

RESEARCH ARTICLE

Genomic Characteristics of Genetic Creutzfeldt-Jakob Disease Patients with V180I Mutation and Associations with Other Neurodegenerative Disorders

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Data Availability Statement: Genome sequencing files of five gCJD patients with V180I will be available in SRA (<http://www.ncbi.nlm.nih.gov/sra>, PRJNA309000). KCDC provides genome information of healthy individuals to researchers who permitted for usage of it. The information of genome variants of healthy Koreans including 134 healthy individuals has just been disclosed for public availability (<http://152.99.75.168/KRGDB/menuPages/intro.jsp>).

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Abstract

Inherited prion diseases (IPDs), including genetic Creutzfeldt-Jakob disease (gCJD), account for 10–15% of cases of prion diseases and are associated with several pathogenic mutations, including P102L, V180I, and E200K, in the prion protein gene (*PRNP*). The valine to isoleucine substitution at codon 180 (V180I) of *PRNP* is the most common pathogenic mutation causing gCJD in East Asian patients. In this study, we conducted follow-up analyses to identify candidate factors and their associations with disease onset. Whole-genome sequencing (WGS) data of five gCJD patients with V180I mutation and 145 healthy individuals were used to identify genomic differences. A total of 18,648,850 candidate variants were observed in only the patient group, 29 of them were validated as variants. Four of these validated variants were nonsense mutations, six were observed in genes directly or indirectly related to neurodegenerative disorders (NDs), such as *LPA*, *LRRK2*, and *FGF20*. More than half of validated variants were categorized in Gene Ontology (GO) terms of binding and/or catalytic activity. Moreover, we found differential genome variants in gCJD patients with V180I mutation, including one uniquely surviving 10 years after diagnosis of the disease. Elucidation of the relationships between gCJD and Alzheimer's disease or Parkinson's disease at the genomic level will facilitate further advances in our understanding of the specific mechanisms mediating the pathogenesis of NDs and gold standard therapies for NDs.

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Competing Interests: The authors have declared that no competing interests exist.

Introduction

Prion diseases (PrDs), also called transmissible spongiform encephalopathies (TSEs), are rare fatal neurodegenerative disorders (NDs) that affect both humans and animals. PrDs are characterized at the pathological level by abnormal accumulation of misfolding prion protein (PrP^{Sc}), affecting the central nervous system (CNS) [1, 2]. In humans, the causative agent is encoded by the prion protein (*PRNP*) gene, found on chromosome 20p13. Several pathogenic mutations in *PRNP*, including P102L, P105L, D178N, V180I, E200K, and V203I, have been reported [3–11]. Pathogenic mutations causing inherited PrDs (IPDs), including genetic Creutzfeldt-Jakob disease (gCJD), fatal familial insomnia (FFI), and Gerstmann-Straussler-Scheinker syndrome (GSS), are responsible for 10–15% of all human PrDs cases [12, 13].

The incubation period for PrDs is influenced by mutations in *PRNP* [14, 15]. Transmission of PrP^{Sc} and the effects of mutations in *PRNP* have been proven in animal models, including primates and rodents [16–18]. In addition to *PRNP*, other genes (loci) are thought to be related to the incubation period of PrDs because of the incomplete penetrance of PrDs [19–23].

PrDs and other NDs, such as Alzheimer's disease (AD) and Parkinson's disease (PD), share several clinical characteristics, including neuropathological features [24–27]. Moreover, in all of these disorders, abnormal accumulation of causative agents, such as prion protein, has been detected in the brains of affected individuals. For example, aggregates of tau and α -synuclein (SNCA) protein have been observed in the brains of patients with AD and PD, respectively [28–30]. Interestingly, SNCA aggregates have also been detected in PrDs-affected brains in sheep and goats [31].

The V180I mutation in *PRNP*, which is the most common cause of gCJD in East Asian patients, was reported first in 1993, and only a few cases of the V180I mutation have been reported in Europe [8, 32, 33]. Most cases of gCJD exhibit onset of symptoms in the late 70s, with the disease lasting 2–3 years [34]. A family history of gCJD with V180I mutation is infrequent, and the mutation has been observed in three non CJD individuals; thus, it is unclear whether this mutation causes PrDs [34, 35]. However, gCJD patients with V180I mutation show several clinical features unique from those of patients with sporadic CJD (sCJD) or gCJD with other mutations, such as less frequent myoclonus, cerebellar signs, and visual dysfunctions and slow progression [34, 36]. Thus, V180I is thought to be a pathogenic mutation in patients with gCJD, but may have low penetrance. Moreover, because of this incomplete penetrance, unknown genes may be involved in the pathogenesis of this disease. Thus, analysis of the relationships between gCJD and other NDs, such as AD and PD, may provide insights into unidentified genes affecting PrDs.

Therefore, in the current study, we aimed to identify genomic differences between healthy individuals and patients with PrDs, particularly gCJD patients with V180I mutation, and improve our understanding of the genetic relationships between other NDs and gCJD by analysis of genetic variants in gCJD patients with V180I mutation.

Results

Patient characteristics

Disease onset occurred between 72 and 77 years of age in four of the patients and at 57 years of age in one of the patients (Table 1). Patient no. 4, who experienced disease onset at 57 years of age, was diagnosed with gCJD in 2004. However, the patient visited the hospital again 8 years later (in 2012) and was still alive, although bedridden (last confirmed on October 13, 2015).

With the exception of this patient, who showed atypical disease onset, the other patients in this study showed disease onset similar to those of other gCJD patients with V180I mutation [36, 37].

Table 1. Clinical features of the five gCJD patients with V180I mutation.

Patient no.	Diseases onset (years)	Disease progression	Clinical features
1	77	Rapid	Symptoms: Rapid progressive cognitive impairment, ataxia, dementia, and gait disturbance EEG: Diffuse slow wave
2	72	Rapid	Symptoms: Rapid progressive cognitive decline, depression MRI: No abnormal enhancing focal lesion in the brain DWI: High signal intensity in cortex of temporoparietal lobe, left frontal lobe, and left insular cortex
3	73	Rapid	Symptoms: Rapid progressive cognitive impairment EEG: Spike in right side frontal lobe
4	57	Slow	Symptoms: Muscle cramping, bedridden state EEG: Diffuse cerebral dysfunction MRI: T2 High signal intensity in superficial cortex and basal ganglia
5	73	-	Symptoms: Encephalopathy symptoms, stammering, and nausea continuously

Abbreviations: MRI, magnetic resonance imaging; DWI, diffusion weighted image; EEG, electroencephalogram

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All five gCJD patients with V180I mutation were positive or weakly positive for 14-3-3 protein in the cerebral spinal fluid (CSF) (Table 2). In three of the patients, total tau (t-tau) protein titers were greater than the recommended cut-off level (1,000 pg/mL) [38]. The phospho-tau/total tau ratio (p/t tau ratio) have been recommended as second screening tau test, and recommended p/t tau ratio in ref. [38] were below than 4×10^{-2} . The p/t tau ratio in the CSF of the four patients ranged from 2.209×10^{-2} to 10.093×10^{-2} . Therefore, according to recommended tau protein detection criteria, patients no. 1 and 3 were within the range established for CJD.

Because the CSF of patient no. 4 was not stored, the RT-QuIC assay with substrate replacement was performed using CSF samples from only four patients, and the sensitivities were 75% (Fig 1).

Table 2. Results of biochemical analysis of the five gCJD patients with V180I.

Patient no.	14-3-3	t-tau (pg/mL)	p-tau (pg/mL)	p/t ratio	RT-QuIC with substrate replacement
1	Positive	3088.005	79.403	0.02571	Positive
2	Positive	780.45	17.24	0.02209	Positive
3	Positive	1136.5	28.2	0.02481	Positive
4	Weakly positive	-	-	-	-
5	Positive	4537.8	458	0.10093	Negative

Abbreviation: RT-QuIC, Real-time quaking induced conversion. 14-3-3 protein, tau protein detection and RT-QuIC with substrate replacement were performed using CSF. Scores within the range of CJD are displayed in bold.

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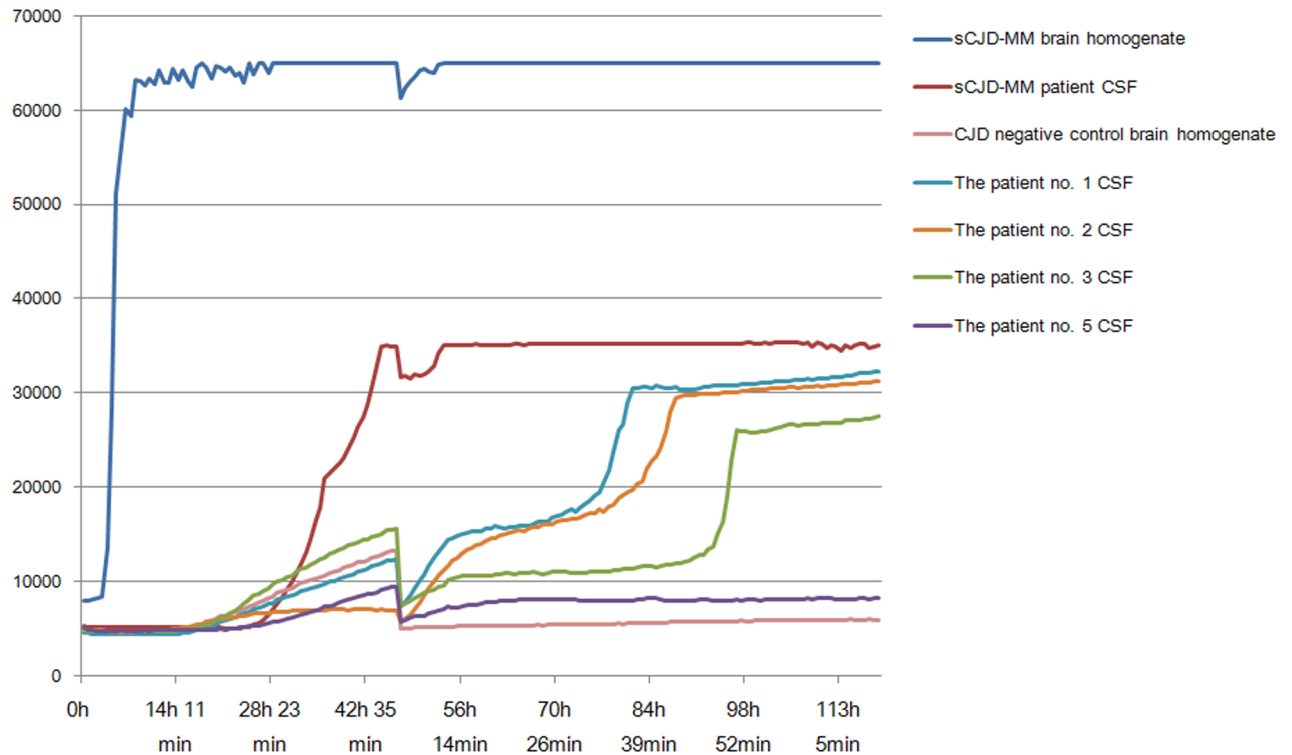


Fig 1. Results of RT-QuIC analysis. ThT fluorescence was measured by relative fluorescence units (rfu) with saturation occurring at 65,000 for every 42 min. The RT-QuIC responses were performed using 10^{-3} dilution of sCJD patient with 129MM type brain homogenate (NHBX0/0001), 10^{-5} CJD negative control brain homogenate (NHBZ0/0005), 15ul of 10^{-1} dilution of CSF from 4 gCJD cases with V180I (the Patients nos. 1, 2, 3 and 5) and 1 sCJD case with 129MM. Each point represents the mean of 4 replicate rfu readings.

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Whole genome data analysis

Raw data for whole genome information are shown in [S1](#) and [S2](#) Tables. A total 72–114 Gbp with Q30 of sequences was generated. The average read length was 126 bp, and about 51–92 Gbp were aligned with mapping quality of Q20 to the reference genome (hg19).

The genomic variants in the five gCJD patients with V180I mutation were compared with 134 of 135 healthy individuals (see [materials and methods](#), [S3 Table](#) and [S1 File](#)), and 125 of 18,648,850 variants were selected for validation ([Fig 2](#)). Of these 125 candidates, 76 were

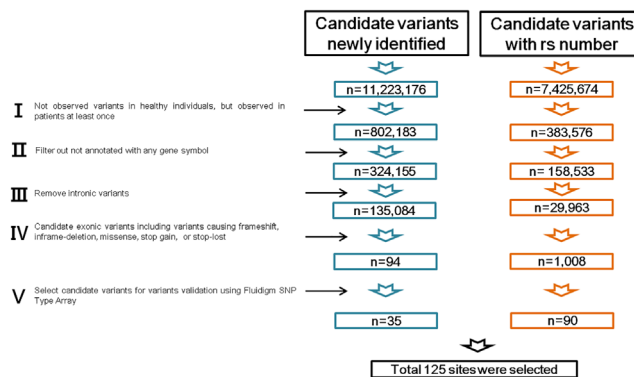


Fig 2. Variant filtering strategy of whole genome sequencing data. Overview of variant filtering to identify gCJD-related genes and gene variants. Each stage was processed followed by stage I–V.

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observed in all five patients, one was observed in four patients, two were observed in two patients, and 46 were observed in only one patient (S4 Table). From these candidates, to eliminate false positives, we performed validation of the 125 candidate variants using Fluidigm SNPtype Assays and Sanger sequencing.

Validation of the 125 candidate variants was performed using Fluidigm SNPtype Assays and Sanger sequencing. Of the 125 candidates, 29 were confirmed as variants, 80 were false positive, five were validated as variants but also observed in healthy individuals, and the remaining 11 failed the validation because of low gDNA quantity from patient no. 5 (Tables 3 and 4 and S4 Table).

Our analysis showed that there were 29 validated variants present in the patients in this study, with one patient harboring as many as 10 variants (patient no. 1). Each validated variant was observed in a single patient. None of the 29 variants were observed in patient no. 3. Variants with known functions with biological terms extracted from gene ontology (GO) analysis of genes harboring each variant are listed in Table 3. Four nonsense candidates were validated as variants, two of which were observed in patient no. 5, and the remaining nonsense variants were observed in patients no. 1 and 2. The other validated variants were missense variants.

Genes harboring validated variants and analysis of biological interactions

Understanding the biological interactions among genes and diseases facilitates the identification of disease mechanisms and target proteins for new drugs. Based on such approaches, we can conveniently sort candidate genes that should be preferentially analyzed before applying other approaches using reverse genetics tools, such as methods for target gene disruption and gene silencing. Thus, genes harboring the 29 validated variants were used as seeds to analyze the biological interaction between genes and NDs, such as PrDs, AD, and PD.

The 29 genes were not found to be directly related to PrDs; however, interactions with other NDs, such as AD and PD, were detected. In addition, indirect relationships with PrDs were observed. Thus, using results from other published studies, candidate genes related to disease onset or susceptibility for CJD with the V180I mutation could be inferred.

Interestingly, *LPA*, *LRRK2*, *TET1*, and *FGF20* were linked directly to AD and/or PD but not to PrDs. However, genes such as *ACO1*, *POSTN*, *LRRK2*, *FGF20*, and *LPA* had indirect associations with PrDs (Figs 3 and 4, S1A–S1E Fig).

Discussion

Characteristics of the patients

14-3-3 protein has been used as a biomarker for CJD, and all five patients in this study were positive for 14-3-3 protein in the CSF. However, positivity for 14-3-3 protein is not always observed in gCJD patients with V180I mutation, as reported previously [37]. Alternatively, to distinguish patients with CJD from those with other diseases having elevated t-tau protein in the CSF, studies have been performed to determine the applicability of the p/t tau protein ratio [38]. In cases of CJD, the p/t tau ratio in the CSF has been shown to be significantly lower than that in patients with other diseases.

Although the diagnostic specificity (100%) of the standard RT-QuIC technique has been proven, the sensitivity of the technique in cases with the V180I mutation has been found to be somewhat lower than that in other cases of CJD [34]. In this study, the sensitivity was 75% by RT-QuIC with substrate replacement which has been used to improve the sensitivity of analysis while maintaining the high degree of specificity of standard RT-QuIC.

Table 3. GO and PANTHER information of genes harboring validated variants.

Gene symbol	Full gene name	PANTHER protein class	GO term	Relationship with NDs
<i>ABCA13</i>	ATP-binding cassette sub-family A member 13	ATP-binding cassette (ABC) transporter	Catalytic activity (GO:0003824) Transporter activity (GO:0005215)	
<i>ACO1</i>	Aconitase 1, soluble	Dehydratase Hydratase	Catalytic activity (GO:0003824)	o
<i>ADAM18</i>	ADAM metallopeptidase domain 18	–	–	
<i>ARAP2</i>	ArfGAP with RhoGAP domain, ankyrin repeat and pH domain 2	Nucleic acid binding G-protein modulator	Binding (GO:0005488) Catalytic activity (GO:0003824) Enzyme regulator activity (GO:0030234)	
<i>ATP13A3</i>	ATPase type 13A3	Cation transporter Ion channel Hydrolase	Catalytic activity (GO:0003824) Transporter activity (GO:0005215)	
<i>C1orf51 (CIART)</i>	Circadian-associated transcriptional repressor	–	–	
<i>DDX1</i>	DEAD (Asp-Glu-Ala-Asp) box helicase 1	RNA helicase Helicase	Binding (GO:0005488) Catalytic activity (GO:0003824) Translation regulator activity (GO:0045182)	
<i>FAM105A</i>	Family with sequence similarity 105, member A	–	–	
<i>FGF20</i>	Fibroblast growth factor 20	Growth factor	Binding (GO:0005488)	o
<i>FNDC1</i>	Fibronectin type III domain containing 1	–	–	
<i>GBP4</i>	Guanylate binding protein 4	Heterotrimeric G-protein	Binding (GO:0005488) Catalytic activity (GO:0003824)	
<i>GPR63</i>	G protein-coupled receptor 63	G-protein coupled receptor	Receptor activity (GO:0004872)	
<i>HACL1</i>	2-Hydroxyacyl-CoA lyase 1	Transferase Dehydrogenase Decarboxylase	Catalytic activity (GO:0003824)	
<i>LPA</i>	Lipoprotein, Lp(A)	Peptide hormone Serine protease Protease inhibitor Annexin Calmodulin	Binding (GO:0005488) Catalytic activity (GO:0003824) Enzyme regulator activity (GO:0030234)	o
<i>LRRK2</i>	Leucine-rich repeat kinase 2	Nonreceptor serine/threonine protein kinase Nonreceptor tyrosine protein kinase;	Catalytic activity (GO:0003824)	o
<i>MYOZ2</i>	Myozenin 2	Structural protein	Structural molecule activity (GO:0005198)	
<i>PDLIM5</i>	PDZ and LIM domain 5	Transcription factor Nonmotor actin binding protein	Binding (GO:0005488) Nucleic acid binding transcription factor activity (GO:0001071) Structural molecule activity (GO:0005198)	
<i>PKP1</i>	Plakophilin 1	Intermediate filament binding protein	Binding (GO:0005488) Structural molecule activity (GO:0005198)	

(Continued)

Table 3. (Continued)

Gene symbol	Full gene name	PANTHER protein class	GO term	Relationship with NDs
<i>POLR1C</i>	Polymerase (RNA) I polypeptide C, 30kDa	DNA-directed RNA polymerase	Catalytic activity (GO:0003824)	
<i>POSTN</i>	Periostin, osteoblast specific factor	Signaling molecule Cell adhesion molecule	Binding (GO:0005488)	o
<i>SFRP4</i>	Secreted Frizzled-related protein 4	Signaling molecule G-protein coupled receptor	Binding (GO:0005488) Receptor activity (GO:0004872)	
<i>SLAIN2</i>	SLAIN motif family, member 2	–	–	
<i>SLC6A5</i>	Solute carrier family 6 (neurotransmitter transporter), member 5	Cation transporter	SLC6A5	
<i>SMC5</i>	Structural maintenance of chromosomes 5	Nucleic acid binding	Binding (GO:0005488)	
<i>TET1</i>	Tet methylcytosine dioxygenase 1	–	–	o
<i>TWF1</i>	Twinfilin actin-binding protein 1	Nonmotor actin binding protein	Binding (GO:0005488) Structural molecule activity (GO:0005198)	
<i>VEPH1</i>	Ventricular zone expressed pH domain-containing 1	–	–	
<i>WDR64</i>	WD repeat domain 64	–	–	
<i>ZRANB2</i>	Zinc finger, RAN-binding domain containing 2	–	–	

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The diagnostic criteria for familial CJD (fCJD or gCJD) include definite or probable CJD plus definite or probable CJD in a first-degree relative and/or a neuropsychiatric disorder plus disease-specific *PRNP* gene mutation (<http://www.cdc.gov/prions/cjd/diagnostic-criteria.html>). Based on these diagnostic criteria, all five of the patients with gCJD in this study could be accurately diagnosed with gCJD because of their neurological symptoms and the V180I mutation, which were further supported by biochemical analyses (Tables 1 and 2).

All guardians of the five patients informed us that they did not have family histories of CJD. Although gCJD with V180I is a genetic form, it is commonly observed in families of gCJD patients with V180I without neurodegenerative symptoms. In a previous study [34], 11 of 186 gCJD patients with V180I confirmed having a family history of the disease. Three of 11 patients had family histories of CJD, and the remaining patients had family histories of dementia. Although gCJD with V180I is not frequently reported in family histories, the possibility that V180I is related to the pathogenicity of the disorder cannot be excluded because the symptoms of gCJD with the V180I mutation have been shown to be unique compared with other types of CJD and with AD [34, 36, 37]. These distinct symptoms led us to postulate that this unique pattern of symptoms and incomplete penetrance were caused by the influence of unknown genes (loci). Thus, we analyzed genomic differences between gCJD patients with V180I mutation and healthy individuals.

Whole genome data analysis

Interestingly, in this study, each of the 29 validated variants was observed only in one patient. Therefore, we concluded that it was difficult to identify gene variants associated with the disease mechanism of CJD and found in more than two patients. Accordingly, we focused on the 29 variants observed in the single patient and analyzed the biological interactions among the

Table 4. Profiles of validated variants found only in the patients.

Chromosome	bp	rs	ref.	alt.	Amino acid change	Type	Gene	Validation methods	Observed patient no.	SIFT	PolyPhen-2
1	71542511	-	T	A	T90S	Missense	ZRANB2	Fluidigm	2	Tolerated, (0.12)	Benign, (0.054)
1	89657064	-	C	A	E266*	Nonsense	GBP4	Fluidigm, Sanger	5	-	-
1	150259289	-	A	G	K361E	Missense	CIART	Fluidigm	2	Tolerated, (1)	Benign, (0)
1	201285776	-	C	A	A266D	Missense	PKP1	Fluidigm	1	Deleterious, (0)	Possibly damaging, (0.588)
1	241946599	rs141496101	G	A	R864H	Missense	WDR64	Fluidigm	1	Deleterious, (0)	Probably damaging, (0.988)
2	15744615	rs137946800	G	C	V203L	Missense	DDX1	Fluidigm	2	Deleterious, (0.04)	Benign, (0.047)
3	15604937	rs147740656	C	G	Q544H	Missense	HACL1	Fluidigm	1	Deleterious, (0.03)	Possibly damaging, (0.617)
3	157160265	-	G	A	A215T	Missense	VEPH1	Fluidigm	4	Tolerated, (0.11)	Possibly damaging, (0.883)
3	194169200	-	A	G	I379T	Missense	ATP13A3	Fluidigm	1	Deleterious, (0)	Benign, (0.063)
4	36230978	-	C	T	S44N	Missense	ARAP2	Fluidigm	1	Tolerated, (0.15)	Benign, (0.014)
4	48422226	rs146342071	G	A	G482D	Missense	SLAIN2	Fluidigm	2	Deleterious, (0)	Possibly damaging, (0.967)
4	95497131	-	A	G	E219G	Missense	PDLIM5	Fluidigm	4	Deleterious, (0)	Possibly damaging, (0.83)
4	120072108	-	G	A	R53H	Missense	MYOZ2	Fluidigm	2	Deleterious, (0)	Probably damaging, (1)
5	14608984	rs74815459	C	G	T252S	Missense	FAM105A	Fluidigm	4	Deleterious, (0.01)	Possibly damaging, (0.827)
6	43488977	-	A	AAGTACTG	K327KVL*	Nonsense	POLR1C	Fluidigm	2	-	-
6	97246455	-	T	C	M385V	Missense	GPR63	Fluidigm	4	Deleterious, (0.02)	Benign, (0.007)
6	159646580	-	C	A	R300S	Missense	FNDC1	Fluidigm	1	-	Probably damaging, (1)
6	161015089	-	C	T	R1177Q	Missense	LPA	Fluidigm	4	Deleterious, (0.03)	Probably damaging, (0.957)
7	37954042	-	T	C	I153M	Missense	SFRP4	Fluidigm	4	Tolerated, (0.34)	Benign, (0.001)
7	48392080	-	G	A	D3562N	Missense	ABCA13	Fluidigm	4	-	Probably damaging, (0.998)

(Continued)

Table 4. (Continued)

Chromosome	bp	rs	ref.	alt.	Amino acid change	Type	Gene	Validation methods	Observed patient no.	SIFT	PolyPhen-2
8	39468116	-	A	G	H138R	Missense	ADAM18	Fluidigm	1	Deleterious, (0)	Probably damaging, (1)
8	16850813	rs200152641	G	C	S135C	Missense	FGF20	Fluidigm	2	Deleterious, (0.05)	Possibly damaging, (0.734)
9	32427337	rs189305274	G	A	V463M	Missense	ACO1	Fluidigm	4	Deleterious, (0.01)	Possibly damaging, (0.841)
9	72912888	-	G	T	E354*	Nonsense	SMC5	Fluidigm, Sanger	5	-	-
10	70333443	-	C	G	P450A	Missense	TET1	Fluidigm	1	Deleterious, (0.03)	Possibly damaging, (0.619)
11	20622957	-	G	A	D96N	Missense	SLC6A5	Fluidigm	2	Deleterious, (0.01)	Probably damaging, (1)
12	40677699	rs34410987	C	T	P755L	Missense	LRRK2	Fluidigm, Sanger	5	Tolerated, (0.73)	Probably damaging, (0.999)
12	44199353	-	G	GA	S38F*	Nonsense	TWF1	Fluidigm	1	-	-
13	38153424	rs200986202	T	A	E578V	Missense	POSTN	Fluidigm	1	Tolerated, (0.07)	Probably damaging, (1)

Abbreviations: bp, base pair; rs, reference SNP ID number; ref, reference allele; alt, alternative allele

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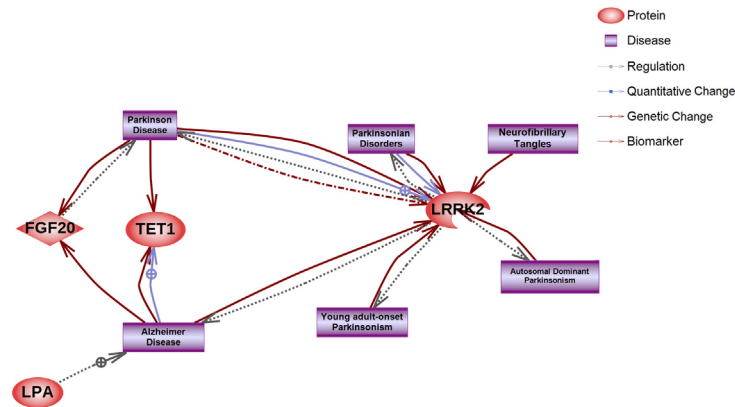


Fig 3. Direct interaction network of genes harboring validated variants with NDs. The red diamonds or circles indicate seed nodes (genes harboring validated variants). Each edge (biological interaction between seeds) status is explained in the legend located in the upper right panel. Terms related to AD and PD are described in purple squares. Because direct interactions of genes harboring validated variants with PrDs were not observed, the term PrDs was excluded.

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gene harboring these variants by GO analysis [39]. Eleven genes were categorized in the GO term of binding (GO:0005488), and 10 were categorized in the term of catalytic activity (GO:0003824). All genes harboring nonsense mutations (*GBP4*, *POLR1C*, *SMC5*, and *TWF1*) were classified into at least one of these two GO terms. Although GO analysis provided specified categories of the core biological functions, these enrichment data could not be used to easily predict disease relationships among genes harboring validated variants.

Four newly identified nonsense variants were detected. Because these variants were observed here for the first time, no functional data for the nonsense variants were available. However, it may be possible to use reported nonsense variants in the same gene to infer the roles of the newly identified nonsense variants. As an example, *SMC5*, which has a role in promoting chromatid homologous recombination, harbored the variant chr9_72912888 (G to T) in patient no. 5. The *SMC5* gene encodes a protein of 1,101 amino acids, and the variant above results in the E354X mutation, encoding a stop codon. Although this variant was reported here for the first time, similar nonsense mutations in tumor samples allow us to infer the phenotype [40]. Additionally, *GBP4*, an interferon signaling-related protein, harbored the newly identified variant chr1_89657064 (C to A) in the same patient, resulting in the E266X mutation. Sixteen stop gain mutations have been reported, as listed in the Ensembl database (GRCh37, release

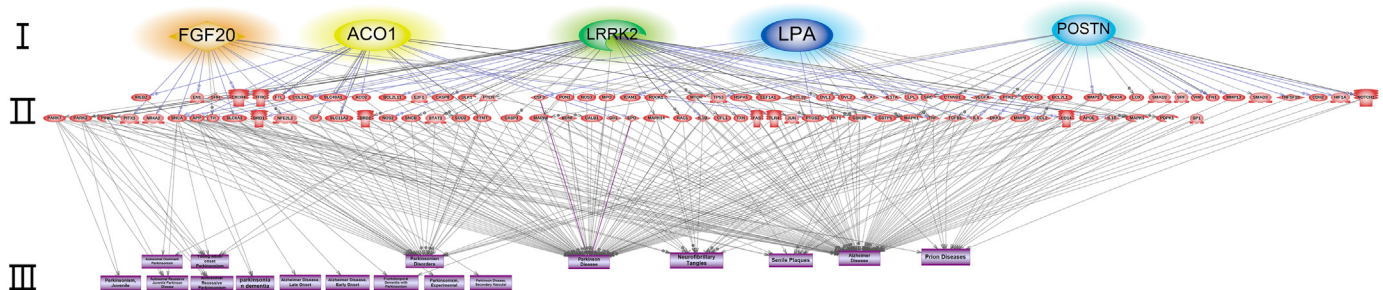


Fig 4. Indirect interaction network of genes harboring validated variants with NDs. Seed nodes (colored orange, yellow, green, blue, or sky blue) are described in layer I. Nodes (proteins or genes) interacting with seeds are listed in layer II. Diseases terms related to PrDs, AD, and PD are described in layer III.

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82). These mutations have been observed in cancer tissues of uterine corpus endometrial carcinoma and lung squamous cell carcinoma. *POLR1C*, a subunit of both RNA polymerase I and III, contained a newly identified nonsense variant (chr6_43488977, which creates K327KVL*) in patient no. 2. Seven similar nonsense mutations, detected in cancer tissues, are listed in the Ensembl database (GRCh37, release 82), and dysfunctions in *POLR1C* activity are associated with Treacher Collins syndrome. Finally, *TWFL1*, an actin monomer binding protein, harbored the newly identified variant chr12_44199353 (S38F*) in patient no. 1. Five similar nonsense variants in *TWFL1* are listed in the Ensembl database (GRCh37, release 82) and were observed in tumors of the large intestine and lung.

Changes in other nucleotides causing nonsense mutations in these four genes have not been reported to have any significant relationships with NDs. The four newly identified variants causing nonsense mutations must be analyzed further to determine their relationships with prion diseases and other NDs using reverse genetic tools, such as gene knockout or gene interference technology.

Genes harboring validated variants and analysis of biological interactions

LPA has been shown to be associated with AD (Figs 3 and 4 and S1A Fig), and we observed a newly identified *LPA* variant (chr6_161015089, R1177Q) in patient no. 4 [41, 42]. Although this mutation is not a form related to the nonsense phenotype, PolyPhen-2 and Sorting Intolerant From Tolerant (SIFT) scores suggested that the mutation caused an abnormal functional change in *LPA* in patient no. 4 (Table 4).

The single-nucleotide polymorphism (SNP) rs189305274, which was observed in patient no. 4, is a variant in *ACO1*. The abnormal functional change in this target resulting from rs189305274 was predicted to be damaging by PolyPhen-2 and SIFT scores (Table 4). *ACO1* was not shown to be directly associated with NDs. However, indirect interactions with NDs, including PrDs, were detected. *ACO1* has been reported to inhibit or regulate amyloid beta (A β) precursor protein (APP) [43, 44], which is involved in the induction of AD and acceleration of PrDs [27, 45, 46]. Thus, mutations that cause functional changes in *ACO1* may delay PrDs progression by regulating APP. These results may explain why patient no. 4 is still alive and suggest that factors inhibiting *ACO1* expression may be promising candidates for repression of CJD.

The S135C mutation (rs200152641) in *FGF20* was observed in patient no. 2. *FGF20*, which was found to interact directly with PD, has been shown to be a candidate therapeutic protein for PD (Fig 3). An indirect interaction was observed between *FGF20* and PrDs (Fig 4 and S1C Fig), and mitogen-activated protein kinase (MAPK) 1 and MAPK3 have been reported to inhibit PrP expression, supporting their potential application in therapeutic strategies against PrDs. Because *FGF20* is known to induce MAPK1 and MAPK3, it may also have applications in the treatment of PrDs. Significant functional changes in *FGF20* protein were predicted by PolyPhen-2 and SIFT (Table 4); such changes may influence MAPK1 and MAPK3 regulation, representing a potential therapeutic strategy for the management of PrDs. Thus, this variant may have influenced the rapid progression of patient no. 2. *POSTN* and *LRRK2* are also involved in the upregulation and activation of MAPK1 and/or MAPK3, respectively (S1B and S1E Fig).

TET1 is associated with late-onset AD (LOAD), and the expression of *TET1* is increased in the hippocampus of patients with preclinical AD and LOAD [47], and the exon region of *TET1* (also called CXXC finger 6 [CXXC6]) has been shown to be related to LOAD [48]. However,

although *TET1* has been shown to be directly related to AD, we did not detect a direct or indirect relationship with PrDs.

The E578V mutation in *POSTN* was observed in patient no. 1. *POSTN* has been reported to promote upregulation of NOTCH-1 [49, 50]. Moreover, NOTCH-1 activation is associated with dendritic atrophy in PrDs, and NOTCH-1 regulation may be controlled by prion infection [51].

LRRK2 has been reported to be related to AD. The SNP rs34410987, which causes a proline-to-leucine mutation (P755L) in *LRRK2*, as observed in this study, was first reported in nine Chinese patients with PD in 2006 and is an important mutation in the diagnosis of PD [52]. However, it is unclear whether this mutation is a causative agent for PD because it has also been observed in healthy individuals, and no significant associations with PD has been reported [53–56]. Despite these previous findings, we could not exclude the possibility that P755L in *LRRK2* may be associated with PrDs. Further studies are needed to analyze the associations between diseases and gene variations. For example, in gastric cancer [57], genotype associations have been analyzed among various types of gastric cancer according to the Lauren's classification system. Additionally, to determine whether P755L may be a causative mutation in PrDs, further analyses are needed. The occurrence of both V180I in *PRNP* and P755L in *LRRK2* may represent a causative haplotype for gCJD. However, the number of patient samples was insufficient to confirm this possibility, and additional studies will be required.

Conclusions

Our results supported the possibility that PrDs may be associated with other NDs through the different gene variants found in five gCJD patients with V180I mutation, including a patient who survived more than 10 years after disease diagnosis. Six of the validated variants were located within genes (*LPA*, *LRRK2*, *TET1*, *FGF20*, *ACO1*, and *POSTN*) that have been shown to be associated with NDs such as PrDs, AD, and PD. Although these gene mutations having significant associations with NDs were observed only in patients with gCJD, more in-depth follow-up studies of the families of these patients are required to definitively conclude that these variants are involved in the pathogenesis of gCJD with V180I. Four variants causing nonsense mutations were newly identified. Further studies are needed to determine the relationships between these mutations and disease development because of the lack of significant phenotype data on the genes harboring these variants (e.g., *SMC5*, *GBP4*, *POLRIC*, and *TWFI*). Despite the lack of data, we cannot exclude the possibility that these genes may contribute to the overall risk of the disease. In order to confirm these data, additional analyses using reverse genetic methods, such as gene knockout or gene interference technologies, are needed.

Materials and Methods

This study was approved by the Institutional Review Board of the Korea Centers for Disease Control and Prevention (IRB No. 2014-06EXP-04-R-A). Written informed consent was obtained from the patients or their legal guardians.

Subjects

All five gCJD patients with V180I mutation described in this study were South Korean natives. Detail information regarding these patients is given in Tables 1 and 2. No patients reported having a family history of CJD. In addition, we also selected the whole genome sequences of 135 healthy Koreans stored at the Division of Center for Genome Science in KNIH as controls for variation filtering. However, the mutation V180I was observed in one individual. Although this individual did not have gCJD, the genomic sequence of this individual was excluded.

Because DNA samples of these controls were not available for further analysis, we also collected DNA samples from 10 healthy individual volunteers (ages 20–40 years) for variant validation. Thus, a total of 145 DNA samples from healthy Koreans were used in this study.

DNA extraction

Genomic DNA was extracted from the patients and 10 control individuals described above. Total DNA was isolated from whole blood samples using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The extracted DNA was quantified using a Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA, USA).

Biochemical analysis of the CSF of patients with gCJD

Detection of 14-3-3 and tau protein. CSF samples from all five patients were analyzed by western blot analysis for 14-3-3 protein expression. Enzyme-linked immunosorbent assays (ELISA) were used to analyze t-tau and p-tau proteins. These biochemical analyses were performed as previously described [58].

Detection of PrP^{Sc} using RT-QuIC with substrate replacement. Purification of recombinant human PrP (rHuPrP: residues 23–231, codon 129M) was performed as previously described [59]. After purification, rHuPrP was stored at -80°C. Brain homogenates (10% [w/v]) were diluted (10^{-3}) with 0.1% SDS/PBS/N2. Next, 85 μ L of RT-QuIC buffer mixture (300 mM NaCl, 160 mM phosphate buffer, 10 μ M thioflavin T (ThT), 1 mM EDTA, and 1 mg rHuPrP) was loaded into each well of a 96-well Optical Bottom Plate (Nalge Nunc International) and mixed with 15 μ L of a CSF sample with 1:10 dilution or diluted brain homogenate. The assay was monitored using an OPTIMA FLUOstar plate reader at 42°C for over 118 h. ThT fluorescence measurements (440 nm excitation and 485 nm emission) were acquired every 42 min. RT-QuIC buffer was replaced with fresh RT-QuIC buffer mixture (as described above) after 48 h. Two human brain homogenates (NHNX0/0001 and NHBZ0/0005) were obtained from National Institute for Biological Standards and Control.

Whole genome sequencing. Whole genome sequencing of the five gCJD patients with V180I (cases 1–5) was performed using the Solexa sequencing technology platform (HiSeq2500; Illumina, San Diego, CA, USA). Briefly, 1–3 μ g of gDNA was fragmented by sonication. Library span size was controlled using a Covaris System to generate ~500-bp inserts. The sonicated DNA was end-repaired using T4 DNA polymerase and Klenow polymerase. Tailing was performed to create sticky ends, and Illumina paired-end adaptor oligonucleotides were ligated to the sticky ends. Adaptor-ligated oligonucleotides were subjected to polymerase chain reaction (PCR) enrichment. Ligated DNA was then size-selected for lengths of about 500 bp, including adaptors of about 180 bp. Cluster generation was performed using amplified library DNA in a flowcell to produce clusters. Purified fragments were loaded onto the Illumina flow cell for sequencing on the Illumina HiSeq2500 instrument.

Raw data are described in S1 and S2 Tables. Reads were aligned to the Human Reference Genome (hg19) using Burrows-Wheeler Aligner (BWA) v0.6.1. Multisample SNP calling was performed using GATK and Picard tools with default parameters.

The genome sequences of the 134 healthy individuals were called and annotated in a manner similar to that of the patients. Next, variant call format (VCF) files were merged using VCF tools v4.1 (<https://vcftools.github.io/>). Differences in variant counts between groups were calculated and analyzed using PLINK v1.9 (pngu.mgh.harvard.edu/~purcell/plink).

Variant filtering. A total of 11,223,176 candidate variants without rs number were identified, of which 802,183 variants were not observed in 134 healthy Korean sequences. Next, intronic variants were removed, and variants causing frameshift, inframe-deletion, missense,

or stop loss mutations were selected. Additionally, 7,425,674 candidate variants with rs numbers were filtered in the same manner. Next, unannotated variants in any gene and intronic variants were removed, and 1,102 candidate exonic variants, including variants causing frameshift, inframe-deletion, missense, or stop loss mutations, were selected for further validation. Of these 1,102 candidates, 110 were found in all five of the patients, and 94 newly identified variants were also selected. Additionally, 20 variants found in 1–4 patients were selected followed by phred scores (over 600). These 224 candidates were selected for variant validation; and after filtering, as described in [S4 Table](#), 125 candidates were processed for further validation.

Validation of selected variants. A total of 125 variants were validated by Fluidigm BioMark Dynamic Array. Variant validation was performed among all five patients and 10 healthy individuals as described above. However, variant validation in patient no. 5 failed. Thus, the variants observed in patient no. 5 were revalidated by Sanger sequencing. However, owing to low gDNA quality, only three variants were revalidated using this method. PCR and sequencing were performed as previously described using the PCR primers listed in [S5 Table](#) [23].

Genome data analysis. All variants that showed different allele frequencies between groups were analyzed using Variant Effect Predictor in ENSEMBL release 81 (based on the GRCh37), and the effects of variants on the annotated canonical transcripts for all genes were assessed using PolyPhen-2 and SIFT [60, 61]. The effects of amino acids substitutions resulting from the different variants were predicted and considered as damaging changes if indicated as ‘probably damaging’ or ‘possibly damaging’ by PolyPhen-2 or if indicated as ‘deleterious’ by SIFT. GO categorization were performed using the Panther Classification System (<http://pantherdb.org>; [Table 3](#)). Known information of analyzed genes was extracted from GeneCards (<http://www.genecards.org>).

Biological network analysis. Biological networks among the genes (proteins) harboring validated variants were created using Pathway Studio 9.0 software (Ariadne, Rockville, MD, USA). The molecular interactions between the genes and NDs, such as AD, PD, and PrDs, were extracted from Elsevier's MedScan text-mining software, which contains biological articles and abstracts. Error information and interactions because of mismatches and mistyping were removed from the output data.

Supporting Information

S1 Fig. Indirect interaction of genes with PrDs, AD, and PD. (A) Indirect interactions of *LPA*, (B) *LRRK2*, (C) *FGF20*, (D) *ACO1*, and (E) *POSTN*. (TIF)

S1 File. Genomic information for the healthy individual with V180I. (DOCX)

S1 Table. Raw data for whole genome sequences. (DOCX)

S2 Table. Alignment summary of raw genome data. (DOCX)

S3 Table. Information of variants in *PRNP* observed in one healthy individual with V180I. (XLSX)

S4 Table. Information for 1,102 variants. All information for the 1,102 variants, including variants generated during the validation process (see stage IV and V in [Fig 3](#)). The Fluidigm array design results are listed in the last two columns. Ninety-nine variants were not designed

for Fluidigm SNPtypeAssays for the following reasons: the target design parameters were not met, and there were regions containing large repeats and/or complex genomic diversity; GC contents were outside of the range of product specification; adjacent variant(s) were found within 30 bp of the target SNP; and target sequences were not supported. 'High' indicates that the target could be designed to the product specifications; 'Medium' indicates that the target could be designed, but with limited support because of problems such as high GC content (over 65%) of the target or indel target.
(XLSX)

S5 Table. Primer sequences for variants validation.
(DOCX)

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Author Contributions

Conceived and designed the experiments: SML YRJ SYK. Performed the experiments: SML MC JWH SWJ. Analyzed the data: SML MC JWH SWJ JL SYK. Contributed reagents/materials/analysis tools: JWH MC SK SSAA SBC SYK. Wrote the paper: SML SYK. Arranged whole genome sequencing data: MC SBC. Helped interpretation of results: HK YSL.

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