Analysis of vesicular monoamine transporter 2 polymorphisms in Parkinson’s disease

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ABSTRACT

Generation of reactive oxygen species during dopamine (DA) oxidation could be one of the factors leading to the selective loss of nigral dopaminergic neurons in Parkinson’s disease (PD). Vesicular monoamine transporter type 2 (VMAT2) proteins in nerve terminals uptake dopamine into synaptic vesicles, preventing its cytoplasmic accumulation and toxic damage to nigral neurons. Polymorphisms in VMAT2 gene and in its regulatory regions might therefore serve as genetic risk factors for PD. In the present study, we have analyzed 8 single-nucleotide polymorphisms (SNPs) located within/around the gene for association with PD in an Italian cohort composed of 704 PD patients and 678 healthy controls. Among the 8 SNPs studied, only the 2 located within the promoter region (rs363371 and rs363324) were significantly associated with PD. In the dominant model, odds ratios were 0.72 (95% confidence interval [CI]: 0.6–0.9, p < 0.005) for rs363371 and 0.76 (95% CI: 0.6–0.9, p = 0.01) for rs363324; in the additive model, odds ratios were 0.78 (95% CI: 0.65–0.94, p = 0.008) for rs363371 and 0.85 (95% CI: 0.7–0.92, p = 0.04) for rs363324. There were no significant relationships between the remaining SNPs (rs363333, rs363399, rs363387, rs363343, rs4752045, and rs363236) and the risk of sporadic PD in any genetic model. This study adds to the previous evidence suggesting that variability in VMAT2 promoter region may confer a reduced risk of developing PD, presumably via mechanisms of gene overexpression.

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1. Introduction

Parkinson’s disease (PD) is a debilitating neurologic disorder characterized clinically by bradykinesia, resting tremor, rigidity, and postural instability with a therapeutic response to levodopa. Pathophysiologically, PD is characterized by the loss of dopamine neurons in the substantia nigra (SN) pars compacta and by the presence of intracellular inclusions known as Lewy bodies, which are composed primarily of alpha-synuclein protein aggregates. Although the disease etiology remains largely unclear, generation of reactive oxygen species during oxidation of dopamine (DA) could be one of the factors leading to the selective loss of nigral dopaminergic neurons in PD (Spina and Cohen, 1989).

The vesicular monoamine transporter type 2 (VMAT2, SLC18A2) uptakes cytosolic monoamines, including dopamine, into intracellular secretory vesicles, preventing their toxicity in the cytosol and discharging them into the extracellular space by exocytosis. Altered function of VMAT2 proteins may therefore cause cytoplasmic accumulation of free DA, leading to dopaminergic neuron death, therefore being a risk factor for PD. VMAT2 is expressed in central, peripheral, and enteric neurons as well as in platelets (Peter et al., 1995). VMAT2-deficient mouse exhibits increased oxidative stress, progressive loss of DA terminals and cell bodies in the SN pars compacta, alpha-synuclein accumulation (Caudle et al., 2007), and increased sensitivity to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity (Mooslehner et al., 2001); interestingly, this model displays also motor and nonmotor symptoms of PD (Taylor et al., 2011). In humans, the involvement of VMAT2 in PD pathogenesis is supported by positron emission tomography studies showing significantly lower VMAT2 densities in the putamen, caudate, and SN of PD patients (Bohnen et al., 2006; Lee et al., 2000;
Martin et al., 2008; Okamura et al., 2010); interestingly, reduced VMAT2 messenger RNA (mRNA) levels were observed in platelets from patients (Sala et al., 2010).

Polymorphisms in the VMAT2 gene or in its regulatory regions that affect its quantitative expression or protein function might therefore represent genetic risk factors for PD. Some common gain-of-function haplotypes in VMAT2 promoter have been reported to confer a protective effect against PD in women (Glatt et al., 2006), whereas no studies have so far addressed specifically the association of intragenic VMAT2 variability with PD in Caucasian populations. In light of these premises, we carried out an association study to evaluate whether genetic variants in VMAT2 gene and in its regulatory regions might influence either susceptibility to PD or age of onset of the disease in a series of Italian sporadic PD patients and unrelated healthy controls.

2. Methods

2.1. Subjects

The study included 704 PD patients recruited consecutively from the outpatient clinic for movement disorders at the Department of Neurology of San Gerardo Hospital in Monza and at the Parkinson Institute in Milan. PD patients were diagnosed according to the standard criteria (Gelb et al., 1999). Among patients, 45 (7.5%) reported a family history of PD in at least a first-degree or second-degree relative. PD cases with an age at onset (AAO) of <40 years were screened to exclude that they were carriers of recognized PD-causing mutations (Parkin, PINK1, and LRRK2 genes); the remaining familial cases were screened for the highly penetrant G0219S mutation in the LRRK2 gene that was absent in all the examined subjects. All the 45 cases with a family history of PD were included in the analysis. Control population consisted of 678 individuals, comprising healthy spouses and former blood donors. Both patients and controls were Caucasians, and the controls were geographically matched to the cases. None of the controls had clinical evidence of neurologic disease (as assessed by neurologic examination) or familial history of neurodegenerative diseases. None of the patients and of the controls had a current or former history of alcohol use disorders. Informed consent for participation was obtained from all the subjects. Full ethical committee approval for this investigation was obtained.

2.2. Markers selection and genotyping

The present analysis comprised a total of 8 single-nucleotide polymorphisms (SNPs). SNPs were selected according to the inter-marker distance, their minor allele frequency (MAF >5%), the Illumina design scores (>0.7), and the previous publications showing associations with other dopamine-related traits. In detail, the 2 SNPs in the promoter region (rs363371 and rs363324) previously identified by resequencing and included in haplotypes have been shown to be associated with alcoholism (Lin et al., 2005), rs363333 and rs363387 have been previously shown to be associated with substance dependence (Schwab et al., 2005), and rs363399 and rs4752045 with depression (Christiansen et al., 2007). Lastly, considering a possible role in VMAT2 mRNA stability, an additional candidate SNP in the 3’-untranslated region (rs3633236) was also selected. Table 1 highlights the genomic positions and the type of the genotyped markers.

Genomic DNA was extracted from peripheral venous blood using a Qiagen Blood kit (Qiagen, Milan, Italy). SNPs were typed using the VeraCode GoldenGate genotyping assay on Illumina BeadXpress Reader Platform according to the manufacturer’s protocol (Illumina, San Diego, CA, USA). The genotyping call rate for the studied SNPs was >97%. Goodness of fit of the genotype frequencies to Hardy–Weinberg (HW) expected proportions in control subjects was examined.

2.3. Statistical analysis

Chi-square tests were used to compare sex distribution and genotype frequencies between patients and controls and to test for deviation from HW equilibrium. Student t test was used to assess differences in age between the 2 groups. We used logistic regression analysis to test for the association between the VMAT2 gene selected variants and PD risk. The estimated odds ratios (ORs) and relative 95% confidence intervals (CIs) were adjusted for sex, age at enrollment, and smoking (ever/never). We performed analyses assuming autosomal dominant, log-additive, and recessive models of inheritance. In the test, all p values for individual SNPs, OR, and 95% CI are presented as noncorrected for multiple testing. Analyses were performed for subjects overall and stratified by family history of PD, age at study (divided in quartiles), and sex. The association of each genetic variant with AAO of PD was assessed using Cox proportional hazard models, adjusted for sex. For each genetic variant, we calculated a hazard ratio, a 95% CI, and a 2-tailed p value. All the analyses were performed using SPSS version 16. Power calculations were carried out using the Quanto software version 1.2, under a dominant model of inheritance. The pairwise linkage disequilibrium (LD) between markers was estimated using Haplovewview software (Barrett et al., 2005). The Ensembl genome browser (http://www.ensembl.org/index.html) was used to identify and to annotate nearby SNPs in LD (proxies) based on HapMap CEU. Haplotype associations were explored using score tests (Schaid et al., 2002) as implemented in the haplo.stats R Package (http://mayoresearch.mayo.edu/mayo/research/schaid_lab/software.cfm). The overall difference in haplotype frequencies between cases and controls was assessed using a global score test. Haplotype-specific tests were performed only including haplotypes with frequencies

### Table 1

Overview of location and type of the genotyped SNPs

| SNP ID   | Genome position (base pair) | Allele 1 | Allele 2 | MAF cases/controls | Type of variant     |
|----------|-----------------------------|----------|----------|--------------------|--------------------|
| rs363371 | 118976386                   | G        | A        | 0.19/0.23          | Flanking 5’ UTR    |
| rs363324 | 118979152                   | A        | G        | 0.29/0.33          | Flanking 5’ UTR    |
| rs363333 | 118994085                   | T        | C        | 0.09/0.10          | Intron 2           |
| rs363399 | 118998861                   | T        | C        | 0.22/0.24          | Intron 2           |
| rs363387 | 119003564                   | T        | G        | 0.07/0.08          | Exon 2 synonymous  |
| rs363343 | 119004938                   | A        | C        | 0.17/0.18          | Intron 6           |
| rs4752045| 119009680                   | G        | C        | 0.46/0.48          | Intron 9           |
| rs363236 | 119028361                   | T        | C        | 0.19/0.18          | 3’ Downstream      |

Allele 1, major frequency allele and allele 2, minor frequency allele.

Key: MAF, minor allele frequency; SNPs, single-nucleotide polymorphisms; UTR, untranslated region.

* Base position according to National Center for Biotechnology Information genome build 36.
>1%, using the additive model with the most frequent haplotype as the referent group in the regression analysis. Correction for multiple testing was performed by applying the simulate=TRUE parameter in haplo.score that gives simulated p values based on 10,000 permutations.

3. Results

The demographic data for the 704 PD patients were as follows: mean age at study was 65.14 ± 9.3 years (range 32–90) and mean AAO of symptoms was 57.28 ± 10 years (range 27–87). There were 415 men and 289 women (male–female ratio 1.43:1). The 678 control subjects consisted of 375 men and 303 women; their mean age at examination was 64.2 ± 7.7 years (range 44–99). There was no statistical difference in the age at study and sex between patients and controls (p > 0.05).

The control genotype distributions were in agreement with the HW equilibrium for all markers (HW p value > 0.05). The allele frequencies of the examined polymorphisms showed close similarity to HapMap CEU data. All the considered SNPs showed an MAF more than 8%. LD between the studied SNPs was low as detailed in Table 2. The LD statistics were similar in PD patients and controls.

Table 3 lists the estimated ORs and associated 95% CIs from the association analysis between each SNP and PD, assuming autosomal dominant and trend (additive) models of inheritance. Two out of the 8 investigated SNPs, both located in the putative promoter region at the 5′ untranslated region, were inversely associated to PD when assuming dominant (rs363371: OR 0.72, CI 0.62–0.97, p = 0.004; rs363324: OR 0.76, CI 0.61–0.95, p = 0.01) and trend model of inheritance (rs363371: OR 0.78, CI 0.65–0.94, p = 0.008; rs363324: OR 0.85, CI 0.72–0.92, p = 0.04). After Bonferroni correction for multiple comparisons, only rs363371 remained significantly associated with PD (corrected p value = 0.03, dominant model). Sensitivity analyses excluding patients with a family history of PD yielded similar results.

The remaining intragenic SNPs were not significantly associated with PD overall or in strata defined by age at study, sex, and family history (data not shown).

Furthermore, we tested the possible association among all the different combinations of alleles at the 8 polymorphic loci with PD. Four common haplotypes (GATTAGT, frequency in cases/controls = 0.30/0.29; GATTACT, frequency = 0.23/0.21; GATTTAC, frequency = 0.07/0.07; and AGTCTAGT, frequency = 0.05/0.06) and several rare ones (with frequency <5%) were inferred. None of the common haplotypes was associated with the overall risk for PD (global haplotype association p value = 0.56). Given the observed LD pattern between the studied SNPs (with poor linkage between the 5′ half and the 3′ half markers), we then partitioned the whole haplotype into smaller segments (composed of 2–4 adjacent markers) and tested them for association with PD. Consistent with the individual SNP analysis, a significant association with PD was observed for the haplotype including the 2 promoter variants, rs363371 and rs363324 (global p value = 0.04). In particular, the all-minor allele haplotype AG (the second most frequent haplotype in both case and control populations) was significantly underrepresented in cases (frequency 19%) as compared with controls (frequency 23%), hence being associated with a reduced PD risk as compared with the most frequent haplotype GA (OR 0.78, 95% CI 0.65–0.94, permutation-based p value = 0.03, additive model).

Finally, in analyses restricted to PD cases, none of the studied SNPs displayed a significant association with age at disease onset under additive or dominant models, either in the overall sample or after stratification by sex (data not shown).

4. Discussion

In our study, we found that 2 polymorphisms located in the putative promoter region of the VMAT2 gene were inversely associated to PD, whereas the other SNPs within the gene were not related to the risk of developing PD or to disease AAO.

The human VMAT2 gene consists of 16 exons and 15 introns and has been localized to chromosome 10q25. The recently launched website PDGene (http://www.pdgene.org/), which systematically reviews genetic association studies of PD including genome-wide association studies (GWAS), confirms that information regarding the association of VMAT2 gene with PD is still deficient. A single case–control study comprising 190 Japanese patients and 190 controls failed to identify any significant association among 3 intronic SNPs in the gene (rs2072362, rs3523, and rs363334) and PD (Mizuta et al., 2006). Although none of the SNPs analyzed in the present study was specifically tested in the publicly accessible GWAS (as assessed using the National Center for Biotechnology Information database of genotypes and phenotypes, http://view.ncbi.nlm.nih.gov/dbgap), the National Institute of Neurological Disorders and Stroke (NINDS) Genome-Wide Genotyping in Parkinson’s Disease (dbGaP study accession number: phs000089.v3.p2) and the Center of Inherited Disease Research (CIDR): GWAS in Familial Parkinson Disease (phs000126.v1.p1) contained respectively 2 (rs363327 and rs1860404) and 1 (rs1860404) SNPs in high LD with our variants rs363343 and rs363333, which similarly were not associated with PD. Hence the present study, extending the analysis to more SNPs inside the gene (both in coding and noncoding regions), adds to previous evidence showing no association between intragenic VMAT2 variability and PD.

A few more studies focused on the association of variants in the VMAT2 promoter region with PD. In stark contrast to the very low degree of variability of the coding sequence, a high degree of genetic variability in the VMAT2 promoter region has been identified (Glatt et al., 2001, 2006). Lin et al. (2005) sequenced the 17.4-kb VMAT2 promoter region in 23 Caucasian individuals and identified 47 polymorphisms conferring 13 haplotypes associated to different promoter activities, as suggested by in vitro luciferase-

| Table 2 |
|---|
| Pairwise linkage disequilibrium measurements between SNPs (r² below and D’ above the diagonal) calculated using the control sample |
| rs363371 | rs363324 | rs363333 | rs363399 | rs363387 | rs363343 | rs4752045 | rs363236 |
| rs363371 | 1 | 0.868 | 0.965 | 0.952 | 0.963 | 0.989 | 0.709 | 0.236 | 0.036 |
| rs363324 | 0.61 | 1 | 0.932 | 0.91 | 0.963 | 1 | 0.989 | 0.709 | 0.236 | 0.036 |
| rs363333 | 0.025 | 0.207 | 1 | 0.91 | 0.963 | 1 | 0.989 | 0.709 | 0.236 | 0.036 |
| rs363399 | 0.878 | 0.535 | 0.035 | 0.91 | 0.963 | 1 | 0.989 | 0.709 | 0.236 | 0.036 |
| rs363387 | 0.025 | 0.165 | 0.743 | 0.026 | 0.989 | 1 | 0.989 | 0.709 | 0.236 | 0.036 |
| rs363343 | 0.198 | 0.116 | 0.012 | 0.22 | 0.012 | 0.001 | 0.001 | 0.001 | 0.012 | 0.024 |
| rs4752045 | 0.001 | 0.005 | 0.007 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.012 | 0.024 |
| rs363236 | 0.002 | 0.001 | 0.001 | 0.002 | 0.002 | 0.001 | 0.001 | 0.001 | 0.012 | 0.024 |

Key: SNPs, single-nucleotide polymorphisms.
transport assays. Of note, both the SNPs in the promoter region (rs363371 and rs363324) genotyped in our study were among the 4 haplotype-defining SNPs identified by Lin et al. (2005) and were likely to be functional. The G allele (wild type) of rs363371 is embedded in a consensus sequence for transcriptional repressor GC-binding factor (GCF) that is expressed in many tissues, whereas the minor A allele is not (Kageyama and Pastan, 1989). On the other hand, rs363324 is in considerable LD with rs363371 \((D' = 1, r^2 = 0.61)\) and with another variant at the 5' untranslated region, \(-2504T>C\) (rs2619096), which is probably a transcription factor binding site, because the switching from T (wild-type allele) to C (variant allele) could double the promoter activity in luciferase assays in SHSY cells (Lin et al., 2005). Interestingly, regulatory haplotypes including these variants have been associated also with alcohol dependence, a different dopamine-related phenotype, might relate to in vivo RNA and protein levels, the reported inverse association might still be biologically meaningful: even slight variations in VMAT2 expression might affect pathogenic mechanisms involved in nigrostriatal degeneration (Chen et al., 2008) or in the defense against PD-related environmental toxins (Lee et al., 2000).

In contrast to Glatt's and our findings, 2 polymorphic sequences upstream the VMAT2 promoter region were not associated to PD in the Japanese population (Kariya et al., 2005); however, the different type (polymorphic microsatellites) and location of the studied polymorphisms as well as the relevant ethnic differences previously observed in the genotype and allele frequencies of the polymorphisms in/around this gene (Crowley et al., 2008; Glatt et al., 2006) may account for the discrepant findings. The aforementioned GWAS NINDS and CIDR examined only 2 variants (rs10886051 and rs2532798) in the VMAT2 putative promoter region, which were in high LD with rs363371 and rs363324, respectively. Whereas in the NINDS study both variants were not significantly associated with PD (association \(p = 0.41, 0.47\), respectively), in the CIDR study only the SNP in LD with rs363371 showed a tendency toward inverse association with PD \((OR = 0.8, p = 0.08)\), which is in favor of a possible involvement of rs363371 rather than rs363324 in disease risk. Although our reported associations of the promoter variants with PD may be spurious, it is also recognized that efforts to minimize the false-positive rates in GWAS may lead to missed associations. Replication of our findings in independent populations as well as marker saturation of this still understudied VMAT2 regulatory region will be required to determine if and how variability in VMAT2 promoter affects susceptibility to PD.

Our study has some strengths. We adjusted our analyses for possible confounders (sex, age at study, and smoking). The sample size employed and the MAF for the selected SNPs provided sufficient statistical power for the main effect analyses. Assuming the population susceptibility allele frequency to be the values observed

### Table 3

| Genotype frequency, \(n(\%)
| Dominant model \(OR(95\% CI)
| Trend model \(OR(95\% CI)
| p Value \(p_{\text{corr}}\) value \(p_{\text{corr}}\) value |
|----------------|------------------|------------------|------------------|
| rs363371 Cases | 464 (66)         | 208 (29.6)       | 31 (4.4)         | 0.72 (0.58–0.90) | 0.004 (0.03) | 0.78 (0.65–0.94) | 0.0088 (0.07) |
| Controls       | 396 (58.5)       | 245 (36.2)       | 36 (5.3)         | 0.76 (0.61–0.95) | 0.01 (0.08) | 0.85 (0.72–0.92) | 0.04 (0.32) |
| rs363324 Cases | 364 (52.5)       | 259 (37.4)       | 70 (10.1)        | 0.97 (0.74–1.28) | 0.85       | 0.94 (0.73–1.21) | 0.62 |
| Controls       | 304 (45.4)       | 291 (43.5)       | 74 (11.1)        | 0.83 (0.66–1.03) | 0.086      | 0.91 (0.76–1.10) | 0.34 |
| rs363333 Cases | 576 (81.9)       | 121 (17.2)       | 6 (0.9)          | 1.01 (0.74–1.37) | 0.95       | 0.97 (0.73–1.28) | 0.81 |
| Controls       | 551 (81.3)       | 116 (17.1)       | 11 (1.6)         | 1.01 (0.80–1.27) | 0.94       | 0.97 (0.80–1.19) | 0.78 |
| rs3633599 Cases| 435 (61.8)       | 229 (32.6)       | 39 (5.6)         | 0.85 (0.67–1.07) | 0.17       | 0.92 (0.79–1.07) | 0.27 |
| Controls       | 387 (57.2)       | 260 (38.5)       | 29 (4.3)         | 1.05 (0.84–1.32) | 0.68       | 1.03 (0.85–1.25) | 0.76 |
| rs363387 Cases | 596 (85.8)       | 96 (13.8)        | 3 (0.4)          | 1.01 (0.74–1.37) | 0.95       | 0.97 (0.73–1.28) | 0.81 |
| Controls       | 577 (85.5)       | 91 (13.5)        | 7 (1)            | 1.01 (0.80–1.27) | 0.94       | 0.97 (0.80–1.19) | 0.78 |
| rs363343 Cases | 481 (68.4)       | 203 (28.9)       | 19 (2.7)         | 0.85 (0.67–1.07) | 0.17       | 0.92 (0.79–1.07) | 0.27 |
| Controls       | 463 (68.6)       | 187 (27.7)       | 25 (3.7)         | 1.05 (0.84–1.32) | 0.68       | 1.03 (0.85–1.25) | 0.76 |

The estimated ORs and relative 95% CIs were adjusted for sex, age at enrollment, and smoking. Key: PD, Parkinson's disease; OR, odds ratio; CI, confidence interval.

- Allele 1 (major frequency/wild-type allele) and allele 2 (minor frequency/variant allele) base pair for each marker is specified in Table 1.
- \(p\) value after Bonferroni correction for 8 single-nucleotide polymorphism tests \((\alpha = 0.006)\). Significant value is indicated in bold.
in controls and a population prevalence of 0.02, our study had 80% power (alpha = 0.05, uncorrected for multiple testing) to detect ORs as small as 1.43 (or 0.71 or smaller) for the SNP with the highest MAF and as small as 1.5 (or 0.63 or smaller) for the SNP with the lowest MAF under a dominant model of inheritance. We also considered multiple genetic models and stratified our sample for multiple variables to explore possible effect modifications, in particular by sex.

Although we planned our study to include most haplotype-defining SNPs based on the previous data from the literature, our coverage of VMAT2 variability is inevitably incomplete, given the low LD across and around the gene, as inferred from HapMap CEU data; furthermore, nonsynonymous variants in coding regions of the gene (most likely to alter its function), as well as rare polymorphisms (MAF < 5%) and copy number variations, have been excluded from our study. Hence, the possibility remains that intragenic VMAT2 variability contributes to PD susceptibility or interacts epistatically with other genes.

Another limitation of our study might be that, because we did not conduct correction for multiple testing, false-positive findings remain possible; hence, our results must be considered as preliminary until replication in additional series is obtained. With use of the Bonferroni criteria to adjust for multiple comparisons, the association with PD of only 1 of the 2 SNPs in the promoter (rs363371) remained significant (corrected p-value = 0.03, dominant model). However, the Bonferroni correction for multiple testing takes no account of LD between adjacent variants, thus leading to overcorrection and possible rejection of true-positive findings (Attia et al., 2009).

In conclusion, the current study suggests that variability within the VMAT2 promoter region may confer a reduced risk of developing PD. If confirmed and supported by further functional studies, genetic association of these variants with PD susceptibility might also support molecular targeting of the VMAT2 gene or gene products as a therapeutic strategy for the disease.

Disclosure statement

The authors have no actual or potential conflicts of interest.

Full approval for this investigation was obtained from the ethical committee. Informed consent for participation was obtained from all the subjects.

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