Common variation in the miR-659 binding-site of \textit{GRN} is a major risk factor for TDP43-positive frontotemporal dementia

Rosa Rademakers$^{1,*}$, Jason L. Eriksen$^1$, Matt Baker$^1$, Todd Robinson$^1$, Zeshan Ahmed$^1$, Sarah J. Lincoln$^1$, Nicole Finch$^1$, Nicola J. Rutherford$^1$, Richard J. Crook$^1$, Keith A. Josephs$^2$, Bradley F. Boeve$^2$, David S. Knopman$^2$, Ronald C. Petersen$^2$, Joseph E. Parisi$^2$, Richard J. Caselli$^3$, Zbigniew K. Wszolek$^4$, Ryan J. Uitti$^4$, Howard Feldman$^5$, Michael L. Hutton$^1$,$^\dagger$, Ian R. Mackenzie$^6$, Neill R. Graff-Radford$^4$ and Dennis W. Dickson$^1$

$^1$Department of Neuroscience, Mayo Clinic, Jacksonville, FL, USA, $^2$Department of Neurology, Mayo Clinic, Rochester, MN, USA, $^3$Department of Neurology, Mayo Clinic, Scottsdale, AZ, USA, $^4$Department of Neurology, Mayo Clinic, Jacksonville, FL, USA, $^5$Division of Neurology and $^6$Department of Pathology, University of British Columbia, Vancouver, Canada

Received March 30, 2008; Revised July 30, 2008; Accepted August 20, 2008

Loss-of-function mutations in progranulin (\textit{GRN}) cause ubiquitin- and TAR DNA-binding protein 43 (TDP-43)-positive frontotemporal dementia (FTLD-U), a progressive neurodegenerative disease affecting \sim 10\% of early-onset dementia patients. Here we expand the role of \textit{GRN} in FTLD-U and demonstrate that a common genetic variant (rs5848), located in the 3'-untranslated region (UTR) of \textit{GRN} in a binding-site for miR-659, is a major susceptibility factor for FTLD-U. In a series of pathologically confirmed FTLD-U patients without \textit{GRN} mutations, we show that carriers homozygous for the T-allele of rs5848 have a 3.2-fold increased risk to develop FTLD-U compared with homozygous C-allele carriers (95\% CI: 1.50–6.73). We further demonstrate that miR-659 can regulate \textit{GRN} expression \textit{in vitro}, with miR-659 binding more efficiently to the high risk T-allele of rs5848 resulting in augmented translational inhibition of \textit{GRN}. A significant reduction in \textit{GRN} protein was observed in homozygous T-allele carriers \textit{in vivo}, through biochemical and immunohistochemical methods, mimicking the effect of heterozygous loss-of-function \textit{GRN} mutations. In support of these findings, the neuropathology of homozygous rs5848 T-allele carriers frequently resembled the pathological FTLD-U subtype of \textit{GRN} mutation carriers. We suggest that the expression of \textit{GRN} is regulated by miRNAs and that common genetic variability in a miRNA binding-site can significantly increase the risk for FTLD-U. Translational regulation by miRNAs may represent a common mechanism underlying complex neurodegenerative disorders.

INTRODUCTION

Frontotemporal lobar degeneration (FTLD) is a progressive neurodegenerative disorder representing \sim 5\% of all dementia patients (1). It is the second most common form of early-onset neurodegenerative dementia after Alzheimer’s disease (AD), affecting 10–20\% of patients with an onset of dementia before 65 years. FTLD patients present with prominent behavioral and...
personality changes, often accompanied by language impairment, which evolve gradually into cognitive impairment and dementia (2,3). FTLD may occur alone or in combination with motor neuron disease (MND) (4). The most common neuropathology associated with clinical FTLD is fronto and anterior temporal lobe atrophy with neuronal inclusions immunoreactive for ubiquitin and TAR DNA-binding protein 43 (TDP-43), but negative for tau and α-synuclein (FTLD-U) (5–7). Neuronal cytoplasmic inclusions (NCIs) in the neocortex, striatum and the dentate fascia of the hippocampus are the pathological hallmarks of FTLD-U. Up to four subtypes of FTLD-U have been delineated that are based on the distribution of NCIs, dystrophic neurites and the presence of neuronal intranuclear inclusions (NIIs) (8–10). Interestingly, all cases with GRN mutations have a common FTLD-U subtype, characterized by NCIs, short, thin neurites in layer II of the cortex and lentiform NIIs (11–13). This subtype is referred to as Type 1 by Mackenzie et al. (8) and Type 3 by Sampathu et al. (10).

FTLD has a high familial incidence with up to 50% of patients reported to have a family history of dementia. Recent molecular genetic advances in the field of FTLD have revealed that the genetic basis of FTLD-U is heterogeneous, and the causative mechanisms are just starting to be unraveled (14). Loss-of-function mutations in the gene encoding the secreted growth factor progranulin (GRN) on chromosome 17 have been identified as a major cause of familial FTLD-U and are present in up to 25% of familial FTLD-U patients worldwide (15–17). In addition, mutations in the valosin containing protein gene (VCP) and the gene encoding the charged multivesicular body protein (CHMP2B) have been reported in a small number of FTLD-U families (18,19). Despite these recent developments, it is evident that additional FTLD-U genes and genetic risk factors remain to be identified to explain the disease in the majority of the familial patients and in apparently sporadic FTLD-U patients.

We previously reported the identification of 23 different pathogenic loss-of-function mutations in GRN in 10% of the patients in our Mayo Clinic FTLD series, including a small number of apparently sporadic patients (17). In the present study, we expand the spectrum of mutational mechanisms that can lead to the loss of functional GRN. Using cell-based systems and in vivo studies we demonstrate that a common genetic variant (rs5848), located in the 3′-untranslated region (UTR) of GRN in a binding-site for micro-RNA (miRNA) miR-659 significantly increases the risk of developing FTLD-U most likely through suppressed translation of GRN. Our findings suggest that translational regulation by miRNAs may present a common mechanism underlying complex neurodegenerative disorders.

RESULTS

Association study of rs5848 with FTLD-U

We previously performed sequencing analyses of GRN in an extended population of FTLD patients (n = 378) derived from the Mayo Clinic FTLD series to assess the genetic contribution of GRN mutations to FTLD (17). A close inspection of our sequencing results in the subgroup of non-GRN mutation carriers (n = 339) revealed a statistically significant deviation from the expected Hardy–Weinberg equilibrium (HWE) for the common polymorphism rs5848 (P = 0.002), which was attributable to an excess of homozygous patients (Table 1). Deviations from HWE were not observed for any of the other genetic variants in GRN. We re-genotyped rs5848 using a pre-designed Taqman genotyping assay and confirmed all rs5848 genotypes in the FTLD patient series. Subsequent analyses of rs5848 in a large cohort of control individuals ascertained at Mayo Clinic Jacksonville (MCJ) and Mayo Clinic Scottsdale (MCS) showed a selective increase in the TT genotype frequency in FTLD patients (16%) compared with control individuals (9%) (Pgenotypic = 0.002) (Table 2).

To further confirm the genetic contribution of rs5848 to the development of FTLD, we focused our analyses on a homogeneous series of patients with a primary neuropathological diagnosis of FTLD-U with confirmed TDP-43-positive neuronal inclusions, derived from the MCJ brain bank and an age- and gender-matched control group ascertained at MCJ and MCS. Of the 81 genealogically unrelated FTLD-U patients identified in our brain bank, 19 (23.5% of the FTLD-U population) carried a pathogenic loss-of-function GRN mutation and were excluded from the study. One VCP and one LRRK2 mutation carrier were also excluded, resulting in a total of 59 FTLD-U patients for the genetic studies. Using logistic regression analyses of rs5848, we showed a highly significant association of rs5848 with FTLD-U (Padj = 0.003), resulting from an increase in the TT genotype frequency of rs5848 in FTLD-U patients (25.4%) compared with control individuals (9.9%) (Table 3). We calculated that within our series, the odds ratio (OR) to develop FTLD-U for carriers homozygous for the minor T-allele of rs5848 compared with homozygous C-allele carriers was 3.18 [Padj = 0.003; 95% confidence interval (CI): 1.50–6.73]. In contrast, individuals heterozygous for rs5848 did not show an increased risk to develop FTLD-U (Padj = 0.74; OR = 1.12; 95% CI: 0.59–2.10) (Table 3). Since MND pathology is rare or absent in GRN loss-of-function mutation carriers, we also re-analyzed the association excluding patients with MND pathology (n = 11),
SNPs was selected for single SNP and haplotype association (Supplementary Material, Results). A panel of 12 additional and performed single SNP and haplotype association analyses in the Supplementary Material, Table S1). Moreover, haplotype analyses more strongly associated with FTLD-U than rs5848 (Supplementary Material, Fig. S2: Fig. 1, SNPs in green). Single SNPs in blue) and five SNPs in considerable LD with mined the LD structure underlying the genetic variant in linkage disequilibrium (LD) with rs5848 underlying the association with FTLD-U or whether another To study whether rs5848 is the likely functional variant been shown sufficient to affect miRNA regulation (21,22). which further increased the OR for homozygous T-allele carriers to 3.76 (95% CI: 1.69–8.39; \( P_{\text{adj}} = 0.001 \)). Comparison of gender, age at death and brain weight of FTLD-U patients by rs5848 genotype groups did not show significant differences (mean age at death was 71.6 ± 7.4 years in CC, 76.0 ± 10.5 years in CT and 75.5 ± 11.3 years in TT carriers).

**Detailed genetic analyses in GRN genomic region**

To study whether rs5848 is the likely functional variant underlying the association with FTLD-U or whether another genetic variant in linkage disequilibrium (LD) with rs5848 could be responsible for the observed association, we determined the LD structure underlying the GRN genomic region and performed single SNP and haplotype association analyses (Supplementary Material, Results). A panel of 12 additional SNPs was selected for single SNP and haplotype association analyses in the FTLD-U patient-control series: seven tagging SNPs identified in the genomic sequencing analyses that together with rs5848 capture 94% of the genetic diversity in the GRN region (Supplementary Material, Fig. S1; Fig. 1, SNPs in blue) and five SNPs in considerable LD with rs5848 selected from the downstream haplotype block (Supplementary Material, Fig. S2; Fig. 1, SNPs in green). Single SNP association analyses did not reveal SNPs that were more strongly associated with FTLD-U than rs5848 (Supplementary Material, Table S1). Moreover, haplotype analyses in the GRN genomic region and in a downstream haplotype block containing rs5848 only showed significant association when the risk T-allele of rs5848 was included (Supplementary Material, Table S2). Sequencing and genotyping analyses further revealed multiple GRN genetic backgrounds for the risk T-allele of rs5848, further favoring rs5848 as the potential functional variant underlying the observed association (Supplementary Material, Results and Table S3).

rs5848 is located in a predicted miRNA binding site of GRN

The rs5848 single base change (c.*78C>T) is located 78 nucleotides downstream of the translation termination codon in the 3′-UTR of the GRN transcript in a predicted binding site for the human specific miRNA miR-659 (Supplementary Material, Table S4). miRNAs are small non-coding RNAs that bind via imperfect base-pairing with target mRNAs to posttranscriptionally modulate their expression (20). We hypothesized that rs5848 may increase the risk for FTLD-U by altering the miRNA regulation of GRN, similar to previous studies in which a single nucleotide change in a miRNA target site had been shown sufficient to affect miRNA regulation (21,22).

By means of *in silico* analyses using the RNA folding and two-state hybridization servers (http://frontend.bioinfo.rpi.edu/applications/mfold/) we predicted that miR-659 binds to the GRN 3′-UTR through a perfect complementarity of the ‘seed’ region at position 2–7 of the miRNA and an additional 3′match of an adenosine anchor at position 1 (23). However, depending on the presence of the C-allele or the T-allele at rs5848, the positioning of miR-659 with respect to the miRNA binding site in GRN was expected to shift, resulting in the formation of three additional base-pairs at the 5′end of the miRNA when the risk T-allele of rs5848 was present (Fig. 2). The stronger binding of miR-659 to the GRN mRNA containing the T-allele was expected to result in a more efficient inhibition of GRN translation leading to reduced GRN expression levels.

**rs5848 affects GRN protein levels but not mRNA levels in FTLD-U patients**

To determine the effect of rs5848 on GRN expression, we performed GRN immunoblot analyses using brain extracts derived from cerebellum of FTLD-U patients homozygous for the C- or T-allele. Using western blot analyses we observed a significant decrease in GRN protein levels in TT carriers compared with CC carriers (\( P < 0.001 \)) (Fig. 3A and B). These data were further supported by immunohistochemical analysis, which showed a significant reduction in GRN burden in the granular cell layer of the cerebellum in TT compared with CC patients (\( P < 0.001 \)) (Supplementary Material, Fig. S3). To further quantify the reduction in GRN, we re-extracted brain homogenates from the cerebellum of FTLD-U rs5848 CC, CT and TT carriers and determined GRN expression using an enzyme-linked immunosassay (ELISA). A significant ~30% decrease in GRN levels was observed in rs5848 TT carriers compared with CC carriers in the TBS-X fraction (\( P < 0.001 \)) (Fig. 3C). Intermediate levels of GRN protein were observed in FTLD-U patients heterozygous for rs5848 supporting a dose-dependent decrease of GRN with each T-allele (data not shown).

As expected from a translational repression by miR-659, real-time mRNA expression analyses did not show a significant difference in GRN mRNA levels between rs5848 CC and TT carriers (Fig. 3D).

**miR-659 inhibits GRN translation at rs5848**

To provide evidence that miR-659 binds to GRN and can regulate the expression of GRN levels *in vitro*, we transiently transfected human M17 cells with 12 nm of miR-659 to mimic the expression of endogenous miR-659, or 12 nm negative control miRNAs, miR-C1 and miR-C2 (Ambion). Forty-eight hours post-transfection, cells were harvested and the endogenous human GRN levels produced by the M17 cells were measured by immunoblot analyses. A highly significant decrease in the expression of endogenous GRN was observed in M17 cells treated with miR-659 (\( P < 0.001 \)) (Fig. 4A and B).

To further study the regulation of GRN expression by miR-659, we inserted the full-length 304 bp GRN 3′-UTR sequence containing the wild-type C-allele at position 78.

### Table 3. Logistic regression analyses of rs5848 in FTLD-U patient–control series

<table>
<thead>
<tr>
<th>rs5848 genotypes</th>
<th>Controls (n = 433)</th>
<th>Patients (n = 59)</th>
<th>( P_{\text{adj}} )-value</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>199</td>
<td>46.0</td>
<td>21</td>
<td>35.6</td>
<td>—</td>
</tr>
<tr>
<td>CT</td>
<td>191</td>
<td>44.1</td>
<td>23</td>
<td>39.0</td>
<td>0.74</td>
</tr>
<tr>
<td>TT</td>
<td>43</td>
<td>9.9</td>
<td>15</td>
<td>25.4</td>
<td>0.003</td>
</tr>
</tbody>
</table>

\[
P_{\text{adj}} = 0.001.
\]
downstream of the luciferase reporter gene in the pMIR-REPORT miRNA expression reporter vector system and transiently transfected the construct into mouse N2A neuroblastoma cells (Fig. 4C). Co-transfection of a high dose (12 nM) of miR-659 or negative control miR-C2 in these cells resulted in significantly reduced expression of luciferase (representing GRN protein) in the presence of miR-659 further confirming the functional potential of the mRNA–miRNA duplex (Fig. 4D). We next constructed a luciferase construct with the GRN 3′-UTR sequence in which the complete 18 bp predicted binding site of miR-659 was deleted (position 71–88 downstream of the termination codon; vectorΔ18) (Fig. 4C). Addition of 12 nM miR-659 to N2A cells transfected with vectorΔ18 failed to repress the luciferase activity (Fig. 4D), supporting the hypothesis that miR-659 binds to the predicted binding-site in the 3′-UTR of GRN, overlapping with rs5848 at position 78.

To further study the differential regulation of GRN expression resulting from the presence of the wild-type ‘C’ or risk ‘T’ allele at rs5848, we created a luciferase construct containing the GRN 3′-UTR including the T-allele at position 78 (Fig. 4C). Co-transfection of the luciferase construct containing either the C-allele or the T-allele with variable low doses (0.01–100 pM) of miR-659 or negative control miR-C2 showed a dose-dependent reduction of luciferase activity derived from constructs containing the T-allele, reaching statistical significance at doses of 5 and 100 pM ($P = 0.02$) (Fig. 4E). A strong translational repression of the T-allele construct was also observed at 20 pM, however this result was non-significant ($P = 0.06$). Translational repression of luciferase activity from the wild-type rs5848-C construct was not observed for cells treated with miR-659 at any of these low doses (0.01–100 pM) (Fig. 4F).

miR-659 is expressed in human brain

To confirm the expression of miR-659 in human cells and in brain, we used a Taqman expression assay specific for miR-659 (Applied Biosystems). Positive expression of mature miR-659 was observed in both M17 cells and cerebellum (Supplementary Material, Fig. S4). Additional analyses using RNA extracted from seven different brain regions of a control brain (amygdala, occipital lobe, temporal lobe, frontal lobe, hippocampus, caudate and cerebellum) showed miR-659 expression in all analyzed regions, including frontal and temporal neocortex, which is most affected in FTLD-U (data not shown).

Correlation of rs5848 genotypes with FTLD-U pathological subtype

To determine whether the neuropathology of rs5848 TT carriers resembles the pathology of GRN loss-of-function mutation carriers, we determined the FTLD-U pathological subtype as proposed by the classification scheme of Mackenzie et al. (8) for all FTLD-U cases included in the genetic analyses of rs5848 with paraffin-embedded tissue blocks available for additional studies ($n = 57$). In addition, we determined the absence or presence of lentiform NIs.
Overall, FTLD-U subtypes could be determined for 54 FTLD-U patients resulting in 23 patients with FTLD-U type 1 (42.6%), 15 patients with FTLD-U type 2 (27.8%) and 16 patients with FTLD-U type 3 (29.6%). For the remaining three patients, the FTLD-U subtype could not be unambiguously assigned in part because inclusions were sparse (FTLD-U type 1 versus type 3). Stratification of FTLD-U patients by rs5848 genotype showed a non-significant increase in the frequency of FTLD-U type 1 pathology (resembling GRN mutation carriers) in TT carriers (66.7% in TT versus 33.3% in CT and CC carriers; \(P = 0.26\); Fisher exact test) (Fig. 5A). Moreover, NILs were significantly more common in the subgroup of TT carriers compared with CT or CC carriers (66.7% in TT versus 23.8% in CT and 27.8% in CC carriers; \(P = 0.02\); Fisher exact test) (Fig. 5B). Finally, MND pathology, which is rare or absent in GRN mutation carriers, was only present in one of the 15 FTLD-U patients homozygous for the risk T-allele (6.7%) compared with five out of 23 CT carriers (21.7%) and five out of 21 CC carriers (23.8%).

**DISCUSSION**

We previously showed that heterozygous loss-of-function mutations in **GRN** are an important cause of FTLD, explaining up to 25% of familial patients with the FTLD-U pathological subtype. In this study we focused our attention on the role of the common genetic variant rs5848, located in the 3'-UTR of **GRN**. This was stimulated by our observation of a deviation from the expected HWE for rs5848 in an extensive Mayo Clinic FTLD patient series, due to a selective increase in the TT genotype frequency in patients compared with control individuals. We hypothesized that if rs5848 is a true genetic risk factor for FTLD, the genetic association would be strongest in a homogeneous population of patients with FTLD-U pathology. To test this hypothesis, we performed genetic association analyses in a pathology-confirmed FTLD-U series derived from the MCJ brain bank and confirmed the genetic risk associated with rs5848. In our series, the OR to develop FTLD-U for carriers homozygous for the minor T-allele of rs5848 compared with homozygous
C-allele carriers was 3.18 (95% CI: 1.50–6.73; \( P \) adjusted = 0.003). Detailed genetic analyses of \( GRN \) and its flanking genomic regions further showed multiple haplotype backgrounds for the risk T-allele of rs5848 and did not reveal other genetic variants or haplotypes that were more strongly associated with FTLD-U, favoring rs5848 as the functional variant underlying the association.

We determined that rs5848 is located in the 3' UTR of \( GRN \) within a predicted binding site for the human specific miRNA miR-659. miRNAs are a widely distributed class of non-coding RNAs that play an integral role in gene regulation by binding to partially complementary sites in the 3' UTR of target mRNA transcripts, thereby inducing translational repression (20). Using \textit{in silico} analyses we predicted a stronger binding (reducing \( GRN \) expression) of miR-659 to the \( GRN \) 3' UTR containing the risk T-allele compared with the wild-type C-allele of rs5848.

To support the hypothesis that rs5848 is indeed a functional variant regulating \( GRN \) expression, we determined \( GRN \) expression levels in brain extracts derived from FTLD-U rs5848 CC and TT carriers by western blot analyses, ELISA and immunohistochemistry. These \textit{in vivo} analyses confirmed...
the functional capacity of rs5848. Compared with CC carriers, a statistically significant ~30% decrease in GRN protein levels was observed in FTLD-U rs5848 TT carriers. Furthermore, intermediate levels of GRN protein were observed in FTLD-U patients heterozygous for rs5848 supporting a dose-dependent decrease of GRN protein levels with each T-allele. As expected from a translational suppression of GRN by miRNAs, GRN mRNA expression levels were not significantly different between FTLD-U rs5848 CC and TT carriers.

Using cell-based systems we further demonstrated that miR-659 binds to the predicted binding site in the 3’-UTR of GRN and is able to suppress GRN expression in vitro. To determine the differential regulation of GRN expression resulting from the wild-type C-allele or risk T-allele of rs5848, we generated luciferase reporter constructs expressing firefly luciferase under the regulation of the GRN 3’-UTR carrying both rs5848 alleles. Our data suggested that miR-659 binds more efficiently to the risk T-allele than the wild-type C-allele, as demonstrated by the dose-dependent translational inhibition of the luciferase reporter (representing GRN protein) from the T-allele construct at low miRNA doses ranging from 5 to 100 pm (Fig. 4E). No translational repression was observed using the wild-type C-allele constructs, further supporting the differential effect of rs5848 (Fig. 4F). Together with the confirmation of positive expression of miR-659 in human brain, these results strongly support the functional potential of the miR-659/GRN mRNA complex. Of note, neither the binding site of miR-659 in the 3’-UTR of GRN, nor the gene encoding miR-659 on human chromosome 22q13, are conserved in other vertebrate or invertebrate logical FTLD-U phenotype resembling proportion of homozygous T-allele carriers to develop a patho-

status, revealed that >65% of patients homozygous for the T-allele (10/15 patients) had FTLD-U consistent with Mackenzie type 1 (12). In addition, compared with the other rs5848 genotype groups, the frequency of lentiform NIIs was significantly higher in homozygous T-allele carriers (P = 0.02). Finally, MND pathology, which is rare or absent in GRN loss-of-function mutation carriers, was only present in one of the 15 FTLD-U patients homozygous for the risk T-allele (6.6%). Re-analyses of the genetic association study excluding patients with a pathological diagnosis of FTD-MND increased the OR for homozygous T-allele carriers to 3.76 (95% CI: 1.69–8.39; \( P_{\text{adjusted}} = 0.001 \)). The strong similarity in pathological presentation between GRN loss-of-function mutation carriers and patients homozygous for the rs5848 T-allele provides additional support for the hypothesis that rs5848 increases FTLD-U risk by reducing the expression of GRN.

In 2 years, 57 different pathogenic GRN loss-of-function mutations have been reported in 160 genealogically unrelated FTLD families (Alzheimer Disease and Frontotemporal Dementia Mutation Database, http://www.molgen.ua.ac.be/FTDmutations/). The majority of these mutations are nonsense, frameshift and splice-site mutations that introduce a premature stop codon leading to the degradation of mutant RNA by the process of nonsense-mediated decay and the subsequent loss of 50% functional GRN (15). The identification in this study of homozygosity of the T-allele of rs5848 as a major risk factor for FTLD-U, represents yet another way in which genetic variation in GRN may lead to FTLD-U via a decrease in the levels of functional GRN. In this case, haploinsufficiency is predicted to result from increased suppression of GRN translation through altered miRNA regulation. Our findings suggest a scenario whereby a decrease in GRN expression below a critical threshold results in the development of FTLD-U. While a single GRN null allele can sufficiently reduce GRN expression levels to cause FTLD-U, two copies of the risk T-allele of rs5848 are required (but may not be sufficient) to reduce the GRN expression below this critical threshold. We propose that a homozygous state of the risk T-allele at rs5848 combined with other environmental and/or genetic factors, will ultimately determine which individuals develop FTLD-U. In this respect, the association of rs5848 with FTLD-U is similar to many other neurodegenerative diseases where genetic variability at the same loci implicated in Mendelian pathogenic forms of the disease predispose to sporadic forms of the disease (24–29). Whether rs5848 may also contribute to the development of other TDP43-proteinopathies (such as amyotrophic lateral sclerosis) or may determine the presence of TDP-43 pathology in more common neurodegenerative conditions such as AD (30) needs further investigation.

The assumption of a role for miRNA pathways in neurodegeneration is intriguing and has been previously suggested based on experiments in mice, flies and cultured neurons in which the enzyme Dicer (required for miRNA maturation) was genetically inactivated (31–33). A role for specific miRNAs in the development of common neurodegenerative diseases has further been suggested by comparing changes in miRNA expression profiles in brain samples derived from patients and controls (32,34). Based on the possible role of miRNA dysregulation of GRN in FTLD presented in this study and the knowledge that overexpression
of neurodegenerative disease proteins, such as the amyloid precursor protein in AD (35) and α-synuclein in Parkinson’s disease (36), is sufficient to cause disease, we speculate that regulatory mutations affecting the interaction between miRNAs and their targets may present a common mechanism underlying complex neurodegenerative disorders.

MATERIALS AND METHODS

FTLD patient and control populations

Mayo Clinic FTLD case–control series. The Mayo Clinic FTLD patient series in which the initial observation of a deviation from HWE for rs5848 was observed has been previously described in detail (17). A total of 934 control individuals (mean age at inclusion $64.8 \pm 10.9$ years) ascertained through MCJ and MCS were employed to determine initial rs5848 control genotype frequencies.

FTLD-U case–control series. The MCJ brain bank comprises >2500 neurodegenerative brain samples primarily ascertained through The State of Florida Alzheimer’s Disease Initiative funded through the Department of Elder Affairs, The Einstein Aging Study (P01-AG03949), The Udall Center for Excellence in Parkinson’s Disease Research (P50-NS40256), CurePSP/The Society for Progressive Supranuclear Palsy, Mayo Clinic ADRC (P50-AG16574), the Alzheimer’s Disease Patient Registry (ADPR) (P30-AG19610) and the Florida ADRC (P50-25711).

We selected all 81 patients from the MCJ brain bank with the neuropathological diagnosis of FTLD-U and positive TDP-43 (ADPR) (P30-AG19610) and the Florida ADRC (P50-25711).

In this series, the mean age at death was 74.4 ± 9.8 years (range 56–97 years). A selection of 433 control individuals matched for age and gender. Only haplotypes with an estimated overall frequency of ≥5% were considered in the analyses. The level of significance was defined as $P < 0.05$.

Estimation of haplotype frequencies in GRN genomic region

The expectation maximization algorithm provided by the Arlequin package was applied in order to estimate GRN haplotype frequencies in 35 FTLD patients homozygous for the rs5848 T-allele.

Immunoblot analyses

Fourteen cerebellar brain samples of FTLD-U cases (seven CC and seven TT carriers) were selected for immunoblot analyses. The quality of each of these brain samples was previously assessed by determining the RNA Integrity Number (RIN) using an Agilent 2100 Bioanalyzer, and only samples with RIN values > 6 were included in this study. Brain samples were sonicated in PARIS cell disruption buffer (Ambion/Applied Biosystems, Austin, TX) supplemented with protease and phosphatase inhibitors, centrifuged and the protein concentrations determined with a BCA protein assay (Pierce, Rockford, IL). Forty micrograms of protein were resolved by SDS–PAGE using pre-cast 8% Tris–Glycine gels (Invitrogen, Carlsbad, CA). Separated proteins were transferred to PVDF membranes and blocked for 1 h at RT with 5% skim milk/TBST. After incubation with anti-human PCDGF (1:1000; Zymed, South San Francisco, CA) primary antibody under blocking conditions, proteins were detected with anti-rabbit HRP conjugated secondary antibody (1:5000; Southern Biotech, Birmingham, AL) and ECL-Plus (Perkin Elmer, Waltham, MA). Quantification of immunoreactive bands was performed by densitometry (Image J, Research Services Branch, NIMH, Bethesda, MD). GRN protein levels were normalized to GAPDH by re-probing blots with anti-GAPDH (1:50 000; Biodesign International, Saco, ME) and anti-mouse HRP conjugated secondary (1:25 000; Southern Biotech) antibody. To compare GRN protein levels assessed on separate immunoblots, a further normalization was performed using a reference sample included on each blot. In addition, all immunoblots were run in parallel on the same day, to minimize variability in experimental conditions.

To determine GRN expression in human M17 cells transiently transfected with miR-659 or negative control miRNAs (Cye-3 dye labeled miRNA Negative Control #1
Cytomaton, Carpinteria, CA) and 3,3 Group, Inc., Chicago, IL) with a DAKO-Autostainer (DAKO for TDP-43 (rabbit polyclonal antibody; 1:3000; ProteinTech)

Immunohistochemical analyses

For expression analyses Applied Biosystems assays were used for GRN (Hs00173570_m1), and for the endogenous controls GAPDH (Hs00266705_g1), YWHAZ (Hs00852925_sH) and HPRT1 (Hs99999909_m1). Real-time PCR was performed on an ABI 7900 using the TaqMan® method. Reactions contained 1 μl cDNA amplified with 0.25 μl primer/probe mix and 2.5 μl TaqMan 2X Universal PCR Master Mix. The cycling parameters as recommended by the manufacturer were followed; 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s/ 60°C for 1 min. All samples were run in triplicate and normalized to the geometric mean of the three endogenous controls as described previously (38). The FAM-fluorescent signal was analyzed using SDSv2.2.2 software, and relative quantities of GRN mRNA were determined using the ΔΔct method.
Generation of human GRN 3′-UTR luciferase constructs

The 3′-UTR of GRN was amplified from genomic DNA from individuals homozygous for the C-allele or T-allele of rs5848 using the following primers: 3′-UTR-Spel-F: AATTACTAG TGGGACAGTACTGAAGACTCTGC and 3′-UTR-HindIII-R: AATTAAGCTTAAGTGACAAGCTTTATGAAAA GC. The 5′ end of each primer was designed to include restriction enzyme digest sites for subsequent digestion and ligation into the multiple cloning site of the pMIR-REPORT Luciferase vector (Ambion) to create pMIR-REPORT-rs5848C and pMIR-REPORT-rs5848T vectors. PCR reactions were performed in 100 μl using 50 ng genomic template and 20 pmol of each primer. Initial denaturation at 95°C was followed by 35 cycles of 94°C for 30 s, 60–50°C touchdown annealing for 30 s and 72°C for 30 s with a final extension at 72°C for 10 min. PCR products were purified using the Qiagen purification system (Qiagen) both before and after digestion with SpeI and HindIII (New England Biolabs, Ipswich, MA).

To create the pMIR-REPORT-D18 luciferase vector in which the 18 bp predicted binding site of miR-659 was deleted, we performed site-directed mutagenesis using the QuikChange protocol (Stratagene, La Jolla, CA) on a full length GRN cDNA clone (Invitrogen) using the following primers D18-F: CAGGCCCTCCTAATTCTCCTGGAC and D18-R: GTCCA GGGAGGATTAGGGAGGCTG. The mutated construct was subsequently used as template for PCR amplification using the 3′-UTR-Spel-F and 3′-UTR-HindIII-R primers, digestion and ligation into the pMIR-REPORT Luciferase vector as described earlier. Inserts of all constructs were verified by direct sequencing.

Luciferase assays

N2A neuroblastoma cells were plated on TC-treated Costar® 6-well cell culture plates (Corning) at 1.0 × 10⁵ cells per well in antibiotic-free Opti-mem reduced serum medium supplemented with 10% fetal bovine serum and 2 mM L-Glutamine (Invitrogen). 4 h after plating, cells were transfected with Lipofectamine 2000 (Invitrogen) using the manufacturer’s protocol. For the first experiment, each well was co-transfected with 100 ng of either pMIR-REPORT-rs5848C or 100 ng pMIR-REPORT-D18 and 100 ng of pRL-CMV-renilla luciferase (Promega, Madison, WI), and treated with either miR-659 or negative control miR-C2 (Ambion) at 12 nM. Six replicates were performed for each treatment. Twenty-four hours after transfection, cells were lysed using 250 μl of Reporter Gene Assay lysis buffer (Roche, Indianapolis, IN) and Luciferase Firefly (LA₅) and Luciferase Renilla (LA₉) activities were measured in triplicate using the dual-luciferase reporter assay (Promega) on a Veritas microplate luminometer using manufacturer’s instructions. To determine the differential regulation of the rs5848 C- or T-allele constructs by miR-659, luciferase experiments were further performed by co-transfection of pMIR-REPORT-rs5848C or pMIR-REPORT-rs5848T with 100 ng of pRL-CMV-renilla luciferase and treatment with increasing but low doses of miR-659 and negative control miR-C2 (0.01–100 pM). Three replicates were performed for each treatment. For each well the relative luciferase activity (RLA) was calculated as RLA = LA₅/LA₉ using the average from three independent measurements. Next, for each quantity of miRNA, the mean RLA was calculated based on all replicates. Statistical analyses using t-tests were performed for each quantity of miRNA by comparing the mean RLA in cells treated with miR-659 with the mean RLA in cells treated with negative control miR-C2.

Real-time miR-659 expression analyses

Tissue from the cerebellum of a control brain (pathologically normal) was dissected and processed using the mirVana PARIS system (Ambion) to extract RNA enriched for small RNA species. RNA from an M17 cell pellet was prepared similarly. A TaqMan microRNA reverse transcription reaction was performed following the manufacturer’s protocol and using the primers specific for miR-659 (RT 1514) and for human control RNU48 (RT1006) (Applied Biosystems). Real-time PCR was performed on the reverse transcription products to confirm the presence of miR-659 in the M17 cells and the control brain; each 5 μl reaction contained 0.33 μl reverse transcription product with 0.25 μl primer/probe mix and 2.5 μl TaqMan 2X Universal PCR Master Mix (Applied Biosystems) and was cycled as recommended by the protocol: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s/60°C for 1 min. Samples were run in triplicate and analyzed using SDSv2.2.2 software. This procedure was repeated on enriched RNA extracted from amygdala, occipital lobe, temporal lobe, frontal lobe, hippocampus, caudate and cerebellum tissue from the same control brain to confirm the presence of miR-659 in these various brain regions.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

FUNDING


ACKNOWLEDGEMENTS

The authors wish to acknowledge and thank the families who contributed samples that were critically important to the completion of this study. We further thank John Gonzalez, David Stroh, Linda Rousseau, Virginia Phillips and Monica...

