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A Novel Splice-Acceptor Site Mutation in GRN (c.709-2 A>T) Causes Frontotemporal Dementia Spectrum in a Large Family from Southern Italy

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Abstract

Heterozygous loss of function mutations in granulin represent a significant cause of frontotemporal lobar degeneration with ubiquitin and TDP-43 inclusions (FTLD-TDP). We report a novel *GRN* splice site mutation (c.709-2 A>T), segregating with frontotemporal dementia spectrum in a large family from southern Italy. The *GRN* c.709-2 A>T is predicted to result in the skipping of exon 8, leading to non-sense mediated mRNA decay. Moreover, the PGRN plasma levels in the *GRN* c.709-2 A>T carriers were significantly lower (24 ng/ml) compared to controls (142.7 ng/ml) or family members non-carriers (82.0 ng/ml) (*p*-value = 0.005, Kruskal Wallis), suggesting progranulin haploinsufficiency. We do not report any potential pathogenic *GRN* mutation in a follow-up cohort composed of 6 FTD families and 43 sporadic FTD cases, from the

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SUPPLEMENTARY MATERIAL

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same geographic area. Our study suggests that *GRN* (c.709-2 A>T) is a novel and likely very rare cause of FTD in this Italian cohort. Finally, in line with previous studies, we show that *GRN* haploinsufficiency leads to a heterogeneous clinical picture, and plasma progranulin levels may be a reliable tool to identify *GRN* loss of function mutations. However, given that a) genetic and environmental factors, gender, and age may regulate PGRN plasma levels and b) plasma progranulin levels may not reflect PGRN levels in the central nervous system, we suggest that the measurement of progranulin in the plasma should always be coupled with genetic screening of *GRN* for mutations.

Keywords

Alzheimer's disease; frontotemporal dementia; progranulin; splice site mutation

INTRODUCTION

Frontotemporal dementia (FTD) refers to a clinical spectrum of disorders that are genetically and neuropathologically heterogeneous. FTD is the second leading cause of early onset dementia, after Alzheimer's disease [1]. The main FTD phenotypic subtypes are behavioral variant FTD (bvFTD) and primary progressive aphasia (PPA), which includes primary non-fluent aphasia (PNFA) and semantic dementia (SD). 40–50% of the FTD cases are apparently familial in nature [2], thus genetics appears to play a significant role in disease development. Most commonly, heterozygous loss-of-function (LoF) mutations in the granulin precursor gene (*GRN*) cause frontotemporal lobar degeneration with TAR DNA-binding protein TDP-43 inclusions (FTLD-TDP) through progranulin haploinsufficiency [3]. In addition, the microtubule-associated tau gene (*MAPT*) [4] and a hexanucleotide repeat expansion in the non-coding region of chromosome 9 open reading frame (*C9orf72*) represent other important genetic factors [5]. Less frequently, mutations in *TDP-43* [6], *VCP* and *CHMP2B* have been reported to cause FTD (http://www.molgen.ua.ac.be/ADMutations/). Finally, two genome-wide association studies identified three main loci associated with FTD [7, 8]. A growing body of evidence has shown a correlation between progranulin mRNA and proganulin plasma levels, suggesting that analysis of progranulin levels in plasma reliably predicts *GRN* LoF mutations in patients with FTD and their asymptomatic family members [9–11]. In this study, we report a novel splice-acceptor site mutation (c.709-2 A>T) in *GRN,* segregating with the disease in a large FTD Italian family, and we aimed to assess whether plasma progranulin levels may have been used to detect mutation status.

MATERIALS AND METHODS

Patients were ascertained as part of an ongoing genetic-epidemiologic study conducted in the Apulia region of Italy. Family A extends over five generations and comprises at least 126 individuals, of whom 22 had a clinical diagnosis of dementia (Fig. 1). The family history was suggestive of an autosomal dominant pattern of inheritance. In this study we have collected information from the spouses and/or first-degree relatives and examined 12 affected and 19 unaffected family members. The average age at onset was 67 years (range:

43–80 years), disease duration was 7 years (range: 2–16 years), and age at death was 74 years (range: 49–85) (Table 1). The typical clinical presentation included bvFTD with behavioral and personality changes at onset, progressive language impairment over the course of the disease, and dementia at the end stages. Furthermore, we have used exome sequencing data to screen *GRN* mutations in a cohort of 6 FTD families (16 familial cases) and 43 sporadic independent cases from the same geographic area (Supplementary Table 1).

A diagnosis of FTD was made according to Neary criteria [12]. The family pedigree was reconstructed by means of interviews with family members. At the time of evaluation, the majority of affected members of this family were deceased, thus, whenever possible, clinical information was obtained by review of medical records and/or family report of progressive behavior/cognitive dysfunction. Written consent for participation was obtained in accordance with institutional review board standards. The independent local ethics committee of the "Ospedale Policlinico Consorziale di Bari" specifically approved this study (3789-13/07/2011).

Genetic screening

In this family we collected blood samples from 6 affected subjects and 19 unaffected first-degree relatives. DNA was extracted from blood following standard procedures. The 25 family members were screened for pathogenic mutations in *GRN* and *C9orf72* repeat expansions.

Sanger sequencing

We sequenced all the *GRN* exons and flanking regions. Primers for target exons were designed in Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/) using UCSC (http://genome.ucsc.edu/) reference sequences NM_002087.2 (*GRN*). We amplified exons and exon-intron boundaries flanking the rare variants of interest using the following PCR reaction mix: 15 ng of genomic DNA, 10 nM forward primer, 10 nM reverse primer, and 10 μl of FastStart PCR Master Mix (Roche, IN, USA). Sequencing was performed on an ABI 3730 DNA Analyzer (Applied Biosystems, CA, USA). Sequence traces were analyzed using Sequencer (version 4.2 Gene Codes Corporation).

Screening for *C9orf72* repeat expansions was performed using repeat prime PCR as previously described [5].

Exome sequencing

We performed exome sequencing on 16 familial and 43 sporadic FTD cases. Library preparation for next-generation sequencing was performed according to the Nextera (Illumina) sample-preparation protocols. DNA libraries were then hybridized to exome-capture probes with NimbleGen SeqCap EZ Human Exome Library, version 2.0 (Roche Nimble-Gen) or TruSeq (Illumina). Exome-enriched libraries were sequenced on the Illumina HiSeq™ 2000 using 2 × 100 bp paired end read cycles.

Bioinformatics

Sequence alignment and variant calling were performed against the reference human genome (UCSC hg19). Paired end sequence reads (2 × 100 bp paired end read cycles) were aligned using the Burrows-Wheeler aligner [13]. Format conversion and indexing were performed with Picard (http://broadinstitute.github.io/picard/). The Genome Analysis Toolkit (GATK) was used to recalibrate base quality scores, perform local re-alignments around indels and to call and filter the variants [14]. VCFtools and ANNOVAR were used to annotate the variants [15]. Variants were checked against established databases (1000 Genomes Project, released 16 October 2014, and dbSNP v.137). The protein coding effects of variants was predicted using Mutation Taster, PolyPhen2 and SIFT.

Progranulin plasma levels

We collected plasma samples from the available Family A members carrying the *GRN* c. 709-2A>T mutation (4 affected and 2 asymptomatic members) and from individuals not carrying the mutation (4 unaffected family members and 28 healthy elderly unrelated controls) (Table 2 and Supplementary Table 2).

Progranulin enzyme-linked immunosorbent assay

To measure progranulin plasma levels, we used an ELISA kit (Human Progranulin Elisa Kit, Adipogen Inc., Seoul, Korea). We used a 1 : 100 dilution of the plasma samples in 1× diluent following the manufacturer's instructions.

Statistical analysis

The non-parametric Kruskal-Wallis test was used to compare plasma progranulin levels and age between three groups (c.709-2A>T carriers, non-mutation carrying family members, and healthy unrelated controls). Exact *p*-values were computed. The Mann Whitney U test was performed as post-hoc comparisons using the Bonferroni correction for multiple testing. Linear regression analysis was used to test for trend across groups. Correlation between age and plasma progranulin levels were assessed using Spearman's rho coefficient. All analyses were performed using SAS 9.3 software.

RESULTS

Genetic analysis

We report a novel predicted pathogenic splice site mutation in *GRN* (c.709-2 A>T), segregating with FTD in Family A. Notably, *GRN* (c.709-2 A>T) does not seem to be a common founder mutation, as it has not been found in a follow-up cohort of 6 FTD families (16 familial cases) and 43 independent sporadic FTD cases geographically matched. No pathogenic mutations in *GRN* have been detected in this follow-up cohort (Supplementary Table 3). None of the patients carries *C9orf72* repeat expansion.

Family A

At the time of study, all the members of the 1st and 2nd generations were deceased. Thus, we collected information and examined members from the III and IV generations. Six affected and 19 unaffected members underwent genetic screening.

The pedigree is shown in Fig. 1. Clinical characteristics are described in Table 1.

In line with the clinical picture associated to the most frequently reported *GRN* LoF mutation (p.R493X) [16], the main symptom at onset for affected family members was behavioral and personality changes (9 members [75%]), followed by memory problems (2 members [16.6%]) and, finally, only one patient (8.3%) displayed language impairment with the classical features of PPA. Typically presenting at the end of the 6th decade, although highly variable in some cases (range: 43–80 years), disease generally progressed with language difficulties and finally, in the more severe outcomes, to a more generalized dementing disorder with spastic hypertonia, dysphagia, and global aphasia. The average disease duration was 7 years (range: 2–16 years), and tended to be shorter in patients with later onset. For 12 individuals, a detailed clinical description was available (Table 1). In addition, member A III 9, presented with a very early onset of bvFTD (diagnosed at 43 years of age). By contrast, member A III 14, displayed the most typical disease progression.

Member A III 14

This patient presented the onset of disease at 63 years of age. Symptoms at onset were confined to daily executive functions (difficulties in cooking, money handling) and behavioral and personalities changes (vulnerability, reduced hygiene, and apathy). During the course of the disease, she developed language impairment with reduced vocabulary, paraphasic errors, and word finding difficulties. At the end stage of the disease, the patient became globally aphasic and bed-ridden. Neurological examination revealed also dysphagia, spastic hypertonia, and increased deep tendon reflex in all 4 limbs. The patient died at 71 years of age.

Notably, spastic hypertonia was present in all the patients that underwent neurological examination except the two members with memory onset, who may display pyramidal signs later in the course of the disease. Importantly, the hypertonia may reflect the involvement of the corticospinal tract and damage to the upper motoneuron. Although extrapyramidal signs and particularly parkinsonism represent a *GRN* LoF mutation hallmark, pyramidal signs have been already reported in an extended FTD kindred from southern Italy and a Canadian FTD cohort [17] and may reflect a different path of pathology spreading (motor cortex and corticospinal tract versuss basal ganglia). However, rigidity and parkinsonism may affect the patients during the disease progression.

GRN c.709-2A>T

We identified a heterozygous A to T transversion that occurs in intron 7 of *GRN* at position −2 relative to the first coding nucleotide of exon 8 (c.709 in coding DNA reference sequence NM_002087.2) (Fig. 2). The heterozygous c.709-2A>T change segregated with disease in Family A. It was found also in two asymptomatic members of generation IV (A IV 15 and A

IV 26), who, at age 62 and 51 years, were likely too young to manifest the phenotype and should be considered at risk (90% of *GRN* mutation penetrance at 70 years old and complete penetrance by 80–85 years old)[18]. The *GRN* c.709-2A>T transversion is likely pathogenic: 1) it has been predicted to most probably alter splicing (score 0.95) (http://www.fruitfly.org/seq_tools/splice.html); 2) a different nucleotide substitution in the same position (c.709-2A>G) causes FTD through *GRN* nonsense-mediated mRNA decay; 3) 7 out of 8 splice-site mutations reported in *GRN* (87.5%) have been described as pathogenic (Supplementary Table 4) (http://www.molgen.ua.ac.be/ADMutations/), suggesting that *GRN* splice sites are well conserved domains and therefore particularly enriched for highly functional variation; 4) the same A-to-T transversion in the splice acceptor site of several genes, although very rare, has been reported to cause Mendelian disorders mainly characterized by autosomal dominant inheritance (Supplementary Table 5) and finally, the phenotype of the affected members overlaps the clinical features associated to the most common *GRN* mutation (p.R493X). Therefore, we predict that the heterozygous splice site mutation identified here may have the same net result. Neither this mutation nor any other *GRN* pathogenic mutation have been detected in 6 FTD families (16 familial cases) and 43 FTD sporadic cases originating from the same geographic area. Thus, suggesting that *GRN* mutations are rare and do not represent a major genetic factor in this Italian cohort, as already reported for two other cohorts from northern and southern Italy, with an overall prevalence of *GRN* pathogenic mutation around 1% among FTD cases [17, 19]. Moreover, *GRN* (c.709-2A>T) was not found in 204 in-house exome elderly controls (age of ascertainment >60 years), Caucasian from North America and UK.

Progranulin plasma levels

We determined whether levels of progranulin in plasma could have been used to identify *GRN* c.709-2A>T carriers.

Plasma PGRN levels were measured in 3 groups: *GRN* c.709-2A>T carriers ($n = 6$), unaffected – *GRN* c.709-2A>T non-carriers from Family A ($n = 4$), and healthy elderly unrelated controls ($n = 28$) (Table 2 and Supplementary Table 2).

The PGRN plasma levels in the *GRN* c.709-2 A>T carriers were significantly lower (24 ng/ml) compared to controls (142.7 ng/ml) or family members non-carriers (82.0 ng/ml) (p-value = 0.005, Kruskal Wallis).

The mean plasma PGRN level in affected patients carrying the *GRN* c.709-2A>T mutation was 26.5 ng/ml. The lowest mean value was detected in the two asymptomatic first-degree carriers (21.5 ng/ml). An intermediate PGRN level has been detected in the unaffected first-degree family members non-carriers (82.0 ng/ml) and the highest in the unrelated elderly controls *GRN* c.709-2A>T non-carriers (142.7 ng/ml) (Fig. 3 and Supplementary Table 2).

A linear association between increasing progranulin levels and the three study groups (*GRN* c.709-2A>T carriers, family members non-carriers and unrelated age-matched controls) was found (p-value for trend <0.0001). Furthermore, no significant differences were found between unaffected first-degree family members non-carriers and unrelated elderly controls (p-value = 0.1980). PGRN plasma levels ≤32.5 ng/ml reliably identified *GRN* c.709-2A>T

carriers (Fig. 3 and Supplementary Table 2). Finally, we observed no pattern of correlation between plasma PGRN levels and age (rho = 0.23; *p*-value = 0.16).

DISCUSSION

Heterozygous LoF mutations in *GRN* represent an important cause of FTLD with TDP-43 inclusions (FTLD-TDP) [3]. These mutations are fully penetrant by the age of 80–85 years old, lead to haploinsufficiency and therefore to a significant reduction of progranulin levels, which appears to be the pathogenic mechanism [9, 20, 21]. On the contrary, *GRN* missense mutations, leading to a modest decrease in GRN secretion or GRN misfolding [22] do not cause FTLD and their role in the disease development remains controversial. The only exception to this general rule is represented by missense mutations clustering within the signal peptide domain (p.A9D) [23].

We report a novel splice site mutation in *GRN* (c.709-2 A>T), segregating with FTD in Family A. This mutation is predicted to cause a nonsense-mediated mRNA decay, leading to *GRN* haploinsufficiency. Most likely, *GRN* (c.709-2 A>T) is not a common founder mutation, as it has not been found in 6 FTD families (16 familial cases) and 43 independent sporadic FTD cases from the same geographic area.

Interestingly, a transition (A>G) in the same position (*GRN* c.709-2) has been reported to lead to a frameshift and premature stop codon (p.A237TfsX6), causing FTD in 7 families [24–28]. Remarkably, the clinical presentation of the patients carrying the c.709-2 A>G differs from the patients bearing the c.709-2 A>T transversion. Although the age at onset (6th decade) and the disease duration (6–8 years) were similar, the main difference was detected in the symptoms at onset. Predominant language impairment and parkinsonism characterize the *GRN* c.709-2 A>G mutation [24, 27, 28]. By contrast, behavioral and personality changes with spastic hypertonia were the main signs in affected members of Family A. The diverse clinical presentation may be related to a different involvement of the hemispheres and spreading of the pathology: the prevalent damage to the left hemisphere, reported in the *GRN* c.709-2 A>G carriers, likely affecting language areas (Broca's area [inferior frontal gyrus, Brodmann area 44 and 45] and Wernicke's area [superior temporal gyrus, Brodmann area 22]) and later the substantia nigra may explain the language impairment and parkinsonism, respectively. By contrast, the main damage to the right hemisphere and later corticospinal tract is likely responsible for the prevalent behavioral presentation with spastic hypertonia. Nevertheless, the evolution to language dysfunction with extrapyramidal signs may appear during the course of the disease. Thus, highlighting the heterogeneous phenotypic spectrum associated to *GRN* mutations. Notably, two of six (33.3%) affected *GRN* mutation carriers were diagnosed with Alzheimer's disease (AD) before our evaluation. They were 65 and 66 years of age and displayed severe forgetfulness. Both of these patients were described as markedly apathetic at onset and showed attentive-executive deficits at neuropsychological examination. Importantly, early-onset dementia and spastic paraplegia at the 4 limbs, that are the main clinical features of the affected members of Family A, can be easily misdiagnosed with AD with *PSEN1* mutations [29]. However, memory dysfunction affects 10–30% of FTD patients with *GRN* LoF mutations in the early stages, resembling AD or an amnestic variant of mild cognitive impairment [30]. Moreover,

a severe degenerative damage of different nodes of the Papez circuit (mainly fornix, anterior thalamus, and anterior cingulate cortex) has been reported in both clinically or neuropathologically diagnosed FTD [31]. Therefore, memory decline should not be regarded as an exclusion criterion for the clinical diagnosis of FTD. Thus, genetic screening is important for the definitive diagnosis of FTD.

Finally, to assess whether progranulin plasma levels may have been a useful tool to detect *GRN* LoF mutation status, we measured progranulin plasma levels in c.709-2 A>T mutation carriers and non-carriers from both Family A and 28 independent elderly controls from the same geographic area.

We report that plasma progranulin levels are significantly lower in patients carrying the *GRN* c.709-2 A>T mutation compared to non-carriers (*p*-value = 0.005, Kruskal Wallis).

In our study, PGRN plasma levels ≤32.5 ng/ml reliably identified *GRN* c.709-2 A>T carriers. Recent lines of evidence established a plasma PGRN cut-off value of 112 ng/ml [9] and 74.4 ng/ml [20] to distinguish *GRN* LoF mutation carriers from non-carriers. Likely, genetic, environmental [32], or biohumoral [33, 34] factors may play a role in regulating plasma progranulin level. The disparity of our cutoff-value from previous studies may suggest that other factors may influence progranulin plasma levels including genetic variants. Importantly, several single nucleotide polymorphisms (SNPs) in *GRN* (rs5848)[35], sortilin1 (*SORT1*[rs646776]) [36], *TMEM106B* (rs3173615, rs1020004, rs1990622, and rs6966915) [7, 37], age, and gender have been shown to regulate plasma progranulin levels [38]. Alternatively, these differences may be due to the different methodology applied, which led to disparate absolute progranulin level measurements.

In summary, we report a novel pathogenic splice site mutation in *GRN* (c.709-2 A>T) causative for FTD and we show that 1) *GRN* LoF mutations are a rare cause of FTD in this Italian cohort; 2) *GRN* (c.709-2 A>T) leads to a heterogeneous clinical phenotype; and 3) progranulin plasma levels may efficiently be used as a first indicator of *GRN* LoF mutations. However, we suggest that measurement of plasma progranulin levels should always be complemented with *GRN* genetic screening and gene dosage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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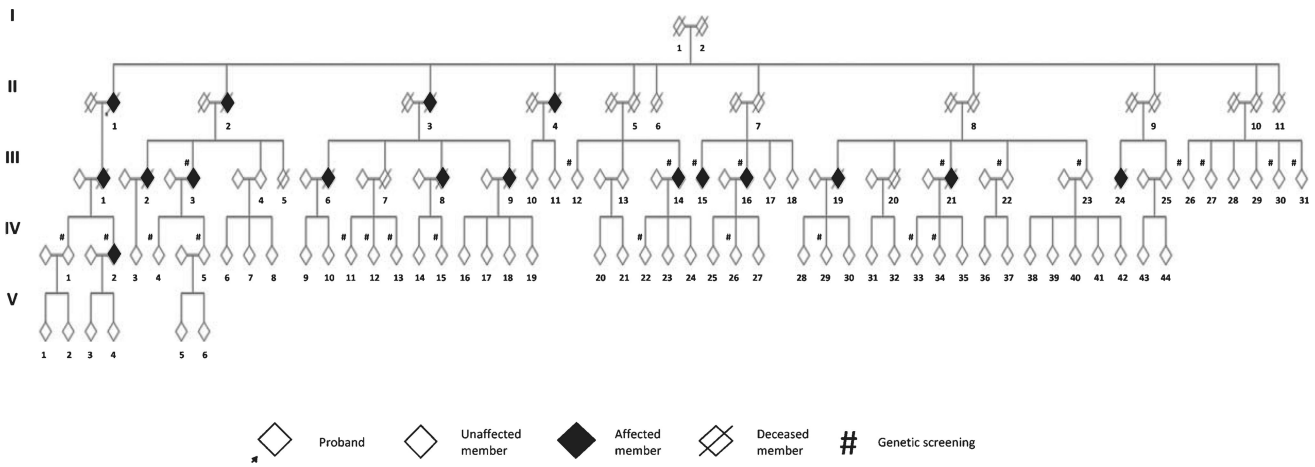

Fig. 1.
Family A pedigree.

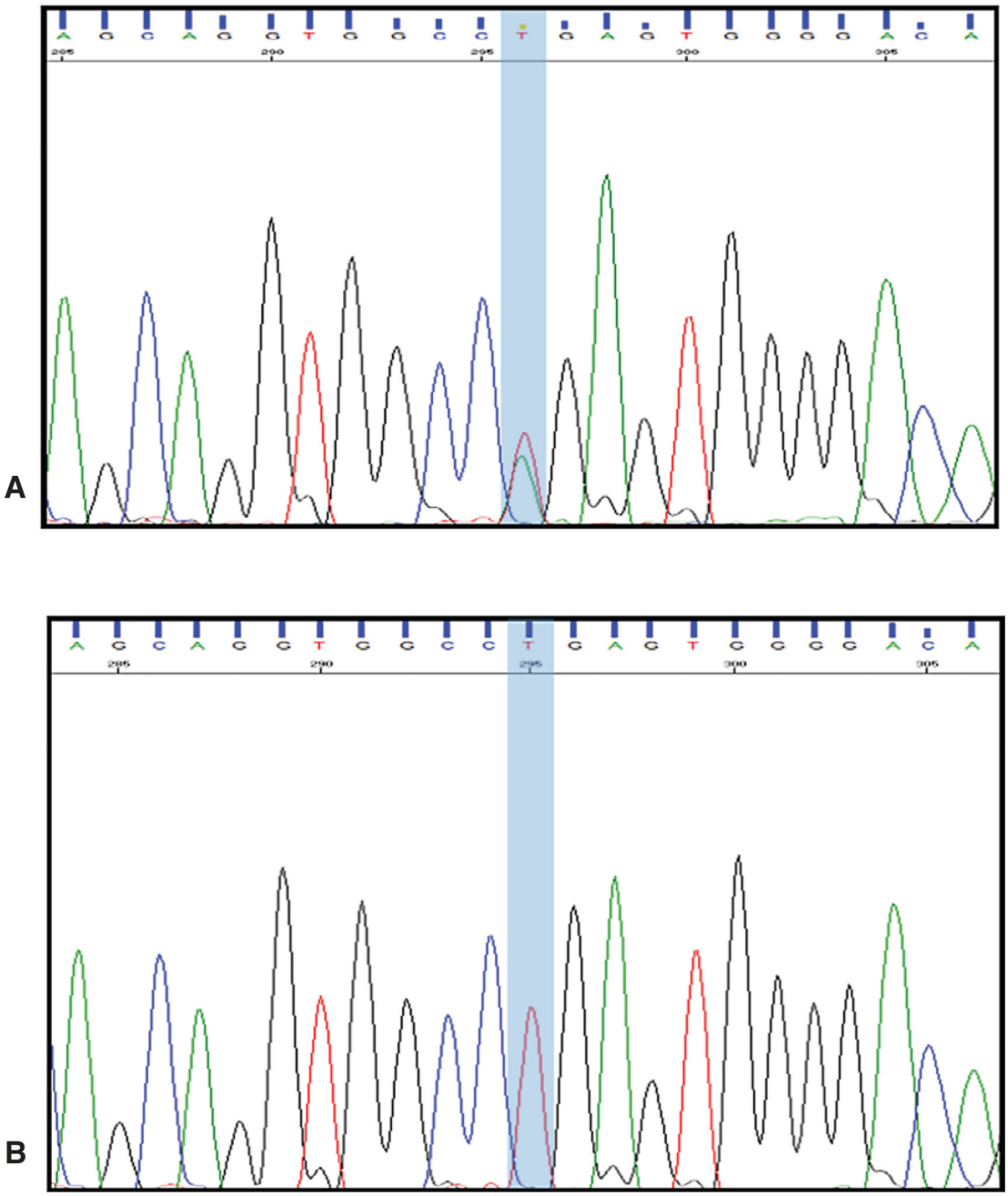

Fig. 2.

Sanger sequencing reverse sequence chromatogram, *GRN* c.709-2A>T mutation. A) Affected member Family A, carrying the heterozygous *GRN* c.709-2A>T mutation. B) Unaffected member Family A, homozygous for the wild type allele *GRN* c.709-2A.

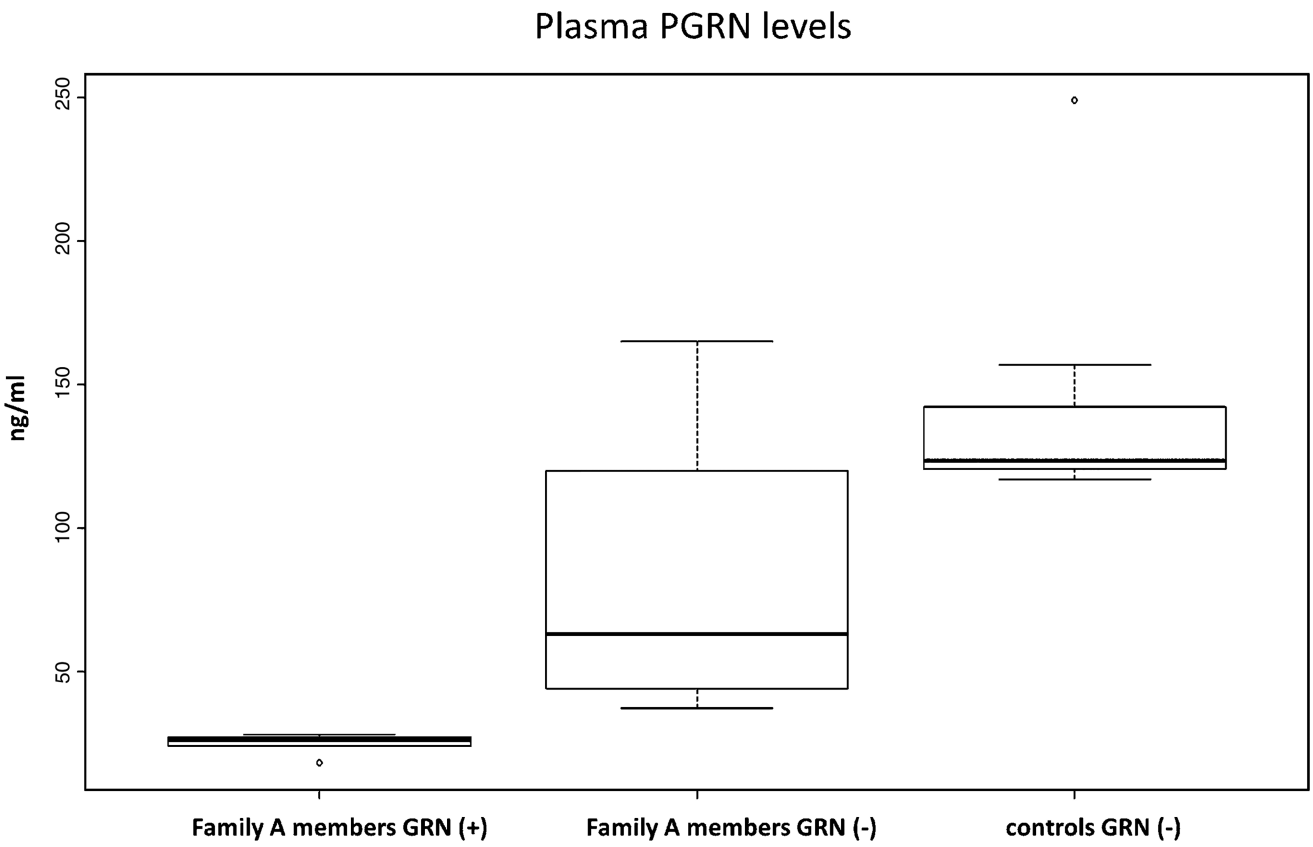

Fig. 3.

Plasma progranulin levels in affected and unaffected members of Family A and controls. PGRN, progranulin; GRN(+), c.709-2A>T; GRN(−), c.709-2A.

Table 1

Family A clinical features

Member	Genetic screening	Gender	AAO-AAD (y)	Duration (y)	First symptom	Behavioral problem	Memory deficit	Language impairment	Neurologic evaluation	CT/MRI findings	Clinical diagnosis
A III 1	not performed	F	71–76	5	behavioral change	reported	none known	none known	NA	NA	bvFTD
A III 2	not performed	F	80–85	5	behavioral change	reported	none known	none known	NA	NA	bvFTD
A III 3	*GRN* c.709-2A>T	F	71-alive	16	behavioral change	apathy	memory deficit	global aphasia	spastic tetraparesis, increased DTR, dysphagia	NA	bvFTD
A III 6	not performed	F	74–84	10	behavioral change	aggressiveness, disinhibition	none known	none known	NA	NA	bvFTD
A III 8	not performed	F	74–79	5	behavioral change	apathy, disinhibition	none known	none known	NA	NA	bvFTD
A III 9	not performed	F	43–49	6	behavioral change	aggressiveness, disinhibition	none known	none known	NA	NA	bvFTD
A III 14	*GRN* c.709-2A>T	F	63–71	8	behavioral change	vulnerability, apathy	none known	global aphasia	spastic hypertonia 4 limbs, increased DTR, dysphagia	NA	bvFTD
A III 15	*GRN* c.709-2A>T	F	65-alive	9	short term memory problems	none known	short term, attentional deficit	none known	normal	NA	FTD with memory onset
A III 16	*GRN* c.709-2A>T	F	66-alive	6	short term memory problems	apathy	short term, visual attentional deficit	none known	normal	bilateral frontal atrophy	FTD with memory onset
A III 19	not performed	F	73–75	2	behavioral change	apathy	none known	none known	NA	NA	bvFTD
A III 21	*GRN* c.709-2A>T	F	71–78	7	behavioral change	verbal aggressiveness, disinhibition	later in the disease	global aphasia	spastic hypertonia 4 limbs, increased DTR, dysphagia	NA	bvFTD
A IV 2	*GRN* c.709-2A>T	F	53-alive	6	language problems	apathy	none known	global aphasia	spastic hypertonia 2 superior limbs, increased DTR	NA	PPA

AAO, age at onset; AAD, age at death; Y, years; CT, computed tomography; MRI, magnetic resonance imaging; NA, not available; bvFTD, behavioral FTD; AD, Alzheimer's disease; PPA, primary progressive aphasia, DTR, deep tendon reflex.

Table 2

Demographic description of our cohort and progranulin plasma levels

	Family A, affected, *GRN* (+)	**Family A, asymptomatic, *GRN* (+)**	**Family A, unaffected, *GRN* (−)**	**Healthy unrelated CTRLS *GRN* (−)**
Age*, years (mean ± SD)	73.0 ± 11.5	56.5 ± 7.8	64.7 ± 9.3	65.8 ± 9.8
Gender (M/F)	0/4	0/2	2/2	8/20
PGRN ng/ml (mean ± SD)	26.5 ± 1.7	21.5 ± 4.9	82.0 ± 57.5	142.7 ± 38.08

GRN (+), *GRN* c.709-2A>T carriers; *GRN* (−), *GRN* c.709-2A>T non-carriers; M, male; F, female.

*Age at the time of the present study.

Age at onset for affected members has been reported in Table 1.