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No *C9orf72* repeat expansion in patients with primary progressive multiple sclerosis

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ABSTRACT

Pathological repeat expansion (RE) of the *C9orf72* hexanucleotide sequence is associated to amyotrophic lateral sclerosis (ALS) and frontotemporal dementia disease *continuum*, although other heterogeneous clinical phenotypes have been documented. The occurrence of multiple sclerosis (MS) in some *C9orf72* carriers with a more severe ALS disease course has suggested a possible modifying role for MS. However, *C9orf72* RE seems not to play a role in MS pathogenesis. In this study, we screened *C9orf72* in 189 Italian patients with primary progressive MS (PPMS), a rare clinical form characterized by less inflammation over neurodegenerative features. We failed to detect *C9orf72* RE, but a significant representation of intermediate alleles ($\geq$ 20 units) was observed in our PPMS cohort (2.1%) compared to healthy controls (0%, $p < 0.05$). In the normal range, allele distribution showed a trimodal pattern (2,5,8-repeat units) in PPMS and healthy controls with no significant difference. Our findings further demonstrate that *C9orf72* RE is not genetically associated to MS spectrum, but suggest that intermediate alleles may represent risk factors as already reported for Parkinson disease.

1. Introduction

A hexanucleotide GGGGCC repeat expansion (RE) in the non-coding region of *C9orf72* gene represents the main genetic cause of familial and sporadic forms of both amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), two neurodegenerative disorders now considered part of the same disease *continuum* (Bennion Callister and Pickering-Brown, 2014; DeJesus-Hernandez et al., 2011; Renton et al., 2011). Interestingly, the occurrence of heterogeneous clinical phenotypes, including parkinsonism, psychosis, Alzheimer's disease (AD), has been reported in ALS and FTD patients carrying *C9orf72* RE and in their relatives, broadening the spectrum of possible *C9orf72*-associated diseases (Akimoto et al., 2013; Cooper-Knock et al., 2014; Harms et al., 2013; Lesage et al., 2013; Ticozzi et al., 2014).

The role of *C9orf72* RE has been also investigated in the pathogenesis of multiple sclerosis (MS), a chronic immune-mediated disorder of the central nervous system characterized by demyelination and neurodegeneration processes. Notably, the disease course of MS is highly heterogeneous, with approximately 85% of patients presenting with relapsing-remitting MS (RRMS), characterized by episodes of acute worsening of function followed by partial or complete recovery (Goodin et al., 2016). The remaining 10–15% of MS cases exhibit a continuous progression of neurological disability since disease onset without relapsing-remitting phases, a condition referred as primary progressive MS (PPMS) (Polman et al., 2011). Even if there is no evidence of genetic differences between RRMS and PPMS, there are distinctive features including a different male: female ratio (from 1:2 to 1:1.3) and age at onset (a decade later in PPMS) (Ebers, 2004). Although the concurrence of ALS and MS is extremely rare, previous epidemiological studies have reported a possible family aggregation between these two diseases, with a several-fold increase of ALS occurrence in the first degree relatives of MS patients and vice versa

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(Etemadifar et al., 2012). MS was firstly associated to *C9orf72* gene as pathological RE was detected in 4/5 of patients with coexisting MS and ALS in a population of North England, in association to a more rapidly progressive form, suggesting that MS status may modify disease manifestation in *C9orf72* carriers (Ismail et al., 2013). However, the same study failed to identify *C9orf72* RE in 215 patients affected only by MS without clinical presentation of ALS (Ismail et al., 2013). Subsequently, two further genetic studies screened *C9orf72* gene in MS cohorts of Italian origin, but no significant genetic association was found (Fenoglio et al., 2014; Lorefice et al., 2015). Nevertheless, these studies included patients being prevalently affected by RRMS (Fenoglio et al., 2014) or being genetically homogeneous because they belonged to the geographical isolate of Sardinia (Lorefice et al., 2015). Based on the distinctive profile of PPMS, in which neurodegenerative changes prevail over inflammation, in this study we aimed to assess whether pathologic *C9orf72* RE is a cause of this rare MS subtype by analyzing a cohort of 189 Italian unrelated PPMS patients.

2. Material and methods

2.1. Study cohort

The study included 189 unrelated Italian patients affected by PPMS, recruited from the Department of Neurology at San Raffaele Scientific Institute, Milan. PPMS was defined as a clinical syndrome that was progressive from the onset, with no evidence of relapses or remissions, according to the international consensus revised McDonald criteria (Polman et al., 2011). Patients of Sardinian origin up to second-degree relatives were excluded in order to avoid any confounding factors and spurious association due to a possible stratification effect. Our PPMS cohort was characterized by a male to female sex ratio of 0.95 and by an age of onset of 40.6 ± 9.7 years (Table 1).

The control group, consisting of 211 healthy controls matched for age and gender, and negative for medical or family history of neurological diseases, was consecutively recruited at IRCCS Istituto Auxologico Italiano from 2007 to 2009.

All participants provided written informed consent and the study was approved by the local Ethics Committees of the two participating Institutions.

2.2. C9orf72 genetic screening

Genomic DNA was isolated from peripheral whole blood by using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. Genetic analysis of *C9orf72* was carried out using a two-step polymerase chain reaction (PCR) strategy, as previously described (Ratti et al., 2012). For *C9orf72* repeat size determination, fluorescent fragment length analysis with primers flanking the hexanucleotide repeat region was performed on an automated ABI 3700 genetic analyzer (Applied Biosystems) and data were visualized using GeneMapper v4.0 software (Applied Biosystems). Only samples showing a single peak at the first analysis were further tested for the presence of RE by repeat-primed PCR. Briefly, amplification was performed with Accuprime GC rich polymerase (Thermo-Scientific), in presence of 7'-deaza-2-deoxy GTP (Roche), using an optimized cycling program and previously published primers (DeJesus-Hernandez et al., 2011). A cutoff value of > 30 repeats was used to define the pathogenic threshold, as previously reported (Renton et al., 2011). A DNA sample from an ALS patient carrying *C9orf72* RE was included as positive control (Supplementary Figure 1).

Table 1
Clinical and demographic data of the Italian PPMS patients and healthy controls included in the study.

	PPMS	HC
Sample size	189	211
Gender (Male/Female)*	92/97	112/99
Age at collection, mean ± SD (yrs)	–	39.9 ± 10.6
Age at onset, mean ± SD (yrs)	40.6 ± 9.7	–
Disease duration, mean ± SD (yrs)	10.7 ± 6.9	–
EDSS score (min-max)	5.5 (2.0–8.5)	–

PPMS, primary progressive multiple sclerosis; HC, healthy controls.
SD, standard deviation; EDSS, Expanded Disability Status Scale.
* not significant between the two groups (Fisher's exact test, $p > 0.05$).

2.3. Statistical analyses

In the non pathological range of repeat number (< 30), case-control differences in the mean repeat length and repeat length distribution were evaluated by using T test and Mann-Whitney U test, respectively. Considering that 20-repeat length has been generally used as cutoff to distinguish intermediate from wild type alleles in previous genetic investigations (Ng and Tan, 2017), in our study we adopted this predefined value and classified alleles in two main categories: wild type (< 20) and intermediate ones (≥ 20 and ≤ 30 units). Allele distributions were analyzed by using Fisher's exact test, and a p value < 0.05 was considered statistically significant. All statistical analyses were conducted using Prism Software (GraphPad).

3. Results

We conducted a genetic analysis of the ALS/FTD-associated gene *C9orf72* in a cohort of 189 patients diagnosed with PPMS and in 211 healthy controls of Italian origin. Demographic and clinical features of patients are reported in Table 1.

No pathological *C9orf72* RE (> 30 units) was detected in either PPMS patients or controls, supporting previous negative findings from other independent Italian MS cohorts (Fenoglio et al., 2014; Lorefice et al., 2015). The range of the hexanucleotide repeat length varied between 2–28 units in PPMS cases and 2–17 in controls (Fig. 1A). A trimodal and not significantly different distribution of hexanucleotide repeats was observed in both groups with 2-, 5-, and 8-unit alleles accounting for about 77,5% of all alleles (Fig. 1B), consistent with previous studies (van der Zee et al., 2013). Comparison of the mean repeat length between PPMS cases (4.6 ± 0.15) and controls (4.7 ± 0.19) revealed no significant difference ($p = 0.66$). Similarly, no differences were found in repeat length distribution between cases and controls by considering either total alleles (Fig. 1A, $p = 0.76$) or only the longer allele ($p = 0.72$, data not shown).

Literature data report that the number of GGGGCC repeats significantly correlates with the transcriptional activity of *C9orf72* promoter (Gijselinck et al., 2016; van der Zee et al., 2013) and that intermediate alleles (≥ 20 and ≤ 30 units) may act as risk factors for different neurodegenerative diseases (Ng and Tan, 2017). Interestingly, our results show that 4/189 (2.1%) PPMS patients carried intermediate alleles compared with none in the control group ($p = 0.049$, Table 2). In particular, 2 patients carried 20 repeat units, 1 patient had 21 units and 1 carried an intermediate allele of 28 units (Fig. 1A, Supplementary Figure 1). The patient carrying 28 repeats had a disease onset at 32 years old, with progressive spastic paraparesis. At the time of sampling (60 years old), the patient had a pronounced involvement of pyramidal and sphincteric functional systems (score 4), and he was unable to walk corresponding to an EDSS score equal to 7.0. Electromyography was negative for motoneuron disease. The patient was treated with several immunosuppressive drugs, with partial efficacy. No familiarity for MS was reported.

4. Discussion

Given the increasing clinical and pathological overlap between various neurodegenerative diseases and the large clinical heterogeneity