ORIGINAL COMMUNICATION

Identification of novel Angiogenin (*ANG)* gene missense variants in German patients with amyotrophic lateral sclerosis

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Abstract Amyotrophic lateral sclerosis (ALS) is a fatal progressive neurodegenerative disease characterized by the selective death of motor neurons in the motor cortex, brain stem and spinal cord. Recently, missense variants in the angiogenin gene (*ANG*), an angiogenic factor expressed in ventral horn motor neurons that is up-regulated by hypoxia, have been found in ALS patients of Irish/Scottish, North American, Italian, French and Dutch descent. To investigate the role of ANG in the German population, we screened for mutations by sequencing the entire coding region of the ANG gene in a large sample of 581 German ALS cases and 616 sex- and age-matched healthy controls. We identified two heterozygous missense variants, F(−13)L and K54E, in two German sporadic ALS cases but not in controls. Both missense variants are novel and have not been previously found in ALS cases. Our results suggest that missense variants in the *ANG* gene play a role in ALS in the German population and provide further evidence to support the hypothesis that angiogenic factors up-regulated by hypoxia are involved in the pathophysiology of ALS.

Keywords Angiogenin · Missense variant ·
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Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that specifically affects the motor neurons in the spinal cord, brain stem and motor cortex. To date, five ALS causative genes have been identified in patients with familial ALS, accounting for 5–10% of all ALS cases. While autosomal dominant mutations in the Cu/Zn superoxide dismutase (SOD1) [20], vesicle-associated protein B (VAPB) [17], and dynactin [19] have been shown to cause adult-onset forms of ALS, mutations in senataxin (SETX) lead to an early onset of the disease [3]. In contrast, mutations in the gene encoding alsin have been associated with juvenile-onset autosomal recessive ALS [10, 24].

In addition, genetic variants in certain genes may play a role in modifying ALS susceptibility. Certain haplotypes in the vascular endothelial growth factor (VEGF) gene have been reported to be a possible risk factor for sporadic ALS [16] in a population based study, whereas a recent meta-analysis has confirmed increased susceptibility for ALS in male carriers of the promoter single nucleotide polymorphism (SNP) −2578AA [15]. Subsequently, seven missense variants in angiogenin (ANG), another angiogenic factor in the ventral horn motor neurons which is up-regulated by hypoxia, have been suggested to be associated with ALS in Irish/Scottish patients [9]. Other ANG missense variants have been identified in ALS patients from North America [23], Italy [4, 7], France [18] and the Netherlands [22]. In addition, recent functional studies have shown that ANG missense variants identified in ALS patients lead to reduced angiogenic activity of ANG in endothelial cells [23], and impairment of neurite extension and survival in motor neurons [14, 21].

To investigate the role of ANG in ALS patients from Germany, we screened for mutations by sequencing the entire coding region of the ANG gene in a large cohort of German ALS patients and controls. We identified two novel heterozygous ANG missense variants in two sporadic ALS cases but not in controls. Our findings suggest that missense variants in the ANG gene play a role in ALS in the German population.

Subjects and methods

Subjects

Informed consent was obtained from 581 sporadic ALS patients and 616 healthy controls. Of these, 401 patients were referred to the Neurology Department of the University Hospital of Ulm, and 180 patients to the Neurology Department of the University Hospital Grosshadern (Munich). Samples from 365 age- and gender-matched healthy controls were obtained from the adult population-based KORA study [13], whereas those from 251 gender-matched controls older than age 60 were obtained from our local DNA database. The ALS sample consisted of 581 sporadic ALS patients with clinical diagnosis of probable or definite ALS according to the revised El Escorial criteria [1, 2]. The male to female ratio was 1.7. Average age-at-onset was 59 years and average survival time was 51 months. 70% of the patients had limb onset, 30% showed bulbar onset.

Genetic analysis

Genomic DNA was isolated from peripheral blood using standard protocols. The GenBank reference sequence accession number of ANG was NM_001145.4. In the cDNA, the A of the ATG translation initiation start site was numbered as nucleotide +1. In accordance with the literature, amino acids from the signal peptide of ANG (24 aminoacids) were named in negative numbers whereas those from the processed protein (123 amino acids) were named in positive numbers, the first Gln residue of the entire sequence being numbered as amino acid +1. Primers were designed using the ExonPrimer software (http://ihg.gsf.de/ihg/ExonPrimer.html) for the ANG coding region. PCR products were sequenced directly using the BigDye Terminator Cycle sequencing kit 3.1 (Applied Biosystems) and were analyzed on an ABI3730 sequencer. The following primers were used for PCR and sequencing: 5′-TGTTCTTGGGTCTACCACACC-3′, 5′-ATGTTGCCACCACTGTTCTG-3′ (amplicon length: 583 bp). The complete ANG coding region was sequenced for both case and control subjects from forward and reverse ends including the exon/intron junction.

Statistical analysis

The statistical analysis of the allelic frequency for SNP rs11701 was conducted using the χ^2 test with one degree of freedom.

Bioinformatics

Missense variants were mapped onto the three-dimensional crystal structure of the mature ANG protein using PyMOL software (Delano Scientific LLC).

Results

In our study, we identified two heterozygous ANG missense variants in two ALS patients of German descent that were negative for mutations in the SOD1 screening

(Table 1). A ca. 36 C>T transition located within the signal peptide of *ANG* results in a F(−13)L substitution whereas the ca. 232 A>G transition causes a K54E amino acid substitution that affects the mature protein. The replacement of the basic Lys by the acid Glu in K54E is represented in Fig. 1. The missense variants F(−13)L and K54E are novel and have not been previously linked to ALS. Neither of these variants were found in 616 controls. Overall, the estimated mutational frequency of the *ANG* gene is 0.3% in our population. Unfortunately, the segregation of missense variants F(−13)L and K54E could not be investigated further due to the unavailability of DNA from family members of the patients. However, none of the carriers had a familial history of ALS. The clinical picture of the F(−13)L and K54E carriers is summarized in Table 1.

Our results also identified the previously reported heterozygous missense variant I46V (ca. 208 A>G) in one ALS patient but not in controls. Originally, I46V was described in three Scottish ALS patients [9] and subsequently in two French cases [18]. Nonetheless, I46V has been suggested to be a benign variant, at least in the Italian population, because it was found in a total of 7 Italian patients and 11 healthy controls [4, 5, 7]. Furthermore, we identified the variant K17I (ca. 122 A>T) in one sporadic ALS case (0.1%) and two controls (35/62 years of age) (0.3%). This finding is in agreement with a previous study that identified K17I in two Irish/Scottish ALS patients and one control (65 years) [9]. In a Dutch pedigree, variant K17I was recently shown to segregate with disease following an autosomal dominant inheritance model with reduced penetrance [22]. Of the 17 different *ANG* missense variants identified in ALS patients up to date (Table 1, supplemental data), only K17I and I46V were found in both cases and controls.

Examination of ANG evolutionary conservation showed that residues Phe(−13), Lys17, Ile46 and Lys54 are conserved in mammals (Fig. 1, supplemental data). None of the identified missense variants were exclusively present in controls.

The sequence analysis confirmed the presence of the common synonymous SNP rs11701 (T/G) in our population. This SNP was shown to be associated with ALS in the Irish population [9] in which the frequency of the allele G was significantly higher in ALS cases compared with controls (0.17 vs. 0.09, $P < 0.001$). Nevertheless, we did not find the association of this allele to disease as previously reported in the Irish/Scottish population (Table 2). In our sample, the distribution of the G allele was 0.12 in cases and 0.13 in controls ($P > 0.05$). The previously identified SNP rs17560 was not observed in our sample. However, we found the allele T of the SNP rs2228653 in one ALS case and none of the controls. This SNP is located

Table 1 *ANG* missense variants identified in German ALS patients and clinical picture

Nucleotide change	Amino acid change	Variant location	Zygosity	Frequency	Sporadic/familial ALS	Gender	Year of birth	Age at onset	Site of onset	Duration of disease	ALS diagnosis	SOD1 screening	Concomitant diseases
ca. 36 C>T	F(−13)L	SP	Hetero-	1/1197	Sporadic	Male	1930	71 years	Limb	38 months	Probable	Negative	No
ca. 232 A>G	K54E	MP	Hetero-	1/1197	Sporadic	Male	1974	28 years	Limb	24 months	Definite	Negative	Frontal deficit

SP signal peptide, *MP* mature protein
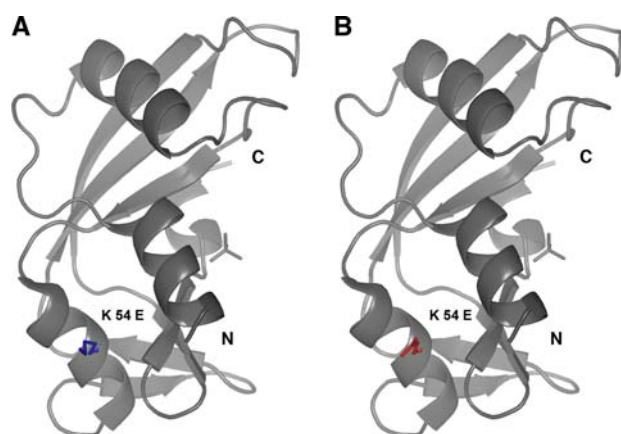
Fig. 1 Structure of angiogenin (PDB-ID 1h53). **a**, **b** Angiogenin is shown as ribbons with the phosphate near the active site and with the mutated residue shown as sticks. The K54 side-chain from the wild-type protein is in *blue* (**a**), the modeled K54E side chain is in *red* (**b**)

Table 2 Genotypic and allelic frequencies of synonymous SNP rs11701 in German ALS patients and controls

	Patients	Controls
TT genotype	0.768	0.747
TG genotype	0.217	0.235
GG genotype	0.015	0.018 ($P > 0.05$)
T allele	0.876	0.864
G allele	0.124	0.136 ($P > 0.05$)

within the coding region of ANG and results in the synonymous substitution T97T, which makes it difficult to predict whether this synonymous variant is disease specific.

Discussion

ANG was first suggested to be a susceptibility gene for ALS when the SNP rs11701, located within the coding region of the gene, was found to be significantly associated with disease in an Irish ALS cohort [8]. Subsequently, seven different heterozygous *ANG* missense variants were identified in a large cohort of 1629 patients from Ireland, Great Britain, the USA and Sweden [9]. Although *ANG* missense variants were present in individuals from all four subpopulations, the majority of the affected individuals were of Irish/Scottish descent, suggesting that ANG might be a specific ALS susceptibility factor in the Irish/Scottish subpopulation. However, another study confirmed the occurrence of *ANG* missense variants in an independent ALS cohort of North American descent [23]. Subsequently, novel ALS-associated *ANG* missense variants were reported in one French [18], one Dutch [22] and two different Italian [4, 7] samples. In contrast, two additional Italian

groups have failed to identify ANG missense variants in their ALS cohorts [5, 6]. To date, no report has assessed the role of ANG in the German ALS population.

In our study, we sequenced the coding region of the *ANG* gene in a German ALS cohort and in controls. Our sample, together with that of Gellera et al. [7], is the second largest sample studied after the one from Greenway et al. [9]. We identified two *ANG* missense variants, F(−13)L and K54E, in two ALS cases (0.3%; 2/581). Both *ANG* missense variants are novel and, to our knowledge, have not been previously documented. The variants F(−13)L and K54E appear to be disease-specific in our population, since both were absent in our 616 controls, as well as in 3107 controls that were sequenced in previous *ANG* mutational screenings [4–7, 9, 18, 22, 23]. Our results indicate that the novel *ANG* missense variants F(−13)L and K54E are linked to ALS in the German population.

In the Scottish/Irish population, several *ANG* missense variants have been suggested to segregate with familial and sporadic ALS [9]. However, segregation of *ANG* missense variants has not been fully reproduced in other populations [5, 7, 18, 23] until the recent screening of a Dutch pedigree carrying the K17I missense variant [22]. In our case, the carriers of the ANG missense variants F(−13)L and K54E were apparently 'sporadic' ALS cases without a family history of ALS. Both mutation carriers were male patients with predominant lower motor-neuron deficits and a reduced life expectancy of less than 3 years. The F(−13)L carrier had a late age at onset (71 years), while the K54E carrier showed an early onset of disease (28 years). Interestingly, the K54E carrier showed clear signs of frontal deficit. Nonetheless, because the K54E carrier was deceased, we were unable to further characterize this patient clinically. Previously, a Dutch patient carrying the missense variant K17I presented ALS with frontotemporal dementia (FTD) [22], whereas another Italian SALS patient with a synonymous ca. 132 C>T variant had a diagnosis of frontal lobe dysfunction [7]. These findings are interesting because they suggest that ANG may also be involved in cognitive impairment.

F(−13)L is located in the signal peptide of ANG, whereas K54E affects the mature ANG protein. Although the effects of these missense variants on the ANG protein are difficult to predict, we performed structural modeling of these variants in the 3-D crystal structure of the protein. We speculate that the F(−13)L variant, located in the signal peptide of ANG which is involved in protein trafficking [12], might lead to abnormal protein transport and secretion or to an altered recognition site for the signal peptide peptidase, leading to mislocalization of ANG. In the case of the K54E variant, the replacement of a basic residue by an acidic glutamate disrupts a patch of positively charged protein surface formed by residues Lys50,

Arg51 and Lys54 that might be of functional relevance for the interaction of ANG with negatively charged molecules like nucleic acids or other proteins.

To date, a total of 15 different *ANG* missense variants have been exclusively identified in ALS patients but not in controls [4, 7, 9, 18, 22, 23], including the novel *ANG* missense variants F(−13)L and K54E reported in this study. Altogether, these results clearly suggest that missense variants in the *ANG* gene, although rare, are potentially causative of disease as recently suggested in discussions regarding other complex neurodegenerative diseases, including the glucocerebroside gene (*GBA*) in Parkinson's disease (PD) [11].

There is increasing evidence that variants in the *ANG* gene play a role in ALS pathophysiology. Recently, the *ANG* missense variants K17I, S28N and P112L identified in ALS patients from North America have been shown to result in complete ANG loss-of-function by reducing angiogenesis due to defects in ribonuclease activity, nuclear translocation, or a combination of both [23]. In addition, an independent study has shown that human ANG is neuroprotective, whereas the ALS-associated ANG variants Q12L, C39W and K40I, which are located in the catalytic center of the protein, compromise neurite extension, pathfinding and survival of motor-neurons [14, 21]. These findings have been confirmed for K40I in another recent report [14]. Furthermore, ANG is highly expressed in the spinal cord ventral horn motor-neurons during both fetal development and adulthood [21].

In summary, we identified two heterozygous missense variants, F(−13)L and K54E, in the *ANG* gene of two sporadic ALS patients of German descent but not in controls. Our results suggest that the F(−13) and K54E *ANG* missense variants are linked to ALS in the German population and support the hypothesis that angiogenic factors up-regulated by hypoxia are involved in the pathophysiology of ALS. Future functional studies will help to decipher the specific role of these *ANG* missense variants in ALS.

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