Published in final edited form as:
Arch Neurol. 2011 May ; 68(5): 581–586. doi:10.1001/archneurol.2010.350.

TMEM106B gene polymorphism is associated with age at onset in granulin mutation carriers and plasma granulin protein levels

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Abstract

Objective—A recent genome-wide association study for frontotemporal lobar degeneration with TAR DNA-binding protein inclusions (FTLD-TDP), identified rs1990622 (*TMEM106B*) as a risk factor for FTLD-TDP. In this study we tested whether rs1990622 is associated with age at onset (AAO) in granulin (*GRN*) mutation carriers and with plasma GRN levels in mutation carriers and healthy elderly individuals.

Design—Rs1990622 was genotyped in *GRN* mutation carriers and tested for association with AAO using the Kaplan-Meier and a Cox proportional hazards model.

Subjects—We analyzed 50 affected and unaffected *GRN* mutation carriers from four previously reported FTLD-TDP families (HDDD1, FD1, HDDD2 and the Karolinska family). GRN plasma levels were also measured in 73 healthy, elderly individuals.

Results—The risk allele of rs1990622 is associated with a mean decrease of the age at onset of thirteen years ($p=9.9\times10^{-7}$), with lower plasma granulin levels in both healthy older adults ($p = 4\times10^{-4}$) and *GRN* mutation carriers (p=0.0027). Analysis of the HAPMAP database identified a non-synonymous single nucleotide polymorphism, rs3173615 (T185S) in perfect linkage disequilibrium with rs1990622.

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Conflict of Interest statement: None declared

SUPPLEMENTARY MATERIAL
Supplementary Material is available at Archives of Neurology online

Conclusions—The association of rs1990622 with AAO explains, in part, the wide range in the age at onset of disease among *GRN* mutation carriers. We hypothesize that rs1990622 or another variant in linkage disequilibrium could act in a manner similar to *APOE* in Alzheimer's disease, increasing risk for disease in the general population and modifying AAO in mutation carriers. Our results also suggest that genetic variation in TMEM106B may influence risk for FTLD-TDP by modulating secreted levels of GRN.

INTRODUCTION

Genome-wide association studies (GWAS) are a powerful tool to identify genetic variants associated with risk for disease.[1–4] In the last two years GWAS for the most common neurodegenerative diseases, such as Alzheimer's Disease (AD), Parkinson's Disease (PD), Progressive Supranuclear Palsy (PSP), and CorticoBasal Degeneration (CBD),[1–4] have found genetic variants associated with risk for disease. The main drawback of these studies is that, the functional and biological mechanism that explains the association is often unknown. This is the case for a recent GWAS for frontotemporal lobar degeneration with TAR DNA-binding protein inclusions (FTLD-TDP)[5]. Frontotemporal dementia is the third most common neurodegenerative disease after Alzheimer's and Parkinson's disease.[6] This study was performed in 515 FTLD-TDP cases, including 89 individuals carrying pathogenic mutations in the *granulin* (*GRN*) gene, a known cause of familial FTLD-TDP. The strongest association was observed with rs1990622, located 6.9 kb downstream of the *TMEM106B* gene (chromosome 7p21). Interestingly this signal was stronger among the *GRN* mutation carriers than in the other FTLD-TDP samples. The authors of this study suggested that rs1990622 or another single nucleotide polymorphism (SNP) in linkage disequilibrium (LD) is modifying the risk for disease through modulation of TMEM106B expression levels because they found a strong association between this SNP and TMEM106B mRNA levels. These studies were performed in lymphoblast cell lines and replicated in a small series of human brain samples. However, rs1990622 is not located in an obvious promoter region or transcription factor-binding site for *TMEM106B.*

Individuals with pathogenic *GRN* mutations have very low mRNA and plasma levels of GRN,[7–13] but the relationship between *TMEM106B*, GRN levels and/or FTLD-TDP pathology is unknown. In this study we tested whether rs1990622 is a disease modifier in *granulin* mutation carriers and examined possible pathogenic mechanisms by which variation in *TMEM106B* influences risk for FTLD-TDP.

METHODS

Subjects

DNA was extracted from 50 individuals from families previously shown to have FTLD-TDP caused by mutation in *GRN* (6 samples from the HDDD1 family[7], 13 from the FD1 family[8], 18 from the HDDD2 family[9] and 13 from the Karolinska family[10], Table 1). All samples were sequenced and confirmed to have a pathogenic *GRN* mutation. All participants gave informed consent and the Washington University School of Medicine IRB, and the Human Subjects Committee of the Karolinska Institute (Stockholm, Sweden) approved the study. Plasma from 73 healthy, elderly individuals and 6 *GRN* mutation carriers was obtained according to standard procedures.

Genotyping and GRN plasma levels measurements

Genomic DNA was isolated from blood using standards procedures. Rs1990622 was genotyped using Kaspar or Taqman technologies. Plasma levels of GRN were measured, in

duplicate, using an ELISA kit (Human Progranulin ELISA Kit, Adipo-Gen Inc., Seoul, Korea).

Gene Expression

Association with expression was carried out using cDNA obtained from the frontal lobes of 40 clinically non-demented individuals (clinical dementia rating (CDR)[14]=0) obtained through the Washington University Knight-Alzheimer Disease Research Center (WU-ADRC) Neuropathology Core. We included samples for which the CDR assessment was done within six months of the date of death. The mean age at death of the included individuals was 85 ± 9 (range: 64–107). Twenty one percent of the individuals carried at least one APOE ε 4 allele. Thirty nine percent of the individuals were male. Twenty four brains had a Braak and Braak score ranging from 1–4 indicating the presence of some tangle pathology. All the brains had a Consortium to Establish a Registry for Alzheimer's Disease (CERAD) score[15] lower than B. Only samples with a postmortem interval lower than 24 hours were included. As explained in the Statistical and Bioinformatic Analyses section postmortem interval was not significantly associated with GRN or TMEM106B mRNA levels. RNA integrity was also checked by agarose gel electrophoresis (data not shown).

Total RNA was extracted from the frontal lobe using the RNeasy mini kit (Qiagen) following the manufacturer's protocol. cDNAs were prepared from the total RNA, using the High-Capacity cDNA Archive kit (ABI). Gene expression was analyzed by real-time PCR, using an ABI-7500 real-time PCR system. Real-time PCR assays were used to quantify *TMEM106B*, *GRN*, *GAPDH* and *cyclophilin* cDNA levels using Taqman assays. Each real-time PCR run included within-plate duplicates. Real-time data were analyzed using the comparative Ct method. The Ct values of each sample were normalized with the Ct value for the housekeeping genes, *GADPH* and *cyclophilin*, and were corrected for the PCR efficiency of each assay,[16] although the efficiency of all reactions was close to 100%. Only samples with a standard error <0.15% were analyzed.

We also used the GEO dataset GSE8919[17] to analyze the association between rs1990622 and *TMEM106B* gene expression. In this dataset there are genotype and expression data from 486 late onset Alzheimer's Disease cases and 279 neuropathologically clean individuals. We extracted the data for normalized *TMEM106B* mRNA levels measured in parietal cortex from neuropathologically confirmed healthy individuals and the genotype data for rs1468804. Only mRNA levels from parietal cortex were used to minimize heterogeneity and maximize sample size (n=105). Genotypes for rs1468804 were used because rs1990622 was not included in this dataset, and rs1468804 is in perfect linkage disequilibrium (LD) with rs1990622 based on the HapMap database.

Statistical and Bioinformatic Analyses

Association with age at onset (AAO) was carried out using the Kaplan-Meier method and tested for significant differences, using a Cox proportional hazards model (proc PHREG, SAS), including gender in the model and family ID in the aggregate option (covs(aggregate)), which creates a robust sandwich estimate, to take into account the relatedness between samples and potential differences in AAO between mutations. This approach has been widely used to analyze the association of genetic variants with both risk for disease and age at onset in family-based analyses in multiple studies.[18–22] The PHREG procedure in SAS can be used to fit the Cox proportional hazards model, and a robust variance estimate that is valid for testing association in the presence of linkage within families of arbitrary size.[18, 19] The advantage of this method is that it is a valid test in the presence of linkage, but does not require the correlation structure within families to be specified. This approach is more powerful than the sibship disequilibrium test of Horvath

and Laird.[20] This test is valid even under extreme residual familial correlation and with no cost in power at the causal locus.[20]. In our analyses minor allele homozygotes (n = 2) were combined with heterozygotes due to the small number of individuals with this genotype (dominant model). Healthy mutation carriers were included in the analyses as censored data. The inclusion of these samples did not change the association (Supplementary Figure 1).

Association between GRN plasma levels and rs1990622 genotypes was carried out using ANCOVA. Rs1990622 was tested using an additive model with minor allele homozygotes coded as 0, heterozygotes coded as 1, and major allele homozygotes coded as 2. GRN plasma levels were not normally distributed and were log-transformed for analysis. We performed a step-wise discriminant analysis to test whether age, *APOE* and/or gender affect GRN plasma levels, but none of these factors were associated in healthy individuals and were not included in the model. *APOE* was tested as a potential covariate because it has been reported that *APOE* is associated with early memory deficits in *GRN* mutation carriers[23]. In *GRN* mutation carriers age was significant and was therefore included in the ANCOVA. In our study *GRN* mutation carriers have GRN plasma levels between 40.5 – 70 ng/ml. The *GRN* gene was sequenced in the 13 healthy individual samples with GRN plasma levels below 100 ng/ml, but no mutations were found in these samples.

Association with mRNA levels was carried out using ANCOVA. Rs1990622 was tested using an additive model with minor allele homozygotes coded as 0, heterozygotes coded as 1, and major allele homozygotes coded as 2. Stepwise discriminant analyses identified postmortem interval and gender as significant covariates for TMEM106B mRNA levels; and *APOE* and age for GRN mRNA levels in the WU-ADRC samples, and were included as covariates in the analyses. Stepwise discriminant analysis identified postmortem interval and site as significant covariates in the GEO dataset and were included in the analyses.

SNPs in LD with rs1990622 were identified using the CEU-HapMap data and the Haploview software.[24] The LD between rs1990622 and rs3173615 was confirmed by direct genotyping in the WU-ADRC samples. The functional implication of each SNP was analyzed using Pupasuite software.[25]

RESULTS

Association with age at onset

To test whether rs1990622 modifies age at onset of dementia in *GRN* mutation carriers we genotyped rs1990622 in 50 *GRN* mutation carriers, from four FTLD families (HDDD1, HDDD2, FD1, and Karolinska, Table 1) associated with three different mutations (Ala9Asp, Gly35fs, and Ala237fs).[7–10]. Although we have included families with different mutations available evidence suggests that each mutation causes disease via haploinsufficiency. In the case of the Gly35fs and Ala237fs mutations the haploinsufficiency is caused by nonsense mediated decay of the mutant mRNA, while the A9D mutation interferes with protein secretion, leading to functional haploinsufficiency.[7–10, 26]

Association with granulin plasma levels

Most pathogenic mutations in *GRN* causing FTLD-TDP are null alleles and have been associated with lower *GRN* mRNA,[7–10] cerebrospinal fluid[13] and plasma levels[11–13] as a result of a haploinsufficiency.[7–10] Therefore, we hypothesized that rs1990622 modifies risk for sporadic FTLD-TDP and age at onset in *GRN* mutation carriers by regulating GRN protein levels. In our series, GRN plasma levels are highly variable in healthy individuals showing as much as 4 fold difference between individuals (n=73, mean= 163 ng/μl ± 61, range = 76 – 314), while *GRN* mutation carriers have very low GRN plasma levels with lower inter-individual variability (n=6, mean 47 ng/μl ± 13, range = 42 – 70). We first tested

whether rs1990622 was associated with GRN plasma levels in healthy non-demented older adults and observed a significant difference in mean GRN plasma levels for each genotype. Consistent with our hypothesis, homozygotes for the risk allele have the lowest GRN plasma levels while heterozygotes have intermediate levels and homozygotes for the protective allele have the highest GRN plasma levels ($p = 4\times10^{-4}$; table 2, Figure 1B). Among the *GRN* mutation carriers we also found a significant difference in the *GRN* plasma levels between the different genotypes (AA 49.6 ng/μl ± 10 (range: 40–66) vs AG 63 ng/μl ± 10 (range: 56–71); p=0.0027).

Association with TMEM106B and GRN mRNA levels

Van Deerlin et al. found that rs1990622 is associated with *TMEM106B* gene expression in lymphoblastoid cell lines and in a small number for frontal cortex samples (7 samples from neurologically unaffected individuals and 18 with FTLD-TDP)[5] suggesting that rs1990622 modifies risk for FTLD-TDP through modulation of TMEM106B *mRNA* expression. To follow up this result we tested whether rs1990622 is associated with TMEM106B mRNA levels in frontal cortex from 40 non-demented elderly individuals. Surprisingly, we found no association between rs1990622 with gene expression (p=0.78. Figure 1C) in a sample size almost two times the size of the original study. We also tried to replicate the association of rs1990622 with *TMEM106B* gene expression by analyzing the publicly available GEO dataset GSE8919.[17] We analyzed *TMEM106B* mRNA levels in parietal cortex samples (n=105) from non-demented individuals, but did not detect evidence for association with rs1468804 (p= 0.56), a SNP in perfect LD with rs1990622 (D′=1; R^2=1, based on the CEU-HapMap data). Despite our large datasets we were unable to replicate the original observation. Since our tissue is from non-demented elderly adults and the original study examined tissue from FTLD-TDP brains it is possible that the association with expression could represent a SNP by environment interaction, in which rs1990622 modulates *TMEM106B* mRNA levels in the presence of TDP pathology.

Given the strong association of *TMEM106B* expression with rs1990622 in GRN carriers in the original study we tested whether rs1990622 is associated with *GRN* mRNA levels, but failed to detect an association (p= 0.35 Data not shown). We also found no correlation between *TMEM106B* and *GRN* mRNA levels (p=0.80; Pearson correlation R^2=0.04). Together these results indicate that the association of rs1990622 with GRN plasma levels is not likely to be driven by modulation of *GRN* expression levels.

Bioinformatic analyses

We used the CEU-HapMap data and bioinformatics tools to identify all putative functional SNPs in high LD (r^2>0.9) with rs1990622, that could explain the association of rs1990622 with risk for FTLD-TDP, age at onset in *GRN* mutation carriers and GRN plasma levels. Thirty-two SNPs within 10 Kb of the *TMEM106B* gene region showed high LD with rs1990622 (Supplementary table 1). Six SNPs are located in transcription factor binding sites that could modify gene expression and a seventh is located in a sequence susceptible to the formation of a DNA triplex, which has also been suggested to regulate gene expression.[27] Analysis of the CEU-HapMap data revealed that the non-synonymous SNP in *TMEM106B*, rs3173615 (Thr-185-Ser), is in perfect LD with rs1990622 (D′=1; R^2=1). Direct genotyping confirmed that these two SNPs are also in very high LD in our population (D′=1; R^2=0.927). Threonine-185 and the entire protein, is very highly conserved between species (supplementary Figure 2), indicating that this variant may have important functional implications. Polyphen2[28] predicts that rs3173615 results in a possibly damaging amino-acid change, suggesting that rs3173615 may affect protein function. Our bioinformatics analysis also identified another SNP, rs1042949 in perfect LD with rs1990622, which

disrupts an exonic splicing enhancer that may affect alternative splicing thereby changing protein function.

DISCUSSION

Our data provide additional support for the role of rs1990622 (Chr. 7p21) or variants in LD with this SNP and genetic risk for FTLD-TDP, especially in *GRN* mutation carriers. We found that rs1990622 is strongly associated with age at onset in GRN mutation carriers. Individuals carrying the risk allele of rs1990622 have a mean age at onset thirteen years earlier than GRN mutation carriers without an rs1990622 risk allele ($p=9.9\times10^{-7}$). Furthermore, the association of rs1990622 with GRN plasma levels in both normal individuals and *GRN* mutation carriers suggests that *TMEM106B* may influence risk for FTLD-TDP by modulating GRN protein levels. We were not able to test directly the association between GRN plasma levels and age at onset, but we found that unaffected *GRN* mutation carriers have higher GRN plasma levels than affected individuals. This analysis was done in a small sample size and should be interpreted with caution. There are several reports in which GRN plasma levels have been measured in affected and unaffected *GRN* mutation carriers as well as in healthy elderly individuals or in individuals with other neurodegenerative diseases.[11, 13, 29, 30] In all of these reports it was found that *GRN* mutation carriers, regardless of their disease status, have very low GRN plasma levels compared with other individuals. However there are inconsistent results regarding GRN levels in affected *GRN* mutation carriers compared with the unaffected mutation carriers. In Ghidoni et al[30] and Sleegers et al[29] unaffected mutation carriers had slightly lower GRN plasma levels compared with the affected individuals. On the other hand, our results and Finch et al,[13] show unaffected carriers with higher GRN levels than the affected carriers. These contradictory results and the fact that all of these studies were carried out in small series; indicate that more studies are necessary. These studies should be designed to address directly whether or not GRN plasma levels are associated with the onset of disease. There are several factors that should be taken into account in the design of these experiments. Our data and others indicate that GRN plasma levels in *GRN* mutation carriers are very low and show much lower inter-individual variability compared with healthy individuals,[11, 13, 29, 30] therefore small differences are expected to be found and very sensitive assays should be used. It will be necessary to avoid external sources of variability that could mask the difference in GRN plasma levels between the affected and unaffected carriers. For example, we found that the number of freeze-thaw cycles and the time that the plasma has been frozen decrease the GRN plasma level measured by ELISA (data no shown). Obviously it is necessary to include the maximum number of samples to have enough power to find a significant difference.

Our data support the hypothesis that rs1990622, rs3173615 or other SNPs in LD with these SNPs are associated with age at onset possibly through a modulation of GRN plasma levels. This mechanism shows some similarities with that proposed for *APOE* in AD. Although the pathogenic mechanism of *APOE* in not entirely understood, it has been demonstrated in multiple studies that the *APOE ε4* allele is associated with both increased risk for Alzheimer's disease and earlier age at onset in the general population[1–3] and earlier age at onset in familial Alzheimer's disease mutation carriers.[31] We have reported in several papers that the *APOE ε4* is strongly associated with lower cerebrospinal fluid $A\beta_{42}$ levels[32–35] increasing the risk for disease and modifying the age at onset. Similarly, the *TMEM106B* polymorphism increases risk for FTLD-TDP and decreases the age at onset through modulation of GRN plasma levels, a protein known to play a causal role in FTLD. The absence of association between rs1990622 and TMEM106B or GRN mRNA levels suggests that TMEM106B affects GRN protein levels, but not through modulation of *GRN* or *TMEM106B* gene expression. TMEM106B is a transmembrane protein, conserved among

mammals with an unknown function. *GRN* encodes a secreted protein, progranulin, which can be cleaved into smaller peptides (granulins). Our results suggest that TMEM106B may influence secretion, clearance or cleavage of granulin protein and that genetic variants in *TMEM106B* could affect this process, modifying the age at onset of dementia in individuals with *GRN* mutations and be a risk factor for disease risk in the general population, similar to APOE and Alzheimer's Disease.

In our dataset, carriers for the protective allele of rs1990622 have a mean age at onset of thirteen years later than risk allele carriers. The effect of this polymorphism on the age at onset is similar in magnitude to APOE genotype on the onset of Alzheimer's disease or the length of the CAG repeat in the huntingtin gene on the onset of Huntington's Disease[36], Given this observation inclusion of the variant should be considered in clinical genetic testing for families with GRN mutations. The identification of the responsible genetic variant in *TMEM106B* that drives the association and the characterization of the functional mechanism implicated in this association could represent a critical step in the development of new therapies for FTLD-TDP. A therapy that could mimic the effect of the protective allele could significantly delay the age at onset of symptomatic disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge the individuals who participated in this study. The authors also acknowledge the contributions of the Genetics and Clinical Cores of the Washington University Knight-Alzheimer's Disease Research Center.

FUNDING

This work was supported by grants from NIH (AG16208, P01AG03991, P50AG05681, P01AG026276), the Alzheimer's Association, the Barnes-Jewish Hospital Foundation, Swedish Brain Power, Swedish Alzheimer foundation, and Marianne and Marcus Wallenberg Foundation, Gun and Bertil Stohnes Foundation, Gamla tjänarinnor Foundation and Karolinska Institutet KID-funding. The authors thank the Clinical Core of the Knight-ADRC for subject recruitment and assessment, the Genetics Core of the ADRC for *APOE* genotypes. CC has a fellowship from "Fundacion Alfonso Martin Escudero". JW is supported by a grant from Alzheimer's Association (IIRG-07-60299).

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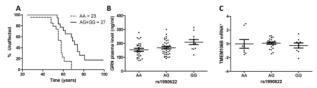

Figure 1. rs1990622 is associated with age at onset of FTLD-TDP in *GRN* mutation carriers and with GRN plasma levels in non-demented individuals

A) Age at onset was analyzed for association with rs1990622 in 50 GRN mutation carriers by the Kaplan-Meier method and tested for significant differences, using a proportional hazards model (proc PHREG, SAS). Family and gender were included in the model to take into account the relatedness between samples and the potential differences in age at onset between mutations. Healthy mutation carriers were included in the analyses as censored data. Homozygotes for the major allele have an earlier AAO than the heterozygotes and homozygotes for the minor allele (mean age at onset 58 vs. 74 years; $p=9.9\times10^{-7}$). **B)** GRN plasma levels were measured in 79 healthy individuals and tested for association with rs1990622. Rs1990622 showed a significant association with GRN plasma levels ($p=4\times10^{-4}$). **C)** *TMEM106B* frontal cortex mRNA levels measured in 40 healthy individuals by Real-Time PCR showed no association with rs1990622 (p=0.78). *TMEM106B* RNA*; levels corrected for gender and postmortem interval.

Table 1

Sample description.

Family	mutation	n	affected	Un-affected	AAO	Age at last assessment
HDDD1	Ala237fs	6	3	3	62 ± 2	66.3 ± 17
FD1	Ala237fs	13	3	10	59.0 ± 7	50.4 ± 10
HDDD2	Ala9Asp	18	14	4	61.6 ± 18	65.6 ± 9.3
Karolinska	Gly35fs	13	7	6	53.5 ± 4	41.8 ± 13
Total	-	50	27	23		

Table 2

Rs1990622 is associated with GRN plasma levels.

Rs1990622	n	GRN plasma levels (ng/μl) Mean (SD)	Min.	Max.
AA	25	156.96 (55.17)	76.50	278.10
AG	37	170.39 (51.30)	91.90	300.20
GG	11	209.25 (59.96)	116.70	314.10
		$P=4\times10^{-4}$		

P value for the additive model.