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Global Long Interspersed Nuclear Element 1 DNA Methylation in a Colombian Sample of Patients With Late-Onset Alzheimer's Disease

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Abstract

Alterations in DNA methylation have implicated as an epigenetic event in the pathogenesis of late-onset Alzheimer's disease (LOAD). The objective of this work was to evaluate global DNA methylation levels for long interspersed nuclear element 1 (LINE-1) repetitive sequences in Colombian patients with LOAD and controls. The LINE-1 DNA methylation levels in peripheral blood samples from 28 Colombian patients with LOAD and 30 healthy participants were assessed using a methylation-sensitive high-resolution melting (MS-HRM) quantitative assay. We did not find differences in LINE-1 methylation levels between patients with Alzheimer's disease (AD; median 76.2%, interquartile range [IQR]: 69.8-81.9) and control participants (median 79.8%, IQR: 73.2-83.8; $P = .3$). Additional stratified analyses did not show differences in LINE-1 methylation levels for male or female patients versus controls nor for apolipoprotein E4 carriers and noncarriers. This is the first report of LINE-1 methylation levels in patients with LOAD using the cost-effective MS-HRM technique, and this is the first global DNA methylation study in Latin American patients with AD.

Keywords

late-onset Alzheimer's disease, DNA methylation, LINE-1, repetitive sequences, epigenetics, MS-HRM, Latin America

Introduction

Late-onset Alzheimer's disease (LOAD) is one of the most common neurodegenerative disorders, and its prevalence is growing, associated with increases in life expectancy around the world.[1] Dementia in Latin America is an ongoing epidemic. It is estimated that almost 2 million people with dementia live in Latin American counties (a figure similar to the number of patients with dementia in the United States and Canada combined).[2] In addition, total societal costs of dementia in Latin American nations are quite high for these developing countries, US$24.5 billions in 2009.[3]

Genome-Wide Association Studies for LOAD have found only a fraction of the risk proposed to be accounted for common genetic variants; this fact highlights the importance of studying additional molecular risk factors for Alzheimer's disease (AD)[4] such as epigenetic changes. Recently, many efforts have focused on the development of epigenetic studies for neurodegenerative disorders.[1,5]

Global DNA methylation differences were found in LOAD brain samples, in comparison with age-matched control samples.[5] Repetitive sequences represent a very important fraction of the genome, being useful to carry out estimations of global DNA methylation in AD samples.[6] In this context, long interspersed nuclear element 1 (LINE-1) repetitive elements constitute around 20% of the human genome.[7] There is very little data about DNA methylation status of repetitive sequences in peripheral blood of patients with LOAD.[8] DNA methylation is a major epigenetic mechanism that interacts with, and is regulated by, both environment and genetic background; for

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this reason, it is important to study DNA methylation status for AD samples from different populations.[1] Global DNA methylation status of patients with LOAD from Latin American populations has not yet been studied. In the present study, we used a quantitative protocol of methylation-sensitive high-resolution melting (MS-HRM)[7,9,10] to evaluate global DNA methylation status in LINE-1 repetitive elements in samples of Colombian patients with LOAD and control participants.

Materials and Methods

Patients

A total of 28 patients with LOAD (mean age: 77.4 $\pm$ 6.5 years, 9 males) and 30 control participants (mean age: 75 $\pm$ 6 years old, 11 males) were included. Clinical assessment of patients was carried out by an interdisciplinary team in a memory clinic in Bogotá, Colombia, led by a senior neurologist.[11] Patients with LOAD fulfilled the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRD) diagnostic criteria.[4] Sex and age-matched participants without familial or personal history of neurological disorders were included in the control group, being similar in age and gender distributions to the AD group. The study was approved by the local ethics committees, and all participants, or their closest relatives, gave written informed consent before participating in this study.

DNA Extraction and Bisulfite Treatment

Genomic DNA was isolated from peripheral blood of participants using a salting-out procedure as previously described.[4] Genomic DNA of 400 ng was bisulfite converted using EZ DNA Methylation Direct Kit (Zymo Research, Orange, California), according to manufacturer's instructions, in a final elution volume of 15 μL. Concentrations of converted DNA were measured[7] using a NanoDrop C2000 (NanoDrop Technologies, Wilmington, Delaware) and normalized to 14 ng/μL.

Human Methylated and nonmethylated DNA Set (Zymo Research) was used as DNA methylation controls. These methylation standards were bisulfite treated and normalized in the same way as the samples. After the normalization step, methylated to unmethylated template ratios of 100%, 75%, 50%, and 0% were prepared using methylated and nonmethylated DNA controls. Such dilutions were used as reference standards in the MS-HRM technique. Normalized DNA samples were stored at -70°C until use.

Methylation-Sensitive High-Resolution Melting

Precision Melting Supermix (BioRad, Hercules, California) was used to perform all MS-HRM assays (final volume of 10 μL), using a CFX96 Touch Real-Time polymerase chain reaction (PCR) Detection System (BioRad). 14 ng of bisulfite converted DNA was used as template per reaction. Assays were carried out in duplicate for each sample and in triplicate for standards. The LINE-1 primer sequences were taken from Tse et al[7]; LINE-1 forward: 5′-GCG AGG TAT TGT TTT ATT TGG GA-3′ and reverse: 5′-CGC CGT TTC TTA AAC C-3′. Both primers (IDT, San Jose, California) were used in a final concentration of 200 nmol/L. Specificity of the assay for converted DNA was verified with the inclusion of nonconverted genomic DNA as a control, nonconverted DNA showed the highest quantification cycle value (Cq) and the highest melt peak temperature.[10]

Conditions in the PCR stage of the MS-HRM assay were 95°C for 2 minutes, 41 cycles 95°C for 10 seconds, 48°C for 30 seconds, and 72°C for 30 seconds. For the HRM stage 95.0°C for 30 seconds, melt curve: 62.0°C to 85.0°C, using increments of 0.2°C with holding steps of 10 seconds. The CFX96 manager software (BioRad) was used for recording and analyzing the relative fluorescence from the sample reactions and to examine the Cq differences between methylated and unmethylated DNA standards. Expected amplification band size was verified on agarose gel electrophoresis (data not shown). Melting profiles were analyzed with BioRad Precision Melt Analysis Software (BioRad). Normalized relative fluorescence units (RFUs) values from the first derivative of the melt curve were exported to a spreadsheet for further analysis. Methylation percentage for each sample was calculated by linear regression analysis, using data from standard curves from each plate.[7,10] Genotyping for apolipoprotein E (APOE) E2/3/4 allelic variants was carried out as previously described.[4] The APOE-ε4 allele was present in 39% of the patients with LOAD (11 patients) and in 20% of the control individuals (6 participants).

Statistics

Statistical tests were performed using R Commander Package 1.7-3 (in R platform, version 2.13.1). The LINE-1 DNA methylation did not show a normal distribution by Shapiro-Wilk analysis (P value = .020). Therefore, Wilcoxon Mann-Whitney U test was used in order to compare LINE-1 DNA methylation levels between the 2 groups. Interassay and intra-assay reproducibilities were verified, with a coefficient of variance below 5% between replicates. Pearson coefficients were calculated to analyze possible significant correlations between variables. Statistical power calculations using a freely available online tool (http://homepage.stat.uiowa.edu/~rlenth/Power/) showed that the sample size used in our study provided a power of 0.8 to identify the standardized mean differences of 0.72 (α value: .05).

Results

In the LINE-1 MS-HRM assay, linear regression analysis from the reference standards (using controls with known methylation status) showed a correlation coefficient of $r^2 = .972$, in concordance with the linearity of the quantitative assay used in the current study. As expected, the Cq difference between the

Table 1. The LINE-1 DNA Methylation Results (Comparisons by Groups).

		LINE-1 Methylation % Median (IQR)				
Group	Age, Years (Mean ± SD)	Case or Control Groups	APOE 3/4 Genotype Carriers	APOE 3/3 Genotype Carriers	Males	Females
AD	77.4 ± 6.5	76.2 (69.8-81.9)	75.80 (69.8-81.9)	77.15 (69.6-80.9)	80.75 (72.5-86.5)	75.80 (69.1-78.6)
CTL	74.9 ± 6	79.80 (73.2-83.8)	83.75 (79.6-86.6)	79.35 (70.1-83.1)	78.68 (76.1-83.8)	79.6 (71.4-83.6)
AD vs CTL, *P* value	.17	.30	.09	.71	.90	.30

Abbreviations: AD, Alzheimer's disease; APOE, apolipoprotein E; CTL, control group participants; IQR, interquartile range; LINE-1, long interspersed nuclear element 1; SD, standard deviation.

methylated and the unmethylated DNA standards was lower than 2 cycles, consistent with the absence of PCR bias.[10]

The median LINE-1 methylation level was 76.2% (interquartile range [IQR]: 69.8-81.9) in the AD group in comparison with 79.8% (IQR: 73.2-83.7) in the control group (CTL) without statistical differences (P value = .3). The LINE-1 DNA methylation levels in patients with LOAD compared to control group did not show differences neither for males ($P = .9$) nor for females ($P = .3$); nor in the presence ($P = .09$) or in the absence ($P = .71$) of APOE-ε4 (risk allele for LOAD), Table 1.

Correlations between age and DNA methylation status were not significant in AD group, control group, or for all patients combined (P values of .5, .99, and .8, respectively). Similarly, no differences were found between APOE-ε4 carriers and non-ε4-carriers (P values of .79, .22, and .65 for AD, CTL, and all patients, respectively). We performed independent comparisons of LINE-1 DNA methylation between males and females, with no significant differences found (P values of .32, .55, and .16 for AD, CTL, and all patients, respectively), Table 1.

Discussion

Recently, many efforts have been focused on the development of epigenetic studies for neurodegenerative disorders.[1,5] Gene expression alterations have been reported in peripheral blood cells of patients with LOAD.[12] Several biomarkers have been found in peripheral blood for other neurological disorders, for example, in Parkinson's disease (usually assumed as an exclusively brain disease) specific changes in peripheral blood correlated with similar findings in brain cells.[13] Consequently, the study of DNA methylation in peripheral blood of patients with LOAD, given its availability in living patients, promises interesting findings of potential application, such as the discovery of biomarkers.[14] In addition, developing countries usually do not have available brain banks that provide neural tissues from patients with AD living in those regions.[2]

A limited number of studies have attempted to assess the methylation profiles in LOAD using peripheral blood, using very few DNA samples (for example, 6 patients with LOAD vs 6 control participants, with the consequent small statistical power).[15] Therefore, global DNA methylation in peripheral blood of patients with LOAD has not been adequately studied. In the present study, we did not find any significant difference in LINE-1 DNA methylation levels between patients with LOAD and control participants. Our findings are in disagreement with a previous pilot study which showed a very small increase in DNA methylation levels in patients with AD (patients with LOAD were pooled with patients with early-onset AD) compared to the control group (83.6% vs 83.1%, with a modest median difference of 0.5%).[8] This divergence may be explained, in addition to population differences, by the dissimilarity in the methods employed, MS-HRM in the present work and pyrosequencing used in the previous study. The MS-HRM brings several advantages for the analysis of epigenetic changes in LOAD, given its cost-effectiveness (it could be easily implemented in an standard molecular genetics laboratory), reproducibility, and its ability to provide quantitative measurements of DNA methylation.[7,10,16]

Absence of differences in global LINE-1 DNA methylation in whole blood cells does not imply lack of alterations in DNA methylation for specific loci. Consequently, global DNA methylation can be useful to complement locus-specific approaches. In addition, the ability to detect DNA methylation in patients with LOAD could be enhanced by new approaches focused on cell subgroups, such as specific cerebral cortex layers.[17]

Overall, we carried out a quantitative assessment to evaluate the global DNA methylation differences in peripheral blood between patients with LOAD and controls. Considering that epigenetic marks probably differ between populations,[1] we highlight the fact that the studied Colombian samples has differences in terms of environment and genetic background, in comparison with other populations.[4,11,18] No differences in global methylation of LINE-1 were found between the groups. This is the first report of LINE-1 methylation levels in patients with LOAD using the cost-effective MS-HRM technique, and this is the first global DNA methylation study in Latin American patients with AD. Sample sizes used in this work are similar to those in the published studies for AD epigenetics.[8,15,17] The present results should be interpreted with caution, and further studies in this field are vital, as they will increase our understanding of the role of DNA methylation in LOAD pathogenesis.

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Declaration of Conflicting Interests

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References

1. Marques SC, Oliveira CR, Outeiro TF, Pereira CM. Alzheimer's disease: the quest to understand complexity. *J Alzheimers Dis*. 2010;21(2):373-383.
2. Maestre GE. Dementia in Latin America and the Caribbean: an overlooked epidemic. *Neuroepidemiology*. 2008;31(4):252-253.
3. Wimo A, Winblad B, Jonsson L. The worldwide societal costs of dementia: estimates for 2009. *Alzheimers Dement*. 2010;6(2): 98-103.
4. Arboleda GH, Yunis JJ, Pardo R, et al. Apolipoprotein E genotyping in a sample of Colombian patients with Alzheimer's disease. *Neurosci Lett*. 2001;305(2):135-138.
5. Rao JS, Keleshian VL, Klein S, Rapoport SI. Epigenetic modifications in frontal cortex from Alzheimer's disease and bipolar disorder patients. *Transl Psychiatry*. 2012;2:e132.
6. Weisenberger DJ, Campan M, Long TI, et al. Analysis of repetitive element DNA methylation by methylight. *Nucleic Acids Res*. 2005;33(21):6823-6836.
7. Tse MY, Ashbury JE, Zwingerman N, King WD, Taylor SA, Pang SC. A refined, rapid and reproducible high resolution melt (HRM)-based method suitable for quantification of global LINE-1 repetitive element methylation. *BMC Res Notes*. 2011;4:565.
8. Bollati V, Galimberti D, Pergoli L, et al. DNA methylation in repetitive elements and Alzheimer disease. *Brain Behav Immun*. 2011;25(6):1078-1083.
9. Wojdacz TK, Dobrovic A. Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation. *Nucleic Acids Res*. 2007; 35(6):e41.
10. Hernandez HG, Tse MY, Pang SC, Arboleda H, Forero DA. Optimizing methodologies for PCR-based DNA methylation analysis. *Biotechniques*. 2013;55(4):181-197.
11. Forero DA, Benítez B, Arboleda G, Yunis JJ, Pardo R, Arboleda H. Analysis of functional polymorphisms in three synaptic plasticity-related genes (BDNF, COMT AND UCHL1) in Alzheimer's disease in Colombia. *Neurosci Res*. 2006;55(3):334-341.
12. Booij BB, Lindahl T, Wetterberg P, et al. A gene expression pattern in blood for the early detection of Alzheimer's disease. *J Alzheimers Dis*. 2011;23(1):109-119.
13. Pellicano C, Buttarelli FR, Circella A, et al. Dopamine transporter immunoreactivity in peripheral blood lymphocytes discriminates Parkinson's disease from essential tremor. *J Neural Transm*. 2007;114(7):935-938.
14. Terry MB, Delgado-Cruzata L, Vin-Raviv N, Wu HC, Santella RM. DNA methylation in white blood cells: association with risk factors in epidemiologic studies. *Epigenetics*. 2011;6(7):828-837.
15. Wang SC, Oelze B, Schumacher A. Age-specific epigenetic drift in late-onset Alzheimer's disease. *PLoS ONE*. 2008;3(7):e2698.
16. Wojdacz TK, Dobrovic A, Hansen LL. Methylation-sensitive high-resolution melting. *Nat Protoc*. 2008;3(12):1903-1908.
17. Bakulski KM, Dolinoy DC, Sartor MA, et al. Genome-wide DNA methylation differences between late-onset Alzheimer's disease and cognitively normal controls in human frontal cortex. *J Alzheimers Dis*. 2012;29(3):571-588.
18. Forero DA, Casadesus G, Perry G, Arboleda H. Synaptic dysfunction and oxidative stress in Alzheimer's disease: emerging mechanisms. *J Cell Mol Med*. 2006;10(3):796-805.