Mutations in the colony stimulating factor 1 receptor (*CSF1R*) cause hereditary diffuse leukoencephalopathy with spheroids

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Abstract

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Hereditary diffuse leukoencephalopathy with spheroids (HDLS) is an autosomal dominantly inherited central nervous system white matter disease with variable clinical presentations including personality and behavioral changes, dementia, depression, parkinsonism, seizures, and others[1,2]. We combined genome-wide linkage analysis with exome sequencing and identified 14 different mutations affecting the tyrosine kinase domain of the colony stimulating factor receptor 1 (encoded by *CSF1R*) in 14 families affected by HDLS. In one kindred, the *de novo* occurrence of the mutation was confirmed. Follow-up sequencing analyses identified an additional *CSF1R* mutation in a patient clinically diagnosed with corticobasal syndrome (CBS). *In vitro*, CSF-1 stimulation resulted in the rapid autophosphorylation of selected tyrosine-residues in the kinase domain of wild-type but not mutant CSF1R, suggesting that HDLS may result from a partial loss of CSF1R function. Since CSF1R is a critical mediator of microglial proliferation and differentiation in the brain, our findings suggest an important role for microglial dysfunction in HDLS pathogenesis.

HDLS typically presents as an autosomal dominant disease associated with variable behavioral, cognitive and motor changes[1-3]. The onset of symptoms is usually in the fourth or fifth decade, progressing to dementia with death within six years. On magnetic resonance imaging (MRI), HDLS is characterized by patchy cerebral white matter abnormalities, often initially asymmetrical but becoming confluent and symmetrical with disease progression[4-12]. The changes predominantly involve the frontal and parietal white matter with evolving cortical atrophy affecting these lobes (Fig. 1a-b). Since neither the clinical symptoms nor the MRI changes are specific, a definite diagnosis of HDLS relies on pathological examination, showing widespread loss of myelin sheaths and axonal destruction, axonal spheroids, gliosis, and autofluorescent lipid-laden macrophages (Fig. 1c-i)[1,4-8,10-12]. Occasionally, brain biopsy has been used to confirm the diagnosis[9].

To identify the genetic basis of HDLS, we established an international consortium with ethical approval from the Mayo Clinic Institutional Review Board and collected clinical data, MRI studies, blood and brain tissue samples from families with at least one patient with autopsy- or biopsy-proven HDLS. In total, we collected 14 kindreds from the United States, Norway, Germany and Scotland (Fig. 2). Family VA was selected for genome-wide linkage studies, and non-parametric linkage analyses identified one locus with a lod-score>2.5 (chromosome 5; lod=2.67) and four loci with lod-scores>1.0 (Supplementary Fig. 1). Subsequent parametric linkage analysis identified significant linkage on chromosome 5q34 (lod=3.71, θ=0 at rs13178296), while none of the other loci reached significance (Supplementary Fig. 1). Obligate recombinants narrowed the candidate region to 30.3cM between rs801399 and rs1445716 (Supplementary Fig. 2), corresponding to a ~25Mb genomic interval containing 233 candidate genes.

To generate a list of potential disease-causing mutations, we performed whole-exome sequencing of two pathologically confirmed patients from family VA (VA-21 and VA-24, Fig. 2). We generated variant profiles for each patient and searched for shared heterozygous variants located within the chromosome 5q candidate region. We further predicted that mutations underlying HDLS are likely to be previously unidentified; therefore, we filtered all of the identified base alterations against dbSNP132. This led to the identification of two

non-synonymous mutations: c.80C>T (p.S27L) in the gene encoding the 5-hydroxytryptamine receptor 4 (*HTR4*) and c.2624T>C (p.M875T) in the macrophage colony-stimulating factor 1 receptor gene (*CSF1R*). Both mutations segregated with disease in the extended family VA and were absent in 660 controls. We therefore searched for additional mutations in a cohort of 13 probands from autopsy- or biopsy-proven HDLS families (Fig. 2). Sanger sequencing of the 6 coding exons of *HTR4* and 22 coding exons of *CSF1R* identified heterozygous *CSF1R* mutations in all 13 probands, whereas no other mutations in *HTR4* were identified (Fig. 3; Supplementary Table 1). Segregation analyses confirmed transmission of the *CSF1R* mutations and co-segregation with the disease phenotype in all families where DNA from multiple affecteds was available (Fig. 2). We further confirmed the *de novo* occurrence of one *CSF1R* mutation in monozygotic twins from family NO, without a family history of HDLS (Supplementary Fig. 3). To confirm the rarity of these mutations, and to provide supporting evidence for pathogenicity, we also sequenced the *CSF1R* gene in 24 unrelated controls and genotyped the 13 novel mutations in at least 1436 Caucasian controls using Taqman genotyping assays. None of the mutations identified in HDLS patients and no other novel *CSF1R* mutations were found in controls.

The 14 *CSF1R* mutations identified in HDLS families are all located in the intracellular tyrosine-kinase domain of CSF1R encoded by exons 12-22. The mutations include 10 missense mutations and one single-codon deletion, all affecting residues highly conserved across species and within members of the CSF1/PDGF receptor family of tyrosine-protein kinases (Kit, FLT3 and PDGFRα/β)[13] (Fig. 3). We further identified three splice-site mutations, leading to the in-frame deletion of exon 13 (NO) or exon 18 (CA2/FL2), deleting up to 40 consecutive amino acids within the tyrosine kinase domain (Supplementary Fig. 4).

Detailed clinical information was available for 24 patients with proven *CSF1R* mutations from 14 HDLS families (Table 1). Mean age at onset was 47.2±14.5 years (range 18-78 years), with mean disease duration of 6.0±3.1 years (range 2-11 years) and a mean age at death of 57.2±13.1 years (range 40-84 years). In some families (FL1/CA1/VA), age at onset or death differed by more than 25 years among family members, whereas a monozygotic twin pair (family NO) showed highly similar disease course with ages at onset and death within one year from each other, suggesting that currently unidentified genetic or environmental factors may be important determinants of the age-related disease penetrance. Presenting features and evolving clinical symptoms also varied significantly within and across families, and *ante mortem* clinical diagnoses in mutation carriers included frontotemporal dementia (FTD), CBS, Alzheimer disease (AD), multiple sclerosis (MS), atypical cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), and Parkinson disease (PD).

Since most patients included in our study were not diagnosed with HDLS, we hypothesized that *CSF1R* mutation carriers may be present in clinical series of early-onset AD, FTD, CBS, MS and PD, or ischemic stroke patients with additional white matter changes. Sequencing analyses of *CSF1R* exons 12-22 encoding the protein tyrosine kinase domain in up to 93 Mayo Clinic patients affected with each of these neurological syndromes led to the identification of an additional *CSF1R* missense mutation c.2509G>T (p.D837Y) in a woman with clinical symptoms resembling CBS (Supplementary Tables 2-3). The identification of a

CSF1R mutation in this limited patient series underscores that HDLS may be an under-diagnosed disease.

CSF1R is a cell-surface receptor primarily for the cytokine CSF-1, which regulates the survival, proliferation, differentiation and function of mononuclear phagocytic cells, including microglia of the central nervous system[14]. CSF1R is composed of a highly glycosylated extracellular ligand-binding domain, a trans-membrane domain and an intracellular tyrosine-kinase domain[15]. Binding of CSF-1 to CSF1R results in the formation of receptor homodimers and subsequent auto-phosphorylation of several tyrosine residues in the cytoplasmic domain[16]. CSF1R autophosphorylation precedes CSF1R-dependent phosphorylation of several proteins, including the phosphatase SHP-1 and the kinases Src, PLC-g, PI(3)K, Akt and Erk[16-18]. In the brain, CSF1R is predominantly expressed in microglial cells, although low levels of CSF1R have been reported in cultured neurons[19-21]. An increase in *CSF1R* copy number and point mutations leading to constitutive activation of the CSF1R receptor have been associated with tumor development, including hematological malignancies and renal cell carcinomas[22,23].

To assess the functional importance of the *CSF1R* mutations identified in this study, we first studied the effect of the mutations on CSF1R *in vitro*. We transiently expressed DDK-tagged wild-type ($CSF1R_{WT}$) and mutant ($CSF1R_{E633K}$, $CSF1R_{M766T}$ and $CSF1R_{M875T}$) CSF1R in cultured cells. Upon stimulation with CSF-1, autophosphorylation on multiple CSF1R tyrosine-residues was observed for $CSF1R_{WT}$, while none of the mutants showed detectable levels of autophosphorylation (Fig. 4 and Supplementary Fig. 5). Since all mutations are in the CSF1R kinase domain, dimerization and/or cell surface expression are unlikely to be affected; however, we cannot exclude this at this time. These preliminary findings suggest that mutant CSF1R kinase activity is abrogated, likely affecting the phosphorylation of downstream targets. We speculate that mutant CSF1R might assemble into non-functional homodimers and wild-type/mutant heterodimers inducing a dominant-negative disease mechanism.

To address whether CSF1R autophosphorylation is also disrupted in HDLS patient samples, we first subjected blood samples from a healthy control and HDLS patient CA1-1 to CSF1R immunoblotting, which revealed no apparent difference in CSF1R total or phosphorylation levels (Supplementary Fig. 6a). Further, CSF1R immunoblotting was performed in frontal cortex brain tissue of healthy controls as well as patients with HDLS. Brain samples from AD and ALS patients were included as neurodegenerative disease controls. Our data showed varied levels of total and phosphorylated CSF1R in these brain samples (Supplementary Fig. 6b); however, statistical analysis did not reveal a significant difference between any of the groups. Although these preliminary *in vivo* studies do not reveal a defect in autophosphorylation, these findings do not necessarily conflict with the data obtained in cultured cells. First, HDLS patients are heterozygous for the *CSF1R* mutations and therefore, in contrast to our *in-vitro* experiments, wild-type receptor is still present in these patients. In our cell culture experiments, CSF1R signaling was down regulated by serum deprivation to minimize basal signaling through this receptor before stimulation with the CSF-1 ligand. CSF-1 is a serum protein, so without this deprivation *in vivo*, immediate changes in CSF-1-induced CSF1R autophosphorylation may not be apparent as we cannot

disregard wild-type receptors at the cell surface that have already been activated. Unfortunately, without access to an immortalized cell line derived from an HDLS patient, we are currently unable to accurately assess acute receptor activation *in vivo*. Finally, the post-mortem brain samples from HDLS patients included in these studies exhibit extensive degeneration, leaving the possibility that cells with greater disruption of CSF1R signaling are underrepresented in the tissue sample.

Unraveling the genetic etiology of HDLS may significantly contribute to the understanding of other adult-onset leukoencephalopathies. *De novo* mutations in *CSF1R* could explain the disease in sporadic patients that have been described with clinical and pathological similarities to HDLS[24-29]. Future *CSF1R* mutation screening may also determine whether HDLS and pigmentary orthochromatic leukodystrophy (POLD) are part of a single clinicopathologic entity, as was recently suggested[2]. Moreover, the discovery of a mutation in a microglial trophic factor receptor may further elucidate the role of microglia in more common white matter disorders, particularly those associated with axonal dystrophy, such as Binswanger's disease[24,30], multiple sclerosis[31] and HIV encephalitis[32].

Interestingly, our findings also shed new light on Nasu-Hakola disease (NHD), a rare condition characterized by systemic bone cysts and dementia with striking similarities to HDLS[33-35]. NHD is caused by recessive loss-of-function mutations in the DAP12/TREM2 protein complex[36,37], which was recently implicated in CSF1R signaling, establishing NHD as a primary microglial disorder[38]. We speculate that a partial loss of the CSF1R/DAP12 signaling cascade in microglia is responsible for the neurological phenotypes observed in HDLS and NHD, whereas a complete loss of this signaling cascade in bone marrow-derived macrophages is needed for the bone-cysts formation observed in NHD. In support of this hypothesis, a partial loss-of-function mutation in *TREM2* in a family with early-onset dementia without bone-cysts was recently reported[39]. Also, no bone-cysts were reported in any of our HDLS patients and a bone scan in a patient CA1-1 did not show bone fractures, hypomineralization or any other bone structure abnormalities.

In summary, we have shown that mutations affecting the tyrosine-kinase domain of CSF1R underlie the white matter disease of HDLS, establishing HDLS as an important novel member of the recently defined class of primary microglial disorders, called 'microgliopathies'[40]. Future molecular studies of CSF1R signaling might offer novel insights into microglial physiology and the involvement of this cell type in HDLS and neurodegeneration. Moreover, *CSF1R* mutation screening in neurodegenerative disease patient series will now allow an accurate diagnosis of HDLS and could facilitate detection of presymptomatic individuals, which is indispensable for therapy development and early treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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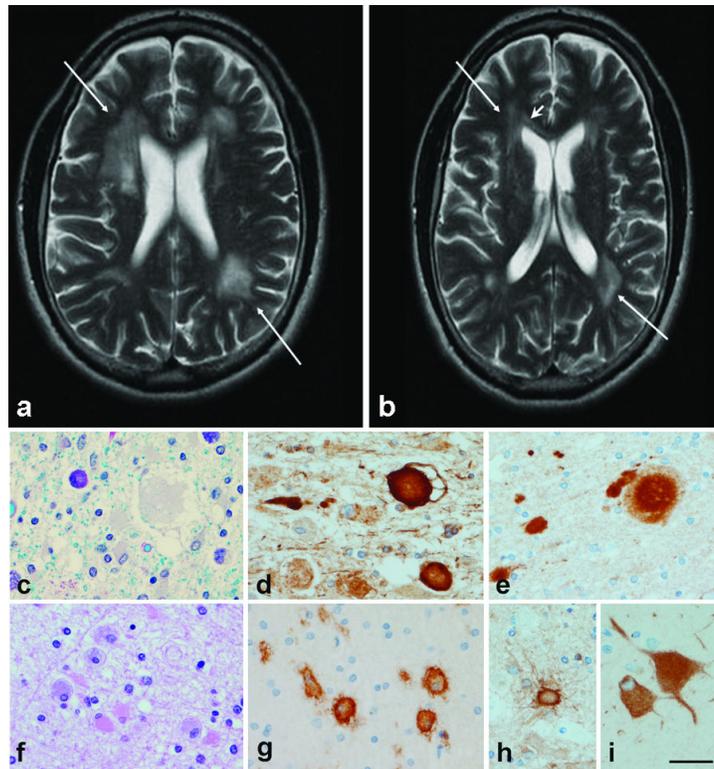
Figure 1. Neuroimaging (a-b) and neuropathological (c-i) findings in HDLS patient FL2-1
Patient FL2-1 (Table 1 and Fig. 2) developed a mild depression followed shortly by
forgetfulness at the age of 50 years. Two years later he had a flat affect, inappropriate
behavior, poor concentration, executive dysfunction, restless legs syndrome, and insomnia.
Examination 3 years after the onset of symptoms demonstrated psychomotor slowing, and
ideomotor and constructional apraxia. The Mini-Mental State Examination (MMSE) score
was 22/30. His gait was slow and shuffling. His postural stability was poor leading to
frequent falls. He had rigidity and bradykinesia in all four extremities symmetrically. (**a-b**)
Axial T2-weighted MR images showed localized hyperintense foci in both frontal and
parietal lobes (long arrows), involving the periventricular, deep and subcortical white
matter, sparing the subcortical U-fibers. Hyperintense focus in the right forceps minor
(arrowhead) was seen. In the final stage of his illness, he became mute, reached a vegetative
state, and died at the age of 55 years. Autopsy was performed. (**c**) Myelin loss in frontal
white matter with a pigmented macrophage and a pale vacuolated axonal spheroid (Luxol
fast blue). (**d**) Spheroids with phosphorylated neurofilament immunohistochemistry. (**e**)
Spheroids with amyloid precursor protein immunohistochemistry. (**f**) Pigmented
macrophages and reactive astrocytes (H&E). (**g**) White matter macrophages with HLA-DR
immunohistochemistry. (**h**) Bizarre white matter astrocytes. (**i**) Ballooned cortical neurons
with alpha-B-crystallin immunohistochemistry. Bar (c-i) = 30 µm
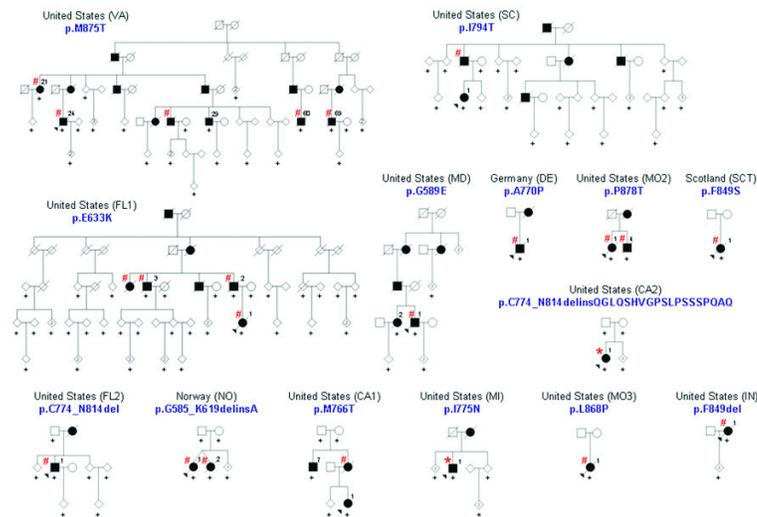
Figure 2. Families with HDLS and *CSF1R* mutations

Abbreviated pedigrees of all families with HDLS included in this study. Filled symbols indicate affected individuals. An arrowhead indicates the proband. To protect confidentiality some individuals are not shown and sex is portrayed using a diamond for all individuals except affected individuals and their spouse. In each family, at least one affected family member received an autopsy (red pound sign) or biopsy (red star) confirmation of HDLS. A '+' sign indicates that DNA was included in the *CSFR1* sequencing analyses to confirm that mutations segregated with disease. For each patient with DNA available for genetic studies, a unique patient number (UPN) corresponding to Table 1 is included above the patient.
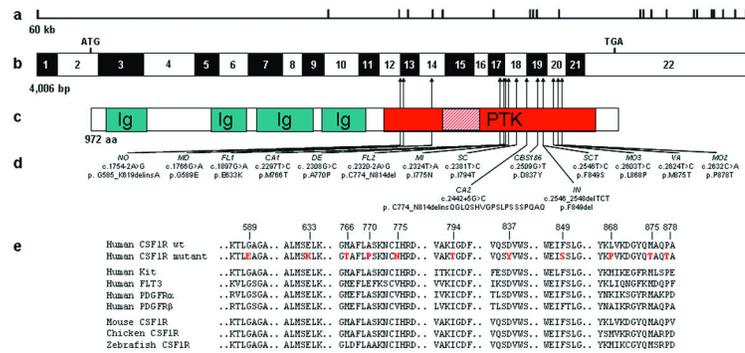
Figure 3. Genomic organization and protein domain structure of CSF1R with summary of *CSF1R* mutations

(a) The *CSF1R* gene extends over 60kb and contains 22 exons (vertical hatches). **(b)** Exon structure of the human *CSF1R* cDNA. Positions of the start codon (ATG) and stop codon (TGA) are indicated. For mutations detected, arrows indicate positions relative to exons and protein domains. **(c)** Domain structure of the CSF1R protein showing the immunoglobulin domains (IG) and the protein tyrosine kinase domain (PTK), interrupted by the kinase insert (shaded). **(d)** Fifteen heterozygote *CSF1R* mutations detected in 14 families with autopsy or biopsy proven HDLS and in one patient clinically diagnosed with corticobasal syndrome. Family identifiers, cDNA numbering (relative to NM_005211.3) and predicted translational changes are indicated. **(e)** ClustalW alignment for the parts of the PTK domain where the mutations occur, including multiple CSF1R homologs and all human CSF1/PDGF receptor family members. Comparison of human CSF1R (NP_005202.2), mouse CSF1R (NP_001032948.2), chicken CSF1R (XP_414597.2), Zebrafish CSF1R (NP_571747.1), human KIT (NP_000213.1), human FLT3 (NP_004110.2), human PDGFRα (NP_006197.1) and human PDGFRβ (NP002600.1). Amino acid positions of the mutations are indicated above the alignment.
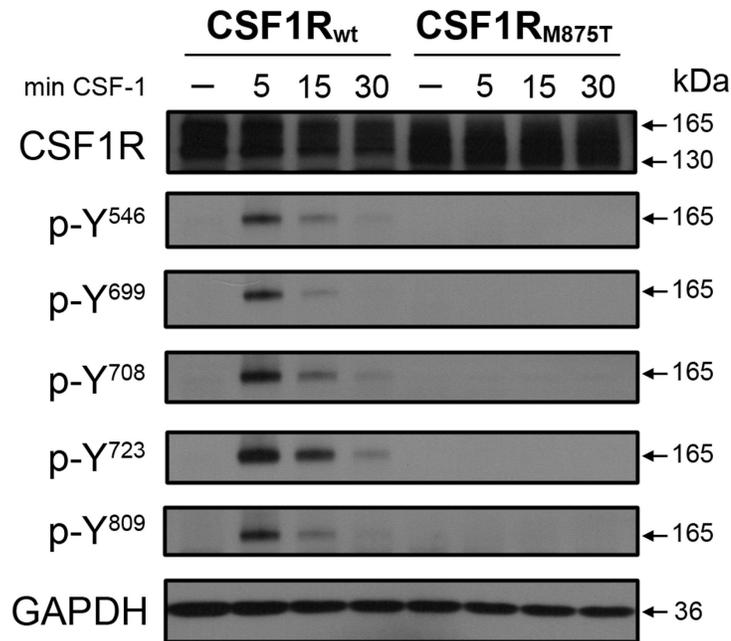
Figure 4. CSF-1 induces autophosphorylation of wild-type but not mutant CSF1R
Autophosphorylation of several tyrosine residues within the kinase domain of CSF1R is critical for its subsequent signaling involved in cell survival and proliferation. We studied CSF1R autophosphorylation in HeLa cells which do not express detectable levels of CSF-1 thereby minimizing endogenous CSF1-induced signaling. A representative Western blot of lysates from CSF1R$_{WT}$ or mutant CSF1R$_{M875T}$ transfected HeLa cells treated with CSF-1 for 5, 15, or 30 minutes is shown. Lysates from untreated CSF1R-transfected cells are included as a control and GAPDH immunoreactivity is shown to ensure equal protein loading. Total CSF1R immunodetection for both DNA constructs was robust. Further, we observed strong phosphorylation of wild-type CSF1R after 5 minutes of CSF-1 treatment, which decreased over the course of 15 and 30 minutes, as determined by immunoblotting using CSF1R phospho-specific tyrosine (p-Y) antibodies. In contrast, no CSF1R autophosphorylation at any of the selected tyrosine residues was detected after CSF-1 treatment in CSF1RM875T transfected cells. Experiments were repeated three times with similar outcome. Comparable results were obtained using CSF1R mutants CSF1RE633K and CSF1RM766T (Supplementary Fig. 5).

Table 1

Clinical characteristics of 24 patients from 14 families with genetically confirmed *CSF1R* mutations.

Family (individual)	Origin	Sex	Onset age	Death age	Initial symptoms	Clinical Features during course of the illness					Clinical Diagnosis	Reference
						Personality/ Behavior changes	Dementia	Depression	Parkinsonism	Seizures		
NO	Norway											new
(NO-1)		F	36	40	Dizziness/Cognitive impairment	+	+	+	+	+	MS	
(NO-2)		F	38	41	Depression	+	+	+	+	+	MS	
MD	US											new
(MD-1)		M	58	61	Cognitive impairment	+	+	–	+	+	FTD	
(MD-2)		F	47	58	Spasticity	+	–	+	+	+	Epilepsy	
FL1	US											4
(FL1-1)		F	42	46	Speech problems/Alien limb	+	+	+	+	–	CBS	
(FL1-2)		M	67[a]	74	Cognitive impairment	+	+	+	+	+	Parkinsonism	
(FL1-3)		M	78	84	Cognitive impairment	+	+	+	+	+	AD	
CA1	US											new
(CA1-1)		F	18	–	Depression	+	+	+	–	–	HDLS[b]	
(CA1-7)		M	43	–	Facial pain	+	+	+	+	–	Psychiatric disease/NPH	
DE	Germany											new
(DE-1)		M	52	63	Cognitive impairment	+	+	+	+	–	FTD[c]/atypical CADASIL[d]	
FL2	US											new
(FL2-1)		M	50	55	Depression	+	+	+	+	+	FTD	
MI	US											new
(MI-1)		M	48	–	Gait disturbances	+	–	+	+	–	MS/ atypical PD	
SC	US											12
(SC-1)		F	35	–	Cognitive impairment	+	+	+	+	–	Atypical CADASIL[d]	
CA2	US											9

Family (individual)	Origin	Sex	Onset age	Death age	Initial symptoms	Clinical Features during course of the illness					Clinical Diagnosis	Reference
						Personality/ Behavior changes	Dementia	Depression	Parkinsonism	Seizures		
(CA2-1)		F	23	–	Dysarthria/apraxia/gait impairments	+	+	+	+	–	Leukoencephalopathy with unknown etiology	
IN	US											new
(IN-1)		F	63	67	Depression/balance issues	–	+	+	+	+	Atypical dementia	
SCT	Scotland											new
(SCT-1)		F	46	51	Depression/epilepsy/cognitive impairment	+	+	+	+	+	FTD/cerebrovascular disease	
MO3	US											new
(MO3-1)		F	55	63	Depression	+	+	+	+	+	FTD	
VA	US											10
(VA-21)		F	n/a	77	n/a	n/a	+	n/a	n/a	n/a	FTD	
(VA-24)		M	58	66	Cognitive impairment	+	+	+	+	+	HDLS[b]	
(VA-27)		M	71	–	Cognitive impairment	+	+	–	–	–	HDLS[b]	
(VA-60)		M	41	43	Cognitive impairment	+	+	–	+	–	HDLS[b]	
(VA-69)		M	46	49	Speech problems	+	+	+	+	–	FTD/ atypical CADASIL[d]	
MO2	US											new
(MO2-1)		F	39	49	Cognitive impairment	+	+	–	–	+	FTD	
(MO2-4)		M	33	43	Cognitive impairment	+	+	+	–	+	FTD	

[a] Patient had an isolated seizure at age 47.

[b] Clinical diagnosis of HDLS was made after autopsy confirmation of HDLS in an affected family member.

[c] Clinical diagnosis was Pick's disease.

[d] Mutations in *NOTCH3* were excluded. MS, multiple sclerosis; FTD, frontotemporal dementia; CBS, corticobasal syndrome; AD, Alzheimer's disease; PD, Parkinson's disease; HDLS, hereditary diffuse leukoencephalopathy with spheroids; NPH, normal pressure hydrocephalus; n/a, not available.