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Clinical features and genetic characterization of two dizygotic twins with *C9orf72* expansion

Francesca Luisa Conforti [a,1], Rosanna Tortelli [b,*,1], Giovanna Morello [a], Rosa Capozzo [b], Maria Rosaria Barulli [b], Sebastiano Cavallaro [a], Giancarlo Logroscino [b,c]

[a] *Institute of Neurological Sciences, National Research Council, Mangone, Cosenza, Catania, Italy*
[b] *Unit of Neurodegenerative Diseases, Department of Clinical Research in Neurology, University of Bari "A. Moro" at Pia Fondazione Card. G. Panico, Tricase, Lecce, Italy*
[c] *Department of Basic Medical Sciences, Neurosciences and Sense Organs, University of Bari "A. Moro", Bari, Italy*

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ABSTRACT

The objective of the study was to present a detailed clinical, genetic, and epigenetic characterization of 2 amyotrophic lateral sclerosis (ALS) concordant dizygotic twins. The described cases underwent clinical and paraclinical examinations according to the motor neuron disease protocol of our referral center. Mutation analysis of the major causative genes related to ALS was performed. The methylation profile of the CpG island located in the promoter region of *C9orf72* and in the repeat region itself was investigated by bisulfite sequencing of *C9orf72* expansion carriers. The described cases presented an overlapping phenotype. Genetic analysis revealed the presence of an abnormal (>50 repeats) G_4C_2-repeat expansion in *C9orf72*. Both the direct bisulfite sequencing–sensitive and the methylation-sensitive HhaI assays did not reveal any DNA methylation at the CpG island 5′ of the G_4C_2 repeat in *C9orf72*. The $(G_4C_2)_n$ methylation assay indicated that also the expansion itself was not methylated in both twins, suggesting a probably intermediate allele expansion. This is the first report of ALS-concordant dizygotic twins carrying a *C9orf72* expansion probably of intermediate length, and with a detailed clinical and genetic characterization. Twin studies add significant information about the mechanisms of *C9orf72* expansion pleiotropism, probably driven by genetic, epigenetic, and environmental factors.

1. Background

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease affecting primary upper (cortical) and lower (spinal/bulbar) motor neurons. In most cases (90%), when no other first- or second-degree relatives are known to be affected, ALS is considered "sporadic" or "apparently sporadic," whereas in up to 10% of cases, it is "familial" ALS (FALS) (Rowland and Shneider, 2001) Nowadays, the definition of FALS has been expanded to include also a family member with a diagnosis of frontotemporal dementia (FTD) in the pedigree (Byrne et al., 2011). The link between ALS and FTD became particularly clear after the discovery of the G_4C_2 hexanucleotide repeat expansion in the first intron of the *C9orf72* gene (GeneID: 203228; OMIM: 614260) on chromosome 9p21 (DeJesus-Hernandez et al., 2011; Renton et al., 2011). This mutation seems to be geographically clustered: in Europe and North America, it accounts for about a third of FALS cases, while its worldwide frequency is now estimated to be 37.6% (95% confidence interval [CI] = 33.7–41.6) for FALS, 6.3% (95% CI = 5.6–7.1) for sporadic ALS (SALS), 25.1% (95% CI = 20.9–29.6) for familial FTD, and 5.8% (95% CI = 4.4–7.4) for sporadic FTD cases (Majounie et al., 2012). The presence of *C9orf72* expansion has been associated with an ALS phenotype characterized by an earlier age of onset, a more malignant course of disease, a bulbar onset, and a frequent occurrence of psychotic symptoms compared to SALS cases without the expansion (Chiò et al., 2012). Anyway, the phenotype associated with *C9orf72* can be extremely pleiotropic, both in term of penetrance and expression (Cruts et al., 2013). With the discovery of *C9orf72* about 60% of the heritability of FALS and 10% of the heritability of SALS can now be explained (Renton et al., 2014). Nevertheless twin studies estimated the genetic component of SALS at 0.61 (range 0.38–0.78) (Al-Chalabi et al., 2010), underlying that a large part of the heritability of ALS is still unknown.

Twin studies are extremely important to delineate the role of genetics, gene-environmental interaction, and epigenetic

* Corresponding author at: Department of Clinical Research in Neurology, University of Bari Aldo Moro, "Pia Fondazione Cardinale G. Panico", via San Pio X, Tricase, Lecce, Italy. Tel.: +39 0883 773904; fax: +39 0883 773909.
E-mail address: r.tortelli@piafondazionepanico.it (R. Tortelli).
[1] These authors contributed equally to this work.

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modifications in determining the phenotypic manifestation of the disease. Previous studies described ALS cases of discordant- and concordant-affected monozygotic (MZ) twins (Al-Chalabi et al., 2010; Graham et al., 1997; Jokelainen et al., 1978; Xi et al., 2014) and of discordant-affected dizygotic (DZ) twins (Al-Chalabi et al., 2010; Graham et al., 1997; Jokelainen et al., 1978). To date, only 2 ALS cases of concordant-affected DZ twins have been reported in literature (Dumon et al., 1971; Estrin, 1977). Notably, few studies on MZ twins analyzed for *C9orf72* repeat expansion have been reported (Dols-Icardo et al., 2014; Pamphlett et al., 2013; Xi et al., 2014). The aim of this study was to present detailed clinical, genetic, and epigenetic findings in a pair of ALS-concordant DZ twins.

2. Methods

2.1. Clinical and paraclinical assessment

A detailed clinical and paraclinical assessment was performed, including a complete neurological examination, with focus on the presence of signs of impairment of upper and lower motor neurons; a standard neuropsychological assessment; a brain and spinal cord magnetic resonance imaging (MRI) scan; an electromyography of the bulbar, cervical, thoracic, and lumbar muscles; and a venous blood sample collection for routine biochemical and genetic analyses.

2.2. Molecular genetic investigations

Both patients (TW1 and TW2), after informed consent, underwent blood sample collection for genetic and epigenetic analyses. DNA was isolated from peripheral blood and extracted using standard protocols. The coding regions and flanking intronic sequences of *SOD1*, *FUS*, and *TARDBP* were amplified, and the presence of mutations was excluded by Sanger sequencing. The G_4C_2 repeat of *C9orf72* was genotyped using a 2-step strategy. First, the repeat number of wild-type alleles was obtained (Fig. 1A), and then it was used as a repeat-primed polymerase chain reaction (PCR) to determine the presence of a G_4C_2 expansion as previously described (Renton et al., 2011). The G_4C_2-repeat expansion, showing the typical sawtooth pattern, was defined as more than 30 repeats (Fig. 1B).

2.3. Methylation assay

The methylation level of the CpG island 5′ of the G_4C_2 repeat was estimated using 2 independent assays (Xi et al., 2013). In the methylation-sensitive restriction enzyme assay, each DNA sample was amplified after incubation with or without HhaI. The PCR product from the digested and undigested DNA was resolved on a 1.5% agarose gel and quantified using black/white inverted gel images to obtain the observed methylation ratio (unpublished data). All CpG sites of the CpG island were investigated using the bisulfite sequencing assay. This analysis is based on the conversion of unmethylated C to T by bisulfite treatment, while methylated C remains unchanged. Bisulfite treatment was performed with 1 μg of DNA by using EpiTect Bisulfite Kit (Qiagen, Milano, Italy), according to the manufacturer's instructions. The investigated region is shown in Fig. 2A. After conversion, bisulfite-modified DNA was dissolved in 20 μL H_2O, and 2 μL of DNA template was used for PCR amplification, as previously described (Xi et al., 2013). In brief, the region 5′ of the G_4C_2 repeat was amplified by a seminested PCR. Bisulfite-treated DNA was amplified by MyTaq DNA Polymerase (Bioline, Taunton, MA, USA) using touchdown PCR (for primers C9ORFmet1_F, C9ORFmet1_R: Tm from 68 °C to 58 °C at a rate of 1 °C/cycle and fixed at 58 °C for 30 cycles; for primers C9ORFmet2_F, C9ORFmet2_F: Tm, from 67 °C to 57 °C at a rate of 1 °C/cycle and fixed at 57 °C for 20 cycles; 3 minutes extension for each cycle). PCR products were resolved on a 1.5% agarose gel and purified with Centri-SEP spin columns (Thermo Fisher Scientific, Waltham, MA USA). Purified PCR products were then sequenced by the ABI Prism BigDye Terminator cycle sequencing ready reaction kit on an ABI PRISM Genetic Analyzer 3130xl (Applied Biosystems Inc, Foster City, CA, USA). The bisulfite sequencing data generated from both DNA strands. Methylation was detected by direct inspection of sequencing chromatograms and the Bisulfite Sequencing DNA Methylation Analysis software (Rohde et al., 2010).

2.4. $(G_4C_2)_n$ methylation assay

The methylation status of the repeat region itself was evaluated by using a $(G_4C_2)_n$ methylation assay that combines repeat-primed PCR and methylation-specific PCR as described for the first time by Xi et al. (2015a,b).

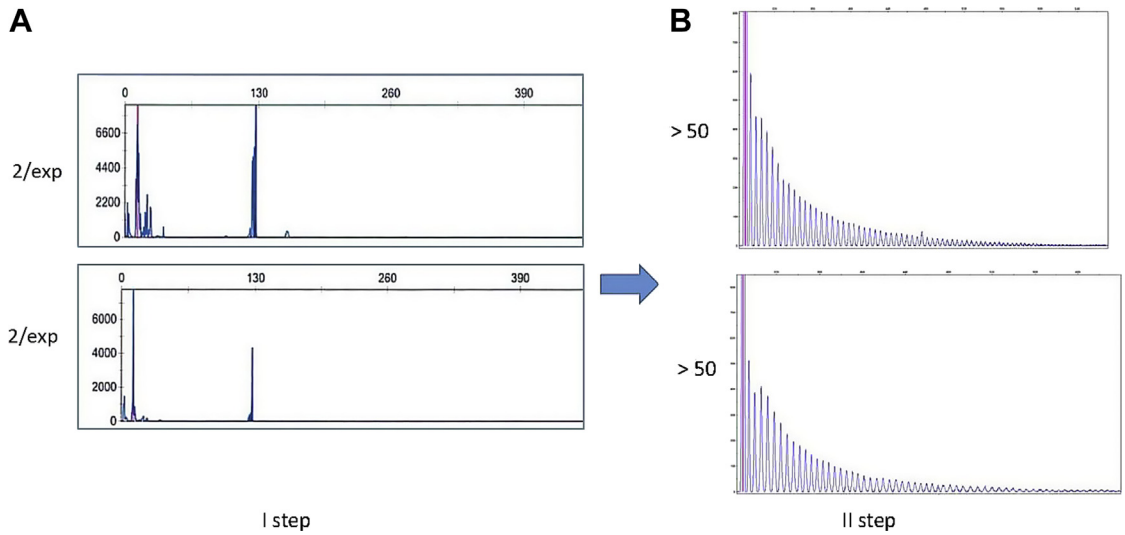

Fig. 1. Results of C9orf72 genotyping. PCR products of amplicon-length analysis (A) and rp-PCR (B) separated on an ABI3130xl DNA Analyzer and visualized by GeneScan software. In both twins, the first-step genotyping detected a 2-repeat allele and the second-step genotyping detected an expansion allele. Abbreviation: PCR, polymerase chain reaction.