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GRN deletion in familial frontotemporal dementia showing association with clinical variability in 3 familial cases

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ABSTRACT

Progranulin (*GRN*) gene mutations have been genetically associated with frontotemporal dementia (FTD) and are present in about 23% of patients with familial FTD. However, the neurobiology of this secreted glycoprotein remains unclear. Here, we report the identification of 3 pedigrees of Southern Italian extraction in whom FTD segregates with autosomal dominant inheritance patterns. We present evidence that all the available patients in these 3 familial cases are carrying the rare *GRN* gene exon 6 deletion *g10325_10331delCTGCTGT* (*relative to nt 1 in NG_007886.1*), *alias Cys157LysfsX97*. This mutation was previously described in 2 sporadic cases but was never associated with familial cases. Our patients demonstrate heterogeneous clinical phenotypes, such as the behavioral variant (bv-FTD) in the affected men and the nonfluent/agrammatic variant of primary progressive aphasia (nfvPPA) in the affected woman. Haploinsufficiency was revealed by both quantitative real-time PCR of the gene and protein analyses. These findings provide further support for a previously proposed role for the *GRN* gene in the genetic etiology of FTD and its phenotypic variability.

1. Introduction

Frontotemporal dementia (FTD) represents a heterogeneous group of progressive neurodegenerative dementias with prominent behavioral alterations and distinct temporal syndromes associated with sporadic nonfluent/agrammatic variant of primary progressive aphasia (nfvPPA) and semantic disorders (Cairns et al., 2007; Warren et al., 2013). Early onset dementia develops in most patients between 45 and 65 years, mostly exhibiting the presence of local atrophy of frontal and/or temporal lobes (Rabinovici and Miller, 2010). About 30%–50% of cases are familial, with men and women equally affected. The main subtypes of FTD are the bvFTD, nonfluent/agrammatic variant of primary progressive aphasia (nfvPPA) and corticobasal degeneration (Gorno-Tempini et al., 2011; Mackenzie et al., 2009).

GRN mutations were recently reported to be associated with 10% of total FTD cases and with 23% of patients with a familial FTD history (Baker et al., 2006; Cruts et al., 2006). To date, the relationship between *GRN* mutations and its disease mechanism has not yet been described, but haploinsufficiency has been suggested to lead to neurodegeneration (Gass et al., 2006; Snowden et al., 2006; Ward and Miller, 2011).

The *g10325_10331delCTGCTGT (relative to nt 1 in NG_007886.1); alias Cys157LysfsX97* mutation, has only been reported in 2 sporadic

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cases to date (Caso et al., 2012; Le Ber et al., 2008). Here, we report the *g10325_10331delCTGCTGT* deletion in 3 familial cases that segregate with FTD and describe the clinical and the molecular characterization of the patients.

2. Methods

2.1. Participants and study design

The study was approved by the ethics committee of our institution and all participants signed informed consent. We enrolled 256 patients and 300 healthy, age-, sex- and geographic region–matched controls in the study. Patient inclusion criteria required a diagnosis of frontotemporal dementia (FTD), as defined by Rascovsky criteria (Rascovsky et al., 2011). Of the study cohort, 252 individuals had a diagnosis of bvFTD and 4 had nfvPPA. Among this cohort, we identified 20 familial cases with at least 2 individuals affected by dementia; only 1 patient from each family, who was affected by dementia, was selected for screening.

2.2. Patients analysis

Blood was collected, and genomic DNA was extracted from whole blood from the 256 enrolled patients by QIAamp DNA Blood Mini Kit (QIAGEN). Candidate genes *granulin, GRN* (NG_007886.1), and Microtubule-Associated Protein Tau, *MAPT* gene (NG_007398.1) were analyzed by sequencing DNA of one patient in each of the 20 families identified as described in the previous section. We sequenced all the coding exons of the *GRN* gene in these twenty patients. For the *MAPT* gene, we opted to start the analysis by sequencing the exons that were reported to carry the most frequent mutations, 1, 9–13. No mutations were found in these exons. Subsequently, in the absence of mutations in these exons, we analyzed the entire gene in the same 20 patients (primers used are detailed in Table 2A and B in SM). We identified the mutation in H72 A-FTD2 first, then we sequenced the DNA of the second patient H73 from the same family.

The remaining patients (256 minus 20) and the 300 controls were then all analyzed by PCR-amplification-refractory mutation system (ARMS) only to reveal the identified mutation g10325_10331delCTGCTG. PCR amplification was performed with SureCycler 8800 Thermal Cycler (Agilent). Primer sequences are reported in Supplementary Materials, Table 2A and B.

2.3. DNA PCR-ARMS

Exon 6 mutation *g10325_10331delCTGCTGT* once identified, was amplified by PCR-ARMS technique using normal and mutated oligonucleotides (Little, 2001; Newton et al., 1989). Amplifications were performed with the following primers: 5′-CAT TTT TTC TCA GGC TTC CTG CTG TG-3′ (forward Wild Type), 5′- GGA CCA TTT TTT CTC AGG CTT CAA G-3′ (forward Mut), and 5′- GCA GCC AGG ATG GAG GAA ACT-3′ (reverse). In each ARMS reaction, 30 ng of DNA was amplified in a final volume of 20 μL containing a mixture of 1X Buffer Taq Pol, 0.2-mM dNTPs, 2.5-mM $MgCl_2$, 0.5 μM of each primer, 0.05 U/μL of Ampli Taq Gold (Applied Biosystem). Amplification conditions were 95 °C for 10′, 35 cycles at 95 °C for 30″, 62 °C for 40″, 72 °C for 1′, and an elongation step of 10′ at 72 °C. PCR-ARMS was analyzed on 1.5% agarose gel electrophoresis, with ethidium bromide 0.5 mg/mL and visualized with UV light (UVITEC-Cambridge). We performed this test on the 256 patients and 300 controls matched by age, sex, and geographic region.

2.4. Brain imaging

Functional neuroimaging studies were performed in the patients from the A-FTD2 family. When we started the study, the affected sister was alive but has since died.

^{18}F-fluorodeoxyglucose Positron Emission Tomography (^{18}FDG-PET) studies were performed in male patient H72. Brain images were acquired for 15 minutes in 3-dimensional mode between 45 and 60 minutes after intravenous injection of 213–250 MBq of ^{18}FDG in a resting state with the eyes closed. The outcome measure was relative glucose metabolism. To highlight the patterns of relative metabolic reduction, we used voxel-based Statistical Parametric Mapping (SPM) analysis of FDG images. Images were normalized in the Montreal Neurological Institute space using SPM2 (Wellcome Department of Imaging Neuroscience, London, UK). The patient ^{18}FGD uptake was compared to that of 16 healthy subjects (mean age 56 ± 12 years, 6 males and 10 females) using the single subject condition covariate model in which age was considered as a nuisance covariate. SPECT study was performed in female patient H73 A-FTD2 40 minutes after the intravenous injection of [^{99m}Tc] ethyl cysteinate dimer. Brain images were acquired for 30 minutes.

3. Results

3.1. Genetic analysis by sequencing candidate genes

We sequenced *GRN* (NG_007886.1) and *MAPT* (NG_007398.1) and identified the *g10325_10331delCTGCTGT* mutation in *GRN* exon 6 in patient H72, from family A-FTD2. Then, we extended the analysis to H73, the female sibling in the same family. We screened the promoter, the coding regions, and the associated intronic splice junction. Although a number of polymorphic variants were found in both the *GRN* and *MAPT* genes, we did not identify other functional polymorphisms and mutations that could have been responsible for the association. Only *g10325_10331delCTGCTGT* clearly proved to segregate with the affected individuals in this family. This deletion was previously reported in 2 sporadic cases, a female that exhibited nfvPPA and a male that exhibited bvFTD (Caso et al., 2012; Le Ber et al., 2008).

We amplified the exon 6 mutation *g10325_10331delCTGCTGT* by the PCR-ARMS test in all of the remaining analyzed recruited patients and identified 2 additional mutation carriers: H225 and H153 (Fig. 1A lanes 6–7 and C and lanes 4 and 5). These 2 mutation carriers were later confirmed to carry the deletion by sequencing *GRN* exon 6 (Fig. 2). To exclude the possibility that this was a common variant, we screened for its presence by PCR-ARMS in the 300 healthy controls matched by age, sex, and geographic origin. We found no deletions carried by any of these individuals. These data strongly support the argument for pathogenicity and provide support for its exclusive association with the disease phenotype already published as a case report (Caso et al., 2012). However, we now describe it in 3 familial cases and focus on the exon 6 mutation *g10325_10331delCTGCTGT.*

The proband, a male patient, H72 in family A-FTD2 remains the only patient still alive in the family (Fig. 3, A-FTD2: H72-III2). The affected sister, H-73, died in 2015 but was alive when we started the study. One male cousin (III-4) was reported to have dementia characterized by cognitive or behavioral disorders, as did his mother, the aunt of the proband (II-2) and his uncle (II-1), and the father of H72-III-2 and H73-III-3 (Fig. 3, A-FTD2).

H72 is a 65-year-old right-handed man who developed problems with memory as the initial symptom of disease onset and later followed a course resembling FTD with frontal behavior disturbances. Neuropsychological assessment confirmed the presence of both short-term and long-term auditory verbal memory deficits in

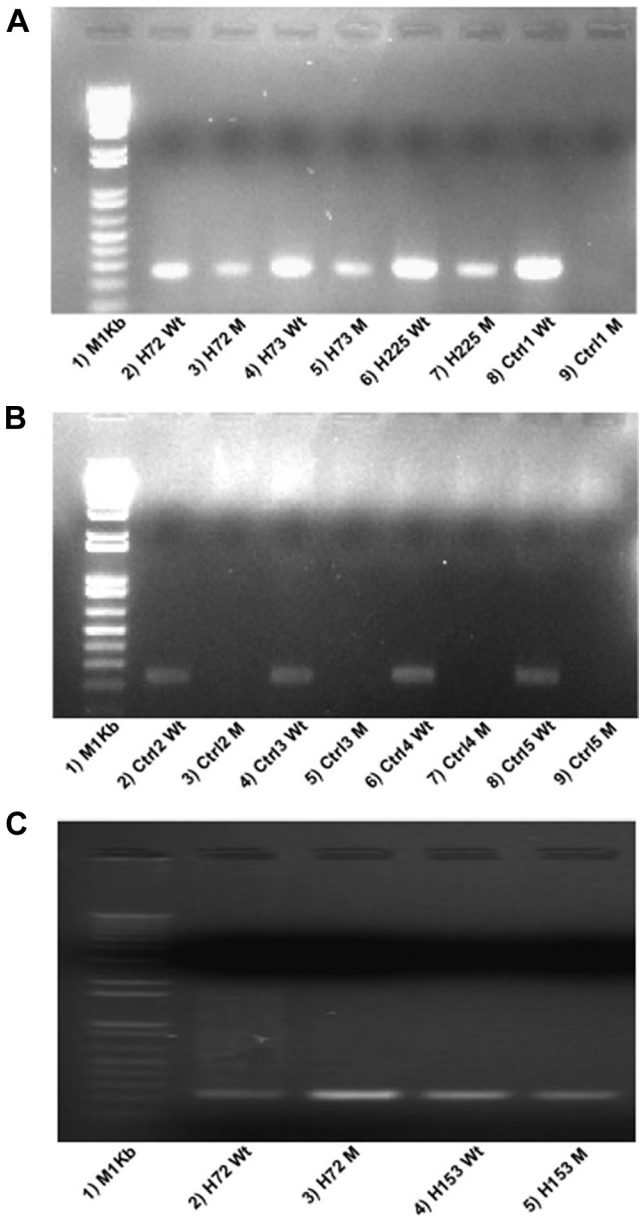

Fig. 1. ARMS-PCR amplifications. (A) Lanes 2, 4, 6, and 8: amplification with Wild type (Wt) forward primer and Wt reverse primers; lanes 3, 5, 7, and 9: amplification with M forward primer and Wt reverse primer. (B) In the absence of the mutation, only the Wt primer set is amplifying, as seen in lanes 2 Ctrl2 Wt, 4 Ctrl3 Wt, 6 Ctrl4 Wt, and 8 Ctrl5 Wt, whereas amplification with the forward M and Wt reverse primer sets fail in lanes 3, Ctrl2 M; 5, Ctrl3 M; 7, Ctrl4 M; and 9, Ctrl5 M. (C) Lanes 2 and 4: amplification with Wt forward and Wt reverse primers; lanes 3 and 5: amplification with M forward and Wt reverse primer. H72 mutant was used as a positive mutant control.

the absence of a significant impairment in social, occupational, or daily living functioning capacity.

The first PET study, performed at the age of 60, revealed a mild decrease in cerebral FDG uptake in the left medial frontal and medial temporal cortices as well as in the left posterior cingulate (Fig. 4A). The second PET study, performed 1 year later at the age of 61, demonstrated that the ^{18}FDG uptake was further reduced in the posterior cingulate and in the frontal cortex, and that at this point, the hypometabolism also involved the right hemisphere. Moreover, reduced ^{18}FDG uptake was also observed in the superior parietal cortex/precuneus bilaterally (Fig. 4C). These changes are clearly highlighted by the SPM statistical analysis as shown in Fig. 4B and D.

The female patient, H73 Family A-FTD2 died in 2015 at the age of 61. She started to have a severe and rapidly progressive language deficit at age 58 years. She became less expressive in speech and began having difficulty in finding appropriate words to context. These impediments were associated with impaired ability to repeat sentences and phrases. Later, she exhibited apraxia and agrammatism and progressive nonfluent aphasia with word-finding difficulty. At the age of 58, the mini-mental state examination score was very low, mostly due to the patient being totally aphasic, with a Progressive Aphasia Severity Scale (PASS = 3) score of only 5/30 (Sapolsky et al., 2014). The diagnosis was logopenic/phonological aphasia according to the Gorno-Tempini classification, presenting a nfvPPA (Gorno-Tempini et al., 2011). SPECT images showed hypoperfusion in the left frontotemporal and parietal cortices (Supplementary Fig. 1).

Male Patient H225, family B-FTD7 (Fig. 3, B-FTD7: H225-II7) was a 59-year-old right-handed man, who was referred at age of 57 years to the Memory Clinic with memory and behavioral disturbances. At 56-year old, he started to suffer behavioral changes with a spectrum that spanned from agitation/wondering to lack of motivation/loss of interest, apathy with language disturbances, and abnormal appetite/eating. Neurologic examination revealed postural instability, loss of reflex, decreased grasping ability of the right hand, and a positive Epstein sign. He had echolalia and a high latency in verbal responses. A family history of dementia was reported. His mother was reported to have behavioral disturbances, affected primarily in speech production.

Male Patient H153, family C-FTD10 was an 81-year-old right-handed man, referred at age 78 years with attention and behavioral disturbances. During young adult life, he suffered depressive moods that were successfully treated with antidepressants. At the age of 73, he had a myocardial infarction. At age 78 years, after his brother's death, he regressed into depressive moods and exhibited FTD symptoms, including loss of initiative, unstable equilibrium, dizziness and confusion. Drug therapy was ineffective and he became passive and purposeless. Neurologic examination exhibited postural instability and loss of reflexes. Two sisters were reported by relatives to be affected by dementia, but we do not have further clinical records in regard.

4. Mutation functional analyses

To understand how the mutation was affecting the protein expression of the patients, we analyzed plasma proteins by Western blot (Fig. 5C–E). Our data demonstrate a decreased protein expression in the plasma of patients H72, H73, and H225 (Fig. 5C, and lanes 1, 2, and 3) when compared to controls (Fig. 5C, and lanes 4–5), corresponding to the described protein haploinsufficiency phenotype in these individuals.

GRN gene expression was later analyzed by quantitative real-time PCR of total RNA extracted from white blood cells (WBC) with a primer set corresponding to a site downstream of the mutation. Specifically, our primers were designed to correspond to sequences within ex7 and ex8 downstream of the mutation (Fig. 5A). Our results demonstrate that the quantity of messenger RNA (mRNA) molecules containing ex7 and ex8 is increased in WBC from patients carrying the g10325_10331delCTGCTGT mutation as compared to controls (Fig. 5A and Supplementary Fig. 2). We further analyzed mRNA expression using a primer set corresponding to sequences within the ex6 g10325_10331delCTGCTGT mutation. The data, in Fig. 5B, show that the quantity of mRNA molecules containing wild type ex6 decreased and strongly correlated with the decreased protein levels observed in the Western blot analysis (Fig. 5C–E). This is also consistent with heterozygosity for the mutation in patient H72.

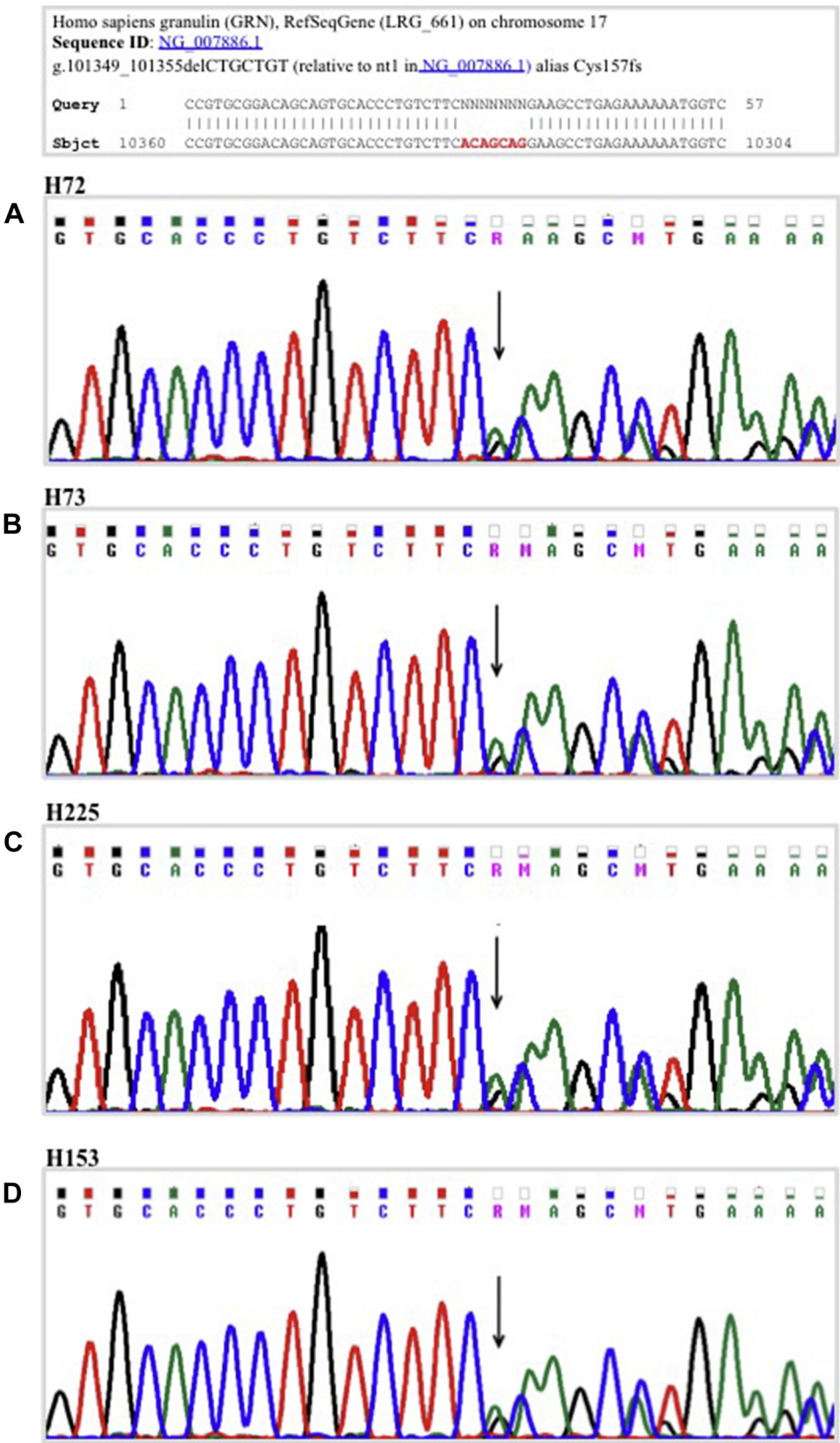

Fig. 2. Sanger sequencing electropherograms of the exon 6 *GRN* gene-mutated region in the FTD patients. Analyses were done by Sanger-based method sequencing. All the 13 exons of the *GRN* gene were investigated (NG_007886.1). Primers were designed to include exon-intron boundaries and 5′ and 3′ regulatory regions. PCR amplifications were performed on SureCycler 8800 Thermal Cycler (Agilent). (A) and (B) Exon 6 *GRN* electropherogram of the mutated region corresponding to H72 and H73 in family A-FTD2. (C) Exon 6 *GRN* electropherogram of the mutated region of Patient H225, family B-FTD7. (D) Exon 6 *GRN* electropherogram of the mutated region of Patient H153, family C-FTD10. The arrows show the position of the mutation. Abbreviations: FTD, frontotemporal dementia; PCR, polymerase chain reaction.

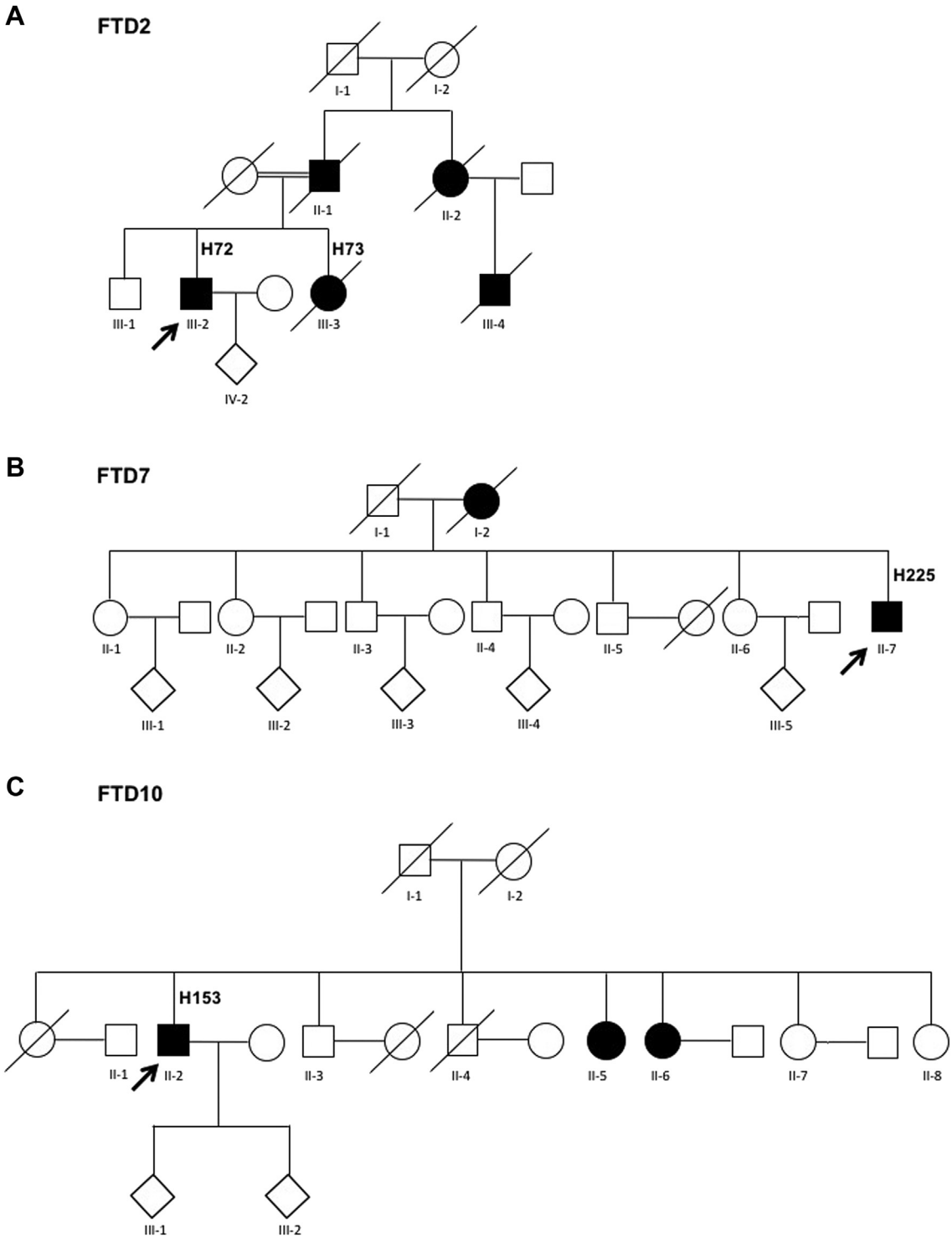

Fig. 3. Pedigrees A-FTD2, B-FTD7, and C-FTD10. Filled symbols represent affected individuals, empty symbols represent unaffected individuals. Symbols with diagonal lines represent deceased individuals. Probands are indicated by arrows; (A) FTD2, Proband H72-III2 is a man with a bvFTD phenotype, H73-III3 is his deceased affected sister with nfvPPA; (B) FTD7, H225-III7 is a man with bvFTD; and (C) FTD10, Proband H153-II2 is a man with bvFTD. Abbreviation: FTD, frontotemporal dementia.

The analysis was not done in H153 for lack of mRNA and proteins. The controls reported in the Fig. 5A and B were prepared from a pool of 5 controls. The same preparation was used in these experiments.

5. Discussion

This study identified a *g10325_10331delCTGCTGT* deletion in exon 6 of the *GRN* gene in 4 patients available for analysis from 3 families from Southern Italy who have positive family histories for FTD. The 2 patients in family A-FTD2 exhibited differing phenotypes, bvFTD in the male and nfvPPA in the female. Male patients H225 in B-FTD7 and H153 in C-FTD10 exhibited bvFTD phenotype, consistent with the clinical heterogeneity associated with the same mutation.

The results of PET and SPECT studies from patients of family A-FTD2 disclosed patterns of hypometabolism and hypoperfusion

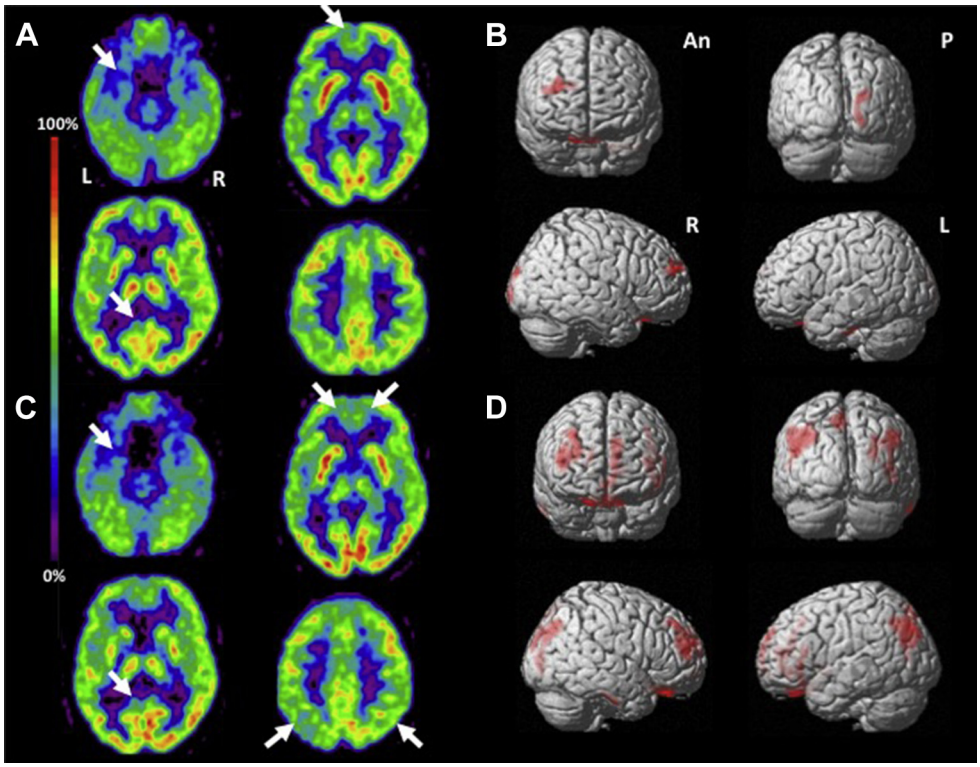

Fig. 4. Patient H72 ^{18}F FDG-PET scans. Axial slices of ^{18}F FDG-PET uptake obtained in patient H72 at different brain levels from the cerebellum to the centrum semiovale at the first (A and B) compared to the second (C and D) study; slight hypometabolism is evident in the left medial temporal cortex, frontal cortex and posterior cingulate at the first study, indicated by the upper and lower arrows. (B) and (D) demonstrate clusters of FDG reduction superimposed on a volume obtained from a normal brain for anatomic reference at the first (B) and the second (D) study. The relative glucose hypometabolism appears more widespread in the second study (C and D) and involves both frontal and parietal cortices. SPM analysis highlights the location of the hypometabolic deficit in our patient as compared to controls ($p < 0.05$ uncorrected for voxel height). The FDG images of each PET study were scaled to the maximum percentage of the global radioactive concentration. The color scale represents the lower (blue) and the higher (red) cerebral radioactive ^{18}FDG concentration values. Abbreviations: An, anterior; FDG-PET, ^{18}F-fluorodeoxyglucose positron emission tomography; FTD, frontotemporal dementia; L, left; P, posterior; R, right. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

associated with the different clinical phenotypes exhibited by each patient. The PET study, initially showed hypometabolism involving the hippocampus and the posterior cingulate in patient H72, which may be related to the prevalent amnesic syndrome observed at the beginning of the symptoms. The subsequent progression of his behavioral and frontal disturbances corresponded to the further reduction of frontal hypometabolism found at the second PET study. This was also observed in prior studies of patients with *GRN* mutations. These studies reported that the disease progressed toward Alzheimer's disease or FTD from an initial amnestic mild cognitive impairment diagnosis (Kelley et al., 2010; Petersen et al., 1999). Other studies reported associations of temporoparietal atrophy with progranulin mutations, demonstrating a pattern analogous to the one observed in our patient. However, hypometabolism patterns reported in association with different gene mutations are quite heterogeneous (Seelaar et al., 2011). Moreover, differentiating these patterns in patients with advanced disease status will likely to be much more difficult. Early imaging used for diagnosis could be much more helpful (Le Ber et al., 2008; Whitwell et al., 2012). It is possible that the left frontotemporal and parietal hypoperfusion observed with the SPECT study in patient H73 is related to the diagnosis of nfvPPA. A similar pattern was observed with the ^{18}F-fluorodeoxyglucose Positron Emission Tomography study in a patient presenting sporadic mutation and nfvPPA.

This mutation was already described in 2 apparently sporadic cases: a female FTD patient presenting nfvPPA phenotype and a male patient with bvFTD (Caso et al., 2012; Le Ber et al., 2008). A speculation that mutations of the *GRN* gene might exert different phenotypes according to the sex of the mutation carrier is premature and not supported by the small number of cases but would certainly merit further study. Functional correlates may be hypothesized from preclinical studies. Progranulin (PGRN) functions as a neurotrophic factor regulating neurite outgrowth and promoting neuronal survival (Ryan et al., 2009; Van Damme et al., 2008). It was also reported that PGRN can play a secretion-independent role because it acts as a promoter-specific transcriptional repressor (Hoque et al., 2010).

Gene expression analysis using a primer set designed to correspond to sequences within the ex6 g10325_10331delCTGCTGT mutation confirms that the *GRN* gene expression corresponded to the heterozygosity in the mutation in patient H72. In fact, our data show that the quantity of mRNA molecules containing wild type ex6 decreased and strongly correlated with the decreased protein levels observed in the Western blot analysis (Fig. 5C–E). The results of the analysis also correlated with the decreased protein quantity observed in the Western analysis of the plasma in the same patients. We do not yet have a complete understanding of the nature of the increase in transcripts observed with a different primer set (ex 7, ex 8 Fig. 5A), and further analyses need to be carried out. Previous studies aimed at examining the relationship between protein and transcript levels revealed that transcript levels in eukaryotic cells provide little predictive value of the extent of protein expression (Guo et al., 2008; Gygi et al., 1999). The mode of inheritance is autosomal dominant with a spectrum of clinical presentations highly heterogeneous (Finch et al., 2009; Sleegers et al., 2009).

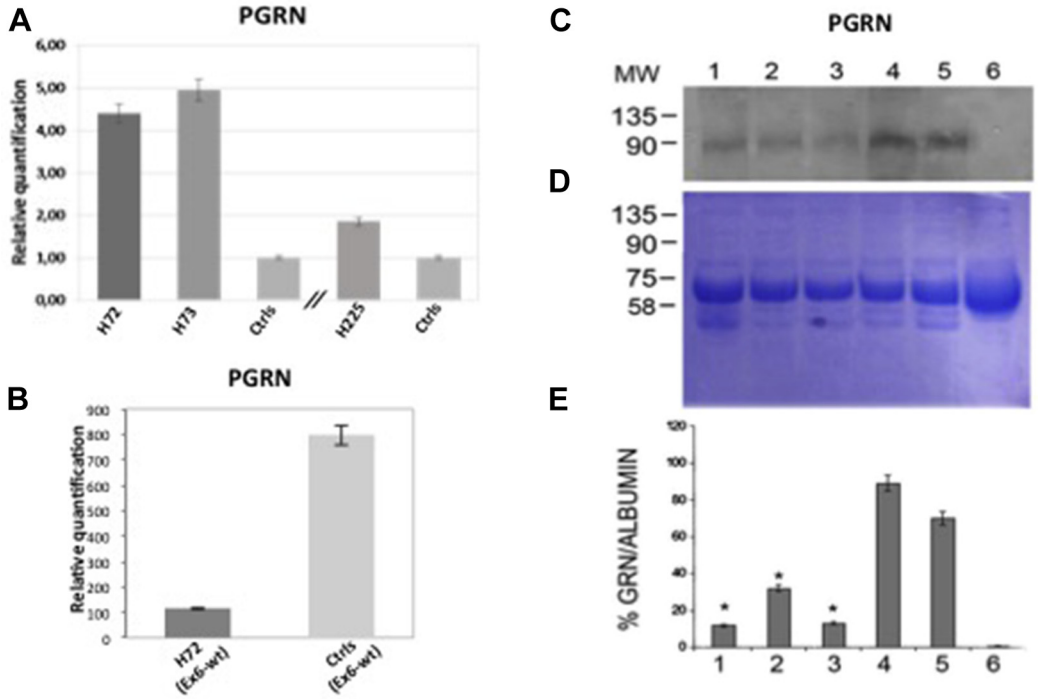

Fig. 5. *GRN* mRNA levels in WBC samples (A, B) and PGRN protein-expression (C, D, and E) in plasma samples from FTD patients. Diagrams in figures (A, B) express the relative level of each mRNA analyzed (A) using primers in ex 7 (forward) and ex 8 (reverse). (A) mRNA analysis in WBC samples from FTD patients H72, H73, and H225 is shown and compared to healthy controls. (B) mRNA analysis results in WBC samples from FTD patient H72 as compared to healthy control subjects when primers in ex 6 (forward) and ex 8 (reverse) were used. Both were compared to the internal standard glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from 3 independent assays. Results are represented as means ± standard error of the mean (SEM) of the 3 experiments. Ctrls represent the mean of the same pool of selected control samples. (C), (D), and (E) Western blotting analysis of PGRN protein in plasma samples from FTD patients H72, H73, and H225 (lanes 1–3) and from healthy controls (lanes 4, 5), with fetal bovine serum (lane 6) as a negative control. Part label C is representative of 3 Western blots; part label D represents Coomassie blue staining of the gel; and E shows a graph of the densitometric analysis of the signal intensity from all 3 assays normalized against albumin (66.5 KDa), the major component of the total plasma protein signal obtained by Coomassie blue staining of the gels. Results are represented as means ± SEM of the 3 experiments. $^{*}p < 0.05$ versus healthy patients. Abbreviations: FTD, frontotemporal dementia; mRNA, messenger RNA; PGRN, progranulin. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Although the function of *GRN* in neurons has not yet been determined, our findings are consistent with a prior hypothesis that it could be essential for neuronal survival (Ward and Miller, 2011). *GRN* is expressed in many tissues and mediates its role in development by activating signaling cascades that control cell-cycle progression and cell motility (Nedachi et al., 2011).

The reported *GRN* mutation was not found in 300 healthy controls without any behavioral and cognitive disturbances, as analyzed by PCR-ARMS, suggesting that this PGRN mutation is characteristic of patients with FTD. Future investigations will focus on understanding how *GRN* is involved in neurodegeneration, specifically on development of different FTD phenotypes.

Disclosure statement

The authors have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2016.12.030.

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