**Functional characterization of a novel CSF1R mutation causing hereditary diffuse leukoencephalopathy with spheroids**

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**Abstract**

**Background:** Colony-stimulating factor 1 receptor is a tyrosine kinase transmembrane protein that mediates proliferation, differentiation, and survival of monocytes/macrophages and microglia. CSF1R gene mutations cause hereditary diffuse leukoencephalopathy with spheroids (HDLS), an autosomal-dominantly inherited microgliopathy, leading to early onset dementia with high lethality.

**Methods:** By interdisciplinary assessment of a complex neuropsychiatric condition in a 44-year old female patient, we narrowed down the genetic diagnostic to CSF1R gene sequencing. Flow cytometric analyses of uncultivated peripheral blood monocytes were conducted sequentially to measure the cell surface CSF1 receptor and autophosphorylation levels. Monocyte subpopulations were monitored during disease progression.

**Results:** We identified a novel heterozygous deletion–insertion mutation c.2527_2530delinsGGCA, p.(Ile843_Leu844delinsGlyIle) in our patient with initial signs of HDLS. Marginally elevated cell surface CSF1 receptor levels with increased Tyr723 autophosphorylation suggest an enhanced receptor activity. Furthermore, we observed a shift in monocyte subpopulations during disease course.

**Conclusion:** Our data indicate a mutation-related CSF1R gain-of-function, accompanied by an altered composition of the peripheral innate immune cells in our patient with HDLS. Since pharmacological targeting of CSF1R with tyrosine kinase inhibitors prevents disease progression in mouse models of neurodegenerative disorders, a potential pharmacological benefit of CSF1R inhibition remains to be elucidated for patients with HDLS.

**KEYWORDS**
clinical diagnostics, disease, DNA, gene, molecular biology, mutation
1 | INTRODUCTION

Hereditary diffuse leukoencephalopathy with spheroids (HDLS, MIM#221820) is an autosomal-dominant neurodegenerative disease characterized by white matter changes and axonal deterioration causing rapid decline of cognitive- and motor functions, as well as personality changes (Axelsson, Roytta, Sourand, Akesson, & Andersen, 1984). Hereditary diffuse leukoencephalopathy with spheroids is summarized together with pigmented orthochromatic leukodystrophy (POLD) as adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP) (Wider et al., 2009). Clinical delineation of this entity is challenging due to variable clinical presentations reminding of Alzheimer’s disease (Sundal et al., 2012), multiple sclerosis (Inui et al., 2013; Sundal et al., 2015), cerebral arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (Kleinfeld et al., 2013), Parkinson’s disease (Lynch et al., 2016), or frontotemporal dementia (Rademakers et al., 2012; Sundal et al., 2012).

Colony-stimulating factor 1 receptor [CSF1R, MIM*164770] mutations cause HDLS (Rademakers et al., 2012). CSF1R is a tyrosine kinase transmembrane protein involved in activation of mononuclear phagocytic cells, for example, microglia that function as immune effector cells with homeostatic and surveillance tasks in the brain (Prinz & Priller, 2014). As microglial dysfunction due to CSF1R mutation is assumed to be the primary disease-causing mechanism, HDLS is classified as microgliopathy (Sasaki, 2017). Most of the mutations are located in the tyrosine kinase domain of CSF1R and are discussed to cause CSF1R loss-of-function (Konno, Kasanuki, Ikeuchi, Dickson, & Wszolek, 2018; Pridans, Sauter, Baer, Kissel, & Hume, 2013; Rademakers et al., 2012). Recently, a clinical and genetic comparison of 122 cases from 90 families with CSF1R mutations revealed no apparent genotype-phenotype correlation but showed a gender-dependent preponderance with a significant younger age of onset in women than men (Konno et al., 2017). The mean onset of manifestation in females and males together was 43 years and the mean disease survival 6.8 years.

Monocytes are a heterogeneous group of cells belonging to innate immunity and like microglia they are part of the mononuclear phagocytic system (Katsumoto, Takeuchi, Takahashi, & Tanaka, 2018). There is consensus about the existence of at least three different blood monocyte subpopulations (Murray, 2018; Sampath, Moideen, Ranganathan, & Bethunaicken, 2018). Classical, intermediate, and nonclassical monocytes are recognized by differential cell surface marker patterns and divergent transcriptomic and proteomic profiles (Wong et al., 2011; Ziegler-Heitbrock, 2015). Of note, monocytes of the nonclassical subpopulation show the highest CSF1R levels (Wong et al., 2011). In particular, the regulation, distribution, and ligand binding capacity of CSF1R along different mononuclear phagocytes in diverse tissues is not fully understood yet (Herz, Filiano, Smith, Yogev, & Kipnis, 2017). Two ligands for CSF1R, CSF1 and IL34, have been identified. Microglia cells certainly do depend on CSF1R, but CSF1R transgene reporter mice systems revealed a heterogeneous expression within microglia with particular lower transgene expression in the cerebellum as compared to other regions of the brain (Hawley et al., 2018). Furthermore, there is evidence for other components in addition to CSF1R involved in the self-renewal of the brain including microglia, such as the IL1R pathway (Bruttger et al., 2015). Interestingly, although the original microglia in the brain are not derived from circulating monocytes but from yolk sac, in conditions of inflammatory brain disorders, monocytes from the periphery enter the brain and take part in the repopulation of microglia (Askew et al., 2017). These aforementioned facts about the role of CSF1R in monocyte/macrophages as well as in microglia biology suggest a fundamental impact and therapeutic potential for HDLS.

2 | MATERIALS AND METHODS

2.1 | Editorial policies and ethical considerations

This study was approved by the local ethics committee and was performed in accordance with the Declaration of Helsinki. The patient gave a written informed consent for the scientific use and publication of medical records and genetic results.

2.2 | Brain magnetic resonance imaging

Initial imaging was performed using a 1.5-Tesla MR machine (Magnetom Sonata Vision, Siemens). For follow-up scans, we used the aforementioned MR machine and a 3-Tesla MR machine (Magnetom Verio, Siemens). Cerebral magnetic resonance imaging included T1-weighted (T1 SE, T1-VIBE), T2-FLAIR-weighted, and diffusion imaging sequences.

2.3 | Samples

DNA and PBMC of the patient as well as age- and gender-matched healthy controls were obtained from EDTA peripheral blood samples. PBMC were gained by Ficoll gradient. Total blood and major immune cell population count were obtained by the use of a hematology analyzer (Cell Dyn, Abbott).

2.4 | Gene sequencing

PCR amplification and Sanger sequencing of all protein coding exons and ±20 bp flanking intronic regions of the CSF1R gene were performed according to standard
proteins were permeabilized with 90% methanol buffer for 30 min paraformaldehyde. Followed by two washing steps, cells were permeabilized with Fcγ block for 15 min. Flow cytometry was performed using a LSR Fortessa™ (Becton Dickinson) flow cytometer and FACS-Diva™ and FlowJo™ (Tree Star) software. To compare CSF1R surface levels between individuals or different blood donations of the patient, we used median fluorescence intensities values. Delta median fluorescence intensities, meaning subtraction of background median obtained by sole secondary antibody staining, were used to display intracellular phospho-CSF1R levels.

Statistical analysis was performed using Graphpad Prism 7.0, and a Student’s t test or Mann–Whitney U test was applied whereat \( p < 0.05 \) was considered significant. Data are summarized in floating bars with line at mean.

3 | RESULTS

3.1 | Case report

The 43-year-old female patient was initially referred to our university hospital because of progressive psychomotor decline during a period of about 1 year. Since the initial magnetic resonance imaging (MRI) of the brain revealed symmetric atrophy pronounced in the frontal lobes and periventricular with matter lesions a neurological examination was initiated (Figure 1a). The complex clinical presentation including progressive spastic-ataxic gait, spastic hemiparesis, apraxia, hand tremor, saccadic eye movements, speech production disorder, and brisk tendon reflexes was topologically correlated with the brain MRI alterations. For further differential diagnostics of an assumed inherited microangiopathy, the patient was referred to our genetic department at age of 44 years. No dysmorphological features suggesting a recognizable syndrome were detected. The pedigree analysis over three generations revealed several affected relatives with neurological disorders, indicating an autosomal dominant mode of inheritance (Figure 2). The patient herself had no children. As far as known, the patient’s mother had passed away at age of 45 years because of cerebral infarction leading to rapid neurological decline with aphasia and paralysis. A maternal aunt of the patient had died after several years of tentative diagnosis of Parkinson’s disease. Two maternal uncles of the patient were also supposed to have cerebral infarctions, one of them already deceased. The maternal grandmother is said to have died by renal insufficiency and polyneuropathy in association with diabetes mellitus. On several cousins, no information on their health conditions was available.

Differential diagnoses including CADASIL (cerebral arteriopathy, autosomal dominant, with subcortical infarcts, and leukoencephalopathy), Fabry disease, Alzheimer’s as well as Parkinson’s disease, and frontotemporal dementia were considered clinically. However, using OMIM database search and the program Phenomizer (Kohler et al., 2009, 2017), we found the best congruence with the clinical synopsis of HDLS and initiated targeted gene analysis of CSF1R which allowed us to confirm the diagnosis of HDLS in our patient.
During follow-up care, our patient was thoroughly examined by a neuropsychologist and a patholinguist at age of 46 years. In accordance with the literature (Freeman et al., 2009; Kohler, Curiel, & Vanderver, 2018), testing revealed rather unspecific cognitive deficits with a score of 28 of 30 points in the Mini Mental State Examination (MMSE). Impairment was proven in selective and divided attention, executive functions, and delayed recall in memory. The speech therapeutic diagnostics revealed hypokinetic dysarthria rather than aphasia as the patient was not able to speak, because phonation and word production

**FIGURE 1** Magnetic resonance imaging (MRI) of the brain. (a) Initial diffuse white matter lesions and gliosis at the age of 43 years. (b) Follow-up MRI at age of 46 years showing increasing diffuse white matter lesions and progression of frontotemporal cerebral atrophy. (c) Diffusion weighted imaging (DWI) of the follow-up examination revealing various isolated spots of hyperintense signal as sign of diffusion restriction and characteristic imaging feature for HDLS

**FIGURE 2** Family pedigree. The accumulation of different neurological disorders over three generations reflects an autosomal dominant inheritance pattern with phenotypic variability. (I:2) polyneuropathy, kidney disease, and diabetes mellitus; (II:1) multiple cerebral infarctions; (II:2) mobilization with walking frame, history of thrombosis, and alcohol abuse; (II:6) aphasia and paresis after cerebral infarction at age of 38; alcohol abuse, smoker, elevated arterial blood pressure; (II:9) clinical diagnosis of Parkinson due to gait disturbance, aphasia, epilepsy; (II:10) cerebral infarction; (III:6) index patient
were so difficult for her. Her understanding was actually quite well. In written form, she was able to produce grammatically correct sentences with orthographically challenging wording. The neuropsychiatric inventory (Schroeter et al., 2011) revealed apathy and depressive symptoms, the latter especially when she was confronted with her disease. In the neurological follow-up examination, 1 month later, the patient showed further progressive psychomotor decline with severe gait bradykinesia, postural instability, and spastic tetraparesis. The speech therapy had led to slight improvement of the hypokinetic dysarthria. However, severely impeded communication skills and labile affect were still present.

Follow-up MRI scans 30–34 months after the initial MRI imaging revealed a drastic progression of leukodystrophy with patchy and confluent bilateral white matter hypointensities predominantly in the frontal and prefrontal white matter (Figure 1b,c; Figure S1). Slightly less intense white matter changes with heterogeneous pattern were also seen in the bilateral parietal white matter. The configuration of some of the changes were tract-shaped and along the corticospinal tract bilaterally. We observed an asymmetry with right-sided accentuation of the bilateral ventricular dilatation as a sign of subcortical atrophy. Focal diffusion restrictions were seen in the bilateral precentral white matter. The configuration of some of the changes were tract-shaped and along the corticospinal tract bilaterally. We observed an asymmetry with right-sided accentuation of the bilateral ventricular dilatation as a sign of subcortical atrophy. 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with typical histological findings of gliosis and axonal spheroids (Kortvelyessy et al., 2015; Rademakers et al., 2012). The white matter lesions can affect different parts of the central nervous system and cause variable neurological symptoms (Konno et al., 2017; Kortvelyessy et al., 2015). As described previously (Sundal & Wszolek, 1993), the following clinical constellation is a sufficient indication for molecular genetic testing of HDLS: First, manifestation at mid-adulthood with rapidly progressive neurological decline including gait, speech and behavioral changes. Second, dispersed white matter changes with signs of gliosis. Third, positive family history for different neuropsychiatric disorders reminding of CADASIL, multiple sclerosis, Parkinson’s, Alzheimer’s, or Fabry disease. Recently, further diagnostic criteria with high sensitivity and sufficient specificity for differentiation from other leukoencephalopathies have been established (Konno, Yoshida et al., 2018). Regarding the different genetic diagnostic methods, for example gene panel analysis of various leukoencephalopathy-associated genes versus clinically guided single gene sequencing one has to consider that a direct approach reduces incidental findings or unclassified sequence variants and facilitates a purposeful genetic counselling. In turn, a gene panel approach might be more cost and time efficient depending on the clinical presetting (Lynch et al., 2018).

The clinical findings of our patient complied with above mentioned diagnostic criteria and led to the identification of a novel CSF1R mutation by single gene sequencing. Segregation analysis was not feasible due to deceased or unreachable relatives of our patient. However, the pedigree is highly suspicious for an autosomal dominant inheritance of HDLS with overlap to findings of Parkinson’s disease, CADASIL, and Fabry disease. Regular clinical monitoring of the patient revealed rapidly progressive psychomotor disturbances in correlation with brain imaging alterations, which is a well-known course in leukodystrophies, especially in HDLS (Konno et al., 2017).

CSF1R is a critical mediator of microglial function (Konno, Kasanuki et al., 2018) and regulates microglia density and distribution in the brain (Oosterhof et al., 2018). Most of the previously identified HDLS-associated CSF1R mutations are missense or splice-site variants leading to amino acid changes in the tyrosine kinase domain and only a few are located in other protein domains (Konno, Kasanuki et al., 2018; Rademakers et al., 2012). The identified amino acid changes of the CSF1 receptor of our patient are located in the tyrosine kinase domain within an α-helix structure without surface access. We reason that p.(Leu844Ile) plays a minor role because of similar chemical and structural characteristics of leucine and isoleucine. Instead, p.(Ile843Gly) is
likely disease-causing for several reasons. First, glycine residues are known to destabilize α-helical structures (Serrano, Neira, Sancho, & Fersht, 1992). Second, isoleucine at position 843 seems to be essential, as the two pathogenic mutations p.(Ile843Asn) and p.(Ile843Phe) had been reported in patients with HDLS before (Battisti et al., 2014; Karle et al., 2013). Third, the importance of this α-helical structure is further supported by the description of the mutations p.(Asp837Tyr) and p.(Phe849Ser) causing HDLS (Rademakers et al., 2012). However, these mutations have not been characterized functionally yet.

So far, in vitro analyses of other CSF1R mutations using transiently transfected HeLa or Ba/F3 cell lines resulted either in absent autophosphorylation or in failure to maintain CSF1 dependent cell survival (Pridans et al., 2013; Rademakers et al., 2012). However, in blood and brain samples CSF1R phosphorylation was not altered (Rademakers et al., 2012). Since mutant CSF1 receptors can be expressed on the cell surface but hamper CSF1-dependent signaling, a dominant negative disease mechanism has been postulated (Pridans et al., 2013; Rademakers et al., 2012). This complies with observations that only multiple combined autophosphorylation mutations or simultaneous inhibition of three downstream signaling pathways completely block induction of D2 in response to CSF-1 (Dey et al., 2000). In turn, based on reduced CSF1R levels in a patient’s brain sample with a CSF1R frameshift mutation, haploinsufficiency is discussed as an alternative molecular mechanism (Konno et al., 2014). However, as shown in their supplemental data, it seems that...
an antibody against the N-terminus (B-8) is more suitable to discard a truncated mutant protein that might interfere with the full-length wild-type protein.

We analyzed blood-derived monocytes for cell surface expression and phosphorylation of CSF1R by flow cytometry. The mutated CSF1 receptor our patient seems to be at least functional in phosphorylation, and thus dimerization and the increased phosphorylation indicates an apparent gain-of-function. We speculate that the amino acid changes in the α-helical region either result in disruption of surface structures important to bind proteins involved in CSF1R degradation, or in locking the enzyme in a conformational state unable to do so. Even mutant homo- and heterodimers with a presumed gain-of-function on the receptor level might override the normal receptor function by perturbing downstream signaling dominantly-negatively causing cell-specific pleiotropic impacts (De et al., 2014; Dey et al., 2000; Hamilton, 1997).

As we do not know whether this finding is mutation specific, other HDLS causing CSF1R mutations should be analyzed in primary immune cells.

Csf1r null mutant mice reveal severe osteopetrosis due to deficient osteoclast activation (Dai et al., 2002; Li, Chen, Zhu, & Pollard, 2006) and structural brain abnormalities with impact on microglia and neural progenitor cells (Erblach, Zhu, Etgen, Dobrenis, & Pollard, 2011; Nandi et al., 2012), but it was not possible to proof neurodegeneration because of early onset high lethality. However, these obvious developmental defects match well with a congenital disorder of two deceased infants assumed to carry a homozygous CSF1R nonsense mutation (Monies et al., 2017). Since the parental heterozygous carrier status was harmless CSF1R loss-of-function mutations seem to display recessive developmental disorders and not dominant alleles causing HDLS by haploinsufficiency (Monies et al., 2017). Moreover, HDLS patients do neither exhibit osteopetrosis nor any other bone structure abnormalities (Rademakers et al., 2012). On the other hand, a mutant Csf1r mouse strain with a haploinsufficient allele resembled HDLS-like symptoms (Chitu et al., 2015), but some neuropathological findings differed from patients with HDLS (Konno, Kasanuki et al., 2018).

Recently, Csf1r knockout rats were established, but, although they survive well into adulthood, they do not reveal an overt phenotype in brain despite the complete absence of microglia (Pridans et al., 2018). The authors suggest that the impact of the loss of neuroprotective function of microglia in the Csf1r knockout rat may be mitigated by the absence of monocytes. Interestingly, recruitment of hematogenous macrophages into the CNS is assumed to take place in patients with HDLS (Tada et al., 2016). Therefore, we focused on the analysis of circulating primary immune cells known to depend on CSF1R signaling. Similar as described earlier (Wong et al., 2011), we found a differential expression of CSF1R on primary monocyte subpopulations of healthy donors and HDLS with the highest levels on nonclassical monocytes. Of particular note, we found a sequential loss of nonclassical monocytes in the HDLS patient over time whereas the total blood monocyte count was increased. Under circumstances of glucocorticoid treatment, the same phenomenon was found where a selective reduction of nonclassical monocytes by apoptosis induction was identified as responsible mechanism (Dayyani et al., 2003). However, the herein investigated HDLS patient did not take glucocorticoids. The absence of nonclassical monocytes in the blood has been identified before in three siblings, where one died and two were healthy. In all three cases, no genetic alteration in CSF1 or CSF1R was found (Frankenberger et al., 2013). However, our observation of CSF1R dysfunction along with highest expression in nonclassical monocytes raises the question whether these monocytes invade into cerebral tissue and whether their removal from blood might be beneficial for HDLS patients. Interestingly, hematopoietic stem cell transplantation halted the disease progression in a patient with HDLS (Eichler et al., 2016). Furthermore, it remains to be elucidated whether levels of CSF1 and IL-34 in the plasma and cerebrospinal fluid as well as ex vivo CSF1R functional studies of patient-derived monocytes might serve to investigate a potential pharmacological benefit of CSF1R inhibition in patients with HDLS. This approach is motivated by observations that CSF1R tyrosine kinase inhibitors prevent disease progression in mouse models of Alzheimer’s disease and amyotrophic lateral sclerosis, among others by reducing invasion of macrophages (Martinez-Muriana et al., 2016; Olmos-Alonso et al., 2016). However, one has to consider that microglia and monocyte response could be both neuroprotective and neurotoxic depending on the stage and progression of the disease (Baufeld, O’Loughlin, Calcagno, Madore, & Butovsky, 2018).

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CONFLICT OF INTEREST

None declared.
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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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