Comprehensive analysis of the mutation spectrum in 301 German ALS families

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Abstract

Objectives Recent advances in amyotrophic lateral sclerosis (ALS) genetics have revealed that mutations in any of more than 25 genes can cause ALS, mostly as an autosomal-dominant Mendelian trait. Detailed knowledge about the genetic architecture of ALS in a specific population will be important for genetic counselling but also for genotype-specific therapeutic interventions.

Methods Here we combined fragment length analysis, repeat-primed PCR, Southern blotting, Sanger sequencing and whole exome sequencing to obtain a comprehensive profile of genetic variants in ALS disease genes in 301 German pedigrees with familial ALS. We report *C9orf72* mutations as well as variants

in consensus splice sites and non-synonymous variants in protein-coding regions of ALS genes. We furthermore estimate their pathogenicity by taking into account type and frequency of the respective variant as well as segregation within the families.

Results 49% of our German ALS families carried a likely pathogenic variant in at least one of the earlier identified ALS genes. In 45% of the ALS families, likely pathogenic variants were detected in *C9orf72*, *SOD1*, *FUS*, *TARDBP* or *TBK1*, whereas the relative contribution of the other ALS genes in this familial ALS cohort was 4%. We identified several previously unreported rare variants and demonstrated the absence of likely pathogenic variants in some of the recently described ALS disease genes.

Conclusions We here present a comprehensive genetic characterisation of German familial ALS. The present findings are of importance for genetic counselling in clinical practice, for molecular research and for the design of diagnostic gene panels or genotype-specific therapeutic interventions in Europe.

Introduction

Genetic factors contribute substantially to the neurodegenerative disease amyotrophic lateral sclerosis (ALS). Approximately 3%–10% of patients newly diagnosed with ALS report a positive family history.[1]

To date, mutations in any of more than 25 genes have been suggested to cause familial ALS (fALS) in a monogenic manner.[2–4] ALS-causing mutations can also manifest as frontotemporal dementia (FTD), sometimes in the same family or even patient (ALS/FTD comorbidity).[5–7]

While a considerable number of ALS/FTD disease genes have been identified since 1993, few common cell biological pathways involved in ALS pathogenesis emerge when grouping these genes according to their known physiological functions.[8] For example, several ALS disease genes are involved in RNA synthesis and processing, protein homoeostasis or cytoskeletal functions. However, beyond novel insights into basic molecular mechanisms of ALS, genetic discoveries may also lead to genotype-specific, improved treatment options in the near future. Examples are knockdown of *SOD1* expression by intrathecal administration of antisense oligonucleotides in a clinical trial (ClinicalTrials.gov: NCT01041222) or reduction in the concentration of SOD1 protein in the cerebrospinal fluid,[9] both studies being performed exclusively in patients with *SOD1* mutations. Consequently, detailed knowledge about the genetic architecture of ALS in a specific population will be important for genetic counselling but also for future gene-specific or even mutation-specific therapeutic interventions. Furthermore, novel mutations identified in known genes represent important starting points and tools to foster research on molecular mechanisms of the disease. Therefore, we here report the spectrum of variants in the consensus splice sites and protein-coding regions of all currently known monogenic ALS genes and their contribution to ALS in a large central European cohort of ALS families.

An estimated 85% of the disease-causing inherited mutations are located in the protein-coding regions of the human genome and in consensus splice sites.[10] Therefore, exome capture and high-throughput sequencing is an efficient method of analysing a patient's DNA to discover the genetic cause of a genetically heterogeneous disease.[11] Consequently, most fALS index patient DNA samples of our cohort were subject to whole exome sequencing (WES) subsequent to screening for mutations in the most frequently mutated ALS genes *C9orf72* and *SOD1*, in order to define the frequency of known mutations and to discover novel mutations in known genes. To define likely pathogenic variants, we applied stringent parameters with regard to the type, frequency and disease cosegregation of the observed variant.

Materials and methods

Study cohort

Overall, 301 pedigrees with familial ALS were recruited at German Clinical ALS Research Centres in Ulm, Berlin, Bochum, Essen, Hannover, Jena, Würzburg, Aachen and Munich from 1995 through 2016. All

patients had been evaluated by neuromuscular specialists and were diagnosed according to the El Escorial criteria.12 The diagnosis of familial ALS was based on the presence of at least one first-degree or second-degree relative with ALS or FTD spectrum disorder. In few cases and if other sources were not available, the diagnosis of familial ALS was based on the patient's or other family members' reporting of symptoms compatible with ALS or FTD. Whenever possible, the information was confirmed by collecting medical records and by scrutinising death certificates and other available documents. In total, 10.5% of the patients included in the German ALS network MND-NET, which was the patient resource for this study, met the definition of familial ALS.

Initially, all patients were screened for mutations in the most frequently mutated ALS genes *C9orf72* and *SOD1*.13 Furthermore, some patients with ALS-associated mutations in other more rare genes were identified in previous studies.14–20 All DNA samples that did not reveal a mutation in a known ALS gene by targeted genotyping were subject to WES, a total of 226 samples from 173 pedigrees.

This study was approved by the local medical ethics committees. All patients gave written informed consent before in accordance with the Declaration of Helsinki (WMA, 1964). In agreement with this approval, patients and healthy probands were informed about positive results only if requested before testing. Moreover, healthy probands (eg, healthy relatives of patients with an ALS mutation) were informed only after undergoing genetic counselling, in accordance with the German gene diagnosis law.

Genetic analysis

DNA was extracted from whole EDTA-containing venous blood samples as described.21 Analysis of the *C9orf72* repeat length was performed by fragment length analysis and repeat-primed PCR (RP-PCR) using previously published primers.22 23 Since PCR-based methods cannot determine the size of larger expanded repeat-alleles, samples with a sawtooth pattern in the RP-PCR were further analysed using Southern blot.24

For the *SOD1* screen and to confirm some variants detected in the WES analysis, the patient's DNA was tested by Sanger sequencing. We designed forward and reverse m13-tailed primers. After the amplification, the fragments covering the variant sites were treated with ExoSAP-IT (Affymetrix). For the sequencing reaction, the BigDye Terminator V3.1 Cycle Sequencing Kit (Life Technologies) was used in accordance with the manufacturer's instructions.

Electrophoresis was performed on an ABI PRISM 3130 Genetic Analyzer (Life Technologies). Data were analysed using the Peak Scanner (fragment length analysis and RP-PCR) and Sequence Scanner V1.0 (sequencing) software, respectively.

The WES was performed as 100 bp paired-end reads on HiSeq2000/2500/4000 systems (Illumina).25 We generated on average 10 gigabases of sequence resulting in an average depth of 125× with 95% of the target regions covered at least 20 times.

Variant analysis

Enrichment for exome sequencing was performed with SureSelect Human All Exon 50 Mb kits, V3, V4, V5 or V6. Burrows-Wheeler Aligner (BWA V0.5.9) with standard parameters was used for read alignment against the human genome assembly hg19 (GRCh37). We performed single-nucleotide variant and small insertion and deletion (indel) calling specifically for the regions targeted by the exome enrichment kit using SAMtools (V0.1.18). Structural variants were analysed with Pindel26 and ExomeDepth.27 Custom scripts and database application are available on request (https://ihg4.helmholtz-muenchen.de/cgi-bin/mysql/snv-vcf/login.pl). The 35 investigated genes are well covered. Overall, 476 and 487 of the 491 target regions were covered at least 20 times in the V5 and V6 kits, respectively. The mean coverage of the 35 investigated genes was 131 (±34 SD) in a representative exome (online supplementary table 1).

We searched for variants in known ALS disease genes (table 1). To define likely pathogenic variants, we applied strict parameters with regard to the type, frequency and disease cosegregation of the variant (see the Results section). To

assess the potential functional consequences of each sequence variation, we used three bioinformatic tools designed to predict possible impacts of an amino acid substitution on the structure and known function(s) of a human protein, PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/) and MutPred (http://mutpred.mutdb.org/). To assess the conservation of affected amino acids for the respective protein, we aligned the sequences within the Mammalia including elephant, chimpanzee, cow, mouse and platypus and within the Vertebrata including *Xenopus tropicalis*, zebrafish, green sea turtle, parrot and lizard.

Table 1

ALS genes investigated in this study, ordered by frequency of mutations in the respective gene

Gene	Chromosomal locus	Inheritance	Putative protein function
C9orf72	9p21.2	AD	DENN protein, autophagy
SOD1	21q22.11	AR and AD	Superoxide metabolism
FUS	16p11.2	AD	RNA metabolism
TARDBP	1p36.22	AD	RNA metabolism
TBK1	12q14.2	AD	Inflammation, autophagy
OPTN	10p13	AR and AD	NfkB signal transduction, autophagy
CHCHD10	22q11.23	AD	Unknown (mitochondrial function?)
UBQLN2	Xp11.21	XD	Ubiquitinated protein degradation
SETX	9q34.13	AD	Transcription/RNA metabolism
NEFH	22q12.2	AD	Neurofilament cytoskeleton
VAPB	20q13.32	AD	Vesicle trafficking
VCP	9p13.3	AD	Ubiquitin-containing autophagosome maturation
ALS2	2q33.1	AR	Vesicle trafficking
ANXA11	10q22.3	AD	Cell membrane repair
NEK1	4q33	AD	DNA damage repair
ERBB4	2q34	AD	Mitogenesis and differentiation
FIG4	6q21	AD	Vesicle trafficking
PFN1	17p13.2	AD	Cytoskeletal function
SQSTM1	5q35.3	AD	Autophagy
HNRNPA1	12q13.13	AD	RNA metabolism
HNRNPA2B1	7p15.2	AD	RNA metabolism
DCTN1	2p13.1	AD	Retrograde axonal transport
ANG	14q11.2	AD	Angiogenesis
ATXN2	12q24.12	AD	Endocytosis, mRNA repair, ribosomal translation
C21orf2	21q22.3	AD	Cilia formation
CCNF	16p13.3	AD	Ubiquitylation, coordination of the cell cycle
CHMP2B	3p11.2	AD	Vesicle trafficking
DAO	12q24.11	AD	Regulation of the levels of D serine
GLE1	9q34.11	AR	RNA metabolism
MAPT	17q21.31	AD	Cytoskeleton
MATR3	5q31.2	AD	RNA metabolism
SIGMAR1	9p13.3	AR	Signal transduction amplifiers
SPG11	15q21.1	AR	DNA damage repair

Gene	Chromosomal locus	Inheritance	Putative protein function
TIA1	2p13.3	AD	RNA metabolism
TUBA4A	2q35	AD	Microtubule cytoskeleton

Results

Patient cohort

Overall, we analysed index patients from 301 ALS families. Additionally, in order to test for cosegregation with disease and penetrance, 81 affected and 25 unaffected individuals from respective families were sequenced. Unaffected individuals were genotyped only if they were informative, that is, older than the latest onset of disease in this family. A subset of the index patients displayed also cognitive or behavioural symptoms of FTD. All patients were of European origin.

Targeted genotyping

Of the 301 ALS families included in this study, a subset of 128 index patients had been screened by Sanger sequencing or fragment length analysis combined with RP-PCR and Southern blotting (for *C9orf72*) for the genes discovered until 2011 in previous projects. Specifically, in 75 out of the 301 index patients, a Southern blot-confirmed *C9orf72* hexanucleotide repeat expansion (HRE) was detected (figure 1). Thirty-seven index patients turned out to carry non-synonymous variants in *SOD1* (variants with a minor allele frequency (MAF) 1:10 000 or lower (according to the ExAC dataset), except for the known pathogenic p.D91A mutation with MAF of 1:891), and further mutations were found in *TARDBP* (three pedigrees detected with two different mutations14), in *FUS* (eight pedigrees detected with six different mutations15 16), in *OPTN* (one pedigree detected with one mutation17), in *PFN1* (one pedigree detected with one mutation18), in *SETX* (two pedigrees detected with two different mutations19) and in *ALS2* (one pedigree detected with one mutation20).

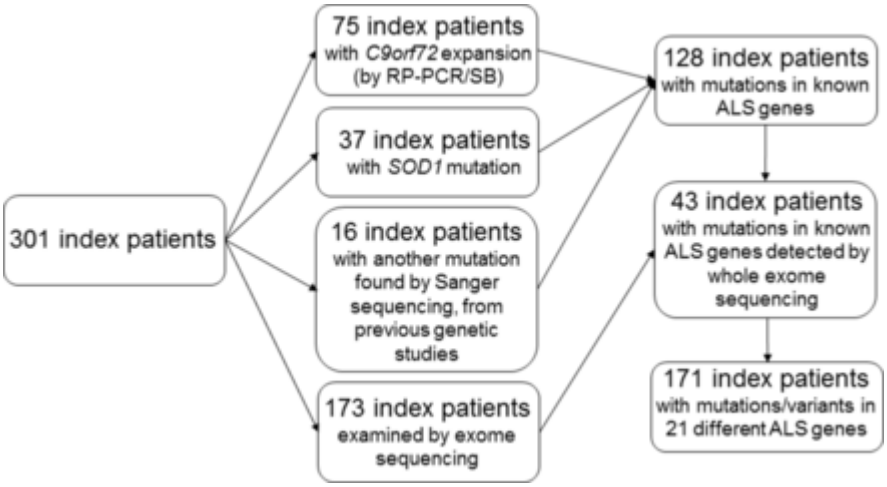

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Figure 1

Overview of the cohort analysis protocol, taking into account likely pathogenic variants and VUS. ALS, amyotrophic lateral sclerosis; RP-PCR, repeat-primed PCR; SB, Southern blot; VUS, variants of uncertain significance.

Whole exome sequencing

Overall, the prescreening of the 301 index patients led to the detection of non-synonymous variants with an MAF <1:10 000 in known ALS genes or a *C9orf72* HRE in a total of 128 index patients (42.5%, figure 1). The remaining 173 index patients as well as 81 affected and 25 informative unaffected relatives of these index patients were subject to WES to obtain a comprehensive mutational profile in this fALS cohort. WES revealed non-synonymous variants with an MAF <1:10 000 in known ALS genes in additional 43 index patients. All rare variants were found in a heterozygous state, except for the homozygous *SOD1* p.D91A in four pedigrees, the homozygous *ALS2* p.T185Lfs*five in one male with juvenile-onset ALS (age of onset 12 years) and an index patient with a homozygous loss-of-function mutation in *OPTN* p.E135*.

Categorisation according to probable pathogenicity

Table 2 summarises all sequence variants we identified in this study with an MAF <1:10 000. To allow an approximation of the relative contribution of each gene to the pathogenesis of fALS in Germany, we grouped the resulting variants in known ALS genes according to their likely pathogenicity. We divided the sequence variants in two groups according to whether they are (1) 'likely pathogenic' or (2) 'variants of uncertain significance' (VUS). The following variants were considered to be 'likely pathogenic': (1) pathologically expanded hexanucleotide repeats in *C9orf72* (all expansions in our cohort displayed a length of 50 to thousands of hexanucleotide repeats), (2) non-synonymous variants in protein-coding regions with an MAF <1:10 000 in the ExAC dataset (http://exac.broadinstitute.org/) that were found in two different families (taken together this and previously published[3 5 9 14 15 17–20 22 28–60] work) and present in all affected members of these families as far as DNA was available for genotyping; (3) any variant in a known ALS disease gene with an MAF <1:10 000 that cosegregates over at least five meioses, that is, is found in two affected relatives separated by at least five meioses and not found in unaffected family members (or otherwise reported as a possible indication for incomplete penetrance).

Table 2

Variants identified in the consensus splice sites and protein-coding regions of all currently known monogenic ALS genes as well as HREs in *C9orf72*

Gene	Variant		Frequency (no of index patients)	Conservation		PolyPhen*	Sift†	MutPred score ‡	Previously reported	Pathogenicity	Comment
				Mammalia	Vertebrata						
C9orf72	(GGGGCC)n, n>50–2600		75×	–	–	–	–	–	22	Likely pathogenic§	
SOD1	c.115C>G	p.L39V	1×	Yes	No	0.998	0.00	0.499	28	Likely pathogenic§	
	c.131A>G	p.H44R	3×	Yes	No	1.000	0.06	0.898	29	Likely pathogenic§	
	c.140A>G	p.H47R	1×	Yes	No	0.997	0.00	0.897	30	Likely pathogenic§	
	c.146A>G	p.H49R	1×	Yes	No	1.000	0.00	0.934	31	Likely pathogenic	

Gene	Variant		Frequency (no of index patients)	Conservation		PolyPhen*	Sift†	MutPred score ‡	Previously reported	Pathogenicity	Comment
				Mammalia	Vertebrata						
	c.217G>A	p.G73S	1×	Yes	Yes	0.970	0.01	0.669	32	Likely pathogenic	
	c.255G>C	p.L85F	1×	Yes	Yes	1.000	0.00	0.846	33	VUS¶	
	c.260A>G	p.N87S	1×	Yes	Yes	1.000	0.00	0.692	34	Likely pathogenic**	
	c.263T>C	p.V88A	1×	Yes	Yes	0.999	0.00	0.786	31	Likely pathogenic	
	c.272A>C heterozygous	p.D91A	2×	No	No	0.000	0.17	0.485	35 36	Likely pathogenic	
	c.272A>C homozygous	p.D91A	4×	No	No	0.000	0.17	0.485	35 36	Likely pathogenic	
	c.301G>A	p.E101K	1×	No	No	0.000	0.61	0.234	37 38	VUS¶	
	c.313A>T	p.I105F	1×	Yes	No	0.999	0.00	0.691	39	Likely pathogenic	
	c.326G>T	p.G109V	1×	Yes	Yes	1.000	0.00	0.864	40	Likely pathogenic§	
	c.341T>C	p.I114T	1×	Yes	No	0.999	0.00	0.725	41	Likely pathogenic**	
	c.346C>G	p.R116G	9×	Yes	Yes	1.000	0.00	0.903	37	Likely pathogenic§	
	c.400G>A	p.E134K	1×	Yes	Yes	1.000	0.17	0.679	9	Likely pathogenic	
	c.435G>T	p.L145F	2×	Yes	Yes	0.999	0.00	0.794	42	Likely pathogenic	
	c.443G>A	p.G148D	1×	Yes	Yes	1.000	0.00	0.955	43	Likely pathogenic	
	c.446T>C	p.V149A	1×	Yes	Yes	1.000	0.00	0.836	no	VUS	
	c.446T>G	p.V149G	1×	Yes	Yes	1.000	0.00	0.925	44	Likely pathogenic	

Gene	Variant		Frequency (no of index patients)	Conservation		PolyPhen*	Sift†	MutPred score ‡	Previously reported	Pathogenicity	Comment
				Mammalia	Vertebrata						
	c.449T>C	p.I150T	1×	Yes	Yes	0.998	0.00	0.911	45	Likely pathogenic	
	c.455T>C	p.I152T	1×	Yes	No	0.969	0.00	0.845	46	Likely pathogenic	
FUS	c.1394-2delA	Direct splice site	1×	Yes	No	–	–	–	3	Likely pathogenic	
	c.1432_1478del47	p.G478Lfs*23	1×	–	–	–	–	–	15	Likely pathogenic	
	c.1483C>T	p.R495*	1×	–	–	–	–	–	47	Likely pathogenic§	
	c.1526G>A	p.G509D	1×	Yes	Yes	1.000	0.03	0.880	48	Likely pathogenic	
	c.1529A>G	p.K510R	3×	Yes	Yes	0.945	0.03	0.329	15	Likely pathogenic§	
	c.1540A>G	p.R514G	1×	No	Yes	0.079	0.01	0.540	49	Likely pathogenic§	
	c.1561C→T	p.R521C	1×	Yes	No	0.002	0.00	0.308	49	Likely pathogenic§	
	c.1562G>A	p.R521H	3×	Yes	No	0.002	0.00	0.186	49	Likely pathogenic§	
	c.1570A>G	p.R524G	1×	Yes	Yes	0.437	0.00	0.589	5	VUS	Patient with an additional *TBK1* p.Y185* and *DCTN1* p.I195L variant
TARDBP	c.881G>T	p.G294V	1×	Yes	No	0.139	0.42	0.797	50	Likely pathogenic	

Gene	Variant		Frequency (no of index patients)	Conservation		PolyPhen*	Sift†	MutPred score ‡	Previously reported	Pathogenicity	Comment
				Mammalia	Vertebrata						
	c.943G>A	p.A315T	2×	Yes	Yes	0.063	0.61	0.622	51	Likely pathogenic§	
	c.1042G>T	p.G348C	1×	Yes	No	0.992	0.07	0.789	14	Likely pathogenic	
	c.1055A>G	p.N352S	7×	Yes	No	0.000	0.48	0.620	52	Likely pathogenic**	Two patients with an additional *ANXA11* p.P87T or p.G162R variant
	c.1132_1146del15	p.(N378_A382del)	1×	4/5	0/5	–	–	–	No	VUS	
TBK1	c.140G>A	p.R47H	1×	Yes	Yes	1.000	0.00	0.633	5 53	VUS	
	c.358+2T>C	p.T77Wfs*3	1×	–	–	–	–	–	5 53	Likely pathogenic§	
	c.555T>A	p.Y185*	1×	–	–	–	–	–	5 53	Likely pathogenic§	Patient with an additional *FUS* p.R524G and *DCTN1* p.I195L variant
	c.1928-1930delAAG	p.E643del	1×	Yes	Yes	–	–	–	5 53	Likely pathogenic	
OPTN	c.403G>T homozygous	p.E135*	1×	–	–	–	–	–	No	Likely pathogenic	
	c.1320delA	p.K440Nfs*	1×	–	–	–	–	–	17	Likely	

Gene	Variant		Frequency (no of index patients)	Conservation		PolyPhen*	Sift†	MutPred score ‡	Previously reported	Pathogenicity	Comment
		8		Mammalia	Vertebrata					pathogenic§	
CHCHD10	c.44C>A	p.R15L	2×	No	No	0.993	0.28	0.380	54	Likely pathogenic**	
UBQLN2	c.1460C>T	p.T487I	1×	Yes	No	0.417	0.24	0.624	55	Likely pathogenic§	
	c.1489C>T	p.P497S	1×	No	No	0.588	0.90	0.510	56	Likely pathogenic	
SETX	c.1166T>C	p.L389S	1×	Yes	Yes	1.000	0.00	0.853	57	Likely pathogenic§	
	c.1374A>C	p.F458L	1×	Yes	Yes	0.999	0.00	0.696	no	VUS	
	c.3056C>T	p.S1019F	1×	Yes	No	1.000	0.01	0.667	no	VUS	
	c.4517A>G	p.M1506T	1×	Yes	No	0.999	0.00	0.821	no	VUS	
	c.4979T>C	p.H1660R	1×	No	No	0.000	0.74	0.051	no	VUS	
	c.5885A>G	p.H1962R	1×	Yes	No	1.000	0.03	0.915	19	VUS	
NEFH	c.1376_1379delAACA	p.E459Gfs*7	1×	–	–	–	–	–	No	Likely pathogenic	
	c.2564_2566delAGA	p.K857del	1×	No	No	–	–	–	No	VUS	
VAPB	c.166C>T	p.P56S	1×	Yes	Yes	1.000	0.00	0.880	58 59	Likely pathogenic§	
VCP	c.464G>A	p.R155H	1×	Yes	Yes	0.849	0.06	0.798	60	Likely pathogenic§	
ALS2††	c.553delA homozygous	p.T185Lfs*5	1×	–	–	–	–	–	20	Likely pathogenic	
ANXA11	c.1087–1G>A	Direct splice site	1×	Yes	Yes	–	–	–	No	VUS	
	c.484G>A	p.G162R	1×	Yes	No	0.001	0.38	0.142	No	VUS	Patient with an additional

Gene	Variant		Frequency (no of index patients)	Conservation		PolyPhen*	Sift†	MutPred score ‡	Previously reported	Pathogenicity	Comment
				Mammalia	Vertebrata						*TARDBP* p.N352S variant
	c.259C>A	p.P87T	1×	Yes	No	0.190	0.52	0.319	No	VUS	Patient with an additional *TARDBP* p.N352S variant
	c.137C>T	p.A46V	1×	Yes	No	0.125	0.16	0.334	No	VUS	
	c.112G>A	p.G38R	1×	Yes	No	1.000	0.51	0.825	No	VUS	
NEK1	c.1634A>G	p.M545T	1×	No	No	0.087	0.23	0.323	No	VUS	
	c.1433C>G	p.G478A	1×	Yes	No	0.936	0.23	0.143	No	VUS	
	c.395T>C	p.Q132R	1×	No	Yes	1.000	0.04	0.777	No	VUS	
ERBB4	c.3809A>G	p.Q1270R	1×	Yes	Yes	0.421	0.56	0.709	No	VUS	
	c.2428G>A	p.E810K	1×	Yes	Yes	0.999	0.31	0.710	No	VUS	
	c.812C>T	p.T271I	1×	Yes	No	0.014	0.35	0.540	No	VUS	
FIG4	c.2095C>T	p.R699C	1×	Yes	Yes	1.000	0.01	0.679	No	VUS	
	c.2096G>A	p.R699H	1×	Yes	Yes	1.000	0.13	0.497	No	VUS	
PFN1	c.326C>T	p.T109M	1×	No	No	0.347	0.03	0.529	18	VUS	
SQSTM1	c.344A>T	p.Q115L	1×	Yes	No	0.052	0.26	0.306	No	VUS	
HNRNPA1	c.1075G>A	p.G359S	1×	No	No	0.957	0.07	0.461	No	VUS	
DCTN1	c.583A>C	p.I195L	1×	Yes	No	0.001	0.54	0.089	No	VUS	Patient with an additio

Gene	Variant	Frequency (no of index patients)	Conservation				MutPred score ‡	Previously reported	Pathogenicity	Comment
			Mammalia	Vertebrata	PolyPhen*	Sift†				nal *TBK1* p.Y185* and *FUS* p.R524G variant

- For the conservation, we aligned the sequences within the Mammalia including elephant, chimpanzee, cow, mouse and platypus and within the Vertebrata including *Xenopus tropicalis*, zebrafish, green sea turtle, parrot and lizard. Yes, highly conserved; no, at least the amino acid in one organism is changed.
- *The lower the score, the more benign the substitution.
- †The Sift score ranges from 0 to 1. The amino acid substitution is predicted as damaging if the score is ≤0.05, and tolerated if the score is >0.05.
- ‡The MutPred pathogenicity score ranges from 0 to 1, with higher scores indicating a greater likelihood that the amino acid variation is pathogenic.
- §The variant cosegregates with the disease.
- ¶The variant does not cosegregate with the disease, there are affected family members without that variant.
- **The variant cosegregates with the disease and shows reduced penetrance.
- ††Mutations in *ALS2* cause autosomal recessive motor neuron diseases, an autosomal-dominant inheritance has so far not been reported.
- ALS, amyotrophic lateral sclerosis; HRE, hexanucleotide repeat expansion; VUS, variant of uncertain significance.
- (4) loss-of-function variants (frameshifts, premature STOP codons/nonsense mutations, consensus splice site mutations, STOP loss) in genes with haploinsufficiency as the likely molecular genetic mechanism of toxicity (ie, *FUS*, *TBK1*, *OPTN*, *NEK1*, *NEFH*).
- All other non-synonymous variants were classified as VUS. Thus, besides cosegregation data, we put emphasis on the low frequency of specific variants for our classification, based on the observation that rare and unique alleles contribute most to the heritability of ALS,[61] and known monogenic causes of familial ALS represent mostly rare or even private mutations. One exception was principally made for loss-of-function variants in *NEK1* with an MAF above 1:10 000, as *NEK1* variants have a greatly reduced penetrance,[62] although loss-of-function variants in *NEK1* were lacking in our German fALS cohort. The second exception is the known pathogenic p.D91A mutation with an MAF of 1:891.
- Based on this classification, we identified likely pathogenic variants in 49% and VUS in 8% of the 301 index patients (figure 2). In the remaining 43% of the families, no rare variant in any of the known ALS genes was detected by our screening approach. Thus, in total, 51% of all families were lacking a likely pathogenic variant according to the definition above. However, it has to be taken into account that a substantial proportion of the other rare variants that were found only in one family so far could also be causal, although this is hard to prove without segregation data supporting their role in ALS pathogenesis.

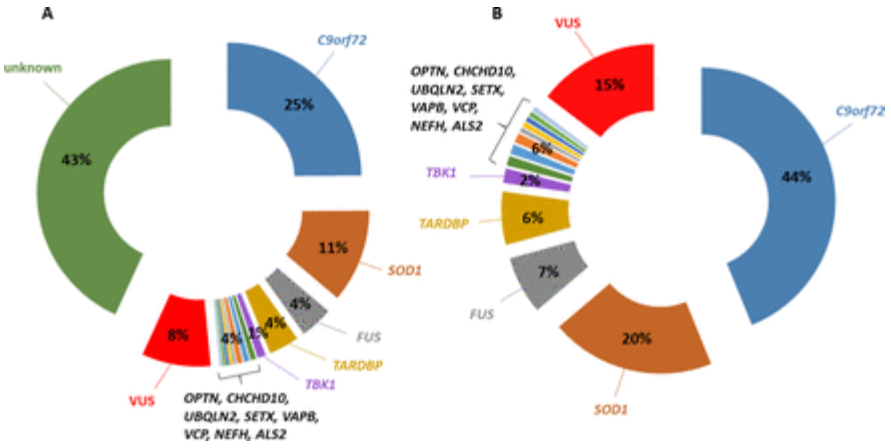

Figure 2

Mutations in known ALS genes in familial ALS in Germany. Values represent the relative contribution of mutations in the known ALS genes (A) in the cohort of patients with familial ALS (B) only in the families with a rare variant or mutation in a known ALS gene. ALS, amyotrophic lateral sclerosis.

We detected no index patient with more than one likely pathogenic mutation. However, double or triple mutations may have escaped detection, as DNA of the patients who were positive in the *C9orf72* or *SOD1* prescreening were not subject to further analysis by WES. Moreover, we observed an index patient with three rare variants, although the trigenic inheritance could not be formally proven. The patient had *DCTN1* p.I195L, *FUS* p.R524G (both according to our strict definition classified as VUS) and *TBK1* p.Y185*. Interestingly, the patient had a substantially earlier onset compared with the other family members with only one of the three genetic alterations. Furthermore, we could identify a *TARDBP* p.N352S and *ANXA11* p.P87T or p.G162R mutation (both classified as VUS) in two index patients.

Overall, based on the likely pathogenic variants, the five most frequently mutated genes in our German cohort were *C9orf72, SOD1, FUS, TARDBP* and *TBK1* (figure 2, table 3). We additionally observed likely pathogenic variants in the more rarely mutated genes *OPTN, CHCHD10, UBQLN2, SETX, VABP, VCP, NEFH* and *ALS2.* Collectively, the latter genes are found mutated in a total of 4% of index patients in our cohort. Moreover, table 3 provides an overview of the clinical features of the study population.

Table 3

Clinical features of the patient cohort according to the mutant gene

Gene	Frequency pedigrees/patients	% relative contribution of mutations	Sex ratio (males/females)	Mean age-at-onset (years)	Mean disease duration (months)	% spinal onset	% bulbar onset	Initial phenotype (upper vs lower MN)	FTD comorbidity
C9orf72	75/107	24.9	1.00	56	34	66	34	L>U	34%
SOD1	37/47 (incl VUS)	12.3	1.35	53	>85*	100	0	L>U	4%†
	34/43 (excl VUS)	11.3	1.39	54	>72*	100	0	L>U	4%†
FUS	13/23 (incl VUS)	4.3	1.30	45	52	88	12	L>U	0%
	12/22 (excl	4.0	1.44	45	52	88	12	L>U	0%

Gene	Frequency pedigrees/patients	% relative contribution of mutations	Sex ratio (males/females)	Mean age-at-onset (years)	Mean disease duration (months)	% spinal onset	% bulbar onset	Initial phenotype (upper vs lower MN)	FTD comorbidity
	VUS)								
TARDBP	12/13 (incl VUS)	4.0	1.17	55	52	100	0	L>U	17%
	11/12 (excl VUS)	3.7	1.40	57	55	100	0	L>U	17%
TBK1	4/4 (incl VUS)	1.3	3.0	51	>87*	100	0	L<U	0%
	3/3 (excl VUS)	1.0	2.0	46	>101*‡	100	0	L<U	0%
OPTN	2/3	0.7	2.00	51	23	100	0	L>U	33%
CHCHD10	2/4	0.7	0.33	48	103	75	25	L>U	0%
UBQLN2	2/3	0.7	2.00	49	37	0	100	NA	NA
SETX	6/9 (incl VUS)	2.0	3.00	35	>276*	88	12	L=U	20%
	1/3 (excl VUS)	0.3	2.00	22	>364*	100	0	L>U	0%
NEFH	2/2 (incl VUS)	0.7	Male	64	19	50	50	L>U	0%
	1/1 (excl VUS)	0.3	Male	76	19	0	100	L	0%
VAPB	1/1	0.3	Male	42	NA	100	0	NA	NA
VCP	1/1	0.3	Female	46	NA	100	0	NA	NA
ALS2	1/1	0.3	Male	12	NA	100	0	U	0%
ANXA11	5/5 (VUS)	1.7	0.67	62	89	80	20	L>U	0%
NEK1	3/3 (VUS)	1.0	2.00	61	81	100	0	NA	NA
ERBB4	3/3 (VUS)	1.0	Female	52	154	50	50	L>U	0%
FIG4	2/2 (VUS)	0.7	1.00	57	35	100	0	L>U	0%
PFN1	1/1 (VUS)	0.3	Female	48	360	100	0	L=U	0%
SQSTM1	1/1 (VUS)	0.3	Female	50	24	100	0	L	NA
HNRNPA1	1/1 (VUS)	0.3	Female	78	24	0	100	L	0%
DCTN1	1/1 (VUS)	0.3	Female	47	>108*	100	0	NA	0%
Unknown	130/151	43.2	1.93	57	45	78	22	L>U	6%
Total	301/382	100	1.39	55	56	79	21	L>U	16%

- ↲*Patient is or some patients are still alive.
- †One patient with a p.H49R mutation in *SOD1* presented symptoms that were consistent with a beginning bvFTD (aggression, emotional lability, reduced working memory and slightly reduced verbal fluency). At the same time, CSF analysis was in agreement with an Alzheimer's disease (increased Tau and decreased a-beta values).
- ‡We have detected four mutations in *TBK1* in four index patients. The disease durations were 46 and 99 months. Two patients are still alive (>96 and>108 months), but their affected relatives died after 36 months and after an average of 54 months, respectively.
- bvFTD, behavioural variant FTD; CSF, cerebrospinal fluid; excl, excluding; FTD, frontotemporal dementia; incl, including; L, predominant lower motor neuron signs ; MN, motor neuron; U, predominant upper motor neuron signs; VUS, variants of uncertain significance.

WES of unaffected relatives

We performed WES also in a total of 25 unaffected relatives of patients from 17 families. We had chosen only informative unaffected family members, defined as individuals who were lacking symptoms of ALS or FTD at an age at least as old as the latest known onset of disease in the same family. In some instances, for example, for variants in *CHCHD10* (p.R15L), *SETX* (p.F458L and p.H1962R) and *ERBB4* (p.T271I), the variant was found not only in the index patient but also in an informative relative without ALS. This argues for possible reduced penetrance of the respective variant (in case of likely pathogenic variants) (table 2). At the same time, a caveat has to be expressed, as the presence of variants in unaffected informative family members could also indicate that the found variant is not causal, and thus, the criteria for likely pathogenicity were still too liberal.

Known ALS genes without mutation in our cohort

We identified several previously described mutations. Moreover, several novel potentially or likely pathogenic variants that have not been described in other families so far were observed (table 2). On the other hand, we demonstrate also the absence of variants in some recently described ALS genes in our cohort. Specifically, no variant with an MAF of <1:10 000 was found in *ANG, ATXN2, C21orf2, CCNF, CHMP2B, DAO, GLE1*, *HNRNPA2B1*, *MAPT*, *MATR3*, *SIGMAR1*, *TIA1* or *TUBA4A*. Moreover, no homozygous variants were found in *SPG11*. *SPG11* mutations are most frequently associated with autosomal recessive spastic paraplegia with thin corpus callosum, an autosomal-dominant inheritance has so far not been reported.

Discussion

In our work, we present the genetic characterisation of a large cohort of patients with ALS from Central Europe, in order to estimate the frequency of known mutations and discover novel mutations important for clinical testing as well as the design of gene-specific therapeutic trials. Moreover, novel mutations described in this work could be the starting point for mechanistic molecular research.

While we identified known pathogenic variants in a subset of index patients, we found also novel variants in established ALS disease genes. In order to be able to classify these variants, we defined two principle categories: ‘likely pathogenic’ and VUS. We chose a strict definition for ‘likely pathogenic’. We put a strong emphasis on classical segregation analysis and rarity of the respective variant, considering that low-frequency alleles contribute most to heritability of ALS.[61] In contrast, we did not take into account bioinformatic prediction results, since bioinformatic algorithms are designed to predict impairment of known protein function, but detrimental effects of a given mutation could also be due to, for example, toxicity by a gain of novel function instead of a loss-of-function of the protein.

All remaining variants not fulfilling our above mentioned criteria were categorised as VUS. We thus perform a dichotomic separation of variants based on a strict, but in our view plausible threshold for pathogenicity. It has to be emphasised that a substantial number of VUS may still be causative. Nevertheless, variants that do not fulfil our high evidence standards for pathogenicity are hard to interpret in clinical settings and are not recommended for experimental work-up because the results would remain inconclusive.

We observed likely pathogenic variants in 49% of the 301 ALS families, whereas 43% and 8% of the families remained genetically unexplained or harboured a VUS, respectively. Generally, this cohort of patients with familial ALS reveals a heterogeneous genetic architecture, with variants in several rarely mutated genes, and a relatively small contribution even of the most frequently mutated genes *C9orf72* and *SOD1* when compared with other populations that historically went through a genetic ‘bottleneck’. For example, the relative contribution of the *C9orf72* mutation to familial ALS is 25% in our study, whereas it reached 46% in populations in Sweden or Finland[23] and even 51.1% in patients of Sardinian ancestry.[63] The genetic heterogeneity of our cohort could also be responsible for the comparably high proportion of

familial patients in whom a genetic cause could not be established, because of the contribution of a relatively high number of very rare and therefore so far undiscovered disease genes. Moreover, polygenic inheritance of variants with lower effect size may account for additional familial ALS cases. Furthermore, an unknown fraction of regulatory variants can only be identified by means of whole genome sequencing.

However, also in this German cohort, some mutations are detected that are found identical in multiple, seemingly unrelated families and most likely represent founder mutations. For example, the most frequent *SOD1* mutation in Germany is p.R116G, which has not been described in any other population so far.[37 64]

We discovered also several novel variants, for example, in *HNRNPA1*, *TARDBP*, *OPTN* and *NEFH*, although in some instances, their pathogenicity will remain unclear until additional evidence for cosegregation with disease or a second patient with the same variant becomes available.

In line with the usually dominant mode of inheritance, the vast majority of mutations were found in a heterozygous state. An index patient with a homozygous *OPTN* loss-of-function mutation represents an exception, in agreement with the biallelic *OPTN* mutations previously observed in patients with ALS.[65 66] The p.D91A mutation in *SOD1* is another rare instance of ALS-causing mutations detected in both heterozygous and homozygous state, as confirmed in this study. The *SOD1* p.D91A mutation carriers are all of German descent.

Moreover, mutations in several of the rarely mutated ALS disease genes were absent in the study cohort. Specifically, no rare variants were observed in *ANG*, *ATXN2*, *C21orf2*, *CCNF*, *CHMP2B*, *DAO*, *GLE1*, *HNRNPA2B1*, *MAPT*, *MATR3*, *SIGMAR1*, *TIA1* and *TUBA4A*, and no homozygous variants were found in *SPG11*. WES did not allow us to scrutinise the *ATXN2* poly-Q-repeat, which is an established risk factor for ALS at an intermediate length.[67]

A higher frequency of patients with mutations in more than one ALS disease gene than expected by chance has been suggested before.[68] In our cohort, we observed only three index patients with more than one rare variant, although the begenic or trigenic inheritance could not be formally proven because, according to our strict definition, the second and third variant(s) in the respective index patient are not classified as ‘likely pathogenic’ but as VUS. Furthermore, it has to be emphasised that patients who were positive in the *C9orf72* or *SOD1* prescreening or patients from previous studies were not subject to further analysis by WES, which concerns a total of 128 index patients (42.5%). Thus, double or triple mutations may have escaped detection.

Overall, the clinical phenotype/genotype association was similar to what had been described before. For example, the high prevalence of FTD comorbidity, more rapid disease progression and more bulbar onsets in patients with the *C9orf72* HRE has been described before.[24 69] As expected, the homozygous *ALS2* mutation was connected to a juvenile-onset motor neuron disease. Interestingly, one of the patients with a *SOD1* mutation (p.H49R) displayed mild symptoms that were principally in agreement with a beginning behavioural variant FTD, which is rarely observed in patients with *SOD1* mutations.[70] In addition, CSF analysis was consistent with Alzheimer’s disease in this patient, therefore possibly representing a rare mixed degenerative phenotype caused by this *SOD1* mutation.

Taken together, we here present a comprehensive genetic characterisation of German fALS. We delineate the contribution of all known Mendelian ALS genes and reveal several novel mutations. Our work should represent a valuable resource for genetic counselling as well as the design of ALS multigene panels for diagnostics. Moreover, the novel mutations described here could be starting points for molecular genetic work-up of ALS disease mechanisms. Finally, the dataset could turn out to be pivotal for the development and clinical evaluation of gene-specific or mutation-specific therapies based on, for example, antisense oligonucleotide techniques in the near future.

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Footnotes

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helped with the implementation. All authors contributed to the refinement of the study protocol and approved the final manuscript.

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