

Rs5848 Variant Influences *GRN* mRNA Levels in Brain and Peripheral Mononuclear Cells in Patients with Alzheimer's Disease

Chiara Fenoglio^{a,*}, Daniela Galimberti^a, Francesca Cortini^a, John S.K. Kauwe^b, Carlos Cruchaga^b, Eliana Venturelli^a, Chiara Villa^a, Maria Serpente^a, Diego Scalabrini^a, Kevin Mayo^b, Laura M. Piccio^{a,c}, Francesca Clerici^d, Diego Albani^e, Claudio Mariani^d, Gianluigi Forloni^e, Nereo Bresolin^a, Alison M. Goate^b and Elio Scarpini^a

^aDepartment of Neurological Sciences, "Dino Ferrari" Center, University of Milan, IRCCS Fondazione Ospedale Maggiore Policlinico, Milan, Italy

^bDepartment of Psychiatry, Washington University School of Medicine, St. Louis, MO, USA

^cDepartment of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO, USA

^dCenter for Research and Treatment on Cognitive Dysfunctions, University of Milan, "Luigi Sacco" Hospital, Milan, Italy

^eIstituto di Ricerche Farmacologiche Mario Negri, Milan, Italy

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Abstract. Mutations in the progranulin gene (*GRN*), causative for Frontotemporal Lobar Degeneration with ubiquitin-immunoreactive neuronal inclusions (FTLD-U), could also be associated with Alzheimer's disease (AD). The influence of *GRN* genetic variability on susceptibility to AD and on expression levels in a series of neuropathologically-confirmed AD patients as well as in peripheral mononuclear cells (PBMC) and in cells isolated from cerebrospinal fluid (CSF) was investigated. An association study of rs9897526 and rs5848 was carried out in an Italian population and in a replication population of European American patients and controls. None of the variants tested act as unequivocal susceptibility factor in both populations although rs9897526 anticipated the onset of the disease in the Italian population. *GRN* expression in the parietal lobe of AD cases showed a 0.76-fold decrease compared with controls (1.31 ± 0.07 versus 1.73 ± 0.12 , $P = 0.0025$). Patients carrying the rs5848 *TT* genotype had the lowest *GRN* expression levels (0.96 ± 0.12 , $P = 0.014$). Despite no significant differences were found in the relative PBMC and CSF *GRN* expression in patients compared to controls, stratifying patients according to the presence of rs5848 *T* allele, a 0.57-fold decrease in *GRN* mRNA levels over *C* carriers was found in PBMC (1.22 ± 0.23 versus 0.70 ± 0.12 , $P = 0.04$). Similarly to data obtained in brain samples, patients carrying the *TT* genotype showed the lowest *GRN* mRNA levels ($TT = 0.46 \pm 0.14$, $CC = 1.22 \pm 0.23$; $P = 0.013$). These data argue against a direct role of *GRN* as a susceptibility factor for sporadic AD but support a role of *GRN* as a disease-modifying gene, possibly contributing to the failure of neuronal survival.

Keywords: Alzheimer's disease (AD), cerebrospinal fluid (CSF), peripheral mononuclear cells (PBMC), progranulin (GRN), single nucleotide polymorphism (SNP)

INTRODUCTION

*Corresponding author: Chiara Fenoglio, Department of Neurological Sciences, "Dino Ferrari" Center, University of Milan, IRCCS Fondazione Ospedale Maggiore Policlinico, Milan, Italy. Tel.: +39 2 5033887; Fax: +39 2 50320430; E-mail: chiara.fenoglio@unimi.it.

Alzheimer's disease (AD) is the most common form of primary degenerative dementia with a complex etiology and a strong genetic component affecting ap-

proximately 15 million people worldwide [1]. The only well-confirmed genetic risk factor for sporadic AD is the apolipoprotein E (*APOE*) gene. Many other functional candidate genes coding for molecules involved in the pathogenic pathway of AD were proposed. Among these, Progranulin (*GRN*) could be considered a strong biological candidate gene as it has attracted significant attention in the scientific community following the recent discoveries of mutations causative for Frontotemporal Lobar Degeneration with ubiquitin-immunoreactive neuronal inclusions (FTLD-U) [2,3]. A number of pathogenic *GRN* variants are predicted to result in a premature termination codon, leading to the degradation of the mutant mRNA through the process of nonsense mediated decay, resulting in a null allele [2].

GRN is a multifunctional secreted growth factor encoded by *GRN* gene on chromosome 17q21. *GRN* encodes for a 593 amino acid glycoprotein containing 7.5 tandem repeats of 12 cysteinyl granulin motifs. Progranulin and the various granulin peptides derived by elastase cleavage are implicated in a range of biological functions [4]. Progranulin is widely expressed in several tissues and has been implicated in development, wound repair, inflammation, and tumorigenesis [5]. It is highly expressed in neurons of the cerebral cortex, the hippocampus, and the cerebellum but its role in the central nervous system (CNS) has not been investigated extensively [6].

Interestingly, *GRN* expression has been found increased in activated microglia as well as in peripheral blood in AD suggesting a potential role in this pathology [2,7]. Furthermore, several patients carrying mutations in *GRN* exhibited a clinical presentation indistinguishable from AD [8]. Recently the same authors investigated the genetic variability within the *GRN* locus in a Belgian population of AD patients and found *GRN* haplotypes associated with increased risk for AD [9]. Furthermore, Cortini and colleagues [10] reported a novel putative *GRN* mutation leading to an amino acidic substitution in an Italian patient with clinical AD.

In the present study, *GRN* mutation scanning was performed in a cohort of Italian patients and an association analysis was performed for two frequent variants, rs9897526 *G* > *A* and rs5848 *C* > *T*, to test whether *GRN* genetic variability contributes to the susceptibility for AD in the Italian population. Results were confirmed in a second replication population.

In addition, a wide transcriptional analysis was carried out in the parietal lobe of 68 neuropathologically-confirmed AD cases and 43 controls as well as in pe-

ripheral blood mononuclear cells (PBMC) and in cells isolated from cerebrospinal fluid (CSF), to investigate the expression profile of *GRN* and the possible influence of the common variants considered on mRNA levels.

SUBJECTS AND METHODS

Subjects

Two set of samples were included in this study. The first series consisted of 355 patients with AD consecutively recruited at the Alzheimer Units of Ospedale Maggiore Policlinico (Milan) and Ospedale L. Sacco (Milan). All patients underwent a standard battery of examinations, including medical history, physical and neurological examination, screening laboratory tests, neurocognitive evaluation, brain magnetic resonance imaging (MRI) or computed tomography (CT), and, if indicated, positron emission computed tomography (PET). Dementia severity was assessed by the Clinical Dementia Rating (CDR) and the Mini Mental Scale Examination (MMSE). Disease duration was defined as the time in years between the first symptoms (by history) and the clinical diagnosis. The presence of significant vascular brain damage was excluded (Hachinski Ischemic Score < 4). The diagnosis of probable AD was made by exclusion according to NINCDS-ADRDA criteria [11]. Fifty AD patients had an early onset of disease (EOAD; < 65 years) whereas remainders had a late onset (LOAD; ≥65 years).

The control group (CON) consisted of 377 subjects matched for ethnic background, age, and gender without memory impairment (MMSE > 28) and psychobehavioral complaints. The age of controls did not significantly differ from that of patients ($P > 0.05$). All patients or their caregivers gave informed consent for this study.

The second series consisted of an American set of samples collected through the Alzheimer's Disease Research Center (ADRC) at Washington University. Cases were diagnosed using the NINCDS-ADRDA criteria, slightly modified to include AD as a diagnosis for individuals aged > 90 years [11,12]. A total of 355 unrelated AD cases were recruited for the study. DNA from 343 age- and sex-matched non-demented controls aged > 60 years at assessment were obtained through the ADRC.

Sixty-eight out of 355 patients as well 43 out of 343 controls included in the American series, underwent neuropathological analysis, which confirmed the clini-

Table 1
Characteristics of patients and controls for both populations

	CON	AD	US CON	US AD
Number of subjects	377	355	343	355
Gender (M:F)	121:256	105:250	131:212	128:227
Mean age, years \pm S.E.M.	73.56 \pm 0.58	76.50 \pm 0.35	78.73 \pm 0.47	83.89 \pm 0.37
Mean age at onset, years \pm S.E.M.		73.03 \pm 0.58		76.69 \pm 0.39
Mean disease duration, years \pm S.E.M.		3.17 \pm 0.22		8.21 \pm 0.25
ApoE ϵ 4 carriers (%)	57 (15)	147 (41)*	59 (21)	189 (56)*

* $P < 0.001$, AD versus CON (OR: 4.35; CI: 3.06–6.19).

cal diagnosis. Transcriptional analysis was carried out from RNA extracted by the parietal lobe of these individuals. All subjects included in the replication population were European Americans. All patients or their caregivers gave informed consent for this study. Information about cases and controls are summarized in Table 1.

DNA isolation and ApoE genotyping

High-molecular weight DNA was isolated from whole blood using a Flexigene Kit (Qiagen, Hildren, Gemany), as described by the manufacturer. For the Italian sample, DNA was amplified using specific primers and then digested with *HhaI*, as previously described [13]. For the U.S. sample, *APOE* genotyping was performed using a Taqman assay (purchased from Applied Biosystems, ABI).

Direct sequencing

The entire open reading frame with the exon-intron boundaries of *GRN* was sequenced in a subgroup of 50 patients with AD as well as in 50 age-matched controls using specific primers, as previously described [14]. Briefly, each PCR was carried out in 50 μ l total volume containing 25 ng genomic DNA, 12.5 pmol of each specific primers, 0.6 μ M of each dATP, dTTP, dCTP and dGTP, 1U Taq DNA polymerase (ABI). Amplification conditions consisted of an initial denaturation step at 94°C for 5 min followed by 38 cycles of 94°C for 30 sec, Tm for 30 sec and 72°C for 45 sec, and a final extension step of 72°C for 10 min.

Fragments were purified using the ExoSAP-IT® Kit (USB, USA), according to instructions of the manufacturer, and then direct sequencing was performed with an ABI PRISM®3100 gene analyzer (ABI). Sequencing PCR was carried out in 20 μ l total volume containing 1 μ l BigDye Terminator v3.1 (ABI), 1.6 pmol of each primer and water to reach the final volume. Thermocycling consisted of 25 cycles of 96°C for 30 sec, 50 for 20 sec and 60°C 3 min. Sequences were analyzed with the Seqscape software.

Genotyping assays

Allelic variants with a frequency > 10% were tested for association in the Italian series were analyzed by using TaqMan methodology. Each Taqman 5'-nuclease assay employed 25 ng of genomic DNA as template. Assay-on-demand products, ABI assay IDs: C_2548248_10, C_7452046_20, were used for rs9897526 and rs5848 genotyping respectively. Probes specific for these variants were labeled with 6-FAM and VIC as reporter dyes and MGB-NFQ (ABI) as quencher. All the assays were performed in 20 μ l reactions in 96-well plates using an ABI PRISM®7000 instrument (ABI) as previously described [15].

Genotyping for rs5848 in the US series was performed using matrix assisted laser desorption/ionization time-of-light (MALDI-TOF) mass spectrometry (Sequenom). PCR primers and primer extension assays were designed by using SPECTROGEN software (Sequenom). SNP assays were designed to generate extension products of different masses resulting in genotype dependent peak appearance [16]. Genotyping for rs9897526 in the US series was done using TaqMan as described above.

RNA isolation and expression analysis

Total RNA from the parietal lobe of 68 neuropathologically-confirmed AD cases and 43 controls from the US series was extracted using the Tissue Kit and RNeasy Lipid Tissue kit (<http://www.qiagen.com>). cDNA was obtained from total RNA using the High Capacity cDNA Archive Kit.

Total RNA from PBMC was obtained from 26 patients (17 females, mean age: 73.42 \pm 1.93 and 9 males, mean age 77.57 \pm 1.67) and 27 control subjects (16 females, mean age: 70.13 \pm 4.20 and 11 males, mean age: 69.29 \pm 3.20) from the Italian series carrying different genotypes.

Additionally, total RNA from cells isolated from CSF was available from 25 subjects of the Italian se-

ries: 17 patients (9 males, mean age 69.75 ± 4.5 and 8 females, mean age: 75.5 ± 3.50) and 8 control subjects (4 males, mean age 70.50 ± 0.20 and 4 females, mean age 74.00 ± 0.89).

RNA purity was measured by optical density and only samples with an OD 260/280 ratio of 1.8 to 2 and an OD 260/230 of 1.8 or greater were used. Then, to confirm the lack of gDNA, PCR was carried out using primers located in different consecutive exons of actin housekeeping gene. cDNA was detected by the presence of an amplicon of 250 bp, whereas gDNA, characterized by the presence of a 444 bp fragment, was absent. In addition, RNA integrity was confirmed by quantified cDNA levels for GAPDH (2 different assays, located in different regions of the gene), B2M and cyclophilin A.

GRN expression levels in parietal lobe were measured by using a custom TaqMan assay for *GRN* exon 12 (primers and probes available upon request) using GAPDH as endogenous control. Each real-time PCR run included within-plate duplicates and each experiment was performed twice for each sample. Correction for sample-to-sample variation was done by simultaneously amplifying GAPDH as a reference. 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 gene, GAPDH, and were corrected for the PCR efficiency of each assay, although the efficiency of all reactions was close to 100%. Only samples with an s.e. < 10% were analyzed.

Total RNA from PBMC was reverse-transcribed using the Ready to go kit (USB, USA). Subsequently, the relative expression of *GRN* was measured by quantitative RT-PCR using premade TaqMan gene expression assays developed in the meantime (ABI, Assay ID: Hs00173570_m1). This custom-made assay underwent rigorous quality control testing, including mass spectrometry to verify the sequence, and further testing ensuring proper formulation of probe and primer mix. As an endogenous control for sample normalization, 18S rRNA was used (ABI, Assay ID: HS99999901_s1). The relative quantity of RNA was measured in duplicate by the $\Delta\Delta$ CT method relative to the data from a control subject [17].

Qualitative transcriptional analysis of rs9897526 has been performed through cDNA amplification of exons 2-7 by using specific primers (forward: 5'-GCCACTCCTGCATCTTTACC-3'; reverse: 5'-TTCTCCTTGAGAGGCACTT-3'). Fragments (wild type length: 586 bp) were visualized by agarose gel-electrophoresis stained with ethidium bromide.

Statistical analysis

Allelic and genotypic frequencies were obtained by direct counting. Haploview v4.1 software was used to test for Hardy Weinberg Equilibrium (HWE), Linkage Disequilibrium (LD), and for differences in allele distribution between cases and controls. Non-parametric Mann-Whitney Rank sum test was used for differences related to age at onset of disease in different genotype carriers. According to the Bonferroni's correction, the threshold for significance was set at $P < 0.025$. One way Anova test, including Holm-Sidak for multiple comparisons, was performed for differences related to mRNA levels.

In the neuropathological series, *GRN* mRNA levels were compared using the Kruskal-Wallis test. Step-wise discriminant analysis did not detect any important covariant (age, gender, *APOE* status).

RESULTS

Genetic analysis

Direct sequencing of *GRN* in 50 Italian AD samples did not show any novel mutation but led to the identification of several known common variants previously reported (Table 2). Among these, rs9897526, which is located 21 bp downstream of the *GRN* intron 2 splice donor site and rs5848, located in the 3'UTR region, showed a minor allele frequency (MAF) > 10% and were subsequently tested for association with AD. Pairwise LD analysis computed with Haploview software indicated no LD between rs9897526 and rs5848 ($D' = 0.40$) thus no haplotype blocks and markers combinations were highlighted according to the definition by Gabriel and colleagues [18], as implemented in Haploview v4.1.

Both SNPs were in HWE in cases and controls for both datasets. A tendency to an increased frequency of rs5848T allele was found in patients as compared with controls although it hardly reaches the significant threshold required according to the Bonferroni's correction (rs5848 MAF: 34.5% versus 29.0% in patients as compared with controls, $P = 0.025$). Allelic and genotype frequencies did not differ between patients and controls for rs9897526 (MAF: 14.4% versus 12.2% in patients as compared with controls, $P \geq 0.05$). Data were replicated in a second independent series from US (rs5848 MAF: 30.0% versus 27.9% in cases and controls respectively, rs9897526 MAF: 12.7% versus

Table 2
PGRN allelic variants identified in Italian patients

Mutation Genomic ^a	Predicted protein ^b	Location	rs number
g.95914delC		5'-untranslated region	rs17523519
g.96286insG		5'-untranslated region	rs11398947
g.100165C>T	p.Asp33Asp	EX1	
g.100169G>A	p.Gly35Arg	EX1	
g.100460G>A		IVS2	
g.100474G>A		IVS2	rs9897526
g.101084insGTCA		IVS3	rs34424835
g.101164T>C	p.Asp128Asp	EX4	rs25646
g.101266G>A		IVS4	rs850713
g.102072G>A		IVS7	
g.103778C>T		3'UTR	rs5848

^aNumbering relative to the reverse complement of GenBank accession number AC003043.1 and starting at nucleotide 1.

^bNumbering according to GenPept accession number NP_002078.1.

Table 3
Allelic and genotype frequencies (%) in AD compared with age-matched controls in the two study samples

SNP	n	Genotype % (n)			Allele % (n)		p-value
Italian series							
Rs9897526		<i>GG</i>	<i>GA</i>	<i>AA</i>	<i>G</i>	<i>A</i>	
Controls	377	77.7 (293)	20.2 (76)	2.1 (8)	87.8 (662)	12.2 (92)	0.10
Cases	355	72.7 (258)	25.9 (92)	1.4 (5)	85.6 (608)	14.4 (102)	
Rs5848		<i>CC</i>	<i>CT</i>	<i>TT</i>	<i>C</i>	<i>T</i>	
Controls	377	51.0 (194)	39.0 (147)	10.0 (36)	71.0 (535)	29.0 (219)	0.025
Cases	355	43.1 (153)	44.9 (159)	12.0 (43)	65.5 (465)	34.5 (245)	
US series							
Rs9897526		<i>GG</i>	<i>GA</i>	<i>AA</i>	<i>G</i>	<i>A</i>	
Controls	343	76.0 (261)	22.4 (77)	1.5 (5)	87.3 (599)	12.7 (87)	0.53
Cases	355	74.1 (263)	23.7 (84)	2.2 (8)	85.9 (610)	14.1 (100)	
Rs5848		<i>CC</i>	<i>CT</i>	<i>TT</i>	<i>C</i>	<i>T</i>	
Controls	302	52.0 (157)	40.0 (121)	8.0 (24)	72.1 (435)	27.9 (169)	0.52
Cases	329	49.2 (162)	41.3 (136)	9.5 (31)	70.0 (460)	30.0 (198)	

14.1% in cases as compared with controls; $P > 0.05$, Table 3).

No association with *APOE* status or gender was found in both populations considering each SNP.

In the Italian series, cases carrying the rs9897526A variant had a significantly earlier age at disease onset compared with patients carrying the G allele (mean age 70.12 ± 1.18 , $n = 71$ versus 74.32 ± 0.69 , $n = 173$ respectively, $P = 0.002$) whereas no influence on age at onset has been found according to the rs5848 status.

Expression analysis

GRN expression in the parietal lobe of 68 neuropathologically-confirmed AD cases showed a 0.76-fold decrease as compared with controls (1.31 ± 0.07 versus 1.73 ± 0.12 , $P = 0.0025$, Fig. 1A). Moreover, among cases, individuals carrying the *TT* genotype presented

the lowest *GRN* expression levels (0.96 ± 0.12 , $P = 0.014$, Fig. 1B). On the contrary, the P -value for association of rs5848 with *GRN* expression in controls' brain was 0.922. The *GRN* mRNA mean level in controls carrying the rs5848 *T* allele was 1.62 and for rs5848C carriers was 1.63.

Although no significant differences were found in the relative PBMC and CSF *GRN* expression in patients as compared with controls in the independent AD Italian series (data not shown), stratifying patients according to the presence of rs5848*T* allele, a statistically significant 0.57-fold decrease in *GRN* mRNA levels over *C* carriers was found in PBMC (0.70 ± 0.12 versus 1.22 ± 0.23 , $P = 0.04$, Fig. 2). This effect was likely dose-dependent, as patients carrying the *TT* genotype showed the lowest *GRN* mRNA levels in PBMC, 0.38 fold decrease over *CC* carriers ($TT = 0.46 \pm 0.14$; $CC = 1.22 \pm 0.23$, $P = 0.013$, Fig. 2). This tendency was

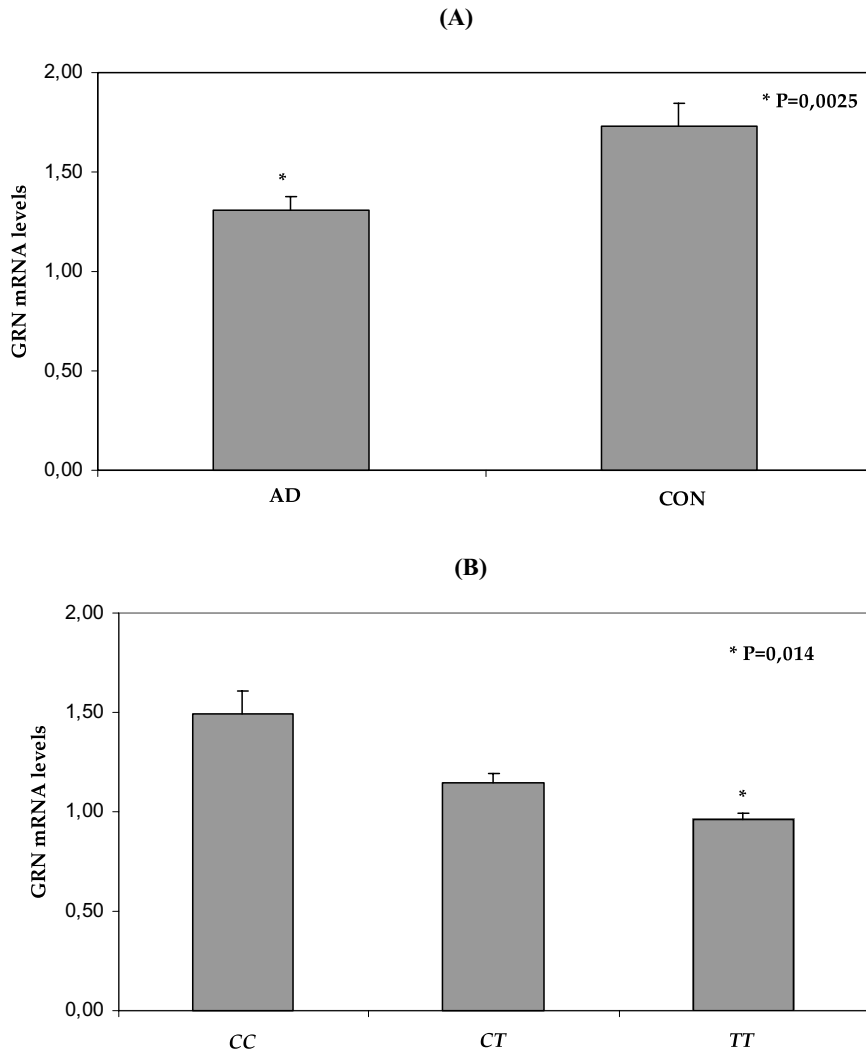


Fig. 1. A) *GRN* expression levels in parietal lobe from patients and controls B) association of rs5848 with *GRN* relative mRNA expression. Values are expressed as mean relative mRNA levels \pm S.E.M. Kruskal-Wallis test was used for differences related to the mRNA levels in rs5848 genotype carriers. Y-Axis represents the relative expression level taking an arbitrary reference sample as 1.

not significant in controls (CC = 1.09 ± 0.22 versus CT/TT = 0.84 ± 0.16). Similarly, considering *GRN* expression levels in cells isolated from CSF, the same effect was observed although the significance threshold was not reached, probably due to the small sample size (data not shown).

Conversely, real time PCR showed no difference in the rate of mRNA levels in carriers of rs9897526 *A* compared with non-carriers (data not shown). In addition, a qualitative agarose gel-electrophoresis of *GRN* PCR amplicons obtained from first-strand cDNA prepared from PBMC revealed no differences in the transcript length comparing subjects carrying different alleles (Fig. 3).

DISCUSSION

According to our results, *GRN* rs9897526 and rs5848 or SNPs in high LD with these variants do not act as susceptibility factors for AD, but rs5848 polymorphism is consistently associated with decreased *GRN* mRNA levels in the parietal lobe of patients with AD. This effect was observed also in PBMC from patients.

The association study revealed no significant differences in the distribution of rs9897526 and rs5848 in cases and controls beneath a tendency towards an increased frequency of rs5848T allele was observed in the Italian AD population. Data were replicated in an independent American set of samples, thus arguing

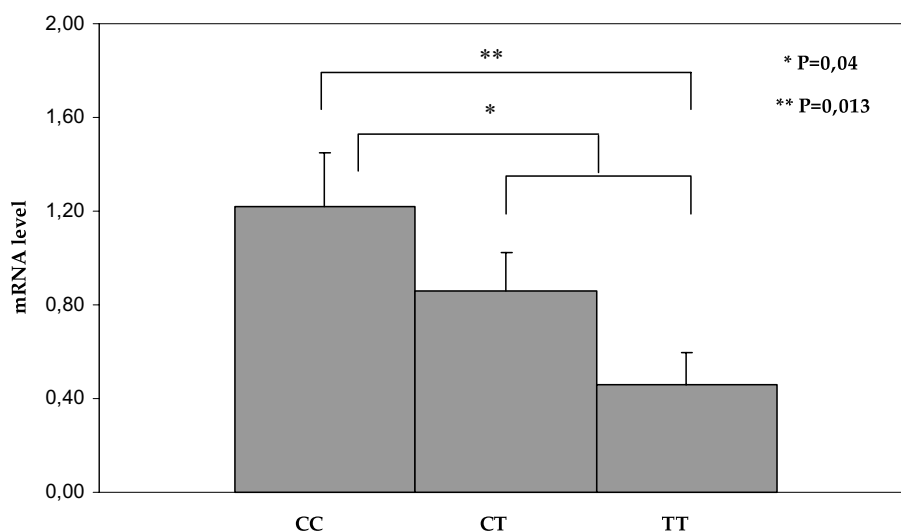


Fig. 2. Association of rs5848 with *GRN* mRNA expression in PBMC from AD patients. Values are expressed as mean relative mRNA levels \pm S.E.M. One way Anova test, including Holm-Sidak for multiple comparisons was performed for differences related to the mRNA levels in rs5848 genotype carriers. Y-Axis represents the relative expression level taking an arbitrary reference sample as 1.

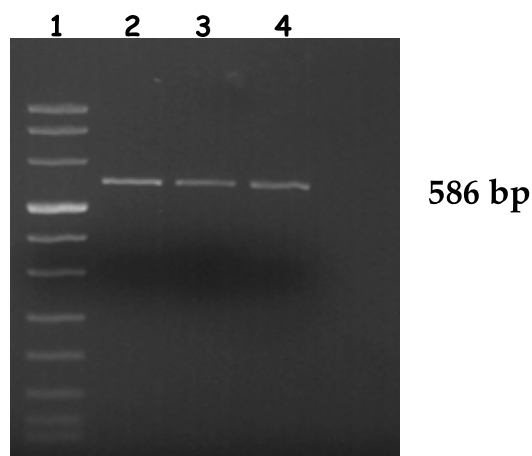


Fig. 3. Transcript analysis of rs9897526 variant. Agarose gel-electrophoresis of *GRN* amplicon obtained from PBMC cDNA from subjects carrying different genotypes: GG lane 2, GA lane 3, AA lane 4. The expected transcript length of *GRN* cDNA exon 2-7 (586 bp) does not differ according to rs9897526 status. DNA weight marker, lane 1: VIII (Boehringer-Roche).

against a direct role of these common variants in susceptibility to AD. On the other hand, some recent findings suggested a role for rs5848 in the risk for FTL-D-U, increasing the OR to develop FTL-D-U for carriers homozygous for the minor T-allele up to 3 times [19]. The apparent negative association found in AD could be due to different pathogenic mechanisms underlying different neurodegenerative diseases, however, the difference observed in rs5848 distribution in the Italian

AD population suggests that further replication studies are required to draw definitive conclusions.

Interestingly, *GRN* expression was decreased in the parietal lobe of AD subjects. A similar trend was found in cells from CSF in the independent Italian series, although significance levels were not reached, possibly due to the small number of CSF samples studied. On the contrary, no significant differences were found in the relative PBMC *GRN* expression levels in patients compared with controls. These results did not confirm findings from Coppola and colleagues [7] who reported increased *GRN* expression levels in PBMC of patients affected from different neurodegenerative disorders, suggesting whole blood gene expression profile as a suitable surrogate for gene expression in the CNS. This discrepancy could be explained by several reasons: first, the large gene expression study from Coppola et al. [7] was performed using microarrays on PBMC without any further validation which is usually performed, since concerns regarding the reliability and comparability of microarray data have been raised [20–22]. Herein, the TaqMan technology, commonly used for validation and comparison of gene expression in microarray quality control studies [23–25] and considered the gold standard for gene expression measurement, was performed.

Moreover, whole blood gene expression profile, although sharing significant similarities with that of the CNS, could not be representative for all brain regions as evinced from the hierarchical clustering results for gene

expression data from whole blood and several CNS tissue including the parietal lobe [26]. Recent evidence described clinical parietal lobe features in many cases of FTLN with a positive family history together with histopathologic evidence of significant parietal lobe involvement in association with *GRN* mutations [27–32]. According to these data it is tempting to consider parietal lobe deficits as salient feature of the progranulin phenotype, thus an imbalance of *GRN* expression in parietal lobe of AD patients would be conceivable.

In the present study, *GRN* expression in the parietal lobe of patients with AD was strongly influenced by the presence of rs5848T allele. A significant decrease of *GRN* mRNA levels was observed in AD cases carrying the polymorphic T allele as compared with non-carriers. Similar results were obtained in PBMC as well as in cells isolated from the CSF, although the small sample size did not allow a definitive conclusion. These data are in accordance with previous evidence suggesting that rs5848 is implicated in differential expression of *GRN* in a database-mining experiment [33]. Moreover, Rademakers and colleagues [19] supported the homozygosity of rs5848T allele as a major risk factor for FTLN-U through a decrease in levels of functional GRN. Haplo-insufficiency was predicted by the authors to result from increased suppression of GRN translation through altered micro (mi)RNA regulation, since rs5848 resulted to be located within a predicted binding-site for the human specific miR-659, which would contribute to translational repression.

According to our results, rs5848 variant showed an influence on *GRN* mRNA levels. This effect could be due to different translational regulation mechanisms by miRNAs peculiar of AD pathology, which in the case of rs5848 could have the same effect in reducing *GRN* expression levels.

The reduction of available GRN could be related with neurodegeneration, as most *GRN* mutations resulting in a reduction of the *GRN* protein levels due to nonsense mediated mRNA decay and consequently reduced GRN levels are causative for neurodegenerative processes [2, 3].

Recently several missense mutations resulted associated, through different molecular mechanisms, to a significantly reduced secretion of the protein [34]. Thus, apparently all *GRN* mutations cause reduced protein expression levels or secretion [34,35]. Furthermore Ghidoni and collaborators [36] analyzed GRN plasma and CSF levels in FTLN and demonstrated that GRN is strongly reduced both in plasma and CSF of affected and unaffected subjects carrying mutations in the gene,

confirming the initial reports of low levels of GRN due to the effect of *GRN* mutations.

Although the exact effects of GRN in the CNS remain to be established, emerging evidence support a role in the CNS as neurotrophic factor, possibly stabilizing neurons injured by deposited proteins such as amyloid- β (A β) [34]. The observation that in AD *GRN* is upregulated in activated microglia around amyloid plaques further support the hypothesis of a protective role towards a defense response to neuritic degeneration [2].

Lastly, a significant association between rs9897526 and disease onset was observed in the Italian series in term of a reduction of the age at onset in patients carrying the rs9897526A allele. Nevertheless, the association was not confirmed in the US series suggesting that other factors such as environmental conditions could act synergistically with the individual genetic background to contribute to the disease.

Interestingly, a number of previous findings suggested that rs9897526 influences the age at disease onset in different neurodegenerative diseases. In particular, Sleegers et al. [37] examined the contribution of eight frequent polymorphisms in risk of amyotrophic lateral sclerosis (ALS) in a Belgian and a Dutch population, finding three common variants in the same LD block, including rs9897526, significantly associated with a reduction of age at onset and a shorter survival after onset of ALS.

In addition, Rademakers et al. [38] demonstrated that patients with FTLN carrying the Arg493X mutation together with the rs9897526A allele on their wild type allele show a delayed mean age at onset of the disease. Despite the influence of rs9897526A allele on age at onset in this last study was opposite to our findings, the authors reported it was not possible to draw definitive conclusions because of the small sample size. However, the molecular mechanism at the basis of this effect is not clear yet, since rs9897526 is not located in the splice site region and does not seem to generate an aberrant transcript in RT-PCR; moreover, when stratifying patients according to the presence of rs9897526A allele, no difference in the relative amount of mRNA has been found. Therefore, a possible hypothesis to be considered is that another true modifying variant should exist in LD with rs9897526.

In conclusion, these results suggest that *GRN* common variants rs5848 and rs9897526 could act as modifier factors, affecting *GRN* mRNA levels and possibly decreasing the age at disease onset, likely contributing to the failure of neuronal survival. However, more extensive functional analysis is needed to confirm this hypothesis.

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