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RESEARCH ARTICLE

Genetic and epigenetic disease modifiers in an Italian *C9orf72* family expressing ALS, FTD or PD clinical phenotypes

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Abstract

Objective: The presence of the hexanucleotide repeat expansion (HRE) in *C9orf72* gene is associated to the ALS/FTD spectrum, but also to parkinsonisms. We here describe an Italian family with the father diagnosed with Parkinson disease (PD) at the age of 67 and the two daughters developing FTD and ALS at 45 years of age. We searched for *C9orf72* HRE with possible genetic and epigenetic modifiers to account for the intrafamilial phenotypic variability. **Methods:** *C9orf72* mutational analysis was performed by fragment length analysis, Repeat-primed PCR and Southern blot. Targeted next generation sequencing was used to analyze 48 genes associated to neurodegenerative diseases. Promoter methylation was analyzed by bisulfite sequencing. **Results:** Genetic analysis identified *C9orf72* HRE in all the affected members with a similar repeat expansion size. Both the father and the FTD daughter also carried the heterozygous p.Ile946Phe variant in *ATP13A2* gene, associated to PD. In addition, the father also showed a heterozygous *EIF4G1* variant (p.Ala13Pro), that might increase his susceptibility to develop PD. The DNA methylation analysis showed that all the 26 CpG sites within *C9orf72* promoter were unmethylated in all family members. **Conclusions:** Neither *C9orf72* HRE size nor promoter methylation act as disease modifiers within this family, at least in blood, not excluding HRE mosaicism and a different methylation pattern in the brain. However, the presence of rare genetic variants in PD genes suggests that they may influence the clinical manifestation in the father. Other genetic and/or epigenetic modifiers must be responsible for disease variability in this *C9orf72* family case.

Keywords: *C9orf72*, genetic modifiers, DNA methylation

Introduction

A hexanucleotide repeat expansion (HRE) in *C9orf72* gene is the most frequent cause of familial and sporadic amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (1,2), ranging from 2–23 units in the normal population to >30–>4000 units in pathological conditions (3). In contrast to other repeat expansion disorders, no clear association between HRE size and phenotype severity or disease state (ALS/FTD) has been

demonstrated so far. Genetic anticipation is not an evident phenomenon and, within the same pedigree, individuals with a similar HRE may manifest indifferently ALS, FTD, or mixed phenotypes (4–11). In addition, *C9orf72* HRE has been reported in a heterogeneous array of neurological disorders, other than ALS and FTD, including parkinsonism and psychosis (12,13). However, also within the ALS/FTD disease spectrum, the wide heterogeneity of clinical features and symptoms even intra-familially suggests that modifiers,

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both genetic and epigenetic, may contribute to influence disease presentation in HRE carriers (14). The presence of additional rare genetic variants in ALS-associated genes (15–18) and of microdeletions in the low complexity region flanking the repeat sequence (9,19) have been described as consistent features in *C9orf72* mutation carriers, possibly influencing disease presentation as well.

As regards epigenetic disease modifiers, *C9orf72* gene promoter was described to be methylated in 30% of both ALS and FTD HRE carriers, in association to decreased V1 and V3 transcripts spanning the HRE and diminished pathological RNA foci number and dipeptide repeat protein (DPR) in autaptic brains (20–24). *C9orf72* promoter methylation was also associated to a reduced cerebral atrophy in neuroimaging data from *C9orf72* mutation carriers (25) and increased survival in *C9orf72*-FTD patients (22), suggesting a neuroprotective role of this epigenetic modification.

Here we investigated possible genetic and epigenetic modifiers in a *C9orf72*-positive Italian family showing very different clinical presentations, including Parkinson disease (PD), ALS and FTD.

Materials and methods

C9orf72 genotyping

The family was clinically investigated at the Department of Neurology-Stroke Unit, Hospital of Crema, Italy. Informed consent was obtained and the study approved by the ethics committee at IRCCS Istituto Auxologico Italiano. Genomic DNA was isolated from whole blood using Wizard® kit (Promega). *C9orf72* repeat expansion was evaluated by a combination of fluorescent amplicon-length analysis and repeat-primed PCR (RP-PCR) as previously described (26). In the first amplification reaction we used primers flanking the repeat sequence (FAM-labeled forward 5'-TGTA AAACGACGGCCAGTCAAGGAGGGAAACAA CCGCAGCC-3' and reverse 5'-GCAGGCACCG CAACCGCAG-3'). For RP-PCR, three primers were used (FAM-labeled forward 5'-TGTA AAC GACGGCCAGTCAAGGAGGGAAACAACCG-CAGCC-3'; reverse 5'-CAGGAAACAGCTATGA CCGGGCCCGCCCGACCGCCCGGCC-CCGGCCCCGG-3'; reverse anchor M13 primer 5'-CAGGAAACAGCTATGACC-3') with AccuPrime™ GC-Rich DNA Polymerase (Invitrogen) and 180µM 7-deaza-2-deoxy GTP (Roche). Amplicons were run on ABI Prism 3500 (Applied Biosystems) and visualized using Gene Mapper v.4 software (Applied Biosystems).

Southern blotting

Genomic DNA (10 µg) was XbaI-digested and run on 0.7% agarose gel. After gel washes in 0.25N HCl for 20 min, in 0.4N NaOH and 0.6M NaCl for 30 min and in 0.5M Tris pH 7.5 and 1.5M NaCl for 30 min, DNA was transferred to a Hybond N+ membrane (Amersham) in 20X Saline Sodium Citrate (SSC) buffer. A unique-sequence probe (466bp) within *C9orf72* first intron was PCR amplified (forward 5'-CTTTCTCCAGA TCCAGCAGCCTCC-3' and reverse 5'-CTGAG TTCCAGAGCTTGCTACAG-3') and α -³²P-dCTP radiolabelled by random primer labeling kit (Thermo Fisher). Probe hybridization was carried out in 50% formamide, 5X SSC, 5X Denhardt, 0.5% SDS and 25 µg/µl salmon sperm at 42 °C overnight. The membrane was washed in 2X SSC/0.1% SDS buffer at room temperature and in 0.5X SSC/0.1% SDS buffer at 60 °C for 20 min each and visualized by autoradiography.

For the non-radioactive Southern Blotting, the same probe was labeled using Digoxigenin-dUTP and PCR DIG Probe Synthesis Kit (Roche). Probe hybridization was performed with Dig Easy Hyb buffer (Roche). The DIG Nucleic Acid Detection Kit (Roche) was used and hybridization signals were visualized by the image system Azure c200 (Azure Biosystems).

Bisulfite-sequencing of *C9orf72* promoter

Blood genomic DNA (200 ng) was treated with sodium bisulfite using the EZ DNA Methylation™ Kit (Zymo Research). A semi-nested PCR was performed with methylation-specific primers (BSP_1F 5'-TTTATTAGGGTTTG TAGTGGAGTTTT-3' or BSP_2F 5'-TATTAGGGTTTG TAGTGGAGTTTT-3', and BSP_1R 5'-AAATCTTTTCTTATTCACCCTCA AC-3'). The methylation status of the 450bp-sequence encompassing 26 CpG sites was assessed by Sanger sequencing on ABI Prism 3500.

NGS target resequencing

The custom-designed NGS panel includes 48 genes associated to ALS (*ALS2*, *ANG*, *DCTN1*, *FUS*, *HNRNPA1*, *HNRNPA2B1*, *MATR3*, *NEK1*, *OPTN*, *PFN1*, *SETX*, *SOD1*, *SPAST*, *SPG11*, *SQSTM1*, *TARDBP*, *TBK1*, *TUBA4A*, *UBQLN2*, *VAPB*), FTD (*CHMP2B*, *GRN*, *MAPT*, *PRNP*, *VCP*), Alzheimer's disease (*APOE*, *APP*, *PSEN1*, *PSEN2*, *TREM2*) and PD (*ATP13A2*, *DJ1*, *DNAJC6*, *EIF4G1*, *FBXO7*, *GBA*, *GCH1*, *LRRK2*, *PARK2*, *PINK1*, *PLA2G6*, *PRKRA*, *SNCA*, *TAF1*, *TH*, *UCHL1*, *VPS13C*, *VPS35*). DNA libraries (Illumina) were sequenced on NextSeq 550 (Illumina).

For data analysis we considered only variants with a Non-Finnish European (NFE) MAF

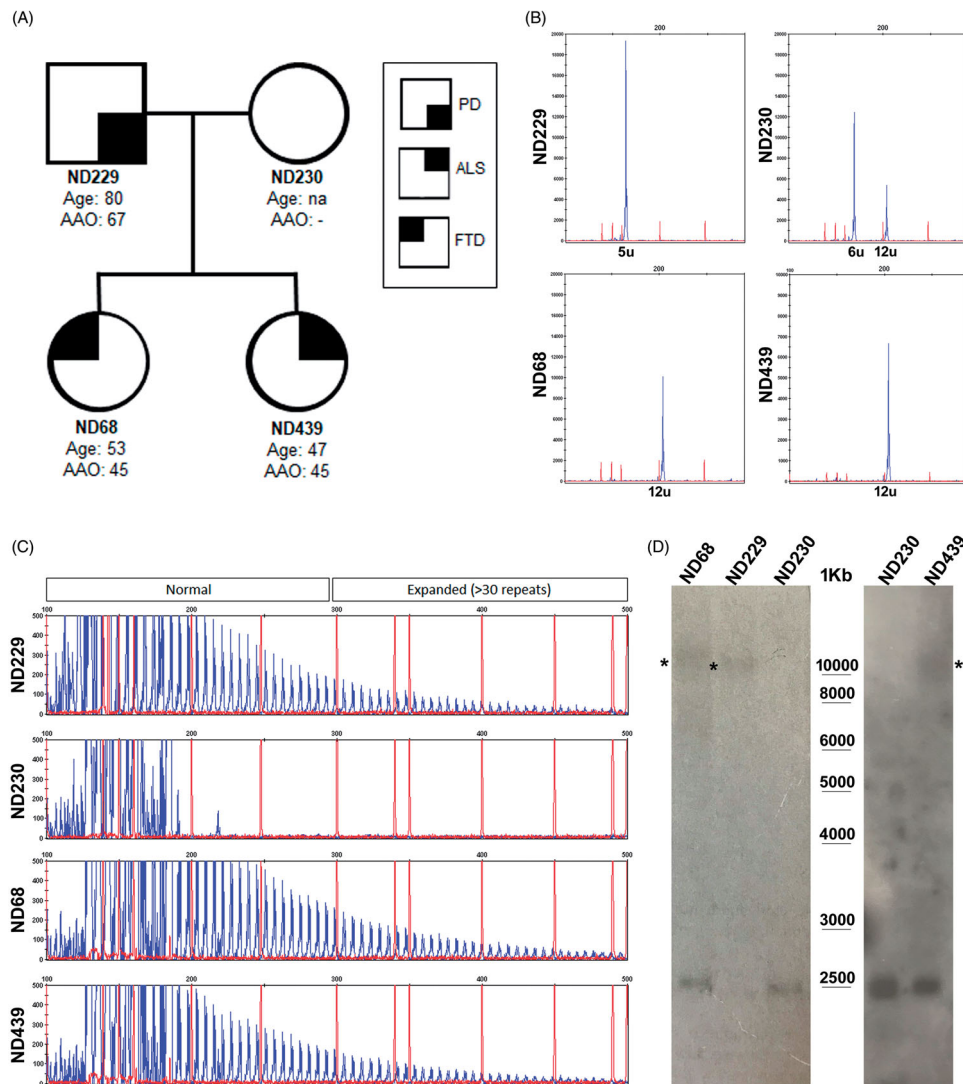


Figure 1. Mutational analysis of *C9orf72* gene in the Italian family case. (A) The pedigree of the investigated family is shown together with the diseases state of each member (PD, FTD, ALS), the age at onset (AAO) and the age at last follow-up; (B) *C9orf72* repeat sizing is shown by GeneScan run of fluorescent amplicons obtained using primers pairs flanking the hexanucleotide repeat; the number of repeat units is shown for each allele; (C) Electrophoregram of the fluorescent amplicons obtained by RP-PCR using a degenerate primer on the repeat sequence; the 6bp-interval peaks and the pathogenic threshold of 30 repeat units are clearly visible in the three affected members. (D) Southern blot analysis was first conducted by using a radioactive P^{32} -labeled probe (left panel) and then with a DIG-labeled probe (right panel). Genomic DNA was XbaI-digested and the 2.3Kb wild-type allele is visible. * expanded alleles; the wild-type mother DNA (ND230) was used as a negative control in the assay.

≤ 0.001 in gnomAD database and predicted to be nonsense, missense, insertions/deletions and splicing.

Results

Clinical report

We report an Italian family with 4 members (parents and two offsprings; Figure 1(A)) who came to our attention because the proband (ND68), at 45 years of age, started to manifest subacute delirium with fluctuating psycho-motor agitation, spatial/temporal disorientation, confusion, confabulation, paranoid/persecution type delusions and multimodal hallucinations. At onset the disorder was fluctuating with periods of remission, but

then it became persistent with night worsening. No fever, headache, brain injury, previous infectious event, vaccinations or psychiatric disorders were reported, neither history of alcohol or psychoactive substances. Routine blood sampling and brain CT scan were normal. She was admitted to the psychiatric ward and treated with neuroleptic drug and benzodiazepine with gradual improvement of psychotic symptoms. She was diagnosed with a suspected major depressive episode but, due to late onset and atypical course, the possibility of a neurological disease was considered. Neurological examination evidenced a generalized and symmetric hypokinesia and extrapyramidal rigidity probably due to drug-induced parkinsonism. No tremor or MND signs were present. Antipsychotic drugs were reduced with reappearing of a severe confusional state.

Neuroleptic drug was resumed with symptoms control. Routine blood tests, thyroid function, anti-thyroglobulin and thyroid peroxidase antibodies, ACTH, serum and urine cortisol, Vitamin B12, folate, ceruloplasmin, serum and urine copper were normal. Antibody tests for HBV, HCV, HIV 1-2 and *Borrelia burgdorferi* were negative. Serologic tests for systemic autoimmune diseases, neoplastic markers, onconeural antigens, and serum anti VGKC-complex, NMDAR and GAD antibodies were negative. Cerebrospinal fluid (CSF) analysis and cytology were normal with no oligoclonal bands. There was a minimal reduction of CSF beta-amyloid protein with Tau, P-Tau and 14.3.3 proteins in normal range. EEG was normal and brain MRI with gadolinium showed a mild widening of subarachnoid spaces without parenchymal signal alterations or contrast-enhancing lesions. Neuropsychological testing showed a mild cognitive impairment with executive dysfunction, attention and judgment deficit, visuospatial alterations, loss of empathy, motivation, planning and difficulty in performing multiple tasks. Short-term memory deficit was also detected. She did not develop MND signs. She was finally diagnosed with a form of FTD and we decided to further inspect her family.

Her father (ND229) was diagnosed with PD associated with delusions of paranoid type. When he was 67, he began presenting resting tremor to the right hand and minimal motor discomfort. He also complained of a memory deficit due to recent events, insomnia, and behavioral alterations. Neurological examination showed a fine tremor at rest and a shaded plastic hypertonus in the upper limbs prevalent on the right. Brain MRI showed suffering of the periventricular white substance as from chronic microcirculation disorders. He started therapy with pramipexole with improvement in tremor and insomnia and good control of motor and cognitive-behavioral symptoms for at least two years. At the age of 69 he developed psychiatric symptoms with delusional ideas. A history of nonspecific psychiatric disorder was reported also in his mother. Neuropsychological testing showed a mild cognitive impairment with executive dysfunction with difficulty in performing multiple tasks (MMSE: 21). In the next years disease worsened and DOPA was introduced with few beneficial effects. The patient began to show a progressive cognitive impairment with hallucinations and delirium. No MND signs were present until the last follow-up (Figure 1(A)).

The proband's mother (ND230) did not report any neurological sign or family history of neurodegenerative diseases. Recently, also the proband's sister (ND439) came to our attention at the age of 47 because in the last two years she started to show a disorder of deambulation due to muscle weakness in the legs with bilateral steppage. She

presented a spinal form of ALS, more severe in lower limb. Spasticity was present in upper and lower limbs. Bilateral hand muscle wasting was evident, bulbar muscles were still spared. EMG showed active denervation in all limb muscles examined. Cerebral and cervical MRI were normal. She started oral therapy with riluzole. Neuropsychological testing showed a constructive apraxia with no global cognitive impairment. She did not develop extrapyramidal signs, at least at the last follow-up two years after disease onset.

Mutational analysis of C9orf72 gene

Given the clinical features of the ND68 proband and of her ND229 father (Figure 1(A)) with a family history of psychiatric symptoms, we performed a mutational analysis of *C9orf72* gene. We found that both ND229 and ND68 carried a single wild-type allele of 5 and 12 repeat units, respectively, and the pathological HRE, while the mother (ND230) carried two wild-type alleles (6 and 12 units) (Figure 1(B,C)). Since the proband showed an earlier disease onset compared to the father, we first evaluated HRE length. We performed a radioactive Southern Blot and found that both the proband and her father carried a similar HRE length (~1500 repeats)(Figure 1(D), Table 1). When also the ALS-affected sister ND439 came later to our attention (Figure 1(A)), *C9orf72* mutational analysis revealed a single 12-repeat wild-type allele and the HRE (Figure 1(B,C)). HRE sizing by DIG-labeled Southern Blot showed that she also carried ~1500 repeat units-expansion (Figure 1(D)).

Analysis of C9orf72 genetic and epigenetic modifiers

We investigated possible genetic and epigenetic disease modifiers, already associated in literature to *C9orf72*, other than HRE size.

We first searched for additional rare variants by using a custom targeted resequencing gene panel which included 48 causative genes associated to ALS, FTD, AD and PD (gene list in Materials and Methods). The proband ND68 and her father ND229 carried a rare heterozygous *ATP13A2* (PARK9) variant (p.Ile946Phe; MAF = 0.001)(Table 1). The father also carried a heterozygous variant in the PD-associated gene *EIF4G1* (p.Ala13Pro), never reported before (Table 1) and predicted to be “deleterious” and “possibly damaging” by SIFT and Polyphen2 prediction tools, respectively. No additional pathogenic variants were identified in the ALS-affected sibling ND439 (Table 1).

We then analyzed the epigenetic state of *C9orf72* promoter by bisulfite sequencing. We found no DNA methylation either in the father or in the two siblings and in the wild-type mother at

Table 1. Summary of phenotypic, genotypic and epigenetic features of the *C9orf72* family members.

Family disease features	ND230	ND229	ND68	ND439
Disease presentation	None	PD	FTD	ALS
Age at onset (years)	na	67	45	45
Age at last follow-up (years)	na	80	53	47
Disease duration at last follow-up (years)	na	13	8	2
<i>C9orf72</i> allele sizing (n. units)	6/12	5/1500	12/1500	12/1500
Gene variants (GnomAD frequency ≤ 0.001)				
<i>ATP13A2</i> ;NM_022089;c.A2836T;p.I946F (MAF =0.001)	na	X	X	–
<i>EIF4G1</i> ;NM_198241;c.G37C;p.A13P (MAF <0.001)	na	X	–	–
<i>C9orf72</i> promoter methylation (26 CpG sites)				
Number of methylated CpG sites at each allele	0/0	0/0	0/0	0/0

MAF, Minor Allele Frequency; na, not applicable.

any of the 26 CpG sites analyzed in the promoter region (Table 1).

Discussion

We here describe an Italian family in which the presence of *C9orf72* HRE segregates with different disease manifestations, including PD, FTD and ALS, and different ages of onset. By investigating whether genetic and/or epigenetic factors might act as disease modifiers, we first analyzed HRE size and found a similar pathological length in all mutated members. A clear relationship between HRE size and disease severity or clinical manifestations in *C9orf72* mutation carriers is not consistently supported in literature (5,27–30), in contrast to the other repeat expansion disorders. Some studies have provided supportive evidence for disease anticipation in families with a *C9orf72* mutation (5,31) although in contrast to other studies (28) and to reports of HRE contraction across generations (7), leaving this issue still inconclusive.

In our family the two siblings showed the same age of onset and HRE size, but different clinical manifestations, thereby excluding a role for HRE size in influencing disease presentation. However, given the high degree of HRE somatic mosaicism (29), we can't exclude a different HRE length in the affected brains compared to blood.

The size of the wild-type alleles in *C9orf72* carriers has also been considered a possible risk factor because the >8-units alleles, rare in the normal population, are functionally associated to a decreased transcription of *C9orf72* gene in a length-dependent manner (5,9). The 12-units wild-type alleles in the two daughters might represent a still undefined risk factor, since no evidence of a differential *C9orf72* gene expression in association to the wild-type allele repeat size was demonstrated in ALS/FTD patients so far.

As a multifactorial disease, ALS is largely characterized by an oligogenic etiology also in familial cases where double mutations in ALS/FTD causative genes have been reported (15–18). In our

NGS-based mutational analysis of a wide array of genes implicated not only in ALS and FTD, but also in PD and AD, we identified two rare variants in heterozygous state in *ATP13A2* and *EIF4G1* genes. Of interest, the father carried both these variants, while the FTD daughter shared only the *ATP13A2* one. Homozygous and compound heterozygous mutations in the *ATP13A2* gene were shown to cause a rare, atypical form of recessive juvenile parkinsonism with dementia, known as Kufor–Rakeb syndrome (32). *ATP13A2* is a lysosomal protein found in Lewy bodies, whose loss-of-function leads to α -synuclein accumulation (33). Single rare *ATP13A2* variants were also associated to sporadic PD patients (34,35) who showed a decreased expression of *ATP13A2* gene in dopaminergic neurons of the substantia nigra (36). In contrast to the *ATP13A2* p.Ile946Phe variant we identified, predicted to be tolerated and previously described also in a healthy control (35), the novel *EIF4G1* p.Ala13Pro variant in the father was predicted to be deleterious by *in silico* analyses. Although variants in *EIF4G1* gene were initially identified in familial and sporadic PD (37,38), their association to PD is now disputed (39). Therefore we can only speculate that the presence the two rare variants in *ATP13A2* and *EIF4G1* genes may confer a higher risk to the father to develop PD. Given that the ALS-affected sister did not carry any rare variant in the selected genes, we can exclude their contribution in influencing disease presentation within the family. However, our targeted NGS approach, although quite extended to comprehend 48 genes, may miss rare variants in still unknown genes for ALS/FTD and other neurodegenerative disorders. Only whole-exome or whole-genome sequencing will allow to definitely clarify whether additional genetic variants may act as disease modifiers in this *C9orf72* family.

Finally, as a possible epigenetic disease modifier, we studied the methylation state of *C9orf72* gene promoter whose hypermethylation occurs in about 30% of *C9orf72* ALS and FTD carriers and

acts as a potential neuroprotective factor, being associated to decreased *C9orf72* gene expression, DPR synthesis and cerebral atrophy (23–25). Although *C9orf72* promoter was unmethylated in all three family members, ruling out an association with this epigenetic modification, methylation is tissue-specific so that we can not completely exclude a different methylation pattern in the affected brains compared to the blood genomic DNA analyzed.

In conclusion, by investigating the possible role of different disease modifiers within a *C9orf72* family, our study failed to find a clear association between disease presentation and HRE length, oligogenic inheritance or promoter methylation, suggesting that the high phenotypic variability of the mutation carriers likely relies on a more complex interplay of still unknown genetic factors, possibly including the wild-type allele repeat size, undefined environmental risk factors and other epigenetic modifications.

Declaration of interest

V.S. is in the Editorial Advisory Board of Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration. All the other Authors report no conflicts of interest.

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