A novel progranulin mutation associated with variable clinical presentation and \tau, TDP43 and alpha-synuclein pathology

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Mutations in the progranulin (GRN) gene have recently been reported as a cause of the frontotemporal dementia (FTD) syndrome. We performed a clinical, neuropathological and molecular genetic study of two families with FTD and the same novel mutation in GRN. Age of onset ranged from 35 to 75 years and all individuals progressed to a severe dementia syndrome with a mean disease duration of 6–10 years. Variable clinical presentations included language impairment, behaviour change or parkinsonism. Seven total autopsies in the families (five in Family 1, two in Family 2) showed gross and microscopic evidence of neuronal loss in the neocortex, striatum, hippocampus and substantia nigra. All cases with material available for immunohistochemistry had cytoplasmic and intranuclear ubiquitin positive, tau negative inclusions that stained best with an antibody to the TDP43 protein. In addition, all but one had evidence of distinctive tau pathology. Two cases in Family 1 also had \alpha-synuclein (SNCA) pathology, one with diffuse neocortical inclusions and neurites and unusual striatal cytoplasmic inclusions. Affected persons in both families had the same mutation in GRN (c.709-2A>G). A minigene construct showed that this mutation alters splicing of exon 7 and results in reduced mRNA message in brain.

A single GRN mutation in these two families was associated with variable clinical presentations consistent with the FTD syndrome. All cases had ubiquitin/TDP43 immuno-positive inclusions and most had additional tau pathology. Two cases had SNCA pathology. These findings suggest a link between mutations in GRN and aggregation of \tau, TDP43 and SNCA.

Keywords: Frontotemporal dementia; progranulin; tau; alpha synuclein; Neurogenetics

Abbreviations: AD = Alzheimer’s disease; FTD = frontotemporal dementia; SNCA = \alpha-synuclein


Introduction

Frontotemporal dementia (FTD) is a clinically, neuropathologically and genetically heterogeneous syndrome. Many families, initially designated as FTD-P-17, are associated with mutations in the MAPT gene and are neuropathologically characterized by immuno-positive tau pathologic change (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). A single Danish family has been demonstrated to have mutations in CHMP2 on chromosome 3 (Skibinski et al., 2005), and FTD associated with bone and muscle disease is caused by mutations in the p97/valosin-containing protein gene (Watts et al., 2004). More recently additional families have been documented to have mutations in the progranulin (GRN) gene whose locus lies very close to MAPT (tau) on chromosome 17q (Baker et al., 2006; Cruts et al., 2006; Gass et al., 2006; Huey et al.,...
2006; Mukherjee et al., 2006; Snowden et al., 2006). These latter GRN mutation-associated cases are characterized by ubiquitin-positive, tau and α-synuclein (SNCA) negative, pathologic change (Mackenzie et al., 2006). Neumann et al. (2006) have recently shown that the ubiquitinated inclusions in FTDU are composed of the protein TAR-DNA binding protein 43 (TDP43).

Here we describe two unique families with the same novel mutation in GRN (c.709-2A>G) and describe the extensive neuropathological material. Individual cases from these families presented with variable symptoms including language impairment, behavioural change and parkinsonism. In addition, six autopsied cases from these families demonstrated ubiquitin/TDP43 immunopositive inclusions and neurites and, surprisingly, all but one had evidence of a distinctive tau pathology. Two cases also had SNCA pathology. The c.709-2A>G mutation alters splicing of GRN and results in a reduction of mRNA. Neither tau nor SNCA pathology has been previously described in confirmed GRN mutation cases.

Methods

Families with a clinical history consistent with a diagnosis of FTD were evaluated and characterized with IRB approval in the University of Washington Alzheimer’s Disease Research Center.

Brains removed at autopsy were fixed in 10% formaldehyde, paraffin embedded and sectioned for microscopic study. Standard microscopic neuropathologic evaluation included staining with haematoxylin and eosin, Luxol Fast Blue, a modified Bielschowsky silver stain and a Gallyas stain. Immunohistochemistry was performed with the following primary antibodies: Aβ peptide (6E10, Signet Labs, 1: 400), α-synuclein (LB509, 1: 1000 and syn 303, 1: 500, generous gifts of J.Q. Trojanowski), phosphorylated tau (Tau-2, Sigma, 1: 500; PHE-1, generous gift P. Davies, 1: 10; AT8, Endogen, 1: 250), TDP43 (Protein Tech, 1: 2000) and ubiquitin (Dako, 1: 150). The immunohistochemical method, in brief, included deparaffinization of ten micron sections in xylene, and then hydration through graded alcohols to distilled water. Sections were then pre-treated with 88% formic acid for 3 (6E10, 6E10, Signet Labs, 1: 400), α-synuclein (LB509, 1: 1000 and syn 303, 1: 500, generous gifts of J.Q. Trojanowski), phosphorylated tau (Tau-2, Sigma, 1: 500; PHE-1, generous gift P. Davies, 1: 10; AT8, Endogen, 1: 250), TDP43 (Protein Tech, 1: 2000) and ubiquitin (Dako, 1: 150). The immunohistochemical method, in brief, included deparaffinization of ten micron sections in xylene, and then hydration through graded alcohols to distilled water. Sections were then pre-treated with 88% formic acid for 3 (6E10, LB509, Syn303) or 10 min (Tau-2), or microwave heated (AT8, TDP43) in an antigen retrieval solution (DakoCytomation Target Retrieval Solution, Dako, Denmark), or underwent no pretreatment (PHE-1, ubiquitin). Sections were then rinsed in PBS, and then with 3% hydrogen peroxide. After a 60-min block in 3% milk, sections were incubated at room temperature with primary antibody for 1 h. After PBS rinse the sections were incubated with secondary antibody for 45 min. After an additional PBS rinse, the sections were incubated in an avidin–biotin complex for 1 h. Sections were incubated in diaminobenzidine for 10 min, rinsed, and then dipped in lithium carbonate solution, rinsed with tap water, dehydrated through graded alcohols, and cleared in xylene.

DNA sequencing and genotyping

The 13 exons of GRN and at least 30 bp of their flanking introns were fully sequenced in both directions. These fragments were Polymerase chain reaction (PCR) amplified from genomic DNA using primers that were selected by Primer3 software Rozen & Skaletsky (2000) (Supplementary Table). PCR were carried out by mixing 20 pmol of primers and 20 ng of genomic DNA with 10 µl HotStarTaq DNA Polymerase master mixture (Qiagen) in a final volume of 20 µl. After 32 cycles, 2 µl of ExoSAP-IT (USB) were added to the PCR products and digested for 1 h at 37°C to remove the residual primers and dNTPs. Four microlitre of the ExoSAP-treated PCR fragments were sequenced using BigDye terminator cycle sequencing kit (Applied Biosystems) in a final volume of 10 µl and 25 cycles. The sequence information was collected and analysed on a 3100 Genetic Analyzer (Applied Biosystems), and nucleotide variants were identified by sequence alignment using SEQUENCER software (Gene Codes Corp.). For the GRN c.709-2A>G mutation, a TaqMan allele discrimination assays (Applied Biosystems) was custom made and genotyped in 384 control individuals. Genotyping were then performed on the 384 wells plates with 5 ng genomic DNA each. For genotyping, 0.075 µl of 20×SNP TaqMan Assay, 1.5 µl of TaqMan Universal PCR Master Mix (Applied Biosystems) and 1.425 µl of DH2O were mixed into each well and then carried out PCR reactions using a 9700 Gene Amp PCR System (Applied Biosystems). PCR was carried out with a profile of 95°C for 10 min and then 50 cycles at 92°C for 15 sec and 60°C for 90 sec. Plates were then subjected to end-point read in a 7900 Real-Time PCR System (Applied Biosystems). The results were first evaluated by cluster variations, the allele calls were then assigned automatically before transferred and integrated into the genotype database.

Analysis of RNA

Total RNA was isolated from frozen brain by RNeasy Kit (Qiagen), and subjected to reverse transcription reaction using SuperScript III Kit (Invitrogen). GRN cDNA-specific PCR primers were synthesized and used to amplified full-length cDNA. The amplified products were subjected to gel electrophoresis and visualized by ethidium bromide staining. GRN mRNA levels were quantified by qRT–PCR using premade TaqMan gene expression assays (Applied Biosystems, Assay ID: Hs00173570_m1) with β-actin (Applied Biosystems, Assay ID: Hs99999903_m1) as an endogenous control for sample normalization.

Splicing assays

GRN exon 7 along with 100 bp of flanking intronic sequences was PCR amplified from genomic DNA obtained from a mutation carrier from Family 1. The primers used were GRN-16-Xhol and GRN-17-BamHI which contain Xhol and BamHI restriction sites (Supplementary Table). The resulting PCR product fragment was digested with restriction enzymes Xhol and BamHI and inserted into expression vector pSPES (D’Souza and Schellenberg et al., 2002). Multiple clones were isolated and fully sequenced to select for clones representing either A or G allele of the c.709-2A>G site without PCR artefacts the in surrounding sequences. These constructs were transiently transfected into neuronal PC12 cells using LipofectAMINE (Invitrogen) for 5 h at 37°C. Total RNA was isolated 48 h post-transfection with TRIzol reagent (Invitrogen). Minigene transcripts from the splicing vectors were analysed by RT–PCR assays using SD6 and SA2 (Table 1) primer pairs. Amplified products were resolved on a FlashGel (Cambrex Bio Science, ME, USA) and visualized with ethidium bromide staining.

DNA from affected persons in Family 1 was also scanned for mutations in MAPT (tau), LRRK2 and TARDBP (TDP43) using previously reported methods (Poorkaj et al., 1998; Zabetian et al., 2006).
<table>
<thead>
<tr>
<th>Individual</th>
<th>Apo E</th>
<th>Age onset (years)</th>
<th>Sex</th>
<th>Age death (years)</th>
<th>Dur (years)</th>
<th>First symptom</th>
<th>Memory deficit</th>
<th>Behaviour problem</th>
<th>Language problem</th>
<th>Parkinsonian features</th>
<th>Neurologic exam</th>
<th>CT/MRI findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-1</td>
<td>62 (F)</td>
<td>66</td>
<td></td>
<td>4</td>
<td></td>
<td>Anxiety, ran away from friends, memory loss.</td>
<td>Yes</td>
<td>Threatened suicide, destructive, agitated, pacing, delusions, heard voices</td>
<td>None known</td>
<td>None known</td>
<td>Normal reflexes</td>
<td>NA</td>
</tr>
<tr>
<td>II-6</td>
<td>60 (F)</td>
<td>65</td>
<td></td>
<td>5</td>
<td></td>
<td>Apathy, difficulty recognizing children, sat staring for long periods of time</td>
<td>Yes Completely disoriented, Confusion</td>
<td>Apathy, overly talkative, hallucinations and delusions, poor personal hygiene</td>
<td>None known</td>
<td>Shuffle, Psycho-motor retardation</td>
<td>'No definite defects in coordination', brisk reflexes, bilateral Babinski</td>
<td>NA</td>
</tr>
<tr>
<td>II-7</td>
<td>75 (F)</td>
<td>78</td>
<td></td>
<td>3</td>
<td></td>
<td>Fell and broke hip</td>
<td>Yes</td>
<td>Dysarthria, expressive aphasia, progressed to mute</td>
<td>None known</td>
<td>None known</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>III-1</td>
<td>3/3</td>
<td>59 (M)</td>
<td></td>
<td>16</td>
<td></td>
<td>Cognitive decline</td>
<td>Yes</td>
<td>Unable to handle money</td>
<td>Expressive aphasia, progressed to mute</td>
<td>Resting tremor, rigidity, Dx atypical PD</td>
<td>Myoclonic jerks, tremulous</td>
<td>Severe diffuse atrophy R&gt;L</td>
</tr>
<tr>
<td>III-4</td>
<td>4/4</td>
<td>52 (M)</td>
<td></td>
<td>11</td>
<td></td>
<td>Difficulty with speech</td>
<td>Yes</td>
<td>Unknown</td>
<td>Expressive aphasia, progressed to mute</td>
<td>Asymmetrical PD features</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>III-6</td>
<td>4/4</td>
<td>69 (F)</td>
<td></td>
<td>9</td>
<td></td>
<td>Anxiety, memory problems</td>
<td>Yes, early and progressive, impaired immediate recall for verbal information and impaired naming ability age 70 Forgetful, left stove on, disoriented</td>
<td>Anxious, fearful, panic attacks, angry outbursts</td>
<td>Dysnomia, expressive aphasia, mute</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III-7</td>
<td>35 (F)</td>
<td>57</td>
<td></td>
<td>22</td>
<td></td>
<td>Apathy, anxiety, self-neglect, confusion</td>
<td>Yes</td>
<td>Expressive aphasia, progressed to mute</td>
<td>Marked rigidity L&gt;R, no tremor</td>
<td>BRisk tendon reflexes, left babinski, L&gt;R arm flexion Mild tremulous, apractic gait, Increased DTRs, L Babinski.</td>
<td>CT and MRI: marked atrophy, L&gt;R</td>
<td></td>
</tr>
<tr>
<td>III-9</td>
<td>3/3</td>
<td>53 (M)</td>
<td></td>
<td>7</td>
<td></td>
<td>Stutter, language impairment</td>
<td>Yes, confused, got lost.</td>
<td>Weight gain</td>
<td>Stutter/ dysarthria, dysnomia, mute</td>
<td>Rigidity R&gt;L</td>
<td>PEG: widened sulci, large ventricles</td>
<td>(continued)</td>
</tr>
</tbody>
</table>
**Western immunoblot**

**Immunoblot analysis**

Urea solubilized brain extracts were prepared as previously described by Sampathu et al. (2006). Briefly, grey matter from the post-mortem cortex was dissected and weighed. Tissue was homogenized in 5 ml/g low salt (LS) buffer (10 mM Tris, pH 7.5, 5 mM EDTA, 1 mM DTT, 10% sucrose, and a cocktail of protease inhibitors) and sedimented at 25,000 × g for 30 min at 4°C. Pellets were washed by re-extraction in LS buffer and sedimentation. Resulting pellets were subjected to two sequential extractions in 5 ml/g Triton-X buffer (LS + 1% Triton X-100 + 0.5 M NaCl) and sedimented at 180,000 × g for 30 min at 4°C. Myelin was removed from pellets by homogenization in TX buffer containing 30% sucrose followed by centrifugation. The resulting pellets were then homogenized in 0.25 ml/g urea buffer [7 M urea, 2 M thiourea, 4% 3-[3-Cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 30 mM Tris-HCl, pH 8.5] prior to centrifugation at 25,000 × g for 30 min at 4°C. Supernatants were saved as the urea fractions. Proteins were resolved in Tris-glycine 5–20% gradient SDS-PAGE, transferred to nitrocellulose and probed with rabbit anti-TDP-43 (Proteintech, Chicago, IL, USA), monoclonal anti-α-synuclein LB 509, or monoclonal anti-tau T14/T46 and secondary antibodies [horse-radish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG (Jackson Immuno Research, West Grove, PA, USA)]. Blots were developed with Renaissance Enhanced Luminol Reagents (NEN Life Science Product, Inc., Boston, MA, USA), and digital images were acquired using a Fujifilm Intelligent Darkbox II (Fuji Systems USA, Stamford, CT, USA).

**Results**

**Clinical characteristics**

**Family 1.** This family was initially described as ‘Seattle Family B’ and was one of the earliest kindreds to show likely linkage to chromosome 17q (Foster et al., 1997; Bird et al., 1998). The updated pedigree is shown in Fig. 1 and a summary of the clinical characteristics is shown in Table 1. The affected family members typically had the onset of a progressive language disturbance beginning in the sixth decade sometimes associated with asymmetrical atrophy of the left hemisphere on brain imaging. The disease progressed to a more generalized dementing disorder with average disease duration of ~10 years. One individual was described as atypical Parkinson’s disease because of asymptmetrical rigidity. A typical family member was individual III-9. He began with stuttering and difficulty finding words at age 53. He had halting, non-fluent, perseveration and poor articulation of words, difficulty naming objects and difficulty reading. CT brain imaging showed bilateral diffuse atrophy, worse in the left hemisphere with the left lateral ventricle larger than the right (Bird et al., 1998). As his language difficulty worsened he was thought to have a variant of primary progressive
aphasia. He developed confusion and memory loss, became uncommunicative and bedridden and died at age 60. Of the nine affected persons in this family about whom we have clinical information, six had marked language difficulty, three early in the clinical course. Various clinical diagnoses in the family included Parkinson’s disease, Alzheimer’s disease (AD), Huntington’s disease, depression and schizophrenia. Mean age of onset in the family was 55.6 years and mean age at death was 65.5 years. Family member III-7 was symptomatic for 22 years.

Family 2. The pedigree of this family is shown in Fig. 2 and a summary of the clinical information in Table 2. Of the 11 siblings in generation II living to adulthood, 4 were probably affected. We have limited historical information about two of the siblings, but one had a clinical diagnosis of Pick’s disease and the other Parkinson’s disease with dementia. We have considerable clinical information on the other two siblings and both came to autopsy. The father (I-1) died at age 72 and was reported to be confused and unable to recognize people. The first symptoms in individuals in the second generation had onset between ages 50 and 67 and included memory loss, apathy, social withdrawal and personality change. Three persons had serious compulsive eating with weight gain. All affected individuals eventually developed language problems that progressed to muteness. All were also described as having tremors, shuffling gait or other parkinsonian features. MRI of individual II-7 showed marked bilateral frontal and temporal atrophy, worse on the right (Fig. 3). The average disease duration was 6.8 years.

These two families were from different states, shared no surname, had no known common ancestor and had different ethnic backgrounds (Norwegian and German).

Neuropathology

Family 1. Five cases had an autopsy with neuropathologic examination, and four were available for additional staining (Table 3). Grossly the brains showed severe generalized, though often asymmetric, cerebral cortical atrophy (mean brain weight 861 g). Atrophy, neuronal loss, and gliosis of the striatum and substantia nigra were generally severe (Fig. 4). Microscopically, all cases had moderate to severe gliosis and neuronal loss in neocortex and striatum, but more variable neuronal loss in the hippocampus and substantia nigra (Table 3). Hippocampal neuronal loss was most severe in the CA1 and subiculum, with relative sparing of other hippocampal subfields. In all four cases with additional tissue available for immunostaining, there were ubiquitin and TDP43 immunopositive (tau and SNCA immuno-negative), neuronal inclusions in dentate granule cells, layer II of neocortex and the striatum (Fig. 5). Silver stains and immunohistochemistry for phosphorylated tau revealed limited or no AD-like neurofibrillary tangle (Braak stage 0–II) and neuritic plaque pathology (absent to sparse).
In fact, all cases were classified as ‘low likelihood’ using NIA-Reagan neuropathologic criteria for AD (NIA and Reagan Institute Working Group, 1997). However, all but one case had evidence of non-AD tau pathology using antibodies (AT8 and PHF-1) to hyperphosphorylated tau (Fig. 6). This pathology was characterized by staining of fibres predominantly in the striatum, amygdala and substantia nigra. This fibre staining was absent or sparse in the neocortex. Neurons were infrequently stained in these regions. Gallyas staining did not detect this pathology. In contrast, immunostaining with the antibody tau-2 revealed staining of glia in all cases. This tau-2 pathology was most severe in the striatum and more variably observed in white and grey matter of the cerebral cortices (Fig. 7). The glial tau-2 pathology was poorly visualized with the AT8 and PHF-1 antibodies and the silver stains. Double immunolabelling revealed a subset of tau-2 positive glia stained with GFAP. Finally, two cases (III-4 and III-9) had SNCA pathologic change. Case III-9 had SNCA immunopositive inclusions and neurites restricted to the brainstem, while case III-4 had anatomically diffuse SNCA immunopositive pathologic changes (Fig. 7). The distribution of the SNCA pathologic change in these two cases was generally consistent with that observed in dementia with Lewy bodies and AD (e.g. inclusions predominantly in cortical layers V and VI). Using the recently published categorization criteria for SNCA pathology, Case III-9 would be ‘brainstem predominant’ and Case III-4 ‘diffuse neocortical’ (McKeith et al., 2005). However, in contrast to most published DLB cases, in Case III-4 there were an large number of SNCA immunopositive inclusions in the striatum with few immunopositive neurites.

**Family 2.** Gross and microscopic pathology was similar to that observed in Family 1. Mean brain weight was 985 g (Table 3). Distribution of neuronal loss and gliosis was similar to Family 1. There were no diffuse or neuritic plaques and neurofibrillary pathology was very mild. Both cases demonstrated dentate granule cell, neocortical and striatal ubiquitin and TDP43 immunopositive inclusions and neurites that were immunonegative for tau and SNCA. Fibre staining with PHF-1 and AT8 antibodies was mild to moderate in these two cases as was the tau-2 immunopositive glial staining. No SNCA immunopositive lesions were observed in the brainstem, limbic or neocortical regions.

**Molecular genetics**

**DNA sequencing**

The 13 exons and their 30-bp flanking introns of *GRN* were fully sequenced in affected and unaffected individuals from both families. We identified an A-to-G transition that occurs in intron 6 of *GRN* at position -2 relative to the first coding nucleotide of exon 7 (c.709 in coding DNA reference sequence NM_002087) (Fig. 8). This nucleotide change was designated as c.709-2A>G (genomic contig NT_010783.14 position at 1082208) using the nomenclature rule for the description of sequence variations from Human Genome Variation Society (den Dunnen et al., 2000) and can also be designated IVS709-2A>G. The heterozygous c.709-2A>G change segregated exactly with the disease, in both families (Figs 1 and 2) and was not observed in 375 control individuals. This A-to-G transition alters the consensus sequence of the 3′ splice acceptor site in intron 6 (Fig. 7), which completely abolishes the intron 6 splice acceptor site as predicted by the Automated Splice Site Analyses (Nalla and Rogan, 2005). We predict that this mutation will prevent exon 7 from being included in GRN mRNA.

**Splicing assay of IVS709-2A>G**

The effect of the c.709-2A>G mutation on splicing was analysed using a cell culture-based *ex vivo* splicing assay.
<table>
<thead>
<tr>
<th>Individual</th>
<th>Apo E</th>
<th>Age onset</th>
<th>Age death</th>
<th>Dur (years)</th>
<th>First symptom</th>
<th>Memory deficit</th>
<th>Behaviour problem</th>
<th>Language problem</th>
<th>Parkinsonian features</th>
<th>Neurologic evaluation</th>
<th>CT/MRI findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-1</td>
<td>Unk (M)</td>
<td>72</td>
<td>Unk</td>
<td>Unk</td>
<td>Yes, confused, did not recognize friends</td>
<td>Unk</td>
<td>Unk</td>
<td>Unk</td>
<td>Unk</td>
<td>Unk</td>
<td>NA</td>
</tr>
<tr>
<td>II-1</td>
<td>65 (F)</td>
<td>72</td>
<td>7</td>
<td>Shaky handwriting</td>
<td>Yes</td>
<td>Unk</td>
<td>Yes, mute</td>
<td>Yes, shaky handwriting, Dx PD, falls, stopped walking</td>
<td>Yes, resting tremor, shuffling gait</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>II-7</td>
<td>3/3</td>
<td>67 (M)</td>
<td>73</td>
<td>6</td>
<td>Forgetful, disoriented</td>
<td>Yes, MMSE 27/30 age 68, 18/30 age 70</td>
<td>Yes, overeating</td>
<td>Yes, non-fluent aphaia, progressed to mute</td>
<td>Yes, non-fluent aphaia, dysnomia progressed to mute</td>
<td>Poor balance, seizure, right brain stroke</td>
<td>Moderate to marked frontal/temporal atrophy, R&gt;L</td>
</tr>
<tr>
<td>(NP0301596)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>II-10</td>
<td>4/4</td>
<td>62 (M)</td>
<td>69</td>
<td>7</td>
<td>Apathy</td>
<td>Yes</td>
<td>Yes, compulsive eating, gained 50 lbs, compulsive rubbing</td>
<td>Yes, non-fluent aphaia, dysnomia progressed to mute</td>
<td>Yes, hand tremors, shuffling gait, progressed to unable to walk</td>
<td>Gegenhalten, increased DTR</td>
<td>Marked cortical atrophy, R&gt;L</td>
</tr>
<tr>
<td>(NP0401543)</td>
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<td>II-11</td>
<td>50 (F)</td>
<td>57</td>
<td>7</td>
<td>Behaviour/personality change</td>
<td>Yes</td>
<td>Poor hygiene, disinhibited weight gain, repetitive movements</td>
<td>Yes</td>
<td>Yes, non-fluent aphaia progressed to mute</td>
<td>'Parkinson's shuffle', per family</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Mean family age onset 61.0 ± 6.6, n = 4, range = 50–67
Mean family age death 68.6 ± 6.0, n = 5, range = 57–73
Mean family duration 6.8 ± 0.4, n = 4, range = 6–7

NA = Not Available; Dx = Diagnosis; Rx = Therapy; ECT = Electroconvulsive therapy; PD = Parkinson’s disease; PEG = Pneumoencephalopan; CT = Computerized tomography; Unk = Unknown; DTR = Deep Tendon Reflexes; MMSE = Mini Mental status exam.
For these assays, GRN exon 7 and flanking sequences with either normal A allele or the mutant G allele was inserted into a minigene construct. PC12 cells were transfected with these constructs and the resulting transcripts analyzed by RT–PCR (Fig. 9). When the normal allele was present, only GRN exon 7 containing transcripts were observed. When the mutant allele was present, only RT–PCR (Fig. 9) revealed lack of GRN exon 7. This mutation resulted in the skipping of exon 7, which contains 127 nt, in the GRN transcript and lead to a frame-shift of the protein and create a premature stop codon.

Fig. 3. T1-weighted MRI imaging of II-7 in family 2. Extensive bilateral frontal and temporal atrophy, right worse than left.

Table 3  Neuropathologic findings

<table>
<thead>
<tr>
<th>Case</th>
<th>Age/Sex</th>
<th>Duration</th>
<th>Brain weight</th>
<th>Braak/CERAD stage</th>
<th>Aβ</th>
<th>Tau (glial)</th>
<th>SNCA</th>
<th>TDP43/ubiquitin</th>
<th>HS</th>
<th>Neuronal loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III-1</td>
<td>75 M</td>
<td>16 years</td>
<td>759g</td>
<td>I-A</td>
<td>+DP NP</td>
<td>++ FC/Str/TC</td>
<td>–</td>
<td>++</td>
<td>+</td>
<td>+++ FC/TC, Str</td>
</tr>
<tr>
<td>III-4</td>
<td>63 M</td>
<td>10 years</td>
<td>889g</td>
<td>0-A</td>
<td>++ DP NP</td>
<td>++ FC/Str ++TC</td>
<td>Neocort*</td>
<td>+</td>
<td>+</td>
<td>+++ FC/TC, Str, SN</td>
</tr>
<tr>
<td>III-6</td>
<td>78 F</td>
<td>9 years</td>
<td>920g</td>
<td>II-A</td>
<td>++ DP NP</td>
<td>++ Str ++FC/TC</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+++ FC/TC, Str, +++SN</td>
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<tr>
<td>III-9</td>
<td>60 M</td>
<td>7 years</td>
<td>875g</td>
<td>0-0</td>
<td>–</td>
<td>++ FC/Str ++TC</td>
<td>BS pred</td>
<td>+</td>
<td>+</td>
<td>+++ FC/TC, Str, SN</td>
</tr>
<tr>
<td>III-10 (report only)</td>
<td>68 F</td>
<td>10 years</td>
<td>860g</td>
<td>0-0</td>
<td>–</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>+++ FC/TC, BG, SN</td>
</tr>
<tr>
<td>Family 2</td>
<td></td>
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<tr>
<td>II-7</td>
<td>73 M</td>
<td>6</td>
<td>970g</td>
<td>0-0</td>
<td>–</td>
<td>++ Str ++FC/TC</td>
<td>–</td>
<td>++</td>
<td>+</td>
<td>+++ FC/TC, BG, +++SN</td>
</tr>
<tr>
<td>II-10</td>
<td>69 M</td>
<td>7</td>
<td>1000g</td>
<td>0-0</td>
<td>–</td>
<td>++ FC ++Str/TC</td>
<td>–</td>
<td>++</td>
<td>+</td>
<td>+++ FC/TC, BG, +++SN</td>
</tr>
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</table>

*Atypical striatal neuronal inclusions (see Fig. 6).

# = absent; + = mild; ++ = moderate; +++ = severe; BS Pred = Brainstem Predominant; C = neocortex; DP = diffuse plaques; FC = frontal cortex/lobe; HS = hippocampal sclerosis; Neocort = Diffuse Neocortical; NP = neuritic plaques; SN = substantia nigra; SNCA = alpha-synuclein; Str = striatum; TC = temporal cortex/lobe; BS Pred = Brainstem Predominant; Neocort = Diffuse Neocortical.

CERAD = Consortium to Establish a Registry for Alzheimer’s Disease.
GRN transcripts in brain
We quantified and compared the GRN transcripts level in the post-mortem frozen brain of III-6 in Family 1 and five normal controls. The real-time RT–PCR analyses indicated a lower message level in both cerebellum and frontal lobe regions from III-6 when compared with controls (Fig. 10). Attempts to isolate the abberantly spliced GRN transcripts lacking exon 7 from the frontal lobe and cerebellum of this Family 1 subject were unsuccessful.

Expression of other FTD-related proteins in brain
Previous studies have shown that pathological tau and α-synuclein accumulate in insoluble fractions solubilized in 8 M urea and/or 2% SDS. More recently, we have demonstrated that the accumulation of ~25 Kd C-terminally cleaved fragments, a high molecular ubiquitinated smear and hyperphosphorylated species as the pathological signature of TDP-43 (Neumann et al., 2006). To assess if biochemical signature of pathological tau, α-synuclein and TDP-43 are recovered from different brain regions from III-6 in Family 1, immunoblots containing urea extracts from temporal, hippocampus and cingulated cortex were probed with mAbs T14/46 (for tau), mAb LB509 (for α-synuclein) and rabbit anti-TDP-43, respectively. Significantly, anti-TDP-43 detected low levels of phosphorylated TDP-43 in disease hippocampus and cingulate cortex as well as a high molecular weight smear and a small amount of the 25 Kd fragment in cingulate cortex (Fig. 11). Similar TDP-43 pathological signature is not present in AD, control and PD brains. A small amount of T14/46 detected pathological hyperphosphorylated tau in urea extracts from III-6 and AD but not control or PD brains whereas α-synuclein was also detected in the urea fractions of III-6 hippocampus, cingulate cortex and PD brain (Fig. 11). Taken together, the immunoblot analyses demonstrate the co-existence in specific brain regions of pathological TDP-43, hyperphosphorylated tau and α-synuclein recovered in the insoluble fractions of affected brain in Family 1 which correlated with immunohistochemical studies.

Haplotype analysis
Members of both families were genotyped for six polymorphic markers adjacent to the mutation site in GRN. All affected persons in both families share an identical haplotype suggesting a common, but unknown, ancestor.

Analysis of MAPT and LRRK2 genes
Sequencing of MAPT (tau), LRRK2 and TARDBP (TDP43) genes in affected persons in Family 1 revealed no mutations.

Discussion
Mutations in the gene encoding progranulin (GRN) are the latest genetic cause to be documented underlying the FTD syndrome. Gass and colleagues (2006) found GRN mutations in 5–10% of a large FTD population and in 23% of those with a positive family history. Huey et al. (2006) found only two subjects with a single GRN mutation (R493X) in a sample of 84 FTD cases. This single mutation (R493X) is relatively common and likely represents a North American founder effect. Bronner and colleagues (2007) found 4–7% of Dutch familial FTD cases had mutations in GRN. We report here a novel GRN mutation (c.709-2, A>G) in two families with notable clinical and pathological characteristics.

To date, MAPT mutations have been associated with tau-immunopositive pathologic changes and GRN mutations
with ubiquitin-immunopositive, tau- and SNCA-negative, pathologic changes. The two families reported here with a novel GRN mutation are the first such families to show extensive staining with an antibody to TDP43 and also to have evidence of a non-AD tau pathology. Furthermore, two cases from Family 1 had SNCA pathology. One case, III-4, in fact, had diffuse Lewy body pathology (LBP) including cytoplasmic inclusions in the striatum. Consistent

Fig. 5 Ubiquitin (A) and TDP43 (B–E) immunostaining of brain tissue from case III-4, Family 1. Ubiquitin and TDP43 immunostaining demonstrate cytoplasmic inclusions in dentate granule cells (A and B, respectively). TDP43 immunostaining also revealed neuritic pathology, and intracytoplasmic (arrows) and intranuclear (arrowheads) inclusions in layer II of the temporal cortex (C) and the striatum (D, E).
Fig. 6  Immunostaining for hyperphosphorylated tau in a control AD case (A and C) and case III-4, Family I (B, D, E, and F). PHF-1 immunostaining demonstrates severe tau pathology in layer II (arrow) of the entorhinal cortex of an AD case (A) and an absence of tau pathology in the entorhinal cortex of case III-4. However, in the striatum (putamen) of the same AD case there is relatively sparse PHF-1 immunostained fibres (C) in comparison with case III-4 (D). Low (E) and high (F) magnification views of AT8 immunostaining reveal a similar density of fibre staining in case III-4.
Fig. 7  α-Synuclein (LB509) immunostaining revealed neuritic pathology and cytoplasmic inclusions in the deep layers of the neocortex (A) and striatum (B) in case III-4, Family 1. Tau-2 immunopositive glia in the striatum of case III-1, Family 1 (C) and in the anterior commissure of case III-6, Family 1 (D).

Fig. 8  Nucleotide change and position of the GRN c.709-2A>G mutation. (A) Location of the c.709-2A>G in the intron 6 of GRN. (B) Nucleotide change of c.709-2A relative to the consensus 3' splice site sequence.
with the immunohistochemical results, Western blotting found hyperphosphorylated tau and increased amounts of TDP43 and SNCA in frozen brain from Family 1.

The only other family with a mutation in \textit{GRN} to have such extensive neuropathological material is the HDDD2 family with eight autopsies (Mukerjee et al., 2006). The pathology in that family was typical of FTDU with ubiquitin-positive, tau- and \(\alpha\)-synuclein (SNCA) negative inclusions and neurites. SNCA and TDP43 immunostaining was not reported in that family. AD-like tau and A\(\beta\) were observed in half of the cases, including one that fulfilled criteria for AD, although the cortical distribution was somewhat atypical. In our cases, the tau pathology was very atypical for AD with absent to mild neurofibrillary tangle pathology in the entorhinal cortex. However, we did observe fibre staining with the phosphodependent antibodies PHF-1 and AT8, particularly in the striatum, amygdala and substantia nigra. Surprisingly, these changes were not observed with silver stains. Thus, the distribution and characteristics of the tau pathology were atypical for both AD and MAPT mutations associated FTD. The additional finding of glial staining with the tau-2 antibody has been described in other diseases with gliosis and is likely non-specific (Odawara et al., 1995; Uchihara et al., 2000; Forno et al., 2002).

SNCA pathologic change was observed in two members of Family 1. One case had this change restricted to the brainstem; however, the other (case III-4) had diffuse severe

**Fig. 10** Comparison of \textit{GRN} transcript expression levels in the brain of III-6 (Family I) and five normal controls by real-time \textit{PCR}. Two brain regions, i.e. cerebellum and frontal lob, from each subject were analysed; and each region/subject was assayed in two independent experiments. Y-axis represents the relative quantification (RQ) to the endogenous control transcript of \(\beta\)-Actin (ACTB).

**Fig. 11** Biochemical analyses of TDP-43, tau and \(\alpha\)-synuclein in III-6 (EF), Family I. (A) Immunoblot analysis of urea extracts from temporal (Tem), hippocampal (Hp) and cingulated (Cing) cortex with rabbit anti-TDP-43 shows pathologic \(\sim 25\) kD bands and high Mr smears, which are not present in CO, AD, or PD. (B) (Top) Immunoblot analysis using mAbs T44/T46 detects pathological hyperphosphorylated tau in urea extracts from III-6 (EF), Family I and AD, but not from CO or PD. (Bottom) mAb LB 509 shows insoluble \(\alpha\)-synuclein in urea extracts from III-6 (EF), Family I Hp and Cing, and PD, but not Temp, CO or AD.
pathology and could be classified as ‘diffuse neocortical’ (McKeith et al., 2005). The distribution of SNCA pathology in this case was generally consistent with that observed in dementia with Lewy bodies. However, there were cytoplasmic inclusions in the striatum, a region typically characterized by neuritic pathology in synucleinopathies (Duda et al., 2002). To the best of our knowledge, SNCA pathology has not been previously associated with GRN mutations.

The clinical presentation associated with this novel GRN mutation can be highly variable. Symptoms typically begin in the 50’s (range 35–69 years) and can include behavioural change, language disturbance, excessive eating and Parkinsonian features. In Family 1, nine subjects had a mean disease duration of 10 years which is greater than the average of 6 years in the Gass et al. study (2006) and demonstrate that some persons can be symptomatic for a long time (22 years in one case). Of interest, initial clinical diagnoses in the present families have included atypical Parkinson’s disease, Pick’s disease, Huntington’s disease, primary progressive aphasia, AD and FTD. These patients can be difficult diagnostic challenges for clinicians. It is noteworthy that FTD and HD share many behavioural characteristics and this likely reflects the common frontal/striatal pathology. Masellis and colleagues (2006) have reported a family in which a mutation in GRN (IVS7 +1G>A or IVS708 G>A) is associated with the corticobasal syndrome. Boeve and colleagues (2006) reported a family with an FTDP syndrome initially thought to have a PS1 mutation but later found to have a mutation in GRN (IVC1 +1 G>A or C.138 +1 G>A). Pickering-Brown et al. (2006) have found GRN mutations in families previously thought to have mutations in MAPT. Snowden and colleagues (2006) reported two families with GRN mutations (Q130SFs X124, Q486X) and associated progressive non-fluent aphasia.

Haplotype analysis in these two families suggests a common ancestral origin of the GRN mutation although they have no known common ancestor. This fits with the notion that this is a rare mutation event that has perhaps occurred only once.

Previously reported mutations in GRN have mostly been nonsense, frameshift or splice-site mutations predicted to produce premature termination of the protein, nonsense mediated decay of RNA and loss of function (haploinsufficiency) (Baker et al., 2006; Cruts et al., 2006; Gass et al., 2006). The mutation in the present two families has not been previously reported. We present evidence that this mutation alters splicing of exon 7 and likely leads to a dysfunctional or truncated protein. GRN is a multifunctional growth factor thought to promote neuronal survival and involved in tumour genesis (He and Bateman, 2003). It is presently unknown how loss of function of GRN results in severe diffuse neuronal loss in cortical and sub-cortical structures, often asymmetric brain atrophy and the typical ubiquitin positive, tau negative neuronal inclusions with marked aggregation of TDP43 and sometimes, as in the present cases, tau and SNCA pathology. Tau and progranulin are likely to be related to each other in a biological pathway that has yet to be elucidated. Better understanding of these phenomena will provide important clues to treatment and prevention.

Supplementary material

Supplementary material are available at Brain online.

Acknowledgements

We thank the members of these two families for their generous support. Support by NIH/NIA P50 AG 005 136-22, AG17586 and VA research funds. Dr P Gambetti provided autopsy material on case III-10, Family 1. Dr S.M. Sumi performed the original neuropathologic evaluation of four cases. We also wish to thank Lynne Greenup, Aimee Schantz, Randy Small and Christiana Ulness for technical assistance.

References


