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Clinical short communication

Transcriptional and epigenetic phenomena in peripheral blood cells of monozygotic twins discordant for alzheimer's disease, a case report

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ABSTRACT

Target genes in Alzheimer's disease (AD) have been identified. In monozygotic twins discordant for AD we analysed the expression of selected genes, and their possible regulation by epigenetic mechanisms in peripheral blood mononuclear cells, possibly useful to discover biomarkers. Amyloid precursor protein, sirtuin 1 and peptidyl prolyl isomerase 1 gene expressions were highly up-regulated in the AD twin *versus* the healthy one. Consistently with sirtuin 1 role in controlling acetylation status, we observed a substantial reduction of the acetylation on histone 3 lysine 9, associated with gene transcription in the AD twin. Noteworthy in the AD twin we also observed an increased gene expression in two histone deacetylases (HDACs) isoforms: HDAC2 and HDAC9. A general DNA hypomethylation of all gene promoters studied was also observed in both twins. Our results unravel transcriptional and epigenetic differences potentially helpful to better understand environmental factors and phenotypic differences in monozygotic twins.

1. Introduction

In the last few years many research efforts have been made to associate specific genes to Alzheimer's disease (AD), the most common form of progressive dementia in older people [1].

The role of gene variants has been better characterized in the early-onset form, but genetic basis of late-onset AD, accounting for the majority of cases, is more complex [2]. Evidence now suggest the importance of susceptibility genes interaction with environmental factors, and thus through epigenetic mechanisms, for disease development [3].

The study of monozygotic (MZ) identical twins discordant for AD provides a unique opportunity to investigate thoroughly the environmental factors that may influence the association between specific genes and AD [4]. We here investigated key genes already known as relevant in AD such as: apolipoprotein E [APOE], β-amyloid precursor protein [APP], presenilin 1 [PSEN1], peptidyl prolyl isomerase 1 [PIN1], sirtuin 1 [SIRT1].

APOE is the most important genetic risk factor associated with AD [5,6]. Aβ plaques formation, the major neuropathological hallmarks of AD, are synthesized from the precursor protein APP [7] and mutations of the APP gene were the first to be recognized to cause AD [8]. Mutations have been also identified in PSEN1 gene, encoding a component of gamma-secretase, responsible for proteolytic cleavage of APP [9]. PIN1 regulates APP biological function [10] and gene polymorphism has been associated with increased AD risk [11]. Finally SIRT1, part of sirtuins, NAD+−dependent histone deacetylases, extensively studied

Abbreviations: AD, Alzheimer's Disease; HADACs, histone deacetylases; APP, amyloid precursor protein; SIRT1, sirtuin 1; APOE, apolipoprotein E; PSEN1, presenilin 1; PIN1, peptidyl prolyl isomerase 1; PBMCs, peripheral blood mononuclear cells; DNMTs, DNA methyltransferases; Aβ, Amyloid-β; Ptau, Phosphorylated tau; CSF, cerebrospinal fluid; MCI, Mild Cognitive Impairment; H3K27me3, Histone 3 lysine 27 trimethylated; H3K4me3, Histone 3 lysine 4 trimethylated; H3K9Ac, Histone 3 lysine 9 acetylated.

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in AD and which overexpression shows protective effects against disease development [12].

Therefore we analysed the epigenetic regulation of these genes in the two twins looking for possible differences in DNA methylation of the promoters, global histone modifications in specific positions of the histone 3 tail and gene expression levels of histone deacetylases (HDACs) and DNA methyltransferase (DNMTs), which are the key enzymes responsible for histone acetylation and DNA methylation, respectively.

The detailed twin history was collected from their relatives to correlate any epigenetic alteration to any environmental event possibly influencing disease development.

2. Material and methods

2.1. Subjects

Two female monozygotic twins were enrolled in the study. The affected twin was visited in the Geriatric Unit of Fondazione IRCCS Ospedale Maggiore Policlinico, when she was 70 years old. The unaffected twin was instead evaluated when she was 71 years old. Both twins underwent a complex longitudinal follow up with neurological, neuropsychological, behavioural, functional and instrumental evaluation. In particular, cognitive screening with the Mini Mental State Examination test was included in the standard neurological examination [13]. The neuropsychological assessment included a test battery (i.e., the Bell's test, digit cancellation test, prose memory, Rey-Osterrieth complex figure, trail making test, digit span forwards and backwards, letter fluency, Raven's coloured progressive matrices, De Renzi's test of apraxia, copy of geometrical figures) aimed at evaluating cognitive functioning across different domains, like attention, psychomotor speed, prefrontal functions, memory, language, praxis, and visuospatial functions [14–18]. Affective state was assessed by means of the Neuropsychiatric Inventory (NPI), and functional status by means of the Activities of Daily Living (ADL) and Instrumental ADL (IADL) scales [19–21]. Both twins performed brain CT-scan and/or magnetic resonance (MR). In addition, the affected twin performed longitudinal brain [^{18}F]-fluorodeoxyglucose-positron emission tomography (FDG-PET). Lumbar puncture was carried out, and the levels of Amyloid-β (Aβ), tau and Phosphorylated tau (Ptau) in the cerebrospinal fluid (CSF) were evaluated by ELISA (Innogenetics, Ghent, Belgium) [22]. A complete blood test (including test for *Treponema pallidum*, dosage of TSH and thyroid hormones, as well as of B12 vitamin and folate) was carried out [22]. Finally, a genetic test for the epsilon 4 allele of apolipoprotein E was performed. Diagnoses of Mild Cognitive Impairment and Alzheimer's dementia were posed according to current diagnostic criteria [23,24]. Both twins and their caregivers gave their informed consent to the study, which had been previously approved by the local ethic committee.

2.2. Real Time RT-PCR

PBMCs were separated by density gradient (Lympholyte-H, Cedarlane, Canada), total RNA was isolated as reported [25], and was reverse-transcribed using the M-MLV Reverse-Transcriptase System and oligo (dT) (Clontech, Italy).

Relative abundance of each mRNA species was assessed by real-time RT-PCR using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) on an DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research, Waltham, MA, USA) and was calculated by the Delta-Delta Ct (ΔΔCt) method, using as internal controls GAPDH, β-actin and 18S genes.

The primers used for PCR amplification of target genes, designed using the Primer 3 software, are reported in Table 1.

[APP], sirtuin 1 [SIRT1], apolipoprotein E [APOE], presenilin 1 [PSEN1], peptidyl prolyl isomerase 1 [PIN1].

2.3. Methylation Specific Primer Real Time PCR

Genomic DNA was extracted using a salting-out method [26]. Analyses of DNA methylation at gene promoters paralleled methods already described in detail elsewhere [27]. Firstly, DNA underwent bisulfite modification to convert unmethylated cytosine residues into uracil, using the CpGenome DNA Modification Kit (Chemicon International, Purchase, NY) according to the manufacturer's instructions. Secondly, methylation analysis was performed by fluorescence-based real-time PCR using MS Opticon 2 Light Cycler Instrument (Roche, Germany). Amplified sequences contained at least five CpG sites and were located within the promoter region of the genes. PCR was also performed for non-CpG-containing regions of myoD that served as control gene. Bisulfite-modified CpGenome™ universal unmethylated DNA (Chemicon International, Temecula CA, USA) was used as negative control. Moreover, the amplification reaction was also performed using unconverted genomic DNA as negative control. The percentage of methylation was calculated by the 2^(−ΔΔCt) method, where ΔΔCt = (Ct,Target-Ct,myoD) sample - (Ct,Target-Ct,myoD) fully methylated DNA, multiplying by 100 [28]. For relative quantification, standard curves were generated separately for each gene and myoD from serial dilutions of bisulfite modified CpGenome™ universal methylated DNA (Chemicon International, Temecula CA, USA). The primers designed using MethPrimer software [29] are reported in Table 1.

2.4. Western Blotting

For protein extraction, PBMCs were lysed in Triton X-114/Tris buffer in the presence of a protease inhibitor cocktail (Sigma, Italy). Extracted proteins (20 μg) were separated by 15% SDS-polyacrilamide-resolving gel under reducing conditions and transferred to 0.45 μm nitrocellulose membranes (Bio-Rad, Italy). Bands were immunovisualized with

Table 1
Primer sequences used for reverse transcription–PCR and Methylation Specific PCR. M_APOE, M_APP, M_PSEN1, M_SIRT1, MYOD are bisulfite-converted sequences.

Human gene	*Forward*	*Reverse*	*Position with respect to TSS*	*RefSeq*
APOE	GGTCGCTTTTGGGATTACCT	TCCAGTTCCGATTTGTAGGC	from + 255 to + 401 [exons 3–4]	NM_000041
M_APOE	TGTGTTTGGTTTGTTTTGAGTATTT	AATAACCTTAAAACCCTACCCTCAA	from −2040 to −1798	
APP	CATCCCCACTTTGTGATTCC	GTTTCGCAAACATCCATCCT	from + 489 to + 605 [exons 3–4]	NM_001204302
M_APP	CGTTTGTTTTATTTTTTTAAATCGA	ACGACCCACCTAAACTTCGTA	from −441 to −288	
PSEN1	TTGCGGTCCTTAGACAGCTT	AGGACAACGGTGCAGGTAAC	from 172 to 301 [exons 2–3]	NM_000021
M_PSEN1	GTTTGTATGTCGGGAGAAGTATAC	GACCTAAAAAAACGATTACGAA	from −255 to −83	
PIN1	AGATCACCCGGACCAAGGA	GCTGAACTGTGAGGCCAGAGA	from + 251 to + 355 [exons 2–3]	NM_006221
M_PIN1	GTCGTTTCGGATTATTTAGGAGTC	TAACTAAACGCGCTCTACAACG	from − 256 to − 54	
SIRT1	GCGATTGGGTACCGAGATAA	GTTCGAGGATCTGTGCCAAT	from + 468 to + 653 [exons 1–3]	NM_012238
M_SIRT1	CGGATTAAAATTTGAGTTGTTTC	CCTTCCTCTTTATAACGAACGTA	from − 324 to − 206	
GAPDH	GATTCCACCCATGGCAAATTC	TGGGATTTCCATTGATGACAAG	from + 346 to + 418 [exon 5]	NM_002046
B-ACTIN	TTCTACAATGAGCTGCGTG	AGAGGCGTACAGGGATAGCA	from + 268 to + 435 [exon 3–4]	NM_001101
rRNA 18S	CGCCGCTAGAGGTGAAATTCT	CGAACCTCCGACTTTCGTTCT	non coding exon 1 [101 bp]	NC_000011.10
MYOD1	TGATTAATTTAGATTGGGTTTAGAGAAGGA	CCAACTCCAAATCCCCTCTCTAT	gene region lacking any CpG sites	NC_000011.10