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Angiogenin Variants in Parkinson Disease and Amyotrophic Lateral Sclerosis

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Abstract

Objective—Several studies have suggested an increased frequency of variants in the gene encoding angiogenin (*ANG*) in patients with amyotrophic lateral sclerosis (ALS). Interestingly, a few ALS patients carrying *ANG* variants also showed signs of Parkinson disease (PD). Furthermore, relatives of ALS patients have an increased risk to develop PD, and the prevalence of concomitant motor neuron disease in PD is higher than expected based on chance occurrence. We therefore investigated whether *ANG* variants could predispose to both ALS and PD.

Methods—We reviewed all previous studies on *ANG* in ALS and performed sequence experiments on additional samples, which allowed us to analyze data from 6,471 ALS patients and 7,668 controls from 15 centers (13 from Europe and 2 from the USA). We sequenced DNA samples from 3,146 PD patients from 6 centers (5 from Europe and 1 from the USA). Statistical analysis was performed using the variable threshold test, and the Mantel-Haenszel procedure was used to estimate odds ratios.

Results—Analysis of sequence data from 17,258 individuals demonstrated a significantly higher frequency of *ANG* variants in both ALS and PD patients compared to control subjects ($p = 9.3 \times 10^{-6}$ for ALS and $p = 4.3 \times 10^{-5}$ for PD). The odds ratio for any *ANG* variant in patients versus controls was 9.2 for ALS and 6.7 for *PD*.

Interpretation—The data from this multicenter study demonstrate that there is a strong association between PD, ALS, and *ANG* variants. *ANG* is a genetic link between ALS and PD.

Amyotrophic lateral sclerosis (ALS), or Lou Gehrig disease, is a neurodegenerative disorder characterized by loss of motor neurons in the spinal cord and motor cortex. Patients typically present in their late 50s with progressive weakness, which can develop in any region of the body and eventually leads to respiratory failure and death within 3 years on average. The drug riluzole has been shown to slow disease progression, but to date there is no cure for this relentless disease.[1,2]

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Potential Conflicts of Interest

Nothing to report.

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

ALS is thought to be caused by both environmental and genetic factors. Although several twin studies have estimated the genetic contribution to the risk for ALS to be quite large (61%),[3] the genetic background remains poorly understood. Recently, genome-wide association studies have identified novel risk loci in *UNC13A* and on chromosome 9p.[4] Variants in several genes, including *SOD1, TARDBP, PON, VCP, OPTN,* and *FUS,* can be found in patients affected by the rare Mendelian form of ALS.[5–9]

There are several lines of evidence that suggest that angiogenic genes may be involved in ALS. Mice with a homozygous deletion in the promoter region of the gene encoding vascular endothelial growth factor (*VEGF*) develop an ALS-like phenotype. Subsequently, an association between genetic variation in the *VEGF* promoter was demonstrated in human ALS patients (although this association was not confirmed in a later meta-analysis).[10–12] This prompted a study on the gene encoding angiogenin (*ANG*) as a functional candidate gene, which demonstrated multiple variants in a large cohort of ALS patients.[13,14] However, follow-up studies identified variants not only in ALS patients, but also in controls. The association between ALS and *ANG* variants therefore remains somewhat unclear, as many studies were not large enough to unequivocally differentiate between benign polymorphisms and disease-associated variants.[15]

Interestingly, several ALS patients carrying *ANG* variants also demonstrated signs of Parkinson disease (PD).[16,17] This is an intriguing observation, as there are several reports describing patients affected by both diseases,[16–21] and epidemiological studies have shown that relatives of ALS patients are at increased risk of developing PD.[22,23] It has therefore been suggested that PD and ALS may share genetic risk factors. Indeed, recent studies have demonstrated expanded *ATXN2* repeats and mutations in *TARDBP* in both ALS and PD.[24–26]

We hypothesized that, in addition to ALS, variants in *ANG* could predispose to PD as well. The aim of this international collaborative study was to explore the hypothesis that variants in *ANG* predispose to both ALS and PD. In total, we analyzed data from 3,146 PD patients, 6,471 ALS patients, and 7,668 control subjects from multiple centers from the USA and Europe.

Subjects and Methods

Study Population

We identified and reviewed all previous studies on *ANG* in ALS by performing a systematic search according to the MOOSE guidelines.[27] A search was performed in the MEDLINE, EMBASE, CINAHL, and Cochrane databases up to March 2011. The search string consisted of a combination of Medical Subject Headings and text words. The search terms for ALS included “motor neurone disease,” “amyotrophic lateral sclerosis,” “progressive spinal muscular atrophy,” “motor neuropathy,” and related synonyms. These were combined with search terms for studies on *ANG* and included “ANG,” “angiogenin,” “candidate gene study,” “gene,” and related synonyms. In total, we identified 10 studies performed in populations from Ireland, Scotland, the United Kingdom, the USA, Sweden, France, Germany, and Italy.[13,14,17,28–34]

We additionally sequenced 310 ALS patients and 487 control subjects from Belgium collected by the University Hospital of Leuven; 941 ALS patients, 947 PD patients (of whom 224 had a positive family history), and 1,582 control subjects from the Netherlands collected by the Academic Medical Center Amsterdam, Leiden University Medical Center, University Medical Center Utrecht, and Radboud University Nijmegen Medical Center; 277 ALS patients and 100 controls from Sweden collected by Umeå University Hospital; 820 PD patients (of whom 76 had a positive family history) and 274 controls from Germany collected by the University of Tübingen, University of Ulm, and University of Lübeck; 916 PD patients and 918 control subjects from Italy collected by the Parkinson Institute of Milan; and 464 PD patients and 454 control subjects collected by the University of Massachusetts Medical School. In total, 8,489 subjects were successfully sequenced in this study.

All ALS patients included in this study were diagnosed according to the 1994 El Escorial criteria. PD patients were diagnosed according to the UK Brain Bank criteria. We excluded all familial ALS with known mutations in *SOD1, FUS,* and *TARDBP*. Familial PD patients with mutations in *Parkin*, *LRRK2, DJ-1,* and *PINK1* were excluded from the study. Controls were spouses of patients, healthy volunteers, and participants from a population-based study on ALS or from prospective cohort studies. All participants gave written informed consent, and approval was obtained from the local, relevant ethical committees for medical research. Baseline characteristics for the study population are provided in Table 1, and additional information is available in the Supplementary Material.

Genotyping Methods

To ensure the comparability of our data to the data from the previous studies, we obtained the raw sequence data from the previously published studies. These data were reanalyzed, and we further checked whether the primers used in the previous studies indeed captured the entire gene by using the BLAT alignment tool in the University of California at Santa Cruz genome browser (http://genome.ucsc.edu/). In all studies, the entire gene was sequenced, and all studies reported high rates of successful genotyping (>95%). This is not surprising, as *ANG* is a small gene consisting of a single coding exon made up of ~470bp. All studies were performed between 2004 and 2011 and included patients diagnosed according to the El Escorial criteria for ALS. All studies only reported subjects who were successfully genotyped. The data from the previous studies were therefore complete and comparable to our own data.

Sequencing experiments were carried out at 2 sites. DNA samples from subjects from the Netherlands, Belgium, Sweden, and Germany were sequenced at the University Medical Center Utrecht, the Netherlands. Sequencing was performed on the single coding exon of *ANG* (NM_001097577), using a 96-capillary DNA Analyzer 3730XL (Applied Biosystems, Foster City, CA) and BigDye Terminator 3.1 chemistry as described previously. At the University Medical Center Utrecht, the following primers used in this study: ANG-1-F, GTTCTTGG GTCTACCACACC and ANG-1-R, AATGGAAGGCAAGGA CAGC. The sequences were aligned using the Phred/Phrap/Consed package, and variants were identified using the software application PolyPhred.

For Italian and US samples, amplification was performed using the following M13-tailed primers: ANGex2-M13F, AGTAAAACGACGGCCAGTTGTTCTTGGGTCTACCA CACC-3 and ANGex2-M13R, GCAGGAAACAGCTAT GACCATGTTGCCACCACTGTTCTG-3. The products were sequenced at Beckman Coulter Genomics (Waltham, MA). The sequences were aligned using the Phred/Phrap/ Consed package, and variants were identified using the software application PolyPhred. At both sites, each plate contained a positive control and a dummy, to monitor genotyping quality. Genotyping was successful for >95% of samples at both sites. We only included samples that were successfully genotyped in this study. When a variant was identified, this was confirmed by independent experiments using newly prepared samples from stock DNA.

Statistical Methods

Tremendous progress in our understanding of the genetics of human disease has been made over the past few years, thanks to projects such as the human genome project, the international HapMap study, and genome-wide association studies. These studies have demonstrated that common genetic polymorphisms confer modest risk for many common diseases (odds ratios [ORs] typically <1.5).[35] Despite the hundreds of novel associations identified by the genome-wide association studies, they only explain a fraction of the heritability of most conditions. It has therefore been hypothesized that the missing heritability (the fraction of the genetic risk for a disease that remains to be accounted for) can be found in rare genetic variation, which is defined as variants with a frequency <1.0% in the general population.[36]

Performing association studies dealing with rare genetic variation poses several statistical challenges. First, the low frequency at which these variants are found makes it impossible to test each variant individually, as statistical power is not sufficient. To overcome this problem, so-called burden tests are performed, in which the total number of variants in a gene in patients is compared to the total number of variants observed in controls.[37]

A second issue is that not all variants in a gene are equally relevant. Some variants may severely affect protein structure and function, whereas others may be essentially neutral. In a burden test, it is therefore possible that neutral variants dilute the signal from disease-associated variants. Many strategies have been proposed to overcome this problem, such as (1) only including variants exclusively observed in either patients or controls, (2) weighting variants inversely to their frequency (which is based on the assumption that rarer alleles are more likely to be pathogenic than common alleles),[38] or (3) setting a fixed frequency for inclusion (eg, only variants found in 0.5% of the population or less).[37] Large scale studies dealing with rare variants are still relatively novel, and to date there is no consensus on which strategy is most appropriate. Considering the possibility that we would encounter many rare variants, we decided to use the test with the best statistical power as the primary outcome measure. A recent paper demonstrated this to be the variable-threshold test.[39] To ensure that the detected associations are indeed robust, we compared the frequency of variants in controls in our own data set to the previously published studies, analyzed the data using the aforementioned different methods, and performed the analyses considering only

our own data set (excluding the previous studies) as well as only considering the familial ALS and familial PD cases (Supplementary Tables 5–8).

In the variable threshold test, an algorithm is applied that empirically derives a frequency threshold for inclusion of variants based on the actual data of a study. The algorithm was developed using large population genetic simulations based on empirical sequencing data that analyzed the relationship between phenotypic effect and allele frequency of a variant within an evolutionary model that incorporates purifying selection. Simply put, the algorithm computes a frequency threshold for inclusion of variants. All variants with a frequency above this threshold in the study population are excluded from the analysis.[39]

Significance was computed through extensive permutation testing (100,000,000 permutations) with case–control labels shuffled among individuals of the same country, which directly protects against false positives due to heterogeneity between countries. We further minimized the risk of population stratification by ensuring that all patients and controls in this study were Caucasian individuals of European ancestry. For the statistical analyses on ALS, we combined the data from the previous studies with data from our sequencing experiments. Statistical analyses for ALS and PD were performed separately. We only included data from a population when data for both cases and controls were available. Therefore, for the analyses in ALS we included data from ALS patients and controls from the Netherlands, Ireland, Scotland, the United Kingdom, the USA, Belgium, Sweden, Germany, France, and Italy. PD samples were available from the Netherlands, Germany, Italy, and the USA. For the statistical analyses in PD, we therefore only included the control samples from the Netherlands, Germany, Italy, and the USA. The control samples from Ireland, Scotland, the United Kingdom, Sweden, and France were not included in the statistical analyses for PD, which explains the difference in the number of controls for the ALS and PD analyses. Analyses were performed using the statistical analysis program R (CRAN; http://www.R-project.org). As an effect estimate, we computed the Mantel-Haenzsel OR. Additionally, we used different protein prediction algorithms (Polyphen-2, Panther, and SIFT) to predict the possible effect of the identified variants on protein function.

Results

Our search identified 10 previous studies on *ANG* in ALS, in which 4,943 ALS patients (of whom 465 had a positive family history) and 3,853 control subjects have been sequenced. We additionally sequenced 1,528 ALS patients, 3,146 PD patients, and 3,815 control subjects (total, 8,489 subjects). This allowed us to analyze sequence data on a total 3,146 PD patients, 6,471 ALS patients, and 7,668 control subjects (total, 17,258 individuals). An overview of the identified variants is shown in Table 2 and in more detail in Supplementary Tables 1–4.

In total 29 unique, nonsynonymous variants were identified. Two variants (K17I and I46V) were observed in all populations in cases and controls at comparable frequencies, suggesting that these are likely to be neutral alleles and should be considered to be polymorphisms. The

variable threshold test algorithm indeed eliminated both the K17I and I46V variants from the analysis.

After exclusion of K17I and I46V, *ANG* variants were found in 0.46% of ALS patients and 0.45% of PD patients, compared to 0.04% of control subjects. This difference in variant frequency is statistically significant, with $p = 9.3 \times 10^{-6}$ for ALS and $p = 4.3 \times 10^{-5}$ for PD. The OR for any *ANG* variant in patients versus controls was 9.22 (95% confidence interval [CI], 3.05–27.89) for ALS and 6.74 (95% CI, 2.10–21.68) for PD (Table 3).

The different protein prediction programs were able to make predictions for 19 variants, of which 13 were probably or possibly damaging to the function of *ANG* (Supplementary Table 9).

We next analyzed the clinical characteristics of the patients carrying *ANG* variants to see whether these patients demonstrated a distinct phenotype. *ANG* variants were not associated with a younger age of onset in PD or ALS.

For ALS patients carrying *ANG* variants, we observed a wide range in age of onset and survival, variable involvement of upper and lower motor neurons, and both bulbar and spinal onset. The PD patients with unique nonsynonymous *ANG* variants were clinically indistinguishable from those without, in terms of onset age, rate of positive family history, and disease features. Please note we only included patients with idiopathic PD according to established criteria. We can therefore only conclude that *ANG* variants contribute to susceptibility to classic PD. PD patients with atypical features were not studied.

An overview of the phenotypic characteristics of all patients carrying *ANG* variants (both previously published and those identified by this study) is provided in Supplementary Table 10–12. In general, it appears that there is no specific phenotype associated with *ANG* variants.

Discussion

The results of our analysis indicate that there is a clear association between *ANG* variants and PD and between *ANG* variants and ALS. *ANG* variants are a susceptibility factor for both diseases, and the risk conferred by these variants is considerable (PD: OR, 6.72; ALS: OR, 9.22).

ANG variants were identified in 0.45% of PD patients and 0.46% of ALS patients. Therefore, although *ANG* variants were identified in only a small percentage of PD and ALS patients, it seems that these variants are highly relevant to those patients carrying them.

Despite the relatively low frequency at which these variants were identified, we consider our findings to be very relevant at a population level, when one considers the large number of people affected by PD. PD is the second most common neurodegenerative disorder after Alzheimer disease and affects 1 to 2% of people older than 65 years. It has been estimated that approximately 6,000,000 people suffer from PD worldwide, and there are ~500,000 PD patients in the USA alone.[34] The prevalence of ALS is lower in comparison to PD. However,

nearly 6,000 people are newly diagnosed with ALS each in year in the USA.[2] Moreover, the incidence of both diseases is rising, as life expectancy in developed countries continues to rise. *ANG* variants may therefore be relevant to thousands of ALS and PD patients.

In this study, we show that variants in a single gene predispose to multiple neurodegenerative disorders. This phenomenon is an emerging theme in neurodegeneration. For instance, it has been shown that genetic variation in microtubule-associated protein tau (*MAPT*) is associated with PD, frontotemporal dementia (FTD), progressive supranuclear palsy, and corticobasal degeneration.[40–42] Recently, a large collaborative study showed that variation in the gene for Gaucher disease, the lysosomal enzyme glucocerebrosidase (*GBA*), is also associated with PD.[43] Interestingly, it has been recently shown that expanded *ATXN2* repeats and mutations in *TARDBP* can be seen in both ALS and PD.[7,24–26]

It could be speculated that cells carrying mutant ANG are more susceptible to degeneration in general and that the selective degeneration or the progression of disease is determined by additional genetic and environmental factors. Several ALS patients carrying *ANG* variants also demonstrated cognitive impairment suggestive of FTD. It would therefore be highly interesting to sequence *ANG* in patients with different forms of dementia.[17,32]

Although the identification of a novel genetic risk factor for PD is a substantial step forward in the study of this relentless disease, the ultimate goal remains to understand the pathophysiological mechanism to develop better treatment. *ANG* (chromosome 14q11) encodes a 123-residue (14.1kDa) protein, which is synthesized with a signal peptide of 24 amino acids that is cleaved to form the mature protein. *ANG* is thought to be involved in RNA metabolism, neovascularization, neurite outgrowth, and axonal path-finding, and is a neuroprotective factor.[44] Several of these functions of *ANG* are of particular interest.

First, the RNA processing function of ANG could be relevant, as recent studies have shown that variants in *FUS* and *TARDBP*[5] cause ALS and that both genes are involved in RNA processing, which could thus be a common pathway.

Second and perhaps most interesting are the potent neuroprotective qualities of ANG, which are lost when the gene is mutated.[34,43,44] It has been shown in in vitro models of ALS (using cells containing *SOD1* variants known to cause ALS) that wild-type ANG reduces neuronal death considerably.[45] Furthermore, it has been shown that cell death is promoted when wild-type ANG is silenced by siRNA.[45] Several studies have shown that motor neurons containing *ANG* variants show increased rates of apoptosis when challenged (for instance with hypoxia) and that these cells can effectively be rescued by administering wild-type ANG.[44,46,47] Mice carrying human mutant *SOD1* develop an ALS phenotype. When these mice are treated with wild-type ANG, the onset of weakness is significantly later and survival is longer.[45]

Studies using motor neurons have provided evidence suggesting that the neuroprotective effect of ANG is due to inhibition of apoptosis via activation of the phosphatidylinositol 3-phosphate (PI3K)-Akt signaling pathway.[45]

Variants and multiplication in the gene encoding alpha synuclein (*SCNA*) are known to cause PD, and alpha synuclein is found in abnormal protein aggregates in the substantia nigra of PD patients.[48,49] A highly interesting microarray study using mice overexpressing human *SCNA* found modest alterations in the expression of approximately 200 genes, but dramatic changes for a single gene, mouse angiogenin-1 (*mAng1*), for which a 7.5-fold reduction was seen compared to wild-type littermates.[45] Additional experiments using dopaminergic cells overexpressing human alpha synuclein confirm reduced levels of ANG in these cells. Furthermore, dopaminergic cells treated with wild-type ANG show reduced cell death when challenged with either rotenone or 1-methyl-4-phenylpyridinium.[50] The protective effect of ANG in the dopaminergic cell lines appears to be mediated through inhibition of apoptosis via the PI3K-Akt signaling pathway as well.[50]

To date, all studied *ANG* variants have been shown to result in a loss of function, including the neuroprotective effects.[34,45,47] It could therefore be that individuals carrying *ANG* variants cannot active the PI3K/Akt pathway, and that this renders their neurons more susceptible to apoptosis by activation of caspase-3. This puts forward the intriguing option of using wild-type ANG as a potential treatment strategy in patients carrying *ANG* variants.

An interesting observation is that ANG can also rescue cells from apoptosis in in vitro and in vivo models of ALS and PD that are not based on mutant *ANG* (ALS: *SOD1* and PD: *SCNA*). This may suggest that treatment with wild-type ANG could perhaps be a consideration in all ALS and PD patients.

In short, we have identified a novel risk gene for PD and firmly establish that *ANG* is involved in the pathogenesis of ALS. We demonstrate that variants in *ANG* confer a large risk for both PD and ALS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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TABLE 1

Baseline Data for the Study Groups, according to Center

Center	Subjects, No.	Positive Family History, No. (%)	Male/Female, No. (%)	Mean Age, yr
Ireland13				
ALS patients	169	15 (8.9)	98/71 (58/42)	56
Controls	171	—	120/51 (70/30)	41
Scotland14				
ALS patients	398	34 (8.5)	229/169 (58/42)	56
Controls	299	—	151/148 (51/49)	48
USA (Boston)14				
ALS patients	360	83 (23.1)	205/155 (57/43)	55
Controls	219	—	74/140 (34/66)	54
Sweden14				
ALS patients	434	100 (23.0)	238/105 (55/45)	63
Controls	309	—	162/147 (52/48)	66
Ireland14				
ALS patients	293	31 (10.6)	163/128 (56/44)	57
Controls	339	—	217/122 (64/36)	44
UK14				
ALS patients	144	11 (7.6)	91/53 (63/37)	60
Controls	98	—	30/68 (31/69)	58
USA (Boston)34				
ALS patients	298	0	—	—
Italy (south)28				
ALS patients	163	8 (4.9)	84/79 (52/48)	55
Controls	332	—	195/137 (59/41)	50
Italy (north)29				
ALS patients	227	12 (4.4)	136/91 (60/40)	56
Controls	636	—	382/254 (60/40)	—
Italy (Milan/Pisa)30				
ALS patients	210	0	—	—
Controls	230	—	—	—
Italy (Milan)32				
ALS patients	737	132 (17.9)	543/194 (74/26)	51
Controls	515	—	376/139	52
France33				
ALS patients	855	0	—	—
Controls	234	—	—	—
The Netherlands				
ALS patients	980	39 (4.0)	555/386 (59/41)	59

Center	Subjects, No.	Positive Family History, No. (%)	Male/Female, No. (%)	Mean Age, yr
PD patients	947	224 (23.7)	578/369 (61/39)	52
Controls	1,582	—	933/649 (59/41)	60
Belgium				
ALS patients	310	0	183/123 (59/41)	59
Controls	487	—	283/204 (58/42)	51
Sweden				
ALS patients	277	0	158/119 (57/43)	60
Controls	100	—	52/48 (52/48)	62
Germany31				
ALS patients	581	0	—	—
PD patients	820	76 (9.3)	492/328 (60/40)	49
Controls	890	—	516/374 (58/42)	51
Italy (Milan)				
PD patients	916	0	550/366 (60/40)	56
Controls	918	—	321/597 (35/65)	63
USA (Boston)				
PD patients	464	0	288/176 (62/38)	56
Controls	454	—	381/73 (84/16)	63

ALS = amyotrophic lateral sclerosis; PD = Parkinson disease;

TABLE 2

Nonsynonymous Variants in *ANG*

Variant	PD, No.	ALS, No.	Controls, No.
M (−24)I	3	2	0
F (−13)L	0	1	0
F (−13)S	0	1	0
V (−12)A	1	0	0
G (−10)D	0	1	0
G (−8)D	1	0	0
P (−4)Q	0	1	0
P (−4)S	4	2	2
Q12L	0	2	0
H13R	1	0	0
K17E	0	2	0
D22V	1	0	0
S28N	0	1	0
R31K	0	1	0
C39W	0	2	0
K40I	0	6	0
K54E	0	1	0
K54R	1	0	0
N63L	0	0	1
T80S	0	1	0
R95Q	1	0	0
F100I	0	1	0
P112L	0	1	0
V113I	0	3	0
H114R	0	1	0
R121C	1	0	0
R121H	0	1	0
Total variants	14	31	3
Total samples	3,146	6,471	7,668
Samples with variants	0.45%	0.48%	0.04%

None of these variants was observed in the pilot data from the 1,000 Genomes Project (http://www.1000genomes.org/).

TABLE 3

Results from Statistical Analysis

Disease	Variants, No. (%)	Patients, No.	Variants, No. (%)	Controls, No.	*p*	Odds Ratio [95% CI]
ALS	31 (0.48)	6,471	3 (0.04)	7,668	9.3×10^{-6}	9.22 (3.05–27.89)
PD	14 (0.45)	3,146	3 (0.05)	5,631	4.3×10^{-5}	6.74 (2.10–21.68)

Exact *p* values were computed by permutation testing, randomizing case–control status of individuals of a single country (100,000,000 permutations were performed). All *p* values are 1-sided, testing the specific hypothesis that the presence of rare variants increases risk of ALS or PD. For the analyses in PD, we included control subjects only from countries from which PD cases were available.

ALS = amyotrophic lateral sclerosis; CI = confidence interval; PD = Parkinson disease;