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Research Report

LINE-1 DNA Methylation, Smoking and Risk of Parkinson's Disease

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Abstract.
Background: Long interspersed nucleotide element-1 (*LINE-1*) retrotransposons are located throughout the human genome. Those retaining an intact 5′ promoter can copy and insert themselves into the DNA of neural progenitor cells that express tyrosine hydroxylase, which may influence differentiation and survival of these cells. *LINE-1* promoter methylation is associated with decreased *LINE-1* propagation.
Objective: To investigate whether *LINE-1* promoter methylation is associated with Parkinson's disease (PD).
Methods: We compared *LINE-1* methylation profiles in blood mononuclear cells between 292 newly diagnosed PD cases and 401 unrelated, neurologically normal controls, all non-Hispanic Caucasians in western Washington state.
Results: Overall, PD was not associated with percent methylation of the *LINE-1* promoter. However, the predictable inverse association between PD and ever smoking tobacco was strongest for men and women with the lowest *LINE-1* promoter methylation, and less apparent as *LINE-1* methylation increased. Underlying this possible interaction, ever regularly smoking tobacco was associated with decreased *LINE-1* methylation in controls (age- and sex-adjusted linear regression $\beta = -0.24$, 95% confidence interval [CI] –0.43, –0.04), but not in cases ($\beta = 0.06$, 95% CI –0.17, 0.28, interaction $p = 0.06$).
Conclusion: PD cases may have innate differences in their ability to respond to tobacco smoke.

Keywords: DNA methylation, idiopathic Parkinson's disease, long interspersed nucleotide elements, smoking

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative movement disorder due to selective loss of dopaminergic neurons in midbrain substantia nigra. The causes of PD remain largely unknown, although both genetic and environmental factors are etiologically relevant [1]. One factor not previously examined in relation to PD is methylation of long interspersed nucleotide element-1 (*LINE-1* or L1). *LINE-1* DNA sequences are repeated throughout the genome, and those with intact 5′ promoters can copy and insert themselves elsewhere in cellular DNA [2]. These insertions potentially affect gene expression [3], and both germline

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and somatic *LINE-1* insertions may result in disease [4]. In the brain, most *LINE-1* insertions are somatic [5]. Notably, they occur in the subtype of human neural progenitor cells that express tyrosine hydroxylase [6], a protein suggestive of differentiation into dopaminergic neurons. Integration of *LINE-1* into neural progenitor cells might affect progenitor cell fate, including differentiation [3] or survival [7]. *LINE-1* expression may be especially important during neurogenesis [8].

The number of *LINE-1* insertions in brain cell DNA varies between persons [5]. One factor that may affect retrotransposition of *LINE-1* within neurons is methylation of DNA in the *LINE-1* promoter [6]. Methylation of individual CG dinucleotides (CpG sites) is mitotically heritable yet is alterable within each cell. Mean percent *LINE-1* methylation, although somewhat stable [9], appears to be affected by a variety of environmental exposures [10]. Recently, a small study found that genome-wide methylation, which is correlated with *LINE-1* methylation, was lower in PD than control brains [11]. However, this study was limited to frontal cortex samples from deceased patients, whose DNA methylation may have been altered by their advanced disease state, treatment [12–14] or physical inactivity [15, 16] in the months preceding death.

We investigated the relation between PD and methylation of the *LINE-1* promoter using blood collected close in time to PD diagnosis in a relatively large case-control study. In addition, because oxidative stress increases *LINE-1* retrotransposition in human neuroblastoma cells [17], we examined whether the known inverse association between PD and tobacco smoking [18, 19] was modified by *LINE-1* methylation. As we were unable to obtain dopaminergic neurons from study participants and assess *de novo LINE-1* insertions, we measured percent *LINE-1* methylation in blood mononuclear cells, as a proxy and as an initial step in directly exploring the relation between PD and *LINE-1*.

MATERIALS AND METHODS

Participant identification and data collection

Idiopathic PD cases ($N = 490$) were newly diagnosed during 1992–2008 at Group Health Cooperative ($N = 387$) or the University of Washington Neurology Clinic ($N = 103$) in western Washington State [19, 20]. Briefly, all cases had at least two of four cardinal signs of PD (bradykinesia, resting tremor, cogwheel rigidity, postural reflex impairment). Diagnoses not made by neurologists were verified by chart reviews by study neurologists (W.T.L., G.M.F., P.D.S.). We excluded patients with an established cause of parkinsonism, such as stroke, recent brain trauma, brain tumor, or use of selected medications. Controls ($N = 644$) were unrelated Group Health Cooperative enrollees who were free of PD and other neurodegenerative disorders. Controls were frequency matched to cases on age, sex, race/ethnicity, clinic and length of enrollment. We made no exclusions for cases or controls with regard to history of cancer or other non-neurological outcomes.

We determined history of tobacco (cigarette, cigar and pipe) smoking through a structured, in-person questionnaire. Most participants (96% of cases, 95% of controls) also provided blood or buccal cells at interview. Because *LINE-1* methylation may differ by race/ethnicity [21, 22] and cell type [23, 24], we restricted analysis to non-Hispanic Caucasians with sufficient DNA from blood-derived mononuclear cells at the time of laboratory analysis, 292 (60%) of cases and 401 (62%) of controls. University of Washington and Group Health Cooperative Institutional Review Boards approved all study procedures, and all participants provided written informed consent.

Assessment of LINE-1 methylation

The Functional Genomics Laboratory at the Center for Ecogenetics and Environmental Health at the University of Washington (Seattle, Washington) isolated DNA using the DNeasy Blood and Tissue Kit (Qiagen) following centrifugation of whole blood to isolate the mononuclear cells and then treatment with RNase. Blind to case-control status, the Center for Environmental Health and Technology at Brown University (Providence, Rhode Island) conducted quantitative *LINE-1* methylation assays by pyrosequencing. Bisulfite conversion of genomic DNA (250 ng) was performed using the EZ DNA Methylation Direct Kit (Zymo Research) per the manufacturer's instructions. Four CpG sites in the 5′ promoter of *LINE-1* (TT[T/C]GTGGTG[T/C]GT[T/C]GTTTTTT AAGT[T/C]GGTTT) were analyzed in triplicate on a PyroMark MD system (Qiagen) using primers as described [25]. We retained replicates with complete data, that is, percent methylation for all four CpG sites. All three replicates were complete for 92% of cases and 94% of controls, and the remainder had at least one complete replicate. We took the mean across the four CpG sites for each replicate, and then the mean of these, hereafter *LINE-1* methylation, expressed as a percentage.

Table 1
Characteristics of Parkinson's disease cases and controls, Group Health Cooperative and University of Washington, 1992–2008

	All participants		Non-Hispanic Caucasians with *LINE-1* data	
	Cases $N=490$ *n* (%)	Controls $N=644$ *n* (%)	Cases $N=292$ *n* (%)	Controls $N=401$ *n* (%)
Male	310 (63)	409 (64)	196 (67)	256 (64)
Non-Hispanic Caucasian	456 (93)	595 (92)	292 (100)	401 (100)
Family history of PD[a]	43 (11)	23 (5)	27 (12)	16 (5)
Age at diagnosis/reference, years				
≥60	359 (73)	526 (82)	206 (71)	325 (81)
Mean (standard deviation)	65.6 (10.2)	68.0 (8.7)	64.9 (10.5)	68.0 (8.7)
Time between diagnosis/reference and DNA collection, years				
<1	175 (36)	230 (36)	100 (34)	146 (36)
1	274 (56)	362 (56)	167 (57)	221 (55)
2–4	41 (8)	52 (8)	25 (9)	34 (8)
Ever tobacco smoking[b]	245 (50)	398 (62)	148 (51)	244 (61)
Cigarette smoking[c]				
Never	272 (56)	276 (43)	159 (54)	179 (45)
Former	196 (40)	307 (48)	116 (40)	192 (48)
Current	22 (4)	61 (9)	17 (6)	30 (7)
Smoking among ever smokers, mean (standard deviation)[c]				
Packs per day	0.88 (0.57)	0.92 (0.55)	0.84 (0.53)	0.92 (0.53)
Years	22.5 (15.2)	26.8 (16.4)	22.5 (15.1)	26.3 (16.4)
Pack-years	21.7 (22.0)	26.4 (23.2)	20.5 (20.7)	25.1 (22.0)
Years since smoked	25.1 (14.9)	22.7 (16.8)	24.2 (14.5)	23.1 (16.5)

[a]Any first degree relative with Parkinson's disease (PD), based on 390 cases and 494 controls with complete data, including 231 cases and 316 controls who were non-Hispanic Caucasian and for whom *LINE-1* methylation was assessed. [b]Ever smoked >100 cigarettes, cigar regularly or pipe regularly. [c]History of cigarette smoking (>100 cigarettes) up to diagnosis/reference. Abbreviations: PD Parkinson's disease, *LINE-1* long interspersed nucleotide element-1.

Statistical analysis

We used Stata 11.1 (College Station, Texas) for all statistical analyses. *LINE-1* methylation was normally distributed, and we compared cases to controls using a *t*-test, followed by multivariable linear regression to adjust for age, sex and tobacco smoking (ever/never regularly smoked tobacco, number of cigarettes per day, and number of years smoking cigarettes). These factors are associated with PD occurrence [18, 19] or *LINE-1* methylation [10]. We also adjusted for assay plate to account for any inter-plate effects.

We then examined whether the known inverse association between smoking and PD risk previously seen in this study [18–20] varied by *LINE-1* methylation. We calculated age- and sex-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) as an estimate of the relative risk of PD in relation to tobacco smoking, overall and by *LINE-1* methylation quartiles. We formally tested interactions in multivariate regression models; we used the *p*-value for the multiplicative interaction term between smoking and *LINE-1* methylation as a continuous variable, while including main effects terms and the same covariates.

RESULTS

Characteristics of participants

Other than the restriction to non-Hispanic Caucasians, cases and controls with *LINE-1* methylation data were similar to those in the parent study [19, 20], including greater tobacco smoking by controls than cases (Table 1). For most cases (91%) we had obtained DNA less than two years after diagnosis.

PD and LINE-1 methylation

Overall, percent *LINE-1* methylation was similar in cases and controls (Table 2; $p=0.40$). We confirmed the well-established association between *LINE-1* methylation and sex [10] among both cases and controls (Table 2; both $p<0.001$); therefore, we repeated *LINE-1* comparisons separately for men and women. Again no association between PD and *LINE-1* methylation was evident, nor when we used multivariate linear regression to adjust for age, sex and smoking (all $p>0.40$, data not shown).

Table 2
Percent *LINE-1* methylation in Parkinson's disease cases and controls, overall and by sex

	All		Men		Women	
	Cases	Controls	Cases	Controls	Cases	Controls
	$N = 292$	$N = 401$	$N = 196$	$N = 256$	$N = 96$	$N = 145$
Minimum	76.83	76.72	77.82	77.39	76.83	76.72
Median	80.33	80.23	80.52	80.41	80.02	79.98
Maximum	83.67	84.14	82.52	83.38	83.67	84.14
Mean	80.33	80.26	80.52	80.44	79.95	79.95
Standard deviation	1.03	1.06	0.94	0.99	1.10	1.11

Abbreviations: *LINE-1* long interspersed nucleotide element-1.

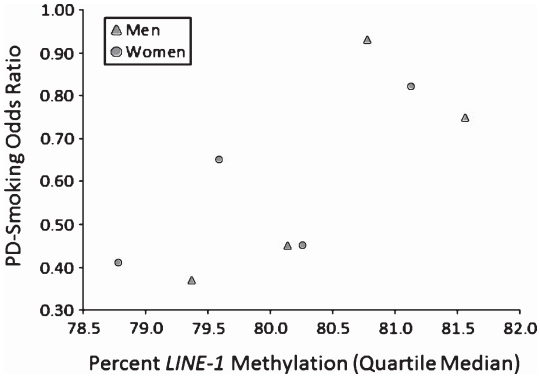

Fig. 1. Age-adjusted odds ratio between Parkinson's disease and ever tobacco smoking, by sex and percent *LINE-1* methylation quartile.

PD, smoking and LINE-1 methylation

In participants with *LINE-1* methylation data, ever smoking tobacco was associated with reduced risk of PD (age- and sex-adjusted OR = 0.63, 95% CI 0.46–0.87, data not shown). Risk was 0.61, 95% CI 0.41–0.91 in men, and 0.67, 95% CI 0.39–1.14 in women. This inverse association between PD and ever smoking was strongest among men and women with the lowest *LINE-1* methylation, and became less apparent with greater *LINE-1* methylation (Fig. 1; interaction $p = 0.14$ for men and women combined). Underlying this potential interaction, ever smoking was associated with reduced *LINE-1* methylation among controls (linear regression $\beta = -0.24$, 95% CI –0.43, –0.04), but ever smoking was not associated with *LINE-1* methylation among cases ($\beta = 0.06$, 95% CI –0.17, 0.28, interaction $p = 0.06$).

DISCUSSION

To our knowledge, this is the first study of PD and *LINE-1* methylation. We observed no difference in *LINE-1* methylation between cases and controls overall, despite studying a reasonably large number of newly diagnosed PD patients and highly comparable, unrelated controls. However, the known inverse association between smoking and PD risk was most evident in men and women with the lowest *LINE-1* methylation, and it markedly diminished with greater *LINE-1* methylation. Although this potential interaction was not statistically significant, it was probably attenuated by our use of non-neural cells. This predictable effect may have been substantial because even the correlation between *LINE-1* methylation in serum vs. buffy coat cells is modest [24]. This conservative bias is a limitation with regard to the lack of overall case-control differences, but the use of cells obtained close in time to diagnosis likely minimized bias related to disease progression and survival. Thus, insofar as mononuclear cell *LINE-1* DNA methylation reflects dopaminergic neural cell *LINE-1* methylation – and hence retrotransposition frequency [6] – the potential PD-smoking-*LINE1* interaction we report may have a biological basis related to neural cell differentiation or survival [3, 7, 8]. Alternatively, lower *LINE-1* methylation in blood may be a surrogate for glutathione depletion [26] or exposure to oxidative stress [27], persistent organic pollutants [28] or heavy metals [29–31]. It is plausible that smoking might be more protective with these exposures.

Underlying the interaction of smoking and *LINE-1* methylation on PD risk was an inverse association between ever smoking and *LINE-1* methylation among controls, which agrees with some but not all epidemiologic studies [9, 32–36]. Experimental studies indicate that cigarette smoke condensate [37] and benzo(*a*)pyrene specifically [38] are associated with reduced *LINE-1* methylation. Heavy metals that are present in tobacco smoke are also associated with reduced *LINE-1* methylation [29–31]. The lack of association between smoking and *LINE-1* methylation in cases probably was not due to lower statistical power, because the β estimate was close to null. Thus, insofar as our results are not simply due to chance, the most

straightforward explanation is that PD cases respond to smoking differently than their counterparts. This possibly includes innate differences in cases' ability to methylate or demethylate DNA. Because DNA methylation may play a role in smoking initiation [39], the inverse association between PD and ever smoking might even simply reflect poor (de)methylation by persons who later develop PD, rather than a true protective effect of smoking. A limitation of our study is that we did not have longitudinal samples to directly assess the effect of smoking on *LINE-1* methylation. However, a recent study with longitudinal blood samples, which examined post-traumatic stress disorder among military personnel deployed in Afghanistan and Iraq, reported findings that parallel ours: No difference in *LINE-1* methylation between cases and controls prior to deployment, but a deployment-related change in *LINE-1* methylation among controls but not cases [22]. In summary, persons with less ability to respond to a changing environment may be at increased risk of developing a variety of neurological disorders.

Additional similar studies of *LINE-1* methylation and PD among newly diagnosed cases and a carefully selected comparison group will be required to confirm our results. Studies that include blood samples collected longitudinally may prove particularly useful, although feasibility for such studies is limited. Targeted examination of PD and methylation of smoking-related genes also may help elucidate potential biological mechanisms pertinent to PD pathogenesis and susceptibility.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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