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Study of the HFE gene common polymorphisms in French patients with sporadic amyotrophic lateral sclerosis

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ABSTRACT

Our objective was to investigate whether the C282Y (p.Cys 282 Tyr) and H63D (p. His 63 Asp) *HFE* polymorphisms were associated with sporadic amyotrophic lateral sclerosis (SALS) in the French population. We searched for a relation of *HFE* polymorphisms with the clinical characteristics of the disease.
The *HFE* polymorphisms were studied in 824 patients with SALS and 583 controls. We compared the frequency of the polymorphisms between SALS and controls groups by univariate and multivariate statistics, taking into account gender, site, age-at-onset and survival.
We did not observe significant difference in the frequency of H63D polymorphism between SALS and control group. We observed a significant difference for C282Y between patients and controls with a low frequency of the Y allele in patients (3.2%) compared to our control group (5.9%). Disease duration, distribution of gender, site-of-onset, age-at-onset did not differ between groups taking into account genotypes of each polymorphism.
Our results in this large cohort of ALS patients indicate that H63D polymorphism is not associated with SALS in the French population. This conclusion does not exclude a weak effect of the *HFE* gene polymorphisms in certain ALS populations, or an effect of other rare *HFE* gene variants.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is the most frequent adult-onset motor neuron disease, characterised by degeneration of both lower and upper motor neurons. This neurodegenerative disease is sporadic in 90% of the cases. Mutations of the *SOD1* gene have been reported in 10–15% of familial ALS (FALS) cases [1] and in 3–5% in sporadic cases [2]. Several other genes like angiogenin, *TARDBP* and *FUS* genes have also been found mutated in FALS [3–6] but only about 5% of all ALS cases can be explained by known genetic mutations.

The causes of the remaining forms of ALS are unknown. In sporadic ALS (SALS), several association studies have searched for possible genetic risk factors. *SMN1* gene copy numbers (one or three) are associated with ALS with an OR of 2.8 (95% CI: 1.8 to 4.4) [7, 8]. Genome-wide association studies identified two polymorphisms in the 9p21.2 locus which were associated with SALS (rs3849942 and rs2814707 with an OR of 1.22 (95% CI: 1.15–1.30)) [9] but this result was not replicated in another study [10] or only for rs3849942 with an OR of 2.16 (95% CI: 1.72–2.70) [11].

Iron homeostasis dysregulation has been regarded as an important mechanism in neurodegeneration and particularly in ALS [12]. The HFE protein plays an important role in iron homeostasis, by participating in the regulation of iron uptake. Two polymorphisms C282Y (p.Cys 282 Tyr, rs1800562) and H63D (p.His 63 Asp, rs1799945) of the *HFE* gene (6p22.2) have been associated with hereditary hemochromatosis of type 1 (OMIM 235200) [13]. To date, four studies showed a significant association between SALS and the D allele of H63D in several populations with different genetic background [14–17]. This association remains puzzling since not all studies observed it [18, 19] and since no clear functional role has been assigned to the H63D polymorphism. On the other side, the known functional polymorphism C282Y was not associated with ALS.

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We asked if this association was also observed in a large French ALS population. We also studied the possible relation of *HFE* polymorphisms with the clinical characteristics of the disease.

2. Methods

2.1. Subjects

Clinical data and blood samples were obtained from 824 patients with SALS and 447 controls. The diagnosis of sporadic ALS was made in one of the French ALS Study Group's centres. All patients met the El Escorial World Federation criteria for the diagnosis of definite or probable ALS [20]. For each patient, clinical data comprised gender, site-of-onset (limb or bulbar), age-at-onset and, if available, disease duration. The site-of-onset was defined as either bulbar or limb-onset. Bulbar-onset was defined as symptoms first occurring at the bulbar level with dysphagia, dysphonia or dysarthria. Limb-onset was defined as symptoms first occurring in the limbs. The age-at-onset was defined as the time at which first motor weakness was reported by the patient. The duration of ALS was defined as the interval between onset (first symptoms) and death or tracheotomy. Controls were matched for age, sex, and ethnicity. All participants gave written informed consent.

In order to enlarge the control population, we analysed also French controls from the literature. We used the review by Merryweather-Clarke et al. [21] to select five French series and pooled controls from these series.

2.2. Genotyping

DNA was extracted by standard procedures from peripheral blood. PCR reactions were carried out in a total of 50 µl containing 200 ng of genomic DNA, 10 pmol of each primers for C282Y or 20 pmol of each primers for H63D, 1.5 mM $MgCl_2$, 200 µM of dNTPs, and 1.75 U AmpliTaq polymerase (PROMEGA). The PCR conditions were 1 cycle at 92 °C for 5 min, 30 cycles at 92 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min, and 1 cycle at 72 °C for 7 min. Amplification primers were as follows : C282Y, forward primer 5′-TGGCAAGGGTAAACAGATCC-3′, reverse primer 5′-CTCAGGCACTCCTCTCAACC-3′; H63D, forward primer 5′-ACATGGTTAAGGCCTGTTGC-3′, reverse primer 5′-GCCACATCTGGCTTGAAATT-3′. The *HFE* polymorphisms were detected in PCR products by restriction enzyme digestion with Rsa1 for C282Y (ROCHE Diagnostics) and Mbo1 for H63D (PROMEGA France) [22]. Restriction reactions were carried out for 3 h at 37 °C using 30 µl of the PCR products and 15 U of Mbo1 or 12 U of Rsa1. The restriction fragments were run on a 2% resophor gel. The 387-bp PCR product digested with Rsa1 showed two fragments of 247- and 140-bp in normal DNA. The C282Y mutation created a new Rsa1 restriction site and the 387-bp PCR product digested showed three fragments of 247, 111 and 29 bp in mutated DNA. The 208-bp PCR product of normal DNA was cut into two fragments of 138 and 70 bp with Mbo1 digestion. The H63D mutation destroyed the Mbo1 site and the mutated DNA was not cut by the restriction enzyme.

2.3. Statistical analysis

Analysis of *HFE* polymorphisms was performed by an investigator blind to the disease status (ALS or control). Allele and genotype frequencies were counted and tested for Hardy–Weinberg equilibrium. A correction for multiple tests was applied to adjust the p values by accounting for the 2 loci (C282Y and H63D) analysed in the study. Differences were considered as significant when $p<0.025$.

We compared the frequencies of alleles and genotypes of the two polymorphisms in controls and patients using the chi^2 test. We also compared the frequency of the *HFE* polymorphisms in our controls group to the control group from literature using chi^2 test [21].

Table 1
Genotype and allele frequency data for the C282Y polymorphism.

	Controls		Patients	
	Frequency (%)	Numbers	Frequency (%)	Numbers
Genotype				
CC	88.4	395	93.7	772
CY	11.4	51	6.2	51
YY	0.2	1	0.1	1
Total	100	447	100	824
Allele				
C	94.1	841	96.8	1595
Y	5.9	53	3.2	53
Total	100	894	100	1648

Chi-square test for genotypes (grouping CY and YY): $\chi^2=10.927$; $p=0.001$ (odds ratio: 1.95 [IC 95%: 1.3–2.92]); chi-square test for alleles: $\chi^2=10.671$; $p=0.001$ (odds ratio: 1.89 [IC 95%: 1.29–2.81])).

Haplotypes of the two variants (C282Y and H63D) were calculated using a maximum likelihood algorithm [23, 24]. We also tested the distribution of the two polymorphisms according to the presence of the APOE ε4 allele [25], in all patients and in each gender group.

t-tests evaluated the influence of gender, site of onset and both polymorphisms on the age-of-onset. We evaluated the distribution of genders, site-of-onset and age-of-onset between D allele carriers and non carriers and between Y carriers and non carriers using chi^2 tests or t-tests. Kaplan Meier methods estimated survival curves. Comparison of survival data between groups defined by gender, site-of-onset, and *HFE* variants and according to age-of-onset was performed using the log-rank test or Cox analysis when appropriate. The different factors having a significant influence on survival were explored with multivariate Cox proportional hazard models. We included only patients without missing data in all statistical survival analyses and $p<0.05$ was considered as significant. The power of this study was sufficient to detect a difference of 5% between controls and patients, accepting an alpha risk of 5% and a beta risk of 20%. Statistical analysis was performed with JMP statistical software version 7.0.2 (SAS Institute, Cary, North Carolina).

3. Results

Gender, site-of-onset and age-at-onset were known for 758 patients (92%), female represented 41.4% (N = 314) of the SALS group and bulbar-onset was determined in 28.9% (N = 219). Median age-at-onset was 61.4 [20.1–89.1] years. The mean age-at-onset for bulbar-onset patients was significantly higher than the age-at-onset for limb-onset patients (mean: 64.4 +/− 11.0 years vs 58.1 +/− 12.9 years respectively; $p<0.0001$) and higher in female (61.9 +/− 12.1 years vs 58.4 +/− 12.9 years; $p=0.0002$).

Table 2
Genotypes and allele frequency data for the H63D polymorphism.

	Controls		Patients	
	Frequency (%)	Numbers	Frequency (%)	Numbers
Genotype				
HH	69.8	312	73.0	601
HD	27.3	122	25.2	208
DD	2.9	13	1.8	15
Total	100	447	100	824
Allele				
H	83.4	746	85.6	1410
D	16.6	148	14.4	238
Total	100	894	100	1648

Chi-square test for genotypes: $\chi^2=2.423$; $p=0.29$; chi-square test for alleles: $\chi^2=2.009$; $p=0.16$.