Decreased mitochondrial DNA methylation in late-onset Alzheimer's disease

Short communication

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Running title: Mitochondrial DNA methylation and Alzheimer's Disease

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Abstract

Mitochondrial impairment is a feature of neurodegeneration and many investigators have suggested that epigenetic modifications of the mitochondrial DNA (mtDNA) might be involved in late-onset Alzheimer's disease (LOAD), but evidence in humans is limited. We assessed the methylation levels of the mtDNA *D-loop* region in blood DNA from 133 LOAD patients and 130 controls, and observed a significant reduction of DNA methylation levels in the group of affected individuals (2.3 vs. 3.1%, $P = 0.04$). Overall, present data indicate that the *D-loop* region is hypomethylated in peripheral blood DNA of LOAD patients, suggesting that mtDNA epimutations deserve further investigations in AD pathogenesis.

Keywords: Alzheimer's disease, epigenetics, DNA methylation, mitochondrial DNA, D-loop region, mtDNA

Introduction

Late onset Alzheimer's disease (LOAD) is a complex disorder resulting from the interaction between genetic and non-genetic factors [1]. Epigenetic mechanisms are able to change gene expression under the influence of environmental factors such as diet, hazardous exposures, and life events and it has been proposed that a deregulation of these mechanisms could contribute to LOAD onset. In fact in recent years a growing number of studies performed *in vitro*, *in vivo* and *ex vivo* in human tissues showed that aberrant DNA methylation, histone modifications and deregulated microRNA (miRNA) expression play a pivotal role in AD pathogenesis [2].

Particularly, DNA methylation represents one of the most studied epigenetic modifications in AD [3]. In mammals this reaction is carried out by DNA methyltransferases (DNMTs) and occurs mainly at CpG site located throughout the genome. When this reaction occurs at the level of gene promoters usually induces repression of gene expression, while methylation of non-CpG islands is associated to the prevention of genomic instability phenomena, such as the movement of transposable elements [4]. DNA methylation changes at both gene-specific level and at global level have been detected in AD [3]. For example, the analysis of post-mortem brain regions of monozygotic twins discordant for AD revealed reduced global levels of DNA methylation in the temporal neocortex of the AD twin [5], and increased methylation of the repetitive element LINE-1, often used as surrogate of global DNA methylation, has been found in blood DNA of AD patients compared to healthy controls [6].

Although mitochondrial DNA (mtDNA) impairment is a feature of AD little attention was given to the mitochondrial epigenome itself, and until now only one study was aimed at searching for the role of methylation modifications in mtDNA of AD patients [7]. Blanch and coworkers investigated methylation levels of the mitochondrial displacement loop (*D-loop*) region and of *MT-ND1* and *MT-ND6* genes (mitochondrial NADH dehydrogenase subunit 1 and 6) and found differential methylation levels, mainly in the *D-loop* region in the entorhinal cortex of AD patients respect to

healthy control individuals, suggesting that the *D-loop* region could be a sensitive epigenetic target in AD pathology.

In the present study we assessed DNA methylation levels of the mtDNA *D-loop* region in blood DNA of a large cohort of LOAD patients and healthy matched controls in order to search for differences associated with the disease status detectable in an easily available tissue.

Materials and methods

Study population

A total of 133 LOAD patients and 130 matched controls were collected at the Department of Neuroscience, University of Pisa (Table 1). Diagnosis of probable AD was performed according to DSM-IV [8] and NINCDS-ADRDA criteria [9]. Based on age at onset above 65 years and absence of a family history of dementia, all the subjects were assumed to be sporadic LOAD cases. As normal controls we recruited healthy volunteer subjects matched to LOAD patients for age and gender, as well as for ethnicity and geographic origin (both LOAD and control individuals were Italian Caucasians, resident in northern Tuscany, and were recruited simultaneously). Cognitive functions and family history of dementia were ascertained in controls, including only healthy subjects with no presence of cognitive impairment and of relatives who developed AD or other dementias. The study was performed in accordance with the declaration of Helsinki and received the approval of the ethics committee of the Pisa University Hospital. A written informed consent was obtained from all participants before enrollment in the study.

Extraction of genomic DNA and bisulfite modification

An aliquot of blood was collected from each subject in EDTA tubes and stored at -20°C until assayed. Genomic DNA extraction was performed using the QIAmp DNA blood Mini Kit (Qiagen, Milan, Italy, Catalog N° 51106) following the manufacturer's protocol. The extracted DNA was quantified using a Nano Drop ND 200c spectrophotometer (NanoDrop Thermo scientific). 200 ng of DNA from each sample were treated with sodium bisulfite in order to convert all unmethylated cytosines into uracil. The EpiTect Bisulfite Kit (Qiagen, Milan, Italy, Catalog N° 59104) was used for this purpose, following the manufacturer's instructions.

Methylation sensitive-high resolution melting (MS-HRM) analysis.

Methylation of the *D-loop* region was assessed by means of methylation sensitive-high resolution melting (MS-HRM) technique using a CFX96 Real-Time PCR detection system (Bio-Rad). The D-loop region (GenBank: J01415.2) was analyzed for the presence of CpG islands by CpG plot software (http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/). We developed in-house MS-HRM protocols according to literature criteria [10], using methylation independent primers (MIP) designed by us by means of the software MethPrimer [11]. The sequences of the primers used were as follows: forward 5'-GGAGTTTTTTATGTATTTGGTATTTT3', reverse 5'-ACAAACATTCAATTATTATTATTATATCCT-3'. These primers amplified a *D-loop* amplicon of 222 bp which included 10 CpG sites. The MS-HRM analyses were performed as follows: 1 cycle of 95°C for 12 min, 50 cycles of 95 °C for 30 s, 56 °C for 45 s and 72 °C for 30 s, followed by an HRM step of 95 °C for 10 s and 50 °C for 1 min, 65 °C for 15 s, and continuous acquisition to 95 °C at one acquisition per 0.2 °C. PCR was performed in a final volume of 10 µl, containing 5 µl of master mix (Qiagen, Milan, Italy, Catalog N° 59445), 10 pmol of each primer and 10 ng of bisulfite modified DNA template. Each reaction was performed in duplicate. Fully methylated and unmethylated DNA (EpiTect methylated and unmethylated humancontrol DNA, bisulfite converted,

Qiagen, Milan, Italy, Catalog N◦59695) was mixed to obtain the following ratios of methylation: 0%, 12.5%, 25%, 50%, 75%, 100%. Standard DNA samples with known methylation ratios were included in each assay in order to generate standard curves to be used for the deduction of the methylation ratio of each sample (Fig. 1). In order to obtain single methylation percentage values from MS-HRM assays, rather than a range, we applied an interpolation method developed and described in our laboratory [12].

Statistical analyses

Demographic data, such as age at sampling and gender, were compared between groups by means of Student's t Test and Fisher Exact Test, respectively. Differences in *D-loop* methylation levels between AD patients and controls were compared by one-way multifactorial ANOVA correcting for age at sampling and gender. Statistical analyses were performed with STATGRAPHICS 5.1 plus software package for Windows. The statistical power of the study was calculated with the free sample size calculator (http://clincalc.com/Stats/SampleSize.aspx). We designed a study with an *a priori* power (>80%) to detect DNA methylation differences of 1% or higher between groups.

Results

D-loop MS-HRM analysis showed the existence of an inter-individual variability with methylation levels ranging from 0 to 9% (Figure1). *D-loop* methylation levels were lower in AD patients (2.3 ± 0.2) respect to control subjects (3.1 ± 0.2) in a statistically significant manner ($P = 0.042$) (Table 2). No significant correlation between *D-loop* methylation levels and age was observed ($r = -0.09$, $P = 0.14$), and no differences in methylation levels between males and females were detected ($P = 0.55$).

Discussion

In the current study we investigated the methylation levels of the mitochondrial *D-loop* region in blood DNA of relatively large cohort of LOAD patients and healthy matched controls. *D-loop* is a non-coding region of the mtDNA of about 1.1 kb, critical for both mitochondrial replication and transcription [13]. We observed significant lower *D-loop* methylation levels in AD patients respect to healthy controls (P= 0.042). Until now only one study investigated mtDNA methylation changes in AD pathology [7]. Blanch and coworkers found increased methylation levels of *D-loop* in the entorhinal cortex of 8 AD patients respect to controls. Moreover they observed a dynamic pattern of methylation of this region in transgenic AD mice (*APP/PS1* mice) along with disease progression. The authors also found decreased methylation levels of *D-loop* in the *substantia nigra* of 10 Parkinson's disease patients with respect to healthy matched controls [7], suggesting that hypomethylation or hypermethylation of this region might characterize different tissues of individuals suffering from neurodegeneration. In the present study, which is the first addressing this issue in peripheral tissues, we observed a decreased methylation of the *D-loop* region in the mtDNA extracted from blood DNA of LOAD patients, strengthening previous evidence suggesting that this region could be a sensitive biomarker of epigenetic modifications in neurodegenerative diseases.

Previous studies conducted in colorectal cancer and adjacent tissues have revealed that *D-loop* methylation could regulate the transcription of mtDNA genes, such as *MT-ND2* (a subunit of NADH) [14]. Furthermore, *D-loop* methylation was found to be sensitive to particulate matter <2.5 μm (PM2.5) in blood DNA of exposed people, and in particular the subjects with higher mtDNA methylation levels were more susceptible to the effect of PM2.5 on Heart Rate Variability (HRV) measures [15]. Similarly an increase in *D-loop* methylation was observed in mtDNA extracted from placental of women exposed to PM2.5 during their gestation [16]. Hypermethylation of *D-loop* region was also observed in polycystic ovaries, together with increased expression of *DNMT1*, one of the enzymes involved in DNA methylation reactions [17].

The focus on blood-based epigenetic AD biomarkers has grown exponentially during the past decade [18]. An important advantage in the use of peripheral tissues, of easier availability, in the study of AD is the opportunity to investigate the molecular events associated with the different stages of the disease. Otherwise the study on post-mortem brains only provides a snapshot of the final result of the pathogenetic processes of the disease, which does not necessarily reflect the mechanisms that lead to the disease [19]. Both gene specific and global DNA methylation analyses have been performed in peripheral blood of AD patients however providing inconclusive results regarding the contribution of DNA methylation changes to the pathogenesis detectable in peripheral tissues [6, 18, 20, 21, 22, , 23]. Interestingly it is well known that mitochondrial impairment is a classical feature of AD condition and mitochondrial alterations and changes in mitochondrial gene expression have been detected in peripheral lymphocytes of AD patients [24, 25], as well as in other neurodegenerative diseases [26, 27]. Moreover mitochondrial dysfunction through the over production of reactive oxygen species (ROS) is able to induce epigenetic modifications [28, 29]. So the analysis of epigenetic modifications in mtDNA, that is more susceptible to oxidative stress than nuclear DNA, of AD patients could be promising in the research of AD peripheral biomarkers. Noteworthy, both present and previous studies [15] revealed that on average *D-loop* methylation levels in peripheral blood DNA are of about 2-3%, so that more than 120 patients and 120 controls should be required to detect with enough power a difference of about 1% as we observed in the present study. Further investigation is now required to clarify how *D-loop* methylation changes affect the methylation levels of other mtDNA regions in AD, as well as to clarify the environmental factors able to induce those changes.

In summary present data indicate that *D-loop* region is hypomethylated in peripheral blood of LOAD patients, suggesting that mtDNA epimutations deserve further investigations in AD pathogenesis.

Acknowledgments (including sources of support)

The present study was supported by the researcher's intramural funds (ATENEO Funds, University of Pisa).

Conflict of Interest/Disclosure Statement

The authors have no conflict of interest to report

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Tables

Table 1. Demographic characteristics of the study population.

	AD patients (n = 133)	Ctrls (n = 130)	*P*-value
Age at sampling (mean ± SEM)	77.66 ± 6.98	78.88 ± 7.65	0.18[a]
Gender (M/F)	50/83	54/76	0.53[b]

[a] Student's t Test.

[b] Fisher Exact Test.

Table 2. Mean D-loop region methylation levels (%) in AD patients and matched healthy controls.

	AD patients (n = 133)	Ctrls (n = 130)	*P*-value[a]
***D-loop* methylation (%, mean ± SEM)**	2.3 ± 0.2	3.1 ± 0.2	0.042

[a] Multifactorial analysis of variance, corrected for age at sampling and gender

Figure

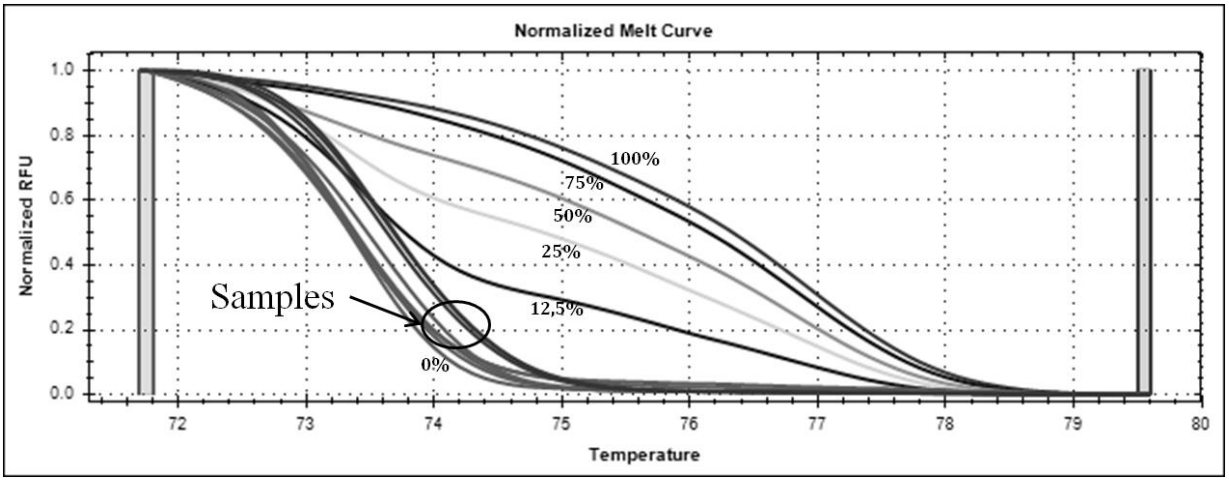

Figure 1. MS-HRM curves of *D-loop*. Standard curves generated by mixing methylated and unmethylated standard DNAs (0%, 12.5%, 25%, 50%, 75%, and 100%) and curves of the samples ranging from 0 to 9% (highlighted) are shown.