Reduced expression of the Kinesin-Associated Protein 3 (KIFAP3) gene increases survival in sporadic amyotrophic lateral sclerosis

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Reduced expression of the Kinesin-Associated Protein 3 (KIFAP3) gene increases survival in sporadic amyotrophic lateral sclerosis


1Department of Neurology, Emory University, Atlanta, GA 30322; Departments of 2Neurology and 3Medical Genetics, Rudolf Magnus Institute of Neurosciences, University Medical Center Utrecht, 3584 CX, Utrecht, The Netherlands; 4Howard Hughes Medical Institute, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139; 5Department of Medicine and Laboratory of Neuroscience, ‘Dino Ferrari’ Center, University of Milan Medical School—Istituto Di Ricovero e Cura a Carattere Scientifico Istituto Auxologico Italiano, 20149 Milan, Italy; 6Department of Molecular Biology and Genetics, Neurodegeneration Research Laboratory, Bologazi University Research, Istanbul, Turkey; 7Faculty of Medicine, Universidad Autonoma de San Luis Potosi, San Luis Potosi, Mexico S.L.P., CP 78210; 8Harvard NeuroDiscovery Center, Harvard Medical School, Boston, MA 02115; 9Neuropsychiatric Institute, University of California, Los Angeles; 10Department of Neurology, Beaumont Hospital, Dublin 9, Ireland; 11Medical Research Council Centre for Neurodegeneration Research, Department of Clinical Neuroscience, PO43, Institute of Psychiatry, King’s College London, London SE5 BAF, United Kingdom; 12Service de Neurologie, Centre Hospitalier Universitaire Bretonneau, 37044 Tours, France; 13Centre National de Genotypage, Institut Genomique, Commissariat à l’Energie Atomique, 91057 Evry, France; 14Unité de Recherche en Épidémiologie Nutritionnelle, l’UFR Santé Médecine et Biologie Humaine, 74 rue Marcel Cachin, 93017 Bobigny, France; and 15Center for Human Genetics Research, Massachusetts General Hospital, Richard B. Simchess Research Building, CPZN-6254, 185 Cambridge Street, Boston, MA 02114

Edited by David E. Housman, Massachusetts Institute of Technology, Cambridge, MA, and approved April 1, 2009 (received for review December 22, 2008)

Amyotrophic lateral sclerosis is a degenerative disorder of motor neurons that typically develops in the 6th decade and is uniformly fatal, usually within 5 years. To identify genetic variants associated with susceptibility and phenotypes in sporadic ALS, we performed a genome-wide SNP analysis in sporadic ALS cases and controls. A total of 288,357 SNPs were screened in a set of 1,821 sporadic ALS cases and 2,258 controls from the U.S. and Europe. Survival analysis was performed using 1,014 deceased sporadic cases. Top results for susceptibility were further screened in an independent sample set of 538 ALS cases and 556 controls. SNP rs1541160 within the KIFAP3 gene (encoding a kinesin-associated protein) yielded a genome-wide significant result ($P = 1.84 \times 10^{-8}$) that withstood Bonferroni correction for association with survival. Homozygosity for the favorable allele (CC) conferred a 14.0 months survival advantage.

Sequence, genotypic and functional analyses revealed that there is linkage disequilibrium between rs1541160 and SNP rs522444 within the KIFAP3 promoter and that the favorable alleles of rs1541160 and rs522444 correlate with reduced KIFAP3 expression. No SNPs were associated with risk of sporadic ALS, site of onset, or age of onset. We have identified a variant within the KIFAP3 gene that is associated with decreased KIFAP3 expression and increased survival. These findings support the view that genetic factors modify phenotypes in this disease and that cellular motor proteins are determinants of motor neuron viability.

genome-wide association study | single nucleotide polymorphism

Amyotrophic lateral sclerosis (ALS) is an age-dependent, degenerative disorder of motor neurons (1) that typically develops in the 6th decade and is uniformly fatal, usually within 5 years (2). Approximately 10% of ALS cases are dominantly inherited (3); 20% of these are caused by mutations in the gene encoding copper/zinc superoxide dismutase 1 (SOD1) (4); mutations in the TARDBP gene (5, 6) account for ~5% of cases. Rare familial cases arise from mutations in genes encoding the vesicle-associated membrane associated protein B (7), alsin (a RAB5-guanine nu-
variants modify susceptibility, survival, site of onset or age of onset in sporadic ALS, we have undertaken a multicenter genetic analysis of 1,821 sporadic ALS cases (SALS) and 2,258 controls.

**Results**

Genotypes were obtained from 3 sources in the U.S. (Boston, Atlanta, National Institute of Neurological Disorders and Stroke) and 3 in Europe (London, France, Netherlands), using Illumina BeadArrays. Survival information was not available for samples from the NINDS and France. No SNPs generated significant \( P \) values for association with susceptibility, site of onset, and age of onset of disease after Bonferroni correction (288,357 SNPs \( \times \) 4 phenotypes) (Fig. 1 C–E, Table 1, see also Table S1). In a further attempt to reveal any SNPs that were associated with susceptibility of sporadic ALS, we elected to genotype those SNPs that yielded a \( P < 5.0 \times 10^{-4} \) (153 in total) in a confirmatory (Stage 2) panel consisting of 538 ALS cases and 556 controls. Survival information was not available for most of the samples. Successful genotypes were obtained for 139 (90.8%) of the SNPs; none of the variants yielded a significant \( P \) value after Bonferroni multiple test correction (Table S1).

Although our study failed to confirm recent reports that susceptibility to sporadic ALS may be mediated by variants in the inositol-triphosphate receptor (ITPR2) (21), DPP6 (22, 23) or a novel, brain-expressed gene (FLJ10986) (24), these discrepancies may reflect differences in methodology or case populations (Table S2).

In contrast to susceptibility, site of onset, and age of onset, SNPs rs1541160 and rs855913 generated significant \( P \) values after Bonferroni correction (288,357 SNPs \( \times \) 4 phenotypes) for association with disease survival, using linear regression (Fig. 1A and Table 1).

For SNP rs1541160, the nominal and Bonferroni-corrected \( P \) values were 1.84 \( \times \) 10^{-8} and 0.021. Within the region of rs1541160, several SNPs (including imputed SNP alleles) yielded a cluster of positive values; 4 of the imputed SNPs were significant after Bonferroni correction (Fig. 1B). SNP rs1541160 maps within intron 8 of the KIFAP3 gene (encoding a kinesin-associated protein) on chromosome 1. For SNP rs855913, the nominal and Bonferroni-corrected \( P \) values were 4.02 \( \times \) 10^{-8} and 0.046. This SNP lies \( \sim \)10 kb upstream of the ZNF746 gene. This gene was not further characterized for 3 reasons. First, a sensitivity analysis of this SNP revealed that it does not replicate within the individual Boston population (\( P = 0.264 \)). Second, in our sensitivity analyses, had we analyzed the U.S. as the Stage 1 population, we would not have identified this variant due to its relatively high \( P \) value (0.0073) and low ranking (2169th). This is in contrast with SNP rs1541160 that emerges as significant in our study, whether considering the aggregate of all cases or each individual population. Finally, for the ZNF746 gene variant in question, the homozygotes for the minor allele are rare (0.7%) so that it is difficult to ascertain the reliability of the results (despite having >1,821 ALS cases in our screening study).

The genotype frequencies of rs1541160 are 9.9% (CC), 39.7% (CT) and 50.4% (TT); the minor allele frequency is 29.7% (Table S3). The rate of genotyping rs1541160 was 100%. Hardy–Weinberg testing revealed that rs1541160 is in equilibrium (controls \( P = 0.541 \), cases \( P = 0.527 \), all \( P = 0.970 \)). Haplotypes defined by 3 SNPs, rs2750014, rs4656729 and rs12123693, but excluding rs1541160, yielded association with survival comparable to that of rs1541160 (\( P = 1.35 \times 10^{-8} \)), indicating that genotyping artifacts specific to rs1541160 are not generating the association. Further tests confirmed that this association is not biased by population stratification (SI Methods). Pairwise linkage disequilibrium (LD) analysis for \( \sim \)50 SNPs showed no enrichment for linkage disequilibrium compared to the entire genome (Fig. 1C–E).

**Fig. 1.** Plot of \(-\log_{10}(P)\) for survival, age of onset, site of onset and susceptibility of sporadic ALS. Analysis for survival, age of onset, site of onset and susceptibility was performed for 288,357 SNPs and the results for the entire genome were plotted as shown in A and (C–E). The x axis represents the chromosomal position and the y axis represents the \(-\log_{10}\) of the \( P \) value for each SNP. The dotted line represents the cutoff for Bonferroni significance. (A) \( P \) values from linear regression analysis of survival. SNPs rs1541160 (circled) and rs855913 were significant after Bonferroni correction. (B) A closer view of the rs1541160 region is shown. Dark points represent SNPs typed in the study, and light points represent SNPs whose genotypes were imputed. (Lower) Imputation certainty for each imputed SNP, defined as the average maximum posterior genotype call probability. The chromosomal region spans 5 Mb on either side of SNP rs1541160. Positions are in National Center for Biotechnology Information build-35 coordinates.
SNPs distributed across the locus defined by KIFAP3 and 5 neighboring genes (SCYL3, Clorf156, Clorf112, and Selectins E and L) revealed disequilibrium that spanned \( \approx 155 \) kb from marker rs2750014 to rs2126443 but was centered on rs1541160 within the gene KIFAP3 (Fig. S1).

Our approach to identify variants associated with increased survival was based on a joint analysis of 4 DNA sets. This approach is more powerful than a 2-staged method in which a set of SNPs within an initial population below a cutoff \( P \) value is verified within a secondary confirmation population (36). However, because several genome-wide association studies (GWAS) have used 2-stage analyses (21, 22, 24, 30, 32, 33), we have investigated how such an approach would influence our results. We performed a sensitivity analysis in which we dropped each of the 4 populations in turn from this sensitivity testing (Table 2) revealed that rs1541160 remained a minimum of 20.51% (Netherlands) and a maximum of 78.54% (United States) survival was based on a joint analysis of 4 DNA sets. This approach approach, both the U.S. and Europe yielded a high ranking for rs1541160 if used as a Stage 1 population (20th and 31st, respectively). Furthermore, both the U.S. and Europe yielded significant individual \( P \) values (\( 5.65 \times 10^{-5} \) and \( 7.70 \times 10^{-5} \), respectively) (Table 2). These results also confirm that the observed association is not due to population stratification, which would not be expected to yield a significant \( P \) value for each individual population.

The absolute median survival for the CC, CT, and TT genotypes were 3.96, 2.84 and 2.67, respectively. The absolute mean survival for the CC, CT, and TT genotypes were 4.60, 3.40, and 3.07, respectively. As assessed by linear regression analysis, the mean and median survival increments for the CC genotype were 14.0 and 14.9 months, respectively, compared with the TT genotype, based on the analysis with SNP rs1541160 alone. With genotypic-based survival curve analysis using the Peto-Prentice generalized Wilcoxon text and deceased ALS cases (Fig. 2A), the \( P \) value for the rs1541160 SNP is \( 3.87 \times 10^{-5} \) (\( n = 1,014 \)). A censored analysis considering all of the cases (Fig. 2B) yielded a \( P \) value for rs1541160 of 1.82 \( \times 10^{-3} \) (\( n = 1,321 \)).

Sequence analysis of the KIFAP3 coding region and exon/intron boundaries of 8 individuals homozygous for the CC and 4 for the TT rs1541160 genotype (12 individuals) did not reveal variants in strong LD with rs1541160, suggesting that the KIFAP3-mediated increase in survival is not due to an alteration in its protein sequence. To determine whether the expression of KIFAP3 is modified by the genotype of rs1541160, we performed real-time PCR on lymphoblastoid cell lines harboring either a CC \((n = 38)\) or TT \((n = 40)\) genotype for rs1541160. KIFAP3 expression in the CC genotypes was 31.9% less than that in the TT genotypes (Fig. 3A) \((P = 0.0084, \text{Wilcoxon } 2\text{-sample test})\). A comparison of

<table>
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<th>Stage 2 population</th>
<th>Stage 1 ( P ) rank</th>
<th>Stage 1 sample size</th>
<th>Stage 2 ( P )</th>
<th>Stage 1 sample size</th>
<th>Stage 2 median Surv. CC</th>
<th>Stage 2 median Surv. TT</th>
<th>Survival increase (CC vs. TT)</th>
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occipital lobe brain samples homzygous for either the C (n = 9) or T (n = 17) alleles again revealed a decrease in expression of KIFAP3 (41.1%) in the CC as compared with TT samples (Fig. S2) (P = 0.025, Wilcoxon 2-sample test). A second real-time PCR probe for KIFAP3 confirmed the findings for both lymphoblast and brain samples. Western blotting of the brain samples confirmed a decrease in KIFAP3 protein (69.8%) in CC phoblast and brain samples. We further confirmed this high level of LD by subcloning the promoter region and 5' UTR (633 bp) were amplified from individuals harboring either the CC or TT genotype and subcloned upstream of the firefly luciferase gene. The constructs, which differ only at the variant position, were transfected into SKN-AS cells and relative luciferase activity was measured. The error bars represent the 95% C.I. A promotorless vector yielded <1% relative activity. The construct containing the G allele displays higher luciferase activity relative to the C allele. *
P < 0.05; ** P < 0.01.

Analysis of the KIFAP3 promoter using transcription element search system (TESS) (37) revealed that the KIFAP3 gene lacks a TATA box and that rs522444 lies within a putative Sp1 binding site (log-likelihood score = 12) (Fig. 3C). Moreover, the C allele of rs522444, which is in linkage disequilibrium with the lower expressing C allele of rs1541160, creates the putative Sp1 binding site, whereas the G allele at rs522444 destroys this site. Comparison with the promoter region in other primates demonstrates that the G allele is evolutionarily conserved suggesting this is the ancestral allele and that the KIFAP3 gene is not normally regulated by a Sp1 binding site. Because Sp1 family members binding to cognate Sp1 binding sites can both repress and activate gene expression (38), we sought to define the influence of variants of rs522444 on KIFAP3 promoter activity by subcloning the promoter region and 5' UTR of KIFAP3 upstream of the firefly luciferase gene. The constructs (Fig. 3D), differing only at the variant position, were transfected into the neuroblastoma cell line SKN-AS and the resulting luciferase activity was measured. This revealed that the promoter harboring the C allele for rs522444 displayed significantly decreased transcriptional activity (19.6%, P = 0.0046, Wilcoxon 2-sample test) (Fig. 3E). This result is analogous to the decreased expression of KIFAP3 in association with the rs1541160 C allele in both brain and lymphoblast tissues. We conclude that rs522444 C variant alone suffices to attenuate expression of the KIFAP3 gene.

Discussion
In a set of 4,079 DNA samples from sporadic ALS cases and controls we have completed an unbiased analysis of ~288,000 SNPs distributed across the genome and identified a SNP (rs1541160) in the KIFAP3 gene that is associated with reduced KIFAP3 expression and longer survival. Other SNPs in the region of
rs1541160 trended toward association (Fig. 1B); the 4th and 10th highest SNPs in the survival analysis were also within the KIFAP3 gene (Table S1). The failure to detect other significant SNPs may be subject to multiple interpretations. Perhaps most importantly, this suggests that there is not a single, readily detectable genetic variant that exerts a preponderant influence on either the risk of developing sporadic ALS or ALS phenotypes other than survival. In the present study, the absence of strongly associated SNPs other than rs1541160 may reflect other factors including inherent heterogeneity in the populations studied, locus and allelic heterogeneity, the inability of our present study design to detect underlying epistatic interactions of multiple gene variants, the effect of a microdeletions or insertions or inadequacies in the power of our study to detect genes of small effect. (For susceptibility studies, assuming conservatively a genome-wide significance level of 5.0 \times 10^{-8} and a minor allele frequency of 0.3, for genotypic relative risks of 1.3 and 1.5 the corresponding powers are 53% and 100%). (Table S4).

That heterozygosity for SNP rs1541160 confers survival variation of \( \sim 14 \) months is of clinical importance in a disorder with a mean survival of only 3–5 years. Why attenuation of KIFAP3 expression should be most pronounced in ALS is unclear. With the kinesin motor proteins KIF3A and KIF3B, KIFAP3 forms a trimeric motor complex, KIF3, that mediates binding between the motor proteins and their cargoes, serving multiple functions such as chromosomal cytokinesis and anterograde transport (39, 40). Presumably, reduced levels of KIFAP3 modulate survival by favorably affecting both the stoichiometry of KIFAP3 and the KIF3 complex and one or more transport functions, such that the CC genotype of rs522444 is beneficial. Heightened expression of KIFAP3 is reportedly an early marker of disease in transgenic mutant SOD1 mice (41), suggesting that levels of KIFAP3 reflect adverse events within motor neurons. In human sporadic ALS, expression of 2 other kinesin-related proteins (KIF3Ab and KIF1Bb) is reportedly reduced (42). A recent report documents that KIFAP3 (also described as KAP3) binds mutant SOD1 protein, slowing axonal transport of choline acetyltransferase in motor neurons; moreover, KIFAP3/KAP3 colocalizes with mutant SOD1 in human motor neurons at autopsy (43). It is conceivable that diminished expression of KIFAP3/KAP3 has a beneficial impact on the SOD1-motor protein interaction in ALS. More generally, mutations in motor proteins are implicated in multiple motor neuron degenerative disorders in both humans (11, 44) and mice (45).

It is encouraging that investigations employing complex genetics may provide fresh insight into sporadic ALS (21–24, 46). Few genetic factors that modify ALS survival are reported (19, 20, 47); none were identified in the previous ALS genome analyses (21–24, 46). The identification of KIFAP3 as a determinant of progression rate of sporadic ALS is therefore promising; insights into this pathway may provide new targets for therapies to slow this devastating disease, for example, by reducing levels of KIFAP3 expression or modifying its interactions with 1 or more protein binding partners.

Materials and Methods

Genotypes were obtained from 3 sources in the U.S. (917 ALS, 912 controls) and 3 in Europe (904 ALS, 1,346 controls) (Table S5). To maximize the power of this study, we combined these into a single set of cases and controls (36). Duration information was obtained from 4 of the sites. A set of 307,776 SNPs common to all sites was used for this analysis. Multiple quality control measures were applied to the set of DNAs and SNPs. 10,360 SNPs were eliminated because they were not in Hardy-Weinberg equilibrium (p < 10^{-4} in controls) or demonstrated call rates <0.95 or minor allele frequencies <0.001. An additional 9,059 SNPs were eliminated by tests for divergence of cases and control call rates and for nonrandom missing genotype data (to determine whether genotypes are missing with respect to the true genotype as defined by the observed genotypes of nearby SNPs). Samples were excluded if their call rates were <95%; if genotypes revealed duplicate samples, relatedness (proportion of genome IBD > 0.5), or excess homozygosity or heterozygosity (inbreeding coefficient >0.05 or deviation <0.025); or if samples were considered spurious because of the genotypically-assessed gender. The sample set was additionally subjected to stratification analysis; based on the distribution of pairwise genome-wide identity-by-state distances, we applied complete linkage hierarchical cluster analysis and classical multidimensional scaling. As a result, 72 outlier samples, defined as 3 standard deviations from the group mean, were eliminated, leaving the cases and controls as in Table S5. For the final analysis, 288,357 SNPs were evaluated (Table S6). After applying quality control metrics to the full set of DNAs and SNPs (SI Methods), 288,357 SNPs remained that were evaluated in 4,079 DNA samples, yielding 1,176,208,203 genotypes (Table S6). Genotypic and phenotypic data are available through the dbGaP database at the National Center for Biotechnology Information web site (www.ncbi.nlm.nih.gov/). Genotypes were used for the analysis of 4 SALS phenotypes: sustainability, site of onset, age of onset and survival of disease. Multiple analyses failed to detect biases introduced by stratification of our case-control cohorts (see SI Methods).

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