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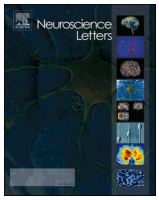

Detection of rare genetic variations in the promoter regions of the ATG16L gene in Parkinson's patients

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ABSTRACT

Mutations in the ATG genes have been related to impair autophagic function, contributing to the sporadic onset of Parkinsońs Disease (PD). However, scarce studies have been performed about ins/del within the regulatory domains of the autophagy genes in sporadic PD patients. This study was aimed to found ins/del within part of the crucial core autophagy promotor gene region of the ATG16L1 in a groups of sporadic PD patients. After developing a genetic marker to find ins/del using fragment size analysis, a rare mutation by insertion (0.45%) was reported in the patients. This mutation was characterized by sequencing. No others ins/del were found. As a results, the frequency of this insertion should be considered as a rare genetic variant. An *in silico* analysis also highlighting the usefulness of a search GDV which revealed multiples ins/del within ATG16L1 promoter. Furthermore, these genetic insertions could be found in patients with sporadic PD in the ATG161L promoter gene. When a breakpoint as deletions, insertions or tandem duplication are located within a functional gene interruption of the gene and a loss of function was expected but removing or altering in the regulatory sequence can influence the expression or the regulation of a nearby gene which may impair healthy due to dosage effects in sporadic diseases.

1. Introduction

Human genome studies have highlighted the enormous variability of human genomes, to the extent that the notion of a single human genomic reference sequence has been discarded [1]. Therefore, a major challenge has also opened up for complex neurological diseases such as Parkinson's disease (PD), Alzheimer's disease (AD) and schizophrenia, as several types of rare DSVs (DNA sequence variation) have been identified, including promoters and non-coding regions that can affect even distant genes. Furthermore, numerous rare mutations are known to cause sporadic disease, but SNPs are associated with common diseases. Recently, few gene rearrangements had been linked to disease. For example, submicroscopic genomic duplications and deletions causing gene CNV (copy number variation) were shown to cause Mendelian traits [2–3]. The extent to which CNVs contribute to human genetic diversity, and may or may not convey phenotypes, is still being unravelled [1].

PD has been described as a progressive neurodegenerative disease that affects the neurons of the substantia nigra (SN), which are involved in synthesis of dopamine [4]. Mainly people over the age of 65 sometimes develop the sporadic form of the disease, although they can appear early from the age of 40 on-wards in less than 5% of cases [5]. Resting tremor, postural instability, rigidity, and bradykinesia [6] are hallmarks, as non-motor manifestations such as apathy, cognitive impairment or dysautonomia symptoms among others [7]. Although sporadic PD is attributed to genetic and environmental factors including its

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interactions, it is known that up to 10% of PD are strictly classified as genetically determined, both sporadic and familial types account for most of the cases [8]. However, genome-wide association, next generation sequencing, and exome sequencing studies have associated several genetic variants with sporadic PD [9–10], which could be useful to developed simplest diagnosis methods.

Furthermore, although the specific underlying molecular pathways are largely unknown, the autophagy is a highly conserved cellular pathway delivering long-lived proteins and organelles to lysosomes for digestion, and it consists of three events: macroautophagy (also autophagy), microautophagy and chaperone-mediated autophagy [11–13], with much of the cell transcriptional is driven from large regulatory elements or (super) enhancers, some related to malignant cells [14]. Several levels of regulation of autophagy have been demonstrated: pre-transcriptional, transcriptional and post-translational [15]. Consequently, animal and human studies have confirmed that dysfunctional autophagy and chaperone-mediated autophagy contributed to PD pathogenesis [12,16–22], but that is mediated by a set of key autophagy proteins (ATG). The ATG proteins found in this process are the ATG1/ULK complex, the ATG9 cycling system, the PtdIns 3-kinase complex, the ATG12 conjugation system and the ATG8/LC3 conjugation system [13,23]. In this molecular context, ATG16L1, along with ATG5, ATG7, ATG10 and ATG12, is a main component of ATG12 conjugation system, which help in the elongation of the phagophore, as a precursor of the autophagosome [24]. Additionally, ATG16L1 form an oligomeric complex with ATG12-ATG5 conjugates to enhanced LC3/ATG8-conjugation to phosphatidylethanolamine by recruiting an LC3-ATG3 intermediate [16–17] and, also, a way that specifies the site of LC3 lipidation [18]. Furthermore, the autophagic disruption and enhanced production of IL-1β and IL-18 involved ATG16L1 in the inflammatory immune response [18]. Moreover, mutation of the ATG16L1 gene has been linked to the retention of the early-stage cells in various tissues during development, as well impair differentiation toward neurons [19]. But is known, regarding this promoter region, as ATG16L1 also performs non-autophagic functions during cellular secretion and exocytosis [20–21].

Moreover, mutations in the ATG genes have been related to a variety of human diseases [25]. It has been speculated that genetic variants in ATG genes may impair autophagic function, contributing to the sporadic onset of PD [26–28]. Previous studies have found and functionally examined some genetic variants within the regulatory domains of the autophagy genes, such as microtubule associated protein 1 light chain 3 beta (LC3B), ATG5 and ATG7, in sporadic PD patients [27,29–30]. In this study, we further genetically analyzed aimed to found ins/del within part of the crucial core autophagy promoter gene region of the ATG16L1 in one group of sporadic PD patients and ethnically matched controls. Moreover, the effect that molecular variation of the promoter could have on transcription was evaluated using bioinformatics tracking approaches aimed to study alteration of the functionality of the variants detected in the promoter regions of these ATG gene.

2. Materials and methods

2.1. Study subjects

From a total of 110 individuals, 73 Caucasian male and female patients with sporadic PD (mean age at debut 59.8 years and mean sampling age 68.8 years) were recruited those with biological material from the Servicio de Neurología del Complejo Universitario Hospitalario de Albacete del SESCAM (Servicio de Salud de Castilla la Mancha). All PD patients were diagnosed by two neurologists. Ethnically matched healthy controls (n = 37, mean age 64.3 years) were recruited from the same hospital and diagnosed as healthy. Initially, patients with PD and controls with a family history of PD were excluded. This study was approved by the Clinical Research Ethics Committee of the health area management of Albacete, as well as the authorisation of the bioethics and biosafety committee of the University of Extremadura. Informed consent was obtained, with prior explanation to the population participating in the study.

2.2. Genotyping by direct DNA sequencing

Total blood was used for genomic DNA isolation. DNA was extracted with Archive pure DNA Blood Kit (5PRIME GmbH). The ATG16L1 gene promoter, from –1095 bp (Location 233250476) to + 153 bp (Location 233251723) (size: 1246 bp), was generated by PCR and directly sequenced in both directions to generate the ATG16L1 promoter region (unpublished data). Sequences were deposited in Gene Bank with Accession Numbers from ON230169 to ON230233.

Sequencing failure was detected in the patient P85, reason why a primer pair from both 3′ end of each sequence reads (forward and reverse strand) were designed to amplify partially the ATG16L1 promoter to investigate the cause. Details of the designed PCR primers are shown in the Table 1, aimed to detect fragment size differences polymorphisms using a 3130 DNA Analyzer and Gene Mapper ver. 4.0 software (Applied Biosystems, Foster city, CA, USA). Expected *in silico* wildtype size should be 342 bp. PCR was performed with initial denaturation at 94 °C for 5 min, followed denaturation for 30 s at 94 °C, annealing at 55 °C for 45 s and extension at 72 °C for 45 s using 35 cycles; and a final extension at 72 °C for 7 min. PCR products were separated by capillary electrophoresis and size estimated using internal size marker GeneScan™ 500 ladder (Applied Biosystems, USA).

2.3. Detecting ins/del by cloning

The sequence composition was studied using a cloning strategy followed by sanger sequencing, which validates the size. PCR product from P85 patient was obtained using unlabelled primers (ATG16insdel-F and ATG16insdel-R) and cleaned using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel) following the manufacturer's recommendations. Clean PCR products were cloned using TOPO TA Cloning® (Invitrogen™ company). According to the manufacturer's recommendations, ten white colonies were resuspended individually in 20 μl of purified water, incubated ten minutes at 90 °C to lyse the cells and inactivated nucleases. One μl of inactivated cells were amplified with the protocol described above. The PCR were visualised on 1.4% agarose gels, stained with Sybr™-Safe™ (Invitrogen™ company) and size estimated with Ladder V (NZYDNA, NZYTECH company). Eight out of ten colonies showed approximately the expected size. These PCR products were purified enzymatically by ExoSAP-IT™ (Thermo Fisher Scientific) and sequenced bidirectionally using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). The raw data were read on a DNA genetic analyser (Applied Biosystems™ 3130 DNA Analyzer). The sequence of seven colonies were aligned using the option ClustalW algorithm in MEGA X [31] but only one colony carrying an 8 bp insertion.

2.4. In silico study of transcription factors affecting ins/del

The analysis of transcription factor (TF) binding sites was performed with the TRANSFAC database to predict those relevant for the ins/del polymorphism in ATG16L1 gene promoter. A comparison between wild and mutant type input sequences was done within a dissimilarity margin less or equal than 15%.

3. Results

3.1. Molecular analysis of ins/del in Parkinson's gene promoter

In this study, the detection of polymorphism due to size variation by insertion in ATG16L1 gene promoter region was performed in a diagnoses sporadic PD Spanish population. The reported polymorphism occurred only in one sporadic PD affected. Fig. 1 showed the results of the fragment size analysis from the patient P85 (a woman 86 years old)

Table 1
Primers designed from partial sequence reads. Named Experimental primers. EM = Electrophoretic motility.

PCR-primers	Experimental Primers	Location Ref seq	*in Silico* bp	EM bp: Wild/Mutant
ATG16insdel-F	FAM-TGAAGACAAACTCTGATACACAA	−1008 to −986	342/350	340 / 348
ATG16insdel-R	ACAGAAACATTACGAACCTGCAA	−689 to −666		

who was a heterozygous carrier for an insertion of 8 bp according to capillary electrophoresis fragment separation (Fig. 1 A). No individual (except P85) carries out this or others ins/del (Fig. 1B). The wildtype allele showed the expected size (Table 1, Fig. 1). The frequency of this insertion was estimated at 0.45% suggesting that should be considered as a rare genetic variant. As a result, it can be assumed that no more than 9 out of 1,000 individuals might be carrier or, alternatively, less than one homozygote per 10,000 cases at HW equilibrium for this insertion.

As this was one single heterozygous case, nor statistical studies nor association analyses to assess the relationship between this insertion and the distribution of sporadic PD risk were performed. However, an *in silico* analysis was performed aimed to find others SNP and ins/del within the studied amplicon using a search in the NCBI Genome Data Viewer (GDV) to visualise size variations within the amplicon from the position 233,250,476 to 233,251,722 in Chr2. (Nc000002.12) (table S1).

3.2. Analysis of the ins/del in the ATG16L1 promoter in the P85 patient

Further study was needed to decipher the exact molecular composition of this mutation. Moreover, Sanger sequencing was performed of the insertion, aimed to validate the size and to identify the nucleotide sequence using unlabelled experimental primers.

Sequencing analysis of the eight positive colonies showed only six sequences with the wildtype (wt) allele showing a 100% identity with the reference sequence (Ref. Seq. NC 000002.12). Another colony showed the mutant type carrying eight additional nucleotides in respect to the wildtype. A Basic Local Alignment Search Tool (BLAST) was performed to compare wildtype and mutant alleles. As a result, it was found the insertion due to eight additional nucleotides, which duplicate AGTTAAGA sequence (Fig. 1A) from position 138 bp to 146 bp in the amplified product (see Fig. 1A). Moreover, an identical insertion was found in NCBI data base, but no other information consistent with Parkinson's disease was reported.

3.3. In silico study for the utility of the experimental primer for to be used as a genetic marker

As part of this study, a search was performed in the NCBI Genome Data Viewer (GDV) to visualise size variations within the analysed amplicon from position 233,250,476 to 233,251,722 in Chr2. (Nc000002.12). Fig. 1B showed a graphical representation of all found ins/del within the amplicon (see details in table S1), which included the rs ID numbers and its expected size using the wildtype as reference (Fig. 1B). In addition, polymorphic sites were found inside both experimental primers, a reason why universal primer pairs (Table 2) were designed to avoid allele dropout. However, no such trouble was detected in the Spanish population tested in this study due to all samples were sequenced for the entire ATG16L promoter (Gomez-Martín et al., in prep.). Experimental primers were revised using alignment of DNA sequence variations in GDV. As a result, it was designed universal primers (Table 2) to overcome failures due to accumulation of DSV like those found within experimental primers. Table 3 showed limitations of the experimental primers due to extreme SNPs and ins/del DNA variation inside its sequence (rs number also indicated), which may make these prone to unspecific amplifications. An *in silico* analysis was performed to estimate the fragment sizes using these universal primers, although some deviations could be expected (table S1).

3.4. Binding sites for transcription factors created by the inserted-Sequence variants

To further study the possible creation of TF binding site of the ATG16L1 gene promoter around the insertion site, the nucleotide sequences of the wild-type and mutant allele were compared *in silico* (see Mat & Meth). The results are shown in Table 4. It shows that one or two additional binding sites were present in the mutant in respect to the wild-type allele for eight of the nine TF. But a new binding site was also found with two new targets for HNF-1C. Although it can be speculated that the short duplication in the ATG16L1 promoter may increase binding sites for transcription factors of PD patient which may unbalance transcription level, it is worthwhile to study them because few studies of short ins/del in PD patients has been performed. Even these variants may be useful to *in vitro* studies.

4. Discussion

The utility of the experimental primers to genotype ins/del at the studied promoter has been uniquely demonstrated for this ethnic population (see material and methods). However, an extensive review of the genetic variability reported for ATG16L1 in the NCBI database (Table S1) suggested us to redesign more universal primers that could collect ins/del genetic variants from different ethnicities. Unfortunately, such research has not yet been conducted. In 2017, Wang et al described the functional composition of the promoter region of the human ATG16L1 gene in 151 Asian PD patients without references to ins/del, and little known about its expression and regulation [21]. Such studies are not performed for Caucasian populations, thus justifying to developpe of molecular tools as conducted here to unravel rare variants in sporadic PD. Most cases of PD are sporadic, with aetiology still unknown, possibly attributable to the interaction of different genetic and environmental risk factors [36]. In autophagy, autophagosome formation is mediated by a number of essential ATG proteins. Wang et al (2017) suggest that autophagy function may be affected by genetic variation that occurs in ATG genes, including specifically the promoter regions of these genes [24]. However, DSV other than SNP variations have never been studied in the sporadic PD [21,37–38] and less for CNV (copy number variation) at promoter regions. For the first time here, a perfect duplication of one stretch of nucleotides suggested short CNV may be involved in sporadic PD, which can convey phenotypes by several molecular mechanisms as gene dosage, gene interruption, gene fusion, position effects, unmasking of recessive alleles or functional polymorphism, and potential transvection effects [39] in the same way as previously described for AD or MR (Profound Mental Retardation). Although CNV changes in less than one individual has been suggests as rare, that do not exclude its implication in functional genes involved in regulation of cell growth and metabolism in human traits, disease, and evolution [40]. It has been suggested that genetic analysis of ATG16L1 gene promoter variants may provide relevant information to understand the transcriptional control of the ATG16L1 gene in development and PD, including *in silico* genetic and functional analysis of the ATG16L1 promoter [24], so in our study.

Other molecular changes involving novel triplication has been identified in PD [41], involving SNCA (synuclein alpha (human) gene) linked to autosomal dominant PD in a large family. The triplicate allele implicates a dosage effect of SNCA gene expression in the PD patients, consequently twofold increase both of this protein in blood and mRNA in brain tissue, and increased deposition of heavily aggregated SNCA

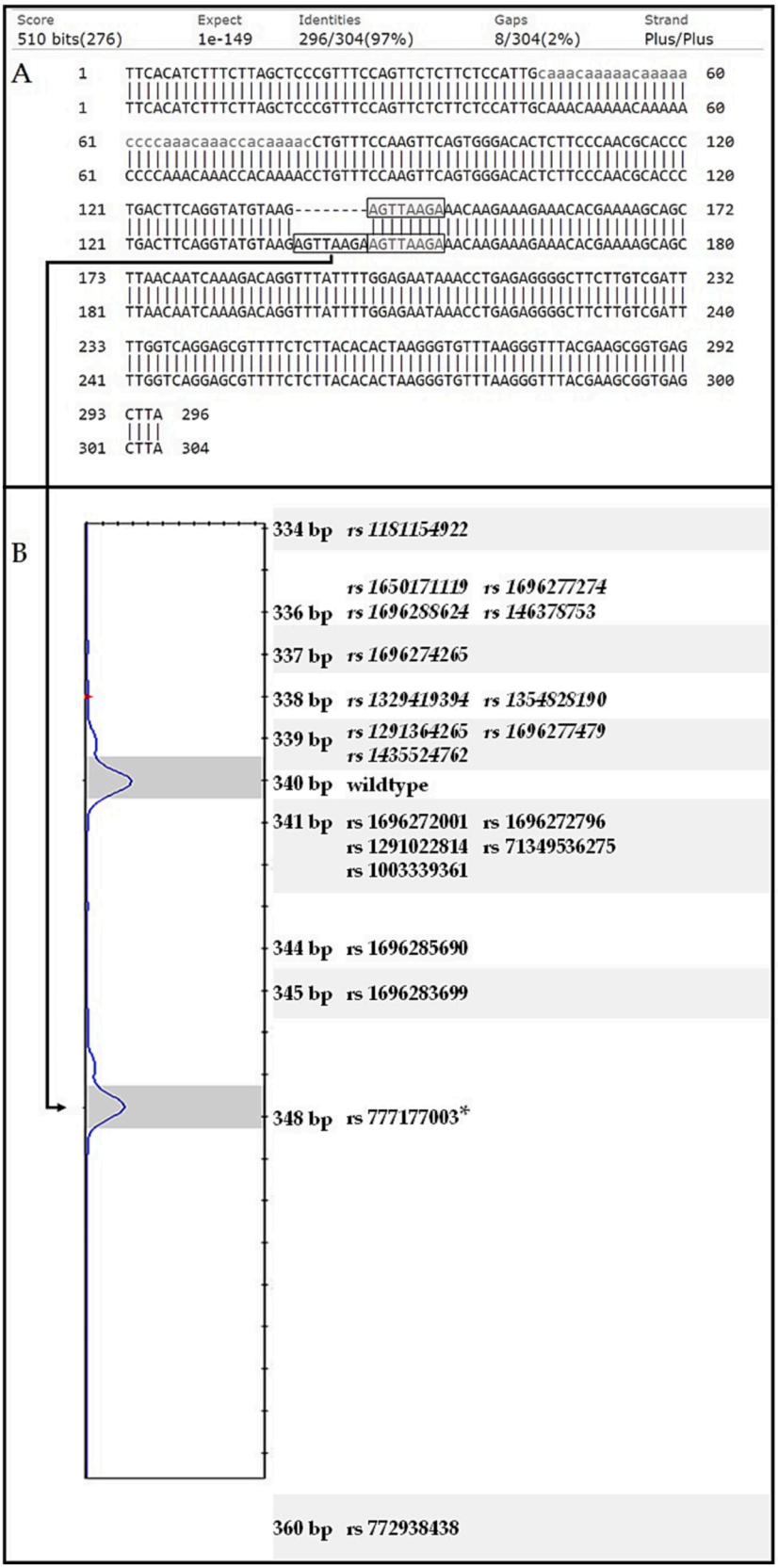

Fig. 1. A. Sequence alignment of wild and mutated allele using BLAST (empty square: inserted sequence). B. Electropherogram of FAM labelled PCR product of both alleles from the patient P85 (mutated*: 8 bp insert) and a representation of all known deletions (above wildtype) and insertions (below wildtype) found *in silico*.

Table 2
Universal primers.

Primer.	Sequence Universal Primer (UP).	Location	Estimated amplicon wt size
ATG16UPi/d-F	YCAGGCCACARYTTCTAGGAT	−1037 to −1017	359 bp
ATG16UP7i/d-R	CRAAMCTGCARTRAGCTCAC	−681 to 661	

Table 3
Revised DSV (bold) inside experimental primers by searching Genome Data Viewer (GDV) at NCBI. (F = ATG16insdel-F and R = ATG16insdel-R).

primer	Sequence primers.	rs in order of appearance (wt/mut)
F	**yg**AAGACRAA**YKCYRWYA**$_{(A)}$CACAA	del/ins rs 1,650,171,119 (lower case); rs 15r4829201(T/C); rs1574829205 (A/G); rs 16,962,720,794 (T/C);rs 1,696,270,951 (T/G) rs 1,408,608,399 (T/C);rs 1,696,271,301 (G/A) rs 1,356,367,914 (A/T);rs 1218322410(T/C) ins/del rs 1,696,272,001 (A/AA) (sub script)

R	A**MRR**AAAC**DTT**$_{(-)}$**ABR**AA**M**CTGCA**R**	rs 747,054,625 (T/C); rs 1696300341(G/T);
		rs 1,696,300,509 (C/T);rs 1388531689(G/A/C); del/ins rs 1435524762(AA/A);
		rs 1,347,862,247 (T/C/A);
		rs1696301371(C/T);
		rs912189048(T/C);
		rs1414604066(G/T)

Table 4
TF predicted in TRANSFAC with dissimilarity margin less or equal than 15%.

Transcription Factors (at 15% dissimilarity)	Fold number in wild vs mutant type (% binding excess)
FOXP3 [T04280]	21/23 (9,5%)
TFIID [T00820]	5/6 (20.0%)
PR B [T00696]	8/9 (12.5%)
PR A [T01661]	8/9 (12.5%)
HNF-3beta [T01049]	7/8 (14.3%)
HNF-1A [T00368]	2/3 (50%)
HNF-1B [T01950]	1/2 (100%)
HNF-1C [T01951]	0/2 (-)
c-Myb [T00137]	2/3 (50%)

protein in brain tissue [42]. Later studies also confirmed the role of SNCA copy number gain, both triplication and duplications with autosomal dominant early-onset PD. Chartier-Harlin et al. [43–44], Fuchs et al.[45] in a large Swedish pedigree and Ibanez et al. [46] in 2 out of 119 individuals, identified SNCA duplication associated to PD. These cases were confirmed by analysis of intragenic and flanking microsatellite markers [46], thus using a similar molecular method to those proposed by us. Furthermore, all these observations strongly suggest a dosage effect of SNCA in selected cases of PD, which should encourage further investigation in the cases described in this study.

The relative small sample size in this and early studies, these de novo CNVs were identified in single cases and each CNV may be different [33–34], suggesting the needed to detect and confirm the roles of rare *de novo* variants in larger data set and *in vitro* studies. Accordingly, our results of *in silico* binding Sites for TF created by the inserted-Sequence Variants, point out for more research about the effects on gene expression of these or others ins/del promoter regions of ATG genes.

Like for PD, as shown in this study, different genetic variants in gene promoters have been associated with neurological diseases. In this way, Theuns et al [32] identified up to eight novel variants in the *APP* promoter in late-onset AD, three of which can cause almost 2-fold specific increase in transcriptional activity in neurons *in vitro*. Also, *de novo* genomic CNV have been identified in patients with MR [33–35]. Thus, it has been suggested that transcriptional regulation may lead to neurological disease due to dosage-sensitive genes [32].

The present study examines the genetic insertion that characterise patients with sporadic PD in the ATG161L promoter gene, but published studied on this topic is rather scarce [47], and similarly, the literature reviews the implications of polymorphisms in the ATG16L1 gene and highlights the association with different diseases [24]. When the breakpoint of a deletion, insertion, or tandem duplication is located within a functional gene, it may interrupt the gene and cause a loss of function by inactivating a gene as exemplified by red-green opsin genes and colour blindness [48]. But removing or altering a regulatory sequence, CNV can have an effect on expression or regulation of a nearby gene. Many of them have been identified to alter gene expression and cause human diseases [49]. Other genomic variation different from SNP has been recognized as the predominant source of variation among human individuals [1].

Dosage effects may be related to convey sporadic diseases, Mendelian and complex traits. Although technical limitations have been stated to uncover genetic variation, we show that it is possible to detect many of these variations when technical flaws are not ruled out, instead revised. That is, conducting further revision will advance our understanding on the location, distribution, formation, convey phenotype or genetic susceptibility, selection, and evolution for all kinds of genetic variation.

Declaration of Competing 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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neulet.2023.137195.

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