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Gene Expression Study on Peripheral Blood Identifies Progranulin Mutations

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Peripheral blood is a readily available tissue source allowing relatively noninvasive screening for a host of medical conditions. We screened total-blood progranulin *(PGRN)* levels in 107 patients with neurodegenerative dementias and related conditions, and 36 control subjects, and report the following findings: (1) confirmation of high progranulin expression levels in peripheral blood; (2) two subjects with reduced progranulin levels and mutations in the *PGRN* gene confirmed by direct sequencing; and (3) greater *PGRN* messenger RNA levels in patients with clinical diagnosis of Alzheimer's disease. This proof-of-principle report supports the use of gene quantification as diagnostic screen for *PGRN* mutations and suggests a potential role for progranulin in Alzheimer's disease.

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Frontotemporal lobar degeneration (FTLD) comprises a group of dementias with related clinical and neuropathological characteristics.[1] FTLD is the second most common cause of presenile dementia after Alzheimer's disease (AD)[1–3] and accounts for 5 to 10% of neurodegenerative dementias in epidemiological samples and between 9 and 16% in autopsy series.[1] Clinical subtypes of FTLD include (1) a behavioral variant with predominant frontotemporal involvement, (2) semantic dementia, and (3) primary progressive aphasia. A family history is present in about 40% of the FTLD patients, and four genes have been discovered as genetic causes. Mutations in *MAPT* have been identified in more than 100 families, and 2 other causative genes (*VCP*[4] and *CHMP2B*[5])

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have been discovered in a much smaller number of families. The recent discovery of mutations in the progranulin gene *(PGRN)* as a major cause of familial FTLD[6,7] constitutes a major breakthrough and has reshaped this research field. To date, 47 mutations have been reported in *PGRN* (http://www.molgen.ua.ac.be/FTDmutations/[8]), all of which lead to the loss of 50% of *PGRN* messenger RNA (mRNA), or haploinsufficiency, a novel pathogenetic mechanism in FTLD.

We are performing a large gene expression study on patients with dementia using microarrays on peripheral blood samples, to identify molecular markers associated with different forms of dementia.[9] Based on the proposed pathogenetic mechanism of *PGRN* mutations, we reasoned that we could identify potential *PGRN* mutation carriers by studying its expression in our microarray dataset.

We report the analysis of *PGRN* expression levels in peripheral blood in 107 patients with clinical diagnosis of FTLD, AD, and related neurodegenerative conditions, and 36 control subjects. We demonstrate that *PGRN* is highly expressed in peripheral blood and that mRNA quantification is a valid approach to identify *PGRN* mutation carriers. In addition, the identification of a significant increase in *PGRN* mRNA levels in AD patients suggests a potential role for *PGRN* in AD pathogenesis.

Subjects and Methods

Patients were enrolled at the Memory and Aging Center at the University of California San Francisco after obtaining informed consent. Diagnosis was based on clinical, laboratory, and neuropsychological examination as part of the standard evaluation at the Center.

Peripheral blood samples were drawn in two PAXgene tubes, stored at room temperature for at least 2 hours, and then at 4°C. Total RNA was extracted using the PAXgene blood RNA kit (PreAnalytix GmbH, QIAGEN, Germany). RNA quantity was assessed with Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE), and quality with Agilent Bioanalyzer Nanochips. Total RNA (200ng) was amplified, labeled, and hybridized on Illumina HumanRef-8 v1 Expression BeadChips (Illumina Inc, San Diego, CA), querying the expression of approximately 24,000 RefSeq-curated gene targets. Slides were processed and scanned using the Illumina BeadStation platform. Data analysis was performed using R (www.r-project.org) and Bioconductor (www.bioconductor.org[10]) packages. Absolute expression values were $\log_2$ transformed and normalized using quantile normalization. Data quality assessment included inter-array Pearson correlation, clustering based on the top 1,000 most variable genes, and detection of outlier arrays.

DNA was extracted from blood using standard protocols. Sequencing of the *PGRN* gene was performed as described previously.[6]

Results

One hundred forty-three subjects were included in the study: 43 patients with clinical diagnosis of FTLD; 46 patients with AD; 13 patients with corticobasal syndrome (CBS); 3 patients with progressive supranuclear palsy; 2 patients with amyotrophic lateral sclerosis; and 36 unaffected control subjects. Family history for dementia or psychiatric illness was positive in 50% of the FTLD patients in this series. One FTLD sample failed the quality-control test because of poor array signal and was excluded. Age, disease duration, and sex were similar across the groups, with the exception that FTLD patients were generally younger (61.2 vs 66.9 years in control subjects).

Progranulin absolute expression levels were high in peripheral blood (average $\log_2$ expression level: 10.7 ± 0.4; range 9.6–11.8; 97th percentile of the normalized intensity distribution of all the genes on the array. We identified two outliers with expression signal lying approximately three standard deviations below the overall average and corresponding to 50% of reference *PGRN* levels (Fig 1). Patient FTLD.20 is a 69-year-old woman with a clinical diagnosis of FTLD-frontotemporal dementia without family history of dementia, and the second subject (C.31) is a 44-year-old clinically normal control subject with a family history positive for dementia.

Sequence analysis of the *PGRN* coding region in these subjects showed a point mutation (IVS7-1G>C) in Patient FTLD.20 and a microdeletion (c.675_676delCA) in Subject C.31 (Fig 2A). Both mutations have been described and are predicted to reduce progranulin levels via nonsense-mediated decay.[11] We sequenced the *PGRN* gene in the remaining 41 subjects with clinical diagnosis of FTLD and normal blood progranulin level, and did not find further progranulin mutations. The *PGRN* patients showed a gene expression profile distinct from the other FTLD patients, suggesting a specific pathogenetic mechanism (see Fig 2B). A total of 412 genes were differentially expressed between *PGRN* mutation carriers and control subjects, most of which were also significantly different when comparing *PGRN* cases with the other FTLD patients. One hundred forty-three were differentially expressed when comparing the remaining 41 FTLD patients with control subjects, and the overlap between these two lists was small (see Fig 2B), showing a *PGRN*-related pattern. Overrepresented gene ontology categories in this gene list include cytoskeleton, axonal transport, and neurogenesis (see Supplementary Table).

We validated the *PGRN* outliers in this study as well as some of the top differentially expressed genes between *PGRN* patients and control subjects using real-time quantitative polymerase chain reaction (qPCR). We used the two *PGRN* patients identified in this study, and an additional *PGRN* case. All the *PGRN* outliers were confirmed, and six of nine genes (66%) differentially expressed between *PGRN* mutation carriers and control subjects were confirmed by real-time qPCR (see Supplementary Figure).

We then looked at progranulin expression levels in

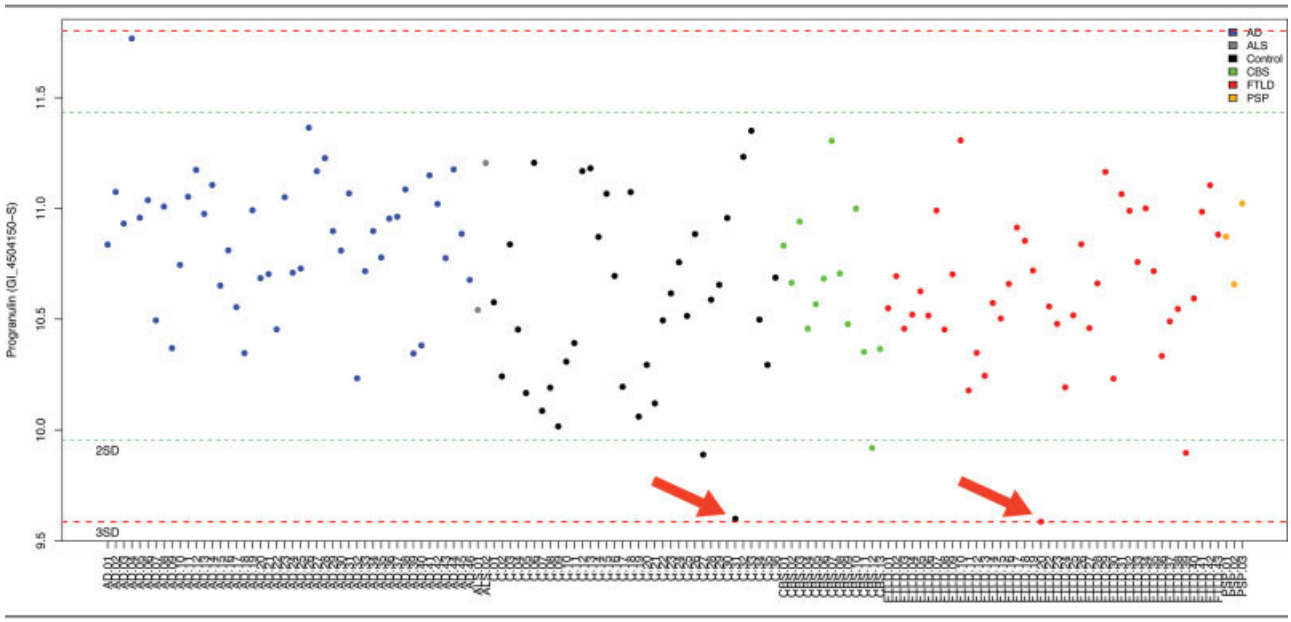

Fig 1. Peripheral blood PGRN *expression levels in 142 subjects. Data are expressed in* log_2*-transformed normalized expression values.* Dotted lines *indicate two and three standard deviations (SDs) from the overall mean. Two samples* (arrows) *were outliers lying at approximately three SDs below the mean, corresponding to approximately 50% of reference progranulin messenger RNA levels. Blue denotes Alzheimer's disease patients; gray, amyotrophic lateral sclerosis patients; black, control subjects; green, corticobasal syndrome; red, frontotemporal lobar degeneration patients; and orange, progressive supranuclear palsy.*

patients with a diagnosis of probable AD. Patients with clinical diagnosis of AD showed greater *PGRN* mRNA levels in peripheral blood (+23%; $p \leq 0.001$) when compared to both control subjects and FTLD/CBS patients. These levels remained significantly greater ($p \leq 0.004$) after exclusion of six outliers (Fig 3).

Discussion

The discovery of mutations in *PGRN* causing FTLD has been a major breakthrough in the field. Twenty-three percent of the FTLD cases with positive family history and 5% to 10% of the total FTLD cases are caused by *PGRN* mutations according to a recent large-scale study.[11] Unlike the other FTLD-causing mutations reported in *MAPT,* mutations in *PGRN* act through a haploinsufficiency mechanism, resulting in reduced *PGRN* expression levels in mutation carriers. This suggests that alternative strategies aimed at quantifying the transcript levels can be effective in patient screening.

Peripheral blood is a widely used source of DNA. Systematic evaluation of comparability of gene expression in blood and brain has shown that whole-blood

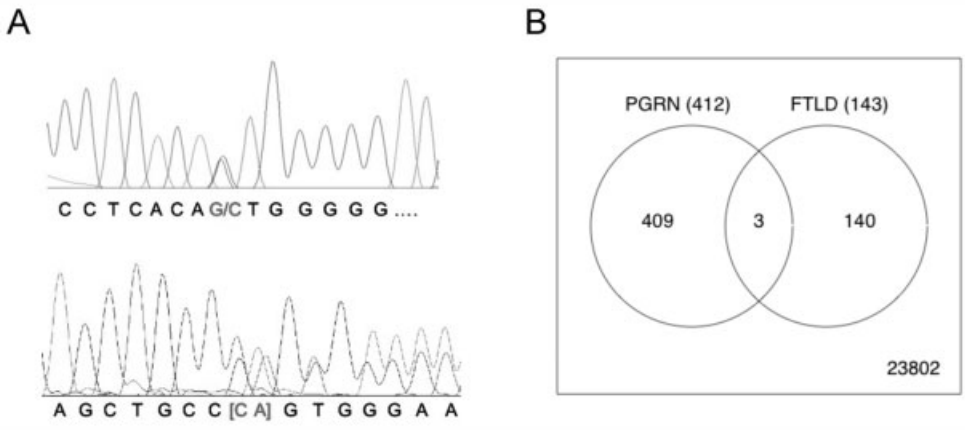

Fig 2. (A) Electropherograms showing two detected mutations: IVS7-1G>C or c.836-1G>C (top) and c.675_676delCA (bottom). (B) Venn diagrams representing the number of genes differentially expressed in 2 subjects with PGRN *mutations (412 genes; left circle) and in 41 frontotemporal lobar degeneration (FTLD) cases (143 genes; right circle) versus control subjects. The numbers of genes are in parentheses. There are 23,802 (bottom right corner) remaining genes on the array.*

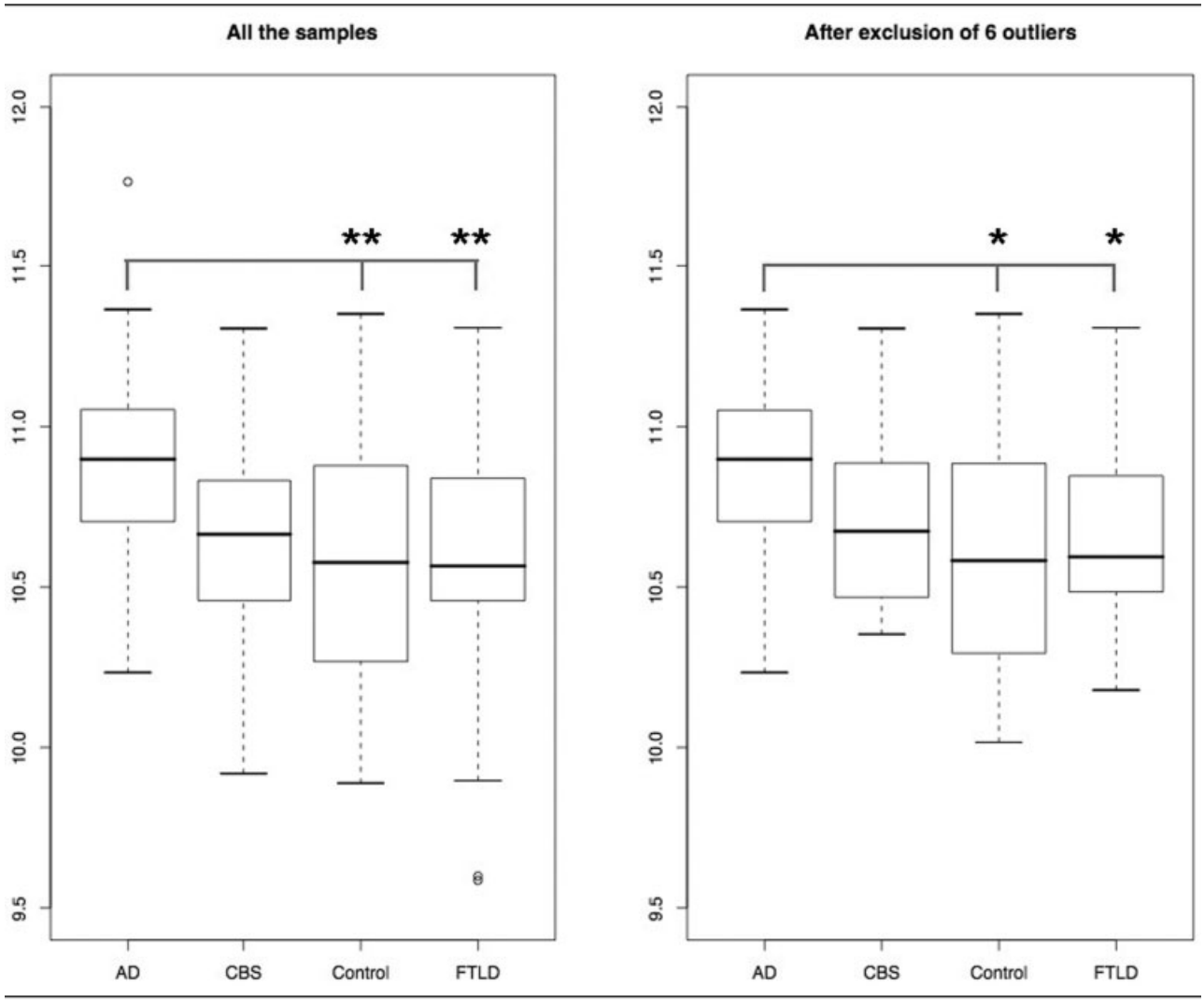

Fig 3. Box plot of log_2-normalized PGRN *expression levels in Alzheimer's disease (AD), frontotemporal lobar degeneration (FTLD), and corticobasal syndrome (CBS) patients, and control subjects. (A) Analysis on all the subjects. (B) Analysis after exclusion of six outliers.* $^{*}p \leq 0.004$; $^{**}p \leq 0.001$.

gene expression profile shares significant similarities with that of multiple central nervous system tissues[12]; therefore it may provide a suitable surrogate for gene expression in central nervous system disease in some cases as well.[13] For example, real-time quantitative PCR on peripheral leukocytes has been proposed as an alternative assay for molecular diagnosis in Friedreich's ataxia, as levels of frataxin are reduced in peripheral blood of patients with this neurodegenerative disease,[14] and microarray studies on peripheral lymphoblasts or peripheral blood have identified candidate genes relevant for AD,[15] Parkinson's disease,[16] and autism[17] pathogenesis.

We found that *PGRN* is highly expressed in peripheral blood, confirming initial reports of high expression levels in rat lymphocytes.[18] This high blood level is consistent with the proposed role for progranulin in wound repair and/or inflammation.[19] High expression in peripheral blood allows reliable quantification of mRNA levels using microarray and real-time qPCR. We screened 143 patients and control subjects and identified reduced levels of *PGRN* mRNA, compatible with haploinsufficiency, in 1 out of 43 (2%) patients with clinical diagnosis of FTLD and in 1 unaffected control subject. This warrants a wide screening for progranulin mutations (including patients with family history negative for dementia) and supports gene quantification as a valid approach, an alternative to direct sequencing.

We confirmed the presence of pathogenic *PGRN* mutations in both subjects with reduced levels of *PGRN* mRNA by direct sequencing. The observed reduced mRNA levels support nonsense-mediated decay as the most likely pathogenetic mechanism and constitutes a proof of principle that it is possible to screen patients for *PGRN* haploinsufficiency on peripheral total blood samples, without deriving cell lines, by using widely avail-

able gene quantification methods. In addition, this method would identify reduced *PGRN* levels caused by out-of-frame single exon deletions and duplications, as well as microdeletions, which are usually not detected through regular sequencing. However, the possibility that reduced *PGRN* levels can be related to active regulation of the gene has to be considered. For example, we observed a trend for *PGRN* increase with age in control subjects (data not shown), and other factors might be involved in regulating *PGRN* levels. Interestingly, three subjects (one FTLD patient, one CBS patient, and one control subject) showed reduced (60%) levels of progranulin, but tested negative for mutations in *PGRN,* suggesting that it might be worthwhile to investigate whether progranulin levels are contributing factors in the pathogenesis/clinical course of FTLD/CBS.

The gene expression profile in the two subjects with progranulin mutations is distinct from other FTLD patients. Although suggestive that specific pathogenetic mechanisms can be involved in this genetic form of FTLD, this observation is based only on two subjects and warrants extended gene expression studies involving a larger number of patients.

We reported increased mRNA *PGRN* levels in peripheral blood of patients with clinical diagnosis of AD, a difference that remains after the exclusion of outliers. This is the first report of quantitative assessment of *PGRN* expression in AD patients. Intense progranulin staining of senile plaques and microglia in AD pathology has been reported,[6] and an increase of *PGRN* mRNA was also reported in spinal cords of amyotrophic lateral sclerosis patients in an early microarray study.[20] This is intriguing and suggests the idea that, whereas decreased levels cause FTLD, increased levels of this gene are associated with distinct, but related neurodegenerative conditions such as AD and amyotrophic lateral sclerosis. Given the proposed roles of *PGRN,* this increase could be related to neuroinflammation associated with granulin peptides and/or a repair strategy. However, this finding needs to be confirmed on a larger series with neuropathological assessment.

In conclusion, quantification of *PGRN* levels in peripheral blood samples is a valid and effective strategy for large-scale first screening in patients with dementia and related conditions. Expression data in AD patients suggest that *PGRN* may play a role in AD pathogenesis.

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