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Clinical short communication

Gene promoter methylation and expression of Pin1 differ between patients with frontotemporal dementia and Alzheimer's disease

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ABSTRACT

Frontotemporal Dementia (FTD) and Alzheimer's Disease (AD) share the accumulation of fibrillar aggregates of misfolded proteins. To better understand these neurodegenerative diseases and identify biomarkers in easily accessible cells, we investigated DNA methylation at Pin1 gene promoter and its expression in peripheral blood mononuclear cells of FTD patients. We found a lower gene expression of Pin1 with a higher DNA methylation in three CpG sites at Pin1 gene promoter analysed in FTD subjects, in contrast to a higher gene expression with a lower methylation in AD subjects and controls. These data suggest an important and distinct involvement of Pin1 in these two types of dementia.

1. Introduction

Alzheimer's Disease (AD) and Frontotemporal Dementia (FTD) are two of the most common neurodegenerative disorders and the leading causes of neurodegenerative dementia [1–3]. These diseases share the accumulation of fibrillar aggregates of proteins that results from protein misfolding [4].

Much alike the late-onset AD is identified by an accumulation of extracellular amyloid beta (Aβ) plaques derived from amyloid precursor protein (APP), and by intracellular tangles comprised of hyperphosphorylated tau protein [5, 6].

The hallmark of FTD is the selective atrophy of the frontal and temporal cortex, with neuronal loss and gliosis of the superficial layers [7]. The pathologic substrate in FTD is an abnormal tau deposition or aggregation in neurons and glia [8].

Pin1 is a ubiquitously expressed protein, belonging to the peptidyl-prolyl isomerase family, and that specifically recognizes phosphorylated Pro-directed Ser/Thr peptide sequences [9–12]. Pin1 regulates the conformation of proteins after their phosphorylation to further control their function [11–15]. Pin1 plays a crucial role in multiple cellular processes and is implicated in pathogenesis of several diseases, including neurodegenerative disorders [16–22]. Indeed, Pin1 regulates APP metabolism and facilitates tau dephosphorylation, thus making a subset of proteins accessible to kinases and phosphatases [23]. Therefore a potential neuroprotective function of Pin1 in neurodegenerative diseases has been suggested [17, 18, 20, 24, 25].

Our group has recently observed a significant increase in Pin1 gene expression along with a decreased promoter methylation in peripheral blood mononuclear cells (PBMCs) of patients with late-onset AD, compared to controls (CT) [26]. Thus, alterations in easily accessible PBMCs may be valuable biomarkers in the early diagnosis of AD, and potentially of other tauopathies [27].

In this preliminary study, we focused on gene and protein expressions of Pin1 in PBMCs of subjects with FTD, as well as of a larger sample of patients with AD and CT compared to our previous study [26]. We decided to investigate DNA methylation of Pin1 gene promoter in FTD

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because epigenetic modifications may explain the altered APP metabolism and tau phosphorylation in neurodegenerative diseases [28].

2. Materials and methods

The study involved 34 FTD (mean age ± standard error: 73.5 ± 1.1 years), 176 late-onset AD patients (79.2 ± 0.5 years) and 107 non-demented gender-matched CT (79.7 ± 0.6 years) (Table 1). Subjects were recruited from outpatients attending Geriatric and Neurological Units of the Fondazione IRCCS Ca' Granda of Milan, Italy. All participants gave their informed consent to the study previously approved by the local ethics committee. The diagnosis of FTD was made according to current criteria [29] and subsequent revision [30]. AD fulfilled the NINCDS-ADRDA criteria [31, 32].

Workup for all patients included: past medical history, general and neurological examination (Katz and Lawton Index - ADL and IADL, Tinetti scale, Hand Grip Strength test, Mini Nutritional Assessment), routine blood tests (vitamin B12, folate, 25-OH vitamin D, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, omocystein, antibodies *Treponema pallidum*), neurocognitive assessment (Mini Mental State Examination - MMSE, Montreal Cognitive Assessment, Frontal Assessment Battery, Trail Making Test, Verbal Fluency Test, Clock Drawing Test, Digit Span Forward and Backward tests, verbal learning tests, Token tests, Corsi's Spatial Test, Boston Naming Test, Rey's Figure Copy and Delayed Recall, Raven Coloured Progressive Matrices), depression evaluation (Geriatric Depression Scale), analysis of Aβ, tau and hyperphosphorylated tau levels in the cerebrospinal fluid, brain Computed Tomography scan or Magnetic Resonance Imaging and 18f-Fludeoxyglucose-Positron Emission Tomography. The presence of vascular brain damage was excluded (Hachinski Ischemic Score < 4).

PBMCs were isolated by density gradient (Lympholyte-H, Cedarlane, Canada). Genomic DNA was extracted from PBMCs using a salting-out method [33] and apolipoprotein E (ApoE) genotypes were determined as previously described [34]. Methylation status of Pin1 promoter region was determined using pyrosequencing of bisulfite converted DNA. Pyrosequencing assays used, including primer sequences and Qiagen (Hilden, Germany) assay names, are available upon request. Briefly, 0.5 μg of DNA from each sample was bisulfite treated (Zymo Research, Orange, CA, USA) and amplified by PyroMark PCR Kit (Qiagen) according to the manufacturer's protocol. Pyrosequencing methylation analysis was conducted using the PyroMark Q24 (Qiagen). Methylation was analysed using PyroMark Q24 Software (Qiagen), which calculates the methylation percentage (mC/(mC + C)) for each CpG site, allowing quantitative comparisons (mC is methylated cytosine, C is unmethylated cytosine) (Supplementary Fig. 1).

Total RNA was extracted from 15 × 10^6 frozen PBMCs using Chomczynski and Sacchi's modified method. Two micrograms of total RNA was reverse-transcribed using the M-MLV Reverse-Transcriptase System and oligo (dT) (Clontech, Italy).

The relative abundance of Pin1 mRNA was assessed by means of Real-Time PCR in a final volume of 50 μl using 25 μl of 2× iQ SYBR Green Supermix (dNTPs, iTaq and MgCl2; Bio–Rad, Italy) and 300 nM of each primer (Pin1: forward 5′-AGATCACCCGGACCAAGGA-3′, reverse 5′-GCTGAACTGTGAGGCCAGAGA-3′; GAPDH: forward 5′-ATTCCACCCA TGGCAAATTC-3′, reverse 5′-TGGGATTTCCATTGATGACAAG-3′). Quantitative relativePCR (qPCR) was performed using a Chromo 4 instrument and analysed using Opticon Monitor 2 (Celbio, Italy). All of the reactions were performed in triplicate, with thermal cycling conditions of 10 min at 95 °C followed by 40 cycles at 95 °C for 15 s, 56 °C for 40 s and 72 °C for 30 s, with a ramp of 5 °C/s. qPCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed simultaneously. The quantification of Pin1 mRNA was carried out by using the comparative cycle threshold (Ct) method and the formula: normalization ratio (NR) = $2^{-\Delta\Delta Ct}$. The ΔCt value of each sample was calculated as the Ct of the target gene minus the Ct of GAPDH, and the ΔΔCt value was obtained as the difference between the ΔCt of the sample and the ΔCt of the calibrator. According to this formula, the normalization ratio of the calibrator in each run is 1. The calibrator in each sample run was the same RNA pool of healthy controls [26].

For protein extraction, 10 × 10^6 frozen PBMCs were lysed in Triton X-114 Tris buffer with a protease inhibitor cocktail (Sigma, Italy). Two μg of total protein extract were used to evaluate Pin1 expression by ELISA (Pin1 EIA kit, Assay Designs, Enzo Life Sciences, USA).

Statistical analysis was performed using the SPSS program (SPSS version 22, Chicago, IL). Data were expressed as mean values ± standard error and/or percentage of distribution. DNA methylation, mRNA and protein levels and continuous variables were compared by using the one-way analysis of variance (ANOVA), with Student's *t*-test applied to paired comparisons. Categorical data were compared by using the chi-square test. Partial correlation analysis adjusted for age and gender were performed to exclude the effect of these parameters on the results. A *p*-value <0.05 was considered significant. The predictive efficacy of Pin1 was assessed using the area under the curve (AUC) generated by a receiver operating characteristic (ROC) analysis.

3. Results

FTD patients (mean age ± standard error: 73.5 ± 1.1 years) were significantly younger than AD (79.2 ± 0.5 years) and CT subjects (79.7 ± 0.6 years) ($p < 0.001$) (Table 1). Moreover, there was a significantly different female distribution among the groups analysed: 44.1% in FTD, 73.3% in AD and 61.7% in CT subjects ($p = 0.002$) (Table 1). Despite these evidences, partial correlation analysis adjusted for age and gender showed that these differences had no effect on our results.

We found a different distribution of ApoE ε4 carriers among the three groups: 33.3% in FTD, 48.8% in AD and 19.6% in CT subjects ($p < 0.001$) with a difference in AD *versus* CT ($p < 0.001$) (Table 1). The frequency of ApoE ε4 was in line with previously published data [35, 36]. All data were similar in both ε4 carriers and non-carriers, indicating that this genetic risk factor for AD [35], but not for FTD [36], does not seem to participate in the modulation of Pin1 (data not shown).

DNA methylation of five CpG sites at Pin1 gene promoter was carried out and showed in Table 2. We found that DNA of FTD patients was significantly more methylated in CpG sites 2, 4 and 5 at Pin1 gene

Table 1
Participants' characteristics. Age and Mini Mental State Examination score are expressed as mean values ± standard error.

	FTD	AD	CT	*p*-Value
No. of participants	34	176	107	
Age	73.5 ± 1.1[a]	79.2 ± 0.5	79.7 ± 0.6	<0.001
Female (%)	15 (44.1%)	129 (73.3%)	66 (61.7%)	0.002
MMSE score	20.9 ± 1.2	18.0 ± 1.2	28.9 ± 0.3[b]	<0.001
Apo ε4 (0/1/2)	12/6/0	66/52/11[c]	86/18/3	<0.001

p-Values for continuous variables are from one-way analysis of variance (ANOVA). *p*-Values for categorical data are from chi-square test.
MMSE: Mini Mental State Examination.
Student's *t*-test: [a]$p < 0.001$ *versus* AD and CT; [b]$p < 0.001$ *versus* FTD and AD; [c]$p < 0.001$ *versus* CT.

Table 2
Methylation percentages of the five DNA sites considered at Pin1 gene promoter in FTD, AD and CT subjects.

	% methylation	% methylation	% methylation	% methylation	% methylation
	Site 1	Site 2	Site 3	Site 4	Site 5
FTD	6.37 ± 0.44	0.94 ± 0.12[a]	2.86 ± 0.47	1.57 ± 0.17[b]	1.65 ± 0.23[c]
AD	7.00 ± 0.30	0.65 ± 0.04	1.91 ± 0.11	1.03 ± 0.06	1.00 ± 0.04
CT	7.12 ± 0.19	0.61 ± 0.04	2.00 ± 0.20	1.00 ± 0.07	1.08 ± 0.08
ANOVA	NS	$p = 0.006$	NS	$p = 0.001$	$p = 0.004$

Methylation data are expressed as mean values ± standard error. NS, not significant.
Student's *t*-test: [a]$p = 0.030$ *versus* AD, $p = 0.014$ *versus* CT; [b]$p = 0.006$ *versus* AD, $p = 0.004$ *versus* CT; [c]$p = 0.009$ *versus* AD, $p = 0.024$ *versus* CT.