Urinary LRRK2 phosphorylation predicts parkinsonian phenotypes in G2019S *LRRK2* carriers

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ABSTRACT

Objective: To test whether phosphorylated Ser-1292 LRRK2 levels in urine exosomes predicts *LRRK2* mutation carriers (*LRRK2*+) and noncarriers (*LRRK2*−) with Parkinson disease (PD+) and without Parkinson disease (PD−).

Methods: LRRK2 protein was purified from urinary exosomes collected from participants in 2 independent cohorts. The first cohort included 14 men (*LRRK2*+/PD+, *n* = 7; *LRRK2*−/PD+, *n* = 4; *LRRK2*−/PD−, *n* = 3). The second cohort included 62 men (*LRRK2*−/PD−, *n* = 16; *LRRK2*+/PD−, *n* = 16; *LRRK2*+/PD+, *n* = 14; *LRRK2*−/PD+, *n* = 16). The ratio of Ser(P)-1292 LRRK2 to total LRRK2 was compared between *LRRK2*+/PD+ and *LRRK2*− in the first cohort and between *LRRK2* G2019S carriers with and without PD in the second cohort.

Results: *LRRK2*+/PD+ had higher ratios of Ser(P)-1292 LRRK2 to total LRRK2 than *LRRK2*−/PD− (4.8-fold, *p* < 0.001) and *LRRK2*−/PD+ (4.6-fold, *p* < 0.001). Among mutation carriers, those with PD had higher Ser(P)-1292 LRRK2 to total LRRK2 than those without PD (2.2-fold, *p* < 0.001). Ser(P)-1292 LRRK2 levels predicted symptomatic from asymptomatic carriers with an area under the receiver operating characteristic curve of 0.844.

Conclusion: Elevated ratio of phosphorylated Ser-1292 LRRK2 to total LRRK2 in urine exosomes predicted *LRRK2* mutation status and PD risk among *LRRK2* mutation carriers. Future studies may explore whether interventions that reduce this ratio may also reduce PD risk. **Neurology® 2016;86:994–999**

GLOSSARY

AUC = area under the curve; **LCC** = LRRK2 Cohort Consortium; **LRRK2** = leucine-rich repeat kinase 2; **MoCA** = Montreal Cognitive Assessment; **PD** = Parkinson disease; **ROC** = receiver operating characteristic; **UPDRS** = Unified Parkinson's Disease Rating Scale.

Genetic studies identified mutations in the leucine-rich repeat kinase 2 (*LRRK2*) gene in late-onset Parkinson disease (PD).^{1–5} The most prevalent known genetic cause of neurodegeneration, the G2019S mutation in the *LRRK2* kinase domain, has an incomplete lifetime penetrance that varies between 30% and 80% depending on the study.^{6,7} A recent fMRI study demonstrated that asymptomatic G2019S mutation carriers may have abnormalities in corticostriatal circuitry.⁸ However, there are currently no known ways to predict which G2019S carriers will develop PD.

LRRK2 encodes a large protein that can enzymatically incorporate phosphates through autophosphorylation (figure 1A).⁹ Antibodies directed to these phosphorylated residues, particularly antibodies to the Ser-1292 residue, have demonstrated that the G2019S mutation enhances kinase activity.^{10,11} However, the effects of the G2019S mutation on *LRRK2* protein phosphorylation have not previously been studied in clinical samples in either G2019S carriers that manifest PD or asymptomatic carriers. Previously, we and others identified urine exosomes as a stable source of *LRRK2* protein in clinical populations.^{12,13} In the present study, we utilized urine exosomes to determine whether Ser(P)-1292 *LRRK2* levels predict *LRRK2* G2019S mutation status and PD diagnosis.

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Figure 1 Ser(P)-1292 LRRK2/total LRRK2 ratio depends on LRRK2 kinase activity

(A) Diagram of the conserved domain structure of LRRK2 that highlights the pathological mutations R1441C, Y1699C, and G2019S in relation to the autophosphorylation residue Ser-1292. (B) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of lysates from HEK-293T cells transiently transfected with WT-LRRK2, S1292A-LRRK2 (prevents phosphorylation of the LRRK2 S1292 residue), or LRRK2 containing the labeled pathogenic mutations. Densitometry results for the Ser(P)-1292 LRRK2/total LRRK2 ratio are calculated from 6 independent experiments normalized with respect to G2019S LRRK2 (mean value defined as 1.0). (C) Representative immunoblots of lysates analyzed by SDS-PAGE from HEK-293T cells transiently transfected with G2019S LRRK2 and treated with the LRRK2 kinase inhibitors HG-10-102, SRI-29451, or vehicle control (dimethylsulfoxide, defined as 1.0) for 24 hours at the indicated concentration. Quantitation and bar graphs represent data from 3 independent replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. p Values are from one-way analysis of variance with Tukey post hoc honestly significant difference test.

METHODS Participants. Urine exosomes were purified from 2 separate all-male cross-sectional cohorts. The first cohort included 14 participants enrolled in the Columbia University Movement Disorders Center. These participants underwent clinical evaluation for motor severity using the Unified Parkinson's Disease Rating Scale (UPDRS) part III, and cognitive functioning was assessed using the Montreal Cognitive Assessment (MoCA). Urine specimens were collected from participants following neurologic assessment. Demographics and clinical features are listed in table 1.

In the second cohort, biobanked urine samples were obtained from the Michael J. Fox Foundation (MJFF) LRRK2 Cohort Consortium (LCC). Participants included 72 men: 18 mutation carriers with PD, 18 mutation carriers without PD, 18 noncarriers with PD, and 18 noncarriers without PD. Participants were selected to match group demographics as closely as possible. Two samples were selected from each of the groups at random and processed in feasibility experiments that consumed the samples. Two additional samples from G2019S carriers with PD were lost during sample preparation to tube breakage, resulting in 62 participants available for study.

Clinical data associated with the MJFF LCC included measures of PD severity (UPDRS, Hoehn & Yahr scale, and modified Schwab & England scale), assessments of cognitive function (MoCA), University of Pennsylvania Smell Identification Test, and a number of tests to assess various aspects of daily life including REM Sleep Behavior Disorder Questionnaire, Epworth Sleepiness Scale, and Geriatric Depression Scale. Participant demographics and clinical information are listed in table 2.

All participants in this study were DNA genotyped for the rs34637584 G2019S LRRK2 mutation using standard genotyping assays as described.⁶ All samples were processed by investigators blinded to genotype and PD status, and final curated datasets were achieved before assigning clinical characteristics or genotype information to the samples.

Standard protocol approvals, registrants, and patient consents. All studies were approved by the local institutional review boards, and all participants signed informed consents.

Exosome isolation. Exosomes were isolated from coded frozen urine by rapid thawing in a shaking heated water bath followed by differential ultracentrifugation as described.¹² Exosome pellets were washed in saline-buffered solution twice before being processed into exosome lysis buffer. See the e-Methods on the *Neurology*[®] Web site at Neurology.org for full protocols.

Protein quantification. Exosome proteins TSG101, total LRRK2, and Ser(P)-1292 LRRK2 were measured by Western blot and LI-COR analysis. Antibodies used were Abcam (Cambridge, UK) rabbit monoclonal clone MJFR-19-7-8 to Ser(P)-1292 LRRK2, rabbit polyclonal to TSG101 (Tumor susceptibility gene 101, Abcam), and Antibodies Inc. (Davis, CA) mouse monoclonal clone N241A/34 to total LRRK2. An exosomal pellet and a corresponding exosome protein marker (TSG101) were detected by Western blot in all lysates from all participants in the study, and LRRK2 was measurable in all preparations (figure e-1). The exosome marker protein TSG101 was quantified from all specimens and the abundance of this protein did not vary between groups (table 3). Total LRRK2 protein levels did not vary between groups. See the e-Methods for full protocols.

Statistical analysis. For biochemistry studies, continuous variables between conditions were compared with one-way analysis of variance with Tukey post hoc honestly significant difference test for individual comparisons or with unpaired t test in the case of comparisons between 2 conditions. For clinical measures, scaled

Table 1 Clinical measures and demographics in a group comparison among G2019S *LRRK2*-positive patients with Parkinson disease (PD), patients with idiopathic PD, and healthy controls attending the Columbia Movement Disorder Clinic

	Healthy controls (n = 3)	G2019S <i>LRRK2</i> PD (n = 7)	Idiopathic PD (n = 4)	p Value
Age, y	67 (60-75)	63 (60-78)	69 (55-73)	NS ^a
Age at onset, y	NA	58 (54-75)	60 (54-67)	NS ^b
UPDRS part III	NA	22 (3.5-24.5)	12 (11-32)	NS ^b
MoCA	27 (27-27)	20.5 (18-24)	27 (24-30)	0.058 ^a
LEDD	NA	700 (565-1,100)	300 (0-1,200)	NS ^b

Abbreviations: LEDD = L-dopa equivalent daily dosage; MoCA = Montreal Cognitive Assessment; NA = not applicable; NS = not significant; UPDRS = Unified Parkinson's Disease Rating Scale.

Values are median (range). p Values > 0.5 are indicated as NS.

^aDetermined by Kruskal-Wallis test.

^bDetermined by comparison of G2019S *LRRK2* PD and idiopathic PD with 2-tailed Mann-Whitney U test.

variables were analyzed with Kruskal-Wallis one-way analysis of variance with the Dunn test for post hoc comparison between individual pairings. For general comparisons between 2 independent conditions, scaled variables were analyzed with Mann-Whitney U test for continuous variables, and association between categorical variables was assessed using Fisher exact test. For primary outcome variables associated with clinical samples (i.e., total *LRRK2*, and Ser(P)-1292 *LRRK2* levels), data were calculated to a ratio Ser(P)-1292 *LRRK2*/total *LRRK2*. Receiver operating characteristic (ROC) curve analysis was performed to assess sensitivity and specificity of this outcome variable in delineating the categorical disease conditions. All statistical analyses and graphs were generated in JMP Pro 10 (SAS

Institute Inc., Cary, NC) and GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Significance was ascribed at p value <0.05. All significance levels reported are 2-tailed.

RESULTS Ser(P)-1292 *LRRK2* levels depend on *LRRK2* kinase activity. Previously, studies using HEK293T cells transfected with *LRRK2* protein containing pathogenic mutations demonstrated that *LRRK2* mutations with the exception of Y1699C increased the proportion of Ser(P)-1292 *LRRK2* to total *LRRK2* protein.¹¹ Since Y1699C is clearly a penetrant mutation that leads to late-onset PD,^{14,15} we tested the effects of Y1699C in addition to R1441C and G2019S on Ser(P)-1292 to total *LRRK2* protein ratios. In contrast with previous results, Y1699C induced a large increase in the Ser (P)-1292 to total *LRRK2* ratio (figure 1A). These results show that a common outcome of pathogenic *LRRK2* mutations is to enhance the proportion of Ser (P)-1292 *LRRK2* to total *LRRK2* protein.

Ser(P)-1292 *LRRK2* levels are known to diminish in the brains of mice treated with a *LRRK2* kinase inhibitor.¹¹ We applied *LRRK2* kinase inhibitors to cells and found that the ratio of Ser(P)-1292 to total *LRRK2* protein is diminished, presumably due to the activity of constitutively active phosphatases that remove the Ser(P)-1292 modification and lack of *LRRK2* kinase activity to replenish the modification (figure 1C). Thus Ser(P)-1292 levels appear to be sensitive to both enhancements in kinase activity caused by pathogenic *LRRK2* mutations as well as

Table 2 Clinical measures and demographics in a group comparison between a selected cohort of patients from the MJFF *LRRK2* cohort consortium with or without G2019S *LRRK2* mutations with or without clinical presentation of Parkinson disease (PD)

	<i>LRRK2</i> -/no PD (n = 16)	<i>LRRK2</i> + /no PD (n = 16)	<i>LRRK2</i> + /PD (n = 14)	<i>LRRK2</i> - /PD (n = 16)	p Value ^a
Age, y	63 (52-66.75)	55.5 (45.5-60.5)	59.5 (52-67.5)	64.5 (56.25-70.25)	—
Age at onset, y	NA	NA	53 (43.5-58.75)	58 (46-60)	NA
UPSIT	34 (29-35)	32.5 (29.5-34.75)	21 (12.5-25)	18.5 (13.5-22.25)	0.012 ^b
RBDSQ	2 (1-3)	0.5 (0-3.75)	3 (2-4.5)	5 (3-7.75)	0.456
ESS	8.5 (5.25-10.75)	5.5 (3.25-7.75)	6 (5-13.5)	7.5 (6-11.75)	—
MoCA	27.5 (25.25-29.75)	27 (25-30)	26 (24.25-29)	28 (25-29)	—
UPDRS part II	0 (0-1)	0 (0-0)	9.5 (7-12.5)	12.5 (5.25-17.25)	<0.0001 ^b
UPDRS part III	0 (0-1.5)	0 (0-1)	15.25 (9-22.5)	31 (21.75-46)	0.0009 ^b
GDS	2 (1-2)	2 (0.25-3)	4 (3-7.5)	2 (1-5)	0.05
H&Y	0 (0-0)	0 (0-0)	2 (2-2.125)	2 (2-3)	<0.0001 ^b
Mod S&E	100 (100-100)	100 (100-100)	90 (85-92.5)	85 (77.5-92.5)	0.011 ^b
LEDD	NA	NA	510 (252.5-1326)	640 (262.5-912.5)	NA

Abbreviations: ESS = Epworth Sleepiness Scale; GDS = Geriatric Depression Scale; H&Y = Hoehn & Yahr scale; LEDD = L-dopa equivalent daily dosage; *LRRK2*- = G2019S *LRRK2* noncarrier; *LRRK2*+ = G2019S *LRRK2* carrier; MoCA = Montreal Cognitive Assessment; Mod S&E = modified Schwab & England scale; NA = not applicable; NS = not significant (p > 0.5); RBDSQ = REM Sleep Behavior Disorder Questionnaire; UPDRS = Unified Parkinson's Disease Rating Scale; UPSIT = University of Pennsylvania Smell Identification Test.

Values are median (interquartile range). Values found not significant across conditions by Kruskal-Wallis were not pursued and are indicated by a dash.

^aDetermined by comparing *LRRK2*+ /no PD and *LRRK2*+ /PD using the Dunn post hoc test following Kruskal-Wallis test of all groups.

^bp < 0.05.

Table 3 Comparative expression of urinary exosomal proteins in patient cohorts with or without *LRRK2* G2019S mutations with or without clinical presentation of Parkinson disease (PD)

Protein	<i>LRRK2</i> −/no PD	<i>LRRK2</i> +/no PD	<i>LRRK2</i> +/PD	<i>LRRK2</i> −/PD	p Value
Columbia cohort	No. = 3	—	No. = 7	No. = 4	
TSG101	1.03 (0.739–1.86)		0.975 (0.657–1.53)	0.758 (0.407–1.22)	0.471 ^a
Total <i>LRRK2</i>	0.642 (0.403–1.27)		0.902 (0.723–1.28)	0.866 (0.511–2.84)	NS ^a
Total <i>LRRK2</i> /TSG101	0.545 (0.345–1.23)		0.922 (0.793–1.60)	1.23 (0.938–2.86)	NS ^a
Ser(P)-1292 <i>LRRK2</i>	0.128 (0.049–0.423)		0.839 (0.663–1.38)	0.187 (0.125–0.594)	0.033 ^{a,b}
Ser(P)-1292 <i>LRRK2</i> /TSG101	0.069 (0.066–0.412)		0.858 (0.388–1.83)	0.320 (0.185–0.631)	0.040 ^{a,b}
Ser(P)-1292 <i>LRRK2</i> /Total <i>LRRK2</i>	0.200 (0.122–0.334)		1.00 (0.779–1.14)	0.221 (0.192–0.269)	0.007 ^{a,b}
MJFF cohort	No. = 16	No. = 16	No. = 14	No. = 16	
TSG101	0.835 (0.385–1.26)	0.695 (0.231–1.13)	1.24 (0.576–1.74)	0.845 (0.558–1.76)	—
Total <i>LRRK2</i>	0.333 (0.204–0.535)	0.625 (0.219–0.892)	0.858 (0.620–1.06)	0.356 (0.192–0.613)	0.409 ^c
Total <i>LRRK2</i> /TSG101	0.418 (0.238–0.686)	0.768 (0.558–1.50)	0.576 (0.513–1.21)	0.343 (0.277–0.577)	NS ^c
Ser(P)-1292 <i>LRRK2</i>	0.221 (0.0767–0.290)	0.359 (0.0315–0.704)	0.880 (0.648–1.29)	0.0299 (0.0005–0.113)	0.026 ^{b,c}
Ser(P)-1292 <i>LRRK2</i> /TSG101	0.235 (0.094–0.458)	0.451 (0.087–0.863)	0.726 (0.498–1.09)	0.033 (0.0002–0.128)	0.459 ^c
Ser(P)-1292 <i>LRRK2</i> /Total <i>LRRK2</i>	0.450 (0.294–0.970)	0.467 (0.145–0.919)	1.00 (0.820–1.17)	0.081 (0.002–0.265)	0.026 ^{b,c}

Abbreviations: *LRRK2*− = G2019S *LRRK2* noncarrier; *LRRK2*+ = G2019S *LRRK2* carrier; NA = not applicable; NS = not significant.

Values are median (interquartile range). p Values > 0.5 are indicated as NS.

^aDetermined by use of Kruskal-Wallis test.

^bp < 0.05.

^cDetermined by comparing *LRRK2*+/no PD and *LRRK2*+/PD using the Dunn post hoc test following Kruskal-Wallis test of all groups. Values found not significant across conditions by Kruskal-Wallis were not pursued and are indicated by a dash.

reductions in *LRRK2* kinase activity via small molecule inhibition.

Higher Ser(P)-1292 *LRRK2* in G2019S carriers with PD.

Urine exosome concentrations of Ser(P)-1292 *LRRK2* and total *LRRK2* are presented in table 3 for the small pilot cohort collected at Columbia University Movement Disorder Center. Levels of Ser(P)-1292 *LRRK2* relative to total *LRRK2* were elevated in *LRRK2* G2019S mutation carriers (table 3). The increase in the ratio of Ser(P)-1292 *LRRK2* to total *LRRK2* due to the G2019S mutation is approximately 4.7-fold and could not be attributed to medications associated with the treatment of PD. In these participants, no difference in the levels of either total *LRRK2* protein or the exosomal protein TSG101 were detected between G2019S carriers and noncarriers. Thus the ratio of Ser(P)-1292 *LRRK2* to total *LRRK2* perfectly distinguished the presence of the G2019S *LRRK2* mutation (area under the curve [AUC] 1.00, figure 2A).

Ser(P)-1292 *LRRK2* levels distinguish clinical PD from nonmanifesting G2019S carriers. Urinary exosome concentrations of Ser(P)-1292 *LRRK2* and total *LRRK2* are summarized in table 3 for the cohort previously collected by the MJFF LCC. Using biobanked urine specimens, we again observed that the *LRRK2* G2019S mutation carriers with PD (*LRRK2*+/PD+) had higher ratios of Ser(P)-1292 *LRRK2* to total *LRRK2*

compared to noncarriers (*LRRK2*−/PD− and *LRRK2*−/PD+, 3.6-fold, p < 0.0001). G2019S carriers with PD had higher ratios of Ser(P)-1292 *LRRK2* to total *LRRK2* protein from asymptomatic G2019S carriers without PD. The control exosome protein TSG101 was similar in both groups. ROC curves were generated to assess the ability of this outcome ratio to distinguish PD status in *LRRK2* G2019S carriers (AUC 0.844, figure 2B). This model demonstrated a sensitivity of 100% and specificity of 63% at the optimal threshold.

DISCUSSION In the current study we identified phosphorylated *LRRK2* in urine exosomes as a potential biomarker for PD risk among *LRRK2* G2019S mutation carriers. Previous studies suggest kinase-activating effects of the G2019S mutation on autophosphorylation activity,¹⁶ with one study suggesting that autophosphorylation of the Ser(P)-1292 residue is required for cellular toxicities caused by pathogenic mutations in the *LRRK2* enzyme in primary neurons.¹¹ In cell culture, we observed that the Ser(P)-1292 *LRRK2* to total *LRRK2* ratio increased due to pathogenic *LRRK2* mutations, including G2019S, and diminished in response to *LRRK2* kinase inhibitors. Although we do not directly assess *LRRK2* kinase activity in our clinical samples, Ser(P)-1292 *LRRK2* may be an effective biomarker surrogate for *LRRK2* kinase activity.

Figure 2 Ser(P)-1292 LRRK2/total LRRK2 predicts Parkinson disease (PD) in LRRK2 G2019S carriers

(A) Receiver operating characteristic (ROC) curve shows Ser(P)-1292 LRRK2/total LRRK2 prediction of the G2019S mutation in PD cases (red line), area under the curve (AUC) = 1.00, sensitivity = 1.00, selectivity = 1.00. (B) ROC curve shows ability of Ser(P)-1292 LRRK2/total LRRK2 to predict PD in G2019S carriers (red line), AUC = 0.844, sensitivity = 1.00, selectivity = 0.625.

To test the hypothesis that Ser(P)-1292 LRRK2 levels may be enhanced in G2019S mutation carriers, we utilized our past observation describing abundant LRRK2 protein in urinary exosomes.¹² Although CSF can also be used to measure LRRK2, we prioritized experiments using urine because of the noninvasive

manner in which the biofluid is collected. We presume that the majority of LRRK2 protein in these exosomes derives from the kidney, although we cannot rule out other sources, including brain-derived exosomes. Ser(P)-1292 LRRK2 levels in urine exosomes may be determined by a combination of genetically encoded factors as well as environmental stimuli.

Ours and other experiments in model systems suggest that the *LRRK2* G2019S mutation should enhance Ser(P)-1292 LRRK2 levels. In a novel small cohort collected in the Columbia Movement Disorders Center using samples directly procured from a routine clinical examination, the average elevation of the ratio of Ser(P)-1292 LRRK2 to total LRRK2 (~5-fold above controls) is achieved with a single *LRRK2* G2019S allele. As the number of participants from this cohort was low, the findings prompted a larger study using biobanked samples. In the MJFF LRRK2 LCC, G2019S carriers with PD again had a higher Ser(P)-1292 LRRK2 to total LRRK2 ratio. Results here cannot be explained by differences between these groups in any clinical measure associated with the cohort. Importantly, samples from both the Columbia and MJFF LRRK2 cohort were provided to the University of Alabama at Birmingham investigators coded, and genotypes were not revealed until after final data curation. Demographics were tightly matched and all participants in this study were male. It will be important to replicate these findings in larger cohorts that include female participants. The utility of our urine test is not to replace the simpler G2019S *LRRK2* DNA test. Rather, our observations provide additional support for the LRRK2 kinase activation hypothesis for *LRRK2*-linked PD, and may provide a novel biomarker for PD susceptibility in *LRRK2* mutation carriers.¹⁷

LRRK2 mutation carriers who will develop PD in their lifetime cannot currently be discriminated from mutation carriers who will not clinically manifest PD. The urine exosome Ser(P)-1292 LRRK2 to total LRRK2 ratio showed a risk prediction statistic of 0.844 for clinical manifestation of PD. Although our sample size was not powered to detect significant clinical differences between asymptomatic and PD-manifesting G2019S carriers, the asymptomatic carriers who showed the 5 highest levels of Ser(P)-1292 LRRK2 had increased geriatric depression and Epworth Sleepiness Scale values compared to G2019S carriers with the 5 lowest levels of Ser(P)-1292 (8.0 ± 2.5 vs 4.0 ± 1.5 , and 3.0 ± 1.4 vs 1.4 ± 0.68 , respectively). Future larger studies that measure Ser(P)-1292 LRRK2 levels over time in asymptomatic carriers will be required to understand the prognostic potential of this new biomarker.

AUTHOR CONTRIBUTIONS

K.B.F., M.S.M., R.N.A., and A.B.W. contributed to the experimental design and analysis of data. K.B.F., R.N.A., and A.B.W. wrote the manuscript. K.B.F. and M.S.M. performed the experiments.

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DISCLOSURE

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