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Association of polymorphism in cytochrome P450 2D6 and N-acetyltransferase-2 with Parkinson's disease

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Abstract. The present case-control study was carried out to investigate the association of polymorphism in cytochrome P450 2D6 (*CYP2D6*) and N-acteyltransferase-2 (*NAT2*), that are involved in the metabolism and detoxification of chemicals causing Parkinson disease (PD) like symptoms, with PD. Our data demonstrated increased frequency of *CYP2D6*2* (1749G/C and 2938C/T), *CYP2D6*4* (1934G/A) and *CYP2D6*10A* (188C/T) polymorphisms in PD cases when compared to the controls. Statistical analysis revealed the significant association of *CYP2D6*4* (1934G/A) and *CYP2D6*10A* (188C/T) polymorphism with PD. Likewise, increased frequency of *NAT2*7* polymorphism that leads to the slow acetylator phenotype was observed in PD patients with more than fivefold increased risk (OR: 5.55; 95%CI: 0.56–54). No change was observed in the frequency of *NAT*5* or *NAT*6* alleles in the cases. Further, cases carrying combination of heterozygous genotypes of *CYP2D6*4* or *CYP2D6*10A*(*188C* $>$ *T*) and *NAT2*5* were found to be at significantly higher risk for PD demonstrating the importance of gene-gene interactions in determining susceptibility to PD.

Keywords: Cytochrome P450 2D6, N-acetyltransferase-2, poor metabolizers, parkinson disease, risk

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder, clinically characterized by bradykinesia, rigidity, resting tremor and postural instability. The main pathological characterstic of PD is the profound loss of pigmented neurons, primarily in the pars compacta of the substantia nigra. This neuronal loss is associated with the presence of large eosinophilic inclusions, called Lewy bodies, within the remaining pigmented neurons, made up of series of proteins, including neurofilaments, α-synuclein fibrils, ubiquitin, parkin, and proteosomal elements. Early symptoms of PD are primarily due to the selective degeneration of dopaminergic neurons of the substantia nigra innervating the neostriatum. The primary cause of PD is unknown. However, mitochondrial failure and oxidative stress, responsible for neurodegeneration in the substantia nigra is one of the most investigated hypotheses for the etiology of idiopathic PD [1].

Poor metabolizers (PM) of enzymes such as cytochrome P450 2D6 (CYP2D6), N-acetyltransferase-2 (NAT-2) etc. involved in the detoxification of endogenous as well as environmental chemicals have been hypothesized to be more susceptible to PD [2,3]. SNPs in *CYP2D6* gene were found to be associated with various neurodegenerative diseases in some of the populations. Individuals inheriting PM genotype of *CYP2D6* may accumulate the neuroactive chemicals or their metabolites in brain, which may render them more suscep-

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tible to develop PD [4–7]. Similarly, polymorphism in *NAT-2*, results in the production of NAT proteins with variable enzyme activity or stability, which leads to slow or rapid acetylation phenotypes, has also been associated with drug-induced toxicities and complex diseases including PD [3,8–10]. Several association studies have been undertaken on *NAT2* sequence variations and PD, but results are conflicting. Some of these studies have reported strong association between slow acetylating genotypes of *NAT2* and PD [9,11–14]. One possible hypothesis is that the slow acetylation may lead to insufficient detoxification and accumulation of toxic chemicals or neurotoxins causing neuronal damage in the substantia nigra and inducing PD, though the mechanism remains speculative.

Since PD has been shown to be influenced by the environmental factors, polymorphism in enzymes like *CYP2D6* and *NAT-2*, which help to protect against toxic environmental compounds may influence susceptibility to PD (15). As inconsistencies exist on the association of polymorphism in *CYP2D6* and *NAT-2* genes with susceptibility to PD, attempts were made in the present case-control study to investigate the association of polymorphism in *CYP2D6* and *NAT-2* genes, found to be present in North Indian population, with susceptibility to PD.

2. Materials and methods

2.1. Study subjects

The study group consisted of 77 Parkinson's disease (PD) patients (61 males and 16 females) and 125 healthy controls (95 males and 30 females). PD patient were recruited from OPD facility of Department of Neurology, Chhatrapati Shahuji Maharaj Medical University (Formerly King George's Medical University), Lucknow, India during the period of 2003–2007. Ethical committee of IITR approved the study and it conforms to The Code of Ethics of the World Medical Association (Declaration of Helsinki), printed in the British Medical Journal (18 July 1964). The diagnosis of idiopathic PD was based on the presence of two or more of the cardinal features of PD (tremor, bradykinesia, rigidity and postural instability). The average age of patients was 58.7 ± 10.7 (mean $\pm$ SD) years (range: 22–82).

The healthy controls were randomly selected from the same geographical location and ethnicity as the patient's group and followed the similar life-style. They were matched for age and sex with the cases and only those controls were included in the study that did not suffered from any chronic disease. The average age of control subjects was 53.8 ± 9.6 (mean $\pm$ SD) years (range: 22–74). Both controls and cases were unrelated North Indians. Informed consent and a detailed questionnaire including family background, urban vs. rural residency, smoking habit and family history of PD were recorded for each individual. Majority of the subjects belonged to the urban areas. Since CYP2D6 and NAT-2 are known to be influenced by environmental factors, it was ensured that only those subjects (controls and cases) were included in the study that were living in the similar environment and followed similar life-style with respect to tobacco smoking, alcohol drinking etc.

Blood samples were drawn and processed for isolation of genomic DNA. Genomic DNA was extracted from whole blood using the QIAGEN spin columns.

2.2. Genotyping

Genetic polymorphisms in *CYP2D6* and *NAT-2* genes were assessed by previously described PCR-RFLP protocols. For identifying the *CYP2D6*2*(*1749 $G > C$*) and *CYP2D6*10A*(*188C $>$ T*) polymorphic sites, the methodology of Yokota et al. [16], was followed. *CYP2D6*2*(*2938C $>$ T*) polymorphism was identified by the method of Tsuneoka et al. [17]. *CYP2D6*4* (1934G $>$ A) polymorphic site was identified by amplifying the intron 3/ exon 4 boundary of CYP2D6 gene using specific primers described by Schur et al. [18]. The Ml, M2, M3 mutant NAT2 alleles, which accounts for most of the slow acetylators in humans, were also identified by PCR based RFLP. The primer sequences used for the DNA amplification were described earlier by Bell et al. [19]. PCR product (1093-bp) obtained after PCR amplification were digested with three different digestive enzymes: *Kpnl* for *Ml* allele (overnight, 37°C), *Taql* for $M2$ allele (4 hours, 65°C), and *BamHl* for $M3$ allele (overnight, 37°C).

The reproducibility of the genotyping methods was confirmed by duplicating the analysis in 20 randomly selected samples. In each case, the duplicated samples were in agreement with the original genotyping results.

2.3. Statistical analysis

All statistical analysis was performed using the software Statistical Package for the Social Sciences (SPSS Version 13.0, Chicago, IL) for windows. The Chi-square-goodness-of-fit test was used to test the distribu-

tion of genotypes and allele frequencies for deviations from Hardy-Weinberg equilibrium between the patients group and controls. Odds ratios (ORs) and 95% confidence intervals (CIs) associated with different variants among cases or controls were calculated by unconditional logistic regression. In this model variables were selected on a theoretical basis and the 'Enter' method of SPSS was used instead of backward-forward. To enter a variable, entry value was 0.05 and removal was at 0.10. Classification cut-off was at 0.5 and maximum iterations were 20. The interaction terms i.e. gene-gene interaction was examined by clubbing the cases and controls which carry a combination of variant forms of CYP2D6 and NAT2 and calculating the odds ratio associated with such a combination. The differences were considered significant if the P-value did not exceed 0.05.

3. Results

Chi square test was performed to test the Hardy-Weinberg Equilibrium (HWE) for genotype distribution of CYP2D6 and NAT2 polymorphism. The two groups were in HWE for 1749G > C, 1934G > A and 2938C > T polymorphism of CYP2D6 gene and 481 C > T, 590G > A, 857G > A polymorphism of NAT2 gene. Hence, there were no significant differences between expected and observed genotype frequencies in the two groups ($\chi 2 < 3.84$). The 188C > T polymorphism of CYP2D6 was not observed to be in HWE in control group ($\chi 2 = 35.25$).

3.1. Genotype analysis for CYP2D polymorphism

No significant difference was observed in the distribution of the frequency of heterozygous genotype of *CYP2D6*2*(*1749G > C*) polymorphism in the cases when compared to the controls. In contrast, the frequency of the homozygous variant genotype was found to be slightly decreased in PD cases (23.3%) when compared to the controls (32.8%). Not much difference was observed in the distribution of heterozygous genotype of another polymorphic site (*2938C > T*) of *CYP2D6*2* in both cases (45.4%) and controls (42.4%). The frequency of the homozygous variant genotype was found to be slightly increased in the cases, which increased the risk (OR: 1.61; 95% CI: 0.71–3.65) for PD, though it was found not to be statistically significant. The frequency of heterozygous genotype of *CYP2D6*4* (*1934G > A*) polymorphism was found to be higher in PD cases (28.5%) when compared to the controls (15.8%) that resulted in an significantly increased risk for PD (OR: 2.27; 95%CI: 1.11–4.6). Occurrence of the homozygous variant genotype was rare in both cases (1.3%) and controls (2.5%). When the heterozygous and homozygous variant genotypes were combined, an increase in the frequency of variant genotypes was observed in cases when compared to the controls. This increase in frequency in cases also resulted in a significantly increased risk (OR: 2.03; 95%CI: 1.02–4.01) to PD (Table 1). Similarly, the frequency of heterozygous genotype of *CYP2D6*10A* polymorphism was found to be higher in PD cases (37.6%) as compared to the controls (18.4%) which resulted in statistically significant increase in the risk (OR: 2.44; 95%CI: 1.24–4.8), for PD. The frequency of homozygous variant genotype was found to be reduced in PD cases (7.8%) as compared to controls (17.6%), though this decrease did not resulted in any significant change in risk to PD (Table 1).

3.2. Genotype analysis for NAT2 polymorphism

As evident from Table 2, the frequency of heterozygous genotype of *NAT2*5*(*481C > T*) was found to be slightly increased in the cases which resulted in slightly increased risk (OR: 1.41; 95%CI: 0.77–2.59) to the PD. The frequency of the homozygous variant genotype was found to be almost equal in both cases (5.2%) and controls (5.6%). Likewise, the frequency of heterozygous genotype of *NAT2*6*(*590G > A*) was found to be slightly increased in the PD cases (46.7%) when compared to controls (44%) while that of homozygous mutant genotype of *NAT2*6* polymorphism was found to be almost equal in both cases (10.3%) and controls (9.6%). The frequency of heterozygous genotype *of NAT2*7*(*857G > A*) polymorphism was also found to be overrepresented in the cases (26%) when compared to the controls (19.2%) which resulted in slight increase in the risk (OR: 1.41; 95%CI: 0.70–2.84) to the disease in the cases. Likewise, the frequency of homozygous variant genotype was also found to be increased in the cases (3.9%) when compared to the controls (0.8%), which increased the risk for PD up to six fold (OR: 5.97; 95%CI: 0.60–59.1) in the cases, though it was not found to be statistically significant (Table 2).

3.3. Gene-gene interaction and risk to PD

Interaction amongst poor metabolizer genotypes of CYP2D6 and NAT2 was also carried out to investigate

Table 1
Genotype and allele frequencies in $CYP2D6$ gene in PD cases and controls

Genotype and allele frequency	Controls N=125	Cases N=77	Crude OR (95%CI)	p value	Adj.OR (95%CI)	p value
*CYP2D6*2* (*1749G > C*)						
GG	32(25.6%)	16(20.7%)	1.0 (ref.)		1.0 (ref.)	
GC	52(41.6%)	43(55.8%)	1.65(0.80–3.4)	0.17	1.59(0.66–3.15)	0.21
CC	41(32.8%)	18(23.3%)	0.87(0.38–1.9)	0.75	0.83(0.35–1.9)	0.65
GC+CC	93(74.4%)	61(79.2%)	1.31(0.66–2.59)	0.43	1.26(0.62–2.51)	0.52
*CYP2D6*2* (*2938C > T*)						
CC	52(41.6%)	25(32.4%)	1.0 (ref.)		1.0 (ref.)	
CT	53(42.4%)	35(45.4%)	1.37(0.72–2.60)	0.33	1.20(0.62–2.3)	0.58
TT	20(16%)	17(22%)	1.76(0.79–3.9)	0.16	1.61(0.71–3.65)	0.25
CT+TT	73(58.4%)	52(67.5%)	1.48(0.82–2.68)	0.19	1.32(0.71–2.4)	0.37
*CYP2D6*4* (*1934G > A*)						
GG	103(82.4%)	54(70%)	1.0 (ref.)		1.0 (ref.)	
GA	19(15.8%)	22(28.5%)	2.21(1.1–4.43)	**0.02***	2.27(1.11–4.6)	**0.02***
AA	3(2.5%)	1(1.3%)	0.63(0.06–6.2)	0.69	0.57(0.05–5.8)	0.63
GA+AA	22(18.3%)	23(29.8%)	1.99(1.02–3.9)	**0.04***	2.03(1.02–4.01)	**0.04***
*CYP2D6*10* (*188C > T*)						
CC	80(64%)	42(54.5%)	1.0 (ref.)		1.0 (ref.)	
CT	23(18.4%)	29(37.6%)	2.40(1.23–4.65)	**0.01***	2.44(1.24–4.8)	**0.01***
TT	22(17.6%)	6(7.8%)	0.52(0.19–1.3)	0.18	0.43(0.16–1.19)	0.10
CT+TT	45(36%)	35(45.4%)	1.48(0.83–2.64)	0.18	1.42(0.78–2.56)	0.24

ref: reference; OR: odds ratio; CI: confidence interval; Adj. OR: adjusted for age and sex;$^*p < \mathbf{0.05}$ is considered as significant.

Table 2
Genotype and allele frequencies in N-acetyl transferase-2 ($NAT2$) gene in PD cases and controls

Genotype and allele frequency	Controls N=125	Cases N=77	Crude OR (95%CI)	p value	Adj.OR (95%CI)	p value
*NAT2*5* (*481C > T*)						
CC	71(56.8%)	38(49.3%)	1.0(ref.)		1.0 (ref)	
CT	47(37.6%)	35(45.4%)	1.39(0.77–2.50)	0.27	1.41(0.77–2.59)	0.25
TT	7(5.6%)	4(5.2%)	1.06(0.29–3.67)	0.92	1.11(0.30–4.11)	0.87
CT+TT	54(43.2%)	39(50.6%)	1.35(0.76–2.3)	0.30	1.39(0.77–2.48)	0.27
*NAT2*6* (*590G > A*)						
GG	58(46.4%)	33(42.8%)	1.0(ref.)		1.0(ref.)	
GA	55(44%)	36(46.7%)	1.15(0.63–2.09)	0.64	1.08(0.59–2.00)	0.78
AA	12(9.6%)	8(10.3%)	1.17(0.43–3.15)	0.75	1.07(0.39–2.94)	0.88
GA+AA	67(53.6%)	44(57%)	1.15(0.65–2.04)	0.62	1.08(0.60–1.93)	0.79
*NAT2*7* (*857G > A*)						
GG	100(80%)	54(70%)	1.0(ref.)		1.0(ref.)	
GA	24(19.2%)	20(26%)	1.54(0.78–3.0)	0.21	1.41(0.70–2.84)	0.33
AA	1(0.8%)	3(3.9%)	5.55(0.56–54.7)	0.14	5.97(0.60–59.1)	0.12
GA+AA	25(20%)	23(29.8%)	1.70(0.88–3.28)	0.11	1.57(0.80–3.07)	0.18

Ref: reference; OR: odds ratio; CI: confidence interval; Adj. OR: Odds Ratio adjusted for age and sex; $^*p < \mathbf{0.05}$ is considered as significant.

the effect of these interactions on modifying the susceptibility to PD. Subjects who carried heterozygous genotypes for CYP2D6*10(188C > T) and NAT2*5 represented significantly higher risk for PD (OR: 2.83; 95%CI: 1.16–6.85). Likewise, subjects with heterozygous genotypes of CYP2D6*4 and NAT2*5 polymorphisms resulted in more than fivefold increased risk for PD (OR: 5.73; 95%CI: 1.15–28.51), which was found to be highly significant. However, combination of genotypes of CYP2D6*10 and NAT2*6 or NAT2*7 were not found to be significantly associated with PD (Table 3).

4. Discussion

Our data have revealed an association of polymorphism in CYP2D6 and NAT-2, the most extensively studied drug metabolizing enzymes, with PD. The risk for PD was found to be significantly associated with individuals carrying heterozygous genotype for

Table 3
Important genotype combinations and PD risk

Gene-gene interaction	Controls (N=125)	Cases (N=77)	Adj. OR (95%CI)	p-value
*CYP2D6*10* × *NAT2*5*				
$w \times w$	45(36%)	23(29.8%)	1.0(ref.)	
$h \times h$	11(8.8%)	14(18.2)	**2.83(1.16–6.85)**	**0.02***
$m \times m$	2(1.6%)	0(0%)	0.0	0.99
*CYP2D6*10* × *NAT2*6*				
$w \times w$	45(36%)	17(22%)	1.0(ref.)	
$h \times h$	12(9.6%)	10(13%)	1.44(0.57–3.6)	0.43
$m \times m$	2(1.6%)	2(2.6%)	1.56(0.20–11.7)	0.66
*CYP2D6*10* × *NAT2*7*				
$w \times w$	64(51.2%)	25(32.4%)	1.0(ref.)	
$h \times h$	5(4%)	3(3.9%)	0.91(0.20–40)	0.89
*CYP2D6*4* × *NAT2*5*				
$w \times w$	53(42.4%)	23(29.8%)	1.0(ref.)	
$h \times h$	2(1.6%)	7(9.1%)	**5.73(1.15–28–51)**	**0.03***
*CYP2D6*4* × *NAT2*6*				
$w \times w$	54(43.2%)	26(33.7%)	1.0(ref.)	
$h \times h$	14(11.2%)	11(14.3%)	1.4(0.58–3.2)	0.46
*CYP2D6*4* × *NAT2*7*				
$w \times w$	86(68.8%)	35(45.5%)	1.0(ref.)	
$h \times h$	7(5.6%)	4(5.2%)	0.98(0.27–3.5)	0.98

CI: Confidence interval; Adj. OR: Odds Ratio adjusted for age and sex;$^*p <$ **0.05** is considered as significant; h: heterozygous; m: mutant.

*CYP2D6*10A*(*188C* > *T*) polymorphism. A much higher increase in risk (∼5 fold) for PD was associated with this polymorphism in Japanese case-control studies while studies in the Caucasians failed to find such association with the disease [4–6,9,17]. The frequency of the variant allele 188T in our controls (0.27) was relatively less when compared to the other Asian (Oriental) populations (0.50) while it is reported to be rare in the Caucasians (0.05–0.08) [20]. As this mutation is involved in abolishing the function of CYP2D6, the carriers of *CYP2D6*10A*(*188C/T*) allele has been classified as poor metabolizers [21,22]. Further, *CYP2D6*10A*(*188C/T*) polymorphism has been reported to be associated with other neurodegenerative disease like Schizophrenia in Japanese and Chinese populations [23–25].

Likewise, consistent with the earlier studies in Caucasians and Japanese [4,20,21], the present study has shown significantly increased risk (OR: 2.27; 95%CI: 1.11–4.6) of the disease in cases carrying *CYP2D6*4*(*1934G* > *A*) polymorphism, present in intron-3/exon-4 boundary. Interestingly the frequency of variant allele (A) of this polymorphism, which accounts for 70 to 90% of all PMs in Caucasians (0.20–0.25), was relatively less in our control population (0.12) but still higher when compared to African, African-American (0.06–0.07) and other Asian (Oriental) populations (0.01) [26]. This G > A change at the last nucleotide of intron 3 along with 188C/T polymorphism is demonstrated to be the dominant cause of poor metabolizing activity or function of CYP2D6 [21]. This lower enzymatic activity in the cases with *CYP2D6*4*(*1934G* > *A*) or *CYP2D6*10A*(*188C* > *T*) or polymorphism may increase the risk for PD as CYP2D6 is known to be involved in the metabolism and detoxication of chemicals involved in the pathogenesis of PD [22,27,28]. An increased OR of more than 1.5, though statistically not significant, was observed in cases with homozygous variant genotype of *CYP2D6*2*(*2938C* > *T*) polymorphism (OR: 1.61; 95%CI: 0.71–3.65). The Arg residue at this polymorphic site is near to the substrate-binding site and is highly conserved in the CYP2D subfamily, suggesting the functional importance of the site [17, 29]. Tsuneoka et al. [17], have earlier shown that cases with the variant 2938T allele have a 5.56 fold higher risk of developing PD amongst Japanese.

Similar to that observed with *CYP2D6*, *NAT2* was also found to display polymorphic distribution in our study. Though *NAT2*5* and *NAT2*6* alleles of *NAT2* were not found to exhibit any significant association with PD in our study, a slightly increased frequency of heterozygous genotype of *NAT2*7* was observed in PD cases (26%) when compared to the controls (19.2%). Frequency of homozygous variant genotypes was also found to be higher in PD cases (3.9%) as compared to controls (0.8%) that resulted in almost six fold-increased risk for PD (OR: 5.97; 95%CI: 0.60–59.1). However, this increase was not found to be statistically significant which can be attributed to the smaller

sample size of our study. When the heterozygous and homozygous variant genotype of *NAT2*7* were combined, a slight increase in variant genotype frequency was observed in PD cases (29.8%) as compared to controls (20%) which also resulted in a slightly increased risk (OR: 1.57; 95% CI: 0.80–3.07). An association of slow acetylators was also reported in the Caucasians and Chinese, though inconsistencies also exist in the literature [3,9,11–14,30].

Our data have further indicated that cases who carried combination of variant genotypes of *CYP2D6* and *NAT-2*, which accounts for the PM status, were at increased risk for PD. Cases who carried heterozygous genotype of *CYP2D6*10* or *CYP2D6*4* and simultaneously carried the *NAT2*5* allele were at significantly increased risk for PD. Nevertheless, our data demonstrating increased risk in PD cases carrying combination of variant genotypes of *CYP2D6* and *NAT2* is of significance and suggests that SNPs in multiple genes may increase the risk to PD.

In conclusion, the present data have shown that polymorphism in *CYP2D6*, the phase I enzyme and *NAT2*, the phase II enzyme, both of which are involved in the detoxification of chemicals, may modify the susceptibility to PD. Though the effect of environmental risk factors cannot be ruled out, our data indicating susceptibility of cases with variant genotypes of *CYP2D6* and *NAT2* is of significance as the subjects (controls and cases) belonged to the same geographical location, ethnicity and followed similar life-style. Interestingly, the risk increased several folds in cases carrying combination of certain variant genotypes of *CYP2D6* and *NAT2*5*, though *NAT2*5* polymorphism alone did not exhibited risk to PD to the extent as that observed with combination genotypes, further providing evidence for the role of gene-gene interaction in the development of PD.

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