

# The human $\delta 2$ glutamate receptor gene is not mutated in patients with spinocerebellar ataxia

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## Abstract

The human glutamate receptor delta 2 gene (*GRID2*) shares 90% homology with the orthologous mouse gene. The mouse *Grid2* gene is involved with functions of the cerebellum and spontaneous mutation of *Grid2* leads to a spinocerebellar ataxia-like phenotype. To investigate whether such mutations occur in humans, we screened for mutations in the coding sequence of *GRID2* in 24 patients with familial or sporadic spinocerebellar ataxia and in 52 normal controls. We detected no point mutations or insertion/deletion mutations in the 16 exons of *GRID2*. However, a polymorphic 4 nucleotide deletion (IVS5-121\_-118 GAGT) and two single nucleotide polymorphisms (c.1251G>T and IVS14-63C>G) were identified. The frequency of these polymorphisms was similar between spinocerebellar ataxia patients and normal controls. These data indicate that spontaneous mutations do not occur in *GRID2* and that the incidence of spinocerebellar ataxia in humans is not associated with *GRID2* mutation or polymorphisms.

**Key Words:** nerve regeneration; spinocerebellar ataxia;  $\delta 2$  glutamate receptor; mutation; gene polymorphism; single nucleotide polymorphism; NSFC grant; neural regeneration

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## Introduction

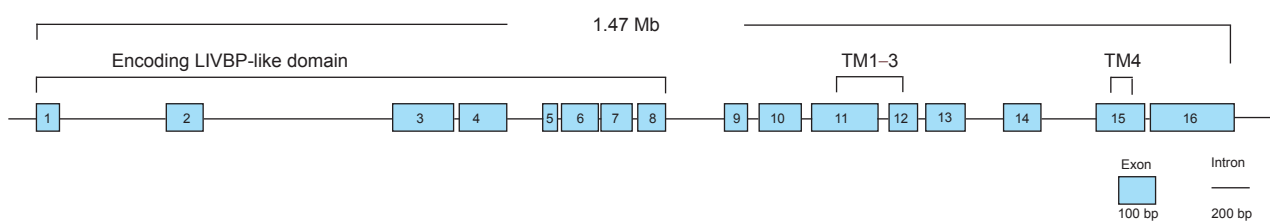
Spinocerebellar ataxia (SCA) is a clinically and genetically heterogeneous group of neurodegenerative disorders, which are characterized by progressive ataxia variably associated with other neurological signs and progressive degeneration of cerebellum and brainstem. Although common pathological changes are believed to be involved in the loss of cerebellar Purkinje cells and brainstem neurons, the detailed mechanisms underlying these diseases remain unclear. In SCA cases, 80% are familial and 20% are sporadic. There are two major types of familial SCA: autosomal dominant cerebellar ataxias and autosomal recessive cerebellar ataxias (also known as Friedreich's ataxia). So far, the disease gene has been identified in 12 genetic variants of familial ataxias (*SCA1-SCA3*, *SCA6-SCA8*, *SCA10*, *SCA12*, *SCA14*, *SCA17*, *FGF14-SCA*, and *DRPLA*) (Orr et al., 1993; Kawaguchi et al., 1994; Koide et al., 1994; Nagafuchi et al., 1994; Imbert et al., 1996; Pulst et al., 1996; Sanpei et al., 1996; David et al., 1997; Zhuchenko et al., 1997). In most cases, the disorder is caused by expanded CAG repeats within the coding sequence of ataxin genes (*SCA1-SCA3*, *SCA6*, *SCA7*, *SCA17*, and *DRPLA*), which are translated into polyglutamine tracts, or by expanded trinucleotide repeats in the promoter (CAG in *SCA12*) or in the non-coding region of the RNA (CTG in *SCA8*). However, not all patients with cerebellar ataxia,

especially sporadic cases, are caused by trinucleotide repeat expansions. Moreover, although there is persuasive evidence that the mutant proteins gain toxic functions, precisely what these new functions might be and how a ubiquitous protein can cause the degeneration of only a highly circumscribed neuronal population remains a fundamental question (Lin et al., 2000; Orr, 2012).

Most familial SCAs are inherited in an autosomal dominant manner and several subtypes of the disorder are linked to trinucleotide expansions within the ataxin gene. However, trinucleotide expansions can only be detected in a proportion of patients, indicating that the pathogenesis of these disorders may involve other causal genes. Recently, a member of the ionotropic glutamate receptor family, glutamate receptor delta 2 (*GRID2*), has been shown to play crucial roles in cerebellar functions in mice. Mouse *Grid2* (also known as *GluR $\delta$ 2*) is predominantly located in postsynaptic dendrites of parallel fiber-Purkinje cell synapses in the cerebellum. When expressed alone or co-expressed with other glutamate receptors,  $\delta 2$  receptors do not form functional glutamate-gated ion-channels or bind to glutamate analogues (Araki et al., 1993; Rossi and Borsello, 1993). Nevertheless, knockout of *Grid2* impairs the formation and stabilization of parallel fiber-Purkinje cell synapse and suppresses parallel fiber-Purkinje cell long-term depression (Selimi et al., 2003; Kuroyan-

**Table 1** PCR annealing temperatures and denaturing high performance liquid chromatography (DHPLC) resolution temperatures for *GRID2* exons

Exon	Primer sequence	Amplicon size (bp)	PCR annealing temperature (°C)	DHPLC resolution temperature (°C)
1	Forward: 5'-CAT CCT CCA AGA GAA TCG GC-3' Reverse: 5'-CAC GAG CTG CAC CAA CAC TT-3'	144	56.0	60.8
2	Forward: 5'-CCA GAG GGG ATG ACA GAA T-3' Reverse: 5'-CCC TTA CCT TCT TGA ACT GC-3'	222	56.0	58
3	Forward: 5'-ACT GTG CTT TCT TGC AGC CTG-3' Reverse: 5'-GCG AGT AAG ACC CTT GAA AC-3'	393	60.0	61.4
4	Forward: 5'-ACA GCC TAC AGT GTG GAA C-3' Reverse: 5'-GTA CAA CTC TCA CCC CAA G-3'	349	60.0	57.5
5	Forward: 5'-GCA AGT CTA CAG TGC ATC-3' Reverse: 5'-GCA GCA TTC ACT CAG TAA G-3'	390	56.0	54.9
6	Forward: 5'-CGC TCT AAT TCT TCC ACC TC-3' Reverse: 5'-CTC TCC TTG CAA TTG CCC AAC-3'	264	56.0	56.9
7	Forward: 5'-GCC TGA TGA CTG GTG TCA ATG-3' Reverse: 5'-GTA AGG AAG CCT CCC CTT T-3'	399	60.0	58.5
8	Forward: 5'-GTT CTC TCC TTT AGG GTG G-3' Reverse: 5'-ACC CCC TGC TTT CTC TTT CA-3'	261	60.0	53.4
9	Forward: 5'-CAG AGG TGA TGG GAC TGT T-3' Reverse: 5'-CCA CGA GGC TCA GAT AAT-3'	185	58.0	58.5
10	Forward: 5'-CGG GAG CAC TAT AGA TTG AC-3' Reverse: 5'-CCA CCA CCT AAT GCT TAA CC-3'	387	58.0	56.3
11	Forward: 5'-GGC AAG CTG AAG TGG CAC AA-3' Reverse: 5'-CGG CTT TGG AAA CAT CAC-3'	446	62.0	58.9
12	Forward: 5'-TGC TTC TTT CTA CAG GCG-3' Reverse: 5'-CTC AGA TAG CTT TGG CCA C-3'	222	54.0	56.6
13	Forward: 5'-GTG TCT CTC TTC TCT CCC A-3' Reverse: 5'-CAA TTC CAG CAT GGG ACA C-3'	283	58.0	60.2
14	Forward: 5'-TCC CCA ACA GAT ACT GAA GG-3' Reverse: 5'-CTC TCC CCT TAT TTG GTC-3'	349	54.0	55.8
15	Forward: 5'-GGA TGT GTT GTG AAC CAG-3' Reverse: 5'-CAG CCT GCT TGT TGA TTC-3'	356	60.0	59.1
16	Forward: 5'-CGC TAG TAC TGA TGA GAA CC-3' Reverse: 5'-TGC TCC TTG CAA AGG GAG T-3'	522	58.0	60

**Figure 1** Genomic organization of the human *GRID2* gene.

The exons and introns are drawn according to different scales. The positions of the putative transmembrane regions (TM1–4) are indicated. LIVBP: Leucine/isoleucine/valine-binding protein; TM: sequence encoding a transmembrane domain.

agi et al., 2009), causing ataxic phenotypes that are similar to spinocerebellar ataxias in humans. The *Grid2* gene is one of the largest brain-expressed genes in mice, spanning 1.4 Mb and comprising 16 exons (Wang et al., 2003). *Grid2* includes several large introns and because of the existence of potential fragile sites within these large introns, frequent spontaneous in-frame deletions within the N-terminal domain, designated as hotfoot mutations, occur in mice (Motohashi et al., 2007). All the deletions are recessive and occur within

an N-terminal extracellular leucine/isoleucine/valine-binding protein-like domain of the protein. This may inhibit the protein's function by impairing its exit from the endoplasmic reticulum. In addition, a semi-dominant point mutation (*Lurcher*) in exon 12 causes constitutive activation of  $\delta 2$  receptors in the absence of ligand, and eventually leads to the death of Purkinje cells (Zuo et al., 1997; Zanjani et al., 2009; Armstrong et al., 2011). These results establish *Grid2* mutations to be involved in the pathology of neurodegeneration

**Table 2 Allelic frequency of mutations or single-nucleotide polymorphisms in *GRID2***

Mutations or single-nucleotide polymorphisms	Controls (2n = 104)	Patients (2n = 48)	$\chi^2$	P
IVS5-121_-118del GAGT				
del <sup>+</sup>	7	6		0.34
del <sup>-</sup>	97	42		
c.1251G>T				
G	85	34	2.29	0.1
T	19	14		
IVS14-63C>G				
C	77	37	0.16	0.43
G	27	11		

Comparisons among alleles of single nucleotide polymorphisms or point mutations were performed using the two-tailed Pearson chi-square test or Fisher's exact test.

involved in cerebellar ataxia. In addition, two studies have reported the detection of anti-*GRID2* antibody in patients with focal epilepsy, dystonia and encephalitis (Fukuoka et al., 2012; Matsumoto et al., 2012). The human gene, *GRID2*, has been mapped to chromosome 4q22, and shares 97.0% amino acid and 90.0% nucleotide sequence identity with mouse *Grid2* (Zuo et al., 1997). The high frequency of spontaneous mutations in the mouse *Grid2* gene and the similarities of the phenotypes of these mutants to human cerebellar ataxia, suggests that *GRID2* is a good candidate gene for mutational screening in SCA. We speculate that a similar association exists between *GRID2* mutations and diseases presenting as ataxias, such as SCA. In this study, we screened for mutations in human *GRID2* in both normal subjects and SCA patients, in an effort to determine whether spontaneous mutations occur in humans and to identify possible mutations/variations that cause or are correlated with the ataxia group of diseases.

## Subjects and Methods

### Subjects

Seventeen patients from four unrelated families with autosomal dominant cerebellar ataxias, seven patients with sporadic cerebellar ataxia and 52 normal controls were included in this study. All patients displayed typical cerebellar ataxia and were variably associated with other symptoms or signs involving the brain or brainstem, such as nystagmus, tremor and dysphagia. Diagnoses were made according to a progressive clinical phenotype in which ataxia was the prominent symptom. Patients with ataxia caused by stroke, central nervous system infection, and chronic alcoholism were excluded from the study. This study was approved by the Ethics Committee of Fujian Medical University, China.

### Extraction of genomic DNA

Genomic DNA was prepared from peripheral blood using a standard phenol/chloroform method (Davoren et al., 2007).

### Identification of the *GRID2* genomic sequence

The genomic sequence of *GRID2* was identified by search-

ing human genomic databases using the full-length *GRID2* cDNA sequence (Genbank accession number: AF009014. <http://www.ncbi.nlm.nih.gov/gene/2895>). The exon-intron boundaries of *GRID2* were defined by aligning its cDNA sequence with the genomic sequence.

### PCR analysis of *GRID2* exons

PCR was performed on a MyCycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) with primers designed from the intronic sequences flanking each exon.

The schedule for PCR was as follows: preincubation at 94°C for 5 minutes; 35 cycles, incubation at 94°C for 45 seconds, at 56°C (or other annealing temperature according to the primers used, as listed in **Table 1**) for 45 seconds and at 72°C for 1 minute; finally, incubation at 72°C for 5 minutes. The PCR products were separated by electrophoresis on 6% polyacrylamide gels. DHPLC analysis for *GRID2* was performed using the WAVE 3500 DNA Fragment Analysis System (Transgenomic Inc., Omaha, Nebraska, USA) with a DNasep cartridge. Prior to analysis, PCR products were denatured for 5 minutes at 95°C, gradually cooled to 50°C and incubated at this temperature for 15 minutes before cooling rapidly to 4°C. Between 50–100 ng (5–10  $\mu$ L) of each PCR product was then separated over a certain column temperature and through a linear acetonitrile gradient to detect the presence of heteroduplexes. The buffers used for DHPLC were buffer A (0.1 mol/L triethylammonium acetate) and buffer B (0.1 mol/L triethylammonium acetate and 25% acetonitrile). The run temperature for each amplicon was determined by the fragment size and GC content of the amplicon, as calculated by Navigator software (Transgenomic Inc.), and resolution was performed at the recommended temperatures and at temperatures near the optimal prediction.

*GRID2* amplicons with heteroduplexes detected by DHPLC were sequenced. PCR products were first purified using Microcon1 PCR Filter Units (Millipore Corporation, Bedford, MA, USA) and then sequenced on an ABI 3730XL sequencer (Applied Biosystems, Foster City, CA, USA) using appropriate sequencing primers. The resulting sequences were analyzed with Lasergene v2 software (DNASTAR, Inc., Madison, WI, USA) and the gene sequences were analyzed using the WU-2 blast program (<http://www.ebi.ac.uk/blast2/>) and the single-nucleotide polymorphism database (<http://www.ncbi.nlm.nih.gov/SNP/>).

### Statistical analysis

Comparisons among alleles of single nucleotide polymorphisms or point mutations were performed using the two-tailed Pearson chi-square test or Fisher's exact test. The differences were considered significant if the *P* value was  $\leq 0.05$ .

## Results

### Genomic structure of the human *GRID2* gene

By searching the Genbank database, the genomic sequence of *GRID2* was found to be contained in a chromosome 4 genomic contig (Genbank accession number: NT\_016354). As depicted in **Figure 1**, the entire gene covers a region of 1.47 Mb, which is slightly larger than that of the mouse

*Grid2* gene (1.4 Mb). All exon-intron junctions were identical to canonical splice donor and acceptor sites. Similar to the mouse *Grid2* gene, human *GRID2* comprises 16 exons, of which the shortest (exon 5) encodes 17 amino acids and the longest (exon 16) encodes 141 amino acids. *GRID2* also contains six large introns including intron 1 (285 kb), 2 (495 kb), 4 (96 kb), 8 (157 kb), 13 (111 kb) and 14 (142 kb). Although the genomic sequence of *GRID2* gene is longer than that of *Grid2*, the length of all 16 exons is almost identical between *GRID2* and *Grid2*. Only a few nucleotide differences in some exons were identified between the two genes. In addition, locations of the N-terminal extracellular leucine/isoleucine/valine-binding protein-like domain and the four transmembrane domains in *GRID2* are the same as those in *Grid2*.

#### No homozygous exonic deletion was detected within *GRID2*

To examine whether homozygous exonic deletions, similar to hotfoot mutations in mice, occur in humans, we PCR-amplified fragments containing all 16 exons of *GRID2* from both controls and patients with cerebellar ataxia. A single band corresponding to each exon was amplified from all samples and no homozygous deletion was detected in either patients or controls, suggesting that spontaneous exonic deletions of *GRID2* are rare in humans. However, heterozygous deletions could not be excluded because quantitative analysis of the PCR products was not performed in this study.

#### Point variations within *GRID2*

To identify possible point mutations or polymorphisms within *GRID2*, DHPLC was performed to screen for heteroduplexes in all 16 exons of the gene, both in patients with cerebellar ataxia and in normal controls. Heterozygous deletion/insertion was determined by two separate peaks at the non-denaturing temperature (50°C) and confirmed by multiple peaks at the partially denaturing temperatures. Heterozygosity for single nucleotide variation was determined by the presence of one to three additional peaks in the elution profile of the amplicons at denaturing temperatures. As a result, a possible heterozygous deletion/insertion was observed in exon 5-containing fragments both in patients and controls (**Figure 2**). Heterozygous duplexes at denaturing temperatures were also observed in both groups in fragments containing exon 9 (**Figure 2B and C**) and exon 13 (data not shown), indicating point mutations or single nucleotide polymorphisms. No heteroduplexes were detected within the remaining 13 fragments that contained individual exons of *GRID2*.

#### Mutations or polymorphisms within *GRID2*

To confirm the variations within *GRID2* detected by DHPLC, we sequenced all the amplicons displaying heteroduplexes at denaturing temperatures. As indicated in **Figure 3A**, a 4 nt deletion in intron 4 (IVS5-121\_-118del GAGT) was revealed in all exon 5-containing amplicons displaying a double peak at 50°C in both controls and patients, indicating that it is a polymorphic deletion. In addition, in exon 9-containing fragments with heteroduplexes, a G to T substitution

in exon 9 (c.1251G>T) was revealed in both patients and controls (**Figure 3B**), suggesting that it is a single nucleotide polymorphism. Similarly, in the exon 14-containing amplicons with heteroduplexes, a C to G substitution in intron 13 (IVS14-63C>G) was found in both controls and patients, suggesting that it is another single nucleotide polymorphism (**Figure 3C**). Although human exon 12 shares 100% identity with the mouse counterpart, neither of the two well-known mouse point mutations that are within this exon, *ho5J* (c.1899delG) and *Lurcher* (c.1960G>A), was identified in patients with cerebellar ataxia.

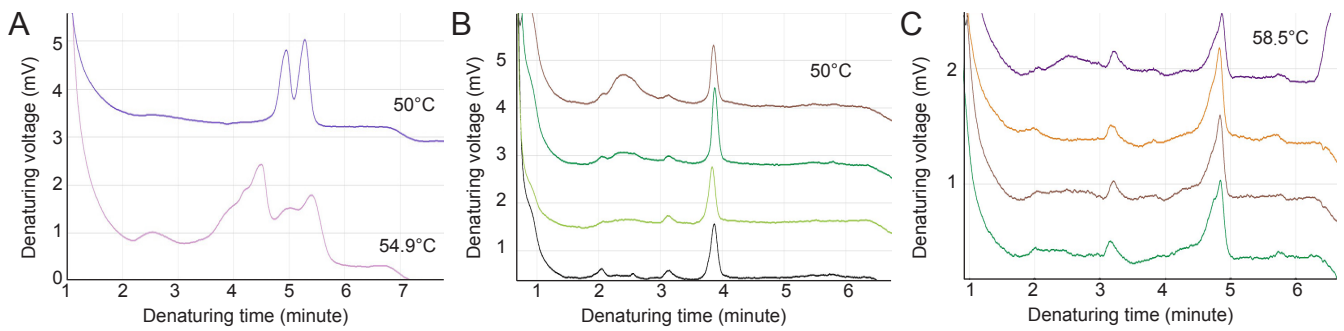
#### Association between the polymorphisms in *GRID2* and spinocerebellar ataxia

To examine the association between the above three polymorphisms and cerebellar ataxia, allelic frequencies of these polymorphisms were compared between normal controls and cerebellar ataxia patients. As indicated in **Table 2**, none of the differences in allelic frequency for the three polymorphisms were statistically significant ( $P > 0.05$ ), suggesting no association between the disease and the polymorphisms in this population.

#### Discussion

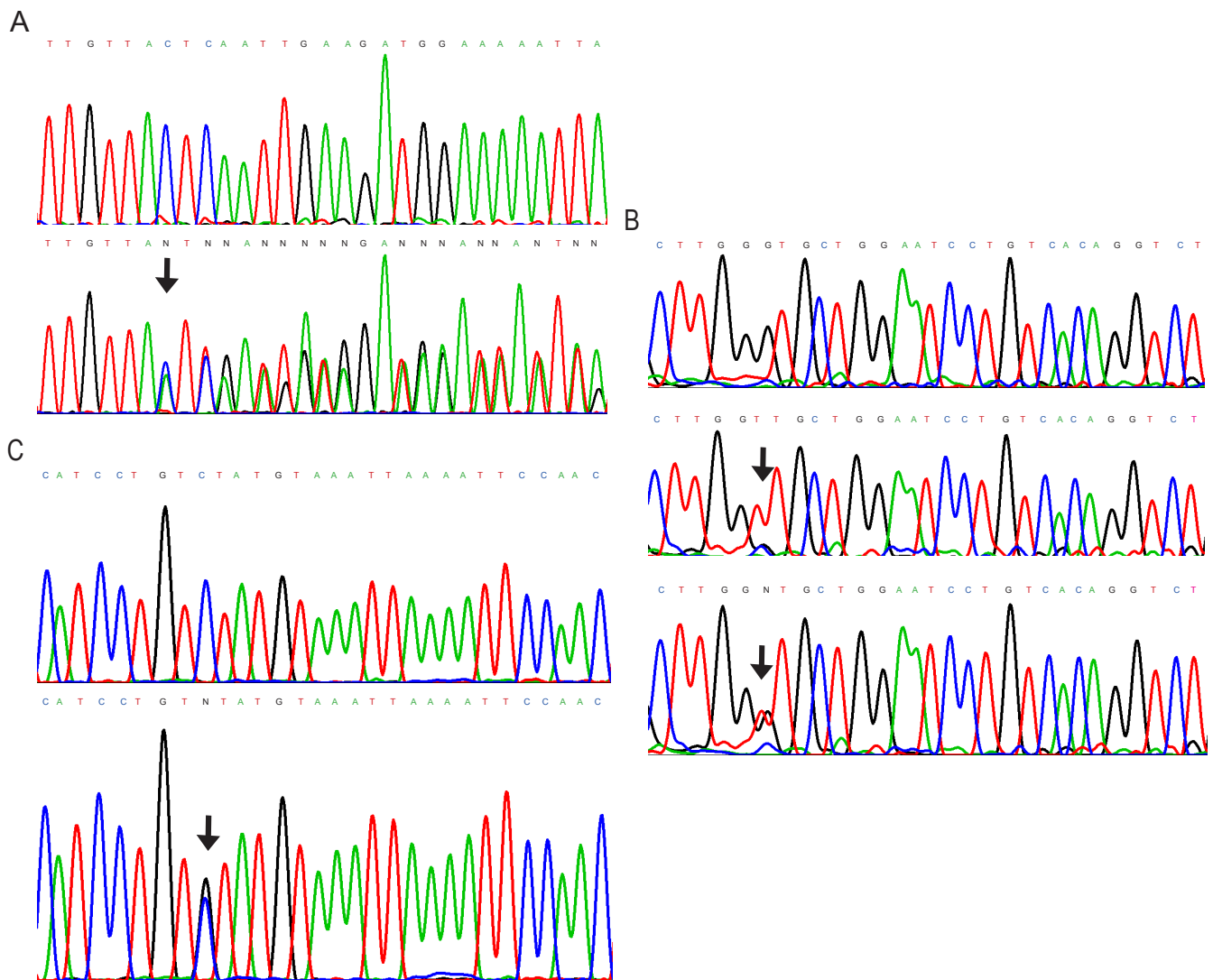
*GRID2* gene is attracting more and more attention as it plays important roles in cerebellar functions, especially those occurring at the parallel fiber-Purkinje cell synapse (Kakegawa et al., 2011; Yamasaki et al., 2011). Although mutations within *Grid2* have been widely studied in mice, no such studies have been undertaken in humans. We first identified the structure of human *GRID2* and found that both the length and sequence of its 16 exons are almost identical to those of the mouse *Grid2* gene, which is subject to high rates of genomic rearrangement and point mutations. Contrary to our hypothesis that similar mutations may also occur in the human *GRID2* gene, especially in patients with cerebellar ataxias, we detected neither hotfoot nor *Lurcher* mutations in patients or controls. The explanation for the relative stability of *GRID2*, which is in sharp contrast to its mouse counterpart, is not clear. However, in the mouse, heterozygous carriers of all known *Grid2* null mutations are normal, and the ataxic phenotype is expressed only in homozygotes (Wang et al., 2003; Motohashi et al., 2007). It is possible that human mutations have not been detected because humans carrying two *GRID2* null alleles are rare and heterozygous deletions could not be detected by the methods used in this study (Robinson et al., 2005; Mandolesi et al., 2009). Another possibility is that the cases included in the present study were spinocerebellar ataxia, in which the pathological changes are not confined to cerebellum, while *GRID2* is relatively specifically expressed in the cerebellum. Thus, patients with ataxic symptoms in which neuronal degeneration is more specifically confined to the cerebellum, such as Holmes type ataxia or primary cerebellar degeneration, are needed to screen for *GRID2* mutations. Alternatively, other conditions with motor discoordination or with other forms of motor learning deficits may be more suitable for screening for *GRID2* muta-





**Figure 2** Sequence alterations detected by denaturing high-performance liquid chromatography analysis.

Analysis of *GRID2* PCR products for exon 5 (A) or exon 9 (B, C) (including flanking sequences) was performed using the WAVE 3500 DNA Fragment Analysis System. (A) Double peaks at the non-denaturing temperature (50°C) (upper) and multiple peaks at partially denaturing temperature (54.9°C) (lower) were detected in exon 5, indicating the presence of a mixture of two fragments with different sizes. (B) Exon 9: Homogeneous peak at the non-denaturing temperature (50°C). (C) Exon 9: Heterozygous duplexes at partially denaturing temperature (58.5°C), indicating the presence of a point mutation within the exon.



**Figure 3** *GRID2* polymorphisms detected by DNA sequence analysis.

PCR amplicons displaying heterozygous duplexes at partially denaturing temperatures were sequenced. (A) A 4 nt deletion was detected in *GRID2* intron 4 (IVS5-121\_-118del GAGT) in both patients (lower) and controls (upper), indicating that it is a polymorphism. (B) A G to T substitution was revealed in exon 9 (c.1251G>T) in both patients and controls, suggesting that it is a single nucleotide polymorphism. Genotypes for different alleles are shown (upper: GG; middle: TT; lower: GT). (C) A C to G substitution in intron 13 (IVS14-63C>G) was detected in both controls and patients, suggesting that it is a single nucleotide polymorphism. Upper: Wild type; lower: mutant. Arrows point to the polymorphism sites.

tions. It is also possible that human mutations have less severe phenotypic effects, perhaps because of different levels of complementation by other glutamate receptors at appropriate steps of brain development. In either case, the high levels of mutability of this and other large genes expressed in the brain may provide a new clue in the quest for genetic causes of neurological disease. We recognize that examination for trinucleotide expansion and other known spinocerebellar ataxia mutations prior to screening for *GRID2* mutations is important, but the purpose of the current study was to identify spontaneous *GRID2* mutations irrespective of the coexistence of other mutations.

Although no causal mutation in *GRID2* was detected in the patients with spinocerebellar ataxias, several polymorphisms were identified for the first time in this gene. The heterozygous GAGT deletion in intron 4 (IVS5-121\_-118del GAGT), which is 121 nt upstream of exon 5, was detected in both controls and patients. Moreover, the frequency of the deletion appeared to be more frequent in patients with cerebellar ataxia (6/24, 25%) than in controls (7/52, 14%), although the difference was not statistically significant. Likewise, the other two single nucleotide substitutions, c.1251G>T and IVS14-63C>G, located in exon 9 and intron 13, respectively, are distributed with no statistical difference among patients and controls. However, because of the small sample size of the current study, exclusion of an association between these polymorphisms and spinocerebellar ataxias should be assigned with caution. Of particular interest, the GATA deletion identified in intron 4 is located in one of the two fragile sites responsible for the frequent occurrence of in-frame deletions within the N-terminal domain in the hotfoot mutant (Robinson et al., 2005). It is plausible that the 4 nt deletion within intron 4 may either create potential fragile sites or increase their fragilities and confers increased risk for cerebellar ataxias or other cerebellar dysfunctions. To test this, an expanded sample size is needed for a further case-control study.

Collectively, our observations suggest that homozygous exonic deletions within the human *GRID2* gene do not exist in patients with spinocerebellar ataxia. However, several novel polymorphisms, including small deletions and single nucleotide polymorphisms are identified for the first time in this gene. Studies with larger samples of patients with cerebellar ataxia or other diseases involving cerebellar dysfunctions are needed to investigate the functions of *GRID2*.

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**Author contributions:** Wang CD participated in study concept and design. Huang JX, Lin AY and Wang CD were responsible for subject recruitment, sample collection and clinical assessment. Huang JX and Dong HY participated in DHPLC and DNA sequencing analysis. Huang JX and Wang CD drafted the manuscript. Wang CD supervised the whole study. All authors approved

the final version of the manuscript.

**Conflicts of interest:** None declared.

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