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PIN1 promoter polymorphisms are associated with Alzheimer's disease

L. Segat [a], A. Pontillo [a], G. Annoni [c], D. Trabattoni [d], C. Vergani [e], M. Clerici [d], B. Arosio [e,*], S. Crovella [a,b]

[a] *Department of Reproductive and Developmental Sciences, University of Trieste, Italy*
[b] *Genetics Service, IRCCS Burlo Garofolo, Trieste, Italy*
[c] *Department of Clinical Medicine, Prevention and Medical Biotechnology, University of Milano-Bicocca, Milan, Italy*
[d] *DISP LITA VIALBA, University of Milan, Milan, Italy*
[e] *Department of Internal Medicine, University of Milan, Geriatrics Unit, Ospedale Maggiore Policlinico, Fondazione IRCCS Milan, Italy*

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Abstract

In our study, we analyzed the coding and promoter regions of the PIN1 gene in a group of 111 Alzheimer's disease (AD) patients looking for a possible genotype–phenotype correlation. The presence of SNPs – which could affect and modify the clinical phenotype of AD patients was also investigated.

We identified two single nucleotide polymorphisms (SNPs) at positions −842 (G → C) and −667 (C → T) in the promoter region of the PIN1 gene. Our results evidenced a significantly higher percentage of −842C allele carriers in AD subjects with respect to healthy controls. We found that this allele significantly raised the risk of developing AD (OR 3.044, CI 1.42–6.52). The −842 and −667 SNPs were in linkage disequilibrium and combined to form haplotypes. The CC haplotype conferred a higher risk of developing AD (OR 2.95, confidence interval 1.31–6.82).

Finally, protein expression analyses revealed that subjects carrying the −842 CC genotype or the CC haplotype showed reduced levels of the PIN1 protein in peripheral mononuclear cells.

Keywords: PIN1; Genetic polymorphisms; Alzheimer's disease; Genotype–phenotype correlation; Neurofibrillary degeneration

1. Introduction

Neurofibrillary tangles (NFTs) are prominent lesions in a large subset of neurodegenerative diseases, including Alzheimer's disease (AD), which are characterized by paired helical filaments (PHFs) composed of the microtubule-associated protein Tau.

In normal situations, Tau plays a role in the modulation of the functional organization and structure of neurons by regulating microtubules assembly [20,7]; in NFTs, instead, Tau is hyperphosphorylated on serine or threonine residues preceding proline and this abnormal phosphorylation is responsible for Tau aggregation and abolishes its ability to bind microtubules and promote microtubule assembly. Interestingly, the increased proline-directed phosphorylation of Tau and other proteins appears to precede tangle formation and neurodegeneration in AD [14,4].

Phosphorylated serine/threonine–proline motifs (like those found in NFTs) can exist in two distinct conformations, whose conversion in some proteins is catalysed by PIN1: PIN1 is in fact a peptidil-prolil-*cis*-trans isomerase that specifically isomerizes phosphorylation of a serine or threonine that precedes proline. The PIN1 protein – characterized by a carboxy-terminal catalytic domain as well as by a WW amino-terminal protein–protein interaction domain –

* Corresponding author at: Department of Internal Medicine, Ospedale Maggiore, Fondazione IRCCS, Via Pace 9, 20122 Milan, Italy.
Tel.: +39 055035405; fax: +:39 050320712.
E-mail address: beatrice.arosio@unimi.it (B. Arosio).

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is mainly expressed in neurons at higher levels than in most other postmitotic cells, where it regulates the dephosphorylation and functioning of several mitotic phosphoproteins, many of which are increased in AD [14,6].

Lu et al. [11] hypothesized that PIN1 can restore the function of phosphorylated Tau and may prevent or reverse the paired helical filaments (PHFs) formation in AD. In their study, they demonstrated that PIN1 WW domain binds hyperphosphorylated Tau from AD brains, but not Tau from age-matched healthy brains; they also proved that PIN1 is capable of restoring the biological function of phosphorylated Tau in vitro.

Overexpression of hyperphosphorylated Tau in AD brains can cause an increased association of these molecules in the tangles that might lead to depletion of the soluble form of PIN1 in neurons; indeed, the level of soluble PIN1 in the brains of AD patients is greatly reduced if compared to that in age-matched control brains.

There are also increasing evidences that AD might be related to an aberrant reactivation of the cell cycle and apoptosis in neurons and that PIN1 can play a pivotal role in this [19,14,15].

Moreover, the gene encoding the PIN1 protein – consisting of four exons and spanning over more than 14 kb – maps on 19p13.2, a locus recently associated with late-onset AD [21].

In our study, we analyzed both coding and promoter regions of the PIN1 gene in a cohort of 111 AD patients looking for a possible genotype–phenotype correlation between PIN1 gene nucleotide sequence variations and AD. We also investigated the presence of SNPs, which could affect and modify the clinical phenotype of AD patients.

2. Methods

2.1. Patients and controls

One hundred and eleven AD patients (79 F/32 M, mean age 79.47 ± S.D. 6.30) and 73 non-demented sex- and age-matched healthy controls (HC 50 F/23 M, mean age 79.98 ± S.D. 6.36) were enrolled for this study. All patients were Caucasian, living in Northern Italy and selected from a larger ambulatory population cared for at the Geriatric Department of the Ospedale Maggiore IRCCS, University of Milan, Italy. There were no significant differences between the groups in age or education level.

Diagnosis of probable AD was performed according to standard clinical procedures and following the DMS IV and NINCDS-ADRDA criteria [17]. The cognitive and functional performances were assessed using mini-mental state evaluation (MMSE), activities of daily living (ADL), instrumental activities of daily living (IADL) as well as an extensive neuropshycological evaluation. Every subject had undergone a recent brain magnetic resonance imaging (MRI)/computed tomography (CT) scan. Criteria for the diagnosis of normal cognition were as follows: (1) no active neurological or psychiatric disorders; (2) any ongoing medical problems or related treatments not interfering with cognitive function; (3) a normal neurological exam; (4) no psychoactive medications; (5) independently functioning community dwellers.

In order to minimize the risk of possible inflammatory processes, all subjects selected showed no clinical signs of inflammation (e.g. normal body temperature, no concomitant inflammatory condition) and normal blood chemistry levels (red blood cell sedimentation rate, albumin, transferrin and C reactive protein plasma levels).

Informed consent was obtained from all subjects or their relatives. The study protocol was approved by the Ethics Committee of the University Hospital.

2.2. Genotyping

Whole blood was collected by venipuncture in Vacutainer tubes containing EDTA (Becton Dickinson Co., Rutherford, NJ).

Genomic DNA was extracted by salting-out method as described in scientific literature [16]. DNA concentration and purity were determined by spectrophotometric analysis.

Amplifications of PIN1 coding (four exons) and promoter regions (1150 bp upstream the ATG codon) were performed by using primers (Table 1) designed using the software Primer Express 2.0 (Applied Biosystems, Foster City, CA) according to the human sequences available in GenBank (NM_006221 range = chr19:9807013–9821356 for the coding region, AF501321 for the promoter).

PCR reactions were carried out in a GeneAmp 9700 Thermal cycler (Applied Biosystems, Foster City, CA) using PCR buffer 1×, 1 unit of Taq Gold, 0.2 mM dNTPs and variable concentrations of $MgCl_2$ (from 1 to 2.5 mM). The cycling was performed with an initial denaturation for 10 min at 95 °C, followed by 36 cycles at 95 °C for 30 s, at the annealing temperature (T_a) for 30 s (see Table 1 for the different T_a used), at 72 °C for 30 s with a final extension to 72 °C for 7 min. PCR products were observed – under UV light – in a 2% agarose gel stained with ethidium bromide.

DNA sequencing of PCR products was performed using the BigDye Terminator Cycle Sequencing Ready Reaction Kit 2.0 (Applied Biosystems, Foster City, CA). DNA sequences were run on an automated ABI Prism 3100 Genetic Analyser (Applied Biosystems, Foster City, CA). Sequences were handled using SeqScape 1.0 Software.

ApoE genotypes were determined by PCR amplification of a 234 base-pair fragment of exon 4 of the ApoE gene, followed by digestion using Cfo1, according to protocols already described in scientific literature [18]. Restriction patterns were revealed by 2% agarose gel electrophoresis.

2.3. Protein expression analysis

Peripheral mononuclear cells (PBMCs) of 25 subjects (AD patients and healthy controls chosen to be representative

Table 1
Primers and annealing temperature (T_a) used to perform the genomic DNA amplification of PIN1 promoter and coding regions

	Forward primer	Reverse primer	T_a
Promoter region			
1	5′-CGCATAGCAAGTGTCAGTCCC-3′	5′-GGTGCCGACATTGACATTCAT-3′	60
2	5′-GCACCCTTTGCTGTCAGTGTC-3′	5′-TGTCCAAAGCTAACCCAGCCT-3′	55
3	5′-CCTGCACCTCCTCCGTGTTCT-3′	5′-TAACGGCGGTCCAGGAGGTAC-3′	55
4	5′-TGGGAAACAGGTGGGAAGAGG-3′	5′-TGAGTGGTCCGAAGCGACG-3′	60
5	5′-AAGCTCTATCCCGCCTGGG-3′	5′-TCGTCCGCCATCTTCCCTC-3′	64
Coding region			
Ex. 1	5′-GCCAATCCGGACCGTTAGG-3′	5′-GAAGAGCCAGGACCCCATG -3′	61[a]
Ex. 2	5′-TGGGAGCACAACCCTAGCTG-3′	5′-TCAGGTCATGCACTGGCGT-3′	55
Ex. 3	5′-AGCATGTGCGCCTGTGAG-3′	5′-GAAGGCCGGTGTGGCA-3′	59
Ex. 4a	5′-AGCCCCATCTGTCGCGGCT-3′	5′-CCACCACACTGCCCTGGGTC-3′	66
Ex. 4b	5′-GTTCCCACAATGGCTGGG -3′	5′-GGAGAACTTGCAGCTGGGAC-3′	58

[a] Auto increment −0.4 °C per cicle.

of all PIN1 promoter genotypes and putative haplotypes) were separated by density gradient using the Lympholyte-H kit (Cedarlane Laboratories Limited, Canada).

Proteins were extracted using Triton X-114 Tris buffer from PBMC. Briefly, after centrifugation (12 000 rpm at 4 °C), supernatant was loaded onto sucrose cushion buffer, and incubated at 37 °C for 3 min. The samples were again centrifuged to obtain two phases. 5.3 μg of the total protein extract of the aqueous phase, were loaded onto electrophoretic 15% polyacrylamide gel (5% stacking gel) according to methods set by Laemnli [5]

Electrophoresis was performed at 20–30 mA for 110 min and proteins were blotted onto PVDF membrane 0.22 μm (Immobilon, Millipore, Italy) at 90 V for 20 min at 4 °C. After transfer, non-specific binding was blocked for 120 min with 5% milk in phosphate buffer saline with 0.1% Tween 20 (PBST); membranes were then incubated overnight with 1 μg/ml of rabbit anti Human PIN1 antibody (Calbiochem, Germany) diluted in PBST with 1% milk. Anti rabbit IgG HRP (GE Healthcare Bio-Sciences, Italy) was added at 1:4000 in PBST 1% milk for 90 min and immunoreactive bands were revealed by chemiluminescent substrate (ECL, GE Healthcare Bio-Sciences, Italy). The relative protein levels were quantified by densitometric scanning (IM1D, GE Healthcare Bio-Sciences, Italy). All western blot experiments were run in duplicate to test the reproducibility of the assay. The antibody specificity was assessed using a HepG2 cell lysate positive control (D.B.A., Italy).

2.4. Statistical analysis

Statistical analysis was performed using the SPSS statistical package (SPSS version 10, Chicago, IL). Genotype frequencies in the study groups were compared using the χ^2 test. Odds ratio (OR) and 95% confidence intervals (95% CI) were calculated as estimates of the risk of AD in carriers of PIN1 polymorphisms compared to non-carriers. Adjusted estimation for an ApoE ε4 carrier status was made by logistic regression analysis. Haplotype frequency and delta values for linkage disequilibrium were calculated using the software Arlequin 1.1 available for non-profit use on the Internet at http://anthropologie.unige.ch/arlequin/.

A comparative study of relative protein levels among the various groups of genotypes and haplotypes taken into consideration was carried out by one-way analysis of variance followed by Bonferroni post hoc test. Differences in mean age of onset and MMSE between patients carrying the different genotypes and alleles were calculated by Student t test or by one-way analysis of variance followed by Bonferroni post hoc test when multiple comparisons were performed. $p < 0.05$ was taken as the cut-off for statistical significance.

3. Results

3.1. Identification of −842(G → C) and −667 (C → T) polymorphisms in the PIN1 gene

The DNA sequencing of the coding and promoter regions of the PIN1 gene in our 111 AD patients and 73 healthy controls allowed us to identify two single nucleotide polymorphisms (SNPs) at position −842 (G → C) and −667 (C → T) in the promoter region. (NCBI refSNP ID: rs2233678 and rs2233679, respectively).

The genotype and allele distributions of these SNPs in AD and HC samples are reported in Tables 2 and 3. The distribution of PIN1 genotypes was in Hardy–Weinberg equilibrium in the two groups analyzed.

In the analysis of the −667 SNP, the percentage of T allele is higher in HC than AD samples (71% versus 62%) (Table 2).

Table 2
Frequency of PIN1 −667 TC genotypes and alleles observed in Alzheimer's disease patients (AD) and in healthy sex- and age-matched controls (HC).

	PIN1 genotypes			PIN1 alelles	
	T/T	T/C	C/C	T	C
AD	46 (41.5%)	46 (41.5%)	19 (17%)	138 (62%)	84 (38%)
HC	38 (52%)	28 (38%)	7 (10%)	104 (71%)	42 (29%)

Genotype: $\chi^2 = 2.957$, d.f. = 2, $p = 0.228$; allele: $\chi^2 = 2.828$, d.f. = 1, $p = 0.093$.

Table 3
Frequency of PIN1 −842 GC genotypes and alleles observed in Alzheimer's disease patients (AD) and in healthy sex- and age-matched controls (HC)

	PIN1 genotypes			PIN1 alleles	
	G/G	G/C	C/C	G	C
AD	78 (70%)	29 (26%)	4 (4%)	185 (83%)	37 (17%)
HC	64 (88%)	9 (12%)	0 (0%)	137 (94%)	9 (6%)

Genotype: $\chi^2 = 8.418$, d.f. = 2, $p = 0.015$; allele: $\chi^2 = 7.948$, d.f. = 1, $p = 0.005$.

Table 4
Frequency of PIN1 haplotypes observed in Alzheimer's disease patients (AD) and in healthy sex- and age-matched controls (HC)

	PIN1 haplotypes		
	CC	GC	GT
AD	36 (16%)	48 (22%)	138 (62%)
HC	9 (6%)	33 (23%)	104 (71%)

$\chi^2 = 8.42$, d.f. = 2, $p = 0.0148$.

AD subjects show a significantly higher frequency of the −842C allele (17% versus 6%; $p = 0.005$) which skews the genotype distribution in AD compared to HC, with a significant decrease of −842GG genotype (70% versus 88%; $p = 0.015$) (Table 3). The presence of −842C allele significantly raised the risk of developing AD (OR 3.044, CI 1.42–6.52).

The −842 and −667 SNPs are in linkage disequilibrium and combine to form haplotypes that show frequencies reported in Table 4; the two cohorts studied were characterized by three putative haplotypes and among these the CC is more frequent in AD than HC (16% versus 6%; $p = 0.015$). The CC haplotype also conferred a higher risk of developing the pathology (OR 2.95, confidence interval 1.31–6.82).

Genotyping of AD patients revealed the presence of Apolipoprotein E4 allele in 42% of cases investigated. Within the cohort of AD patients, we analyzed the frequencies and distribution of the C allele in subjects with or without the Apo E4 allele. No significant difference was found between AD patients (Table 5).

We then evaluated the distribution of the −842C allele by comparing all AD patients (stratified for the presence/absence of the E4 allele) to healthy controls. Our results show that the presence of the C allele conferred a higher risk of developing the pathology in patients carrying or not the E4 allele (OR 2.639, confidence interval 0.52–13.29 and OR 3.606, confidence interval 1.4–9.29, respectively).

Table 5
Relationship of Apolipoprotein E4 carrier status to −842C allele observed in Alzheimer's disease patients (AD)

		Allele C carriers	Allele C non-carriers
AD	Apo E4+	10 (22%)	36 (78%)
	Apo E4−	23 (36%)	41 (64%)

$\chi^2 = 1.94$, d.f. = 1, $p = 0.16$.

3.2. Protein expression analysis

Twenty-five subjects (AD patients and healthy controls) representing all the PIN1 promoter genotypes and putative haplotypes were selected for the protein expression analysis. The presence of PIN1 proteins in PBMC was tested by immunoblotting.

Fig. 1 shows results obtained for protein expression rates. PIN1 concentration varied significantly with −842 genotype ($p = 0.008$) in an apparent allele dose-effect manner, being lower in CC subjects than in individuals with other genotypes ($p < 0.05$ versus GG and GC) (Fig. 1). The same is not so evident for the −667 genotype.

It must be noted that the PIN1 levels showed a significant variability in the various groups of haplotypes ($p < 0.05$): the CC haplotype was associated with the lowest PIN1 protein concentration (Fig. 1).

3.3. Clinical evaluation

AD subjects carrying the −842C SNP show a lower mean age at onset (74.7 ± 9.05 versus 77 ± 6.24; $p = 0.140$) if compared to individuals not carrying this SNP. Also MMSE is lower in −842C carriers (18.5 ± 6.8 versus 20.6 ± 4.56; $p = 0.131$) if compared to individuals not carrying the SNP. Individuals carrying the CC haplotype also have an earlier onset of AD if compared to GT and GC carriers (74.6 ± 8.60, 76.2 ± 7.12 and 77.7 ± 5.80, respectively; $p = 0.172$). Even if these data are not statistically significant, they anyhow indicate a trend towards a worse clinical prognosis in AD subjects carrying the −842C SNP and the CC haplotype. Interestingly, by analysing data according to gender, the statistical significance was obtained in women for the MMSE (16.94 versus 20.19, respectively, in C and non-C carriers; $p = 0.032$). It must be noted that the distribution of C carriers does not differ between male and female AD subjects (data not shown).

4. Discussion

Hyperphosphorylation of Tau is involved in the pathogenesis of many neurodegenerative diseases; more precisely, in Alzheimer's disease, the neuronal cytoskeleton is progressively disrupted and replaced by tangles of paired helical filaments which are composed mainly of hyperphosphorylated forms of Tau [1]. The PIN1 protein, that specifically regulates the conformational changes following phosphorylation of several proteins, targets phosphorylated Tau on the Thr231-Pro motif and directly restores its biological function after Tau is inactivated by hyperphosphorylation; interestingly, Tau is a major substrate for PIN1 in neurons [11].

More significantly, PIN1 is the first gene whose knockout in mice causes progressive age-dependent neuropathies characterized by motor and behavioural deficits, Tau hyperphosphorylation, Tau filament formation and neuronal degeneration [9].

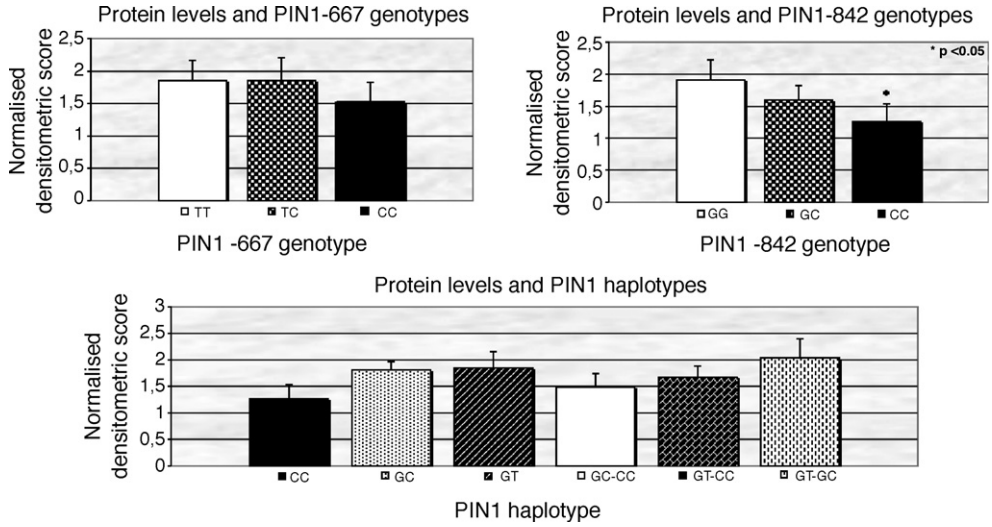

Fig. 1. PIN1 protein levels in individuals grouped by different PIN1 SNPs genotypes and haplotypes. $^{*}p < 0.05$ vs. GG and GC.

Increasing evidences are emerging that AD might also be related to an aberrant reactivation of the cell cycle and apoptosis in neurons: indeed, mitotic events are aberrantly activated in the brains of AD patients, including the re-expression of cdc2 kinase that is able to phosporylate many proteins that are known to be the PIN1 substrate [19,4,23,2].

It is noteworthy that PIN1 can also facilitate dephosphosphorylation of many mpm-2 antigens (which are mitosis-specific phosphoproteins capable of regulating the cell cycle) that are known to be hyperphosphorylated in AD [22].

Soluble PIN1 levels are reduced in AD brains [11] and PIN1 depletion induces mitotic block and apoptosis in cancer cells.

In normal human brains, PIN1 is present in the neuronal cytoplasm and nucleus [10,11,6]. In the hippocampus, its expression is relatively higher in CA4, CA3, CA2 and presubiculum, and lower in CA1 and subiculum; in the parietal cortex, the expression is relatively higher in layer IIIb-c neurons and lower in layer V neurons [9]. The subregions with low expressions of PIN1 coincide with the subregions that are more susceptible to neurofibrillary degeneration in AD brains, whereas those containing high PIN1 expressions are not, showing an inverse correlation between PIN1 expression and predicted vulnerability [9].

Altogether, these observations show that PIN1 may play a role in AD acting at different levels; its reasonable to believe that a well-expressed and fully functioning protein can prevent or slow down AD onset, whereas PIN1 dysfunction or under-expression could accelerate tangle formation or neurodegeneration.

In our study, we identified SNPs in the promoter region of the PIN1 gene that showed a different distribution in AD patients and healthy controls. Our results evidenced a significantly higher percentage of −842C allele carriers in AD subjects. We found that this allele significantly raised the risk of developing AD.

The −842 and −667 SNPs are in linkage disequilibrium and combine to form haplotypes: the CC haplotype conferred a higher risk of developing AD.

Based on the genetic data we have and given the fact that polymorphisms in the promoter region of genes frequently affect gene transcription and expression levels, we can hypothesize that inheritance of the −842C allele (and the CC haplotype) might alter PIN1 production levels. This fact might increase the risk of developing AD and favour an earlier onset of clinical symptoms if we take into consideration the proposed role of PIN1 in neurodegeneration.

This hypothesis is supported by our findings concerning PIN1 protein expression analyses in subjects representative of all PIN1 promoter genotypes and haplotypes: in our study individuals carrying the −842CC genotype and CC haplotype showed reduced levels of PIN1 proteins in PBMC. For the −842 polymorphism, an apparent allele dose-effect seems to be present where PIN1 protein levels correlate to C allele presence.

Indeed, −842C AD carriers showed a 2-year difference in mean age at onset of AD clinical features and a lower MMSE at baseline (particularly AD women).

It must be noted that a significant correlation between cerebro-spinal fluid phosphorylated Tau231 levels at baseline and the annual point loss in MMSE score was found in mild cognitive impairment subjects [3]; moreover, in agreement with the analysis of rates of cognitive decline, increased levels of phosphorylated Tau231 were correlated with conversion to AD [3].

The significative loss in MMSE underlined in women – but not in men – may be due to the different number of subjects recruited in the two groups. However, a stimulating explanation may also come from the study of centenarians, who are clearly less prone than younger people to age-related diseases, describing gender differences in the impact of genetic factors on human longevity [8].

As a conclusion, we could say that in neurons, PIN1 might be normally needed to control the function of phosphoproteins when they become phosphorylated; however, during the development of neurodegeneration this balance might be disrupted [14,9,12,13].

The results of our study indicate that polymorphisms in the PIN1 gene – which influence the protein expression – may be involved in the pathogenic mechanisms of neurodegeneration and predispose to AD.

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