrating the role of *GRN* in EOAD. In addition, our data suggest that it may be important to re-examine AD patients diagnosed when the AD spectrum was not well recognized and the causative AD genes had not yet been identified.

Acknowledgements This research was supported by a National Research Foundation of Korea (NRF) Grants awarded by the Korean government (MEST, No. 2017R1A2B 4012636 & 2017R1C1B5017807). The authors would like to express their gratitude to the proband patient and his family members for their time and support.

Conflict of Interest Lingyan Shen, Seong Soo A. An, Eva Bagyinszky, Vo Van Giau, Seong Hye Choi, SangYun Kim declares that they have no conflicts of interest.

Human and animal rights All human DNA samples were approved by the Institutional Review Board of the Seoul National University Bundang Hospital and Inha University Hospital.

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