

Mutations of the ANG Gene in French Patients With Sporadic Amyotrophic Lateral Sclerosis

Agathe Paubel, MD; Jeremy Violette, MD; Maité Amy, PhD; Julien Praline, MD; Vincent Meininger, MD, PhD; William Camu, MD, PhD; Philippe Corcia, MD, PhD; Christian R. Andres, MD, PhD; Patrick Vourc'h, PhD; for the French Amyotrophic Lateral Sclerosis (ALS) Study Group

Background: Mutations in the angiogenin gene, ANG, have been associated recently with familial and sporadic forms of amyotrophic lateral sclerosis (ALS). However, the cellular and molecular mechanisms that link ANG, a multidomain protein, to ALS are still unknown.

Objective: To assess the frequency of ANG gene mutations in 855 French patients with sporadic ALS.

Design: We analyzed by direct sequencing the full coding region of the ANG gene in a cohort of French patients with sporadic ALS. The clinical characteristics of patients carrying ANG mutations are detailed.

Setting: French ALS Study Group.

Patients: A total of 855 patients with sporadic ALS.

Main Outcome Measures: Results of genetic analyses.

Results: We observed a previously identified mutation (pI46V) in 2 patients with ALS without a known family link and found a novel mutation (pR121H) in 1 patient who developed ALS with rapid progression. We did not observe an association between patients with ALS and the rs11701 polymorphism, as previously reported in certain ALS populations of other ethnic origins.

Conclusion: Overall, our findings support the implication of ANG gene mutations as a rare but widespread cause of ALS.

Arch Neurol. 2008;65(10):1333-1336

Author Affiliations: Laboratoire de Biochimie et Biologie Moléculaire (Drs Paubel, Violette, Andres, and Vourc'h) and Centre SLA (Drs Praline and Corcia), CHRU de Tours, and INSERM U930, Université François-Rabelais (Dr Amy, Praline, Corcia, Andres, and Vourc'h), Tours, Fédération des Maladies du Système Nerveux, Centre référent SLA (Dr Meininger), Paris, and Centre SLA, CHRU de Montpellier (Dr Camu), Montpellier, France.
Group Information: The French ALS Study Group members are listed at the end of this article.

SEVERAL STUDIES¹⁻⁴ ON THE VASCULAR endothelial growth factor gene have suggested a link between angiogenesis and the pathogenesis of amyotrophic lateral sclerosis (ALS). Recently, Greenway et al⁵ identified 7 missense mutations in a second hypoxia responsive gene, the ANG gene (OMIM 105850), in 15 Irish and Scottish patients with familial ALS or sporadic ALS. The ANG gene, located in 14q11.2, encodes angiogenin, a 14.1-kDa enzyme that belongs to the pancreatic ribonuclease A superfamily.⁶ Angiogenin is synthesized as a preprotein that contains a signal peptide of 24 amino acids. The mature angiogenin protein contains a putative receptor binding region, a domain implicated in immunomodulation, a nuclear localization sequence, and a catalytic site responsible for a low ribonuclease activity. It is expressed in several cells, including glial cells and motoneurons in the spinal cord and dorsal root ganglia.⁷ Angiogenin, whose expression is induced by hypoxia, mediates neovascularization and has been recently involved

in neurite pathfinding.^{8,9} Thus, we analyzed the coding region of the ANG gene in a large French cohort of 855 patients with sporadic ALS.

METHODS

Clinical data and blood samples were obtained from 855 patients with sporadic ALS. The ALS was diagnosed according to the El Escorial World Federation criteria by physicians in the French ALS Study Group centers. Clinical data comprised the sex, site of onset, age at onset, and duration of ALS. Control subjects were spouses of patients, who were matched with patients by age and sex. All patients and controls gave written informed consent.

DNA was extracted from peripheral blood samples using a standard procedure. The unique exon of the ANG gene was amplified by polymerase chain reaction using the following forward and reverse primers: 5'-tgttctgggtctacacacc-3' and 5'-aatggaaggcaaggacagc-3', respectively. Bidirectional sequencing (ABI 3130xl sequencer; Applied Biosystems, Courtaboeuf, France) and analysis (Seqscape software; Applied Biosystems) were then performed. Muta-

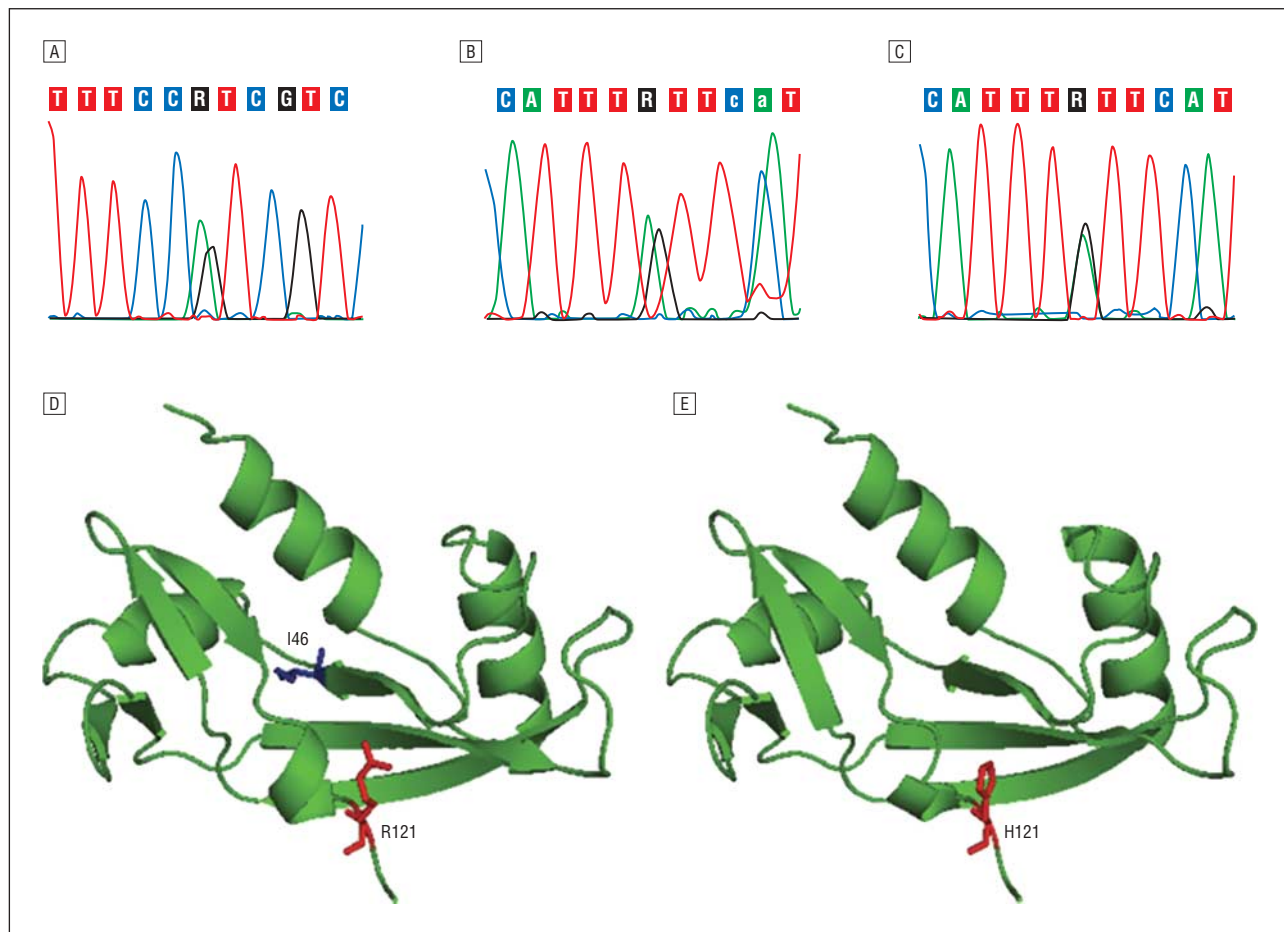


Figure. *ANG* mutations and 3-dimensional structure of angiogenin. DNA sequences of the patients with amyotrophic lateral sclerosis with G434A (A) and A208G (B and C) mutations. Three-dimensional x-ray diffraction structure of the normal human angiogenin protein (PDB ID 1b1i) (D). Model of the angiogenin variant Arg121His (E).

tions were confirmed by reamplification and sequencing. We also analyzed by sequencing the *ANG* coding region in 234 controls. Modelization of the angiogenin variant was performed using the crystal structure of the normal angiogenin protein (PDB ID 1b1i), the Swiss model server (<http://swissmodel.expasy.org/>), and the molecular visualization system Pymol (<http://pymol.sourceforge.net/>).

RESULTS

We found coding mutations in the *ANG* gene in 3 patients with sporadic ALS (**Figure**, A-C) (estimated frequency, 0.35%; 95% confidence interval, 0.07%-1.02%). A novel mutation was found in 1 male patient with sporadic ALS. This mutation, which is a heterozygous substitution G434A, changes the amino acid (Arg121His) in the C-terminal region of the mature angiogenin protein (Figure, A). The modelization of the variant angiogenin suggests that histidine 121 may affect the environment of the catalytic center, in that a shorter and cyclic amino acid replaces a long and linear one (Figure, D and E). We also found the heterozygous substitution A208G (Ile46Val) in 2 patients with sporadic ALS without a known family link (Figure, B and C). No mutation was observed in a control population of 234 healthy individuals.

We confirmed the presence of the rs11701 single-nucleotide polymorphism in the *ANG* gene in ALS and control populations. This polymorphism consists of a substitution T > G. We did not observe significant differences in allelic distributions between patients with sporadic ALS (T: n = 1538 [9.9%]; G: n = 170 [90.1%]) and controls (T: n = 408 [12.4%]; G: n = 58 [87.6%]) ($P = .12$; χ^2 test = 2.42). Similarly, no variation in genotype distributions was found between patients with sporadic ALS (TT: 81%; TG: 18%; GG: 1.0%) and controls (TT: 76%; TG: 23.1%; GG: 0.9%) ($P = .21$; χ^2 test = 3.13). The clinical characteristics of the 3 *ANG* mutations carriers are summarized in the **Table**.

COMMENT

We report a novel mutation of the *ANG* gene in a patient who developed ALS with rapid progression and observed a previously known mutation in 2 patients with sporadic ALS. The novel mutation (G434A) changes the amino acid (R121H) in the C-terminal region of the mature angiogenin protein. This G434A mutation is rare; to date, it has been found in only 1 of 3155 patients with ALS, including our results and all published data.^{5,10-12} The fact that it has not been observed in 2135 healthy individuals suggests that

Table. Clinical Characteristics of the Patients With Amyotrophic Lateral Sclerosis With Mutations in the ANG Gene

Characteristic	Mutations		
	A208G	A208G	G434A
Amino acid substitution	I46V	I46V	R121H
Sex	Male	Female	Male
Age at onset, y	46	73	31
Site of onset	Lower limbs	Lower limbs, bilateral, distal	Upper limbs, distal
Upper motoneuron signs			
Bulbar	-	-	+
Upper limbs	+	+	+
Lower limbs	+	+	+
Lower motoneuron signs			
Bulbar	-	-	+
Upper limbs	+	+	+
Lower limbs	+	+	+
Denervation at EMG			
Bulbar	+	+	+
Upper limbs	+	+	+
Lower limbs	+	+	-
Disease progression	Tetraplegia, bulbar involvement	Dysarthria, choking	Evolution to tetraplegia with bulbar involvement
Duration of evolution, y	9.5	2.1	2.7
Cause of death	NA	Respiratory failure	Respiratory failure

Abbreviations: EMG, electromyography; NA, not available; +, present; -, absent.

it is not a neutral polymorphism. However, functional studies will be necessary to confirm this theory. We observed that the R121H change may alter the conformation of the catalytic center. Wu et al¹² recently identified a P112L mutation in 1 patient with ALS; therefore, this novel R121H mutation represents the second mutation identified in the C-terminal region of the ANG gene.

The A208G substitution that we observed in 2 unrelated patients with sporadic ALS already has been found in 2 Scottish patients with familial ALS and 1 Scottish patient with sporadic ALS.⁵ Patients described in the present study are not of Scottish descent. We did not observe this mutation in our 234 French controls. This substitution was observed in 2% of Italian controls, and thus it may represent a rare polymorphism.¹¹ In our meta-analysis, we found a frequency of 0.2% for this polymorphism in apparently healthy individuals, which is in the range of the estimated life span incidence of ALS (1/800). It may be possible that this mutation is associated in some populations with other genetic variations, modulating its effect. The A208G substitution changes the amino acid in position 46 (I46V). A recent biochemical study¹³ of ANG mutations indicated that this I46V variation was associated with a 10-fold decrease in ribonuclease activity.

Greenway et al⁵ observed an association with the G allele of rs11701 in Irish or Scottish populations with ALS. A similar association was found in an Italian ALS population.¹¹ However, no association was observed with another Italian,¹⁰ a US, an English, and a Swedish population.⁵ In our study, we did not observe a difference in allelic and genotypic distributions for rs11701 between French populations of controls and patients with ALS. These results suggest either that the observed associations were false-positive results or that linkage disequilibrium exists between this polymorphism and an unknown mutation in some populations.

The identification of new mutations in the ANG gene brings further support for a role for angiogenin in the physiopathologic mechanisms of ALS. The implication of the G434A and A208G substitutions in ALS will have to be assessed by biological assays on angiogenesis and neurite path finding and molecular assays on nuclear transport, ribonuclease activity, and ribosomal RNA synthesis. Indeed, angiogenin has been shown to enhance ribosomal RNA transcription via binding to DNA in ribosomal RNA genes.¹⁴ Interestingly, we and others^{15,16} have reported that the SMN1 gene, also presumed to act on ribosomal RNA synthesis (maturation), is a risk factor for ALS.

Accepted for Publication: March 18, 2008.

Correspondence: Christian R. Andres, MD, PhD, INSERM U930, Université François Rabelais Tours, CHRU de Tours, Faculté de Médecine, 10 boulevard Tonnellé, BP3223, 37032 Tours CEDEX, France (andres@med.univ-tours.fr).

Author Contributions: *Study concept and design:* Paubel, Violette, Praline, Corcia, Andres, and Vourc'h. *Acquisition of data:* Paubel, Violette, Amy, Meininger, Camu, Corcia, Andres, and Vourc'h. *Analysis and interpretation of data:* Paubel, Amy, Andres, and Vourc'h. *Drafting of the manuscript:* Paubel, Violette, Praline, and Vourc'h. *Critical revision of the manuscript for important intellectual content:* Amy, Meininger, Camu, Corcia, Andres, and Vourc'h. *Statistical analysis:* Andres and Vourc'h. *Obtained funding:* Violette, Camu, Andres, and Vourc'h. *Administrative, technical, and material support:* Paubel, Violette, Amy, Praline, Meininger, Camu, Andres, and Vourc'h. *Study supervision:* Corcia, Andres, and Vourc'h.

Financial Disclosure: None reported.

Funding/Support: The Association pour la Recherche sur la Sclérose Latérale Amyotrophique provided financial support for this study.

Group Information: The French ALS Study Group is composed of Fredric Dubas, MD, and Guillaume Nicolas, MD, PhD (Angers); Gwendal Lemasson, MD, PhD, and Emmanuelle Salort, MD (Bordeaux); Fausto Viader, MD, and Laurence Carlier, MD (Caen); Pierre Clavelou, MD, PhD, and Nathalie Guy, MD (Clermont-Ferrand); Maurice Giroud, MD, and Cecile Maugras, MD (Dijon); Gerard Besson, MD, PhD (Grenoble); Alain Destée, MD, PhD, and Veronique Danel, MD (Lille); Philippe Couratier, MD, PhD, and Matthieu Lacoste, MD (Limoges); Emmanuel Broussole, MD, Christophe Vial, MD, and Nadia Vandenberghe, MD (Lyon); Jean Pouget, MD, Dominique Lardiller, MD, and Annie Verschuren, MD (Marseille); William Camu, MD, PhD, Guillaume Garrigues, MD, and Nicolas Pageot, MD (Montpellier); Marc Debouverie, MD, PhD, and Sophie Pition, MD (Nancy); Claude Desnuelle, MD, PhD, and Marie Helene Soriani, MD (Nice); Vincent Meininger, MD, PhD, Francois Salachas, MD, Pierre-François Pradat, MD, PhD, Michel Dib, MD, Gaelle Bruneteau, MD, and Nadine Leforestier, MD (Paris); Christine Tranchant, MD, PhD, and Marie-Céline Fleury, MD (Strasbourg); Jean-Christophe Antoine, MD, PhD, and Jean Philippe Camdessanche, MD, PhD (Saint-Etienne); Marie-Christine Arnebes, MD and Pascal Cintas, MD (Toulouse); and Philippe Corcia, MD, PhD, and Julien Praline, MD (Tours).

Additional Contributions: Maryse Guillonnet, Jeanine Le Garrec, Isabelle Besson, Catherine Cherpi-Antar, and Rose-Anne Thépault provided technical help.

REFERENCES

- Oosthuysen B, Moons L, Storkebaum E, et al. Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. *Nat Genet.* 2001;28(2):131-138.
- Lambrechts D, Storkebaum E, Morimoto M, et al. VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death. *Nat Genet.* 2003;34(4):383-394.
- Storkebaum E, Lambrechts D, Dewerchin M, et al. Treatment of motoneuron degeneration by intracerebroventricular delivery of VEGF in a rat model of ALS. *Nat Neurosci.* 2005;8(1):85-92.
- Azzouz M, Ralph GS, Storkebaum E, et al. VEGF delivery with retrogradely transported lentivector prolongs survival in a mouse ALS model. *Nature.* 2004;429(6990):413-417.
- Greenway MJ, Andersen PM, Russ C, et al. ANG mutations segregate with familial and "sporadic" amyotrophic lateral sclerosis. *Nat Genet.* 2006;38(4):411-413.
- Fett JW, Strydom DJ, Lobb RR, et al. Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma cells. *Biochemistry.* 1985;24(20):5480-5486.
- Kieran DM, Greenway MJ, Hardiman O, Prehn J. The effects of angiogenin on motor neuron degeneration [abstract]. *Amyotroph Lateral Scler.* 2005;6(6)(suppl 1):42.
- Adams SA, Subramanian V. The angiogenins: an emerging family of ribonuclease related proteins with diverse cellular functions. *Angiogenesis.* 1999;3(3):189-199.
- Subramanian V, Feng Y. A new role for angiogenin in neurite growth and path-finding: implications for amyotrophic lateral sclerosis. *Hum Mol Genet.* 2007;16(12):1445-1453.
- Del Bo R, Scarlato M, Ghezzi S, et al. Absence of angiogenic genes modification in Italian ALS patients. *Neurobiol Aging.* 2008;29(2):314-316.
- Conforti FL, Sprovieri T, Mazzei R, et al. A novel angiogenin gene mutation in a sporadic patient with amyotrophic lateral sclerosis from southern Italy. *Neuromuscul Disord.* 2008;18(1):68-70.
- Wu D, Yu W, Kishikawa H, et al. Angiogenin loss-of-function mutations in amyotrophic lateral sclerosis. *Ann Neurol.* 2007;62(6):609-617.
- Crabtree B, Thiyagarajan N, Prior SH, et al. Characterization of human angiogenin variants implicated in amyotrophic lateral sclerosis. *Biochemistry.* 2007;46(42):11810-11818.
- Xu ZP, Tsuji T, Riordan JF, Hu GF. The nuclear function of angiogenin in endothelial cells is related to rRNA production. *Biochem Biophys Res Commun.* 2002;294(2):287-292.
- Veldink JH, Kalmijn S, Van der Hout AH, et al. SMN genotypes producing less SMN protein increase susceptibility to and severity of sporadic ALS. *Neurology.* 2005;65(6):820-825.
- Corcia P, Camu W, Halimi JM, et al. SMN1 gene, but not SMN2, is a risk factor for sporadic ALS. *Neurology.* 2006;67(7):1147-1150.

Research Letters

The Research Letter is intended to provide a means to communicate short original research in a highly focused manner. Important, fast-breaking research that lends itself to a short communication and that can be reviewed rapidly is our objective. Papers should not exceed 600 words of text and should have fewer than 6 references. A single table or figure may be included. In general, Research Letters should be divided into the following sections: an introduction (with no heading), Methods, Results, and Comment. Research Letters should be double spaced and a word count should be provided with each letter.