

VALIDATION OF BLOOD PRKN MRNA EXPRESSION AS A BIOMARKER FOR PRKN-RELATED PARKINSON'S DISEASE

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ABSTRACT

Background: Mutations in the *PRKN* gene, encoding the E3 ubiquitin ligase Parkin, are a major cause of early-onset, autosomal recessive Parkinson's disease (PD). Identifying carriers currently requires complex genetic testing. Prior work suggested that *PRKN* mRNA levels in peripheral blood mononuclear cells (PBMCs) may discriminate PRKN-related PD from idiopathic PD (iPD) and controls. **Objective:** To validate and expand previous findings using a partially independent cohort and a novel RT-PCR primer set, assessing the diagnostic potential of *PRKN* mRNA qualification as a screening tool for PRKN-related PD. **Methods:** PBMCs were isolated from 7 PRKN-related PD patients (3 biallelic, 4 heterozygous), 8 iPD patients and 7 matched healthy controls. *PRKN* mRNA expression was quantified using RT-PCR with newly designed primers. Genetic characterisation employed Multiplex Ligation-dependent Probe Amplification (MLPA) and Whole Exome Sequencing (WES). Statistical comparisons were performed using ANOVA with post hoc tests and diagnostic accuracy was assessed by ROC analysis. **Results:** PRKN mRNA levels were significantly reduced in PRKN-related PD compared to controls (181-fold or 99,45% lower, $p=0,0041$) and iPD (74-fold or 98,7% lower, $p=0,021$). Expression levels were uniformly low in both biallelic and heterozygous carriers. ROC analysis showed 100% sensitivity and specificity for distinguishing PRKN mutation carriers from non-carriers (PPV=100%, 95% CI: 72,3-100%). **Conclusion:** Peripheral PRKN mRNA quantification is a highly sensitive and specific marker for PRKN mutation carrier status in PD, including in the heterozygous state. This non-invasive assay holds promise as a rapid screening tool, but warrants larger, multicentre validation.

Keywords: Parkinson's disease, Parkin, PRKN, PBMCs, RT-PCR

ΑΞΙΟΛΟΓΗΣΗ ΤΗΣ ΕΚΦΡΑΣΗΣ MRNA ΤΟΥ ΓΟΝΙΔΙΟΥ PRKN ΣΤΟ ΠΕΡΙΦΕΡΙΚΟ ΑΙΜΑ ΩΣ ΒΙΟΔΕΙΚΤΗ ΓΙΑ ΤΗ ΝΟΣΟ ΤΟΥ ΠΑΡΚΙΝΣΟΝ ΠΟΥ ΣΧΕΤΙΖΕΤΑΙ ΜΕ ΜΕΤΑΛΛΑΞΕΙΣ ΤΟΥ ΓΟΝΙΔΙΟΥ ΤΗΣ ΠΑΡΚΙΝΗΣ

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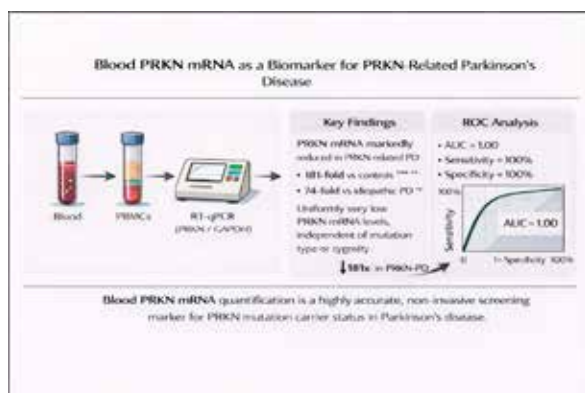
ΠΕΡΙΛΗΨΗ

Υπόβαθρο: Μεταλλάξεις στο γονίδιο της παρκίνης (PRKN), που κωδικοποιεί την E3 ουβικουιτίνη-ηλιγάση παρκίνη, αποτελούν σημαντική αιτία πρώιμης έναρξης, αυτοσωμικής υπολειπόμενης νόσου του Πάρκινσον (NP). Η αναγνώριση φορέων απαιτεί σήμερα πολυπλόκο γενετικό έλεγχο. Προηγούμενες μελέτες έδειξαν ότι τα επίπεδα mRNA του PRKN σε μονοπύρνα κύτταρα περιφερικού αίματος μπορεί να διακρίνουν τη νόσο του Πάρκινσον που σχετίζεται με PRKN από την ιδιοπαθή μορφή και από υγιείς μάρτυρες. **Σκοπός:** Η επικύρωση και επέκταση προηγούμενων ευρημάτων με τη χρήση μερικώς ανεξάρτητου δείγματος και ενός νέου σετ εκκινητών RT-PCR, αξιολογώντας τη διαγνωστική αξία της ποσοτικοποίησης του mRNA του PRKN ως εργαλείου προσυμπτωματικού ελέγχου για τη νόσο που σχετίζεται με PRKN. **Μέθοδοι:** Απομονώθηκαν PBMCs από 7 ασθενείς με νόσο Πάρκινσον σχετιζόμενη με PRKN (3 με ομόζυγες μεταλλάξεις, 4 ετερόζυγοι), 8 ασθενείς με ιδιοπαθή νόσο Πάρκινσον και 7 υγιείς μάρτυρες. Η έκφραση του mRNA του PRKN

ποσοτικοποιήθηκε με Real time – Polymerase Chain Reaction (RT-PCR) χρησιμοποιώντας νέους εκκινητές. Ο γενετικός χαρακτηρισμός πραγματοποιήθηκε με MLPA και αλληλούχιση ολόκληρου εξώματος (WES). Οι στατιστικές συγκρίσεις έγιναν με ANOVA και δοκιμές post hoc, ενώ η διαγνωστική ακρίβεια αξιολογήθηκε με ανάλυση ROC. **Αποτελέσματα:** Τα επίπεδα mRNA του PRKN ήταν σημαντικά μειωμένα στη νόσο Πάρκινσον που σχετίζεται με PRKN σε σύγκριση με τους υγιείς μάρτυρες (181 φορές ή 99,45% χαμηλότερα, $p=0,0041$) και με την ιδιοπαθή μορφή (74 φορές ή 98,7% χαμηλότερα, $p=0,021$). Τα επίπεδα έκφρασης ήταν ομοιόμορφα χαμηλά τόσο σε ομόζυγα άτομα με μεταλλάξη όσο και σε ετερόζυγους φορείς. Η ανάλυση ROC έδειξε 100% ευαισθησία και ειδικότητα στη διάκριση φορέων μεταλλάξεων PRKN από μη φορείς (PPV=100%, 95% CI: 72,3–100%). **Συμπεράσματα:** Η ποσοτικοποίηση του mRNA του γονιδίου PRKN στο περιφερικό αίμα αποτελεί έναν εξαιρετικά ευαίσθητο και ειδικό δείκτη για την παρουσία μεταλλάξεων PRKN στη νόσο Πάρκινσον, ακόμη και στην ετερόζυγη κατάσταση. Η μη επεμβατική αυτή μέθοδος παρουσιάζει προοπτικές ως γρήγορο και εύχρηστο εργαλείο προσυμπτωματικού ελέγχου, αλλά απαιτείται επιβεβαίωση με μεγαλύτερες, πολυκεντρικές μελέτες.

Λέξεις-κλειδιά: Νόσος Πάρκινσον, Parkin, PRKN, PBMCs, RT-PCR

Graphical Abstract



INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting 1% of individuals over 60 years old.^[1] Mutations in **PRKN**, encoding the mitochondrial quality –control protein Parkin, cause up to 50% of early –onset autosomal recessive PD (ARPD).^[2–5] PRKN-associated PD is characterised by early onset, slow progression and marked dopaminergic responsiveness, but genetic confirmation requires advanced laboratory methods.^[4,5]

Parkin acts in the PINK1/Parkin pathway to remove damaged mitochondria via mitophagy. Loss-of-function PRKN mutations reduce mitochondrial quality control, contributing to selective degeneration of substantia nigra pars compacta dopaminergic neurons.^[6,7] In addition to biallelic pathogenic variants, the role of heterozygous variants as disease susceptibility factors is under investigation.^[8]

We previously demonstrated that **PRKN** mRNA levels in PBMCs could accurately differentiate PRKN-related PD from iPD and controls.^[9] The present study aimed to independently validate these findings using

a novel RT-PCR primer design and additional subjects with **PRKN** mutations, exploring the potential of **PRKN** mRNA quantification as a clinically feasible screening tool.

MATERIALS AND METHODS

Participants

These groups were enrolled at the First Dept of Neurology, Eginition University Hospital, Athens, Greece:

- PRKN-related PD (n=7, 3 biallelic, 4 heterozygous)
- Idiopathic PD (n=8)
- Healthy controls (n=7)

Groups were matched for age and sex where possible. All participants provided written informed consent. Ethical approval was obtained from the Scientific Committee of Eginition Hospital.

Genetic analysis: **PRKN** variants were identified using MLPA (Neurogenetics Unit, Eginition Hospital) and WES (Greek Genome Centre, BRFAA). Variant classification followed ACMG guidelines. Further details on the methods of genetic testing are provided by Papagiannakis et al (2024).^[9]

PBMC Isolation, RNA extraction and Reverse Transcription: PBMCs were isolated by Ficoll gradient classification. Total RNA was extracted, quantified (NanoDrop), and assessed for integrity. Reverse transcription was performed using random hexamers. Further details on these methods are provided by Papagiannakis et al (2024).^[9]

Quantitative RT-PCR: A novel primer pair targeting PRKN with the following sequences:

- PRKN forward primer: 5' - AAA ACC ACC AAG CCC TGT C-3'
- PRKN reverse primer: 5' TGC GGA CAC TTC ATG TGC -3'

was designed to optimise amplification efficiency. The aforementioned primer pair produced better

meting curves in the subsequent trial of RT-PCR compared to the ones used in Papagiannakis et al. (2024),^[9] and therefore it was used in all subsequent analyses. GADPH served as the reference gene. Relative expression levels were calculated using the $\Delta\Delta C_t$ method. Apart from the novel primer set, the methodology followed the lines of Papagiannakis et al (2024),^[9]

Statistical Analysis: Group comparisons used one-way ANOVA with Bonferoni correction. ROC analysis determined sensitivity, specificity and predictive values.

RESULTS

Participant characteristics

No significant difference in age or sex distribution was observed between groups ($p>0.05$). PRKN-related PD patients had an earlier mean age of onset ($38,3 \pm 10,2$ years) compared to iPD ($56,5$

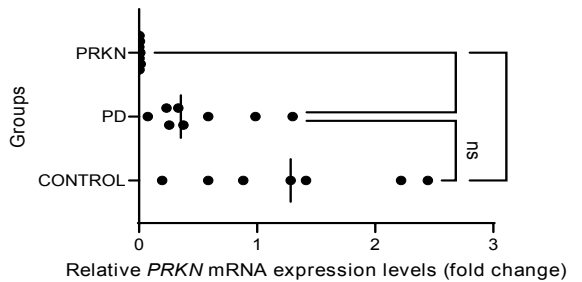


Figure 1. Relative PRKN mRNA expression (fold change) among groups (Controls, PD, PRKN).

$\pm 7,7$ years) (Table 1). The PRKN mutation types and their clinical significance are depicted in Table 2. The samples with the genetic background of c101_102delAG;Exon 7 del (7), Exon 2 del (3) and Exon 6: c540delinsGC:p.V181Rfs*16 (5) have already been tested with the PRKN mRNA assay in our previous study,^[9] while the samples with the variants c401T>C (6), c758G>A; exon 2 deletion (4), c101_102delAG homozygous (1) and c376C>T (2) were tested for the first time, which permits us to verify, using yet another primer set, and extend our previous findings.

Table 1. Demographic characteristics of study participants.

Characteristics	CT (n=7)	iPD (n=8)	PRKN (n=7)
Mean age (years)	59.9 (11.6)	59.9 (7.5)	46.7 (14.4)
Mean age of onset	-	56.5 (7.71)	38.29 (10.19)
Sex (M/F)	1/6	5/3	4/3
Disease duration (years)	-	2.88 (1.22)	8.67 (8.03)
PRKN mutations (Bial/Het)	-	-	3/4

CT: controls; iPD: idiopathic Parkinson disease patients; PRKN: Parkin-related Parkinson’s disease patients; M: male; F: female; Bial: biallelic; Het: heterozygous; Standard Deviation in parentheses.

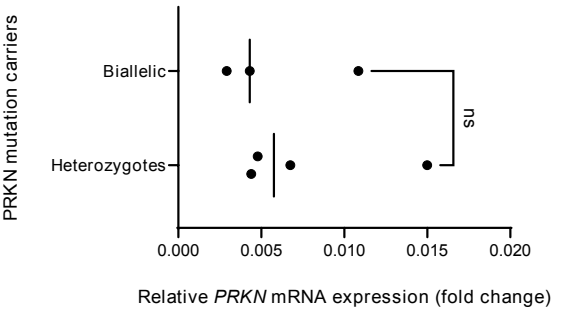


Figure 2. Relative PRKN mRNA expression levels (% fold change) among PRKN mutation carriers (biallelic, heterozygotes).

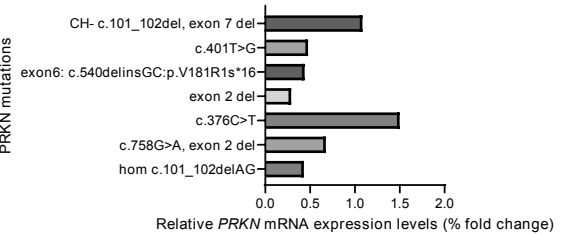


Figure 3. Relative PRKN mRNA expression levels (% fold change) among PRKN mutations carriers (regarding type of mutation).

PRKN mRNA Expression: PRKN mRNA levels differed significantly between groups ($p<0.0001$). Expression was markedly reduced in PRKN-related PD samples compared to both controls and iPD (Figure 1). PRKN expression levels were decreased in biallelic and heterozygous PRKN mutation carrier PD patients compared to healthy controls (181-fold or 99,45% lower) and this relation was statistically significant ($p\text{-value}=0.0004$). Similarly, mRNA levels were significantly decreased in PRKN-related PD compared to iPD (74-fold or 98,7% lower, $p\text{-value}=0.0210$). PRKN mRNA levels were slightly decreased in iPD compared to healthy controls (2,44-fold or 59% lower), but this relationship was not statistically significant ($p\text{-value}=0.6497$) (Table 3). PRKN mRNA levels were uniformly very low in all PRKN mutation carriers, even heterozygotes (Figures 1,2). In fact, heterozygote mRNA levels were not significantly different from biallelic carriers ($p\text{-value}=0.6458$). Individual values

Table 2. Type of mutation of *PRKN*-related PD patients and clinical significance.

Patient	Mutation	Status	Clinical Significance	Protein
1	c.101_102delAG	Homozygous	Pathogenic	p.Gln34Argfs*5
2	c.376C>T (exon 4)	Heterozygous	Pathogenic-likely pathogenic	p.Arg126Trp
3	Exon 2 del	Heterozygous	Pathogenic	-
4	c.758G>A; exon 7 del	Compound heterozygous	Pathogenic/likely pathogenic	p.Cys253Tyr
5	Exon 6: c540delinsGC:pV181Rfs*16	Heterozygous	Uncertain significance	p.V181Rfs*16
6	c401T>C	Heterozygous	Uncertain significance	p.Leu.134Pro
7	c101_102delAG; exon 7 del	Compound Heterozygous	Pathogenic	p.Gln34fs

Table 3. Fold-change of expression levels and % *PRKN* expression reduction between the 3 groups.

Groups	Fold-change	% <i>PRKN</i> expression	p-value
iPD vs CT	2,44-fold lower	59% lower	0.6497 (ns)
PRKN vs CT	74-fold lower	98,7% lower	0.0004 (***)
PRKN vs iPD	181-fold lower	99,45% lower	0.0210 (*)

CT: controls; iPD: idiopathic Parkinson disease patients; PRKN: Parkin-related Parkinson's disease patients and their statistical significance (p-value).

for each PRKN-PD subject are presented in **Figure 3**.

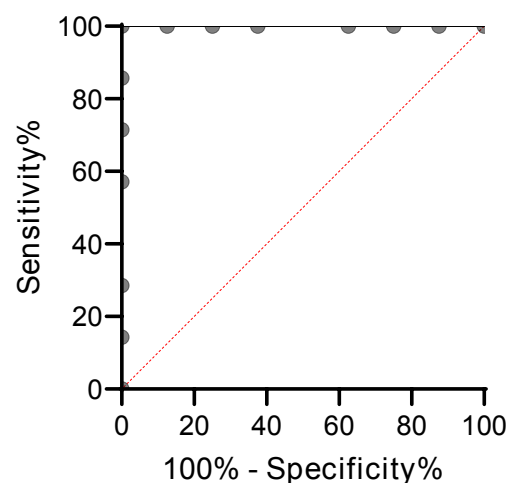
Diagnostic Performance

ROC analysis was performed to assess the usefulness of *PRKN*-expression levels as discriminator of the presence of *PRKN* variants in PD patients. The area under the curve was 1000 (95% confidence intervals 1.000 to 1.000), for the cut-off value of *PRKN* mRNA expression Ct=32.85, yielding a sensitivity of 100 % (64.57-100%) and a specificity of 100% (67.56-100%). In the context of PD, the positive predictive value of low *PRKN* expression levels for the presence of heterozygote or biallelic *PRKN*-variants was 100% (72.3%-100%) (**Figure 4**).

DISCUSSION

Our study provides new insights into the differential expression of *PRKN* mRNA in PBMCs across various groups, including healthy controls, idiopathic PD patients and PD patients carrying various *PRKN* variants. The findings underscore the critical role of *PRKN* expression in the pathophysiology of PD and its potential as a biomarker for the identification of *PRKN*-variant carriers.

A major result of our study is that pathogenic variants in the *PRKN* gene result in reduced expression of the respective mRNA in PBMCs. This result


Figure 4. ROC analysis.

was evident not only in biallelic individuals, but also in heterozygous PD patients. Although the role of heterozygous *PRKN* variants in the pathogenesis of PD remains controversial,^[8] our results suggest that a profound *PRKN* expression impairment, beyond what would be expected from a single allele disruption, is present in most heterozygote *PRKN*-variant carriers who have already developed early-onset PD. The sig-

nificant reduction in **PRKN** mRNA expression among **PRKN** variant carriers, compared to both healthy controls and iPD, corroborates the prevailing idea that these genetic variations are strongly associated with reduced **PRKN** activity. Such reduced activity is linked to compromised mitochondrial quality control and dopaminergic neuronal death.^[6,7] Interestingly, iPD patients also exhibited slightly lower **PRKN** mRNA levels compared to healthy controls. This non-significant trend may suggest a broader involvement of **PRKN** dysregulation in iPD cases or reflect heterogeneity within this subgroup. Alternatively, it might just be the result of the limited number of samples studied; indeed, in the previous study from the lab, no difference was discerned between iPD and controls regarding PBMC **PRKN** mRNA levels, although again levels in controls were slightly higher.^[9] It would be important to perform such studies in a larger scale in the future.

It is striking that, in all cases with **PRKN** mutations examined, whether heterozygous or biallelic, **PRKN** mRNA levels were extremely low. This is true not only for large deletions, and the small deletion c.101_102delAG (which creates a premature translational stop signal in the **PRKN** gene), but is also the case even for 3 single nucleotide variants (c.376C>T, c.401T>C, c.758G>A). A possible mechanism of mRNA degradation in the case of frameshift mutations produced by deletions is the process of non-sense mediated mRNA decay.

It has been suggested that heterozygous **PRKN**-mutation carriers, even when asymptomatic, carry a small degree of dopaminergic nigrostriatal dysfunction. It is possible that participants in our cohort did not have the necessary compensatory mechanisms, as they have already developed PD. The fact that in most cases in our study these mutations lead to an important reduction of **PRKN** mRNA levels close to those in biallelic carriers suggests that additional related variants may exist in the other allele, possibly in regulatory regions for **PRKN** expression. Long read sequencing of 4 heterozygote carriers in our original study, however, did not reveal any additional causal genomic alterations. Long read sequencing for the two new heterozygote samples in the current study has not been performed. It remains quite puzzling why heterozygote mutation carriers have such low **PRKN** mRNA levels, quite similar to those with biallelic mutations. An explanation, apart from a yet unidentified second genetic hit, could be that in these cases there are further epigenetic mechanisms that lead to a substantial reduction of **PRKN** mRNA levels and the resultant early disease manifestation.

These results align with the findings of our previous study.^[9] In 3 samples the results of the prior study were verified using another primer pair, different from the ones in our previous work, providing an

independent verification. The primer pair evaluated in the current work gave the best melting curves and thus would appear to be the best choice at this point for assessing accurately **PRKN** mRNA levels in PBMCs. Furthermore, results of low **PRKN** mRNA levels in **PRKN** mutation carriers were extended to 4 additional samples with the following genetic background: the homozygous c.101_102delAG mutation, the heterozygous c.376C>T mutation, the heterozygous c540delinsGC mutation in exon 6, and the heterozygous 401T>G mutation. All these samples had very low **PRKN** mRNA levels, which is especially notable for the heterozygous cases, as these 3 additional mutations had not been previously assessed.

As far as diagnostic implications are concerned, PBMCs are easily accessible blood cells, while RNA isolation, reverse transcription and quantitative PCR are rather standard laboratory methods; as such, the methodology described here holds promise as a first screening tool to assess for the presence of **PRKN** pathogenic variants. The ROC analysis we have performed shows high sensitivity and specificity for the recognition of a PD patient with a **PRKN** variant. With a ROC area under the curve of 1.00 and sensitivity and specificity both at 100%, **PRKN** mRNA levels appear to be a robust biomarker for detecting the presence of **PRKN** variants. In fact, the positive predictive value of low **PRKN** levels with this assay is 100% indicating that the current protocol may be extremely useful for screening for **PRKN**-mutation carrier status in the PD population. This finding has significant implications for clinical practice, as it offers a non-invasive and reliable method for identifying genetic subtypes of PD, which may inform personalised therapeutic strategies. To our knowledge, there are no other studies reporting an easily accessible screening test for **PRKN** mutation status. This could simplify the genetic work-up of PD patients, especially in underserved areas, with limited access to genetic testing.

Limitations of our study include a small sample size and single-centre design. Larger, multi-ethnic cohorts are needed to validate clinical applicability at a larger clinical scale.

CONCLUSION

This report, using a new set of primers and additional cases, confirms that **PRKN** mRNA quantification in PBMCs is a promising biomarker for **PRKN** mutation status in PD. With its high diagnostic accuracy, it may become a useful screening tool for genetic testing, enabling earlier identification and, in the future, targeted management of **PRKN**-related PD. Furthermore, the present report extends on previous findings that suggest that severe suppression of **PRKN** mRNA levels is a common feature of **PRKN**-related

PD, irrespective of heterozygote or biallelic status or the particular mutation type. This intriguing biological phenomenon warrants further investigation into the underlying mechanisms regulating *PRKN* transcription.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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