

Hot-spot *KIF5A* mutations cause familial ALS

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*Appendix 1.

Heterozygous missense mutations in the N-terminal motor or coiled-coil domains of the kinesin family member 5A (*KIF5A*) gene cause monogenic spastic paraplegia (HSP10) and Charcot-Marie-Tooth disease type 2 (CMT2). Moreover, heterozygous *de novo* frame-shift mutations in the C-terminal domain of *KIF5A* are associated with neonatal intractable myoclonus, a neurodevelopmental syndrome. These findings, together with the observation that many of the disease genes associated with amyotrophic lateral sclerosis disrupt cytoskeletal function and intracellular transport, led us to hypothesize that mutations in *KIF5A* are also a cause of amyotrophic lateral sclerosis. Using whole exome sequencing followed by rare variant analysis of 426 patients with familial amyotrophic lateral sclerosis and 6137 control subjects, we detected an enrichment of *KIF5A* splice-site mutations in amyotrophic lateral sclerosis (2/426 compared to 0/6137 in controls; $P = 4.2 \times 10^{-3}$), both located in a hot-spot in the C-terminus of the protein and predicted to affect splicing exon 27. We additionally show co-segregation with amyotrophic lateral sclerosis of two canonical splice-site mutations in two families. Investigation of lymphoblast cell lines from patients with *KIF5A* splice-site mutations revealed the loss of mutant RNA expression and suggested haploinsufficiency as the most probable underlying molecular mechanism. Furthermore, mRNA sequencing of a rare non-synonymous missense mutation (predicting p.Arg1007Gly) located in the C-terminus of the protein shortly upstream of the splice donor of exon 27 revealed defective *KIF5A* pre-mRNA splicing in respective patient-derived cell lines owing to abrogation of the donor site. Finally, the non-synonymous single nucleotide variant rs113247976 (minor allele frequency = 1.00% in controls, $n = 6137$), also located in the C-terminal region [p.(Pro986Leu) in exon 26], was significantly enriched in familial amyotrophic lateral sclerosis patients (minor allele frequency = 3.40%; $P = 1.28 \times 10^{-7}$). Our study demonstrates that mutations located specifically in a C-terminal hotspot of *KIF5A* can cause a classical amyotrophic lateral sclerosis phenotype, and underline the involvement of intracellular transport processes in amyotrophic lateral sclerosis pathogenesis.

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Abbreviations: ALS = amyotrophic lateral sclerosis; CMT = Charcot-Marie-Tooth disease; HSP = hereditary spastic paraplegia; MAF = minor allele frequency; SNV = single nucleotide variant

Introduction

KIF5A is a member of the kinesin family of proteins that is mainly expressed in neurons (Niclas *et al.*, 1994; Fagerberg *et al.*, 2014). As part of a multi-subunit complex, it acts as a microtubule motor in intracellular protein and organelle transport, including mitochondria (Hirokawa *et al.* 2009). Missense mutations in the kinesin family member 5A (KIF5A) gene at 12q13.3 are the third most frequent cause of autosomal dominant hereditary spastic paraplegia (HSP10, SPG10, OMIM#604187) in European populations, affecting primarily the long pyramidal tracts and sometimes also the peripheral nervous system (Reid *et al.*, 2002; Goizet *et al.*, 2009; Morais *et al.*, 2017). Additionally, the known phenotypic spectrum of KIF5A mutations comprises also an autosomal dominant axonal sensorimotor peripheral neuropathy (Charcot-Marie-Tooth disease type 2; CMT2) (Liu *et al.*, 2014) and a complex infantile neurological syndrome with leukoencephalopathy, myoclonus, hypotonia, optic nerve abnormalities, dysphagia, apnoea, hearing loss, and early developmental arrest [neonatal intractable myoclonus (NEIMY; OMIM# 617235); Duis *et al.*, 2016; Rydzanicz *et al.*, 2017].

Some 5% of patients with the motor neuron disease amyotrophic lateral sclerosis (ALS) self-report a positive family history (familial ALS), most frequently as a

Mendelian autosomal dominant trait. Since 1993, mutations in over 36 genes have been associated with ALS pathogenesis, and mutations in several of these have been predicted to disrupt cytoskeletal function and intracellular transport (*PFN1*, *NEFH*, *PRPH*, *ALSIN*, *DCTN1*; *TUBA4A* Figlewicz *et al.*, 1994; Yang *et al.*, 2001; Puls *et al.*, 2003; Gros-Louis *et al.*, 2004; Wu *et al.*, 2012; Smith *et al.*, 2014). Datasets of two large studies based on whole exome sequencing or genome-wide association testing suggested also an association between variants in *KIF5A* and ALS (Kenna *et al.*, 2016; McLaughlin *et al.*, 2017). In both studies, the association did not achieve genome-wide statistical significance and the studies also lacked data on a possible co-segregation of *KIF5A* variants with ALS. Furthermore, detailed clinical information beyond the phenotype ‘ALS’ was not available with regard to the possibly *KIF5A*-linked patients. Altogether, we hypothesized that mutations affecting *KIF5A* can also be a cause of ALS. Consequently, we here assessed a possible association between *KIF5A* and ALS by first comparing the mutation burden of *KIF5A* in a cohort of 426 familial ALS patients with 144 769 control individuals (comprising 6137 in-house controls and the gnomAD dataset) followed by co-segregation analysis, detailed clinical description of the patients, as well as RNA expression and splicing analysis of *KIF5A* mutations in patient-derived cell lines.

Material and methods

Patients and ethics statement

All ALS patients were diagnosed according to the EFNS Consensus criteria (Andersen *et al.*, 2005, 2012). With informed written consent and approval by the national medical ethical review boards in accordance with the Declaration of Helsinki, EDTA blood samples were drawn from controls, ALS patients, and their unaffected relatives. DNA was extracted from EDTA blood samples according to standard procedures.

Genotyping for *SOD1* and *C9orf72* mutations

Mutations in *SOD1* and *C9orf72* were excluded prior to exome sequencing of familial ALS cases as described before (Freischmidt *et al.*, 2015).

Whole-exome sequencing

We sequenced exomes of 426 European familial ALS index patients and 6137 control subjects. Controls comprised healthy parents of children with various diseases, healthy tissues of individuals with tumour diseases, and 200 individuals from the KORA studies (Kooperative Gesundheitsforschung in der Region Augsburg) (Herder *et al.*, 2013). Sequencing, read mapping and variant calling was performed on HiSeq2000/2500 systems (Illumina) as described previously (Freischmidt *et al.*, 2015).

RNA expression and splicing analysis

RNA was isolated from the immortalized lymphoblast cell lines derived from the mutation carriers and their unaffected mutation-negative relatives. To test the effect of the variants on mRNA level, fragments were amplified using the cDNA template with primers binding to exon 25 and 28/29. Primer sequences are available on request. PCR products were sequenced on ABI 3130xl Genetic Analyzer using BigDye v3.1 cycle sequencing kit (Life Technologies), according to the manufacturer's protocol. Mutation nomenclature is according to the transcript NM_004984.

To calculate the relative expression of *KIF5A* mRNA in the subjects, quantitative real-time PCR was performed with SYBR[®] Green chemistry. Primers spanning exons 2/3 and exon 3/4 were used. TBP (TATA-binding protein) was used as an internal control for normalization. The $\Delta\Delta Ct$ method was used for quantification (Livak and Schmittgen, 2001).

Statistics

Fisher's exact test was used to compare sequence variant frequencies between ALS and control groups. A significance level $\alpha < 0.05$ was applied in all tests statistical tests (two-tailed).

For linkage analysis of Families A–C, we assumed an autosomal dominant model. Penetrance was set at 0.8. The frequency of the deleterious allele was set at 0.0001, the phenocopy rate at 0.003, and the marker allele frequency to 0.0001. Linkage analysis was performed using Merlin software (version 1.1.2). We set the phenotype to unknown if an unaffected individual was either younger than 60 years or had died before the age of 60 years.

Results

Exome sequencing and association analysis

To assess a possible association between *KIF5A* variants and familial ALS we analysed whole exome sequence data of 426 familial ALS index patients. The frequency of *KIF5A* variants was compared to 6137 in-house whole exome datasets from control individuals with non-neurological diseases and the 138 632 exomes and genomes of the gnomAD dataset (<http://gnomad.broadinstitute.org>, Lek *et al.*, 2016). The familial ALS index patients were selected for whole exome sequencing from families with at least two individuals affected by ALS or frontotemporal dementia from the six European countries: Germany, Denmark, Finland, Sweden, Switzerland, and Portugal, subsequent to a negative screen for pathogenic mutations in *SOD1* and *C9orf72*.

We analysed missense variants of *KIF5A* at a minor allele frequency (MAF) below the thresholds of 1% and 0.1%, respectively. We did not observe a significant overall enrichment of *KIF5A* rare missense variants in the familial ALS group when compared to our in-house control group ($n = 6137$) or the gnomAD dataset ($n = 138\,632$; Tables 1 and 2).

Although we did not detect a significant enrichment of rare missense variants in the patient group, the non-synonymous single nucleotide variant (SNV) rs113247976 showed a trend towards enrichment in ALS patients in a previous GWAS (McLaughlin *et al.*, 2017). We found this SNV highly enriched in our familial ALS cohort [29/426 patients; allele frequency (AF) = 3.4%] compared to in-house controls (123/6137 individuals; AF = 1.00%; $P = 1.28 \times 10^{-7}$) or the gnomAD database (3132/138 632 individuals; AF = 1.13%; $P = 3.11 \times 10^{-7}$). This SNV predicts an amino acid exchange in *KIF5A* [hg19: g.57,975,700C>T; c.2957C>T; p.(Pro986Leu)] (Table 2). Rs113247976 represents the only non-synonymous variant with a frequency >0.1% in the normal population (gnomAD dataset and in-house controls); thus the rest of *KIF5A* displays high evolutionary conservation. Remarkably, 11 of 29 patients carrying rs113247976 also had a heterozygous genetic variant in one of the following ALS genes (Supplementary Table 2): *HNRNPA1* (p.M137V), *VCP* (p.R95C), *ERBB4* (p.T271I), *TARDBP* (p.A315T and p.N352S each in one patient), *FUS* (p.G405R and p.R514T each in one case),

Table 1 KIF5A splice site and rare missense variants (MAF < 1%) found in this study (426 index patients) and basic clinical characteristics of index patients

| Variant ^a c: NM_004984.2: g: NC_000012.11 | Predicted consequence at protein level | MAF, % Allele count (gnomAD) | Onset | Age at onset, years | Disease duration, months | Phenotype |
|--|--|---|--------------------------|---------------------------|--------------------------------|----------------------------|
| Missense variants | | | | | | |
| c.1238A>G | p.Glu413Gly | 4.062 × 10 ⁻⁶ (1/246 210) | Spinal, right UL both MN | 35 | 28 | Classical ALS LMN > UMN |
| g.57968879A>G | | | | | | |
| c.1422A>T | p.Gln474His | 4.065 × 10 ⁻⁶ (1/246 010) | Spinal, left LL both MN | 68 | 41 | Classical ALS plus FTD |
| g.57965903A>T | | | | | | |
| c.1729A>G | p.Ser577Gly | 4.062 × 10 ⁻⁶ (1/246 210) | Bulbar | 35 | 60 | ALS |
| g.57968879A>G | | | | | | |
| c.3019A>G | p.(Arg1007Gly) ^b | 0 | Spinal, LMN right UL | 53 | 45 | Classical ALS LMN > UMN |
| g.57976411A>G | | | | | | |
| Splice site | | | | | | |
| c.2993-1G> Ag.57976384G>A | c | 4.061 × 10 ⁻⁶ (1/246 246) | Spinal, UMN left LL | 56 | >36 (alive) | Classical ALS UMN = LMN |
| c.3020+2T>C | p.(Asn999Valfs*39) | 0 | Spinal, LMN left UL | 29 | 34 | Classical ALS UMN > LMN |
| g.57976414T>C | | | | | | |
| c.3020+1G>A ^d | p.(Asn999Valfs*39) | 0 | n.a. | n.a. | n.a. | n.a. |
| g.57976413G>A | | | | | | |

^aGenomic positions according to the GRCh37/hg19.

^bPredicted missense mutation, experimentally shown to abrogate function of splice donor site in intron 27 resulting in the predicted change p.Asn999Valfs*39 (see 'Results' section).

^cSplice acceptor site predicted to be abrogated, resulting protein change unpredictable.

^dSplice site variant found in the familial ALS exome data of the ALS Variant Server (AVS) (<http://als.umassmed.edu/>), no clinical information available.

LL = lower limb; LMN = lower motor neuron; n.a. = not available; UMN = upper motor neuron; UL = upper limb.

Table 2 KIF5A variants with the respective allele frequencies in case and control whole exome datasets

| | Familial ALS | In-house exomes (AF) | P-value (Fisher's exact test) | gnomAD dataset (AF) | P-value (Fisher's exact test) |
|------------------------------------|-----------------|-------------------------|----------------------------------|----------------------------------|----------------------------------|
| <i>n</i> | 426 | 6137 | - | 138 632 | - |
| Loss-of-function | 2 (0.23%) | 0 (0%) | 4.2 × 10 ⁻³ | 13 (4.7 × 10 ⁻³ %) | 9.6 × 10 ⁻⁴ |
| Missense (MAF ≤ 1%) | 5 (0.59%) | 88 (0.72%) | n.s. | 1596 (0.58%) | n.s. |
| Missense (MAF ≤ 0.01%) | 4 (0.47%) | n.a. | n.a. | 714 (0.26%) | n.s. |
| SNV rs113247976 [p.(Pro986Leu)] | 29 (3.40%) | 123 (1.00%) | 1.28 × 10 ⁻⁷ | 3132 (1.13%) | 3.11 × 10 ⁻⁷ |

n.a. = not applicable; n.s. = not significant.

FIG4 (p.R699H), SPG11 [c.7152-1G>C (acceptor splice site) and p.V2426M, each in one case; only bi-allelic mutations regarded to be pathogenic], as well as NEK1 (p.Ser1036Ter) and UBQLN2 (p.P509S) in the same individual.

Furthermore, we separately analysed loss-of-function variants, defined as nonsense, canonical splice sites (within two nucleotides of exon boundary), read-through, and frameshift variants. We identified a significant enrichment of KIF5A loss-of-function variants in the patient group (2/426 patients; AF = 0.23%) compared to the in-house control group (0/6137 individuals; AF = 0%; $P = 4.2 \times 10^{-3}$) or the gnomAD dataset (13/138 632 individuals; AF = 4.7 × 10⁻³; $P = 9.6 \times 10^{-4}$) (Tables 1 and 2). Consistent with the low

abundance of loss-of-function variants in control datasets, the pLI score of KIF5A is 1 (<http://exac.broadinstitute.org/gene/ENSG00000155980>), which indicates a high probability of KIF5A loss-of-function mutation intolerance. Mutations in established ALS disease genes were not detected in the patients with KIF5A loss-of-function mutation.

Segregation analysis

Next, we aimed to further corroborate the observed association of deleterious mutations with ALS by segregation analysis. We extended the two families with loss-of-function mutations and reanalysed them using Sanger sequencing. As shown in Fig. 1A and B, both splice site variants

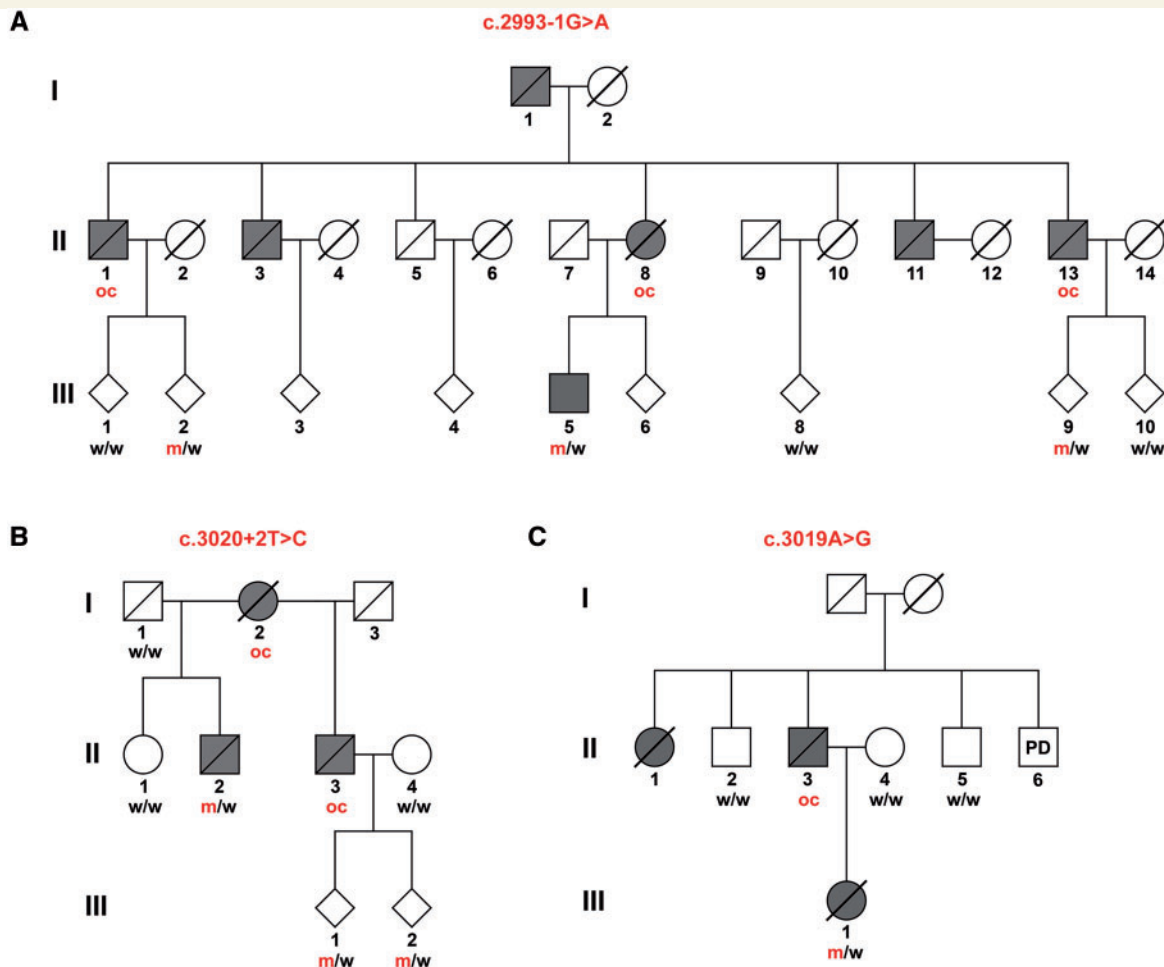


Figure 1 Genetic analysis shows co-segregation of the *KIF5A* splice site variants and the apparent missense variant *c.3019A>G* with ALS. (A) *c.2993-1G>A* (B) *c.3020+2T>C*. (C) *c.3019A>G*. Obligate carriers of the respective variant are abbreviated as 'oc'. All currently asymptomatic mutation carriers are 45 years old or younger. m = mutant allele; PD = Parkinson's disease; w = wild-type allele.

(*c.2993-1G>A* and *c.3020+2T>C*) co-segregate with the disease. Overall, in two families seven patients were either DNA-proven loss-of-function mutation carriers or could be deduced to have been obligate loss-of-function mutation carriers (Fig. 1A and B).

HSP- and CMT-linked *KIF5A* mutations reside in the N-terminal *KIF5A* domains (Fig. 3). In contrast, both the ALS-linked loss-of-function mutations described above, the ALS-associated non-synonymous SNV rs113247976 as well as another splice site mutation (*c.3020+1G>A*) found in familial ALS exome data of the ALS Variant Server (AVS) (<http://als.umassmed.edu/>), reside in close proximity and are predicted to affect the C-terminal exons 26 or 27 (Figs 2E and 3). The same holds true for a rare apparent missense variant detected in our familial ALS exome dataset [*c.3019A>G*; p.(Arg1007Gly)], directly adjacent to the above-mentioned splice site mutations *c.3020+1G>A* and *c.3020+2T>C* (Fig. 1C and Table 1). The variant was detected in the index patient and was absent in the patient's healthy mother. This suggests that the affected father (deceased; no DNA available) of the patient also carried the

variant, although a *de novo* mutation cannot be completely ruled out. Moreover, two unaffected uncles of the patient, one paternal, one maternal, of the patient did not carry the variant. Linkage analysis of Families A–C (Fig. 1) resulted in LOD scores of 0.68, 0.52 and 0.44, respectively, leading to an aggregate logarithm of odds (LOD) score of 1.64.

RNA analysis in patient-derived lymphoblast cell lines

Availability of immortalized lymphoblast cell lines from patients and controls allowed us to further delineate the functional effects of the above-described mutations at the mRNA level. *KIF5A* protein expression is tightly restricted to neurons preventing *KIF5A* protein analysis by western blotting; however, *KIF5A* mRNA was detectable in lymphoblast cell lines by PCR. Sequencing of the PCR products confirmed the presence of wild-type but failed to detect mis-spliced mRNA in lymphoblast cell lines from patients with the splice site variants *c.2993-1G>A* and *c.3020+2T>C* (Fig. 2A). This is most likely attributable

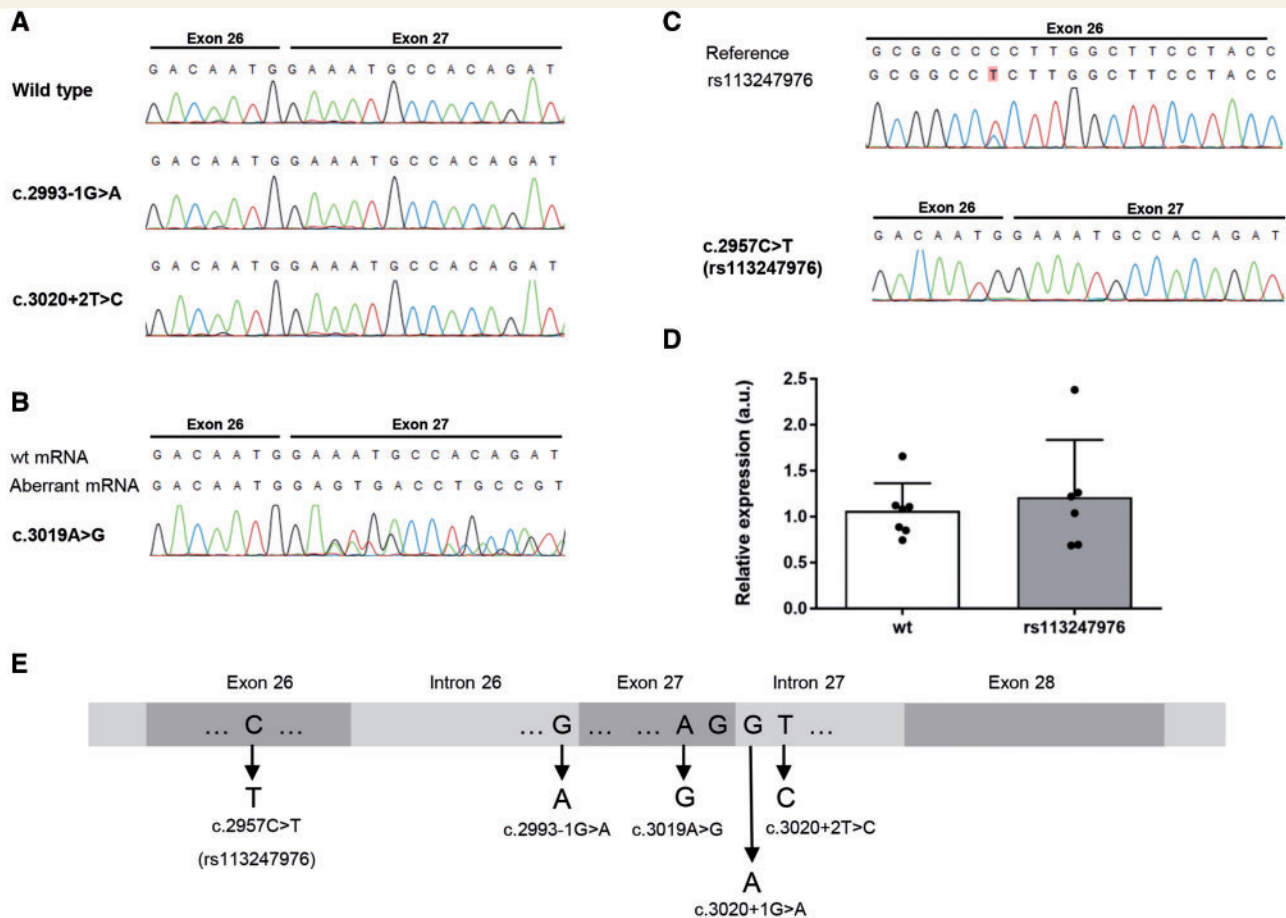


Figure 2 Sequencing and qPCR analysis of mRNA of lymphoblast cell lines from *KIF5A* SNV and mutation carriers. (A) mRNA sequencing of the splice site mutations c.2993-1G>A and c.3020+2T>C shows absence of the mutated allele. (B) mRNA sequencing of the apparent missense variant c.3019A>G reveals a disruption of the splice donor site of intron 27. (C) mRNA sequencing of the SNV rs113247976 [c.2957C>T; p.(Pro986Leu)] suggests unaltered pre-mRNA splicing. (D) As analysed by quantitative PCR, heterozygous carriers of the SNV rs113247976 show equal *KIF5A* mRNA levels as wild-type carriers. (E) Overview of the mutational hotspot around exons 26 and 27 linked to ALS.

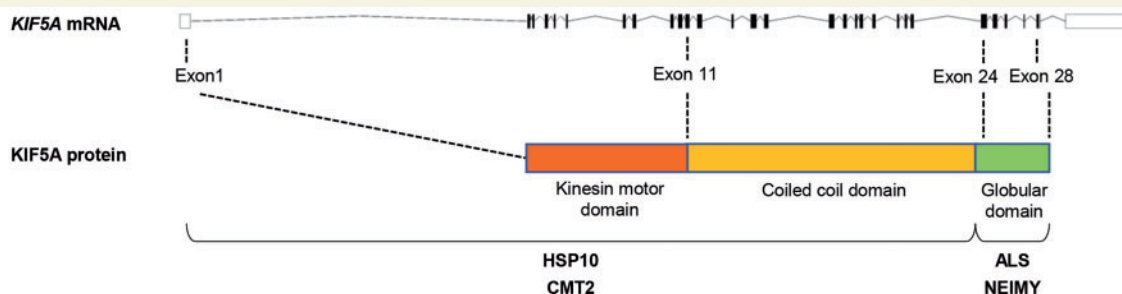


Figure 3 Localizations of mutations in the *KIF5A* gene in the respective diseases caused by *KIF5A* mutations. HSP10 = hereditary spastic paraplegia type 10; NEIMY = neonatal intractable myoclonus.

to nonsense-mediated decay (NMD) of the RNA transcribed from the mutant alleles. To confirm a loss of expression of the splice site mutant alleles, we tried to quantify the total *KIF5A* mRNA levels in c.2993-1G>A and c.3020+2T>C splice site mutant and wild-type lymphoblast cell lines. However, possibly as a result of generally low *KIF5A* expression in non-neuronal cells, the

results were too variable for quantification. Interestingly, the above-mentioned C-terminal rare variant, which theoretically leads to a single amino acid exchange in exon 27 [c.3019A>G; p.(Arg1007Gly)], deviates from the expected effect at the mRNA level: Bioinformatic splice site analysis (Berkeley Drosophila Genome Project; Celniker *et al.*, 2002; http://www.fruitfly.org/seq_tools/splice.html) already

predicts a decrease of the splice site score from 0.79 for the wild-type splice donor site in intron 27 to 0.16 upon the c.3019A>G change. In agreement with this prediction, sequencing of the PCR product from the respective lymphoblast cell line shows that this variant, separated only by one nucleotide from the 5' end of intron 27, disrupts the splice donor site of intron 27 (Fig. 2B). This results in a skipping of exon 27 and the predicted truncating change p.Asn999Valfs*39 at the protein level. Thus, this variant, one of the ALS-linked canonical splice site mutations from this study (c.3020+2T>C) and the splice site mutation (c.3020+1G>A) found in familial ALS exome data of the AVS, are all expected or experimentally proven to disrupt the splice donor site of intron 27. However, contrary to the canonical splice site mutations, mis-spliced mRNA resulting from the c.3019A>G [p.(Arg1007Gly)] missense mutation is detectable in the lymphoblast cell lines from the respective patient (data not shown). We speculate that this result may be explained by a differential stability of the respective pre-mRNAs before or during the splicing event.

In our analysis of lymphoblast cell line RNA, the above-described SNV rs113247976 [p.(Pro986Leu)] did not have an impact on splicing of introns 26 or 27, and the respective mRNA level was unaltered compared to wild-type lymphoblast cell lines (Fig. 2C and D). Together with our data and the splice site mutation derived from the AVS, we identified four different mutations predicted or experimentally shown to affect splicing of introns 26 or 27 of *KIF5A* in familial ALS (Fig. 2E).

Clinical phenotypes of *KIF5A* mutation carriers

The phenotypes of the patients with a canonical *KIF5A* splice site mutation or with the c.3019A>G predicted missense variant that led to abrogation of the intron 27 splice donor site were compatible with a classical ALS syndrome: Specifically, adult-onset of initial focal asymmetric onset of affection of both the upper and lower motor neuron systems with later generalization, bulbar motor involvement, emotional lability, absence of vegetative symptoms and sensory symptoms in most patients, as well as rapid disease progression and early death—all of which were fully compatible with a typical ALS phenotype rather than HSP or CMT (Table 1 and [Supplementary Table 1](#))

Patients carrying the SNV rs113247976 [c.2957C>T; p.(Pro986Leu)] found to be enriched in familial ALS tended to have a (non-significantly) shorter disease duration and a significantly decreased proportion of FTD co-morbidity compared to the background cohort of German patients with familial ALS ([Supplementary Table 3](#)). However, the number of and clinical information on SNV carriers rs113247976 needs to be extended before it can be definitely ascertained whether rs113247976 is modifying the disease phenotype.

Finally, we screened nine patients with sporadic ALS, from whom post-mortem brain tissue was available, for the SNV rs113247976. One of nine patients carried this SNV. Neuropathological examination revealed typical stage 2 TDP-43 neuropathology (Braak *et al.*, 2013, 2017; Brettschneider *et al.*, 2014; Tan *et al.*, 2013) ([Supplementary Fig. 1](#)).

Discussion

The present data, supported by previous suggestive findings (Kenna *et al.*, 2016; McLaughlin *et al.*, 2017), indicate that *KIF5A* is a novel ALS gene. In our study, the association of rare *KIF5A* variants with ALS was predominantly carried by loss-of-function mutations, in particular the two splice site mutations found among the studied 426 ALS families. Notably, in the familial ALS group of the AVS, another *KIF5A* splice variant is reported in a familial ALS patient (c.3020+1G>A), affecting the same splice donor site as the c.3020+2T>C mutation found in our cohort.

Corroborating the link to ALS, both of the splice site mutations (c.2993-1G>A and c.3020+2T>C) we identified co-segregated with the disease. The splice site mutations were present in all seven patients from whom DNA or indirect information about their mutation status (obligate carriers) was available. Furthermore, no healthy individual older than the expected age of disease onset and carrying the mutation was identified in the two families studied. This information, although in its present form limited, is suggestive of a high penetrance of *KIF5A* loss-of-function mutations.

In contrast to loss-of-function mutations, we found no significant association between familial ALS and rare missense variants (MAF < 1%). Nevertheless, it is well possible that at least a subset of missense variants is pathogenic. *KIF5A* has a z-score of 4.38 (<http://exac.broadinstitute.org/gene/ENSG00000155980>), i.e. fewer missense variants than expected, which indicates a general intolerance towards variation in this gene. Remarkably, RNA analysis of patient-derived cell lines revealed that one of the rare (predicted) missense variants (c.3019A>G; predicting p.Arg1007Gly) turned out to abrogate the splice donor site in intron 27, the same donor site that is affected by two of the above mentioned canonical splice site mutations (c.3020+1G>A and c.3020+2T>C).

The *KIF5A* mutations previously described in HSP or CMT2 patients are restricted to missense mutations in the kinesin motor domain (amino acid positions 9–327) and in the alpha-helical coiled-coil domain (amino acid positions 331–906) (summarized in Kaji *et al.*, 2016; Guinto *et al.*, 2017). By contrast, the ALS-associated mutations described here (Fig. 2E) are predicted to affect the C-terminal part of *KIF5A* or to represent loss-of-function mutations, as experimentally shown for two of them (overview in Fig. 3). Moreover, deletion mutations in the same C-terminal domain that are predicted to cause a frame-shift with stop loss and an elongated protein (c.2854delC, c.2921delC, c.2934delC), have been associated with the

severe developmental syndrome neonatal intractable myoclonus (NEIMY) (Duis *et al.*, 2016; Rydzanicz *et al.*, 2017). Given our and previously reported data, it is thus possible to link the N-terminal and the C-terminal mutational hotspots in KIF5A to HSP and to CMT or to ALS and NEIMY (Fig. 3). The C-terminal globular tail has been demonstrated to be necessary for the binding of cargo-adaptor proteins (e.g. TRAK1/2, GABARAP) (Nakajima *et al.*, 2012; Randall *et al.*, 2013). Mutations predicted to affect the C-terminal tail, if translated into protein, might therefore lead to altered binding of cargo to KIF5A, although this awaits experimental confirmation. By contrast, mutations in the N-terminal kinesin motor domain linked to HSP and CMT decrease the velocity and flux of cargo transport (Wang *et al.*, 2010). This could explain why missense mutations in the N-terminal kinesin motor or the central coiled-coil domain cause the milder HSP/CMT2 phenotype, whereas mutations in the C-terminal small globular domain induce the more severe ALS and NEIMY syndromes, possibly owing to altered cargo binding, haploinsufficiency or a dominant-negative effect of truncated KIF5A.

In this study, we only included ALS patients who had been diagnosed by experienced ALS clinical specialists adhering to stringent ALS criteria (Andersen *et al.*, 2005, 2012). This is important, not the least because two previous reports suggested a possible link between ALS and KIF5A, but lacked precise clinical information (Kenna *et al.*, 2016; McLaughlin *et al.*, 2017). Considering the relative phenotypic similarities between ALS, HSP, and CMT, it cannot be excluded that part of the association signal in the previous large studies arose from HSP or CMT patients misdiagnosed as (slowly progressive) ALS. The patients in our study showed a classical ALS phenotype. Nevertheless, overlapping syndromes may exist and could complicate the distinction between ALS, CMT and HSP in some instances. Along this line, it remained unclear if the pronounced sensory symptoms of Patient A/II.13 were the consequence of a paraneoplastic syndrome or indicated CMT2 co-morbidity.

Interestingly, we observe a co-occurrence of rs113247976 with rare genetic missense variants in other known ALS genes in 11 of 29 patients carrying this particular SNV. Half of these variants have been reported to be pathogenic for ALS in earlier studies (Gitcho *et al.*, 2008; Kimonis *et al.*, 2008; Kühnlein *et al.*, 2008; Chiò *et al.*, 2009; Örlén *et al.*, 2009; Deng *et al.*, 2011; Brenner *et al.*, 2016; Nguyen *et al.*, 2018), while the rest are of uncertain significance (Supplementary Table 2). Supporting our observation of a bigenic effect for ALS causality, one of the patients who carried the rs113247976 also had a TARDBP p.N352S mutation, in agreement with van Blitterswijk *et al.* who described that 50% of patients with familial ALS carrying the TARDBP p.N352S mutation also have a mutation in another ALS gene (Van Blitterswijk *et al.*, 2012). Moreover, only homozygous or compound heterozygous SPG11 mutations have been shown to cause juvenile ALS, HSP or CMT2. Nevertheless, considering that both

SPG11 and KIF5A are involved in axonal transport and cause the same phenotypic spectrum when mutated, one is inclined to speculate about an additive effect of the observed co-occurrence of heterozygous SPG11 mutations and the SNV rs113247976 in KIF5A. Similarly, the NEK1 variant (p.Ser1036Ter) observed in combination with rs113247976 and the UBQLN2 mutation p.P509S—previously described by Deng *et al.* (2011)—shows incomplete penetrance (Brenner *et al.*, 2016; Nguyen *et al.*, 2018). Thus, penetrance of NEK1 mutations may require one or more additional genetic variants in the same patient. Taken together, we therefore hypothesize that the SNV rs113247976 lowers the threshold for phenocopy in carriers of additional ALS gene mutations. This would be compatible with an oligogenic mode of inheritance and could possibly also explain a substantial proportion of sporadic ALS cases.

A patient with the rare KIF5A missense variant (c.1422A>T; p.Gln474His, Table 1) suffered from ALS and FTD. Co-segregation analysis of this variant was unfortunately not possible. Preliminary results suggested a reduced frequency of FTD co-morbidity among ALS patients carrying the SNV rs113247976 (Supplementary Table 3); however, this result requires replication in larger cohorts.

In conclusion, we demonstrate here that highly penetrant C-terminal KIF5A splice site mutations can cause ALS and we present detailed clinical information on the KIF5A-linked ALS phenotype. The type of mutations together with RNA analysis in patient-derived cell lines indicates that haploinsufficiency is the most likely molecular genetic mechanism for highly penetrant KIF5A mutations. In addition, we report that the SNV rs113247976 is associated with familial ALS and possibly involved in digenic/polygenic inheritance of the disease, representing thus far, to our knowledge, the most frequent genetic factor contributing to ALS pathogenesis. Our findings underline the importance of intracellular transport molecules for ALS pathogenesis. Finally, we outline a hypothesis on how the type and location of KIF5A variants determine the manifestation of four different neurological syndromes.

Web resources

ALS Variant Server, Worcester, MA (<http://als.umassmed.edu/>).

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Supplementary material

Supplementary material is available at *Brain* online.

Appendix I

List of participants of The German ALS network MND-NET (see also [Supplementary material](#)): Ute Weyen, Andreas Hermann, Tim Hagenacker, Jan Christoph Koch, Paul Lingor, Bettina Göricke, Stephan Zierz, Petra Baum, Joachim Wolf, Andrea Winkler, Peter Young, Ulrich Bogdahn, Johannes Prudlo, and Jan Kassubek.

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