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Caught in the act: LRRK2 in exosomes

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Abstract

Mutations in the *leucine-rich repeat kinase 2* (LRRK2) gene are a frequent genetic cause of late-onset Parkinson's disease (PD) and a target for therapeutic approaches. LRRK2 protein can influence vesicle trafficking events in the cytosol, with action both in endosomal and lysosomal pathways in different types of cells. A subset of late endosomes harbor intraluminal vesicles that can be secreted into the extracellular milieu. These extracellular vesicles, called exosomes, package LRRK2 protein for transport outside the cell into easily accessed biofluids. Both the cytoplasmic complement of LRRK2 as well as the exosome-associated fraction of protein appears regulated in part by interactions with 14–3–3 proteins. LRRK2 inside exosomes have disease-linked post-translational modifications and are relatively stable compared with unprotected proteins in the extracellular space or disrupted cytosolic compartments. Herein, we review the biology of exosome-associated LRRK2 and the potential for utility in diagnosis, prognosis, and theragnosis in PD and other LRRK2-linked diseases.

Introduction

Genome-wide association studies have identified variants in or nearby the *leucine-rich repeat kinase 2* (LRRK2) gene in Parkinson's disease (PD) [1]. In unbiased transcriptome databases, LRRK2 mRNA expression is usually highest in circulating immune cells of the innate immune system, with tissue expression in lung and kidney [2]. In the healthy brain, LRRK2 expression appears relatively confined to a few neuronal subpopulations with a minor fraction of protein in astrocytes in some brain regions [3–5]. In cells, LRRK2 is largely a soluble cytoplasmic protein associated with membranous structures and vesicles, but is largely excluded from the nucleus [6]. LRRK2 does not encode a *trans*-membrane domain and is not a secretory protein, but a fraction of LRRK2 protein appears resistant to soluble extraction and associates with endosomes and lysosomes [7].

Mutations in LRRK2 are the most prevalent genetic cause of PD [8]. In model systems, all tested pathogenic mutations increase both *cis* LRRK2 kinase activity (autophosphorylation) as well as *trans*phosphorylation activity towards Rab substrates [9,10]. This gain of LRRK2 function with respect to these serine and threonine phosphorylation events are consistent with the autosomal-dominant inheritance pattern of pathogenic LRRK2 mutations observed

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Competing Interests

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in many families with genetic PD worldwide. Therefore, inhibitors that block excess LRRK2 activity have been developed as potential interventions for PD [2].

Biomarkers that might reflect pathways underlying LRRK2 pathogenic mechanisms are highly sought after to confirm target engagement for therapeutics and select patients for treatment [2]. The identification of aberrant increased phosphorylation associated with pathogenic LRRK2 mutations has been difficult to study in clinical samples. Tissues with LRRK2 expression such as lung, kidney, and brain are not easily procured in the clinic. Furthermore, LRRK2-post-translational modifications linked to pathogenic mechanisms like autophosphorylation do not appear well preserved in post-mortem tissue samples [11]. The discovery of LRRK2 in exosomes, which capture a diversity of cytosolic proteins not normally secreted through canonical pathways to preserve them in biofluids, may address the biomarker needs for LRRK2-related enquires [7].

In this mini-review, we give an overview of the discovery and localization of extracellular LRRK2 protein to the exosome fraction present in biofluids. We further summarize recent observations in biospecimens from clinical populations, highlight the use of LRRK2 as a biomarker for PD, stress the need for independent replication, and speculate on the potential for exosome-LRRK2 in the successful development of LRRK2-targeted therapeutics.

Exosomal LRRK2

Exosomes are a subset of small extracellular vesicles that originate from within the endosomal pathway. Intraluminal vesicles derive from either the plasma membrane or inward endosomal budding that occurs in a subset of late endosomes called multi-vesicular bodies (MVBs). Originally thought to exclusively assist in degradation pathways via MVB fusion with lysosomes, MVB fusion to the plasma membrane can result in the release of the intraluminal vesicles (Figure 1). These exosomes can bind the plasma membrane of other cells through an often poorly understood network, entering cells through a variety of receptor binding events, endocytosis, or other types of fusion. Exosomes can encapsulate a spectrum of DNA, RNA, and proteins that are not normally secreted or otherwise stable in the extracellular milieu and can readily pass through the blood–brain barrier and specialized compartments throughout the body [12]. The lipid bi-layer protects these molecules from proteases and other degradative enzymes.

As few protein markers have been proposed to specifically label and distinguish exosomes or MVBs from other vesicles within the endolysosomal compartment, electron microscopy studies remain the gold-standard technique to identify MVBs. Several studies suggest that LRRK2 protein can co-localize within MVBs in intraluminal vesicles [6,13]. Some LRRK2 protein in the cytosol complexes with 14–3–3 proteins [14] and 14–3–3 proteins together with annexins and tetraspanin proteins represent some of the most abundant molecules in exosomes [7,14]. From exosomes elicited from HEK-293 cells, LRRK2 protein closely co-eluted with 14–3–3 in gradient separations, together with canonical exosome markers (Figure 1). In subsequent studies in cerebral spinal fluid and urine cleared of cells and other debris, all of the LRRK2 protein we could detect was localized to exosome fraction exclusively, and there is no evidence of LRRK2 secretion in exosome-independent pathways [7,15,16]. In the

heterologous HEK-293 overexpression system, LRRK2 secretion into exosomes could be partially manipulated through 14–3–3 inhibition, LRRK2 kinase inhibition, or overexpression of 14–3–3 proteins [7]. In the exosome fraction of LRRK2, autophosphorylated levels of LRRK2 were similar to that of cytosolic LRRK2, implying once-active LRRK2 protein and not misfolded protein abundant in degradative vesicles [16]. These observations suggest that the release of LRRK2 in exosomes is at its original state (e.g. not misfolded) and depends on regulation processes that involve 14–3–3.

In understanding the biogenesis of exosomes and pathways regulating their release, indirect perturbations far upstream in the endosomal–lysosomal pathway (e.g. lysosomal inhibition) have a cascading effect in exosome formation and release. A subset of Rab GTPases that are important in trafficking of various membrane compartments in the endosomal–lysosomal pathway are shown to interact with LRRK2, but so far there is no evidence of such interactions that affect LRRK2 exosome release [10]. Future experiments with more advanced live cell imaging technology together with sensitive protein interaction classifications may be more revealing as to the protein interactors and pathways responsible for LRRK2 secretion in exosomes.

Exosomal LRRK2 in the diagnosis and prognosis of LRRK2-linked PD

Diagnostic accuracy in PD is typically high in mid-stages of disease, with more than ~90% or greater accuracy, for example using United Kingdom Brain-Bank criteria [17,18]. In early disease, or the so-called prodromal phase, prior to the onset of motor symptoms, diagnostic certainty is much lower [19,20]. As it is common practice in clinical trials evaluating disease-modification to target as early disease as possible, better diagnostic certainty in patients that lack motor symptoms may open the door to a new patient population for neuroprotection therapies. Biochemical biomarkers that address some of these obstacles are in demand [21]. Biomarkers measure some substance that reliably correlates with or predicts some phenomenon of interest is needed for both idiopathic PD patients as well as LRRK2 mutation carriers. In genetic cases of PD positive for pathogenic LRRK2 mutations, DNA analysis cannot currently predict with confidence which G2019S-LRRK2 mutation carriers will go on to develop PD in their lifetime [2]. In a 4 year follow-up of 32 LRRK2 mutation carriers, 12% converted to PD, but the slope of DaT binding decline was similar between the converters and non-converters, suggesting routine imaging approaches may not predict disease [22]. Urinary exosome pS1292-LRRK2, in contrast, highlighted LRRK2 mutation carriers at higher risk of converting to PD [15,16]. In the evaluation of exosomes isolated from donated urine from LRRK2 mutation carriers, a first small pilot cohort of patients seen at the Columbia Movement Disorder Clinic revealed that male G2019S-LRRK2 mutation carriers with PD demonstrated a significant increase in autophosphorylated LRRK2 protein levels (4.6-fold, $P < 0.001$). In a randomized and investigator-blinded experiment using a larger cohort of Ashkenazi Jewish patients, the same trend was identified in male LRRK2 mutation carriers with PD, but age-matched male mutation carriers without PD showed only 2.2-fold over control, less than mutation carriers with PD ($P < 0.001$). Using pS1292-LRRK2 levels in urinary exosomes alone, PD risk prediction receiver operator characteristic (ROC) is 0.84 with 100% sensitivity and 62% specificity in discriminating the diagnosis of PD among mutation carriers [15]. In the third cohort from Norwegian LRRK2 mutation

carriers, the same trend was again identified in male LRRK2 mutation carriers that had the highest levels of pS1292-LRRK2, with less abundance in mutation carriers without PD [16]. LRRK2 levels appear higher in male versus female urinary exosomes [23]. In Norwegian females with the G2019S-LRRK2 mutation, the same test distinguished carriers from non-carriers with higher pS1292-LRRK2 levels, but within carriers, non-manifesting carriers had ~5.4-fold elevations in pS1292-LRRK2 compared with controls versus ~3.6-fold in carriers with PD [16]. These observations were the first made in clinical samples tying LRRK2 kinase-dependent autophosphorylation to the effects of the G2019S-LRRK2 mutation, predicted in numerous model systems to increase kinase activity.

In Norwegian LRRK2 mutation carriers, CSF was isolated within hours of urine donation, but pS1292-LRRK2 measured from CSF did not correlate with urine pS1292-LRRK2, and LRRK2 levels overall in CSF failed to discriminate LRRK2 mutation carriers from non-carriers, or PD from neurologically normal controls [16]. In this first cohort, it was noted that overall pS1292-LRRK2 levels were high in everyone using stoichiometric estimations with total LRRK2 protein, implicating a ceiling effect may prevent group discrimination. Future studies with LRRK2 kinase substrates or autophosphorylation sites in CSF exosomes that do not suffer from saturation or other detection issues, or studies in serum or plasma, may help further shed light on whether LRRK2 activity is enhanced in a proportion of PD cases and whether elevated LRRK2-associated phosphorylation events predict disease risk.

The use of LRRK2 interactors as a diagnostic biomarker for LRRK2 mutation carriers are not established yet. *Trans*-phosphorylation of other Rab substrates like Rab10 may also not be ideal because the most common mutation, G2019S, does not robustly increase levels over WT-LRRK2, and other kinases may target the same site. In contrast, LRRK2 autophosphorylation appears increased in levels via the action of all pathogenic mutations, dramatically in some cases, and this appears true for Rab7L1 *trans*-phosphorylation at least in model systems [9,24].

Overall, these results support the utility of urinary exosome measurements but results should be interpreted with caution until independent replication in other laboratories and longitudinal studies with LRRK2 mutation carriers to assess changes over disease states.

Exosomal LRRK2 in the diagnosis of idiopathic PD

Candidate biomarkers in PD revolve around proteins identified in genetic studies as driving disease in some cases, like α -synuclein and LRRK2, and proteins that are more generalized in neurodegenerative disease, like β -amyloid ($A\beta_{42}$), and tau protein. Current biomarker studies largely focused on CSF detection of α -synuclein, β -amyloid, and tau protein [25]. Cross-sectional and longitudinal studies suggest a slight decrease in some of these CSF biomarkers in PD patients compare with controls, but the clinical meaningful cutoff are still unmet and there are conflicting reports [19,26–29].

With other proteins important in PD, both α -synuclein, $A\beta_{42}$, and tau form aggregates with seeding activity and have substantive post-translational modifications that can be measured in biofluids. Exosomes have likewise been explored in the pathobiology of aggregation,

templating, and propagation [12,30–36]. Preliminary data showed small but significant decreases of CSF exosome α -synuclein that harbor seeding activities in model systems [37]. In contrast, plasma exosome α -synuclein, although not correlated with CSF levels, are increased in PD patients and moderate ability to distinguish PD from controls (70.1% sensitivity and 52.9% specificity) [38]. It is also important to note that α -synuclein is also expressed in red blood cells and contamination during sample collection procedures are subject to alter the result in large clinical cohorts [39].

In contrast, LRRK2 is not aggregating or subject to any conformation changes in the disease phenotype. The increased kinase activity of LRRK2, which is reflected by autophosphorylation or its downstream substrates, is clearly contributing to PD susceptibility and LRRK2 seems to be exclusively enriched in exosome in biofluids. Genetic association studies demonstrate common variability in *LRRK2* is also linked to susceptibility to idiopathic PD, independent from pathogenic mutations [40]. To evaluate the urinary exosome pS1292-LRRK2 biomarker in the first cross-section of idiopathic PD cases, 80 PD cases and sex and age-matched controls were enrolled into the Parkinson's Disease Biomaker Program and screened for LRRK2 mutations [23]. In donated urine samples, the median pS1292-LRRK2 levels were 30% higher in a fraction of the PD patients compared with healthy controls, but the group overlap was much higher than that between mutation carriers and controls, with the difference not particularly useful for diagnostic biomarker development (ROC = 0.64). A recent study showed total LRRK2 is increased in neutrophils in idiopathic PD patients by 25%, which is similar to what was observed in urine exosome pS1292-LRRK2 [23,41]. However, pT73-Rab10, a substrate of LRRK2, was not significantly up-regulated accordingly and was not correlated with LRRK2 expression, suggesting other kinases besides LRRK2 may phosphorylate Rab10 [41,42].

Exosomal LRRK2 in the prognosis of PD

Clinical markers that are promising for diagnosis may not necessarily qualify as progression markers [43]. Although the biochemical biomarkers α -synuclein, A β 42, and tau are extensively evaluated, conclusive biomarker panels are not yet successful as prognostic biomarkers [26,43]. So far, A β 42 is correlated with cognitive decline [19,26,43–45], but no biomarkers or their combination was able to predict the conversion of newly diagnosed PD patients to mild cognitive impairment PD [39,43,46]. PD medications may further skew CSF α -synuclein levels [39]. Thus, novel biomarkers, such as LRRK2, Rabs, and recently reported synaptic proteins could be prioritized for better prediction of PD progression [47].

PD patients with the highest levels of pS1292-LRRK2 (autophosphorylated LRRK2) significantly correlated with the lowest cognitive performance (MoCA, ROC = 0.73 for bottom quartile) and impairments in daily living (Mod S&E, ROC = 0.79 for the bottom quartile). Longitudinal studies are needed to evaluate the potential of LRRK2 as prognostic biomarker for PD. Compared with common genetic markers present in *LRRK2* linked to PD susceptibility, it is also proposed that the biochemical measurement of pS1292-LRRK2 is potentially a more powerful and meaningful way to identify idiopathic PD patients that may benefit most from LRRK2-directed therapeutics, should kinase activity and LRRK2 kinase inhibitors prove beneficial in LRRK2 mutation carriers.

Exosomal LRRK2 as a theragnostic biomarker

The gain of function characteristic of LRRK2 contribution in PD pathogenesis attracts industry to develop potent, selective, blood–brain barrier permeable LRRK2 inhibitor approaches to block kinase activity or expression. The goal is not to alleviate symptoms associated with PD but to slow or block the progression of disease. To date, clinical trials for neuroprotective agents in PD have not included successful integration of theragnostic markers, for example in demonstrating target engagement. Theragnostic biomarkers should serve two purposes: First, a theragnostic biomarker can stratify patient suitable in the treatments. The patient stratification approach for LRRK2-targeted therapeutics can optimize the effect size of LRRK2 inhibition in finding patients with elevated (compared with others) LRRK2 autophosphorylation. Second, a theragnostic biomarker can be used to access target engagement efficiency in both the CNS and periphery. Future LRRK2-targeted trials should include strong theragnostic biomarkers to ensure the disruption of LRRK2 kinase activity or expression in the brain. Without theragnostic markers to determine whether the target of interest had been successfully engaged or biochemical effect achieved, many trials have ended without knowing whether a specific endpoint had been achieved. Historical examples include trials with coenzyme Q10 that did not attempt to demonstrate increased complex I energy production in PD patients [48], inhibition of mixed-lineage kinases that did not measure kinase inhibition in PD patients [3], as well as current ongoing trials that include calcium channel blockers [49] and c-Abl kinase inhibitors that both do not incorporate attempts to measure on-target effects [50,51].

In the periphery, there are several opportunities to directly measure LRRK2 inhibition in the context of a clinical trial. LRRK2 expression in circulating immune cells is high and the direct procurement of immune cell subpopulations may prove to be a powerful approach to measure LRRK2 inhibition in clinical populations [52]. However, autophosphorylated LRRK2 has proved difficult to measure in immune cells for reasons that are not clear. Lower constitutive phosphorylation (pS935 or pS910-LRRK2) of LRRK2 is often used to measure LRRK2 inhibition, but this phosphorylation is also suppressed by several pathogenic LRRK2 mutations (e.g. R1441C, Y1699C) that active kinase activity, thereby confounding interpretation. So far, *ex vivo* LRRK2 inhibitor treatment of human peripheral immune cells showed dose response for its downstream substrate pT73-Rab10 [41,53]. But *in vivo* dosing treatment with LRRK2 inhibitor in rodent failed to show correlated inhibition [42]. In general, biomarkers centering on immune cell subpopulations may be difficult to translate because LRRK2 is highly responsive to pro-inflammatory stimuli so that immune cells from subjects with altered immunity may respond differently to LRRK2-targeting therapeutics [2]. Furthermore, peripheral inhibition in an LRRK2-positive immune cell may not predict LRRK2 inhibition in the brain. Future studies with periphery-CNS inhibition profiles for LRRK2-targeting therapeutics in model systems may help clarify some of these issues.

For a direct measure of LRRK2 in the brain, CSF may provide a convenient biofluid for early clinical studies in validation as well as correlation studies with peripheral markers that predict CSF effects, such as LRRK2 measured in urine. All of the LRRK2 protein, we could detect in fractionated CSF resides in the exosomeenriched fraction, with abundant phosphorylated LRRK2 protein available for measurement [16]. In a critical assessment of

the interaction of 14–3–3 and LRRK2 with respect to exosome localization, a loss of 14–3–3 binding or interaction with LRRK2 caused by kinase inhibition may generally shift LRRK2 cytoplasmic solubility or localization. These changes may indirectly change the proportion of LRRK2 that is released in exosomes (Figure 1). If *in vitro* studies are predictive of what to expect in the brain with LRRK2 inhibition, we would anticipate both diminished phospho-LRRK2 (e.g. pS1292-LRRK2) as well as total LRRK2 protein, possibly due to a loss of 14–3–3 binding and trafficking to exosomes [54]. As vesicles for measuring proteins, CSF exosomes preserve LRRK2 at a stable stage until uptake, but the secretion and turnover rate of CSF exosomes is yet unknown [31]. While procurement of enough CSF in rodent models presents technical challenges, initial LRRK2 inhibitor studies in non-human primates could be informative to understand what to expect in a phase clinical trial. While hundreds of studies have been conducted with exosomes in the research space, few have used CSF as a source of exosomes and thus further studies are required to assist in protocol development and interpretation. Direct validation of LRRK2 kinase inhibition in the brain may be a critical step in interpreting the results of a clinical trial, whether positive or negative results emerge.

Conclusions

Exosomes are ubiquitous in biofluids and serve as a robust source of proteins for biomarker development. Little is yet known regarding the specific mechanisms that lead to the recruitment of LRRK2 protein into exosomes, but the mechanism appears at least in part kinase-sensitive and dependent on interactions with 14–3–3 proteins. Measurements of LRRK2 in exosomes provides some evidence to link phosphorylation events that characterize LRRK2 mutation carriers with PD and with a proportion of idiopathic PD that lack LRRK2 mutations, suggesting that exosomal LRRK2 measures may help identify strata of PD patients that could benefit from LRRK2-targeting therapeutics. Initial cross-sections of patients reveal that the patients with higher levels of pS1292-LRRK2 may have a more severe disease course, although future studies are required in longitudinal series to test this hypothesis. Finally, LRRK2-targeted therapeutics should proceed in the clinic together with validated theragnostic markers of LRRK2 inhibition. The exosome fraction of LRRK2 protein may provide a robust resource for these efforts.

Future perspectives

There are pressing questions surrounding the nature of most candidate biomarkers in neurodegenerative disease as well as in health, and exosomal LRRK2 is no exception. Presently, it is not known whether pS1292-LRRK2 levels are dynamic with disease or a more stable trait with lower lifetime variability. The source of LRRK2-containing vesicles in urine is also not clear, whether they come from the brain, kidney, or some combination of tissues. The implementations of newer and more sensitive standardized and scalable assays that can reliably detect exosome-LRRK2 are needed. Last, it is unlikely that one biomarker will stand on its own for effective prediction of disease progression, so incorporation of the most important biomarkers in informative panels may be the best way to help ensure the success of future clinical trials and improve standards of care.

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Abbreviations

LRRK2	leucine-rich repeat kinase 2
MVBs	multi-vesicular bodies
PD	Parkinson's disease
ROC	receiver operator characteristic

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Figure 1. LRRK2 in exosomes.

LRRK2 can be secreted into easily accessed biofluids through exosomes released upon the fusion of MVBs to the plasma membrane. Without active degradative enzymatic activities occurring in exosomes (e.g. like in lysosomes), LRRK2 is well preserved inside exosomes, a surrogate for its cellular levels. LRRK2 inhibitors that disrupt the LRRK2 and 14-3-3 interaction may diminish LRRK2 secretion through exosomes into biofluids.