Published in final edited form as:

Neurobiol Aging. 2016 February ; 38: 215.e1–215.e12. doi:10.1016/j.neurobiolaging.2015.10.029.

Missense mutations in progranulin gene associated with frontotemporal lobar degeneration: study of pathogenetic features

Celeste M Karch^{a,b}, Lubov Ezerskiy^a, Veronica Redaelli^c, Annarita Giovagnoli^c, Pietro Tiraboschi^c, Giuseppe Pelliccioni^d, Paolo Pelliccioni^d, Dimos Kapetis^e, D'Amato Ilaria^c, Elena Piccoli^c, Maria Giulia Ferretti^c, Tagliavini Fabrizio^c, and Giacomina Rossi^c

^aDepartment of Psychiatry, Washington University School of Medicine, 425 S Euclid Avenue, St Louis, MO 63110, USA

^bHope Center Program on Protein Aggregation and Neurodegeneration, Washington University School of Medicine, 425 S Euclid Avenue, St Louis, MO 63110, USA

^cDivision of Neurology V and Neuropathology, Fondazione IRCCS Istituto Neurologico Carlo Besta, via Celoria 11, 20133 Milano, Italy

^dDivision of Neurology, Geriatric Hospital, INRCA IRCCS, Ancona, Italy

^eBioinformatics Unit, Fondazione IRCCS Istituto Neurologico Carlo Besta, via Celoria 11, 20133 Milano, Italy

Abstract

GRN, the gene coding for the progranulin (PGRN), was recognized as a gene linked to frontotemporal lobar degeneration (FTLD). The first mutations identified were *null* mutations giving rise to haploinsufficiency. Missense mutations were subsequently detected but only a small subset has been functionally investigated. We identified missense mutations (C105Y, A199V and R298H) in FTLD cases with family history and/or with low plasma PGRN levels. The aim of this study was to determine their pathogenicity. We performed functional studies, analyzing PGRN expression, secretion and cleavage by elastase. *GRN* C105Y affected both secretion and elastase cleavage, likely representing a pathogenic mutation. *GRN* A199V did not alter the physiological properties of PGRN and *GRN* R298H produced only moderate effects on PGRN secretion, indicating that their pathogenicity is uncertain. In the absence of strong segregation data and neuropathological examinations, genetic, biomarker, and functional studies can be applied to an algorithm to assess the likelihood of pathogenicity for a mutation. This information can improve our understanding of the complex mechanisms by which *GRN* mutations lead to FTLD.

Correspondence to: Giacomina Rossi, PhD, Division of Neurology V - Neuropathology, Fondazione IRCCS Istituto Neurologico Carlo Besta, via Celoria 11, 20133 Milano, Italy. Tel.: +39 02 2394 4647; Fax: +39 02 2394 2101; grossi@istituto-besta.it.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

progranulin; GRN; mutation; frontotemporal lobar degeneration; pathogenetic; functional analysis

1. Introduction

Frontotemporal lobar degeneration (FTLD) is a group of heterogeneous neurodegenerative diseases that constitute the second most common cause of presenile dementia after Alzheimer's disease (AD) (Rademakers et al., 2012). FTLD is clinically characterized by behavioural changes, executive dysfunctions and language impairment, giving rise to three main clinical syndromes: behavioural variant (bvFTLD), progressive non-fluent aphasia (PNFA) and semantic dementia. It is often associated with parkinsonism or corticobasal syndrome (CBS) and more rarely with motor neuron disease. The neuropathology of FTLD is also heterogeneous, showing abnormal deposits made of different misfolded proteins: tau and transactive response DNA binding protein 43 are the most commonly detected protein inclusions, while fused-in-sarcoma, and dipeptide-repeat proteins are more rare (Rademakers et al., 2012; Mori et al., 2013). Approximately 40% of all FTLD patients have a family history of dementia but only 10% exhibit autosomal dominant patterns of inheritance (Goldman et al., 2011). Three major genes causally linked to FTLD, microtubule-associated protein tau (*MAPT*), progranulin (*GRN*) and chromosome 9 open reading frame 72 (*C9ORF72*), in addition to other more rarely associated genes, underlie the clinical and neuropathological heterogeneity (Rademakers et al., 2012).

Since the discovery, in 2006, of *GRN* as a gene linked to FTLD, it has clearly emerged that the *null* mutations were the major pathological determinants. These mutations cause premature termination of the *GRN* coding sequence and lead to degradation of mutant *GRN* mRNA by non-sense mediated decay and to reduced progranulin protein (PGRN) production. Thus, FTLD is predicted to arise from haploinsufficiency in these mutation carriers (Baker et al., 2006; Cruts et al., 2006). In fact, PGRN levels in the cerebrospinal fluid (CSF) and plasma are significantly lower in subjects carrying a *GRN null* mutation compared with normal controls (Ghidoni et al., 2012). PGRN protein is a 68kDa glycoprotein made up of a leader peptide and 7.5 tandem repeats of 12 cysteine-rich domains. It is secreted as a full-length protein and subsequently cleaved by proteolytic processing into multiple 6–25kDa fragments, termed granulins (GRNs) (He and Bateman 2003). PGRN is a growth factor involved in the regulation of development, wound repair and inflammation and has been implicated in tumorigenesis. It is expressed by many cell types including neurons and microglia (Batemann et al., 2009). Several studies have demonstrated the neuroprotective and neurotrophic properties of PGRN or GRN peptides (van Damme et al., 2008; Xu et al., 2011).

In addition to *null* mutations, missense mutations have been reported in *GRN*, whose pathogenicity was not obvious and required more extensive investigation. For several missense *GRN* mutations, *in silico* analysis predicts that the mutations are damaging; however, only a small number of *GRN* mutations have been subjected to more extensive *in vitro* functional analyses. Missense mutations at codon M1 destroys the native Kozak

sequence that is required for translation initiation and results in a reduction of mutated RNA, likely via the nonsense-mediated mRNA decay (Baker et al., 2006; Cruts et al., 2006). The *GRN* A9D mutation is localized in the leader peptide and produces reduced levels of mutant RNA (Gass et al., 2006) and cytoplasmic missorting of PGRN leading to protein degradation and reduced secretion (Shankaran et al., 2008; Mukherjee et al., 2008). *GRN* P248L and R432C results in intracellular PGRN degradation and reduced secretion (Shankaran et al., 2008), possibly due to misfolding (van Der Zee et al., 2007). *GRN* C521Y and C139R impair the physiological processing of PGRN into GRNs by elastase cleavage without altering PGRN production or secretion (Wang et al., 2010).

Only a small subset of individuals carrying missense *GRN* mutations have been investigated for changes in plasma PGRN levels. Some missense *GRN* mutations produce normal PGRN plasma levels (A324T, R433W, P34S, P451L and C521Y; Sleegers et al., 2009; Finch et al., 2009; Almeida et al., 2014; Wang et al., 2010), while other missense *GRN* mutations produce intermediate PGRN plasma levels, between values typical of normal and *null GRN* mutations (C139R, R432C, R564C, C126W and A266P; Finch et al., 2009; Sleegers et al., 2009; Bernardi et al., 2012). Only the clearly pathogenic A9D mutation produces plasma PGRN levels typical of *null GRN* mutations (Wang et al., 2010).

We recently identified three missense *GRN* mutations in our cohort of patients affected by FTLN: C105Y, A199V, and R298H. *GRN* C105Y is a novel mutation. *GRN* A199V and R298H were described previously but no functional characterization was performed (Beck et al., 2008; Yu et al., 2010). Owing to the presence of positive FTLN family history and/or plasma PGRN dosages typical of *GRN null* mutations, we performed functional analyses to determine whether these missense *GRN* mutations induce functional impairments typical of pathogenic *GRN* mutations. We found that the novel *GRN* C105Y mutation affects both secretion and elastase cleavage. On the other hand, *GRN* R298H only moderately reduced PGRN secretion, while *GRN* A199V behaved similarly to *GRN* WT. Thus, genetic and functional data suggest that *GRN* C105Y is likely a pathogenic mutation, while pathogenicity of *GRN* A199V and R298H is uncertain. Determination of the functional impact of novel genetic variants is important, as distinguishing a likely pathogenic mutation from a benign polymorphism has important implications for genetic counselling of affected families.

2. Materials and methods

FTLN Cohort

Italian patients affected by neurodegenerative diseases of the FTLN spectrum (n = 332; age of onset: range: 30–89 years; mean $\pm$ SD: 62 $\pm$ 9) underwent clinical and neurological examination at the Istituto Neurologico Carlo Besta, Milano. A clinical diagnosis of FTD (n = 261), FTD-MND (n = 25), PPA (n = 19), CBS (n = 3) and PSP (n = 24) was made according to international guidelines (Neary et al., 1998; Mesulam, 1982; Riley et al., 1990; Litvan et al., 1996). Genetic screenings were performed for *MAPT* (228 patients analyzed, 8 pathogenic mutations found), *GRN* (81 patients analyzed, 23 *null* and 3 missense mutations found), *C9ORF72* (159 patients analyzed, 9 with the pathological expansion). Here we

report in detail the clinical data of the patients carrying the three *GRN* missense mutations and the functional analyses performed.

2.1 Case Reports

2.1.1 Family 1R (Fig. 1A)—The proband (III-8), a right handed woman, presented at age 61 with apathy and indifference to family affairs and housekeeping, and memory deficits. A magnetic resonance imaging (MRI) showed brain atrophy prevalent in temporal lobes and a perfusion single-photon emission computed tomography (SPECT) revealed hypoperfusion in frontotemporal lobes. Neuropsychological evaluation disclosed cognitive rigidity, perseverations, planning deficits and apathy (Mini Mental State Examination (MMSE): 21/30). A diagnosis of FTLT was hypothesized. One year after diagnosis, she presented with spatial disorientation, no longer exiting her home alone. Two years after initial diagnosis, she was admitted to Neurological Institute Carlo Besta. At this time, her prominent behavioural and cognitive signs included apathy, disinhibition, fatuity, reduction of speech, reduction of motor planning and memory deficits. Neurological examination revealed palmomental reflex, mild postural bilateral tremor, motor bradykinesia, motor stereotypies (repeatedly scratching the back of the left hand with the right hand), and tendon reflex hyperreflexia with bilateral clonus. CSF analysis showed a slight decrease in Amyloid-beta 42 ($A\beta_{42}$) level (437 pg/ml; normal values >500 pg/ml) while total tau and T181-phosphorylated tau (P-tau) were normal. A second MRI revealed severe cortical-subcortical fronto-temporo-insular atrophy prevailing in the right lobes and ventricular enlargement with white matter mild periventricular alterations, small hemorrhage in left parietal lobe and mild shrinking of midbrain. She was diagnosed with bvFTLD.

There was a family history of dementia (Fig. 1A). The proband's sister (III-9) presented at age 62 with slow-onset loss of initiative and interests in common activities and altered memory, orientation and verbal expression. Three years after symptom onset, the patient was unable to initiate and execute serial actions and habitual gestures in the correct sequence, and she made unmotivated and/or illogical choices. She also compulsively touched and used objects. Her verbal output was repetitive and eating conduct was fast and excessive. Affectivity was flattened and social behaviour was often disinhibited or poorly controlled. Neurological examination revealed severe impairments in awareness, attention, working memory, orientation, and constructive praxis. The MMSE provided an overall normal score (26/30). Movements were slow, face expression was reduced, the muscles were rigid diffusely, and arm movements were not evident during walking. MRI, performed two years after disease onset, showed multiple focal T2-weighted hyper-intense areas in the white matter, associated with mild dilatation of the cortical sulci, suggesting a chronic vascular damage. Four years after disease onset, MRI revealed marked worsening of cortical atrophy and white matter hyper-intensities in the frontal lobe. A diagnosis of bvFTLD was made.

The proband's mother (II-2) and a sister of the mother (II-3) presented with a history of dementia starting at approximately 70 years. Patient II-4 was diagnosed with Parkinson's disease and II-5 with epilepsy. Information about the first generation was not available.

2.1.2 Family 2T (Fig. 1B)—The proband (III-8), a right handed man, at age 71 started complaining of weakness and clumsiness of left limbs, grasping and walking defects, denomination and memory deficits, echolalia, psychomotor agitation and tearfulness. The proband had a history of prostate cancer, which was successfully treated and remained negative at follow up. A brain computed tomography (CT) showed ventricular enlargement more evident on the right side. CSF analysis revealed a slight decrease in A β ₄₂ level (471 pg/ml), while total tau and P-tau were normal. Neuropsychological evaluation showed attention, inhibition, visuospatial memory, speech and praxic-constructive abilities deficits. Neurological examination disclosed postural instability, asymmetric akinetic rigid parkinsonism with gait disorders. Left hand apraxia with left limb rigidity and eyelid apraxia and alien left hand with limb phenomenon were evident. MRI showed marked asymmetric cortical atrophy, more evident in the right posterior frontal and in the parietal regions, on the side contralateral to the clinical symptoms. A follow-up neurological evaluation, 15 months after the first one, showed a clinical picture of asymmetric cortical deficits with a mixture of pyramidal and extrapyramidal features that were gradually progressive. The patient was less mobile, unable to rise from a chair unaided, with a marked impairment in speech, echolalic and palilalic, frequently unintelligible. Asymmetric extrapyramidal bradykinesia and left limb rigidity, more pronounced in the arm with dystonia and stiffness, were evident. An unusual and interesting feature was also hemidysphagia, with dripping only in the left side of the mouth when drinking water. Impaired eye movements, with difficulties in saccadic and pursuit eye movements, prevalent in the horizontal plane, were also observed. He was diagnosed with CBS.

A history of dementia was reported in the proband's family. The father (II-3) was diagnosed with AD and a sister (III-4) as affected by AD with parkinsonism. A cousin (III-2) and a sister (III-6) were reported to be affected by a CBS phenotype very similar to the proband. The sister III-9 presented at age 63 with language impairment diagnosed as PNFA; 18F-fluorodeoxyglucose positron emission tomography (18F-FDG-PET) showed hypometabolism in the left frontotemporal lobe. No information is available regarding the first generation nor for patient II-2.

2.1.3 Patient 3—A diagnosis of FTLT at age 62 with positive family history was made in another hospital but unfortunately no additional details were available.

2.2 PGRN dosage

To measure the level of PGRN in plasma, an ELISA kit (Human Progranulin ELISA kit, Adipogen Inc., Seoul, Korea) was used, according to the manufacturer's instructions. PGRN plasma levels were measured in patients III-8 and III-9 of Family 1R, in the proband of Family 2T (III-8) and in Patient 3. This kit was also used to measure the levels of PGRN in conditioned media from cell cultures.

2.3 Genetic analysis

The work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). The informed consent to genetic

analysis for diagnostic and research purposes was obtained from all subjects and the privacy rights were always observed.

Genomic DNA was extracted from peripheral blood lymphocytes (PBL) of patients III-8 and III-9 of Family 1R, proband of Family 2T (III-8), and patient 3 using standard protocols.

For patient III-8 of Family 1R, all coding exons, flanking intronic sequences and 5' and 3' untranslated regions of *GRN* gene were amplified using previously published primers with minor modifications (Baker et al., 2006; Cruts et al., 2006). Amplified fragments were sequenced in both directions using the Big Dye terminator v 3.1 cycle sequencing kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) and analyzed on an ABI 3100 gene analyzer (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). In addition, exons 9–13 of *MAPT* gene (Poorkaj et al., 1998) and the region containing the hexanucleotide GGGGCC repeat in the first intron of *C9ORF72* gene (DeJesus-Hernandez et al., 2011) were analyzed. To evaluate deletions or duplications in *MAPT* and *GRN*, we performed multiplex ligation-dependent probe amplification (MLPA) using the SALSA MLPA P275-B1 MAPT-PGRN kit (MRC-Holland, Amsterdam, the Netherlands), following the manufacturer's instructions. Only exon 3 of *GRN* was sequenced for patient III-9 (sister of III-8).

For the proband of Family 2T (III-8), the genetic analyses described above were performed (*GRN*, *MAPT*, *C9ORF72* and MLPA). Additionally, due to the reports of family members with clinical presentations of AD, sequencing of *amyloid- β precursor protein* (exons 16 and 17), *Presenilin 1* (exons 3–12) and *Presenilin 2* (exons 4–7,11,12) genes was performed.

For Patient 3, *GRN*, *MAPT*, *C9ORF72* were analyzed and MLPA was performed as described above.

We screened 100 healthy controls for the *GRN* mutations identified (C105Y, R298H, and A199V). The healthy controls were the spouses of subjects affected by various neurological disorders: (age range, 22–73 years; age mean $\pm$ SD, 41 $\pm$ 12). Additionally, we screened 95 patients affected by non-FTLD neurodegenerative diseases for the novel *GRN* C105Y mutation (29 patients affected by Gerstmann-Sträussler-Scheinker disease, carrying the *PRNP* P102L mutation, and 66 affected by Creutzfeldt-Jakob disease, carrying the *PRNP* V210I mutation).

2.4 Bioinformatics

To determine whether the *GRN* mutations represent rare or common polymorphisms, we investigated two publicly available databases with population-based exome sequencing data: (i) the Exome Variant Server (EVS), which includes exomes from 4300 unrelated European Americans; (ii) the Exome Aggregation Consortium (ExAC) browser, which contains exomes from 60,706 unrelated individuals.

Polymorphism Phenotype v2 (PolyPhen-2) (Adzhubei et al., 2010) and Sorting Intolerant from Tolerant (SIFT) in-silico softwares were employed to evaluate *GRN* mutation pathogenicity. PolyPhen2 (Adzhubei et al., 2010) was used to assess the effects of missense

mutations on protein structure and function by weighing features including sequence, phylogenetic, and structural information. A mutation is assigned a qualitative score of benign, possibly damaging, or probably damaging based on false positive rate (FPR). SIFT predicts whether an amino acid substitution affects protein function based on evolutionary conservation of amino acid residues and assigns a score that ranges from 0 to 1. The amino acid substitution is predicted to be damaging if it is less than 0.05 and is predicted to be tolerated if it is more than 0.05.

2.5 PGRN homology modelling and in silico mutagenesis

Homology modelling to construct the tertiary structure of the PGRN was performed. For this purpose, we constructed an atomic model of individual GRN domains (GRN G and GRN A, containing C105 and R298 amino acid residues, respectively) using SWISS-MODEL (Biasini et al., 2014). GRN G and GRN A models were generated based on the NMR structure of GRN A (PDB ID: 2jye) (Tolkatchev et al., 2008). The constructed models were subjected to energy minimization and model refinement using the YASARA force field (Krieger et al., 2009). *In silico*-mutagenesis (*GRN* C105Y and R298H) and stability calculations of the free energy unfolding (ΔG) of the GRN G and GRN A were estimated with the YASARA FoldX plugin (Van Durme et al., 2011). The changes in native stability upon mutation were estimated as ΔG difference between the energy of the mutant protein and that of the wild-type (WT) protein ($\Delta G = G_{\text{mut}} - G_{\text{WT}}$). The default FoldX parameters were used (Temperature: 298 K; Ion strength: 0.05 M; pH: 7; Van der Waals Design: 2); if the mutation destabilizes the structure, ΔG is increased, whereas stabilizing mutations decrease the ΔG . Since the FoldX error margin is around 0.5 kcal/mol, changes in this range are considered insignificant.

2.6 Cell culture and transient transfection

2.6.1 Lymphoblastoid cell lines (LCL)—LCL were obtained by Epstein Barr virus immortalization of PBL from patient III-8 of Family 1R (carrying the *GRN* C105Y mutation), 1 positive control (carrying the *GRN* Q341X mutation) and 5 negative controls (carrying *GRN* WT). LCL were cultured in RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 µg/mL penicillin/streptomycin. LCL were only available from patient III-8 of Family 1R.

2.6.2 Immortalized cell lines and transient transfection—The full-length *GRN* cDNA (Life Technologies, Carlsbad, CA, USA; Wang et al., 2010) was cloned into the pcDNA3.1 myc/his vector (Life Technologies, Carlsbad, CA, USA). The C105Y, R298H and A199V missense mutations were introduced into the *GRN* cDNA using the Quick-Change II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). Clones were sequenced to confirm the presence of the mutation and absence of additional modifications. *GRN* WT and previously analyzed FTLN-associated *GRN* mutations (A9D, C139R and C521Y) were included as controls (Wang et al., 2010; Karch et al., 2013).

Human embryonic kidney 293-T (HEK293T) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, USA), containing 10% FBS, 2 mM L-Glutamine, and 100 µg/mL penicillin/streptomycin. Upon reaching confluency, cells

were transiently transfected with Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). Culture media were replaced after 24 hours with serum free media, and cells were incubated for another 24 hours prior to analysis. Three independent transfections were performed for each construct and used for subsequent analyses.

2.7 Biochemical analysis

A total protein fraction was extracted from cell pellets (LCL and HEK293T). Cell pellets were incubated on ice in lysis buffer (50mM Tris-HCl, 2mM EDTA, 150mM NaCl, 1% NP40, 0.5% Triton X-100, protease inhibitor cocktail). After centrifugation at 14,000×g, supernatant was recovered for further analysis.

2.7.1 LCL

2.7.1.1 PGRN expression and secretion: Total protein concentration was measured in LCL lysates and media by the BCA assay (Pierce-Thermo, Rockford, IL, USA). Cell lysates and media were normalized based on total protein concentration prior to immunoblotting. The samples were incubated in Laemmli buffer supplemented with β -mercaptoethanol, boiled, and run on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The goat polyclonal antibody raised against full-length PGRN (anti-PGRN^{FL}, 1:300 dilution; R&D Systems, Minneapolis, MN, USA) and the rabbit polyclonal antibody against human progranulin (1:500 dilution; Genetex, Irvine, CA USA) were used. An antibody against β -tubulin (dilution 1:20,000; Sigma-Aldrich, St Louis, MO) was used in parallel for further normalization.

2.7.1.2 PGRN ELISA: LCL were incubated with serum-free media for 24 hours. Conditioned media was then concentrated 15X using a 30kDa cut-off size exclusion column (Amicon Ultra-15 Centrifugal Filter; Merck-Millipore, Billerica, MA, USA). PGRN levels were then measured using the ELISA kit described above (AdipoGen, San Diego, CA, USA). PGRN values were normalized based on total intracellular protein levels (calculated using the BCA assay). PGRN values of ng/ml were obtained, similar to values detected in plasma from patients. For each sample, three independent dosages were performed. The mean and standard deviation (SD) were calculated. Student's t-test was used to calculate statistical significance.

2.7.2 HEK293T transfected cells

2.7.2.1 PGRN expression and secretion: Cell lysates and conditioned media were subjected to protein dosage and immunoblot analyses as described above, using 4–20% Criterion Tris-HCl gels (Bio-Rad, Hercules, CA, USA). Immunoblots were probed with a rabbit polyclonal antibody raised against the C-terminus of PGRN (anti-PGRN^{C-term}, 1: 500 dilution; formerly Zymed; Life Technologies, Carlsbad, CA, USA). An antibody against β -tubulin (dilution 1:2000; Sigma-Aldrich, St Louis, MO, USA) was used as a loading control. Quantification of PGRN in cell media was performed by measuring the band intensity of PGRN in each lane using a Syngene Imaging system (Syngene, Frederick, MD, USA). An empty well served as a background. The mean and standard deviation (SD) were calculated. Student's t-test was performed.

2.7.2.2 PGRN ELISA: PGRN levels were measured in the media of transfected cells using the human Progranulin ELISA kit (AdipoGen, San Diego, CA, USA). Technical replicates were analysed for each sample. PGRN measurements were given as ng/mL. The mean and standard error of the mean (SEM) were calculated. Student's t-test was performed.

2.7.2.3 Elastase cleavage assay: To measure the rate at which elastase cleaves PGRN (WT, C139R, C521Y, A199V, C105Y, R298H), serum-free culture media was collected and treated with 1U elastase diluted in 100 mM Tris-HCl and 960 mM NaCl to achieve a final concentration of 5U/ul. Elastase-treated media was then incubated at 37°C for 5, 15, 30, 60, and 90 minutes. PGRN and GRN protein fragments were assessed using SDS-PAGE and immunoblotting as described above. Immunoblots were probed with the anti-PGRN^{C-term} antibody or, in parallel experiments performed under non-reducing conditions, with a goat polyclonal antibody raised against full-length PGRN (anti-PGRN^{FL}, 1:1000 dilution; R&D Systems, Minneapolis, MN, USA).

3. Results

3.1 Plasma PGRN dosage

PGRN plasma levels for patients III-8 and III-9 of Family 1R and Patient 3 were 42, 41.4 and 40.1 ng/ml, respectively, all of which fall below the calculated cut-off level for *GRN* mutation prediction in FTLN patients (61.55 ng/ml; Ghidoni et al., 2012). Patient III-8 of Family 2T produced normal PGRN plasma levels (129 ng/ml) (Table1).

3.2 Genetic analysis

In the proband of Family 1R (III-8), direct sequencing of DNA revealed a single base substitution (TGC to TAC) at codon 105 in exon 3 of *GRN* gene (g.100617, GenBank accession number AC003043.2, reverse complement; c.314, GenBank accession number NM002087.3 starting at the translation initiation site), resulting in a cysteine to tyrosine change (C105Y) (Fig 1C). This is a novel mutation (AD&FTD Mutation Database; Human Genome Mutation Database, Stenson et al., 2014). This mutation was absent in our populations of 100 healthy subjects and 95 patients affected by non-FTLD neurological disorders. All the other genetic analyses performed were normal. The same mutation was detected in the proband's sister III-9.

In patient III-8 of Family 2T, sequencing revealed a single base substitution (CGT to CAT) at codon 298 in exon 8 of *GRN* gene (g.102321, GenBank accession number AC003043.2, reverse complement; c.893, GenBank accession number NM002087.3 starting at the translation initiation site), resulting in an arginine to histidine change (R298H) (Fig. 1D). This mutation was previously reported and was absent in several hundreds of healthy controls (Yu et al., 2010). *GRN* R298H was not detected in our population of 100 healthy subjects. The other genetic analyses performed were normal in patient III-8.

In patient 3, the sequencing revealed a single base substitution (GCA to GTA) at codon 199 in exon 5 of *GRN* gene (g.101476, GenBank accession number AC003043.2, reverse complement; c.596, GenBank accession number NM002087.3 starting at the translation initiation site), resulting in an alanine to valine change (A199V) (Fig. 1E). This mutation

was previously described and was absent in 90 healthy controls (Beck et al., 2008). *GRN* A199V was not found in our population of 100 healthy subjects. The other genetic analyses performed were normal in patient 3.

Screening of C105Y, R298H, and A199V mutations gave negative results in healthy control group; C105Y mutation was also absent in non-FTLD group.

3.3 Bioinformatics

To assess whether *GRN* C105Y, A199V, and R298H represent common polymorphisms or rare, potentially pathogenic mutations, we applied two bioinformatics methods. *GRN* C105Y and A199V were absent in the EVS and in the ExAC Browser. *GRN* R298H was absent in the EVS but found in the ExAC browser (3/121,148 alleles; minor allele frequency (MAF) in European non-Finnish = 4.5×10^{-5}). PolyPhen2 predicted that *GRN* C105Y and R298H are damaging to the PGRN protein, while A199V is tolerated. SIFT predicted that C105Y is damaging and A199V and R298H are tolerated (Table 1).

3.4 In silico mutagenesis

Homology models of the WT *GRN* domains (*GRN* G and *GRN* A) were generated based on the human *GRN* A domain template. Next, YASARA FoldX plugin was used to perform *in silico* mutagenesis to evaluate the impact of C105Y and R298H mutations (Fig. 2).

Structural protein modeling of the mutant residues on the *GRN* peptides predicted that C105Y has a destabilizing effect ($\Delta G = 4.08$ kcal/mol) on *GRN* folding by disrupting a disulfide bridge between C105 and C92 (Fig. 2 A, B). Modeling of the *GRN* R298H mutation predicts an interruption of the hydrogen bond between R298 and D285, producing a destabilizing effect ($\Delta G = 2.15$ kcal/mol) (Fig. 2 C, D). A199 is located in a region between *GRN* motifs for which no structural modelling is possible. Pairwise alignment between PGRN orthologues (> 75% sequence identity) showed that C105, A199 and R298 residues are highly conserved across species (NCBI HomoloGene Database, NCBI Resource Coordinators, 2015) (data not shown).

3.5 Biochemical analysis

3.5.1 *GRN* missense mutations do not affect PGRN production—A few *GRN* missense mutations have been described to reduce PGRN protein production (Shankaran et al., 2008). To determine whether the mutations identified in this study could alter PGRN production, we first analyzed the intracellular PGRN levels in LCL from patient III-8 carrying the *GRN* C105Y mutation. We found that PGRN C105Y levels were comparable to PGRN WT, while the *null* mutation Q341X clearly showed PGRN reduction (Fig. 3A).

Because LCL were not available from *GRN* A199V and R298H carriers, we investigated the effects of these mutations and *GRN* C105Y, on PGRN expression using a cell-based assay (Wang et al., 2010; Karch et al., 2013). HEK293T cells were transiently transfected with an expression vector containing *GRN* WT, the mutations identified in this study, A9D, or C521Y. PGRN was measured in the cell lysates and media. As previously reported, A9D caused a reduction of the intracellular PGRN levels (Shankaran et al., 2008; Karch et al., 2013), while C521Y did not influence PGRN levels (Wang et al., 2010; Karch et al., 2013).

(Fig. 4A). *GRN* C105Y, A199V, and R298H produced intracellular PGRN levels similar to WT (Fig. 4A).

3.5.2 GRN missense mutations partially affect PGRN secretion—A subset of missense *GRN* mutations influence PGRN secretion (Shankaran et al., 2008). Thus, we measured PGRN levels in the media of LCL carrying the *GRN* C105Y mutation. These LCL demonstrated reduced PGRN secretion compared with *GRN* WT-expressing LCL, as detected by both immunoblot analysis and ELISA dosage (Fig 3 B, C).

To confirm these findings in the cell-based assay and to determine whether *GRN* A199V and R298H also impact PGRN secretion, immunoblot and ELISA analyses of the media from transfected cells were performed. In addition to *GRN* A9D, *GRN* C105Y and R298H mutations produced significantly less secreted PGRN protein than *GRN* WT, as detected by immunoblotting (Fig. 4 B,C). Measurement of PGRN in cell media by ELISA, however, did not confirm the immunoblotting findings for *GRN* C105Y and R298H (Fig. 4 D).

3.5.3 GRN C105Y affects PGRN cleavage by elastase—Because some FTLN-associated *GRN* mutations are known to influence PGRN processing and GRN production (Wang et al., 2010), we examined whether the *GRN* mutations identified in this study influenced elastase cleavage of PGRN and GRN production and turnover. Media from cells expressing *GRN* WT, C105Y, A199V, R298H and C521Y mutations were treated with elastase for various time points. Samples were then run under reducing conditions, and immunoblots were probed with the anti-PGRN^{C-term} antibody. Elastase cleavage of PGRN WT produced protein fragments of 35kDa, 19kDa, and 10kDa (Fig. 5). In contrast, cells expressing *GRN* C521Y failed to produce the 35kDa fragment and displayed accelerated turnover of the 19kDa fragment (Fig. 5). The pattern of PGRN and GRN fragments from C105Y, A199V and R298H was similar to WT (Fig. 5). However, because N-terminal GRN peptides produced by elastase cleavage may not be detected by the anti-PGRN^{C-term} antibody, we investigated the N-terminal GRN peptides in cell culture media using the anti-PGRN^{FL} antibody. As previously described, under non-reducing conditions, we observed GRN fragments of 45kDa, 35kDa and 25kDa in media from cells expressing PGRN WT (Fig. 6). The 45kDa and 25kDa GRN fragments have previously been demonstrated to be N-terminal-derived GRN fragments (Wang et al., 2010). The 45kDa fragment was absent in media from cells expressing the *GRN* C139R mutation as well as in media from cells expressing the novel *GRN* C105Y (Fig. 6). Additionally, PGRN C139R and C105Y produced a 25kDa GRN fragment that was detected at much lower levels and turned over more quickly than in cells expressing WT (Fig. 6).

4. Discussion

A large number of pathogenic mutations have been identified in *GRN* that are associated with dominantly inherited forms of FTLN (Cruts et al., 2012). The most common pathological mechanism is the creation of a mRNA carrying a premature STOP codon, leading to its degradation by non-sense mediated mRNA decay, thus reducing the PGRN production and giving rise to haploinsufficiency (*null* mutations) (Baker et al., 2006; Cruts et al., 2006). Non-sense, splicing and frameshift mutations are the vast majority of

pathogenic *GRN* mutations described to date and fall into this category. However, a small number of *GRN* missense mutations have been described in FTLT families and functionally characterized. *GRN* M1 and A9D in the leader peptide also initiate premature mRNA degradation, due to their localization in a region of PGRN that is critical for translation (Baker et al., 2006; Gass et al., 2006). Intracellular protein degradation and reduced secretion are additional mechanisms leading to haploinsufficiency, shared by *GRN* A9D, P248L and R432C mutations (Mukherjee et al., 2008; Shankaran et al., 2008; Van Der Zee et al., 2007). Impairment of elastase-mediated PGRN cleavage has been reported for *GRN* C139R and C521Y mutations (Wang et al., 2010). *GRN* P248L, R432C, C139R and C521Y are also defective in PGRN-mediated neurite outgrowth (Wang et al., 2010; Gass et al., 2012). Other missense mutations appear to be benign polymorphisms (Karch et al., 2013). However, most missense *GRN* mutations remain uncharacterized. The absence of functional data in addition to the small number of individuals carrying these mutations makes interpretation of the pathogenicity and subsequent genetic counselling challenging.

In our cohort of FTLT patients, we identified three *GRN* missense mutations: C105Y, A199V and R298H. While the number of individuals carrying these mutations was small, we used bioinformatic analysis and functional characterization to determine the likelihood of pathogenicity. In addition, based on a previously published algorithm designed to determine whether rare mutations in *APP*, *PSEN1* and *PSEN2* are benign or pathogenic for Alzheimer's disease (Guerreiro et al., 2010), we propose a very similar algorithm to classify the pathogenicity of *GRN* missense mutations, in the absence of large pedigrees with strong segregation data (Fig. 7).

GRN C105Y is a novel mutation. *GRN* C105R mutation was previously described in the proband of a family affected by FTLT; however, it did not segregate with the disease in the family and was dismissed as a probable rare, benign variant (Gass et al., 2006). In a later report, C105R was identified in a case diagnosed as probable AD or possible dementia with Lewy bodies, whose family history is unknown (Meeus et al., 2012). C105R is also present in the ExAC Browser, further supporting its role as a benign polymorphism ($MAF = 1.5 \times 10^{-5}$). We identified *GRN* C105Y in two related individuals exhibiting FTLT clinical phenotypes. This mutation was absent in all control and population-based datasets that we investigated. Limited family history and patient material was available, making segregation analysis for this mutation impossible. Plasma PGRN levels from both C105Y carriers were similar to levels in *GRN null* mutation carriers.

C105 is conserved across PGRN orthologues and *in silico* analysis predicts that the mutation is damaging. Cysteine residues are well-known structural determinant of PGRN folding, in particular of the GRN-fold, bearing structural resemblance to another growth factor, EGF (He and Bateman, 2003). Based on structural modelling, the *GRN* C105Y mutation strongly destabilizes the normal folding of PGRN possibly leading to misfolding and degradation. In two cell models, LCL from a mutation carrier and transfected HEK293T cells, *GRN* C105Y induced significantly lower levels of secreted PGRN. Furthermore, it affected the physiological cleavage of PGRN by elastase. In fact, the 45kDa PGRN fragment was absent, indicating an abnormally rapid processing to produce the 25kDa fragment, which itself was unstable and turned over quickly. We hypothesize that *GRN* C105Y widely destabilizes the

N-terminal domain of PGRN, favouring its degradation. This resembles the behaviour of *GRN* C139R, which is localized in an adjacent GRN domain and similarly involves a cysteine residue (Wang et al., 2010). Figure 8 illustrates the proposed processing of PGRN by elastase, based on previous studies (Wang et al., 2010; Zhu et al., 2002). Based on the overall findings and according to our algorithm (Fig. 7), *GRN* C105Y mutation can be classified as “definitely pathogenic”.

GRN R298H was identified in one FTLN patient in our clinic with a strong family history of dementia and was previously reported in a single FTLN patient with an unknown family history of dementia (Yu et al., 2010). *GRN* R298H was not found in several hundred control subjects (Yu et al., 2010) but was detected in the ExAC browser with a very low MAF. Based on structural modelling, the substitution of histidine for arginine causes the disruption of a hydrogen bond raising the PGRN G and destabilizing protein folding. In our cell model, *GRN* R298H produced significantly lower levels of secreted protein as measured by immunoblotting, which may indicate that the mutated protein is misfolded in such a way that reduces its ability to get out of the cell; however, we could not confirm this observation in an ELISA-based assay of PGRN levels. These findings, taken together with plasma PGRN levels from the mutation carrier that fell within the normal range and in the absence of a detailed family history and plasma/DNA samples from other family members, suggest that *GRN* R298H is not likely to be a pathogenic mutation. Applying the suggested algorithm (Fig. 7), as this mutation was found in 2 unrelated cases (our case and the case described by Yu et al., 2010) and not in several hundred control subjects (Yu et al., 2010), and gave negative answers to functional questions, it may be classified as “possibly pathogenic”. However, *GRN* R298H mutation was also found in the ExAC database. Thus, if it was considered as “present in controls”, it would be classified as “not pathogenic/risk factor”. The presence of a mutation in ExAC database does not prove *per se* the benign/risk factor nature of the mutation, as the subjects in the ExAC database may be presymptomatic for the disease; affected by the disease but undiagnosed; or participants of another, unrelated study. These large genomic databases are very useful in determining the frequency of a mutation, discriminating between common and rare mutations. However they can not help in defining the pathogenicity, as both rare and common variants may be risk factors and it is not excluded the possibility of a pathogenic mutation present in a presymptomatic/undiagnosed individual: thus, supportive functional data are needed. In summary, *GRN* R298H mutation remains of uncertain nature.

GRN A199V was previously reported in a large United Kingdom series of FTLN. It was found in a patient with familial CBS of Bangladeshi origin; samples from other family members were not available. *GRN* A199V was not detected in 90 control subjects of the same ethnicity (Beck et al., 2008). The most interesting finding for *GRN* A199V is that the clinical affected mutation carrier exhibited low plasma PGRN levels, similar to values typical of *GRN null* mutations. On the other hand, we had no detailed information about the family history. Bioinformatic analyses based on evolutionary conservation and structure (PolyPhen2 and SIFT) predict that *GRN* A199V is benign. Functional studies in our cell model displayed no pathological effects on PGRN secretion levels nor on elastase cleavage. Thus, the low plasma PGRN level may be ascribed to unknown modifying factors, such as

genetic modifiers and/or regulators of PGRN expression and level (Rademakers et al., 2012). In summary, *GRN* A199V is not likely to be a pathogenic mutation and, based on our algorithm (Fig. 7), it may be classified as “possibly pathogenic.”

5. Conclusions

We sought to determine whether missense *GRN* mutations found in our FTLD cohort disrupt PGRN function via previously described mechanisms. The *GRN* A199V mutation was identified in one affected individual and did not affect PGRN expression or secretion in cultured cells. The *GRN* R298H mutation was identified in a single affected patient with a family history of dementia who did not produce plasma PGRN levels typical of *GRN null* mutations, and the *in vitro* studies failed to produce a clear pathological phenotype. Thus, the pathogenicity of these mutations remains uncertain. (Fig. 7). On the contrary, *GRN* C105Y exhibited altered PGRN secretion in cultured cells, which could possibly lead to haploinsufficiency, although to a lesser extent than what is found in *GRN null* mutations. We also found evidence that *GRN* C105Y mutation disrupts the physiological elastase-mediated cleavage of PGRN into GRNs. Similarly to *GRN* C139R (Wang et al., 2010), *GRN* C105Y affected the production of N-terminal fragments: the 45kDa fragment was absent and the 25kDa fragment was reduced and unstable. Proteolytic cleavage of PGRN is required to produce mature GRNs. Mature GRNs may have pleiotropic functions in the human brain (Van Damme et al., 2008; Ahmed et al., 2007). Thus, lower levels of mature GRNs could contribute to disease pathogenesis. In conclusion, the clinical and functional findings strongly suggest that *GRN* C105Y is likely a pathogenic mutation: according to our algorithm it can in fact be classified as “definitely pathogenic” (Fig. 7).

We think that in genetic counselling of FTLD patients it is unsatisfactory to be able to provide no information about a mutation identified in a patient. While co-segregation is the gold-standard in determining pathogenicity of a mutation, in absence of this type of data, we can at least provide a high likelihood of a mutation's benign or pathological nature by aggregating genetic, clinical, biomarker, and *in vitro* findings. The results from this study provide a more complete picture of the functional effects of previously identified and novel *GRN* mutations, which taken together may provide insight into the mechanisms by which mutations in *GRN* lead to FTLD.

Acknowledgements

This work was supported by access to equipment made possible by the Hope Center for Neurological Disorders, and the Departments of Neurology and Psychiatry at Washington University School of Medicine. Funding provided by: NIH-K01 AG046374 (CMK); Ricerca Corrente, Italian Ministry of Health (GR). The authors would like to thank the Exome Aggregation Consortium and the groups that provided exome variant data for comparison. A full list of contributing groups can be found at <http://exac.broadinstitute.org/about>.

References

- Ahmed Z, Mackenzie IR, Hutton ML, Dickson DW. Progranulin in frontotemporal lobar degeneration and neuroinflammation. *J. Neuroinflammation*. 2007; 4:7. [PubMed: 17291356]
- Almeida MR, Baldeiras I, Ribeiro MH, Santiago B, Machado C, Massano J, Guimarães J, Resende Oliveira C, Santana I. Progranulin peripheral levels as a screening tool for the identification of

- subjects with progranulin mutations in a Portuguese cohort. *Neurodegener. Dis.* 2014; 13:214–223. [PubMed: 24022032]
- Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR. A method and server for predicting damaging missense mutations. *Nat. Methods.* 2010; 7:248–249. [PubMed: 20354512]
- Baker M, Mackenzie IR, Pickering-Brown SM, Gass J, Rademakers R, Lindholm C, Snowden J, Adamson J, Sadovnick AD, Rollinson S, Cannon A, Dwosh E, Neary D, Melquist S, Richardson A, Dickson D, Berger Z, Eriksen J, Robinson T, Zehr C, Dickey CA, Crook R, McGowan E, Mann D, Boeve B, Feldman H, Hutton M. Mutations in progranulin cause tau-negative frontotemporal dementia linked to chromosome 17. *Nature.* 2006; 442:916–919. [PubMed: 16862116]
- Bateman A, Bennett HP. The granulin gene family: from cancer to dementia. *Bioessays.* 2009; 31:1245–1254. [PubMed: 19795409]
- Beck J, Rohrer JD, Campbell T, Isaacs A, Morrison KE, Goodall EF, Warrington EK, Stevens J, Revesz T, Holton J, Al-Sarraj S, King A, Scallan R, Warren JD, Fox NC, Rossor MN, Collinge J, Mead S. A distinct clinical, neuropsychological and radiological phenotype is associated with progranulin gene mutations in a large UK series. *Brain.* 2008; 131:706–720. [PubMed: 18234697]
- Bernardi L, Frangipane F, Smirne N, Colao R, Puccio G, Curcio SA, Mirabelli M, Maletta R, Anfossi M, Gallo M, Geracitano S, Conidi ME, Di Lorenzo R, Clodimiro A, Cupidi C, Marzano S, Comito F, Valenti V, Zirilli MA, Ghani M, Xi Z, Sato C, Moreno D, Borelli A, Leone RA, St George-Hyslop P, Rogaeva E, Bruni AC. Epidemiology and genetics of frontotemporal dementia: a door-to-door survey in southern Italy. *Neurobiol. Aging.* 2012; 33:2948.e1–2948.e10. [PubMed: 22819134]
- Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, Kiefer F, Cassarino TG, Bertoni M, Bordoli L, Schwede T. SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res.* 2014; 42(Web Server issue):W252–8. [PubMed: 24782522]
- Cruts M, Gijsels I, van der Zee J, Engelborghs S, Wils H, Pirici D, Rademakers R, Randenbergh R, Dermaut B, Martin JJ, van Duijn C, Peeters K, Sciot R, Santens P, De Pooter T, Mattheijssens M, Van den Broeck M, Cuijt I, Vennekens K, De Deyn PP, Kumar-Singh S, Van Broeckhoven C. Null mutations in progranulin cause ubiquitin-positive frontotemporal dementia linked to chromosome 17q21. *Nature.* 2006; 442:920–924. [PubMed: 16862115]
- Cruts M, Theuns J, Van Broeckhoven C. Locus-specific mutation databases for neurodegenerative brain diseases. *Human mutation.* 2012; 33:1340–1344. [PubMed: 22581678]
- DeJesus-Hernandez M, Mackenzie IR, Boeve BF, Boxer AL, Baker M, Rutherford NJ, Nicholson AM, Finch NA, Flynn H, Adamson J, Kouri N, Wojtas A, Sengdy P, Hsiung GY, Karydas A, Seeley WW, Josephs KA, Coppola G, Geschwind DH, Wszolek ZK, Feldman H, Knopman DS, Petersen RC, Miller BL, Dickson DW, Boylan KB, Graff-Radford NR, Rademakers R. Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. *Neuron.* 2011; 72:245–256. [PubMed: 21944778]
- Finch N, Baker M, Crook R, Swanson K, Kuntz K, Surtees R, Bisceglia G, Rovelet-Lecrux A, Boeve B, Petersen RC, Dickson DW, Younkin SG, Deramecourt V, Crook J, Graff-Radford NR, Rademakers R. Plasma progranulin levels predict progranulin mutation status in frontotemporal dementia patients and asymptomatic family members. *Brain.* 2009; 132:583–591. [PubMed: 19158106]
- Gass J, Cannon A, Mackenzie IR, Boeve B, Baker M, Adamson J, Crook R, Melquist S, Kuntz K, Petersen R, Josephs K, Pickering-Brown SM, Graff-Radford N, Uitti R, Dickson D, Wszolek Z, Gonzalez J, Beach TG, Bigio E, Johnson N, Weintraub S, Mesulam M, White CL 3rd, Woodruff B, Caselli R, Hsiung GY, Feldman H, Knopman D, Hutton M, Rademakers R. Mutations in progranulin are a major cause of ubiquitin-positive frontotemporal lobar degeneration. *Hum. Mol. Genet.* 2006; 15:2988–3001. [PubMed: 16950801]
- Gass J, Lee WC, Cook C, Finch N, Stetler C, Jansen-West K, Lewis J, Link CD, Rademakers R, Nykjaer A, Petrucelli L. Progranulin regulates neuronal outgrowth independent of sortilin. *Mol. Neurodegener.* 2012; 7:33. [PubMed: 22781549]
- Ghidoni R, Stoppani E, Rossi G, Piccoli E, Albertini V, Paterlini A, Glionna M, Pegioani E, Agnati LF, Fenoglio C, Scarpini E, Galimberti D, Morbin M, Tagliavini F, Binetti G, Benussi L. Optimal

- plasma progranulin cutoff value for predicting null progranulin mutations in neurodegenerative diseases: a multicenter Italian study. *Neurodegener. Dis.* 2012; 9:121–127. [PubMed: 22123177]
- Goldman JS, Rademakers R, Huey ED, Boxer AL, Mayeux R, Miller BL, Boeve BF. An algorithm for genetic testing of frontotemporal lobar degeneration. *Neurology.* 2011; 76:475–483. [PubMed: 21282594]
- Guerreiro RJ, Baquero M, Blesa R, Boada M, Brás JM, Bullido MJ, Calado A, Crook R, Ferreira C, Frank A, Gómez-Isla T, Hernández I, Lleó A, Machado A, Martínez-Lage P, Masdeu J, Molina-Porcel L, Molinuevo JL, Pastor P, Pérez-Tur J, Relvas R, Oliveira CR, Ribeiro MH, Rogaeva E, Sa A, Samaranch L, Sánchez-Valle R, Santana I, Tàrraga L, Valdivieso F, Singleton A, Hardy J, Clarimón J. Genetic screening of Alzheimer's disease genes in Iberian and African samples yields novel mutations in presenilins and APP. *Neurobiol. Aging.* 2010; 31:725–731. [PubMed: 18667258]
- He Z, Bateman A. Progranulin (granulin-epithelin precursor, PC-cell-derived growth factor, acrogranin) mediates tissue repair and tumorigenesis. *J. Mol. Med.* 2003; 81:600–612. [PubMed: 12928786]
- Karch CM, Jeng AT, Skorupa T, Cruchaga C, Goate AM. Novel progranulin variants do not disrupt progranulin secretion and cleavage. *Neurobiol. Aging.* 2013; 34:2538e2540. [PubMed: 23759146]
- Krieger E, Joo K, Lee J, Lee J, Raman S, Thompson J, Tyka M, Baker D, Karplus K. Improving physical realism, stereochemistry, and side-chain accuracy in homology modeling: Four approaches that performed well in CASP8. *Proteins.* 2009; 77(Suppl 9):114–122. [PubMed: 19768677]
- Litvan I, Agid Y, Calne D, Campbell G, Dubois B, Duvoisin RC, Goetz CG, Golbe LI, Grafman J, Growdon JH, Hallett M, Jankovic J, Quinn NP, Tolosa E, Zee DS. Clinical research criteria for the diagnosis of progressive supranuclear palsy (Steele-Richardson-Olszewski syndrome): report of the NINDS-SPSP international workshop. *Neurology.* 1996; 47:1–9. [PubMed: 8710059]
- Meeus B, Verstraeten A, Crosiers D, Engelborghs S, Van den Broeck M, Mattheijssens M, Peeters K, Corsmit E, Elinck E, Pickut B, Vandenberghe R, Cras P, De Deyn PP, Van Broeckhoven C, Theuns J. DLB and PDD: a role for mutations in dementia and Parkinson disease genes? *Neurobiol. Aging.* 2012; 33:629.e5–629.e18. [PubMed: 22118943]
- Mesulam MM. Slowly progressive aphasia without generalized dementia. *Ann. Neurol.* 1982; 11:592–598. [PubMed: 7114808]
- Mori K, Weng SM, Arzberger T, May S, Rentzsch K, Kremmer E, Schmid B, Kretschmar HA, Cruts M, Van Broeckhoven C, Haass C, Edbauer D. The C9orf72 GGGGCC repeat is translated into aggregating dipeptide-repeat proteins in FTL/ALS. *Science.* 2013; 339:1335–1338. [PubMed: 23393093]
- Mukherjee O, Wang J, Gitcho M, Chakraverty S, Taylor-Reinwald L, Shears S, Kauwe JS, Norton J, Levitch D, Bigio EH, Hatanpaa KJ, White CL, Morris JC, Cairns NJ, Goate A. Molecular characterization of novel progranulin (GRN) mutations in frontotemporal dementia. *Hum. Mutat.* 2008; 29:512–521. [PubMed: 18183624]
- NCBI Resource Coordinators. Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res.* 2015; 43(Database issue):D6–17. [PubMed: 25398906]
- Neary D, Snowden JS, Gustafson L, Passant U, Stuss D, Black S, Freedman M, Kertesz A, Robert PH, Albert M, Boone K, Miller BL, Cummings J, Benson DF. Frontotemporal lobar degeneration: a consensus on clinical diagnostic criteria. *Neurology.* 1998; 51:1546–1554. [PubMed: 9855500]
- Poorakaj P, Bird TD, Wijsman E, Nemens E, Garruto RM, Anderson L, Andreadis A, Wiederholt WC, Raskind M, Schellenberg GD. Tau is a candidate gene for chromosome 17 frontotemporal dementia. *Ann. Neurol.* 1998; 43:815–825. Erratum in: *Ann. Neurol.* 44, 428. [PubMed: 9629852]
- Rademakers R, Neumann M, Mackenzie IR. Advances in understanding the molecular basis of frontotemporal dementia. *Nat. Rev. Neurol.* 2012; 8:423–434. [PubMed: 22732773]
- Riley DE, Lang AE, Lewis A, Resch L, Ashby P, Hornykiewicz O, Black S. Cortical basal ganglionic degeneration. *Neurology.* 1990; 40:1203–1212. [PubMed: 2381527]
- Seelaar H, Rohrer JD, Pijnenburg YA, Fox NC, van Swieten JC. Clinical, genetic and pathological heterogeneity of frontotemporal dementia: A review. *J. Neurol. Neurosurg. Psychiatry.* 2011; 82:476–486. [PubMed: 20971753]

- Shankaran SS, Capell A, Hruscha AT, Fellerer K, Neumann M, Schmid B, Haass C. Missense mutations in the progranulin gene linked to frontotemporal lobar degeneration with ubiquitin-immunoreactive inclusions reduce progranulin production and secretion. *J. Biol. Chem.* 2008; 283:1744–1753. [PubMed: 17984093]
- Sleegers K, Brouwers N, Van Damme P, Engelborghs S, Gijselinck I, van der Zee J, Peeters K, Mattheijssens M, Cruts M, Vandenberghe R, De Deyn PP, Robberecht W, Van Broeckhoven C. Serum biomarker for progranulin-associated frontotemporal lobar degeneration. *Ann. Neurol.* 2009; 65:603–609. [PubMed: 19288468]
- Stenson PD, Mort M, Ball EV, Shaw K, Phillips AD, Cooper DN. The Human Gene Mutation Database: building a comprehensive mutation repository for clinical and molecular genetics, diagnostic testing and personalized genomic medicine. *Hum. Genet.* 2014; 133:1–9. [PubMed: 24077912]
- Tolkatchev D, Malik S, Vinogradova A, Wang P, Chen Z, Xu P, Bennett HP, Bateman A, Ni F. Structure dissection of human progranulin identifies well-folded granulin/epithelin modules with unique functional activities. *Protein Sci.* 2008; 17:711–724. [PubMed: 18359860]
- Van Damme P, Van Hoecke A, Lambrechts D, Vanacker P, Bogaert E, van Swieten J, Carmeliet P, Van Den Bosch L, Robberecht W. Progranulin functions as a neurotrophic factor to regulate neurite outgrowth and enhance neuronal survival. *J. Cell Biol.* 2008; 181:37–41. [PubMed: 18378771]
- van der Zee J, Le Ber I, Maurer-Stroh S, Engelborghs S, Gijselinck I, Camuzat A, Brouwers N, Vandenberghe R, Sleegers K, Hannequin D, Dermaut B, Schymkowitz J, Campion D, Santens P, Martin JJ, Lacomblez L, De Pooter T, Peeters K, Mattheijssens M, Vercelletto M, Van den Broeck M, Cruts M, De Deyn PP, Rousseau F, Brice A, Van Broeckhoven C. Mutations other than null mutations producing a pathogenic loss of progranulin in frontotemporal dementia. *Hum. Mutat.* 2007; 28:416. [PubMed: 17345602]
- Van Durme J, Delgado J, Stricher F, Serrano L, Schymkowitz J, Rousseau F. A graphical interface for the FoldX forcefield. *Bioinformatics.* 2011; 27:1711–1712. [PubMed: 21505037]
- Wang J, Van Damme P, Cruchaga C, Gitcho MA, Vidal JM, Seijo-Martínez M, Wang L, Wu JY, Robberecht W, Goate A. Pathogenic cysteine mutations affect progranulin function and production of mature granulins. *J. Neurochem.* 2010; 112:1305–1315. [PubMed: 20028451]
- Xu J, Xilouri M, Bruban J, Shioi J, Shao Z, Papazoglou I, Vekrellis K, Robakis NK. Extracellular progranulin protects cortical neurons from toxic insults by activating survival signaling. *Neurobiol. Aging.* 2011; 32:2326.e5–16. [PubMed: 21820214]
- Yu CE, Bird TD, Bekris LM, Montine TJ, Leverenz JB, Steinbart E, Galloway NM, Feldman H, Woltjer R, Miller CA, Wood EM, Grossman M, McCluskey L, Clark CM, Neumann M, Danek A, Galasko DR, Arnold SE, Chen-Plotkin A, Karydas A, Miller BL, Trojanowski JQ, Lee VM, Schellenberg GD, Van Deerlin VM. The spectrum of mutations in progranulin: a collaborative study screening 545 cases of neurodegeneration. *Arch. Neurol.* 2010; 67:161–170. [PubMed: 20142524]
- Zhu J, Nathan C, Jin W, Sim D, Ashcroft GS, Wahl SM, Lacomis L, Erdjument-Bromage H, Tempst P, Wright CD, Ding A. Conversion of proepithelin to epithelins: roles of SLPI and elastase in host defense and wound repair. *Cell.* 2002; 111:867–878. [PubMed: 12526812]

Web references

- AD&FTD Mutation Database. <http://www.molgen.ua.ac.be/admutations/>. Last accession 05-04-2015
- Exome Variant Server (ESP6500). <http://evs.gs.washington.edu/EVS/>. Last accession 05-04-2015
- Exome Aggregation Consortium (ExAC) browser. <http://exac.broadinstitute.org>. Last accession 05-04-2015

HIGHLIGHTS

- 1-** Three *GRN* missense mutations were identified in individuals clinically affected with FTLT: *GRN* C105Y, A199V and R298H.
- 2-** Pathogenetic features of *GRN* missense mutations are not obvious and require in vitro functional investigation
- 3-** An algorithm is proposed to classify the pathogenicity of *GRN* missense mutations
- 4-** *GRN* A199V is possibly pathogenic
- 5-** *GRN* R298H has uncertain pathogenicity
- 6-** *GRN* C105Y affects PGRN secretion and elastase cleavage: it is definitely pathogenic

Figure 1. Pedigrees of the families and *GRN* sequencing chromatograms
 (A) Family 1R. (B) Family 2T. (C–E) Sequencing chromatograms of the novel C105Y mutation present in Family 1R (C), R298H mutation in family 2T (D) and A199V mutation (E). Probands are indicated by arrows. Black filled symbols represent subjects clinically affected by FTLT. Gray filled symbols represents subjects with non-FTLT or uncharacterized form of dementia. Diagonal lines indicate the subject is deceased. Asterisks represent patients carrying a *GRN* mutation.

Figure 2. Structural GRN modelling

(A, B) Structural modelling of GRN G. (A) GRN WT; the arrow indicates the disulfide bridge between C105 and C92. (B) GRN C105Y; The disulphide bridge is disrupted by the Y105 substitution. (C, D) Structural modelling of GRN A. (C) GRN WT; the arrow indicates the hydrogen bond between R298 and D285. (D) GRN R298H; The hydrogen bond is disrupted by the H298 substitution.

Figure 3. LCL carrying the *GRN* C105Y mutation exhibit reduced PGRN secretion
 (A, B) LCL cell lysates and media were collected and analyzed by SDS-PAGE. (A) Cell lysates were probed with the anti-PGRN^{FL} antibody and the anti-beta-tubulin antibody. (B) Media were probed with the rabbit polyclonal antibody against human progranulin (Genetex). (C). Media were analyzed by PGRN ELISA. Graphs represent mean $\pm$ SD. *, $p < 0.01$; Student's t-test.

Figure 4. *GRN* missense mutations do not affect PGRN expression and partially affect PGRN secretion

GRN WT or *GRN* mutations were expressed in HEK293T cells for 48 hours. (A, B) Cell lysates and media were collected and analyzed by SDS-PAGE under reducing conditions. Immunoblots were probed with the anti-PGRN C-term antibody. (A) Cell lysates. (B) Media. (C) PGRN protein was quantified in media by measuring the band intensity for each sample. PGRN levels were corrected for background and expressed as arbitrary units (A.U.). Graphs represent mean $\pm$ SD. (D) Media were analyzed by PGRN ELISA. Graphs represent mean $\pm$ SEM. Data is representative of 3 replicate experiments. *, $p < 0.03$; Student's t-test.

Figure 5. *GRN* missense mutations do not disrupt C-terminal PGRN cleavage by elastase
GRN WT or *GRN* mutations were expressed in HEK293T cells for 24 hours and serum-free media was replaced for an additional 24 hours. Media from cells were treated with elastase (5U/ul) and incubated at 37°C for varying time points. Samples were analyzed by SDS-PAGE under reducing conditions. Immunoblots were probed with the anti-PGRN^{C-term} antibody. Data is representative of 3 replicate experiments.

Figure 6. *GRN* C105Y influences N-terminal PGRN cleavage

GRN WT or *GRN* mutations were expressed in HEK293T cells for 24 hours and serum-free media was replaced for an additional 24 hours. Media from cells were treated with elastase (5U/ul) and incubated at 37°C for varying time points. Samples were analyzed by SDS-PAGE under non-reducing conditions. Immunoblots were probed with the anti-PGRN^{FL} antibody. Data is representative of 3 replicate experiments.

Figure 7. Algorithm to classify the benign or pathogenic nature of *GRN* missense mutations
 This algorithm is modelled on the algorithm previously reported by Guerreiro et al. (Guerreiro et al., 2010). The number of controls has been defined according to classical genetics' criteria, that conventionally considered 100 healthy controls the minimal number to be analyzed in order to discriminate between pathological and benign mutations. We now know that there are several mutations with very low frequency that are even so benign polymorphisms, and wide databases such as ExAC allow us to detect an even more growing number of them. In absence of updated guidelines about the appropriate number of controls in the genetics field, in our algorithm we still considered the 100 controls.

Figure 8. Diagram of PGRN processing by elastase

Full-length PGRN protein is cleaved by elastase at the AC linker, which produces a 45kDa N-terminal fragment and a 35kDa C-terminal fragment. The 35kDa fragment is cleaved at CD and DE linkers. This produces 19kDa and 10kDa fragments, which are detectable by the C-terminal PGRN antibody. These fragments are altered in PGRN carrying the C521Y mutation. The 45kDa fragment is cleaved at the FB linker, which yields a 25kDa fragment detected by the full-length PGRN antibody. The 45kDa fragment is absent and the 25kDa fragment is disappearing quickly from PGRN carrying the C139R and C105Y mutations. Mutations examined in this study are marked. Green, pathogenic mutations with functional impact on PGRN. Blue, novel mutation with functional impact on PGRN. Black, mutations with no obvious impact on PGRN. Yellow asterisk, site of cleavage.

Table 1

Mutation, plasma dosage and bioinformatic data of patients

Patient	Family	Mutation	Plasma PGRN (ng/ml)	PolyPhen2 (Score [*])	SIFT (Score ^{**})
III-8	1R	C105Y	42	Probably Damaging (1)	Damaging (0)
III-9	1R	C105Y	41.4	Probably Damaging (1)	Damaging (0)
III-8	2T	R298H	129	Probably Damaging (0.996)	Tolerated (0.13)
3	na	A199V	40.1	Benign (0.058)	Tolerated (0.53)

The amino acid substitution is predicted to be damaging if the score is less than 0.05 and tolerated if the score is greater than 0.05.
na, not applicable.

* Score represents false positive rate thresholds.

** Score ranges from 0 to 1.