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ATXN2 is a modifier of phenotype in ALS patients of Sardinian ancestry

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ATNX2 is not a regulatory gene in Italian ALS patients with *C9ORF72* GGGGCC expansion

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Abstract

There are indications that both familial ALS and sporadic ALS phenotype and prognosis are partly regulated by genetic and environmental factors, supporting the theory that ALS is a multifactorial disease. The aim of this paper was to assess the role of *ATXN2* intermediate length repeats in a large series of Italian and Sardinian ALS patients and controls carrying a pathogenetic *C9ORF72* GGGGCC hexanucleotide repeat. A total of 1972 ALS cases were identified through the database of the Italian ALS Genetic consortium (ITALSGEN), a collaborative effort including 18 ALS centers throughout Italy. The study population included: (1) 276 Italian and 57 Sardinian ALS cases who carried the *C9ORF72* expansion; (2) 1340 Italian and 299 Sardinian ALS cases not carrying the *C9ORF72* expansion. A total of healthy 1043 controls were also assessed. Most Italian and Sardinian cases and controls were homozygous for 22/22 or 23/23 repeats or heterozygous for 22/23 repeats of the *ATXN2* gene. *ATXN2* intermediate length repeats alleles (≥28) were detected in 3 (0.6%) Italian ALS cases carrying the *C9ORF72* expansion, in none of the Sardinian ALS cases carrying the expansion, in 60 (4.3%) Italian cases not carrying the expansion, and in 6 (2.0%) Sardinian ALS cases without *C9ORF72* expansion. Intermediate length repeat alleles were found in 12 (1.5%) Italian controls and 1 (0.84%) Sardinian controls. Therefore, ALS patients with *C9ORF72* expansion showed a lower frequency of *ATXN2* polyQ intermediate length repeats than both controls (Italian cases, p=0.137; Sardinian cases, p=0.0001) and ALS patients without *C9ORF72* expansion (Italian cases, p=0.005; Sardinian cases, p=0.178). In our large study on Italian and Sardinian ALS patients with *C9ORF72* GGGGCC hexanucleotide repeat expansion, compared to age-, gender- and ethnic-matched controls, *ATXN2* polyQ intermediate length does not represent a modifier of ALS risk, differently from non-*C9ORF72* mutated patients.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive degenerative disorder of the central nervous system, almost invariably fatal, characterized by a loss of cortical, bulbar and spinal motor neurons. In 10-15% of cases it is genetically transmitted (familial ALS, fALS), while in the remaining cases it appears sporadically in the population (sporadic ALS, sALS) (Renton et al, 2014). More than 20 major genes have been related to ALS, the most common in the Caucasian population being *C9ORF72, SOD1, TARDBP* and *FUS* (Renton et al, 2014). However, there are now indications that both familial ALS and sporadic ALS phenotype and prognosis are partly regulated by genetic and environmental factors, supporting the theory that ALS is a multifactorial disease (Al Chalabi et al, 2014).

ATNX2 intermediate length repeats have been identified as a risk factor for ALS (Neuenschwander et al, 2014) and their presence are additionally associated with reduced survival in ALS patients (Chiò et al, 2015). More recently, it has been reported that *ATXN2* is also a risk factor for ALS patients carrying the GGGGCC hexanucleotide repeat in the first intron of the *C9ORF72* gene (van Blitterswijk et al, 2014a). This gene accounts for 40% of familial ALS and 7% sporadic ALS in European and American series (Majounie et al, 2012). Phenotypes associated with this repeat expansion include ALS and/or frontotemporal dementia (FTD), psychotic symptoms (hallucinations and delusions), and extrapyramidal signs. The wide and heterogeneous symptomatology related to *C9ORF72* has yet not been fully explained (Roher et al, 2015).

The aim of this paper was to assess the role of *ATXN2* intermediate length repeats in a large series of Italian and Sardinian ALS patients and controls carrying a pathogenetic *C9ORF72* GGGGCC hexanucleotide repeat.

2. Methods

2.1 Patients

A total of 1972 ALS cases were identified through the database of the Italian ALS Genetic consortium (ITALSGEN), a collaborative effort including 18 ALS centers throughout Italy. The study population included: (1) 276 Italian and 57 Sardinian ALS cases who carried the *C9ORF72* expansion; (2) 1340 Italian and 299 Sardinian ALS not carrying the *C9ORF72* expansion.

2.2 Controls

The 1043 controls were included in the analysis. This included: (1) 686 regionally-matched, unrelated Italian subjects, reported in previous papers (Corrado et al 2011; Conforti et al, 2012). These individuals were predominantly blood donors; (2) 243 regionally-matched, unrelated Sardinian subjects; (3) 114 matched subjects identified through the patients' general practitioners (population-based controls) (Chiò et al, 2015).

2.3. Genetic analysis

Genomic DNA was isolated from peripheral blood lymphocytes using a standard protocol. The *ATXN2* CAG repeat in exon 1 (NM_002973.3) was amplified using a fluorescent primer and sized by capillary electrophoresis on an ABI 3130 genetic analyzer (Applied Biosystem, Foster City, CA, USA) (Cancel et al, 1997). As reported in recent guidelines for molecular genetic testing of Spinocerebellar Ataxias (SCA), capillary electrophoresis is the preferred method.to size alleles as it allows resolution of alleles that are one triplet apart (Sequeiros et al, 2010). As a quality control, 20 samples have been genotyped in the six laboratories that performed the molecular genetic testing for the present study. The results showed a consistent allele assignment for all the samples.

To compare our findings to those of van Blitterswijk and colleagues (2014a), we used a threshold of 28 repeats (or greater) as the definition of intermediate size repeats. However, data using a

threshold of 27 repeats (the most common used cut-off for *ATXN2* intermediate length repeats in the literature) are reported as supplementary table.

All ALS cases were also tested for *SOD1* (all exons), *TARDBP* (exon 6), *FUS* (exons 14 and 15), and *C9ORF72* using the methodology described elsewhere (Chiò et al, 2012a).

2.4. Statistical analysis

The difference between *ATXN2* polyQ intermediate length repeats in cases and controls was assessed with Fisher's exact test.

2.5. Standard protocol approvals and patient consents

The study was approved by the Ethical committees of participating centers. Patients and controls signed a written informed consent.

3. Results

The demographic and clinical characteristics of patients and controls are reported in Table 1. Most Italian and Sardinian cases, as well as most Italian and Sardinian controls, were homozygous for 22/22 or 23/23 repeats or heterozygous for 22/23 repeats of the *ATXN2* gene. *ATXN2* intermediate length repeats alleles (≥28) were detected in 3 (0.6%) Italian ALS cases carrying the C9ORF72 expansion, in none of the Sardinian ALS cases carrying the expansion, in 60 (4.3%) Italian cases not carrying the expansion, and in 6 (2.0%) Sardinian ALS cases without *C9ORF72* expansion. Intermediate length repeat alleles were found in 12 (1.5%) Italian controls and 1 (0.84%) Sardinian controls. Therefore, ALS patients with *C9ORF72* expansion showed a lower frequency of *ATXN2* polyQ intermediate length repeats than both controls (Italian cases, p=0.137; Sardinian cases, p=0.0001) and ALS patients without *C9ORF72* expansion (Italian cases, p=0.005; Sardinian cases, p=0.178). In patients with *C9ORF72* expansion the presence of *ATXN2* polyQ intermediate length

repeats did not modify the age at onset of ALS (*ATXN2* expanded, 55.8 years [SD 13.7]] vs. non-expanded, 58.0 years [9.1], p=0.56).

4. Discussion

In our large series of Italian and Sardinian ALS patients, we did not find evidence of increased occurrence of *ATXN2* polyQ intermediate length repeats in patients with *C9ORF72* hexanucleotide repeat expansion. In contrast, we confirmed that in patients without *C9ORF72* expansion *ATXN2* polyQ intermediate length repeats are associated with a higher risk of ALS.

C9ORF72 GGGGCC expansions have been related to a quite wide spectrum of clinical presentations, going from pure ALS to pure FTD, but also including psychotic and extrapyramidal signs and symptoms (Rohrer et al, 2015). This wide range of clinical expressions is reflected by neuropathological, MRI and PET studies, showing in *C9ORF72* patients an extension of TDP43 pathology extends toward non-motor areas including prefrontal cortex, cingulate cortex, basal ganglia and cerebellum (Cooper-Knock et al, 2012; Bede et al, 2013; Cistaro et al, 2014). The widespread diffusion of alterations is considered a hallmark of *C9ORF72* mutations in neuroimaging and functional studies.

The reasons of the heterogeneous symptom constellation in patients carrying a GGGGCC expansion on the first intron of the *C9ORF72* gene are still unclear. All studies up to date have shown that the expansion pattern of GGGGCC in different brain areas was not related to the clinical picture and that no correlation was found between expansion size in frontal lobe and occurrence of cognitive impairment (van Blitterswijk et al, 2013a; Dols-Icardo et al, 2104; Nordin et al, 2015).

Another possibility could be an interaction between the presence of *C9ORF72* expansion and one or more regulatory genes. The presence of mutations of other ALS- and FTD-related genes (*GRN, MAPT, TARDBP, FUS, SOD1*) in patients carrying the *C9ORF72* expansion has been reported as possible modifiers of patients' clinical picture (Chiò et al, 2012b; van Blitterswijk et al, 2014b; van

Blitterswijk et al 2013b). A study on 36 common genetic variants found that three variants were significantly associated with age at onset (rs7018487, *UBAP1*; rs6052771, *PRNP*; and rs7403881, *MT-Ie*) and six variants were significantly associated with survival after onset (rs5848, *GRN*; rs7403881, MT-Ie; rs13268953, *ELP3*; the ε4 allele of *APOE*; rs12608932, *UNC13A*; and rs1800435, *ALAD*) (van Blitterswijk et al, 2014b). Finally, it has been shown that *TMEM106B* protect *C9ORF72* expansion carriers from developing FTD (van Blitterswijk et al., 2014c).

Our data contrasts a recent publication based on 331 U.S. patients and 376 U.S. controls reporting that *ATXN2* polyQ intermediate length repeats act as a disease modifier in *C9ORF72* carriers (van Blitterswijk et al, 2014a). This discrepancy may be explained by (1) the larger size of the control cohort in our series, reducing the risk of false-negative association, and (2) the ethnic-matching of patients and controls, avoiding a possible mismatch related to the different frequency of *ATXN2* polyQ intermediate length repeats according to ethnic background (Chiò et al, 2015).

In our large study on Italian and Sardinian ALS patients with *C9ORF72* GGGGCC hexanucleotide repeat expansion, compared to age-, gender- and ethnic-matched controls, *ATXN2* polyQ intermediate length does not represent a modifier of ALS risk, differently from non-*C9ORF72* mutated patients. Our findings highlight the importance of having complete genetic information on ALS patients when assessing putative genetic modifiers.

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Table 1. Clinical characteristics of ALS cases

	Italian cases (n=1616)	**Sardinian cases (n=356)**
Gender (female)	743 (46%)	128 (36%)
Mean age at onset (years)	61.5 (11.8)	61.3 (11.5)
Site of onset (bulbar)	465 (28.8%)	86 (24.2%)

Table 2. *ATXN2* polyQ intermediate length repeats (<28 vs. ≥28) in *C9ORF72* and non-*C9ORF72* cases

	Italian cases			**Sardinian cases**			**Overall**		
	C9ORF72 cases	Non-*C9ORF72* cases	Controls	*C9ORF72* cases	Non-*C9ORF72* cases	Controls	*C9ORF72* cases	Non-*C9ORF72* cases	controls
<28	549	2620	1588	114	592	485	663	3212	2073
≥28	3	60	12	0	6	1	3	66	13
p value (cases vs controls)	0.62	0.0001	-	0.63	0.10	-	0.61	0.0001	-