Association of the H63D polymorphism in the hemochromatosis gene with sporadic ALS

Abstract—Iron misregulation promotes oxidative stress and abnormally high iron levels have been found in the spinal cords of patients with ALS. The authors investigated whether HFE gene polymorphisms, linked to hemochromatosis, are associated with ALS using two independent populations of patients with sporadic ALS and controls (totaling 379 patients and 400 controls). They found that the H63D polymorphism is overrepresented in individuals with sporadic ALS (odds ratio 1.85, CI: 1.35 to 2.54).

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Many theories of ALS pathogenesis have been proposed, among them that of increased oxidative stress. Elevated levels of iron have been found in ALS spinal cord tissue,¹ and this may contribute to oxidative damage via the ability of iron to generate reactive oxygen species through the Fenton reaction.

Iron homeostasis in humans is tightly controlled as both iron overload and deficiency have severe physiologic consequences. Hereditary hemochromatosis (HH) is a genetic disorder resulting in accumulation of unchelated iron in parenchymal tissues and organ damage. Two principal polymorphisms in the *HFE* gene, C282Y and H63D, have been identified as the cause of classic HH.² Although the exact function of HFE is unknown, reports suggest a role in sensing body iron levels via its interaction with the transferrin receptor (TfR).³ The most frequently identified polymorphism in HH is the C282Y variant, which disrupts cell surface expression of HFE and therefore the interaction with TfR.³ Another common HFE polymorphism is the lowly penetrant H63D, often occurring in HH patients with a heterozygous C282Y variant.² Both of these polymorphisms in the heterozygous state have been reported in association with higher iron concentrations.

Given the evidence of both oxidative stress and disrupted iron homeostasis in ALS, we investigated whether this disease is associated with the HFE polymorphisms. We report an association between the H63D polymorphism and ALS, suggesting that dis-

rupted iron metabolism may be one factor that initiates or propagates motor neuron damage in ALS.

Methods. *Subjects.* A total of 166 individuals with sporadic ALS were recruited through the Birmingham Motor Neurone Disease (MND) Care and Research Centre, Queen Elizabeth Hospital, U.K. An additional 213 patients with sporadic ALS were enlisted from an independent Irish population. All conformed to a diagnosis of definite or probable ALS, according to the El Escorial criteria for ALS. Control samples were obtained from healthy individuals (192 in Birmingham and 208 in Ireland). Fifty-one percent of the Birmingham controls and 10% of the Irish controls were spouses of patients with ALS. The other controls in each population were healthy, unrelated individuals. Informed consent was obtained before blood sampling, and the study was approved by the South Birmingham Local Research Ethics Committee and Beaumont Hospital Ethics Committee. All patients and controls were white.

Genotyping. DNA was extracted from venous blood samples according to standard protocols using NucleonII kits (Amersham). DNA (50 ng) from the Birmingham samples was amplified by PCR primers flanking the H63D and C282Y polymorphisms (Alta Bioscience, University of Birmingham, UK), as described by others.⁴ The *HFE* polymorphisms were detected by restriction enzyme digestion with RsaI and MboI for C282Y and H63D (New England Biolabs). DNA samples from six patients with HH known to be homozygous or heterozygous for the H63D and C282Y polymorphisms were amplified and digested as controls. DNAs from the Irish samples were amplified and genotyped using an alternative method.⁵ Consistency of genotyping was assessed by reanalysis of 10% of the Birmingham samples by this alternative method.

Statistical analysis. The observed genotypes were compared using the Fisher's exact test. Analyses were interpreted as significant if the distributions differed by $p < 0.05$. Presentation with bulbar or limb symptoms and the men vs women breakdown of patients with ALS with and without the HFE variants were compared using Fisher's exact test. Age at disease onset was investigated using the Student *t* test. All statistical analyses were performed using the SPSS software package.

Results. Genotype and allele frequency results for the HFE polymorphisms in the study patients and controls are shown in tables 1 and 2. Genotype frequencies did not deviate from those predicted by Hardy-Weinberg equilibrium in either patients or controls in both data sets, and no sex differences were apparent (table 1). Genotypes at the H63D locus of patients with ALS and controls were compared using Fisher's exact test, pooling individuals either heterozygous (CG) or homozygous (GG) for the disease allele because of the low numbers of homozygous individuals (five patients with ALS vs two controls from Birmingham and 10 patients vs three controls from Ireland). This showed a significant difference in genotypes, with 130 (34%) of the patients with ALS in the combined data set

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Table 1 Genotype and allele frequency data for the H63D polymorphism

	Birmingham		Ireland		Combined	
	ALS, n = 166	Controls, n = 192	ALS, n = 213	Controls, n = 208	ALS, n = 379	Controls, n = 400
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Genotypes						
Wild type (CC)	113 (68)	151 (78.6)	136 (63.8)	161 (77.4)	249 (65.7)	312 (78.0)
Heterozygous (GC)	48 (28.9)	39 (20.3)	67 (31.3)	44 (21.2)	115 (30.3)	83 (20.8)
Homozygous (GG)	5 (3.0)	2 (1.0)	10 (4.7)	3 (1.4)	15 (4.0)	5 (1.3)
Pooled (GC, GG)	53 (31.9)	33 (21.4)	77 (36.0)	47 (22.6)	130 (34.3)	88 (22.0)
	<i>p</i> = 0.030		<i>p</i> = 0.003		<i>p</i> < 0.001	
Allele frequency						
C	274 (82.5)	341 (88.8)	339 (79.6)	366 (88.0)	613 (80.9)	707 (88.3)
G	58 (17.5)	43 (11.2)	87 (20.3)	50 (12.0)	145 (19.1)	93 (11.6)
	<i>p</i> = 0.018		<i>p</i> = 0.001		<i>p</i> < 0.001	
Analysis by gender of patients	Male, n = 104	Female, n = 62	Male, n = 119	Female, n = 94	Male, n = 223	Female, n = 156
Wild type (CC)	69 (66.3)	44 (71.0)	73 (61.3)	63 (67.0)	142 (63.7)	107 (68.6)
Pooled (GC, GG)	35 (33.7)	18 (29.0)	46 (38.7)	31 (33.0)	81 (36.3)	49 (31.4)
	<i>p</i> = 0.607		<i>p</i> = 0.473		<i>p</i> = 0.379	
Gender of controls	Male, n = 59	Female, n = 133	Male, n = 133	Female, n = 75	Male, n = 192	Female, n = 208
Wild type (CC)	45 (76.3)	106 (79.7)	101 (75.9)	60 (80.0)	146 (76.0)	166 (79.8)
Pooled (GC, GG)	14 (23.7)	27 (20.3)	32 (24.1)	15 (20.0)	46 (24.0)	42 (20.2)
	<i>p</i> = 0.574		<i>p</i> = 0.605		<i>p</i> = 0.399	

having at least one G allele compared to only 88 (22%) of controls. Evaluation of the allele frequencies at the H63D locus likewise showed an association between the G allele and ALS ($p < 0.001$). The control group H63D genotype frequencies did not significantly differ between the Birmingham and Irish populations and were consistent with those of other studies of control individuals in these populations.^{6,7} The odds ratio (OR) conferred by the presence of a mutant G allele for the combined data set was evaluated as 1.85 (CI: 1.35 to 2.54).

In contrast, there was no difference between patients and controls for the C282Y polymorphism ($p = 0.466$ for the Birmingham group and $p = 0.905$ for the Irish group) (see table 2). No significant differences were found between HFE genotype and mode of onset (limb vs bulbar) nor in age at onset of disease in either population or in the combined data set (table 3).

Discussion. There is increasing evidence to suggest a role for disrupted iron metabolism in a range

Table 2 Genotype and allele frequency data for the C282Y polymorphism

	Birmingham		Ireland		Combined	
	ALS, n = 166	Controls, n = 192	ALS, n = 213	Controls, n = 208	ALS, n = 379	Controls, n = 400
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Genotypes						
Wild type (GG)	143 (86.1)	159 (82.8)	167 (78.4)	165 (79.3)	310 (81.8)	324 (81.0)
Heterozygous (GA)	22 (13.3)	31 (16.1)	46 (21.6)	40 (19.2)	68 (17.9)	71 (17.8)
Homozygous (AA)	1 (0.6)	2 (1.0)	0 (0)	3 (1.4)	1 (0.3)	5 (1.3)
Pooled (GA, AA)	23 (13.9)	33 (17.2)	46 (21.6)	43 (20.7)	69 (18.2)	76 (19.0)
	<i>p</i> = 0.466		<i>p</i> = 0.905		<i>p</i> = 0.783	
Allele frequency						
G	308 (92.8)	349 (90.9)	380 (89.2)	370 (88.9)	688 (90.8)	719 (89.9)
A	24 (7.2)	35 (9.1)	46 (10.8)	46 (11.1)	70 (9.2)	81 (10.1)
	<i>p</i> = 0.748		<i>p</i> = 0.913		<i>p</i> = 0.607	

Table 3 Age at disease onset and mode of disease onset in patients with ALS with and without the H63D polymorphism

	Birmingham			Ireland			Combined	
	Mean	SD	Range	Mean	SD	Range	Mean	SD
Age at onset (y)								
H63D genotypes								
Wild type (CC)	59.91	10.96	28–81	56.68	12.69	22–84	57.81	12.18
Non-wild type (GC, GG)	58.53	10.53	38–77	54.64	14.26	25–90	56.14	13.04
	$p = 0.493$			$p = 0.304$			$p = 0.249$	

	Birmingham		Ireland		Combined	
	Limb, n = 110	Bulbar, n = 56	Limb, n = 164	Bulbar, n = 49	Limb, n = 292	Bulbar, n = 111
Mode of onset	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
H63D genotypes						
Wild type (CC)	78 (70.9)	35 (62.5)	106 (64.6)	30 (61.2)	198 (67.8)	70 (63.1)
Non-wild type (GC, GG)	32 (29.1)	21 (37.5)	58 (35.4)	19 (38.8)	94 (32.2)	41 (36.9)
	$p = 0.294$		$p = 0.735$		$p = 0.409$	

of neurodegenerative disorders.⁸ Considering ALS, a previous report investigating the prevalence of C282Y and H63D polymorphisms in American patients with ALS and controls did not find any associations, but this study was limited by low numbers (51 patients, 47 normal controls) of ethnically diverse cases and controls.⁹ Another recent report, comprising 121 patients with ALS and 133 controls did report an association of the H63D polymorphism with ALS.⁴ However, there were difficulties with the control group used in this later study as it consisted of individuals with neuromuscular diseases other than ALS, and the control group allele frequencies were different from those previously reported.

Key issues to be addressed in any association study include the number of cases and controls investigated; the validity of the control population; the replication of the findings in an independent population and a consideration of whether the association are biologically plausible. In this study, we first investigated polymorphisms in the *HFE* gene in 166 patients with sporadic ALS and 192 control individuals from Birmingham and found differences in the H63D variant at the $p = 0.03$ level of significance. We then replicated our results in an independent Irish population, and the combined data set gives an OR for the H63D polymorphism of 1.85. Given the key role of iron in oxidative reactions, there is biologic plausibility for iron homeostasis proteins to have an influence in ALS.

It is not clear why the H63D polymorphism and not C282Y is linked to ALS in our study. The molecular mechanisms whereby either polymorphism leads to HH are not fully understood. The C282Y polymorphism seems to alter the interaction of HFE with $\beta 2$ microglobulin, with secondary loss in intracellular transport of HFE leading to decreased ex-

pression on the cell surface.³ H63D is thought to effect the formation of a salt bridge within HFE, altering binding to the transferrin receptor.³ Of possible relevance to this mutation and ALS, the mechanism for iron transport across the blood-brain barrier is thought to be via receptor-mediated endocytosis of the iron-transferrin complex by capillary endothelial cells.¹⁰

Our study, the first to show a significant association of *HFE* gene polymorphisms in ALS compared with appropriate controls in two populations, suggests that further investigation of iron homeostasis pathways in ALS pathogenesis is warranted. This may also have implications for the use of antioxidants as therapeutic agents in ALS, as patient responses may differ based on HFE genotype.

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NeuroImages

Figure. (A) CT scan shows voluminous frontal cyst. (B) Multiple hypodense lesions corresponding to fatty droplets (dashed arrows). (C) T1-weighted MRI shows hyperintense images in the Sylvian valley corresponding to fatty droplets due to dermoid cyst rupture in subarachnoid space (dashed arrows).

Chemical meningitis in reaction to subarachnoid fatty droplets

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A 50-year-old man with no medical history presented to our department with a month-long history of worsening headaches with fever. Clinical examination revealed hyperthermic meningeal syndrome. CSF examination found 900 white cells/mm³ including

95% neutrophils, hyperproteinorachia (125 mg/dL), and hypoglycorachia (1.6 mmol/L) with normoglycemia.

Encephalic CT scan and MRI revealed an aspect of ruptured frontosellar dermoid cyst, with evidence of fatty material within the subarachnoid space (figure). This condition can mimic septic meningitis and is a rare cause of recurrent puriform aseptic meningitis^{1,2}

The tumor was surgically removed and the patient was discharged without further problems.

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