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H63D HFE genotype accelerates disease progression in animal models of amyotrophic lateral sclerosis

Wint Nandar [a], Elizabeth B. Neely [a], Zachary Simmons [b], James R. Connor [a,*]

[a] Department of Neurosurgery, The Pennsylvania State University, M. S. Hershey Medical Center, Hershey, PA 17033, USA
[b] Department of Neurology, The Pennsylvania State University, M. S. Hershey Medical Center, Hershey, PA 17033, USA

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ABSTRACT

H63D HFE is associated with iron dyshomeostasis and oxidative stress; each of which plays an important role in amyotrophic lateral sclerosis (ALS) pathogenesis. To examine the role of H63D HFE in ALS, we generated a double transgenic mouse line (SOD1/H67D) carrying the H67D HFE (homologue of human H63D) and SOD1(G93A) mutations. We found double transgenic mice have shorter survival and accelerated disease progression. We examined parameters in the lumbar spinal cord of double transgenic mice at 90 days (presymptomatic), 110 days (symptomatic) and end-stage. Transferrin receptor and L-ferritin expression, both indicators of iron status, were altered in double transgenic and SOD1 mice starting at 90 days, indicating loss of iron homeostasis in these mice. However, double transgenic mice had higher L-ferritin expression than SOD1 mice. Double transgenic mice exhibited increased Iba-1 immunoreactivity and caspase-3 levels, indicating increased microglial activation which would be consistent with the higher L-ferritin levels. Although both SOD1 and double transgenic mice had increased GFAP expression, the magnitude of the increase was higher in double transgenic mice at 110 days, suggesting increased gliosis in these mice. Increased hemeoxygenase-1 and decreased nuclear factor E2-related factor 2 levels in double transgenic mice strongly suggest the accelerated disease process could be associated with increased oxidative stress. There was no evidence of TAR-DNA-binding protein 43 mislocalization to the cytoplasm in double transgenic mice; however, there was evidence suggesting neurofilament disruption, which has been reported in ALS. Our findings indicate H63D HFE modifies ALS pathophysiology *via* pathways involving oxidative stress, gliosis and disruption of cellular functions.

1. Introduction

Amyotrophic lateral sclerosis (ALS), commonly known as Lou Gehrig's disease, is characterized by degeneration of lower and upper motor neurons in the brainstem, spinal cord, and the motor cortex. The worldwide incidence of ALS is 1–2 per 100,000 and the average age of clinical onset is 55–60 years with an average survival of 3 to 5 years after symptom onset [1,2]. However, the range of survival is from a few months to more than a decade after onset [3]. Because of the high variability in age of onset and in survival, ALS is proposed to be a heterogeneous disease. The majority of ALS cases (90-95%) are sporadic (SALS), whereas 5-10% are inherited (familial ALS, or FALS). Despite identification of mutations in a number of genes associated with FALS and SALS [1,2] including superoxide dismutase (SOD; [4]), TAR-DNA-binding protein 43 (TDP 43; [5]), fused in sarcoma/translated in liposarcoma (FUS/TLS; [6]) and chromosome 9 open reading frame 72 (C9ORF72; [7]), the etiology in most patients with ALS remains inconclusive, and the molecular mechanisms contributing to motor neuron degeneration in ALS have not been elucidated.

Loss of iron homeostasis and the associated oxidative stress are significant parts of the disease processes in neurodegenerative diseases including ALS [8]. Higher iron levels in the central nervous system [9,10] and elevated serum ferritin have been reported in ALS patients [11–13]. Treatment with iron chelators delayed onset, extended survival and prevented motor neuron degeneration in ALS mouse models [14,15]. These reports suggest an important role of iron metabolism in ALS pathogenesis. Therefore, we began studies to determine if there were polymorphisms associated with iron metabolism that could influence the ALS phenotype.

One of the genes involved in iron homeostasis is the HFE gene. Two common HFE polymorphisms are H63D and C282Ywith worldwide allelic frequencies of 8.1% and 1.9% respectively. The C282Y HFE polymorphism is mostly associated with hereditary hemochromatosis (HH), the most common iron overload genetic disorder in Caucasian population (1/200). The occurrence of the H63D HFE in HH is lower than C282Y [16]. However, increasing evidence suggests an association

Abbreviations: GSH, glutathione; HH, hemochromatosis; HO-1, hemeoxygenase-1; NFH, heavy-chain neurofilament protein; Nrf2, nuclear factor E2-related factor 2; SOD, superoxide dismutase; TDP-43, TAR DNA-binding protein 43; TfR, transferrin receptor

* Corresponding author at: Department of Neurosurgery, The Pennsylvania State University, College of Medicine, 500 University Drive (H110), Hershey, PA 17033-0850, USA. Tel.: +1 717 531 4541; fax: +1 717 531 0091.
E-mail addresses: wnandar@hmc.psu.edu (W. Nandar), eneely@psu.edu (E.B. Neely), zsimmons@hmc.psu.edu (Z. Simmons), jconnor@psu.edu (J.R. Connor).

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of H63D HFE with neurodegenerative diseases including ALS [17]. Five independent groups in the United States [18], the United Kingdom [19], Italy [20], the Netherlands [21] and China [22] have reported a positive association between H63D HFE and ALS. Although three studies [23–25] reported no association between H63D HFE and ALS, in all studies, there is agreement that H63D HFE is present in as many as 30% of ALS patients [18–21,23–25]. Moreover, a meta-analysis indicates that the presence of the H63D HFE variant increases the risk of developing ALS by 4-fold [26].

The existing paradigm regarding HFE gene variants and brain function holds that the brain is protected from iron accumulation associated with the HFE polymorphisms because of the blood-brain-barrier. Recent MRI studies, however, suggest that people with HFE polymorphisms have more brain iron and increased cognitive impairment with age [27–30]. In an animal model, the presence of H67D HFE (homologous to H63D in human) disrupts brain iron homeostasis and is associated with increased oxidative stress in the brain [31] and significant disruptions in cholesterol metabolism [32]. The alterations in iron homeostasis and increased oxidative stress are also seen at the cellular level [33], along with increased glutamate release [34] and increased endoplasmic reticulum (ER) stress [35]. Each of above mechanisms is considered a contributing factor to ALS pathogenesis [1,36]. Thus, the data strongly argue that H63D HFE is a genetic modifier for the risk of ALS, and warrant the development of an animal model as presented herein.

Based on findings from our previous *in vitro* and *in vivo* studies, we hypothesized that H63D HFE increases the risk of ALS by establishing a permissive milieu that promotes the convergence of disease mechanisms in ALS. To directly test our hypothesis, we generated a double transgenic mouse line (SOD1/H67D) that carries both H67D HFE (homologous to H63D in humans) and SOD1(G93A) mutations. We found that H63D HFE shortens survival and disease duration in double transgenic mice. Elevated oxidative stress, microglial toxicity and dysregulation of iron homeostasis contribute to an accelerated disease in these mice. Given the data that indicate 1/3 of patients with ALS carry the H63D gene variant, the double transgenic mouse model could serve as a critical preclinical model to evaluate how the H63D HFE genotype can impact the disease process and treatment strategies for ALS patients.

2. Materials and methods

2.1. Generation of double transgenic mice (SOD1/H67D)

SOD1(G93A) male mice (strain name: B6SJL-Tg(SOD1-G93A) 1Gur/J; #002726) purchased from Jackson Labs (Bar Harbor, ME) were crossbred with H67D/H67D (homologue of human H63D) or wild-type HFE female mice (strain name: B6;129X1-Hfe^{tm1Jrco}/J) to generate a double transgenic mouse line, that carries H67D HFE as well as SOD1(G93A) mutation. The H67D colony is maintained at Penn State Hershey Medical Center. Cohorts used for crossbreeding were chosen from littermates. SOD1(G93A) and wild-type (WT) mice from the same litters as the double transgenic mice were included in all experiments. Both males and females were included in all experiments.

Animals were maintained under normal housing conditions with *ad libitum* access to food and water. All experiments were performed according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee.

2.2. Genotyping

The H67D HFE genotyping was performed as previously reported [31]. Briefly, DNA was extracted from tail biopsies using DNeasy blood and tissue kit (QIAGEN, Valencia, CA). PCR was performed using following forward and reverse primers: (5′ AGGACTCACTCTCTGGCAGCAGGAGGTAACCA3′) and (5′TTTCTTTT ACAAAGCTATATCCCCAGGGT3′). Following PCR, DNA was digested with *Bsp*HI restriction enzyme for 2 hours at 37 °C to detect H67D point mutation. The PCR product was separated by 1.5% agarose gel electrophoresis. DNA from WT mice digested by *Bsp*HI resulted 240 and 260 bp and DNA from wt/H67D mice resulted 500, 240 and 260 bp. Genotyping for SOD1(G93A) mutation was performed using primers that specifically amplifying a 236-bp DNA fragment carrying a G93A mutation. The forward and reverse primers are: 5′CATCAGCCCTAATCCATCTGA-3′ and 5′-CGCGACTAACAATCAAAG TGA-3′. PCR conditions for SOD1(G93A) genotyping are 95 °C for 3 minutes, 95 °C for 30 s, 60 °C for 30 s, 35 cycles of 72 °C for 45 s and 72 °C for 2 min.

The SOD1/H67D mice are heterozygous for H67D HFE and also carry G93A mutation while SOD1(G93A) mice carry wild-type HFE and G93A mutation (data not shown).

2.3. Behavior and survival

2.3.1. Rotarod

Starting at 49 days of age, motor performance was tested on a rotarod apparatus (Columbus Instruments, Columbus, OH) rotating at 15 rpm. The amount of time that the mouse could stay on the rotarod before the first fall was recorded to determine disease onset. The duration of the rotarod test was 180 s, and was performed twice every week. A mouse was considered to fail the test when it could not stay on the rotarod for more than one standard error mean (>1 SEM) below the mean time period it stayed on the rotarod during the presymptomatic phase. The probability of passing the rotarod test was analyzed by Kaplan–Meier (n = 19 to 32 per genotype).

2.3.2. Grip strength

Hindlimb and forelimb strength were measured by a grip strength meter (Columbus Instruments, Columbus, OH) to determine disease progression. Mice were held by the base of the tail and were allowed to grasp a horizontal metal bar attached to the grip strength meter with their forelimbs or hindlimbs. They were gently pulled back horizontally. Mice resist the increasing force by clinging onto the metal bar until they can no longer resist the force. The force applied to the bar at the moment the mouse released the bar was recorded as its maximum force. The test was repeated three times and an average determined for each animal. The grip test was performed once each week from 80 days to 127 days of age (n = 5 to 10 per genotype).

2.3.3. Survival and disease duration

End stage of the disease was defined as the inability of the animal to right itself within 30 s after being placed on its side. Kaplan–Meier survival analysis was performed to compare survival between experimental groups (n = 22 to 32 per genotype). Disease duration was the mean time between age of disease onset, determined by rotarod test, and end stage of the disease (n = 19 to 32 per genotype).

2.4. Measurement of iron

The lumbar region of the spinal cord samples was harvested from 90-day (presymptomatic age) and 110-day-old (symptomatic age) SOD1 and SOD1/H67D mice. Presymptomatic and symptomatic ages were chosen based on behavior studies. Lumbar spinal cord samples from age-matched wild-type (WT) littermates were also harvested. The tissues were homogenized in RIPA buffer (Sigma, St. Louis, MO) with protease inhibitor cocktail (1:100; Sigma, St. Louis, MO). Total protein concentration was determined with Pierce BCA protein assay kit (Thermo scientific, MA). Iron concentrations (μg Fe/g of protein) were measured by graphite furnace atomic absorption spectrometry (model 5100AA, Perkin-Elmer, Norwalk, CT) according to standard protocol