Next Generation Sequencing Data Analysis Evaluation in Patients with Parkinsonism from a Genetically Isolated Population

Radek Vodicka1, Radek Vrtel1,∗, Katerina Mensikova1, Petr Kanovsky1, Iva Dolinova2, Kristyna Kolarikova1, Martin Prochazka1

1University Hospital and Palacky University, Olomouc, Czech Republic
2Department of Nanomaterials in Natural Sciences, Technical University of Liberec, Czech Republic

∗ Correspondence: Tel: +420 585854461; email: vrtel@fnol.cz

Received 2016-10-06; Accepted 2017-03-13

ABSTRACT

Parkinson's disease (PD) can be caused by genetic changes in a lot of genes. The effect of these changes is determined by the nature of the mutation and ranges from weak associations to pathogenic mutation which leads to loss of protein function. Our study is based on epidemiological data which show significantly increased prevalence of PD (2.9 %) in an isolated population of South-Eastern Moravia in the Czech Republic. We compared two different Next Generation Sequencing (NGS) data analysis approaches in DNA from 28 PD patients in the genes responsible for Parkinsonism (ADH1C, ATP13A2, EIF4G1, FBXO7, GBA + GBAP1, GIGYF2, HTRA2, LRRK2, MAPT, PARK2, PARK7, PINK1, PLA2G6, SNCA, UCHL1 and VPS35) using: 1) already described missense rare variants or pathogenic mutations 2) twelve control DNA samples from the same isolated population. Ion Torrent NGS data processing and trimming from Fastaq through “bam” to “vcf” files was done parallely by Torrent Suite/Ion Reporter and NextGENe software. After filtering out, three missense mutations were found in LRRK2 gene:

rs33995883 in 6/0 patients/control (p/c); rs33958906 in 1/1p/c; rs781737269 in 3/0p/c; one missense mutation in MAPT gene rs63750072 in 6/1p/c; and one mutation in HTRA2 gene rs72470545 in 3/1p/c. Both the results from NextGENe with Ion Torrent adaptation and from Ion Reporter significantly correlated in variant calling. Our study may contribute to further explain the genetic background of Parkinsonism.

KEYWORDS
Parkinsonism; Next Generation Sequencing; LRRK2 gene; MAPT gene; HTRA2 gene

INTRODUCTION

Parkinson's disease, the second most common neurodegenerative disorder affecting 1% of the population over 65 years, was described in 1817 by James Parkinson [1, 2]. The disease is characterized by a selective loss of dopamine in the substantia nigra pars compacta which leads to abnormal motor symptoms such as resting tremor, stiffness (rigidity), general slowness of movement (bradykinesia) and decreasing of facial expression [3, 4]. The cause of PD is not known, but several neurotoxins have been shown to cause parkinson's-like neuropathology in animals [5]. Further, several genes associated with autosomal dominant and autosomal recessive PD have been described (SNCA, UCHL1, LRRK2, GIGYF2, OMI/HTRA2, PARK2, PINK1, PARK7, ATP13A2) [6]. The segregation analyses which were published by independent studies confirmed the influence of genetic background on the age of PD onset. In general, stronger major effect genes have been described for penetrance or the age of onset than for disease susceptibility [7, 8]. It has been described that prevalence in small isolated families is higher in Europe [9, 10]. A higher prevalence of Parkinsonism has been recently identified in the South-Eastern Moravia (Czech Republic). Particularly, aggregation of Parkinsonism was found in two large family trees. This is probably caused by the isolation of the regional population due to the very low migration rate of its inhabitants to neighbouring regions in the last two centuries [11].

The aim of the study is:

1. to compare two different Next Generation Sequencing (NGS) data analysis approaches in PD patients in the genes responsible for Parkinsonism.
2. to describe the most important findings in the patients with comparison to controls.

METHODS

A set of twenty eight patients with Parkinson's disease and 12 healthy controls were analyzed.

Patients were diagnosed by common neurological tests at the Department of Neurology, Faculty of Medicine and Dentistry, Palacky University Olomouc and University Hospital Olomouc, Czech Republic. A three-stage case ascertainment method was used [10]. At first, questionnaires were delivered to general
practitioners (GP). During three-months, questionnaires were filled in by patients who visited GP for any reason. Further, respondents screened as positive for parkinsonism (after their signing the informed consent with further examination) were investigated by primary care neurologists. The motor part of the Unified Parkinson's Disease Rating Scale (UPDRS) was completed. Subsequently a preliminary parkinsonism's diagnosis was made and or rejected. Finally, individuals with parkinsonism were examined in a tertiary movement disorders centre in University Hospital Olomouc. There they were examined by a neurologist with detailed movement disorders expertise [12]. Average age of patients was 67 years (from 45 to 90 years) and controls from the same region were older than 70 or, in case of known pedigree, 5 year older than individual with the disease symptoms. Study was approved by Ethical Committee of University Hospital Olomouc, Czech Republic. The patients were informed in detail about the study and signed informed consent. DNA was isolated from peripheral blood by isolation kit (QiAamp DNA Mini Kit - Qiagen). DNA concentration was measured fluorometrically (Qubite - Qiagene). Each DNA sample was diluted in water to a final concentration 10 ng/µL. Ampliprobe library was in silico designed by Ion AmpliSeq Designer (Thermo Fisher Scientific) for ADH1C, EIF4G1, FBXO7, GBA + GBA, P1, GIYF2, HTRA2, LRRK2, MAPT, PARK2, PARK7, PINK1, PLA2G6, SNCA, UCHL1 and VPS35 genes. In total 617 amplicons covered 92.5% of CDS including 100bp into the introns and both 3' and 5' UTRs of the regions. Libraries were prepared using Ion AmpliSeq™ Library Kit 2.0 (Ion Torrent™), emulsion PCR was done on the Ion OneTouch™ 2 Instrument (Ion Torrent™) with Ion PGM™ Template OT2 200 Kit. Samples were barcoded to enable to load 8 samples on one Ion 316™ Chip. The amplicons were sequenced by massive parallel sequencing (MPS) on Personal Genome Machine - PGM (Ion Torrent™) using Ion PGM™ Sequencing Kit (Ion Torrent™) and Ion 316™ Chip Kit v2. Coverage of amplicon control: Amplicons were checked in Torrent Suite using plugin Coverage Analysis. 1000 Genomes Project (1000G) and Exome Aggregation (ExAC) population allele frequency databases were used for MAF filtering and for assessment of allele frequency differences [13, 14].

Data analysis and variant searching (Mapping, Variant calling, Annotation) were done using:

1) Torrent Suite and Ion Reporter: Data from PGM were collected and converted to Fastq and BAM formats using Ion Torrent Suite software. The "Bam/Bai" and "vcf" files were reloaded to Ion Reporter v4.6 and variant calling workflow was in the first level relaxed to minimal amplicon coverage 4 and minimal ratio of alternative/common variant 0.1. Variants were then filtered using following parameters: AQ>20; Read coverage>20; MAF from 1000G and/or from ExAC<1%; for missense polymorphisms SIFT: 0-0.05 and/or PolyPhen-2: 0.2-1. MAF was relaxed to 5% if both the SIFT and PolyPhen predictors indicate gene impairment.

2) Torrent Suite and NextGENe (Softgenetics): Data obtained from PGM were saved in Fastq, then using NextGENe software data were converted to Fasta format, adapted for Ion Torrent platform and mapped to the specific reference sequences. Variant was assessed using GRCh37 dbSNP135 and ClinVar reference genome databases. Variants were then filtered as mentioned above in 1).

All filtered in variants were then verified and confirmed by Sanger sequencing. SIFT and PolyPhen-2 prediction softwares were used for missense variants evaluation. PhyloP algorithm was used to assess the phylogenetical conservation.

To assess population frequency differences between patients and control and between patients and 1000G and/or ExAC data we used Pearson’s chi-squared test.

RESULTS

Basic sequencing coverage statistics are displayed in Table 1. Almost 100% of targets were sequenced with coverage 1x-19x and circa 97% with coverage 20x-100x. Percentage of completely sequenced amplicon (end-to-end) was lower (from 38.5% - to 80%).

Variant calling statistics

Before filtering out (mostly population common variants) we found an average of 70 and 72 variants per sample (maximum 101 and 100/minimum 48 and 55)

### Table 1: Description of basic NGS sequencing characteristics.

| Description                                           | Minimum | Maximum | Average |
|-------------------------------------------------------|---------|---------|---------|
| Number of mapped reads                                | 150.000 | 832.000 | 360.000 |
| Percent reads on target                               | 92.5%   | 97.9%   | 96.7%   |
| Percent reads on target                               | 209     | 1035    | 670     |
| Uniformity of base coverage                           | 84.7%   | 96.6%   | 93%     |
| Mean reads per amplicon                               | 225     | 1280    | 570     |
| Uniformity of amplicon coverage                       | 83.5%   | 94.7%   | 90%     |
| The percentage of all amplicons that had at least 1 read| 99.8%   | 100%    | 99.9%   |
| The percentage of all amplicons that had at least 20 reads| 93.4%   | 98.7%   | 97%     |
| The percentage of all amplicons that had at least 100 reads| 79.6%   | 93%     | 89%     |
| The percentage of all amplicons reading end-to-end     | 38.5%   | 80%     | 60.5%   |
using Ion Reporter and NextGENe, respectively.

After filtering out, three missense mutations were found using Ion Reporter and NextGENe in LRRK2 gene: rs33995883 (p.Asn2081Asp), rs3958906 (p.Pro1542Ser), rs781737269 (p.Ser633Phe), one missense mutation in MAPT gene rs63750072 (p.Gln230Arg) and one mutation in HTRA2 gene rs72470545 (p.Gly399Ser). Table 2 describes particular mutations and summarises allelic frequency in patients and control also frequency from 1000G and ExAC databases.

Rare missense variant calling from the NextGENe with Ion Torrent adaptation and Torrent Suite/ Ion Reporter had a 100% correlation.

DISCUSSION

We used two variant calling approaches in order to compare both methods and in order to assess the ability of each method to capture particular rare variant. Another reason is to ensure that we, if possible, do not miss any rare variant.

Most variants were common and in non-coding regions. We did not observe any previously described causal mutation or variant which could be considered as founder mutation in our group of patients. So we suppose that an accumulation of several rare variants could together lead to increasing susceptibility to the disease.

Missense LRRK2 mutations belong to the most common cause of familial forms of Parkinsonism [15–17]. While common variant can increase the risk of sporadic form of Parkinsonism [18, 19], mutation p.Gly2019Ser occurs in both familial and sporadic forms [20, 21]. The substitutions p.Arg1441Cys and p.Tyr1699Cys are associated with loss of neurons [17, 22]. Furthermore, mutations p.Asn1437His, p.Ile2020Thr and p.Arg1441Gly were found to be associated with the disease [23, 24].

The missense variant described in our study (rs33995883; p.Asn2081Asp) was identified in 6/0 patient/control (p/c) and it is located in phylogenetically conserved region (phyloP score 7.2). Prediction tools evaluated it as benign (SIFT score 0.081) and as pathogenic (PolyPhen-2 score 0.983). The exchange uncharged polar for acidic amino acid supported pathogenic role. On the other hand, the study from Benitez et al. 2016 found the allelic frequency 1.8% in patients and 2.2% controls, which supports it’s benign role [25]. Also, the variant was described by the study Foo et al., 2014 in none of patient and in 0.25% of the controls [26].

So, it could be concluded that the variant can be either population specific in association with Parkinsonism or has no influence on PD.

The second LRRK2 variant found was rs33958906; p.Pro1542Ser. The variant is located in the COR domain LRRK2 [27] which is phylogenetically conserved (phyloP

---

Table 2: Description of rare variants and comparison between patients and controls and between patients and 1000G/ExAC databases.

| Variant | Amino Acid Change | SIFT | PolyPhen-2 | Minor Allele Frequency | Number of missense mutations in patients | Number of missense mutations in controls | p.v.c | p.v. MAPT ExAC | p.v. MAPT 1000G |
|---------|-------------------|------|------------|------------------------|----------------------------------------|-----------------------------------------|-------|----------------|-----------------|
| LRRK2 rs33995883 (p.Asn2081Asp) | 0.081/0.983 | 0.0159/0.982 | 0.0520/0.871 | 0.003/0.009 | 3 | 0 | none | none | none |
| LRRK2 rs3958906 (p.Pro1542Ser) | 0.053/0.982 | 0.003/0.009 | 0.0020/0.871 | 0.003/0.009 | 3 | 0 | none | none | none |
| LRRK2 rs781737269 (p.Ser633Phe) | 0.030/0.982 | 0.003/0.009 | 0.0020/0.871 | 0.003/0.009 | 3 | 0 | none | none | none |
| MAPT rs63750072 (p.Gln230Arg) | 0.053/0.982 | 0.003/0.009 | 0.0020/0.871 | 0.003/0.009 | 3 | 0 | none | none | none |
| HTRA2 rs72470545 (p.Gly399Ser) | 0.053/0.982 | 0.003/0.009 | 0.0020/0.871 | 0.003/0.009 | 3 | 0 | none | none | none |
score 5.3) and it was identified in 1/1 p/c. It leads to exchange nonpolar for uncharged polar amino acid, so it could be pathogenic character which is supported by PolyPhen-2 prediction (probably damaging, PolyPhen-2 score 0.962) and predicted benign by SIFT (SIFT score 0.159). Di Fonzo et al. (2006) found allele T in 1.7% from 60 patients [28]. Further, study Foo et al. (2014) confirmed variant in 1 of controls (0.25%) and none of patients [26].

The third mutation was p.Ser633Phe. The variant changes polar amino acid for nonpolar in evolutionarily conserved region (phyloP score 4.2). It was found in 3/0p/c. Both the SIFT (score 0.002) and the PolyPhen-2 (score 0.971) evaluated the variant as a pathogenic.

Concerning the tau gene MAPT missense mutations p.Gly272Val, p.Asn279Lys, p.Pro301Leu, p.Pro301Ser, p.Arg406Trp, p.Gly389Arg, p.Val337Met and p.Ser305Asn were found in the patients with frontotemporal dementia type of Parkinsonism [29–37]. The substitution mutation (rs63750072; p.Gln230Arg) found in our study exchanges uncharged polar amino acids for alkaline in MAPT gene. The variant was evaluated by prediction tools as a pathogenic (SIFT score 0.003, PolyPhen-2 score 0.992) and the region is evolutionarily conserved (phyloP score 2.2). Jin et al., 2012 identified this variant in 10% patients and in 5.8% controls [38]. According to Deters et al. (2014), the variant is in association with the level of tau protein expression in cerebrospinal fluid [39].

Mutation p.Arg404Trp in HTRA2 gene can increase risk for Parkinsonism [40]. Mutation p.Gly399Ser that has been identified in sporadic form can contribute to the development of the disease probably due to disturbing of protease activity of the enzyme [41]. Omi/HtrA2 interacts in vitro with PINK1 and may undergo to phosphorylation which is PINK1 dependent and the substitution of phosphorylationable with non-phosphorylationable sites (p.Ser400Ala) reduces the protease activity [42]. Another mutation p.Ala141Ser which changes the protease activity was also described in patients with Parkinsonism [43].

The variant (rs72470545; p.Gly399Ser) we described is located in evolutionarily conserved site (phyloP score 5.7) in HTRA2 gene and LOXL3 gene. Prediction tools evaluated the variant as a pathogenic (SIFT score 0.020 and PolyPhen-2 score 0.986). According to Gulsuner et al. (2014), p.Gly399Ser is associated with heredity of essential tremor and the variant in homozygote state have influence in the development of Parkinson disease [43]. Strauss et al. (2005) found the variant in 4 patients and none of controls. On the other hand, Simon-Sanchez et al. (2008) found the variant in 5 patients (0.77%) and in 6 controls (0.72%) [44]. Further, study Tzoulis et al. (2015) also did not confirm association between the variant and tremor [45].

None of found variants has unambiguously pathogenic nature and has not yet been repeatedly described as pathogenic in literature. Effect size for 40 samples (alpha=0.05) with 80% probability of variant clinical effect was calculated to be 0.44. So to reach this value the frequency of mutation should be 6 times higher in patients compare to controls for MAF 0.01 [46]. Our results and comparisons (Table 2) due to the small number of controls only suggest that the variants LRRK2 rs33995883 (p.Asn2081Asp, LRRK2 rs33995883 (p.Asn2081Asp), LRRK2 rs781737269 (p.Ser633Phe), MAPT rs63750072 (p.Gln230Arg) and HTRA2 rs72470545 (p.Gly399Ser) could be considered as a potentially risk alleles. The risk level of variants can be influenced by the type of population and by the presence of other risk genetic, epigenetic and environmental factors.

Our findings together with further detailed clinical characteristics of patients could contribute to further understanding of molecular pathogenesis of Parkinsonism and to the creation of clinically applicable diagnostic procedure.

ACKNOWLEDGEMENTS

This study was supported by grant MZ- NV15-32715A from the Ministry of Health of the Czech Republic.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

Ala: alanine
AQ: alignment quality
Arg: arginine
Asn: asparagine
Asp: aspartic acid
BAM: binary version of a SAM file
CDS: coding sequences
ClinVar: archive of clinical variants
Cys: cysteine
dbSNP135: database single nucleotide polymorphisms 135
Consortium database
Consortium database
ExAC: allele frequency based on Exome Aggregation
Fastq: FASTA/QUAL files
NGS: next generation sequencing
Gln: glutamine
Gly: glycine
GP: general practitioner
GRCh37: genome reference consortium human 37
His: histidine
Ile: isoleucine
Leu: leucine
Lys: lysine
MAF: minor allele frequency
Meth: methionine
MPS: massive parallel sequencing
PD: Parkinson disease
PGM: personal genome machine
Phe: phenylalanine
phyloP: tool for evaluation of phylogenetic conservation
PolyPhen-2: prediction tool, Polymorphism Phenotyping v2
Pro: proline
REFERENCES

1. Polymeropoulos MH, Higgins JJ, Golbe LJ, Ide SE, Iorio GD, Sanges G, et al. Mapping of a gene for Parkinson's disease to chromosome 4q21-q23. Science. 1998;1996(274):1197–1199.

2. Yang YX, Wood NW, Latchman DS. Molecular basis of Parkinson's disease. NeuroReport. 2009;vol.20(issue 2):150–156. doi:10.1093/NeuroReport/19.32831.32830.

3. Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. Neurosci. 2003;39(6):889–909.

4. Zhang ZX, Roman GC. Worldwide occurrence of Parkinson's disease: an updated review. Neuroepidemiology. 1993;12(issue 4):195–208.

5. Tolwani RJ, Jakowec MJ, Petzinger GM, Green S, Waggie K. Experimental models of Parkinson's disease: insights from many models. Lab Anim Sci. 1999;49(issue 4):363–371.

6. Lesage S, Brice A. Parkinson's disease from monogenic to genetic susceptibility factors. Human Molecular Genetics. 2009;vol.18(issue R1):R48–R59. doi:10.1038/Primdp1012.

7. Maher NE, Currie LJ, Lazzarini AM, Wilk JB, Taylor CA, Saint-Hilaire MH, et al. Segregation analysis of Parkinson disease revealing evidence for a major causative gene. American Journal of Medical Genetics. 2002;vol.109(issue 3):191–197. doi:10.1002/ajmg.10335.

8. McDonnell SK, Schaid DJ, Strain JH, Ahlskog JE, Maraganore DM, Rocca WA. Complex segregation analysis of Parkinson's disease: The Mayo Clinic Family Study. Ann Neurol. 2006;59(issue 5):788–795.

9. Barbosa MT, Caramelli P, Maia DP, Cunningham MC, Guerra HL, LinaCosta MF, et al. Parkinsonism and Parkinson's disease in the elderly: a community-based survey in Brazil (the Bambuí study). Mov Disord. 2006;21(6):800–808.

10. Kis B, Schrag A, Klein C, Ben-Shlomo Y, Gasperi A, Schoenhuber M, et al. A distinct familial presenile dementia with a novel CLN1 mutation in a family of south-eastern Moravia, Czech Republic. Neurogenetics. 2009;vol.6(issue 4):171–177. doi:10.1007/s10048-005-0005-1.

11. Murrell JR, Koller D, Foroud T, Goedert M, Spillantini MG, Saint-Hilaire MH, et al. Segregation of the LRRK2 gene in Parkinson's disease-related pathogenic and susceptibility genes in Asian populations. Human Molecular Genetics. 2014;vol.23(issue 14):3891–3897. doi:10.1038/hmg2015.

12. Murrell JR, Koller D, Foroud T, Goedert M, Spillantini MG, Saint-Hilaire MH, et al. Segregation of the LRRK2 gene in Parkinson's disease-related pathogenic and susceptibility genes in East Asian populations. Human Molecular Genetics. 2014;vol.23(issue 14):3891–3897. doi:10.1038/hmg2015.

13. Murrell JR, Koller D, Foroud T, Goedert M, Spillantini MG, Saint-Hilaire MH, et al. Segregation of the LRRK2 gene in Parkinson's disease-related pathogenic and susceptibility genes in East Asian populations. Human Molecular Genetics. 2014;vol.23(issue 14):3891–3897. doi:10.1038/hmg2015.

14. Murrell JR, Koller D, Foroud T, Goedert M, Spillantini MG, Saint-Hilaire MH, et al. Segregation of the LRRK2 gene in Parkinson's disease-related pathogenic and susceptibility genes in East Asian populations. Human Molecular Genetics. 2014;vol.23(issue 14):3891–3897. doi:10.1038/hmg2015.

15. Murrell JR, Koller D, Foroud T, Goedert M, Spillantini MG, Saint-Hilaire MH, et al. Segregation of the LRRK2 gene in Parkinson's disease-related pathogenic and susceptibility genes in East Asian populations. Human Molecular Genetics. 2014;vol.23(issue 14):3891–3897. doi:10.1038/hmg2015.
36. D’Souza I, Poorkaj P, Hong M, Nohlin D, Lee VM, Bird TD, et al. Missense and silent tau gene mutations cause frontotemporal dementia with parkinsonism chromosome 17 type, by affecting multiple alternative RNA splicing regulatory elements. Proc Natl Acad Sci USA. 1999;96(10):5598 – 5603.

37. Murrell JR, Spillantini MG, Gusazzelli M, Smith MJ, Hasegawa M, Crowther RA, et al. Tau gene mutation G389R causes a tauopathy with abundant pick body like inclusions and axonal deposits. J Neuropathol Exp Neurol. 1999;58(12):1207–1226.

38. Jin S, Pastor P, Cooper B, Cervantes S, Benitez BA, Razquin C, et al. Pooled-DNA sequencing identifies novel causative variants in PSEN1, GRN and MAPT in a clinical early-onset and familial Alzheimer’s disease Ibero-American cohort. Alzheimer’s Research. 2012;vol. 4(issue 4):34–42. doi:10.1186/alzrt137.

39. Deters K, Nho K, Kim S, Weiner MW, Trojanowski JO, Green RC, et al. Association analysis of MAPT with cerebrospinal fluid tau using targeted sequencing data in older adults with mild cognitive impairment or Alzheimer’s disease. American Society of Human Genetics 64th Annual Meeting: American Society of Human Genetics; 2014.

40. Bogaerts V, Nuytemans K, Reumers J, Pals P, Engelborghs S, Pickut B, et al. Genetic variability in the mitochondrial serine protease HTRA2 contributes to risk for Parkinson disease. Human Mutation. 2008;vol. 29(issue 6):832–840. doi:10.1002/humu.20713.

41. Strauss KM, Martins LM, Plun-Favreau H, Marx FP, Kautzmann S, Berg D, et al. Loss of function mutations in the gene encoding Omi/HtrA2 in Parkinson’s disease. Human Molecular Genetics. 2005;vol. 14(issue 15):2099–2111. doi:10.1093/hmg/ddi215.

42. Plun-Favreau H, Klupsch K, Moisoi N, Gandhi S, Kjaer S, Frith D, et al. The mitochondrial protease HtrA2 is regulated by Parkinson’s disease-associated kinase PINK1. Nature Cell Biology. 2007;vol. 9(issue 11):1243–1252. doi:10.1038/ncb1644.

43. Gulsuner HU, Gulsuner S, Mercan FN, Orol OE, Walsh T, Shahin H, et al. Mitochondrial serine protease HTRA2 p.G399S in a kindred with essential tremor and Parkinson disease. Proceedings of the National Academy of Sciences. 2014;vol. 111(issue 51):18285–18290. doi:10.1073/pnas.1419581111.

44. Simon-Sanchez J, Singleton AB. Sequencing analysis of OMI/HTRA2 shows previously reported pathogenic mutations in neurologically normal controls. Hum Mol Genet. 2008;17(13):1988–1993. doi:10.1093/hmg/ddn096.

45. Tzoulis C, Zayats T, Knappskog PM, Möller B, Larsen JP, Tysnes OB, et al. HTRA2 p.G399S in Parkinson disease, essential tremor, and tremulous cervical dystonia. Proceedings of the National Academy of Sciences. 2015;vol. 112(issue 18):E2268–E2268. doi:10.1073/pnas.1503105112.

46. CHAPTER 7 - Chi-Square Tests for Goodness of Fit and Contingency Tables. In: Statistical Power Analysis for the Behavioral Sciences (Revised Edition). 1st ed. New York: Academic Press; 1977. p. 215–271.