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ATP13A2 variability in Parkinson disease

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Abstract

Recessively inherited mutations in *ATP13A2* result in Kufor-Rakeb syndrome, whereas genetic variability and elevated *ATP13A2* expression have been implicated in Parkinson disease (PD). Given this background, *ATP13A2* was comprehensively assessed to support or refute its contribution to PD. Sequencing of *ATP13A2* exons and intron-exon boundaries was performed in 89 probands with familial parkinsonism from Tunisia. The segregation of mutations with parkinsonism was subsequently assessed within pedigrees. The frequency of genetic variants and evidence for association was also examined in 240 patients with non-familial PD and 372 healthy controls. *ATP13A2* mRNA expression was also quantified in brain tissues from 38 patients with non-familial PD and 38 healthy subjects from the US. Sequencing analysis revealed 37 new variants; seven missense, six silent and 24 that were noncoding. However, no single *ATP13A2* mutation segregated with familial parkinsonism in either a dominant or recessive manner. Four markers showed marginal association with non-familial PD, prior to correction for multiple testing. *ATP13A2* mRNA expression was marginally decreased in PD brains compared with tissue from control subjects. In conclusion, neither *ATP13A2* genetic variability nor quantitative gene expression in brain appears to contribute to familial parkinsonism or non-familial PD.

Keywords

Parkinson/Parkinsonism; Kufor-Rakeb syndrome; ATP13A2

Introduction

Over the last decade the identification of genetic causes of parkinsonism has provided insight into the pathophysiology of this neurodegenerative syndrome [Farrer, 2006]. Several chromosomal loci have been implicated, designated as *PARK1-13* [Bagade, et al., August 2007]. *PARK9* has been assigned to Kufor-Rakeb Syndrome (KRS; MIM# 606693), a juvenile onset form of autosomal recessive, L-DOPA responsive parkinsonism [Najim al-

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Din, et al., 1994]. The syndrome typically manifests with behavioral problems, akinetic-rigidity, pyramidal tract dysfunction, supranuclear gaze paresis and dementia, and is generally recognized as a pallidopyramidal disorder albeit with considerable phenotypic heterogeneity.

KRS was genetically mapped to chromosome 1p36 in a consanguineous Arabic family from Jordan. A homozygous c.1632_1653dup22 (NM_022089.1) mutation within exon 16 of the *ATPase type 13A2* (*ATP13A2*; MIM# 610513) leading to a p.552LfsX788 has now been identified as the pathogenic cause of disease in this family [Najim al-Din, et al., 1994; Ramirez, et al., 2006]. In the same report, compound heterozygous mutations were described in affected subjects of a Chilean kindred that effect the splicing efficiency of exon 13 (c.1306+5G>A) and cause a frameshift in exon 26 (c.3057delC leading to p.1019GfsX1021) [Ramirez, et al., 2006]. Most recently a homozygote missense mutation in exon 6 (c.546C>A, p.F182L) has been described in a Japanese sample with KRS [Ning, et al., 2008]. Additionally, homozygous mutations of exon 15 (c.1510G>C leading to p.G504R) have been found in a sporadic patient with juvenile parkinsonism (<20 years at onset) from Brazil, and heterozygous missense mutations (exon 2 c.35C>T, p.T12M and exon 16 c.1597G>A, p.G533R) were identified in two Italian patients with early-onset parkinsonism (<50 years at onset) [Di Fonzo, et al., 2007]. In brain *ATP13A2* mRNA expression appears highest in the *substantia nigra* and to be greatly elevated in idiopathic PD [Ramirez, et al., 2006]. Together these findings suggest *ATP13A2* genetic variability may play a role in PD and not just in atypical forms with juvenile or early-onset [Lees and Singleton, 2007].

To determine whether genetic variability in *ATP13A2* is etiologically relevant to the pathogenesis of PD, we sequenced *ATP13A2* in probands with familial parkinsonism from Tunisia. The Arab-Berber population investigated has a high frequency of consanguineous marriage that favors recessively inherited disease and the identification of rare homozygous mutations. We examined whether any *ATP13A2* mutations segregated with disease and their frequency in control subjects. Employing novel polymorphisms and haplotype-tagging SNPs (tSNP), we have also assessed evidence for genetic association in non-familial PD. Finally, we have quantified mRNA expression of *ATP13A2* in the *substantia nigra* and cerebellum of patients with PD compared with healthy subjects.

Methods

Study population

A total of 89 families from the Institut National de Neurologie, Tunis, were included in the study. This center provides a specialized neurological service to the entire country of Tunisia [Ishihara, et al., 2007]. The series included 76 multiplex families comprised of 208 patients with parkinsonism, 340 unaffected subjects and 27 with an uncertain diagnosis. The remaining 13 familial index cases (singleton families) had DNA available from one only affected subject and unaffected family members. In addition, 240 non-familial patients with PD and 372 control participants from the same geographic region were assessed (case-control series). The site obtained local ethics committee approval before beginning subject recruitment. Subjects were informed of all aspects pertaining to their participation in the study, and gave either written or proxy consent prior to recruitment.

Physical examinations were performed by neurologists specialized in movement disorders. Patients with PD and control subjects without a family history of parkinsonism were collected from all regions of Tunisia through the Institut National de Neurologie, Tunis. For our family-based study the proband was examined at the study site, and additional family members were recruited via the proband. Inclusion criteria were age at assessment older than 18 years with at least one other affected first to third degree blood relative (excluding a

monozygotic twin). Individuals were diagnosed as “affected” if they satisfied the United Kingdom PD Society Brain Bank (UKPDS) criteria [Hughes, et al., 1992], “unaffected” if all signs of parkinsonism were absent, “controls” if all signs of parkinsonism were absent and there was no family history of parkinsonism, and “uncertain” if only one parkinsonian sign or abnormal feature was present. Most of the latter were diagnosed with essential tremor. Affected members did not show atypical features suggestive of KRS. Standardized case report forms were used for clinical and demographic data collection and included Hoehn and Yahr staging [Hoehn and Yahr, 1967], the Unified Parkinson Disease Rating Scale (UPDRS) [Fahn and Elton, 1987] and Epworth scale [Johns, 1991] (data not reported). In addition to *ATP13A2*, this series was screened for *LRRK2* (MIM# 609007) c.6055G>A (p.G2019S) (NM_198578.2), *Parkin* (MIM# 602544) and *PINK1* (MIM# 608309) mutations; identified carriers were not excluded from the present analysis [Gouider-Khouja, et al., 2003; Ishihara-Paul, et al., 2008; Ishihara, et al., 2007].

DNA sequencing of *ATP13A2*

Genomic DNA was extracted from peripheral blood lymphocytes using standard protocols. Primer pairs for *ATP13A2* were used to sequence all 29 exons and exon-intron boundaries by polymerase chain reaction (PCR) in all familial probands [Ramirez, et al., 2006]. PCR products were purified from unincorporated nucleotides using Agencourt bead technology (Beverly, MA) with Biomek FX automation (Beckman Coulter, Fullerton, CA). Sequence analysis was performed as previously described [Mata, et al., 2005]. All novel variants were examined for disease segregation in affected and unaffected family members by additional sequencing.

Genetic association analysis

The population frequency of 31 novel *ATP13A2* variants was assessed in a case-control series involving 240 non-familial patients with PD and 372 control subjects from Tunisia. The mean ages at exam and gender ratios (M:F) were comparable in patient and control groups (64±11 SD (range: 25-85), M:F 0.9:1 in patients, and 56±11 SD (range: 29-90), M:F 1:1 in controls). Selection of additional haplotype tagging single-nucleotide polymorphisms (tSNP) was based on HapMap Phase II data using Haploview software [Barrett, et al., 2005]. In total seven tSNPs were selected across the *ATP13A2* locus to capture all polymorphic variation with a minor allele frequency >5% ($r^2 = 1.0$ across Caucasian, Asian and African population standards). Genotyping of tSNPs and novel variants, as identified in proband sequencing, was performed on a Sequenom MassArray iPLEX platform (San Diego, CA); all primer sequences are available on request. For each variant genotyping error was assessed by deviation from Hardy-Weinberg equilibrium expectation. Where appropriate, associations between tSNP alleles and PD were investigated using logistic regression models, adjusted for age and gender; otherwise Fisher's exact test was employed.

Autopsy material

Brain material was available from 38 cases with clinical and autopsy confirmed brainstem Lewy body PD (mean age at death (AAD) 77±7, range 60-91, M:F 4.4:1) and 38 control subjects (mean AAD 72±13, range 50-97, M:F 1.3:1). Frozen tissue was regionally dissected to include material from cerebellar cortex for all subjects. *Substantia nigra* was only available from a subset of these brains (n=5 cases, 8 controls). Subjects were of North American/Northern European ethnicity.

Gene expression analyses

Total human RNA was isolated using TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA). High-quality, non-degraded RNA was confirmed by RNA integrity number

(RIN; arbitrary values are on a scale from 0-10, higher values indicating the best quality RNA) using Agilent technology (Santa Clara, CA). One microgram of tissue RNA was reverse transcribed using Applied Biosystems High Capacity Archive Kit (Foster City, CA) according to manufacturer's instructions. Real-time quantitative PCR was performed using on-demand TaqMan gene expression assays purchased from Applied Biosystems. Two different probes were used to assess and corroborate *ATP13A2* expression (Hs01119556_m1 and Hs01119444_m1). Multiple house-keeping genes were used for quantitative normalization across samples, as previously reported [Dachsel, et al., 2007]. Thus Ct values for house-keeping genes (*GAPDH*, *HPRT* and *YWAZ*) were also measured. For each brain, standardized *ATP13A2* expression was calculated by dividing 2^{-Ct} by the geometric mean of the expression levels of the house-keeping genes for that tissue. Standardized expression for each brain was then divided by the geometric mean of standardized expressions for all brains so that the scale of each probe was centered about a value of one, and this final measure of expression was used in all statistical analysis. Linear regression models adjusted for RIN, age of death, and gender were used to investigate the association between expression and PD, where expression was considered on the natural logarithm scale. Kendall's tau was used to investigate the degree of correlation between *ATP13A2* expression in the cerebellum and *substantia nigra*. Statistical significance was determined at the 5% level in all analyses. A Bonferroni correction for multiple testing was made when appropriate.

Results

Mutation screen and segregation analysis

Sequencing analysis of *ATP13A2* in 89 familial probands of Arab-Berber descent from Tunisia, North Africa revealed 31 new variants (six missense, five silent and twenty non-coding (two insertion/deletion)). Sequencing of additional family members to study the segregation of these variants with the disease phenotype identified yet another six novel variants (one missense, one silent and four non-coding) (Figure 1 and Supplementary Table S1). In addition, ten known polymorphisms were also detected (six silent and four non-coding; rs9435736, rs2076603, rs2076604, rs9435662, rs3738815, rs761421, rs7531163, rs9435659, rs2076605, rs3170740). Segregation analyses showed that none of the 37 newly identified variants co-segregated with parkinsonism within families, in either a homozygous or heterozygous manner.

Genetic association analysis

Genotype analysis of 31 novel variants, identified through proband sequencing, and seven tSNPs was performed in the case-control series. In the case-control series, deviations from Hardy-Weinberg equilibrium expectation ($P < 0.01$) were noted for two variants because of rare homozygous genotypes for rs41273157 ($n=1$) and rs56004722 ($n=2$) in the PD group. These chance observation were not considered further. With the exception of two insertion/deletions, all variants were rare with minor allele frequencies $< 5\%$. Four markers showed marginal association with PD at the 5% level (rs56351817, rs56010635, rs56165294 and rs15786), but were not significant after correction for multiple testing ($P > 0.0014$) (Supplementary Table S2). Five variants (rs56275621, rs56186751, rs55633553, rs56164593 and rs56170027) were not observed in the case-control series.

Analysis of gene expression in cerebellum and substantia nigra

Associations with *ATP13A2* gene expression in the cerebellum are shown in Supplementary Table S3. Overall, there was a small but statistically significant decrease in *ATP13A2* gene expression in patients with PD for both *ATP13A2* mRNA probes in comparison to control subjects (Figure 2A and Supplementary Figure S1A). Estimated median expression in PD

cases was 0.82 (CI: 0.73-0.91, $P < 0.001$) times that of controls for Hs01119456, and 0.90 (CI: 0.83-0.98, $P = 0.020$) for Hs01119444. An increase in expression was observed for Hs01119444 as RIN decreased ($P < 0.001$) (Figure 2B); the same trend, albeit non-significant, was noted for Hs01119456 ($P = 0.076$) (Supplementary Figure S1B). Degraded mRNA is more efficiently synthesized into short cDNA fragments and can erroneously lead to an apparent increase in gene expression. There is no statistically significant evidence of an association between *ATP13A2* expression and gender or AAD.

Expression of *ATP13A2* in *substantia nigra* was not significantly different between PD cases and controls (Hs0111444, $P = 0.62$; Hs0111456, $P = 0.58$), however the power to detect a difference was low due to small sample sizes. The estimated median expression in PD cases was 1.19 (CI: 0.55-2.58) times that of controls for Hs01119444, and 1.01 (CI: 0.78-1.31) for Hs01119456. The level of correlation in *ATP13A2* expression between cerebellum and *substantia nigra* was assessed in 8 controls and 5 PD cases from which RNA was available from both tissues. Significant correlation was observed for Hs01119444 (Tau: 0.74, $P < 0.001$) (Figure 2C) and Hs01119456 (Tau: 0.46; $P = 0.028$) (Supplementary Figure S1C).

Discussion

Recessively inherited mutations in *ATP13A2* are linked to KRS, a pallidopyramidal syndrome encompassing behavioral, cognitive and movement disorders [Najim al-Din, et al., 1994]. However homozygote and heterozygote substitutions found in juvenile and young onset patients with parkinsonism have suggested *ATP13A2* plays a role in the development of idiopathic PD [Di Fonzo, et al., 2007]. Herein we sequenced *ATP13A2* in 89 probands with familial parkinsonism and discovered 37 novel variants, seven of which encode missense substitutions for evolutionary conserved amino acids (Figure 3); p.G49S and p.I946F are located in transmembrane domains while the remainder are in cytoplasmatic domains; no variants were identified in the predicted functional domains. Of these, p.P389L, p.V578G and p.V776I are rare mutations that were absent in 607 ethnicity matched samples (Supplementary Table S2). However, no *ATP13A2* variant was found to segregate with disease within families suggesting none may contribute directly to disease risk (Supplementary Table S1). Although reduced penetrance and/or phenocopies may obscure the segregation of *ATP13A2* variants with disease, within most families there were several unaffected carriers and affected non-carriers making this scenario unlikely. Furthermore, case-control analysis of variants identified by sequencing and tSNPs showed no evidence of genetic association with disease. Ideally, segregation of *ATP13A2* mutations with parkinsonism within families, or association within a case-control series, or functional proof, should be demonstrated before a mutation is considered pathogenic. Of note, none of the previously reported *ATP13A2* substitutions p.T12M, p.G533R or p.G604R were found [Di Fonzo, et al., 2007]. While these are also evolutionary conserved, with low minor allele frequencies, segregation with disease was not presented and evidence for pathogenicity remains equivocal. It should be noted that the previously reported PD mutations [Di Fonzo, et al., 2007] have been described in a Brazilian and Italian subjects whereas this study analyzed Tunisian subjects, therefore generalization of this data to other populations may be questionable. However, if PD mutations are found in Italy and Brazil [Di Fonzo, et al., 2007], and KRS pathogenic mutations in Jordanian, Chilean, and Japanese families [Najim al-Din, et al., 1994; Ning, et al., 2008; Ramirez, et al., 2006], it is not unreasonable to expect mutations in *ATP13A2* to be global and present in Tunisia.

Brain expression of *ATP13A2* was similar or marginally decreased in PD versus control subjects. Despite the small sample size, expression in cerebellum mirrored expression in *substantia nigra*, and may provide a reasonable surrogate source of tissue for an area of the

brain that is generally lost in advanced disease. However, RNA quality remains an important issue as degraded RNA, with lower RINs, appears to have higher levels of gene expression. The methodology used to analyze the expression of *ATP13A2* in this study differs from that previously reported, laser microdissection [Ramirez, et al., 2006], therefore the data is not directly comparable. Nevertheless, a 10-fold increased expression in *substantia nigra* from patients with PD patients seems unlikely.

In summary, we have identified 37 novel variants in *ATP13A2* although none segregate with familial parkinsonism within kindreds. Analysis of *ATP13A2* polymorphic variants, including tSNPs, provided no evidence for disease association in a case-control series. Thus genetic variability in *ATP13A2* is unlikely to influence susceptibility to non-familial PD. Nor did gene expression analysis show an increase in *ATP13A2* mRNA in PD brains in comparison to controls. Thus we conclude that *ATP13A2* genetic variability is unlikely to cause or influence the development of PD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Exons are represented as grey boxes with untranslated regions in black. All introns < 500bp and exons are drawn to scale. Positions of all novel variants are indicated; coding variants are given above the gene (black, missense; grey, silent) noncoding variants are given below the gene.

Figure 2. *ATP13A2* expression (Hs01119444_m1)

	G49S	R294Q	P389L	V578G	R762W	V776I	I946F
H. sapiens	WRVIG-----YHVV	KLSMRVCVC	AYVGEHVLA	DLKMVESTG	VTVARGCGM	HLIIIVHATH	LTQFTSVLI
P. troglodytes	LRRARRDCGSVGAA	KLSMRVCVC	AYVGEHVLA	DLKMVESTG	VTVARGCGM	HLIIIVHATH	LTQFTSVLI
M. mulatta	WRVIG-----YHVV	KLSMRVCVC	AYVGEHVLA	DLKMVESTG	VTVARGCGM	HLIIIVHATH	LTQFTSVLI
M. musculus	WRAIG-----YHAA	KLSVRVQVC	AYVGERVLA	DLKMVESTG	VTVARACGM	HLAVIHATH	LTQFTSVLI
R. norvegicus	WRAIG-----YHAA	KLSVRVQVC	AYLGERVLA	DLKMVESTG	VTVAKACGM	HLAIHATH	LTQFTSVLI
D. rerio	WKVWL-----CRIG	CLIVNVTVR	GGPGKGAIA	ELKMIESTG	VNVARVCGM	KVIFVHASP	LIQFASVLI
D. melanogaster	NRPDQDGDSEENIT	YNTGNAWVV	YIGSKKVL	DLKMFESTG	ISVARDCGI	SVITVHADP	LVQFTSVMI

Figure 3. Conservation of novel amino acid substitutions

Protein homologues were aligned using ClustalW. GenBank accession numbers:

NP_071372.1 (ATPase type 13A2, *Homo sapiens*), XP_513111.2 (ATPase type 13A2, *Pan troglodytes*), XM_001087655.1 (Similar to ATPase type 13A2, transcript variant 2, *Macaca mulatta*), NP_083373.1 (ATPase type 13A2, *Mus musculus*), XP_342963.3 (ATPase type 13A2 (predicted), *Rattus norvegicus*), XP_697103.2 (zgc:136762, *Dario rerio*), NP_995587.1 (CG32000, *Drosophila melanogaster*).