PARKINSON'S PROGRESSION MARKERS INITIATIVE

Play a Part in Parkinson's Research

Validation of a Blood Based Gene Expression Assay for Monitoring Disease Severity and Aid in the Early Diagnosis of Parkinson's Disease

Nir Dotan, Ph.D., Danit Mechlovich Ph.D., Jennifer Yarden, Ph.D., J. Martin Rabey, M.D.
BioShai Ltd.

PPMI Project ID: 119

Summary

Parkinson's disease (PD) biomarkers are crucial for earlier certain diagnosis of PD, predicting disease severity/progression, monitoring treatment and development of neuroprotective therapies. Previous research identified 20 genes as being differentially expressed in substantia nigra (SN) of sporadic parkinsonian patients compared to normal controls[1]. Following, two separate studies measured the level of the most significant genes from the SN study, in blood samples from PD patients and controls. Expression of these genes in blood distinguished PD patients from healthy controls and from Alzheimer patients[2,3]. Further analysis revealed a significant difference in the gene expression between de novo and advanced PD patients ($p<0.05$, Mann-Whitney U). In the current study, we measured the gene expression of ALADH1A1, SKP1A, UBE2K, PSMC4, HSPA8 and LAMB2, in 995 blood samples of patients and controls enrolled in the Parkinson's Progression Markers Initiative (PPMI) study. The gene expression was measured by real time quantitative PCR[4]. PD patients' were from baseline: n=279; Year 1: n=87; Year 2: n=189 and Year 3: n=202, SWEDD patients' were from baseline: n=39, Year 1: n=12 and Year 2 + 3: n= 34 and healthy controls: n=153. The aims of this study is to validate the association of the genes' expression to PD severity, investigate the biomarkers ability to predict future disease course, validate the specificity of the biomarkers for differentiating PD from healthy controls and investigate the ability of the biomarkers to differentiate PD from SWEDD.

Method

Quantitative PCR Assays

Samples with RNA integrity values >5.0, RNA concentration >20ng/µL and absorbance 260/280 between 1.7 and 3.0 were used in this study. One microgram of RNA was reverse transcribed into cDNA using a mix of random hexamer primers (High Capacity cDNA Synthesis Kit, Life Technologies). Real time quantitative polymerase chain reaction assays (RT-qPCR) were performed using the StepOnePlus real time PCR system (Applied Biosystems, USA). Each 28 microliters reaction contained Power SYBR Green (Life Technologies, USA) and primers at a concentration of 0.3µM. The amplification conditions used were as follows: denature at 95ºC for

15 sec, annealing at 60ºC for 1 min, extension at 72ºC for 30 sec for 36 cycles of amplification. Melting curves were run in each qPCR experiment to ensure product specificity. PCR products were sent for sequencing analysis to confirm that the expected product was amplified. Using these amplification conditions we have observed primer efficiencies higher than 90%. Each sample was run in duplicates and no cDNA template controls and a calibrator sample for plate-to-plate normalization were included in each plate. A complete list of the primers used in this study is shown in Table 1. Expression data will be analyzed using the comparative ΔΔCt method. In this method the amount of target is normalized to an endogenous reference and relative to the calibrator sample. GAPDH and PGK1 were used in our studies as reference transcripts. We have verified that the efficiency of the reference transcript amplification and the efficiency of the target transcript is approximately equal. For the Ct calculation, the threshold was set to 0.8 and baseline calculation was based on the signal in cycles 3-15.

Table 1: Primers Used in Study

Biomarker	**5' (Forward)**	**3' (Reverse)**
SKP1A	CATCCATTCCCAAATCTTCCAA	CCTTAACACCGAACACCATGC
PSMPC4	TGGAGAAGGCTCAGGATGAGA	AGCTTCTTGTAGCGGCTGTACAG
ALDH1A1	TGGCTTATCAGCAGGAGTGTTTAC	GCCATAGCAATTCACCCACACT
HSPA8	GGAGGTGTCTTCTATGGTTCTGACA	CACAGCATTGGTAACAGTCTTCCC
UBE2K	CTGTAGTAGCAAATCAGTACAAACAAAATC	CCTATCAAAGCCCATAGCACATAG
LAMB2	TTGGTGGCAGTCAGAGAATGG	AGCAGGGCGAAATGTCTTGA
GAPDH	TCTCTGCTCCTCCTGTTCGAC	CCCAATACGACCAAATCCGT
PGK1	GAACAAGGTTAAAGCCGAGCC	GTGGCAGATTGACTCCTACCA

Statistical Analysis

The association between the gene expression values and PD rate of severity progression (fast vs. slow progression) will be evaluated in cross-sectional and in serial measurement statistics. Endpoints for disease progression/severity will be changes in disease scores: MDS-UPDRS[5],

Hopkins Verbal Learning, Hoehn & Yahr (H&Y) Stage[6], MoCA total score, Modified Schwab and England activities of daily living (SE-ADL)[7] and other similar measurements available from the PPMI study. Patients will be grouped into mild and severe sub-cohorts according to the Endpoint values. Mann-Whitney U test will compare the patient groups (mild v. severe) gene expression levels. Wilcoxon paired test will compare patients' paired gene expression values between two different time points. Friedman test will compare patients' gene expression values from more than two points. Results will be depicted in box plots and dot and line diagrams. Logistic regression will be used to build a classifier of the combined gene expression levels. Other variables available from the PPMI study will also be tested for inclusion in the classifier. Confounding demographic and clinical status factors will be tested. Time-to-event analyses will investigated by Kaplan Meir and Cox-Proportional Hazards Regression tests. The endpoint will be time to reach clinically important differences (CID)[8], determined severity scores [H&Y score ≥2, or large MDS-UPDRS total score increase (≥ 17.1 over baseline) or 10% decrease in Modified SE-ADL from baseline or similar].
The gene expression assay's specificity for early/mild PD population vs. healthy controls and SWEDD patients will be evaluated for the baseline samples by calculation of specificity, sensitivity, positive and negative predictive values, and likelihood ratios. The correlation between the gene expression assay values and demographic and clinical status variables in the different cohorts will be tested. The association between PDx and other CSF, blood variables, DaT-SPECT measurements available in the PPMI database may be tested. Tests will be performed for sporadic PD patients and familial PD patients together and separately. Sub-cohorts from the SWEDD group of patients will be tested together and separately.

References

1. Grünblatt E, Mandel S, Jacob-Hirsch J, Zeligson S, Amariglo N, Rechavi G, Li J, Ravid R, Roggendorf W, Riederer P, Youdim MB. Gene expression profiling of parkinsonian substantia nigra pars compacta; alterations in ubiquitin-proteasome, heat shock protein, iron and oxidative stress regulated proteins, cell adhesion/cellular matrix and vesicle trafficking genes. J Neural Transm. 2004, 111:1543–1573.
2. Grünblatt E, Zehetmayer S, Jacob CP, Müller T, Jost WH, Riederer P. Pilot study: peripheral biomarkers for diagnosing sporadic Parkinson's disease. J Neural Transm (Vienna). 2010,117:1387-1393.
3. Molochnikov L1, Rabey JM, Dobronevsky E, Bonucelli U, Ceravolo R, Frosini D, Grünblatt E, Riederer P, Jacob C, Aharon-Peretz J, Bashenko Y, Youdim MB, Mandel SA. A molecular signature in blood identifies early Parkinson's disease. Mol Neurodegener. 2012, 7:26.
4. Livak KJ and Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-[Delta][Delta]CT Method. Methods. 2001, 25:402-408.

5. Fahn S, Elton R, UPDRS Development Committee: Unified Parkinson's disease rating scale. In Recent Developments in Parkinson's Disease. Volume 2. Edited by Fahn S, Marsden CD, Goldstein M. New York: Macmillan; 1987:153-167.
6. Hoehn MM, Yahr MD: Parkinsonism: onset, progression and mortality. Neurology. 1967, 17:427-442.
7. Schwab RSEA. Projection technique for evaluating surgery in Parkinson's disease In: DI Gillingham FJ, editor. , ed. Third Symposium on Parkinson's Disease. Edinburgh, Scotland: E & S Livingstone, 1969:152-157.
8. Shulman LM, Gruber-Baldini AL, Anderson KE, Fishman PS, Reich SG, Weiner WJ. The clinically important difference on the unified Parkinson's disease rating scale. Arch Neurol. 2010;67: 64-70.

About the Authors

This document was prepared by Dr. Nir Dotan and Dr. Jennifer Yarden**, BioShai Ltd., R&D**. For more information please contact **Jennifer Yarden** at **(972) 4909-0016** or by email at **jenyarden@bioshai.com**.