Title

Positional cloning and comprehensive mutation analysis of a Japanese family with lithium-responsive bipolar disorder identifies a novel *DOCK5* mutation

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Conflict of Interest Statement

The authors have nothing to disclose.

Abstract

Bipolar disorder (BD) is a severe psychiatric disorder characterized by the recurrence of depressive and manic episodes. Its heritability is high, and many linkage and association studies have been performed. Although various linkage regions and candidate genes have been reported, few have shown sufficient reproducibility, and none have identified the pathogenic genes based on the results of the linkage analysis. To find functional variants that are expected to be rare and have strong genetic effects, we recruited 10 healthy individuals, two individuals with unknown status, and six patients with BD or recurrent major depressive disorder (MDD) from a Japanese family consisting of 21 members. We performed a genome-wide linkage analysis using a 100K single nucleotide polymorphism (SNP) array and microsatellite markers to narrow linkage regions within this family. Subsequently, we performed whole exome sequencing for two patients with BD to identify genetic mutations in the narrowed linkage regions. Then, we performed co-segregation analysis for DNA variants obtained from the results of the exome sequencing. Finally, we identified a rare heterozygous mutation in exon 31 of *DOCK5* (c.3170A>G, p.E1057G). Convergent functional genomics analysis revealed that *DOCK5* was listed as one of the biomarkers for mood state and suicidality. Although *DOCK5* is still a functionally unknown gene, our findings highlight the

possibility of a pathological relationship between BD and *DOCK*5.

Keywords

DOCK5; lithium responder; whole-exome sequencing; linkage study

1. Introduction

Bipolar disorder (BD) is a severe psychiatric disorder characterized by the recurrence of depressive and manic episodes. In the general population, the lifetime prevalence of BD is around 1%, and those of subthreshold BD and bipolar spectrum disorder are 1.4% and 2.4%, respectively (1). In Japan, the prevalence of bipolar disorder is much smaller, at 0.2% (2). Although molecular genetic research indicates that BD is a multifactorial disorder in which both genetic and environmental factors play a role (3), the etiology of BD is not clearly understood. The overall heritability of bipolar spectrum disorders is estimated to be 0.71 (4). Many linkage and association studies have been performed so far, and various linkage sites have been reported (5). However, many of these results do not have sufficient reproducibility, and the linkage analyses have not yet identified the pathogenic gene. Furthermore, with advances in gene technology, several genes have also been identified by genome-wide association studies (GWAS). However, their functional correlations remain largely unknown. A study by Lee et al. showed that the cumulative impact of small general alleles can only account for about 40% of BD phenotypic differences (6). Therefore, it can be hypothesized that rare and highly penetrant mutations may contribute to BD susceptibility. To evaluate this hypothesis, a family with a high frequency of BD can be studied to extract and evaluate genetic

mutations inherited from a common ancestor (7). Therefore, conducted this study with the aim of finding functional variants that are expected to be rare and have strong genetic effects in a family in which BD and related diseases frequently occur. In this study, we recruited 10 unaffected individuals, two individuals with unknown status, three patients with BD, and three patients with recurrent major depressive disorder (MDD) from a Japanese family consisting of 21 members. To narrow linkage regions, we used the family's genetic material to perform a genome-wide linkage analysis using a 100K single nucleotide polymorphism (SNP) array and microsatellite markers. Subsequently, we performed whole exome sequencing on two of the patients with BD to identify genetic mutations on the narrowed linkage regions. Then, we performed co-segregation analysis for DNA variants obtained from the results of exome sequencing.

2. Materials and methods

2.1. Samples

Participants across three generations in a Japanese family including patients with BD and recurrent MDD were enrolled in this study (Figure 1). All affected individuals were diagnosed independently according to the criteria in the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV). Written informed consent was

obtained from all participants. The clinical diagnoses of patients II-2, II-3, and III-5 were BD-I and those of III-1, III-2, and III-6 were recurrent MDD. II-8, with alcoholic psychosis, as well as I-1 and I-2, with no clinical information, were classified as unknown for this linkage study. Interestingly, all affected six individuals were successfully treated with lithium carbonate (Table 1). The pedigree structure of the family recruited in this study is shown in Figure 1. Participants' genomic DNA was extracted from peripheral leukocytes using standard methods after informed consent and approval by the local ethics committee were obtained. Altogether, the 18 available family members were studied, including the six affected patients, 10 unaffected individuals, and two individuals with unknown status.

2.2. Genetic analysis

Written informed consent was obtained at the time of blood sampling from all study participants, including these 18 family members (Figure. 1) and 50 unrelated BD-I patients and 67 healthy controls. The research protocol and consent form were approved by the Institutional Review Boards of Kagoshima University.

2.3. Linkage and haplotype analysis

Genome-wide two-points linkage analysis was performed on six affected family members, 10 unaffected family members, and two family members with unknown status (Figure 1)

using the Gene Chip Human Mapping 100K set (Affymetrix, Santa Clara, CA, USA). After genotyping, a two-points linkage analysis was performed using the GeneSpring GT2.0 (Agilent Technologies, Santa Clara, CA, USA) for narrowing and screening of candidate linkage regions of BD in this family. In this family, the autosomal dominant mode of inheritance was assumed because phenotypes appeared in all generations with no gender differences. Data were analyzed for dominant mode of inheritance with 90% penetrance values and the disease allele frequency was assumed to be 0.001. We extracted genotypable 114,816 SNPs out of the 116,204 SNPs in the Human Mapping Array 100K set. For the extracted SNPs, we removed 825 SNPs which were inconsistent with autosomal dominant inheritance pattern in this family and performed parametric analysis using the remaining 113,991 SNPs for the genome-wide two-points linkage analysis. Following preliminary haplotype and sequencing analysis revealed a candidate disease mutation, c.3170A>G in *DOCK5* on chromosome 8p, whose proximate region showed the highest LOD score in the genome-wide two-points linkage analysis. For chromosome 8p, we performed a multipoint linkage analysis using microsatellite markers using MERLIN (Multipoint Engine for Rapid Likelihood Inference) v.1.1.2. We set the penetrance to 80% and the disease allele frequency to 0.002, taking into account the penetrance rate of the c.3170A>G in this family and the incidence of bipolar disorder in

123 Japan (2), respectively. In addition, multipoint linkage analysis and haplotype analysis
124 were performed on chromosome 8p to show genetic evidence of recombination using 41
125 SNPs from the Gene Chip Human Mapping 100K set and the Genome-Wide Human SNP
126 Array 6.0 (Affymetrix), and seven microsatellite markers on chromosomes 8p23.1 to
127 8q12.2: D8S277, D8S549, D8S258, D8S136, D8S1771 D8S1443, and D8S260 in the 18
128 available family members (Figure 2).
129 The microsatellite markers that are highly polymorphic in Japanese populations were
130 selected from the map of the human genome (8). The primer sequences were taken from
131 the Genome Database. We genotyped the seven microsatellite markers (Figure 2). The
132 microsatellite sequence was amplified with PCR. The forward primer in each marker set
133 was labeled with a fluorescent dye at the 5' end. The 12.5 μl PCR mixture contained 1
134 μl of template DNA and 0.25 μl of each forward and reverse primer (10 μmol), and the
135 DNA region was amplified using TaKaRa Taq DNA polymerase under the following
136 conditions: denaturation at 94°C for 2 min, 35 cycles at 94°C for 30 s, 58–62°C for 30 s
137 and 72°C for 30 s, followed by a final extension step of 5 min at 72°C. The amplified
138 products were observed on 1% GelRed (Biothium Inc, Hayward, CA, USA) pre-stained
139 agarose gel to determine the efficacy of PCR amplification.
140 The PCR products were then denatured and subjected to capillary electrophoresis on an

ABI PRISM 3130 Avant Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). DNA fragment sizes were analyzed relative to Gene Scan 500 RIZ Dye size-standard (Applied Biosystems, Foster City, CA, and USA) using the Genemapper software (Applied Biosystems).

2.4. DNA sequence analysis using Whole-Exome sequencing

Whole-exome sequencing was performed in II-2 and III-5 (both diagnosed with BD-I) according to the manufacturers' protocol. Genomic DNA was captured with the SureSelect Target Enrichment System Human All Exon V5 Kit (Agilent Technologies, Santa Clara, CA, USA) and sequenced per lane on an Illumina HiSeq 2000 (Illumina, San Diego, CA, USA) with paired-end 101 bp reads. Image analysis and base calling were performed using sequencing control software with real-time analysis and CASAVA ver.1.8.2 software (Illumina). Generated sequence data (FastQ files) were processed using the pipeline with BWA (version 0.5.9), SAMtools, Picard (version 1.59, http://picard.sourceforge.net/) and GATK32 (version 1.6–5). Variant calls were made using the GATK best practices recommendations. To prioritize variations, we applied several filtering steps. First, we included non-synonymous variations. Second, we included variations inherited in an autosomal dominant manner, considering the inheritance pattern of this family. Third, we included variations that were co-segregated

159 in the two family members with BD-I who underwent whole exome sequencing. Fourth,
160 we included variations with mutant allele frequency less than 0.01 according to the
161 Japanese reference panel project of the Tohoku Medical Megabank (4.7KJPN) and
162 NCBI databases. Fifth, we included mutations that exist in the candidate region based
163 on the results of the linkage analysis described above (Figure 4). To validate prioritized
164 variations, Sanger sequencing was performed using a 3130 × l Genetic Analyzer
165 (Applied Biosystems).

166 **2.5. DNA sequence analysis using Sanger sequencing**

167 Sanger sequencing of the mutations on the candidate genes was performed for the 18
168 family members based on the results of linkage analysis and whole exome sequencing.
169 Then, we identified a mutation (c.3170A>G) in *DOCK5* gene. For candidate mutations,
170 sequences in *DOCK5* from 50 unrelated Japanese patients with sporadic BD-I and 67
171 unrelated Japanese healthy controls were searched.
172 For Sanger sequencing, polymerase chain reaction primers (sequences available upon
173 request) were designed to amplify all coding exons (non-coding RNA exons),
174 5'untranslated regions, and intronic regions surrounding each exon. All PCR products
175 were subjected to Sanger sequencing. PCR reactions were performed in a total volume
176 of 12.5 μl containing 10–30 ng of each DNA sample, 0.2 μM each of the forward and

reverse oligonucleotide primers, 0.2 mM each of dATP, dGTP, dTTP, and dCTP, and 0.5 units of TaKaRa Taq. The PCR program was 94°C for 2 min, 35 cycles of 94°C for 30 s, 58–64°C for 30 s and 72°C for 1 min, followed by 72°C for 5 min. The amplified PCR products were separated on 1% agarose gels at 100 V for 30 min and were labeled using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with the same primers used in the initial PCR and following the protocol described above. The products were then directly sequenced on an ABI PRISM 3130 Avant Genetic Analyzer (Applied Biosystems).

2.6. Copy-number variants (CNV) analysis using Genome-Wide Human SNP Array

Whole genome CNV analysis was performed for the 17 (one data set from poor quality DNA of patient III-1 was not usable) family members using the Genome-Wide Human SNP Array 6.0 (Affymetrix), which features 1.8 million genetic markers, including more than 906,600 SNPs and more than 946,000 comparative genomic hybridization probes. Labeling and hybridization were performed according to the Affymetrix protocol. Raw data were acquired from the scanner and transferred to a Genotyping Console 3.0.2 (Affymetrix) to determine the genotypes and CNVs from fluorescence intensity data. For all chromosomes, signal intensities for the copy-number predictions

of both duplications and deletions were investigated. CNV aberrations of more than 100 kbp were automatically detected by the genotyping console 3.0.2 software. In addition, small CNV aberrations of a few hundred to several thousand bp were visually detected in detail from the raw data. Because DNA from III-1 was unavailable for the Genome-Wide Human SNP Array 6.0 analysis due to low quality, this array was not used in genome-wide linkage analysis.

3. Results

3.1. Linkage and haplotype analysis

We performed a genome-wide scan using the DNA arrays to confirm the evidence for linkage. In preliminary two-point linkage analysis, the highest LOD score of 2.3 (θ = 0) was obtained for marker rs10503492 on chromosome 8p22. The second highest LOD score of 2.10 (θ = 0) was obtained for marker rs10491029 on chromosome 10q26. Multipoint linkage analysis using SNPs and microsatellite markers revealed a consistently high LOD score at around D8S136 at 8p (Figure 3). Haplotype analysis using SNPs and additional microsatellite markers analysis revealed a common haplotype delimited by rs10503492 and rs10504053 on chromosome 8p23.1 to 8p11.1, which was segregated in all the available patients (Figure 2). The proximal recombination site was between rs10503452 and rs10503492, and the distal site was

between rs10504053 and rs1474386. These results demonstrate that the region linked to BD and its related diseases was possibly within the approximately 30 Mb section spanned by rs10503492 and rs10504053 on chromosomes 8p23.1 to 8p11.1 (Figure 2). Chromosome 10 was analyzed in the same way, but no mutation that was co-segregated in the patients was found (described in next section).

3.2. DNA sequence analysis

A region of approximately 69.35 Mb was captured from each of the II-2 and III-5 exomes (Supplementary Table). The average reading depth was 82, and about 93.1% of the target area was covered by more than 10 reads. In II-2, 74,386 mutations including 65,858 SNPs and 5,089 indels were detected in the coding region, and in III-5, 70,947 mutations including 68,615 SNPs and 5,771 indels were detected in the coding region. Filtering was performed on the detected mutations, as described in the Materials and Methods section. Only three mutations were found under the linkage peak region on chromosome 8p. In addition, for the three variants, Sanger sequencing revealed that only a g.25224432A>G (E1057G) mutation on *DOCK5* was co-segregated in the affected individuals; it was also found in two healthy family members.

In 67 healthy controls and 50 patients with BD-I, Sanger sequencing was performed for the *DOCK5* mutation (E1057G). However, no similar mutation was found. According to

4.7KJPN, this is an extremely rare mutation with a frequency of 0.0002. This mutation was not found in the gnomAD (The Genome Aggregation Database). Additionally, in *DOCK5*, Sanger sequencing for all 52 coding exons and exon-intron boundaries was performed for 50 patients with BD-I. However, there were no pathological mutations, except for high minor allele frequency (MAF) polymorphisms (>0.01).

We evaluated the E1057G mutation in *DOCK5* using the following five *in silico* tools: poyphen2, SHIFT, PANTHER, Mutation Taster, and PROVEAN. In poyphen2 and SHIFT, the results were “Benign” and “tolerated”, respectively. The results of PANTHER, PROVEAN, and Mutation Taster were “potentially damaging”, “deleterious”, and “disease causing”, respectively.

3.6. Copy-number variants (CNV) analysis using Genome-Wide Human SNP Array

In all chromosomes, the signal intensity of both the replication and deletion copy-number predictions was examined in detail from the raw data. However, no specific CNVs were found in affected individuals.

4. Discussion

The aim of this study was to identify potential susceptibility variants that contribute to the risk of BD. In this study, we identified an E1057G mutation in *DOCK5*, which was

carried by all family members affected by BD-I and recurrent major depression. However, two healthy individuals also carried this mutation. Thus, we considered two possibilities. The first was penetrance rate. If an odds ratio of three, frequency of 0.0002, a dominant model and a one-tailed test for $p = 0.05$ were assumed, then power is > 80% for a sample of 15,000 BPD patients and 15,000 controls. With an odds ratio of five, 5,700 case-control pairs are needed. Such odds ratios would be compatible with two unaffected carriers found in this family. The second was that individuals with this mutation that were healthy at the time of analysis may be affected later. II-9 was a 50-year-old woman without history of psychiatric treatment and detailed clinical information although she was considered to be depressive state with under threshold of diagnose at the interview of the informed consent for the DNA analysis. II-10 was a 47-year-old woman without history of psychiatric treatment and detailed clinical information although she showed hyperthymia with under threshold of diagnose of mood disorder at the interview of the informed consent for the DNA analysis. *In silico* analysis showed inconsistent results. The inheritance of this family suggests an autosomal dominant form, which is generally explained by a gain-of-function mutation. It has been reported that polyphen2 and SHIFT are better at predicting loss-of-function mutations than gain of function mutations (9), suggesting that these *in silico* programs

268 may not be ideal for predicting the effects of the E1057G mutation in *DOCK5*. In
269 general, in order to avoid missing causative mutations and to take the best advantage of
270 in silico analysis, different computational platforms should be used (10). Although
271 further functional analysis is required, one of the rationales of the c.3170A>G mutation
272 in *DOCK5* as risk for BD was that the results of other three platforms, PANTHER,
273 PROVEAN, and Mutation Taster showed “potentially damaging”, “deleterious”, and
274 “disease causing”, respectively.
275 Although there was no significant association between genes on chromosome 8 and BD
276 in the data base of the second GWAS of the multinational Psychiatric Genomics
277 Consortium BD Working Group (11), in a study of approximately 400 families affected
278 by BD, Park et al. reported that the highest LOD score (higher than 3) from a linkage
279 analysis was obtained on 8p21 at the D8S382 region, whose physical position is from
280 25,952,418 to 25,952,386 (GRCh37) (12). The distance between D8S382 and the
281 E1057G mutation in *DOCK5* is approximately 700,000 bp, which is a remarkably close
282 genetic distance equivalent to 0.7 cM. Another study reported that a *DOCK5* mutation
283 was significantly associated with various psychiatric disorders, according to a whole
284 exome analysis of 32 patients from two families with various psychiatric disorders,
285 including bipolar disorder, and 33 controls (13). Furthermore, Le-Niculescu et al.

reported that variable expression levels of *DOCK5* were associated with mood disorders, psychosis and suicidality, and that *DOCK5* may be a key genetic biomarker reflecting those diseases (14).

DOCK5 is a member of a family of genes encoding an atypical Rho guanine nucleotide exchange factor (GEF) for Rac and/or Cdc42 GTPases. The Dock family is genetically conserved from *Drosophila melanogaster* and *Caenorhabditis elegans* to mammals (15). In addition, 11 members of the Dock family have been identified and divided into four groups (Dock-A, B, C, and D) based on their domain structure differences. It has been reported that the Dock family is associated with several neurodegenerative and neuropsychiatric disorders (16). *Dock2,* belonging to the Dock-A group (the same group as Dock5) is a regulator of microglia and is associated with Alzheimer's disease (17). Recently, an association between microglial activation and mental disorders such as schizophrenia and depression was identified (18), suggesting that DOCK5 may be involved in microglial activation. In addition, DOCK5 is an indispensable molecule for transmitting GSK3β signals in mast cells during allergic reactions (19). A similar mechanism may also exist in the central nervous system, because GSK3β-related proteins and *DOCK5* are expressed in the central nervous system. It is also interesting to note that all affected individuals in the present study responded to lithium carbonate. In a GWAS study using 2,000 BD lithium carbonate responders, only ch8 rs7833426 was associated with the BD when the study was restricted to subjects with Asian ancestors, although at just a nominal significant level (20). rs7833426 is within the linkage region in the present study. These results suggest that *DOCK5* is associated with lithium-reactive BD. Although further genetic analysis of additional family members

and functional analysis are required, our results suggest that the DOCK5 protein is a possible pathological molecule for BD and related disorders.

5. Conclusions

Positional cloning of the genes of a Japanese family affected by BD was performed using a SNP array and whole exome sequencing. The analysis revealed an E1057G mutation in the *DOCK5* gene, which was located in the region with the highest LOD score. *DOCK5* may be involved in the pathology of BD and its related diseases.

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Declaration of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure legends

Figure 1

Pedigree of a Japanese family having a high frequency of bipolar disorder and recurrent major depressive disorder. Affected status is indicated with a filled square (male) or circle (female). Gray squares and circles show unknown disease status. Black spots indicate individuals who were sampled and analyzed.

Figure 2

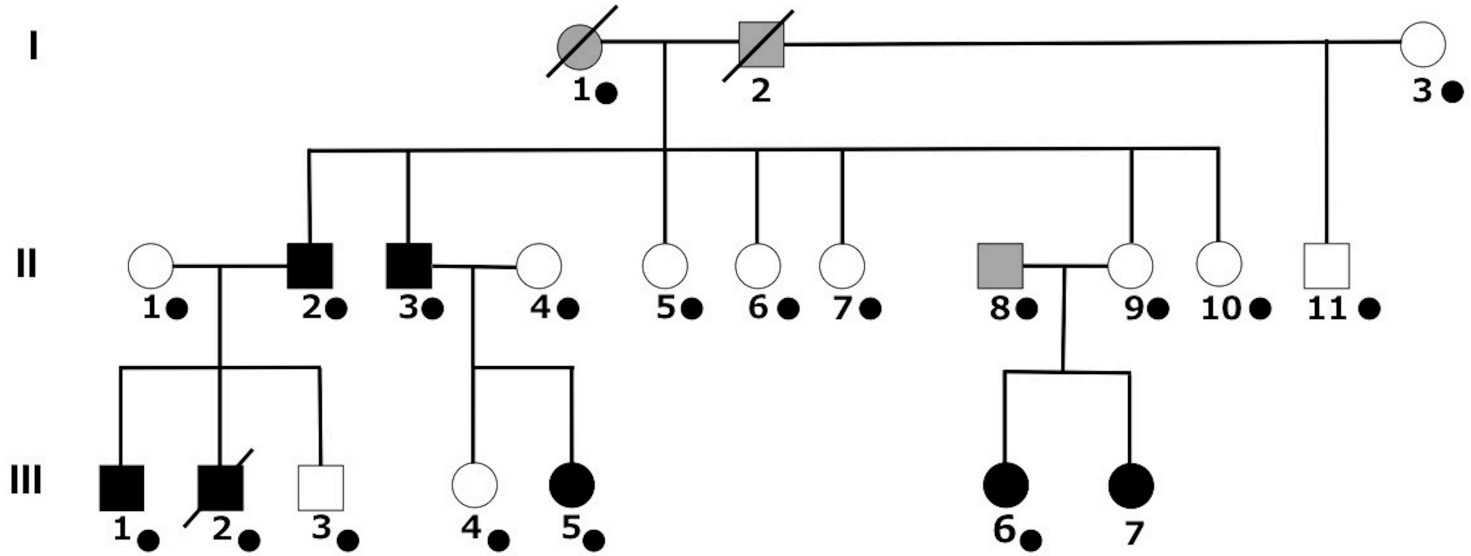
Haplotypes of each individual, with the disease haplotype boxed. The absence of a haplotype indicates that the individual was not genotyped at that microsatellite marker or single-nucleotide polymorphism locus.

Figure 3

Multipoint logarithm of odds (LOD) scores for genetic locations on chromosome 8q21.2.The maximum LOD score was 2.1 for marker D8S136.

Figure 4

Filtering steps applied to variations called from the whole exome sequencing of II-2 and III-5.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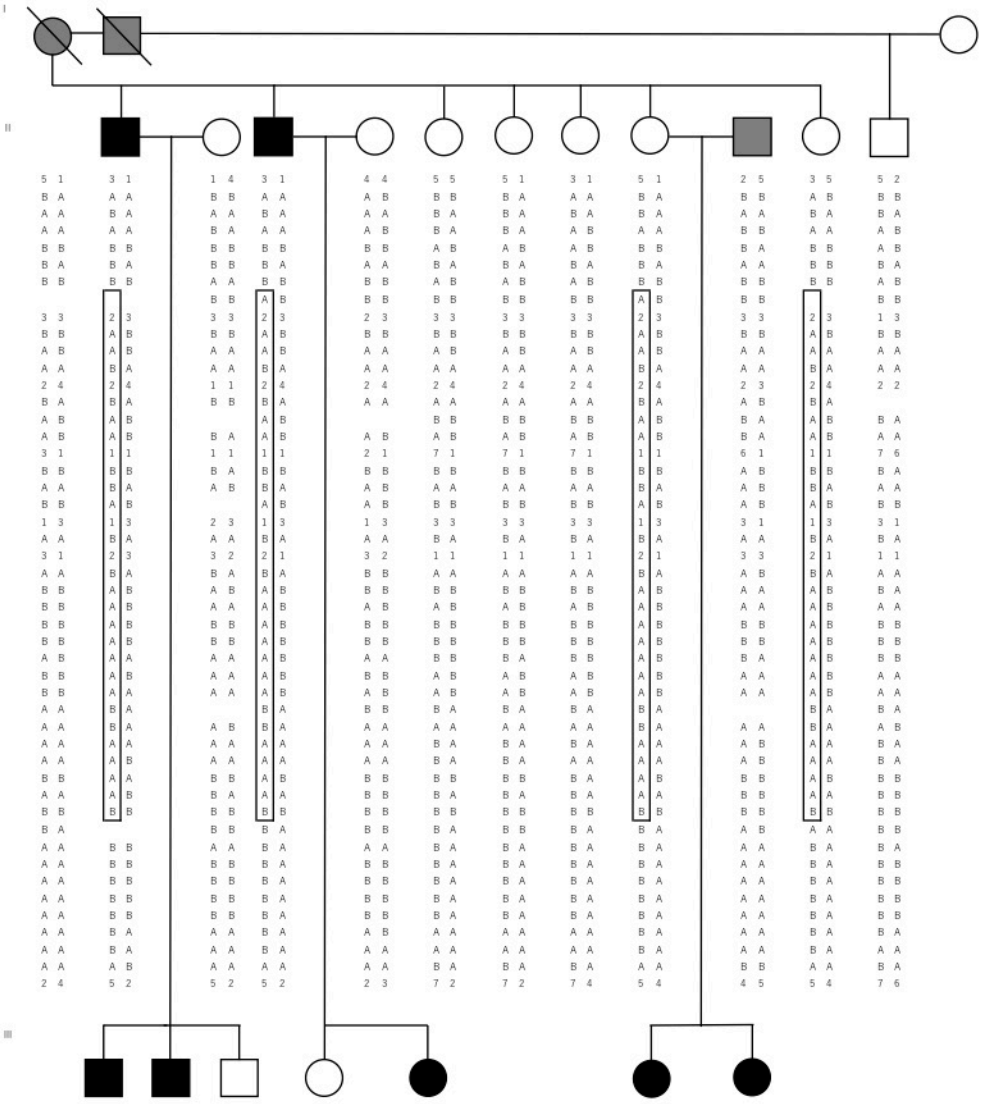
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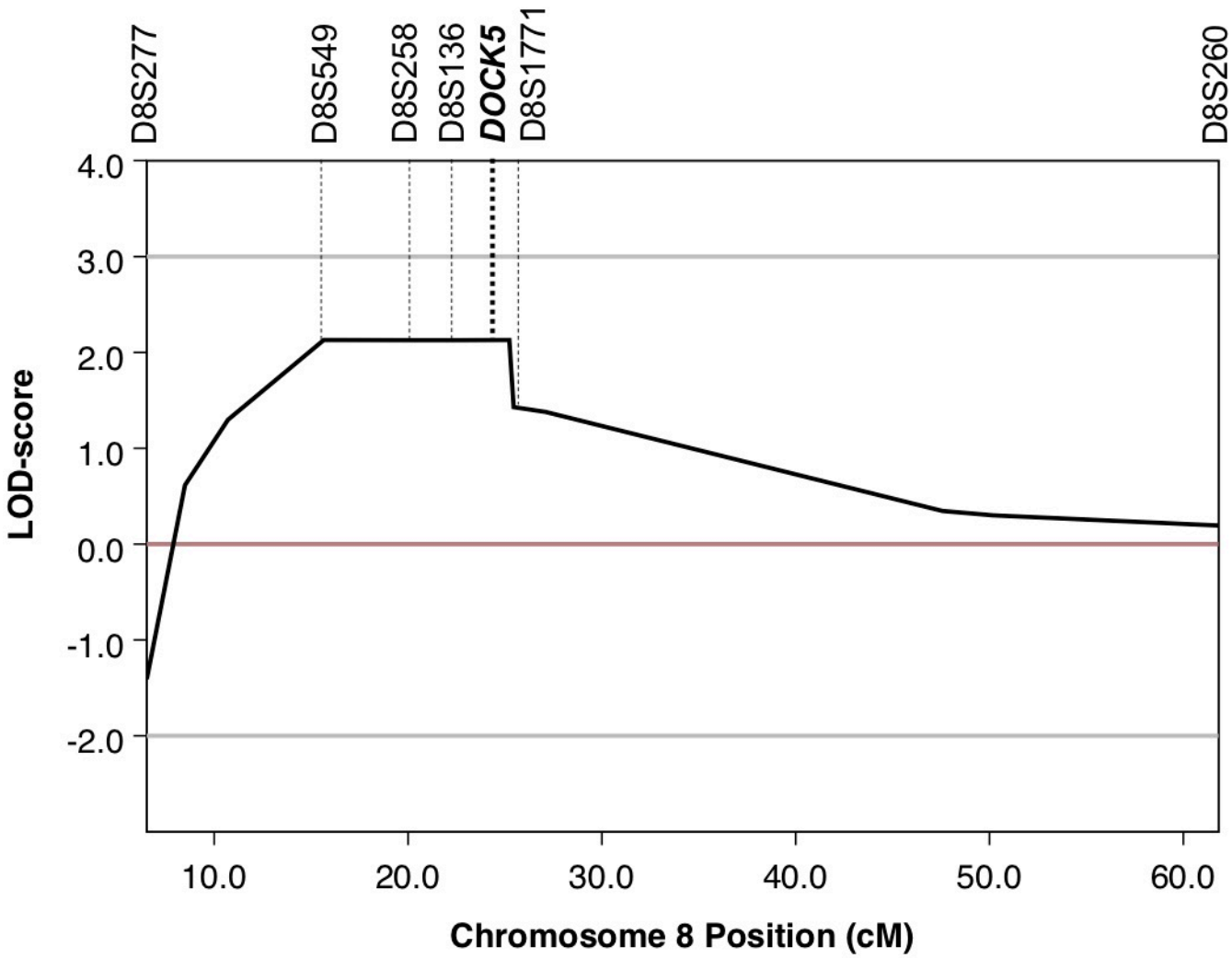
D8S277
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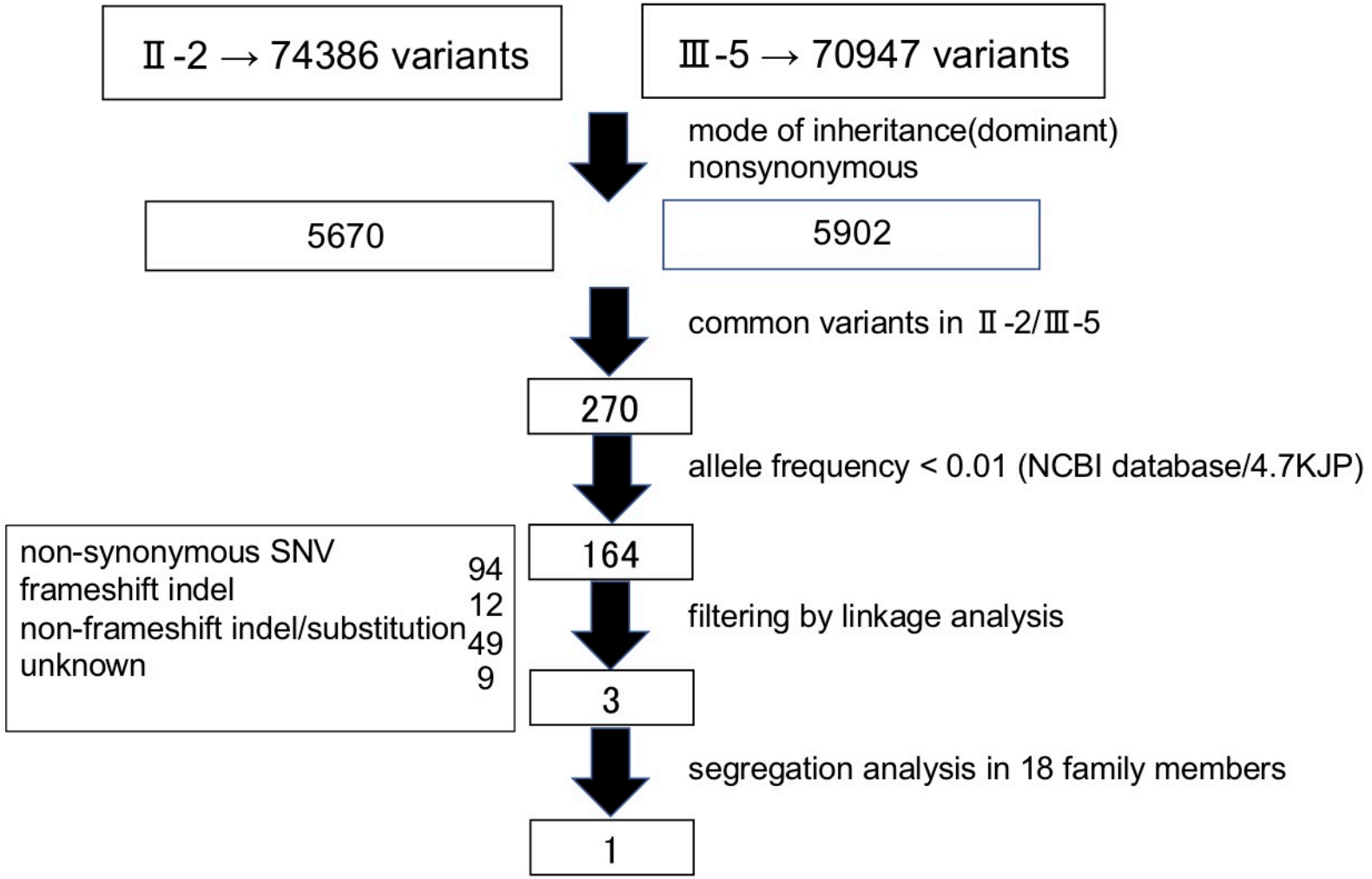
Ⅱ-2 → 74386 variants
Ⅲ-5 → 70947 variants
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5902
common variants in Ⅱ-2/Ⅲ-5
270
allele frequency < 0.01 (NCBI database/4.7KJP)
non-synonymous SNV 94
frameshift indel 12
non-frameshift indel/substitution 49
unknown 9
164
filtering by linkage analysis
3
segregation analysis in 18 family members
1