Reduced mtDNA Copy Number in the Prefrontal Cortex of C9ORF72 patients

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Research Article

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Abstract

Hexanucleotide repeat expansion in *C9ORF72* gene is the most common genetic cause of amyotrophic lateral sclerosis and frontotemporal dementia (C9ALS/FTD). Loss of C9ORF72 protein function and a toxic gain-of-function directly by the RNA or RAN translation have been proposed as triggering pathological mechanisms, along with the accumulation of TDP-43 protein. In addition, mitochondrial defects have been described to be a major driver of disease initiation. Mitochondrial DNA copy number has been proposed as a useful biomarker of mitochondrial dysfunction. The aim of our study was to determine the presence of mtDNA copy number alterations in C9ALS/FTD patients. Therefore, we assessed mtDNA copy number in *postmortem* prefrontal cortex from 18 *C9ORF72* brain donors and 9 controls using digital droplet PCR. A statistically significant decrease of 50% was obtained when comparing *C9ORF72* samples and controls. This decrease was independent of age and gender. The reduction of mtDNA copy number was found to be higher in patient samples presenting abundant TDP-43 protein inclusions. A growing number of studies demonstrated the influence of mtDNA copy number reduction on neurodegeneration. Our results provide new insights into the role of mitochondrial dysfunction in the pathogenesis of C9ALS/FTD.

Introduction

In 2011, abnormal expansion of a GGGGCC (G_4C_2) hexanucleotid repeat in a non-coding region of the *C9ORF72* gene was identified as the most common genetic cause of familial and sporadic forms of both amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (C9ALS/FTD) [1, 2].

Although the mechanisms of disease of C9ALS/FTD remain unknown, three pathological mechanisms of *C9ORF72* gene mutation have been described. First, loss of function of the C9ORF72 protein through haploinsufficiency [1-3]. Second, a toxic RNA gain-of-function suggested by the accumulation of sense and antisense RNA transcripts containing the G_4C_2 repeat within nuclear foci [4]. Third, gain of function by repeat-associated non-ATG initiated (RAN) translation of dipeptide-repeat protein (DPR) from the sense and antisense strand [reviewed in 5]. Five potential DPRs, called ‘poly(GA)’, ‘poly(GP)’, ‘poly(GR)’, ‘poly(PA)’ and ‘poly(PR)’, can be produced by RAN translation [6]. Although the role of DPR in the development of the disease remains uncertain, aggregation of poly(GR) has recently been observed in the brain of patients and associated with neurodegeneration [7, 8]. Apart from DPRs, *postmortem* examinations of C9ALS/FTD brain tissue exhibit cellular inclusions containing transactive response DNA-binding protein 43 kDa (TDP-43). The topographical distribution of this pathology usually determines the phenotype of the disease, i.a. ALS vs FTLD and correlates with the degree of degenerative changes [reviewed in 9]. Of these features, DPR pathology and nuclear RNA foci are unique and highly specific to C9ALS/FTD. In addition to these three molecular mechanisms, many more downstream cellular pathways have been described to be affected in C9ALS/FTD, including nucleo-cytoplasmic transport, RNA processing, function of nucleolus, formation of membrane-less organelles, translation and ubiquitin proteasome system alterations, and DNA damage repair pathways impairment [reviewed in 5, 10]. Despite all efforts, nowadays it is still unclear which molecular events initiate the disease.

Mitochondrial dysfunction is known to be a hallmark of a broad spectrum of diseases, including neurodegenerative disorders, and variation in the mitochondrial DNA (mtDNA) copy number is used as a biomarker of mitochondrial dysfunction. In fact, it is known that mtDNA levels fluctuate in response to the physiological environment surrounding the cell [11], and they have been an attractive, blood-based, non-invasive biomarker for various diseases such as Parkinson's disease and Alzheimer disease [12, 13]. There are several studies supporting a role for mitochondrial dysfunction in the pathogenesis of C9ALS/FTD. It has been shown that poly(GR) in *C9ORF72* neurons compromise mitochondrial function and cause DNA damage in part by increasing oxidative stress [14, 15]. Importantly, these *in vitro* studies revealed that when reducing the levels of poly(GR) or the induced oxidative stress, disease phenotypes were partially suppressed or reversed [14, 15]. Overall, these studies pointed out that mitochondrial defects are a major driver of disease initiation in C9ALS/FTD. In an attempt to provide more insights into the role of mitochondrial dysfunction in C9ALS/FTD, we assessed the mtDNA copy number in human *postmortem* samples of the prefrontal cortex of *C9ORF72* patients in order to decipher its role in the pathogenesis of the disease.

Materials And Methods

Subjects

Deep frozen *postmortem* prefrontal cortex was obtained from 18 C9ALS/FTD brain donors (8 males and 10 females) and 9 controls (5 males and 4 females) with no clinical evidence or neuropathological finding of ALS/FTD. All donors were of Caucasian origin. The disease duration, defined as the time between symptoms onset and death, in the C9ALS/FTD group ranged from 1 to 21 years. Table 1 summarizes clinical, molecular and neurophathological findings of subjects recruited in the study.

No significant difference was observed in gender composition between both groups (p=0.6). However, significant differences were detected in terms of C_4C_2 repeat length and age. While all C9ALS/FTD samples exhibited a G_4C_2 repeat expansion greater than 145 repeats, control samples had normal G_4C_2 repeat size ($\leq$23 G_4C_2). The mean age ± standard deviation (SD) in years was 79±11 for C9ALS/FTD patients (range 55-88) and 67±9 for controls (range 58-90) (p=0.004). Therefore, age was taken into account as a co-variable for statistical correction.

The study was approved by the Ethics Committee of the Hospital Clinic of Barcelona. Brain samples were obtained from the Hospital Clinic-IDIBAPS Brain Bank under approved protocols of the Ethical Committee (Hospital Clinic Barcelona, Spain).

Brain sample preparation

Total mitochondrial and genomic DNA was extracted for each sample using a method that preserves the *in vivo* ratio between them as previously described [16]. Briefly, the frozen brain tissue was rapidly homogenized in 1ml of 100ST-DNA, RNA and protein solubilization buffer (#DCQ100ST, DireCtQuant, Spain) per 10mg of brain tissue using Dounce homogenizer with 25 µm clearance. The samples were

incubated at 90ºC for 3min and centrifuged at 10.000 rcf for 10min. The clear supernatant was transferred to a fresh tube and used to measure the absolute mtDNA copy number and nuclear genomes by digital droplet PCR (ddPCR).

Neuropathological examination

Immunohistochemistry was performed on formalin-fixed and paraffin-embedded tissue blocks applying Anti-C9RANT (Novus Biologicals, Centennial, CO, USA; dilution 1:1,000) and Anti-phosphorylated TDP-43 antibodies (Cosmo Bio, Tokyo, Japan; dilution 1:20,000). The DAKO EnVision© detection kit (Dako, Glostrup, Denmark) was used for visualization of antibody reactions. The frequency of TDP-43 aggregates was scored following a semiquantitative analysis in the frontal cortex. A four-point grading scale (0.5= isolated; 1= sparse; 2= moderate and 3= abundant) was used to evaluate the severity of TDP-43 immunoreactivity. No attempt was made to score cytoplasmic and neuritic lesions separately, but most cases with a high TDP-43 score had dystrophic neurites in addition to neuronal cytoplasmic inclusions.

mtDNA copy number assessment by digital droplet PCR

Digital droplet PCR (ddPCR) technology was chosen to measure the absolute number of mtDNA copies per diploid genome as previously described [16]. Briefly, mtDNA copy number was measured using QX200 Droplet Digital PCR System following manufacture's recommendations (Bio-Rad Laboratories, Inc.) Specific primers were used to amplify a 92 bp fragment from the human mitochondrial sequence and TBP-1 and TEFM genes were used as reference genes.

The presence or absence of amplification per droplet was evaluated using a QX200 Droplet Reader and analyzed using the QuantaSoft Analisys Pro (Bio-Rad Laboratories) software. An assumption that a diploid genome contains 2 copies of each single copy gene *TBP-1/TEFM* was used. mtDNA copy number was calculated by dividing the number of mtDNA copies detected by number of diploid genomes (measured by *TBP-1/TEFM*).

Statistical analysis

Results were expressed as mean ± standard error means (SEM). For comparisons of the means, the statistical significance of the differences was examined using general linear model univariate analysis, the parametric independent *t*-Test or the non-parametric Mann–Whitney U and Kruskal-Wallis tests. Correlations were studied using Pearson correlations. All statistical analyses were performed by the SPSS program, version 25 (SPSS, Inc. Chicago, IL, USA). Significance was accepted for p-value <0.05.

Results

We studied the mtDNA copy number in *postmortem* prefrontal cortex obtained from 18 C9ALS/FTD patients compared to 9 control samples. The amount of mtDNA copy number was analyzed by ddPCR and results are shown as the mtDNA copy number per diploid genome (Figure 1). A statistically

significant decrease was observed when comparing C9ALS/FTD samples (3,923 mtDNA copies/diploid genome ±566) with controls (7,364 mtDNA copies/diploid genome±909) (p=0.003) (Figure 1). Moreover, taking into account the age of individuals as a co-variable the reduction in the mtDNA copy number in *C9ORF72* prefrontal cortex was statistically significant (linear model univariate analysis C9ALS/FTD *vs*. controls with age as a covariate, marginal means for C9ALS/FTD: 3,818±648 and controls: 7,574±968; p=0.006). We further performed Pearson correlation analysis to determine the association between mtDNA copy number and age in C9ALS/FTD patients and controls. There was no correlation between mtDNA copy number and age (R^2=0.05, p=0.26).

Having shown a significant 50% decrease in the mtDNA copy number in prefrontal cortex of C9ALS/FTD patients, we investigated whether this decline was correlated with the abundance of DPR inclusions or TDP-43 aggregates. Neuropathological examination evidenced DPR inclusions and TDP-43 aggregates in all frontal cortex of C9ALS/FTD patients. While no differences could be determined regarding the abundance of DPR inclusions since all samples exhibited high abundance of these inclusions across all cortical layers, the semiquantitative analysis of TDP-43 aggregates showed differences in its frequency (Figure 2). The correlation analysis with mtDNA copy number evidenced that, C9ALS/FTD patients with isolated or sparse TDP-43 inclusions (n=6) showed a reduction of 35% in the mtDNA copy number compared to controls (Figure 3 and Table 2). This reduction was more evident in the group of C9ALS/FTD patients with moderate/severe inclusions (n=12), in which a mtDNA copy number reduction of 63% was obtained when comparing to the control group and of 42% when comparing to C9ALS/FTD patients with isolated or sparse TDP-43 inclusions (Figure 3 and Table 2). Statistical analysis identified only significant differences when comparing mtDNA copy number between controls and C9ALS/FTD patients with moderate/abundant TDP-43 inclusions (p=0.009) (Figure 3 and Table 2). These results suggest that C9ALS/FTD patients with isolated/sparse TDP-43 inclusions in prefrontal cortex brain region might represent an intermediate state in terms of neuronal cell degeneration or death.

Finally, we further analyzed whether there was a relationship between mtDNA copy number and clinical manifestation of *C9ORF72* expansion patients. Patients were divided into three groups based on clinical manifestations: C9ALS, C9FTD and C9ALS/FTD. Although no statistically significant differences were obtained, those patients diagnosed as C9ALS/FTD showed the lowest mtDNA copy number value (Figure 4).

Discussion

Mitochondria are subcellular organelles which have multiple important roles, including ATP production, Ca^2+ homeostasis, and reactive oxygen species (ROS) production [reviewed in 17]. Each mitochondrion contains multiple copies of mtDNA that encode the transfer RNAs and mitochondria specific ribosomal RNAs necessary for the mitochondrial protein synthesis, as well as the essential proteins required for oxidative phosphorylation. Mitochondrial DNA integrity is necessary for mitochondrial maturation during differentiation of neural stem cells, and in fact, several quality control systems counteract processes that lead to mitochondrial dysfunction [18]. Loss of mtDNA integrity by altered mtDNA copy number and

increased mutations is implicated in cellular dysfunction with aging and disease [19]. A growing number of studies demonstrated the influence of mtDNA copy number reduction on neurodegeneration [eg. 20-22].

Although the exact pathogenesic mechanism of C9ALS/FTD is still elusive, several evidences suggest that damaged mitochondrial likely play a fundamental role [23-25]. The relevance of DPR pathology in causing C9ALS/FTD has also been widely discussed and it is currently believed that its production is a key molecular event downstream of *C9ORF72* repeat expansion [24, 26-28]. In *C9ORF72* cellular and animal models it has been reported that DPR proteins, specially poly(GR), have a toxic effect in an accumulative and age-dependent manner; compromising several cellular pathways such as mitochondrial function and causing DNA damage by increasing oxidative stress [7, 8, 14, 15, 29]. It has been suggested that DPR protein aggregation precedes and instigates TDP-43 pathology [30; reviewed in 31]. Recently it has been shown that toxic poly(GR) proteins mediate sequestration of full-length TDP-43 in an RNA-independent manner, promoting TDP-43 proteinopathy [32].

This study investigated the changes in mtDNA copy number in prefrontal cortex of patients with C9ALS/FTD. Additionally, the study evaluated the relationship between mtDNA copy number and the clinical phenotype. For these purposes, we used a ddPCR technique since it is a highly sensitive and specific method for absolute quantification of mtDNA copy number and the ratio of mtDNA per cell. Results showed a statistically significant mtDNA copy number reduction of 50% in C9ALS/FTD patients compared to controls ($p=0.006$). All our samples showed marked DPR pathology; making it difficult to establish differences, whereas the frequency of TDP-43 aggregate was different among the C9ALS/FTD samples analyzed. Even the sample size was relatively small, we found a positive correlation between the abundance of TDP-43 inclusions and the mtDNA copy number decrease, meaning that those patients presenting with moderate or abundant TDP-43 inclusions showed an even more pronounced mtDNA copy number reduction than those with isolated or sparse inclusions (Figure 3). The relationship between mitochondrial dysfunction and TDP-43 proteinopathy has been previously documented. Alterations in mitochondrial dynamics, trafficking and mitophagy have been reported in association with TDP-43 pathology [reviewed in 33]. To our knowledge, this is the first study that shows a reduction of mtDNA copy number in human C9ALS/FTD *postmortem* brain samples that correlates with the abundance of TDP-43 inclusions. Our results might reflect a late-stage of mitochondrial impairment and suggest that TDP-43 inclusion formation contributes in an accumulative manner reducing the mtDNA content and finally, further compromising survival or disease progression.

The *C9ORF72* expansion is associated with clinical heterogeneity, with affected patients displaying symptoms of either ALS or FTD, or mixed features of both. Although several factors have been analyzed, such as the repeat expansion length and the distribution and amount of TDP-43 and DPR protein aggregation, results are inconclusive and the molecular basis for this clinical variation is still unknown [reviewed in 31]. Reports on clinical and neuropathological correlation with DPR protein pathology have been conflicting. While some authors found no relationship between specific DPR species and clinicopathologic subtypes of *C9ORF72*-related disease [34], others describe either a correlation with

DPR distribution [35] or neurodegeneration [8]. In our experience the distribution of TDP-43, and not DPR pathology, determines the clinical phenotype (unpublished data). In this line, although our results need validation in a larger series of cases, they showed a positive tendency correlation between the mtDNA copy number and the clinical phenotype. mtDNA copy number in the prefrontal cortex of *C9ORF72* patients was lower in cases with ALS/FTD than in cases with ALS and FTD (Figure 4). Thus, although interpreted with caution, these results suggest that an impaired mitochondrial function could be more pronounced in *C9ORF72* patients with ALS/FTD phenotype.

Conclusions

Together, our results are in agreement with similar reductions in mtDNA copy number reported in a number of other age-related neurological disorders where mitochondrial dysfunction is also a pathogenic hallmark. We have observed a significant reduction of mtDNA copy number in C9ALS/FTD patients, particularly associated with the amount of TDP43 pathology. This novel finding might help to unravel the role of mitochondrial dysfunction in the pathogenesis of C9ALS/FTD. Overall, these data suggest that the preservation of mitochondrial genome copy number may play a crucial role in replacing damaged mitochondrial complexes to maintain ATP levels and preserve cell viability under stress. As in other neurodegenerative disorders, C9ASL/FTD should be considered as a complex progressive disease with multifactorial pathophysiology, in which different biological, environmental and genetic factors interplay in neuronal degeneration. The increased understanding of C9ALS/FTD pathogenesis casts hope for finally making available a true neuroprotective therapeutic strategy that can be applied at early stages of the disease.

Declarations

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Conflicts of interest: the authors declare no conflict of interest.

Ethics approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the Ethics Committee of the Hospital Clinic of Barcelona. Brain samples were obtained from the Hospital Clinic-IDIBAPS Brain Bank under approved protocols of the Ethical Committee (Hospital Clinic Barcelona, Spain).

Availability of data and material (data transparency): The datasets of the current study are available upon request with no restriction.

Code Availability: Not applicable.

Consent for Publication: Not applicable.

Consent to Participate: Not applicable.

Author contribution:

Maria Isabel Alvarez-Mora: formulation and evolution of overarching research goals. Preparation and presentation of the published work

Petar Podlesniy: development and design of methodology

Teresa Riazuelo and Nuria Serra: conducting research and investigation process

Laura Molina: provision of study materials and preparation of the published work

Ellen Gelpi: preparation and presentation of the published work and critical review.

Laia Rodriguez-Revenga: formulation and evolution of overarching research goals. Preparation and presentation of the published work

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Tables

Table 1. Clinical, molecular and neuropathological findings of patients and controls included in the study. TDP-43 inclusions were semiquantitatively assessed in the prefrontal cortex as: 0= absent, 0.5= isolated, 1= sparse; 2= moderate and 3= abundant.

ID sample	Gender	Age at disease onset (years)	Age at death (years)	Diagnostic group	C9orf72 (GGGGCC)n	TDP-43 inclusions in prefrontal cortex
Control_1	Male	-	78	control	2,2	0
Control_2	Male	-	83	control	2,7	0
Control_3	Female	-	81	control	2,8	0
Control_4	Male	-	64	control	2,6	0
Control_5	Female	-	90	control	2,2	0
Control_6	Male	-	58	control	2,13	0
Control_7	Female	-	88	control	2,4	0
Control_8	Female	-	83	control	5,10	0
Control_9	Male	-	86	control	2,2	0
*C9ORF72*_1	Male	51	61	FTD	>145	2
*C9ORF72*_2	Female	65	66	FTD/ALS	>145	2
*C9ORF72*_3	Female	68	71	FTD/ALS	>145	1
*C9ORF72*_4	Female	63	69	FTD/ALS	>145	3
*C9ORF72*_5	Male	61	66	FTD	>145	3
*C9ORF72*_6	Female	52	55	FTD	>145	3
*C9ORF72*_7	Male	87	88	FTD	>145	1
*C9ORF72*_8	Male	55	69	FTD	>145	2
*C9ORF72*_9	Female	53	57	ALS	>145	0.5
*C9ORF72*_10	Female	65	75	FTD	>145	0.5
*C9ORF72*_11	Female	55	58	FTD/ALS	>145	3
*C9ORF72*_12	Male	72	73	FTD	>145	2
*C9ORF72*_13	Female	67	69	FTD	>145	0.5
*C9ORF72*_14	Female	56	57	FTD/ALS	>145	3
*C9ORF72*_15	Male	52	55	ALS	>145	3
*C9ORF72*_16	Male	75	80	FTD	>145	1
*C9ORF72*_17	Male	65	69	FTD	>145	2

*C9ORF72*_18	Female	35	61	FTD	>145	3

FTD: frontotemporal dementia, ALS: amyotrophic lateral sclerosis.

Table 2. MtDNA copy number observed in prefrontal cortex from 9 controls and 18 C9ALS/FTD donors. Statistical analysis was performed by univariate linear model analysis. Data are expressed as marginal means estimated at 71 years of age ± standard error of the mean (SEM).

	Mean	SEM	p-value *vs* control
Controls (n=9)	7,883	960	
C9ALS/FTD (n=18)			
With isolated/sparse TDP-43 inclusions (n=6)	5,105	1,035	0.082
With moderate/abundant TDP-43 inclusions (n=12)	2,943	841	0.009

Figures

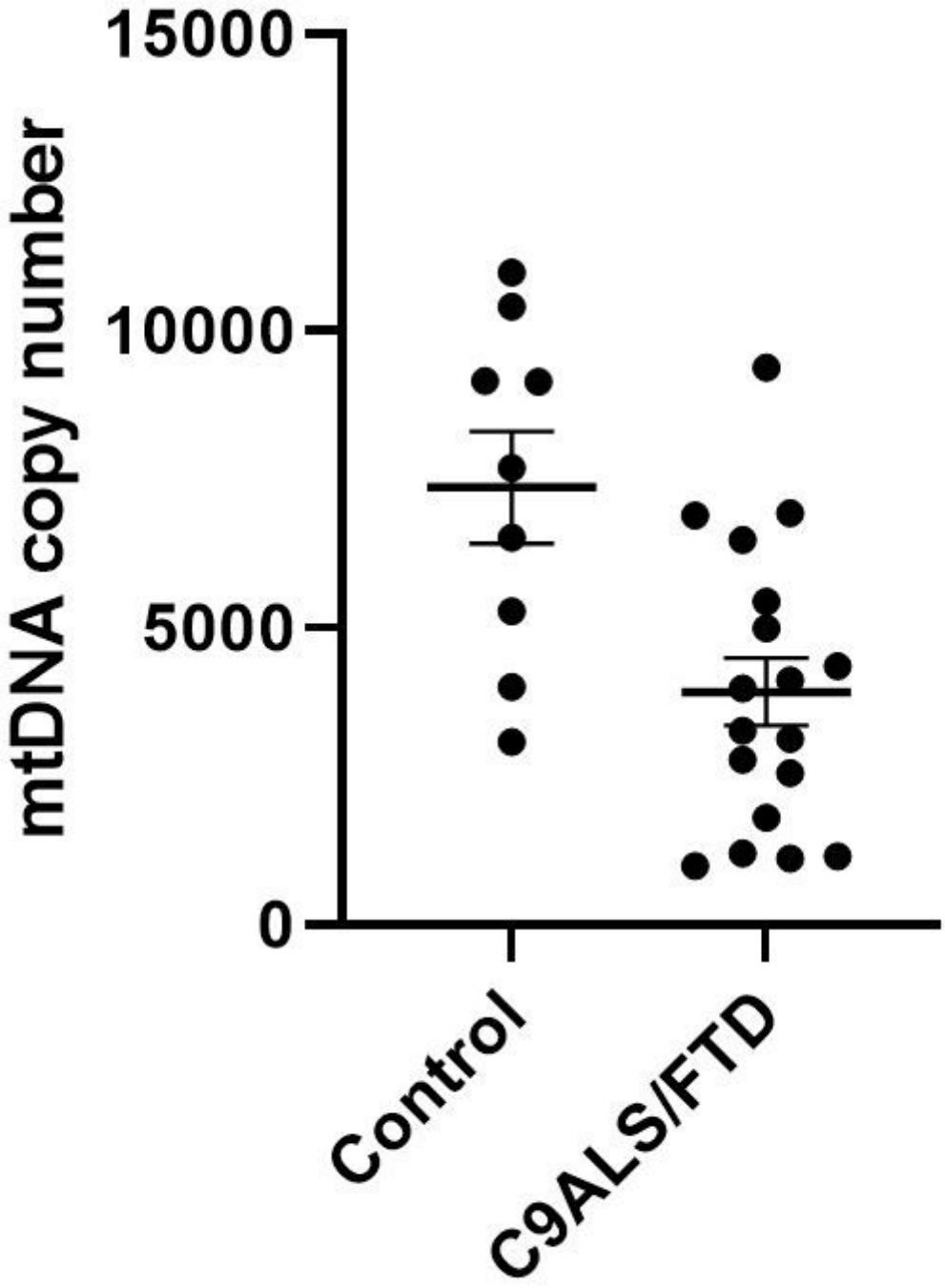

Figure 1

Comparison of mtDNA copy number in human postmortem prefrontal cortex from controls (n=9) and C9ALS/FTD (n=18) brain samples. Data is represented as mtDNA copies/diploid genome. The mtDNA copy number of C9ORF72 patients was significantly lower than that of the control group (p=0.003)

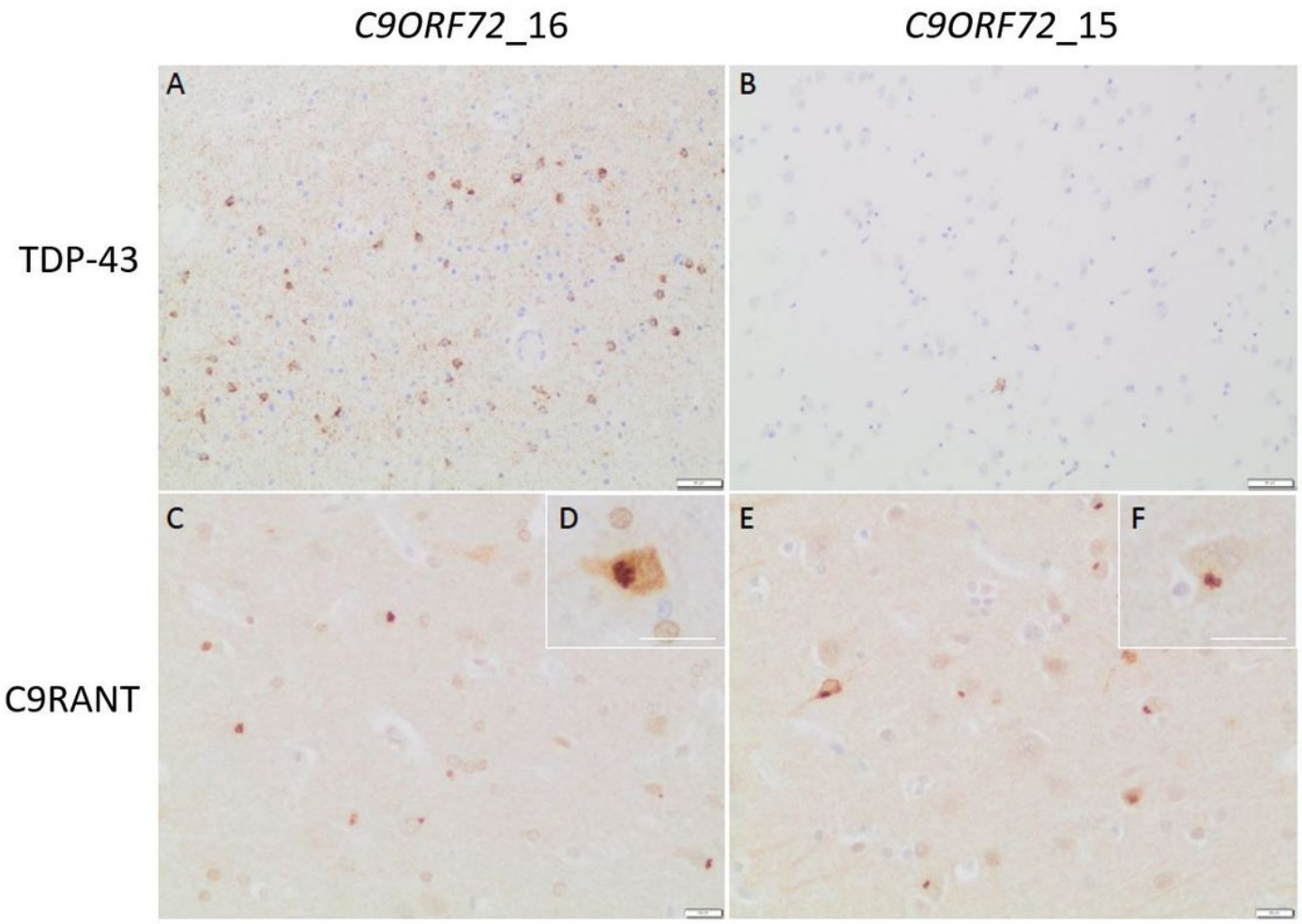

Figure 2

Representative postmortem neuropathological image of two C9ORF72 patients (C9ORF72_16 and C9ORF72_15 in Table 1) showing different frequency of TDP-43 inclusions. (a) Significant wispy threads and abundant small neurons with diffuse granular cytoplasmic TDP43 staining in C9ORF72_16 patient with moderate/abundant TDP-43 inclusions. (b) C9ORF72_15 patient with isolated/sparse TDP-43 inclusions in cortical areas. Isolated neuron with diffuse granular cytoplasmic TDP43 staining. No differences in the frequency of C9RANT inclusions were observed between the C9ORF72_16 patient with abundant (c) and the C9ORF72_15 with sparse (e) TDP-43 pathology. Detail of cytoplasmic C9RANT inclusions in neurons in C9ORF72_16 (d) and C9ORF72_15 (f) patient. Scale Bar A-B: 50um;C-F: 20um

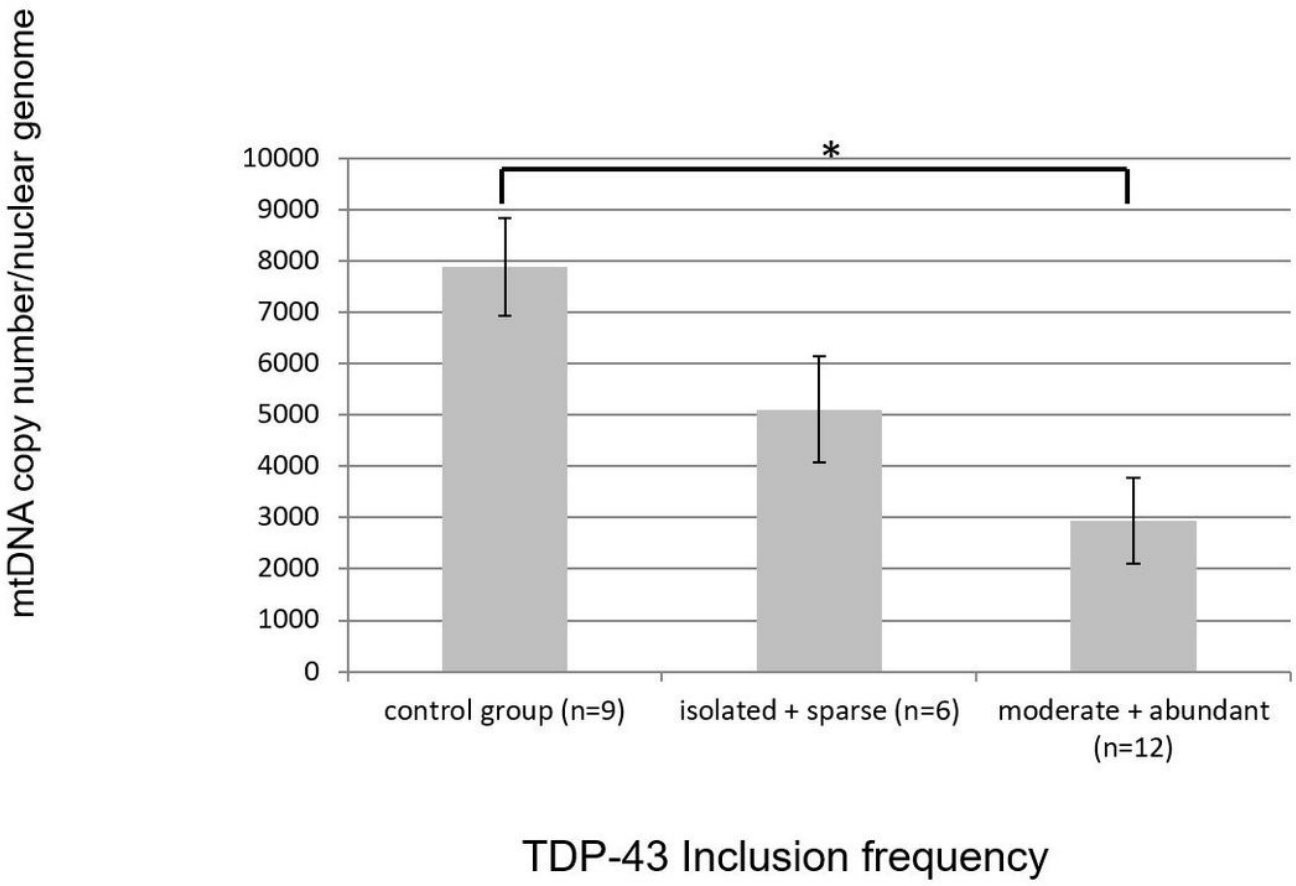

Figure 3

Relationship of prefrontal cortex mtDNA copy number and frequency of TDP-43 inclusions in C9ALS/FTD brain samples

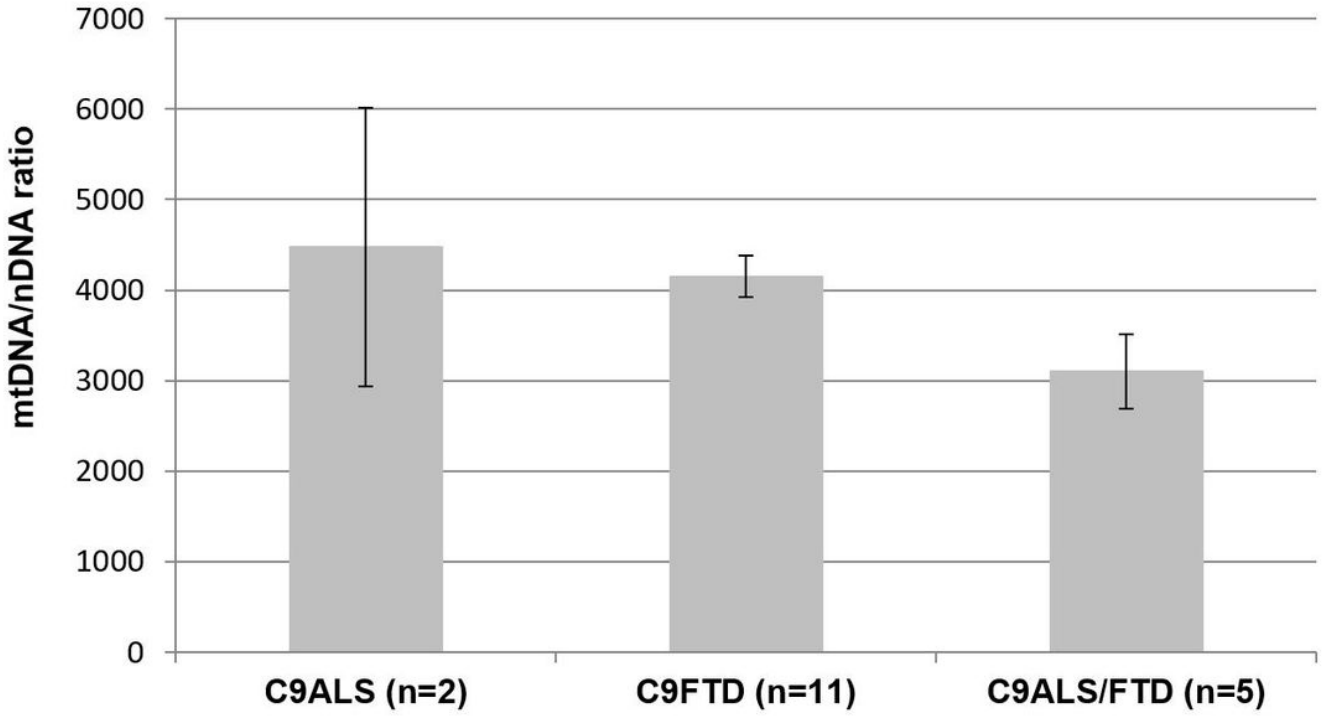

Figure 4

Effect of C9ORF72 expansion clinical phenotype on mtDNA copy number in ALS patients (n=2), FTD patients (n=11) and ALS/FTD patients (n=5). Data are expressed as mean±SEM