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Short communication

Differential expression of gut miRNAs in idiopathic Parkinson's disease

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ABSTRACT

Objective: In the present work, we aimed to investigate the expression of microRNAs (miRNAs) in routine colonic biopsies obtained from patients with idiopathic Parkinson's disease (PD) and to address their value as a diagnostic biomarker for PD and their mechanistic contribution to PD onset and progression.
Methods: Patients with PD (n = 13) and healthy controls (n = 17) were prospectively recruited to undergo routine colonic biopsies for cancer screening. Total RNA was extracted from the biopsy material and the expression of miRNAs was quantified by Illumina High-Throughput Sequencing.
Results: Statistical analysis revealed a significant submucosal enrichment of the miRNA hsa-miR-486–5p in colonic biopsies from PD patients compared to the control subjects. The expression of miR-486–5p correlated with age and disease severity as measured by the UPDRS and Hoehn & Yahr scale. miRNA gene target analysis identified 301 gene targets that are affected by miR-486–5p. A follow-up associated target identification and pathway enrichment analysis further determined their role in distinct biological processes in the enteric nervous system (ENS).
Interpretation: Our work demonstrates an enrichment of submucosal miR-486–5p in routine colonic biopsies from PD patients. Our results will support the examination of miR-486–5p as a PD biomarker and help to understand the significance of the miR-486–5p gene targets for PD onset and progression. In addition, our data will support the investigation of the molecular and cellular mechanisms of GI dysfunction in PD.

1. Introduction

Recent research suggests the affection of the enteric nervous system (ENS) in Parkinson's disease (PD) since previous biopsy and autopsy studies demonstrated Lewy-type Synucleinopathy (LTS) in the ENS of PD patients [1]. In addition to these pathological findings, gastrointestinal (GI) symptoms such as e.g. constipation are common in PD and sometimes appear years prior to the motor signs of the disease [2]. Because the appearance of LTS in enteric neurons may precede LB pathology in the brain, those results prompted the hypothesis that PD is caused by an environmental pathogen that breaches the mucosal barrier of the GI tract to initiate a pathological process in enteric neurons and that this process progresses towards the CNS via the autonomic nerves of the gut [3].

As a consequence of this hypothesis, previous studies examined the diagnostic value of LTS in gut tissue as an early disease biomarker in PD. However, many of these reports have raised concerns regarding the specificity of enteric α-Syn since its immuno-reactivity was also ob-

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served in healthy individuals [4,5]. This discrepancy may, in part, result from technical difficulties inherent in the preparation of human tissue samples for the detection of α-Syn. Therefore, the overall diagnostic value of detecting LTS in GI biopsies to diagnose PD currently remains uncertain. In addition, investigating enteric α-Syn has not contributed further insight into the specific cause of GI symptoms. Thus, there is a need for alternative GI biomarkers in PD that support the understanding of GI dysfunction and the molecular mechanisms of disease onset in PD.

Here, we investigated the expression of miRNAs in routine colonic biopsies from 13 PD patients and 17 healthy control subjects. When comparing both groups, we found a number of differentially expressed, submucosa-enriched miRNAs with hsa-miRNA-486–5p having the highest specificity for PD. The expression of miRNA-486–5p was found to correlate with age and disease severity in PD. The follow-up bioinformatics analyses identified a number of miRNA-486-5p-target genes and their associated cellular pathways in the enteric nervous system (ENS). In summary, our results will support the examination of miRNAs as diagnostic biomarkers in PD and contribute to the understanding of GI symptoms in PD.

2. Methods

2.1. Study subjects

A total of n = 30 patients completed the study. N = 13 subjects had a history of PD (PD group) and n = 17 subjects had no history of prior neurological or gastrointestinal (GI) disease (control group). PD patients undergoing routine colon cancer screening were recruited from the Movement Disorder Outpatient Clinic at the Ludwig Maximilian University Hospital. Control subjects without PD were recruited at from the GI outpatient clinic and the Department of Medicine III at the Ludwig Maximilian University Hospital. Individuals meeting the UK Parkinson's Disease Society Brain Bank criteria for PD without known primary gastrointestinal illness were eligible as PD subjects. All subjects with PD were assessed using the Unified Parkinson's Disease Rating Scale, Part II and III (UPDRS-II/-III) in the “on” state during the subjects' regular clinic visit. All participants provided written informed consent and all procedures were conducted in accordance with a protocol that received prior approval by the University Health Network Research Ethics Boar at Ludwig Maximilian University Munich, Germany.

2.2. Colonic mucosal biopsy

Colonoscopy was performed according to standard procedures. Biopsies were obtained using standard biopsy forceps (BDL-1 x Biopsy Forceps without spikes, Ø 2.3 × 2300 mm, coated, 155-929-02, Pauldrach) in the sigmoid colon (approx. 20 cm from the anal verge. From this region, 3–4 biopsies were collected from each subject and pooled. The tissue was immediately transferred into a tissue preservative (DNA/RNA-Shield, Zymo Research), frozen and stored at −80 °C until used. In order to assess tissue-specific miRNA expression in mucosal vs. submucosal tissue, biopsies from additional 3 healthy control subjects were obtained as described above. The tissue was transferred into saline and the mucosa stripped from the submucosa. Either tissue compartment (i.e. mucosa and submucosa) was then transferred into tissue preservative separately, frozen and stored at −80 °C until used.

2.3. RNA isolation and sequencing

Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, Massachusetts) according to the instructions of the manufacturer. Sequencing libraries were prepared using 10–50 ng of total RNA with the NEXTflex Small RNA Seq Kit v 3 (Bio Scientific) according to the small RNA protocol of the manufacturer. A pool of libraries was used for sequencing at a concentration of 10 nM. Sequencing of 1 × 75 bp was performed with an Illumina NextSeq 550 sequencer at the sequencing core facility of the IZKF Leipzig (University of Leipzig Medical School) according to the instructions of the manufacturer. Sequenced reads were mapped against miRBase release 22 reference [6] including 2656 mature miRNAs using Bowtie 2 [7].

2.4. Statistical analysis

Differential expression analyses were performed using generalized linear models for univariate and multivariate analyses fitting miRNA expression by a negative binomial distribution as implemented in the DESeq2 package [8]. Significance levels for univariate analyses, modelling miRNA expression by groups of patients, were computed using Wald's test. P-values for multivariate analyses also adjusting for sex and age were computed using likelihood ratio test. Results were corrected for multiple testing using Benjamini-Hochberg procedure by computing adjusted p-values. Correlation of miRNA expression and clinical disease phenotypes was performed using Spearman's non-parametric rank correlation. Receiver operating characteristic was computed using U-statistics as implemented in the pROC package. Statistical analyses were performed using R version 4.0.2.

2.5. miRNA target and enteric nervous system enrichment analysis

Two major curated databases of miRNA targets, miRTarBase and mirDIP were used to extract validated targets of hsa-miR-486–5p. The microRNA target network was visualized using Cytoscape v 3.7.1. Pathway enrichment analysis of the network was conducted using METASCAPE and enriched biological process (BP) annotations were obtained from gene ontology. We considered the BP as statistically significant at p-value < 0.05. To determine which targets of hsa-miR-486–5p microRNA are related to enteric nervous system we mapped them to the transcriptome signature profile obtained by Roy-Carson et al. [9]. Zebrafish-related gene symbols from this dataset were mapped to human homologs using gProfiler tool. We mined the cellular localization of overlapped genes between enteric nervous system and microRNA targets from https://www.proteinatlas.org/humanproteome/cell/organelle.

3. Results

3.1. Patient demographics and colonic biopsy

As expected, the PD group more often reported on GI symptoms, as measured by the constipation module (C3) of the ROME-III questionnaire. Moreover, PD patients had higher scores on the REM-Sleep Disorder Questionnaire (RBDSQ) and a poorer performance in the 12-item Sniffin' Stick assessment (Suppl. Table S1).

3.2. miRNA profiling in colonic biopsies

To identify differentially expressed miRNAs in colonic biopsies from the PD and control group, expression levels of miRNAs were analysed based on the miRNA database miRBase release 22. miRNA-Seq identified n = 13 differentially expressed miRNAs that were up-regulated in the PD group ($\log_2$FC ≥ 1; p-value < 0.05) and n = 16 differentially expressed miRNAs that were down-regulated in the in the PD group ($\log_2$FC ≤ −1; p-value < 0.05) using the control group an as reference (Fig. 1A, Table S2). Adjusting for multiple testing revealed a single miRNA, termed hsa-miR-486–5p, to be significantly up-regulated in the PD group ($\log_2$ FC = 1.40; adjusted p-value = 0.049) (Fig. 1B and C). Multivariate analysis confirmed > 2-fold up-regulation of hsa-miR-486–5p in the PD group, which was however not significant for multiple-testing ($\log_2$FC = 1.02; adjusted p-value > 0.05). Analyzing the

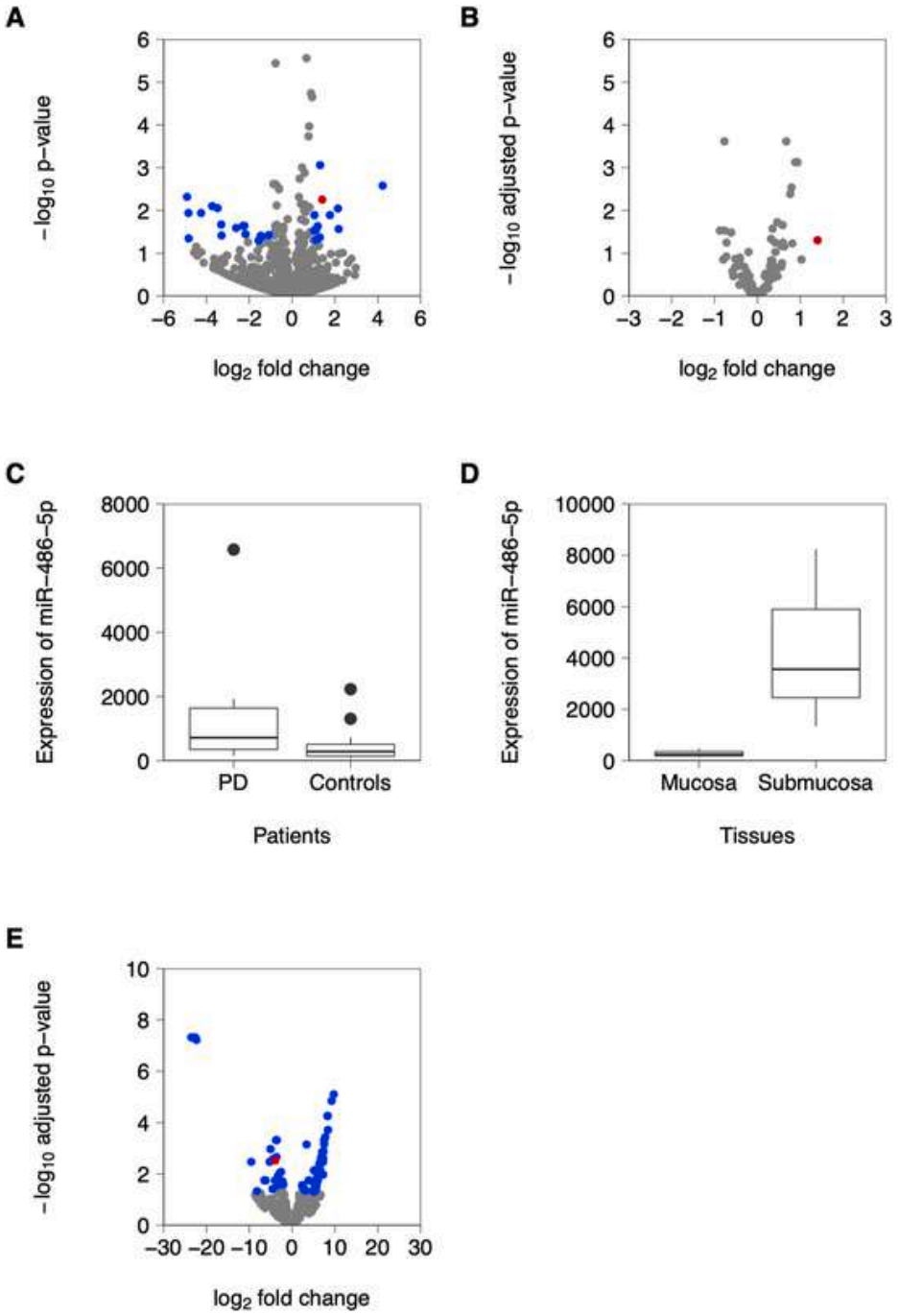

Fig. 1. miRNA sequencing reveals a significant enrichment of miR-486-5p in colonic biopsies from PD patients. (**A**) Volcano plot illustrating the up- and down-regulation of distinct miRNAs in the PD group using healthy controls as reference. Differentially expressed miRNAs are indicated in blue and miRNA-486–5p is shown in red. (**B**) Volcano plot of differentially expressed miRNA adjusted for multiple testing. miRNA-486–5p is shown in red. (**C**) Expression of miR-486–5p in patients with PD and healthy controls. Expression values are given as normalized counts. (**D**) Tissue specific expression miR-486–5p in mucosal and submucosal tissue illustrating normalized counts. (**E**) Volcano plot illustrating the tissue specific changes in miRNA expression level using submucosa as reference. Differentially expressed miRNAs corrected for multiple testing are shown in blue and miRNA-486–5p is shown in red. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

receiver operating characteristic curve for hsa-miR-486–5p revealed an area under the curve of 0.729 with a 95% confidence interval of $CI_{95\%}$ = [0.544, 0.913].

When we analysed the tissue specific expression of miR-486–5p, we found an upregulation of miR-486–5p (log_2 FC = 3.97, adj. p-value = 0.003) in the submucosa compared to mucosa (Fig. 1D and E; Table S3), thus suggesting a predominantly submucosal origin.

3.3. Correlation with clinical disease parameters

In order to examine the relationship between the expression of miR-486–5p with clinical disease phenotypes we have performed Spearman's non-parametric rank correlation. Expression levels of miR-486–5p were shown to correlate with age and disease severity in the PD group as measured by the UPDRS part II and III and the Hoehn & Yahr scale (Fig. S1A,D,E,J; Table S4). Conversely, other established early-

disease clinical biomarkers (Rome III Questionnaire, RBDSQ, Sniffin' Sticks) showed no significant correlation with the expression of miR-486–5p (Fig. S1H,I,K,L; Table S4).

3.4. Analysis of miR-486–5p gene targets and their biological relevance

Using the established data archives (Fig. 2A), 301 potential gene targets of miR-486–5p are identified and visualized as a network (Fig. 2B). Using a gene ontology approach, we next identified key enriched biological processes among these potential miR-486–5p gene targets (Fig. 2C) with "brain development" and "postsynapse organization" being the most enriched biological categories. An increased submucosal miR-486–5p expression may affect a variety of ENS-associated processes in the PD GI tract. In order to examine the relevance of miR-486–5p for the ENS, we profiled our 301 miR-486–5p gene targets back to the GI-ENS transcriptomic signature obtained by Roy-Carson et al. in Zebrafish [9] and its human homologs with specific up- or downregulated target genes (Fig. 2D) that segregate to distinct cellular compartments and pathways (Fig. 2E). In our analysis (Fig. 2E), we also identified *LINGO4* as an interesting novel target of miR-486–5p, to which no previous pathway information is available yet. Because the ENS is assumed to contribute to PD onset and progression, our results will support the examination of miR-486–5p as an early pathogenic molecular target in PD.

4. Discussion

A large number of studies have investigated the role of α-syn in the gut [10]. In addition, other peripheral sites are under active consideration, including skin, stomach, esophagus, pharynx, and salivary glands. However, other reports have raised concerns regarding the specificity of enteric α-Syn [4,5], thus questioning the overall diagnostic value of GI LTS to diagnose PD. Here, we identified miR-486–5p as a specific miRNA to be up-regulated in biopsies from PD cases. miR-486–5p may therefore represent a novel gut biomarker for PD. Although the sample size of the current study is comparably low and the findings thus have an exploratory character, we believe that our results will permit prospective studies to confirm the disease specific expression of miR-486–5p in larger cohorts and in distinct biological material along the entire GI tract.

Routine colonic biopsies typically encompass mucosal and submucosal tissue including submucosal ENS ganglia and neurites [10]. Interestingly, analyzing the expression of miR-486–5p in mucosal and submucosal tissue separately revealed a segregation of miR-486–5p to the submucosa (Fig. 1D and E). In our follow-up analysis, we identified a total number of 301 genes as target genes of miR-486–5p and the gene-associated biological processes (Fig. 2B and C). In line with a role of miR-486–5p in the ENS, we found "brain development" and "postsynapse organization" to have the strongest functional association with

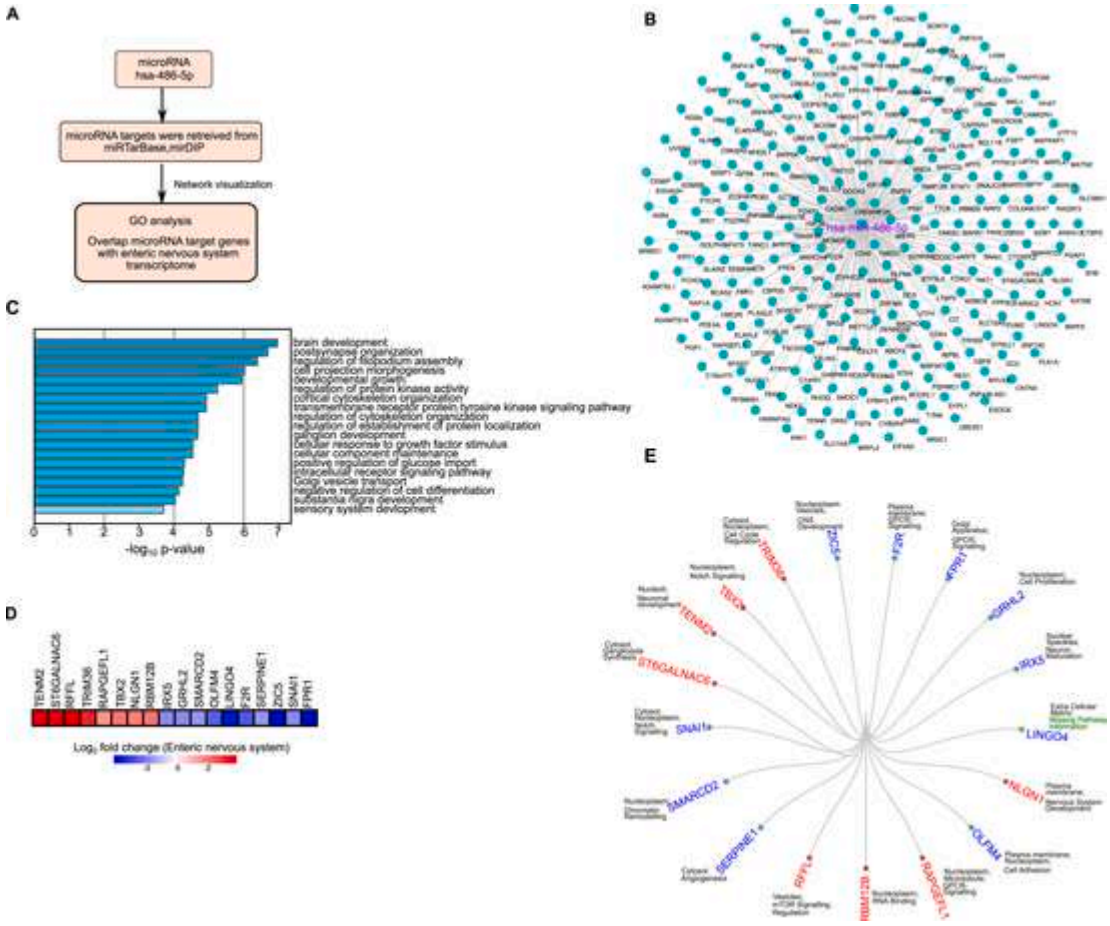

Fig. 2. microRNA hsa-miR-486-5p target network and its association with enteric nervous system. (**A**) Schematic representation of the workflow for generating the microRNA target network and its analysis. (**B**) Network visualization of hsa-miR-486–5p target genes which were identified using miRTarBase and mirDIP. (**C**) Bar graph illustrating the biological processes affected by the miR-486–5p target genes ($P < 0.05$) (**D**) Heat map representing selected target genes of hsa-miR-486–5p as related to the enteric nervous system transcriptome signature. Gene expression values are expressed as fold changes and are colored from blue (down-regulation) to red (up-regulation). (**E**) The network plot illustrates hsa-miR-486–5p microRNA targets based on enteric nervous system and their cellular localization and physiological functions. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the miR-486–5p target gene network. Taken together, these results thus support a novel regulatory role of miR-486–5p for ENS physiology. Because of the enrichment of miR-486–5p in PD, our results have disease-relevant implications for PD and future studies should aim to connect miR-486-5p-regulated functional pathways (Fig. 2C) to the molecular mechanism of GI dysfunction in PD. In addition, these prospective studies should address the relevance of miR-486-5p-related gene targets for PD onset in the gut, such as their contribution to the endocytosis and propagation of environmental pathogens or toxins that may initiate PD-related pathological changes in the gut.

The enteric nervous system (ENS) consists of neurons but also enteric glial cells (EGCs) that reside within the smooth muscle wall and submucosa and that largely outnumber enteric neurons and are easily captured by routine gastrointestinal biopsies. EGCs within the submucosa contribute to the integrity of tight junctions. In line, there is growing evidence that the pathological changes in the gut in PD are not limited to enteric neurons but also involves EGCs. In line with a role of the enteric glia and the enteric extracellular matrix (ECM) in LTS onset or progression, we found for instance the miR-486-5p-target *LINGO4* (Leucine-rich repeat and immunoglobulin domain containing, Nogo receptor-interacting protein-1) (Fig. 2E). Interestingly, LINGO protein family members have been demonstrated to play a role in the structural plasticity and integrity of dopaminergic neurons as well as their survival in animal models of PD. Recently, variants of *LINGO1* and *LINGO2* have been reported as PD risk factors [11] and *LINGO4* has appeared as a novel candidate in this study. Additional examples of PD-associated genes include *TRIM36*, which has been found to be down-regulated in a gene expression profiling study of human SNc from PD patients and FR2, which is highly expressed in SNc DA neurons and is involved in the maintenance and survival of these neurons in the adult brain [12]. In summary, these results suggest that the changes observed in the gut in PD exceed Lewy pathology, but involve enteric neurons and glial cells and the enteric ECM, although further experimental work is needed to clarify the specific role of these genetic factors in the PD gut. These results thus support the hypothesis that this so-called 'neuro-glio-epithelial unit' might constitute an unparalleled source of biomarkers in PD beyond the sole assessment of α-syn aggregates in enteric neurons or neurites. In summary, investigating gut homeostasis in PD will support the understanding of the molecular factors possibly contributing to the initial induction of pathology in PD.

Declarations

Ethics approval and consent to participate: The study was approved by the Ethics committee at the Ludwig Maximilian University of Munich (#343–15). All participants gave written consent to participate.

Consent for publication

Not applicable.

Availability of data and material

All data are part of the manuscript and its supplementary information.

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Authors' contributions

T.K. & K.B. conceived the study. A.K., R.K., B·H·N., C·W., S.D. performed the experiments. Jo.S. & V.R. contributed patients. Jö.S. & R.H. conducted colonoscopy. G.U.H., L.H., K.B. and T.K. supervised the work. A.K., R.K. & T.K. wrote the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parkreldis.2021.05.022.

References

[1] T.G. Beach, C.H. Adler, L.I. Sue, L. Vedders, L. Lue, C.L.W. Iii, H. Akiyama, J.N. Caviness, H.A. Shill, M.N. Sabbagh, D.G. Walker, A.P.D. Consortium, Multi-organ distribution of phosphorylated alpha-synuclein histopathology in subjects with Lewy body disorders, Acta Neuropathol. 119 (2010) 689–702, https://doi.org/10.1007/s00401-010-0664-3.

[2] R.F. Pfeiffer, Gastrointestinal dysfunction in Parkinson's disease, Curr. Treat. Options Neurol. 20 (2018) 54, https://doi.org/10.1007/s11940-018-0539-9.

[3] C.H. Hawkes, K.D. Tredici, H. Braak, A timeline for Parkinson's disease, Park. Relat. Disord. 16 (2010) 79–84, https://doi.org/10.1016/j.parkreldis.2009.08.007.

[4] N.P. Visanji, C. Marras, D.S. Kern, A.A. Dakheel, A. Gao, L.W.C. Liu, A.E. Lang, L.-N. Hazrati, Colonic mucosal a-synuclein lacks specificity as a biomarker for Parkinson disease, Neurology 84 (2015) 609–616, https://doi.org/10.1212/wnl.0000000000001240.

[5] S.J. Chung, J. Kim, H.J. Lee, H.-S. Ryu, K. Kim, J.H. Lee, K.W. Jung, M.J. Kim, Y.J. Kim, S.-C. Yun, J.-Y. Lee, S.-M. Hong, S.-J. Myung, Alpha-synuclein in gastric and colonic mucosa in Parkinson's disease: limited role as a biomarker, Mov. Disord.: Official Journal of the Movement Disorder Society 31 (2016) 241–249, https://doi.org/10.1002/mds.26473.

[6] S. Griffiths-Jones, R.J. Grocock, S. van Dongen, A. Bateman, A.J. Enright, miRBase: microRNA sequences, targets and gene nomenclature, Nucleic Acids Res. 34 (2006) D140–D144, https://doi.org/10.1093/nar/gkj112.

[7] B. Langmead, S.L. Salzberg, Fast gapped-read alignment with Bowtie 2, Nat. Methods 9 (2012) 357–359, https://doi.org/10.1038/nmeth.1923.

[8] M.I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2, Genome Biol. 15 (2014) 550, https://doi.org/10.1186/s13059-014-0550-8.

[9] S. Roy-Carson, K. Natukunda, H.-C. Chou, N. Pal, C. Farris, S.Q. Schneider, J.A. Kuhlman, Defining the transcriptomic landscape of the developing enteric nervous system and its cellular environment, BMC Genom. 18 (2017) 290, https://doi.org/10.1186/s12864-017-3653-2.

[10] T. Lebouvier, E. Coron, T. Chaumette, S. Paillusson, S.B. des Varannes, M. Neunlist, P. Derkinderen, Routine colonic biopsies as a new tool to study the enteric nervous system in living patients, Neuro Gastroenterol. Motil. 22 (2010) e11–e14, https://doi.org/10.1111/j.1365-2982.2009.01368.x.

[11] Y. Chen, B. Cao, J. Yang, Q. Wei, R.W. Ou, B. Zhao, W. Song, X. Guo, H. Shang, Analysis and meta-analysis of five polymorphisms of the LINGO1 and LINGO2 genes in Parkinson's disease and multiple system atrophy in a Chinese population, J. Neurol. 262 (2015) 2478–2483, https://doi.org/10.1007/s00415-015-7870-9.

[12] Q. Zhou, J. Li, H. Wang, Y. Yin, J. Zhou, Identification of nigral dopaminergic neuron-enriched genes in adult rats, Neurobiol. Aging 32 (2011) 313–326, https://doi.org/10.1016/j.neurobiolaging.2009.02.009.