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COMMON POLYMORPHISMS OF *CHEMOKINE (C-X3-C MOTIF) RECEPTOR 1* GENE MODIFY AMYOTROPHIC LATERAL SCLEROSIS OUTCOME: A POPULATION-BASED STUDY

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Abstract

Introduction—In the brain, the *chemokine (C-X3-C motif) receptor 1 (CX3CR1)* gene is expressed only by microglia, where it acts as a key mediator of the neuron–microglia interactions. We assessed whether the 2 common polymorphisms of the *CX3CR1* gene (V249I and T280M) modify amyotrophic lateral sclerosis (ALS) phenotype.

Methods—The study included 755 ALS patients diagnosed in Piemonte between 2007 and 2012 and 369 age-matched and sex-matched controls, all genotyped with the same chips.

Results—Neither of the variants was associated with an increased risk of ALS. Patients with the V249I V/V genotype had a 6-month-shorter survival than those with I/I or V/I genotypes (dominant model, $P = 0.018$). The T280M genotype showed a significant difference among the 3 genotypes (additive model, $P = 0.036$). Cox multivariable analysis confirmed these findings.

Discussion—We found that common variants of the *CX3CR1* gene influence ALS survival. Our data provide further evidence for the role of neuroinflammation in ALS.

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Additional supporting information may be found in the online version of this article.

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Keywords

amyotrophic lateral sclerosis; *CX3CR1* gene; survival; microglia; neurodegeneration

The pathogenesis of amyotrophic lateral sclerosis (ALS) remains largely unknown.¹ Recently, neuroinflammation has come under increasing study, particularly with regard to the propagation of the disease. Microglia are a central protagonist of the neuroinflammatory component of neurodegeneration in ALS. There are indications that microglia have a dual effect on the course of ALS. Initially, microglia in the form of alternatively activated M2 cellular phenotype have neuroprotective functions, but, in the chronic phase, microglia have distinctly toxic functions (classically activated M1 phenotype).²

The *chemokine (C-X3-C motif) receptor 1 (CX3CR1)* gene, also known as *fractalkine receptor* (OMIM: 601470), is expressed by macrophage populations in systemic tissues,^{3,4} whereas it is expressed only by microglia in the brain,⁵ where it is a key mediator of the neuron–microglia interactions that are upregulated in many inflammatory conditions.^{6,7} The *CX3CR1* gene has 2 main functional variants (V249I, rs3732379 and T280M, rs3732378),^{8,9} both of which impair the activity of the CX3CR1 protein and have been associated with several inflammatory diseases, including multiple sclerosis,¹⁰ age-related macular degeneration,¹¹ and coronary artery disease.¹² Recently, it was reported that the p.V249I and p.T280M polymorphisms of the *CX3CR1* gene may modify ALS phenotype.¹³ A study of 187 ALS patients and 378 control participants recruited at an ALS clinic in Barcelona, Spain found that patients homozygous for valine at the 249 amino acid of *CX3CR1* (V249I V/V) had a 25-month-longer survival.¹³ The current study assesses whether the 2 common polymorphisms of the *CX3CR1* gene, V249I and T280M, alter the risk of developing ALS and/or modify phenotype in a large population-based series of ALS patients.

MATERIALS AND METHODS

This study includes ALS patients diagnosed in Piemonte, Italy, between 2007 and 2012 and healthy age-matched and gender-matched controls from the same geographic area. ALS patients were recruited through the Piemonte and Valle d'Aosta Register for ALS (PARALS), a prospective epidemiologic registry involving all the neurological departments of the 2 regions of Northern Italy. Epidemiological data regarding the 1995–2004 period have been published elsewhere.¹⁴ Both familial and apparently sporadic ALS patients were included in the present study. Patients with definite, probable, and probable laboratory-supported ALS by El Escorial revised criteria¹⁵ were included in the registry. The 369 control participants were identified through the patients' general practitioners (population-based controls).

Genotyping

ALS cases and controls were genotyped by using Illumina Infinium II HumanHap550 SNP chips (Illumina, San Diego, CA), which assayed 555,352 common single-nucleotide polymorphisms (SNPs) across the genome.¹⁶ Quality control parameters for genotype calling and filtering were as previously described.¹⁷ Genotype data for V249I (rs3732379;

build hg19, chr3:39307256C>T) and T280M (rs3732378; chr3:39307162G>A) were extracted from the larger data set. SNP genotypes were not confirmed on another platform, such as Sanger sequencing. However, the quality of genotyping has been assessed with polar and Cartesian cluster plots for SNPs V249I (rs3732378) and T280M (rs3732379). The quality control metric of genotyping accuracy (GenTrain score) for this SNP was 0.835, indicating a high level of precision in assigning genotypes to samples (Supporting Information Fig. 1). GenTrain score is a proprietary score instituted by Illumina to evaluate SNP calls. It ranges from 0.0 to 1.0 and, by convention, SNPs with scores greater than 0.4 are considered to be accurate. ALS patients were also tested for superoxide dismutase 1 (SOD1; all exons), TAR DNA binding protein (TARDBP; only exon 6 sequenced), fused in sarcoma (FUS; exons 14 and 15), angiogenin (ANG), and chromosome 9 open reading frame 72 (C9ORF72) by using methodology described elsewhere.¹⁸

Statistical Analysis

Two-tailed Fisher's exact test was used to evaluate the association between the polymorphisms and site of onset. Student's *t* test or ANOVA was used to assess the effect of polymorphisms on age at onset. Pooled odds ratios were calculated for the association between ALS and the 2 polymorphisms according to the dominant, recessive, heterozygote, homozygote, and allelic models. The effect of the 2 polymorphisms on survival was analyzed according to dominant, recessive, and additive models. Survival was calculated from onset to death, tracheostomy, or censoring date (December 31, 2015) with the Kaplan-Meier method and compared by using the log-rank test. No patients were lost to follow-up. Multivariable analysis was performed with the Cox proportional hazards model (stepwise backward), with a retention criterion of $P < 0.1$. Significance level was set at $P < 0.05$ after correction for multiple models. Reported *P* values are those after correction. Data were processed in SPSS version 22 (IBM, Armonk, NY).

Sample Size

Sample size and statistical power were calculated in Quanto software (<http://biostats.usc.edu/Quanto.html>).¹⁹ This study has *a priori* statistical power of 80% ($\alpha = 5\%$) for detecting a difference of 5 months in survival between groups of patients (main population survival, 33.5 ± 29.3 months; minor allele frequency, 0.25), and a β of 98% ($\alpha = 5\%$) for detecting a difference of 5 months.

Ethical Approval

The study was approved by the Città della Salute e della Scienza of Torino ethical committee. All patients and controls signed written informed consent. Databases were treated according to Italian privacy regulations.

RESULTS

This study included 755 ALS patients and 369 controls. The demographic and clinical characteristics of cases and controls are presented in Table 1, and the distribution of *CX3CR1* genotypes among cases and controls is shown in Table 2.

Risk of ALS and Age at Onset and Site of Onset

Neither V249I nor T280M variants were associated with increased risk of disease under any of the tested statistical models (Supporting Information Table 1). Age at onset and site of onset of ALS were not influenced by either the V249I variant or the T280M variant under any of the considered statistical models (Supporting Information Table 2).

CX3CR1 Genotypes and Survival

We found that survival was 6 months shorter in patients who were homozygous for valine at the 249 amino acid of *CX3CR1* (V249I VV genotype) compared with those with V249I I/I and V249I V/I genotypes (Fig. 1, Supporting Information Table 1). No differences in survival were observed in familial ALS patients ($P = 0.95$) or in patients with bulbar onset ($P = 0.96$). In contrast, there was an 11-month difference in survival among patients with spinal onset carrying the V249I V/V genotype (V249I V/V genotype median survival time 2.8 years, interquartile range [IQR] 1.8–4.7 years; V249I I/I and V249I V/I genotype median survival time 3.7 years, IQR 2.1–6.1 years; Fig. 2). The T280M genotype was associated with a significant difference in survival under the additive model (Fig. 3).

Because the presence of the minor allele for the 2 tested SNPs (249I and 280M) was associated with improved survival, we assessed the interaction between the V249I and the T280M genotypes. To do this, we subdivided the cases according to their genotypes into 2 groups. The first group consisted of patients that carried 3 or 4 minor alleles (V249I I/I and T280M M/M + V249I I/I and T280M T/M + V249I V/I and T280M M/M), and the second group consisted of patients with 2 or fewer minor alleles (V249I V/V and T280M T/T + V249I V/I and T280M T/M + V249I V/V and T280M M/M + V249I I/I and T280M T/T). The group of patients with 3 or 4 minor alleles had a significantly better survival (3 or 4 minor alleles median survival time 4.1 years, IQR 2.4–6.8 years; 2 or fewer minor alleles median survival time 2.7 years, IQR 1.8–4.6 years; Fig. 4).

Additional analysis revealed that this difference was present only in the subgroup of patients with spinal onset ($P = 0.027$).

In Cox multivariable analysis, the interaction between the 2 functional variants of the *CX3CR1* gene remained independently significant (the presence of 3 or 4 minor alleles has a hazard ratio of 0.69, with a 95% confidence interval of 0.46–0.93). The full model is presented in Supporting Information Table 2.

DISCUSSION

In this population-based cohort of Italian patients from the Piemonte and Valle d'Aosta ALS registry, we found that the V249I I variant of the *CX3CR1* gene increased survival by 6 months. This effect was limited to spinal onset patients. The T280M M functional variant of the same gene also significantly influenced survival under an additive genetic model. We also found an interaction between the 2 variants of the *CX3CR1* gene, with a significantly better survival in patients carrying 3 or 4 minor alleles. These findings were confirmed by Cox multivariable analysis. The tested variants did not differ in frequency between ALS patients and matched controls, indicating that the *CX3CR1* gene is not a risk factor for ALS.

Our findings directly contradict the findings of a smaller study involving 232 Spanish ALS patients that showed that the presence of the V249I I variant was associated with a 25-month-shorter survival.¹³ This disparity may be due to the population-based composition of our cohort, which is more representative of the whole ALS population. The highly selected nature of the clinic-based patients included in the Spanish study is demonstrated by the patients' young mean age at onset and the very long survival compared with the data from epidemiological studies on ALS.²⁰ In the case of *KIFAP3* gene polymorphisms, it has been clearly demonstrated in ALS that the use of a highly selected population may give erroneous conclusions about the outcome effect of gene polymorphisms.^{21–23}

Alternatively, it is possible that the observed discrepancy between the 2 studies indicates that the association of ALS with *CX3CR1* gene common polymorphism is not real. It is noteworthy that these SNPs did not show an effect on survival in ALS patients in a previous genome-wide association study (GWAS) involving over 4,000 patients, although the large number of variants that were tested in that study resulted in a more stringent *P*-value threshold for declaring genome-wide significance.²⁴ Although this is the most likely explanation, we do not want to dismiss prematurely the survival effect of *CX3CR1* functional polymorphisms because 6 months represents a substantial 20% change in survival in a disease associated with rapid progression and death. Our data suggest that this gene is worthy of additional study in even larger cohorts and also highlight the importance of appropriate selection of cases and controls in such studies.

Assuming that the effect of functional *CX3CR1* polymorphisms is not spurious, the modifying effect of the *CX3CR1* gene on ALS survival implicates microglia regulation in the pathological process underlying ALS⁴ and, more generally, in the neuroinflammatory component of motor neuron degeneration. The *CX3CR1* protein is a key element of the neuron–microglia interactions. It is notable that the 2 variants, V249I and T280M, affect functionality and activity of the protein through different mechanisms. The V249I I variant causes a reduced function through the reduction of the number of *CX3CR1* binding sites and its binding affinity on peripheral mononuclear cells,¹² whereas the 280M variant reduces cell-to-cell adhesion.^{8,12} However, other mechanisms related to systemic immunity in addition to microglia function can also be considered.^{3,4}

The disparity between our findings and those reported in *CX3CR1*^{−/−} knockout mice inbred with the SOD1G93A transgenic ALS model, which demonstrated a worsened disease outcome and increased microglial activation,⁶ is only apparent. Because of the dual effect of microglia in ALS,⁴ it is likely that, in the *CX3CR1*^{−/−} knockout mouse, the increased microglia activity worsens the disease, whereas, in the case of the V249I I variant in humans, only a reduction of function is observed, with a consequent modulating effect on microglia activity.

In the past few years, several studies of genetic modifiers of ALS phenotype or prognosis have been conducted. The rs12608932^{CC} genotype of the *UNC13A* gene has been demonstrated in several populations to be related to a shorter survival than the A/C and A/A genotypes.^{24–27} More recently, a large international meta-analysis of GWAS found 2 loci (at 10q23, SNP rs139550538 and at 1p36, rs2412208) in which the presence of the minor

alleles in the homozygous state were significantly associated with a reduction of survival of 7 and 4 months, respectively.²⁸ Also, the presence of at least 1 minor allele of the rs3011225 SNP located on chromosome 1p34.1 was associated with earlier age of onset in a meta-analysis of 13 GWAS cohorts.²⁹ Finally, the presence of an intermediate CAG repeat expansion in the *ATXN2* gene not only is a risk factor for ALS²⁴ but also is associated with a spinal phenotype and reduces survival by 1 year.^{30,31}

In summary, we used a large population-based cohort of ALS patients to show that functional variants of the *CX3CR1* gene potentially influence ALS survival. A limitation of our study is that it is based on a candidate-gene approach. Although this approach is less powerful than data-driven, genome-wide techniques, several attributes enhance the value of our data. First, this study was performed with an adequately powered cohort. Second, this cohort is population based, so it is representative of the more general ALS community. Third, the evaluated SNPs are known to alter significantly the function of the protein encoded by the gene. Our data suggest that functional modification of this gene influences survival in ALS patients and, by extension, implicate neuroinflammation as an important determinant of the rate of progression, perhaps opening new avenues for ALS treatment. Nevertheless, we recognize the disparate results compared with the previous study of this gene¹³ and recommend that even larger cohorts of appropriately collected ALS patients be studied. Large multicenter studies that are currently ongoing in Europe and the United States may help to clarify this issue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALS	amyotrophic lateral sclerosis
ANG	angiogenin
C9ORF72	chromosome 9 open reading frame 72
CX3CR1	chemokine (C-X3-C motif) receptor 1

FUS	fused in sarcoma
GWAS	genome-wide association study
IQR	interquartile range
PARALS	Piemonte and Valle d'Aosta Register for ALS
SNP	single nucleotide polymorphism
SOD1	superoxide dismutase 1
TARDBP	TAR DNA binding protein

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FIGURE 1.

Kaplan-Meier survival curves for *CX3CR1* V249I genotypes according to a dominant model ($P = 0.018$). Genotypes VI + II (368 cases; blue line) and genotype VV (387 cases; green line).

FIGURE 2.

Kaplan-Meier survival curves for *CX3CR1* V249I genotypes according to a dominant model in patients with spinal onset ($P = 0.016$). Genotypes VI + II (262 cases; blue line) and genotype VV (268 cases; green line).

FIGURE 3.

Kaplan-Meier survival curves for *CX3CR1* T280M genotypes according to an additive genetic model ($P = 0.036$, χ^2 for trend test). Genotype MM (22 cases; blue line), genotype TM (175 cases; green line), and genotype TT (558 cases; red line).

FIGURE 4.

Kaplan-Meier survival curves for *CX3CR1* assessing the interaction between V249I and T280M genotypes ($P = 0.008$). Three or 4 minor alleles (V249I II and T280M MM + V249I II and T280M TM + V249I VI and T280M MM; 42 cases; green line) and 2 or fewer minor alleles (V249I VV & T280M TT + V249I VI & T280M TM + V249I VV & T280M MM + V249I II & T280M TT; 713 cases; blue line).

Table 1

Demographic and clinical characteristics of cases and controls

Factor	ALS cases (<i>n</i> = 755)	Healthy controls (<i>n</i> = 369)	<i>P</i>
Mean age at onset (years, SD)	63.7 (11.1)	63.5 (10.9)	0.76
Women (%)	357 (47.3)	177 (48.0)	0.93
Site of onset bulbar (%)	226 (29.9)	na	na

ALS, amyotrophic lateral sclerosis; na, not applicable.

Table 2Genotypes of *CX3CR1* gene polymorphisms V249I and T280M in ALS cases and healthy controls

SNP	Genotype			Hardy-Weinberg (P)
V249I	V/V	V/I	I/I	
Cases [*] (n)	387	305	63	0.79
Controls [*] (n)	206	133	27	0.39
T280M	T/T	T/M	M/M	
Cases [†] (n)	558	175	22	0.07
Controls [†] (n)	281	77	8	0.19

SNP, single-nucleotide polymorphisms.

^{*} P = 0.295, cases vs. controls.[†] P = 0.561, cases vs. controls.