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Genetic analysis of VCP and WASH-complex genes in a German cohort of sporadic ALS-FTD patients

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Abstract

Objective: Mutations of the human VCP and WASH complex genes cause motor neuron and cognitive impairment disorders. Here, we analyzed a cohort of German patients with sporadic amyotrophic lateral sclerosis and frontotemporal lobar degeneration (ALS/FTD) for VCP and WASH complex gene mutations.

Methods: Next generation panel sequencing of VCP, WASH1, FAM21C, CCDC53, SWIP, strumpellin, CAPZα1 and CAPZβ genes was performed in 43 sporadic ALS/FTD patients. Subsequent analyses included Sanger sequencing, in silico analyses, real-time PCR, and CCDC53 immunoblotting.

Results: We identified one patient with the heterozygous variant c.26C>T, p.(Ser9Leu), in CAPZα1 and a second with the heterozygous variant c.2T>C, p.?, in CCDC53. In silico analysis predicted a structural change in the N-terminal region of CAPZα1, which may interfere with the CAPZα:CAPZβ dimerization. Though the translation initiation codon of CCDC53 is mutated, real-time PCR and immunoblotting did neither reveal any evidence for a CCDC53 haploinsufficiency nor for aberrant CCDC53 protein species.

Conclusions: With exception of a putatively pathogenic heterozygous c.26C>T CAPZA1 mutation, our genetic analysis in 43 sporadic ALS/FTD patients did not reveal mutations in VCP and the remaining WASH complex subunits.

Introduction

The genetic background of amyotrophic lateral sclerosis is complex, and mutations in a wide variety of genes coding for proteins involved in protein homeostasis, cytoskeleton, nuclear transport, mitochondrial function, cell cycle, and DNA repair have been described (for review see (1)). One out of the still growing number of ALS-causing genes codes for the transitional endoplasmic reticulum ATPase VCP (valosin-containing protein, p97) (1, 2). Notably, mutations in the VCP gene also have been shown to cause IBMPFD (Inclusion Body Myopathy associated with Paget disease of bone and Fronto-temporal Dementia) (3, 4), and moreover were implicated in the pathogenesis of Parkinson's disease (5), Charcot-Marie-Tooth disease type 2 (HMSN2) (6), and Hereditary Spastic Paraplegia (7). The VCP protein is involved in a plethora of cellular processes including membrane dynamics and protein quality control (8-11). Since VCP has a role in both ALS and frontotemporal dementia, a first aim of this study was to screen our cohort of 43 sporadic amyotrophic lateral sclerosis and frontotemporal lobar degeneration (ALS/FTD) patients for VCP mutations. The second aim was to analyses this cohort with respect to mutations in the strumpellin gene. Strumpellin and VCP are direct protein interaction partners (12), and both proteins are components of pathological protein aggregates in a wide variety of neurodegenerative and muscular diseases (4, 11, 12). Moreover, mutations in the human strumpellin gene cause a pure motor form of Hereditary Spastic Paraplegia (13) as well as Ritscher-Schinzel Syndrome 1 (RTSC1, 3C syndrome) (14). As strumpellin is an integral component of the WASH complex (Wiskott-Aldrich syndrome protein and SCAR homolog complex; subunits: WASH1, FAM21C, CCDC53, SWIP, strumpellin, CapZα, CapZβ) (15, 16), the third aim was to perform a genetic analysis of the other six WASH complex subunit genes. The WASH core complex together with the associated capping proteins CapZα and β has a central role in the endosomal protein sorting machinery. Together with the Arp2/3 complex, the WASH complex mediates the formation of crucial actin structures on endosomes thus directing cargo proteins e.g. to the plasma membrane, tans-Golgi network and lysosomes (15-17). The relevance of the WASH complex with regard to neurodegenerative disorders is further highlighted by the observation that genetic alterations in the KIAA1033 subunit have been implicated in the pathogenesis of ARID (non-syndromic autosomal-recessive intellectual disability) (18) and Alzheimer's disease (19).

Methods

Patients and Ethics statement

43 ALS/FTD patients were recruited at the Department of Neurology, University Hospital Ulm, Germany, between 20XX and 20XX [Weishaupt]. The study was approved by the Ethics Committee of the University Hospital Ulm. Written informed consent was obtained from all patients and all tests were conducted according to the principles expressed in the Declaration of Helsinki. ALS was diagnosed based on clinical and electrophysiological findings according to the revised El Escorial criteria (20). FTD was diagnosed by XXX [Weishaupt]. Age of onset, age at death, disease duration, and initial presentation (spinal or bulbar) as well as the level of diagnostic certainty (clinically definite, probable, or possible; laboratory-supported) were recorded according to the information given by patients or their relatives and on the basis of the clinical examination (Tab. 1).

Genetic testing

Genomic DNA was extracted from blood samples using standard methods. Enrichment for the genes *VCP* (NM_007126; TER ATPase, p97), *WASH1* (NM_182905; WASH complex subunit 1), *FAM21C* (NM_015262; WASH complex subunit 2C), *CCDC53* (NM_016053; WASH complex subunit 3), *KIAA1033* (NM_015275; WASH complex subunit 4, SWIP), *KIAA0196* (NM_014846; WASH complex subunit 5, strumpellin), *CAPZA1* (NM_006135), and *CAPZB* (NM_001206540) was performed using a custom Ion AmpliSeq design (Ion AmpliSeq Designer, ThermoFisher, Darmstadt, Germany). Coding regions including the conserved splice sites not considered in the design (*WASH1:* 1478 bp, *FAM21C:* 70 bp, *KIAA1033:* 72 bp, *CAPZB:* 17 bp) or with a less than 10x sequence depth were covered by Sanger sequencing. Sequencing was carried out on an Ion Torrent Next-Generation Sequencing Systems (<device name> [Thiel]; ThermoFisher Scientific, Darmstadt, Germany). After initial quality assessment, duplicate reads were removed with the Picard tools (http://broadinstitute.github.io/picard). Read alignment to the hg19 reference genome (assembly date Feb. 2009) and variant calling was performed with the SeqPilot software (JSI medical systems, Ettenheim, Germany). Variants were assessed based on conservation and population frequency in online databases (ESP, 1000 genomes, ExAC, CADD) and evaluated for their biological plausibility. Significant variants were confirmed by Sanger sequencing.

Quantitative real-time PCR

For CCDC53 mRNA quantitation, cDNA was prepared from lymphoblastoid cell lines derived from patient 14 and normal controls using the Superscript II Reverse Transcriptase Kit with random hexamer primers (Invitrogen, Carlsbad, California, USA). Real-time PCR (Taqman probe Hs00211387_m1, <primer sequences> [Thiel]) was performed in quadruplicates in 384-well plates with a final volume of 20 µl each on an ABI 7900HT system using the TaqMan Gene Expression Mastermix according to the manufacturer's instructions (Applied Biosystems, Foster City, California, USA). The relative amount of CCDC53 was calculated using the $\Delta\Delta C_t$ method.

Immunoblotting

Pellets of patient 14 and control derived lymphoblastoid cell lines were used for quantitative CCDC53 (rabbit polyclonal antibody, ABT69, Merck Millipore, Darmstadt, Germany) immunoblotting according to (21).

In-silico analyses

The three-dimensional crystal structures of *Gallus gallus* actin capping protein in the absence (PDB accession code: 3aa7) and presence of a CP-binding peptide from hepatitis C virus (3aa0) or myotrophin (3aaa) were obtained from the PDB, processed with ASSP (Wang and Hofmann, 2015) and visualised using the molecular graphics programs O (Jones et al., 1991) and PyMol (DeLano, 2002). Amino acid sequence identity between chicken (3aa7) and human CAPZα1 (Genbank entry U56637) was calculated using the Needleman-Wunsch module of the Emboss package (Rice et al., 2000). Appraisal of possible phosphorylation sites was carried out with NetPhos 3.1 (Blom et al., 1999) and Scansite 3beta (Obenauer et al., 2003).

Results

For the genetic analysis of VCP and WASH-complex genes, a cohort of 43 sporadic ALS/FTD-patients was enrolled (clinical data is summarized in Table 1). The cohort consisted of 18 female and 25 male patients. The median age of disease onset was 66 years (28 to 78 years), the median disease duration was 24 months (3 to 153 months), and the median age at death was 68 years (47 to 80 years). Next generation panel and Sanger sequencing of all 43 patients revealed no mutations in the genes coding for VCP and the WASH-complex proteins WASH1, FAM21C, SWIP, strumpellin, and CAPZβ. However, in two patients we identified putative pathogenic sequence variants.

In patient 22, the heterozygous c.26C>T variant was found in *CAPZA1* (Tab. 2). This mutation is predicted to lead to a single amino acid change from serine to leucine at position 9, p.(Ser9Leu), of the CAPZα1 protein. This sequence variant has previously solely been described in East Asian population (1:113162492 C/T; ExAC browser; http://exac.broadinstitute.org/) with an allele frequency of 0.0005278. While the Polymorphism Phenotyping v2 tool (http://genetics.bwh.harvard.edu/pph2/) and the ACMG classification (<URL> [Thiel]) considered this change as benign and variant of unknown significance, respectively, SIFT prediction (http://sift.jcvi.org/) indicated a putative deleterious effect on protein function. Three-dimensional crystal structure information for the actin capping protein of muscle Z-line (CAPZ) comprising subunits α1 and α2 in the absence and presence of inhibitory peptides/proteins has previously been reported by the group of Maéda (Takeda et al., 2010). *G. gallus* CAPZα1 shares 87% amino acid sequence identity with its human orthologue and, importantly, strict conservation of amino acid residues in the region around position 9. Structural appraisal using the chicken CAPZα1 structure revealed that serine 9 is the N-terminal capping residue on the first α-helix of thus subunit. With its side chain hydroxyl group, serine 9 accepts a hydrogen bond from the backbone-NH_2 at position (i+2) of the α-helix forming an ST-turn. As a consequence, mutation of serine 9 into leucine may have one or several of the following primary effects: i) The loss of the N-terminal capping may compromise folding of the first α-helix, which may disturb the helical conformation and, in turn, compromise packing of the first α-helix of CAPZα against the helix of CAPZβ. Ultimately, this could de-stabilize the functional CAPZα:CAPZβ heterodimer. ii) The loss of the side chain mediated hydrogen bond

of serine 9 to the following first α-helix may lead to disengagement of the N-terminal tail (residues 1 to 9) from the core of the CAPZ heterodimer and enable new interactions of the tail with other proteins. iii) The disengagement of the N-terminal tail from the core could provide access to the region spanning residues asparagine 30 to leucine 35 of CAPZβ, thus making this region accessible for protein interactions.

Alternatively, a secondary effect of the serine→leucine mutation may arise from the loss of crucial post-translational modification. Within the assembled CAPZ complex, serine 9 is in a surface-exposed position and may be subject to phosphorylation. Previous studies investigating recombinant actin capping protein (Kim et al., 2007; Kuhn and Pollard, 2007; Takeda et al., 2010) did not report on the presence or absence of post-translational modifications; however, since all reported recombinant proteins have been produced in bacterial culture, the native post-translational modifications (if any) may not be possible to occur. Whereas *in silico* analysis using Scansite did not predict any phosphorylation sites in CAPZα, we found strong support for CAPZa phosphorylation on serine 9 with NetPhos 3.1 (score: 0.998). A possible scenario would thus involve a signaling role of serine 9 by means of phosphorylation. This signal may either manifest itself by a conformational change/allosteric regulation of the assembled CAPZ or by direct interactions of phospho-serine 9 with a CAPZ-binding protein. Clearly, mutation of serine 9 to leucine abolishes this post-translational modification and thus lead to pathological dysfunction.

The second variant was found in patient 14 harboring the heterozygous variant c.2T>C in *CCDC53*. This mutation resides in the translation initiation codon of CCDC53. Theoretically, this mutation could result in i) a non-functional allele (p.0), ii) a N-terminally elongated protein with a threonine at this position, or iii) a shortened protein species lacking at least the first eight N-terminal amino acids. This variant is not listed in the ExAC data base, classified pathogenic according to the ACMC criteria, and predicted to be deleterious on protein function by Polymorphism Phenotyping and SIFT prediction tools. For further analysis addressing a putative CCDC53 haploinsufficiency, we performed real-time PCR and immunoblotting. These experiments neither provided any evidence of reduced CCDC53 mRNA nor protein levels when compared to normal controls (data not shown). Since the rabbit polyclonal antibody, which was generated against GST-tagged recombinant human CCDC53 (ABT69, Merck Millipore), did not detect additional protein species in the

patient as compared to normal controls, this experiment strongly argues against the expression of elongated or shortened CCDC53 variants. In due course, a pathological hexanucleotide repeat expansion in C9orf72 was identified in this patient, but not in patient 22.

Discussion

Our genetic analysis of 43 sporadic ALS/FTD patients did not reveal mutations in any of the disease-related genes established so far, comprising VCP (ALS, IBMPFD, Parkinson's disease, HMSN2, HSP) and the two WASH complex genes strumpellin (HSP, RTSC1) and SWIP (ARID, Alzheimer's disease). Furthermore, no alterations could be identified in the WASH complex genes WASH1, CAPZB, and FAM21C. Notably, the FAM21C protein has a dual role as subunit in both the WASH complex and the retromer complex, which e.g. executes essential functions in endosome to Golgi transport (16). Mutations in VPS35, a retromer complex subunit that directly interacts with FAM21C, cause a rare autosomal-dominant form of Parkinsons's disease (22). Subsequent analyses demonstrated that mutant VPS35 affects the retromer-WASH complex interaction eventually compromising the WASH complex recruitment to endosomes (23).

However, a heterozygous mutation (c.26C>T) was detected in the WASH complex subunit gene CAPZA1 in a single patient. Together with CapZβ, the CapZα protein forms a functionally active dimer, which caps the barbed end of actin filaments (24). This previously in East Asian population reported sequence variant is predicted to have a deleterious effect on protein function by SIFT prediction, whereas the Polymorphism Phenotyping v2 and ACMG classification tools considered it as benign and variant of unknown significance, respectively. Our *in silico* analysis of a p.Ser9Leu CAPZα1 protein indicated several scenarios with conformational changes, which may subsequently lead to defects in protein-protein interactions and CAPZα:CAPZβ heterodimer formation. Thus, the c.26C>T, p.(Ser9Leu), CAPZA1 mutation can be considered as a putatively disease-related mutation in Caucasian ALS/FTD patients.

In a second patient a heterozygous mutation (c.2T>C) was detected in the translation initiation codon of the gene coding for the CCDC53 protein, which is a core subunit of the WASH complex. Our real-time PCR and immunoblotting analysis, however, did neither provide any evidence for a CCDC53 haploinsufficiency nor the presence of aberrant CCDC53 protein species. As a consequence, we consider the effect of the c.2T>C CCDC53 mutation, which most likely results in a non-functional allele (p.0), as benign. In due course, this patient was found to carry a pathological hexanucleotide repeat expansion in the ALS-associated C9orf72 gene.

Conclusion: With exception of a putatively pathogenic heterozygous c.26C>T CAPZA1 mutation, our genetic analysis in 43 sporadic ALS/FTD patients did not reveal mutations in VCP and the remaining WASH complex subunits. The pathogenicity of the c.26C>T CAPZA1 mutation is currently unclear and awaits further genetic and functional evaluation.

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Declaration of interest statement

The authors declare no conflicts of interest. [alle: ggf. ergänzen]

Tables

Table I. Clinical characteristics of 43 sporadic ALS/FTD patients
(bitte in endgültige Form bringen [Türk/Weishaupt])

No.	Ulmer ID	Sex	Age of onset (y)	Age of death (y)	Diesease duration (month)	initial ALS symptoms	diagnostic certainty
1	12	F	58	70	153	spinal	clinically definite
2	23; C9ORF72	M	66	70	56	spinal	clinically possible
3	74	M	78	80	20	bulbar	clinically definite
4	76	F	69	70	24	bulbar	clinically probable
5	152	M	62	63	21	spinal	clinically definite
6	179	M	51	alive	68	spinal	laboratory-supported
7	267	M	49	57	103	spinal	clinically definite
8	1055	M	67	68	23	spinal	clinically definite
9	1245	M	66	68	24	spinal	clinically definite
10	1274	M	2	alive		spinal	clinically definite
11	1295	F	46	53	88	spinal	laboratory-supported
12	1297	M	61	63	31	bulbar	clinically possible
13	1385	M	67	alive	105	spinal	clinically probable
14	1418; C9ORF72	M	55	56	19	spinal	clinically probable
15	1435	F	72	72	21	spinal	clinically definite
16	1798 (statt 1748)	F	28	alive	80	bulbar	clinically definite
17	1850	F	54	56	25	spinal	clinically definite
18	1921	M	68	69	17	spinal	laboratory-supported
19	2053	M	65	67	32	spinal	clinically definite
20	2115	M	67	68	18	bulbar	clinically definite
21	2128	F	48	50	24	spinal	clinically definite
22	2130	M	64	alive	6	bulbar	clinically definite
23	2144	F	70	72	35	bulbar	clinically possible
24	2145	M	69	77	93	spinal	clinically probable
25	2247	F	64	65	15	spinal	clinically probable
26	2312	M	61	alive	66	spinal	atypical MND
27	2318	F	71	74	35	spinal	clinically definite
28	2511	M	72	73	12	bulbar	clinically definite
29	2515	F	62	64	35	spinal	clinically definite
30	3187	F	68	69	13	spinal	laboratory-supported
31	3194	M	65	67	29	bulbar	clinically probable
32	3493	F	70	alive	35	spinal	laboratory-supported
33	3521	F	69	70	19	spinal	clinically possible
34	3661	M	53	54	24	bulbar	clinically definite
35	3742	F	67	68	14	spinal	clinically definite
36	3836	M	70	71	22	spinal	clinically definite
37	4011	F	71	72	16	bulbar	clinically definite
38	4019	F	52	54	32	spinal	clinically definite
39	4119	F	77	alive	3	bulbar	laboratory-supported
40	4148	M	66	68	32	spinal	clinically probable
41	4172	M	77	77	14	bulbar	clinically definite
42	4330	M	46	47	10	spinal	clinically definite

43	4405	M	71	71	18	spinal	clinically definite

Table II. Results of VCP and WASH complex genetic analysis in 43 cases of sporadic ALS/FTD

No.	Gene	Genomic position (hg19)	reference	cDNA	Protein	CADD	Polyphen2	SIFT	ExAC frequency	ACMG
22	CAPZA1	chr1:113162492C>T	NM_006135	c.26C>T	p.(Ser9Leu)	26.9	benign	deleterious	4,05E-05	VUS
14	CCDC53	chr12:102455739A>G	NM_016053	c.2T>C	p.0	27.1	deleterious	deleterious	0	pathog

CADD: Combined Annotation Dependent Depletion Score; ExAC: Exome Aggregation Consortium; ACMG: American College of Medical Genetics and Genomics; VU

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