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pNAT AND CYP2D6 GENE POLYMORPHISM IN EPILEPTIC PATIENTS

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Abstract—Certain anticonvulsant drugs require N-acetylation as a major route of metabolic clearance. Single point mutations of the polymorphic *N*-acetyltransferase gene (pNAT) are the primary cause for impaired drug acetylation. Pharmacokinetic parameters are altered in slow acetylator phenotypes and this may compromise drug safety. Genetic analysis of allelic frequencies of individual pNAT genotypes point to significant increases in carriers of the S1/wt and S3/wt ($P < 0.05$) allele and a significant reduction in carriers of the S2/S2 ($P < 0.01$) allele, when control and epileptic patients are compared. Furthermore, the presumed link between the cytochrome P450 CYP2D6 polymorphism and the pathogenesis of Parkinson's disease led us to investigate, whether a similar relationship can be expected for other CNS disorders. Our findings indicate that poor metabolizers are more frequent ($P < 0.05$) amongst epileptic patients, when compared with a control population. An estimate of the odds ratio may suggest an increased risk [95% CI (confidence interval) 1.043–4.734] of up to 5-fold in epileptic patients carrying this mutation. This provides further evidence for a potential link between the debrisoquine hydroxylase gene polymorphism and CNS disorder and therefore warrants further study.

Key words: pNAT; CYP2D6; polymorphic drug metabolism; CNS disorder; drug safety; epileptic patients

Single point mutations of the pNAT§ gene are the primary cause for compromised drug acetylation, which may lead to unwanted drug related side effects [1, 2]. In particular, individuals that are slow acetylators require an altered dosing regime to avoid adverse drug reactions, because their metabolic clearance is impaired. Furthermore, several drugs may be administered to patients all of which are metabolized via the pNAT isoenzyme, e.g. isonazide, iproniazide, hydralazine, phenelzine, dapsone and aromatic amines.

Current advances in molecular biology provided the tools to determine the genotypes linked to the *N*-acetyltransferase polymorphism in humans by utilizing a DNA based genetic assay. This assay requires small quantities of DNA obtained from nucleated blood (as little as 100 μL whole blood) cells as a template for the oligomer specific amplification of DNA catalysed by the polymerase chain reaction followed by restriction enzyme digestion of the amplified product [3]. The results obtained enable an assessment of the S1, S2 and S3 mutations within this single exon gene. Carriers of the S-mutations are identified by a single base replacement mutation, i.e. the pyrimidine base cytosine is replaced by thymine (S1) or the purine base guanine is replaced by adenine (S2 and S3 mutations).

In addition, the allelic frequency of the mutant genotype responsible for poor debrisoquine hydroxylation is increased in patients with Parkinson's disease [4, 5]. The enzyme responsible for the metabolism of debrisoquine is termed cytochrome P450 CYP2D6 and is involved in the metabolism of a wide range of drugs such as debrisoquine, sparteine, dextrometorphan, tricyclic antidepressants, opioids, β-adrenergic receptor antagonists etc. [2]. Homozygote carriers of the mutant cytochrome P450 CYP2D6 DNA are termed poor metabolizers (PM) as they are unable to express the CYP2D6 protein responsible for the catalysis of the above named drugs.

The molecular mechanism leading to this polymorphism has been resolved [6]. The link between the cytochrome P450 CYP2D6 polymorphism and the pathogenesis of Parkinson's disease led us to investigate whether a similar relationship can be expected for other CNS disorders. For this purpose, the single base replacement mutation of the purine base guanine to adenine in the cytochrome P450 CYP2D6 gene was determined. A crude DNA extraction assay suitable for the oligomere specific amplification of wild type and mutant DNA was used and then digested with the endonuclease BstNI, as reported previously [6, 7].

MATERIALS AND METHODS

Patients and Methods. All blood samples were

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§ Abbreviations: CI, confidence interval; CYP2D6, cytochrome P450 CYP2D6, isoenzyme responsible for the oxidation of debrisoquine; NAT, *N*-acetyltransferase; pNAT, polymorphic *N*-acetyltransferase; PCR, polymerase chain reaction.

obtained from unrelated Caucasian individuals. Blood samples were obtained as surplus material during routine laboratory measurements. A total of 59 male and 41 female patients took part in this study. The average age of male patients was 15.3 years and that of females was 16.8 years. Of the total cohort 78 patients were below the age of 21 years and 59 patients were below the age of 16 years. Epileptic patients were selected at random on the basis of clinical manifestations of the disease. Less than N = 10 cases were diagnosed as idiopathic, as the majority of patients had pre-, peri- or postpartum cerebral alterations and were diagnosed as residual epileptic cases. Most patients had focal seizures but clinical manifestations included secondary generalised seizures, including grand mal. About 2/3 of the patient population did not respond to common pharmacotherapy and the clinical mangement of the remaining patients was difficult in nature. Most patients had at least N = 2 seizures per week with an average duration of up to 2 min. But it must be pointed out that there was large patient variation in the frequency and duration of seizures. Blood samples from controls were obtained at random from unrelated Caucasian individuals. DNA was isolated from whole blood as crude cell extracts suitable for the polymerase chain reaction. Details of the preparation of crude DNA extracts were previously published [3].

pNAT assay. The acetylator genotype was determined by oligomer specific amplification of the alleles responsible for slow and fast acetylation [3]. Details of the nucleotide sequence and the assay conditions are detailed elsewhere [3]. The PCR amplified products were digested with three different endonucelases (KpnI, TaqI and BamHI) to differentiate between wild-type and mutant DNA and with Hind III to differentiate between the polymorphic and monomorphic locus [3].

CYP2D6 assay. Details of the nucleotide sequence and the assay conditions are detailed elsewhere [6, 7]. The poor and extensive metabolizer genotypes were determined by oligomer specific amplification of the alleles responsible for poor and extensive metabolism [7]. The PCR amplified product was digested with BstNI to differentiate between wild-type and mutant DNA [7].

Statistical analysis. The distribution of pNAT and CYP2D6 alleles in epileptics and controls were compared using χ^2 goodness of fit test. Confidence intervals at the 95% level were estimated according to Pearson–Clopper and the confidence bounds for the odds ratio were computed assuming asymptotic log normality. These computations were done with the SAS Version 6.07 using the FREQ procedure.

RESULTS AND DISCUSSION

The frequency of the pNAT alleles of 243 unrelated Caucasian controls and 93 epileptic patients, which could be evaluated, is shown in Table 1. The overall allelic frequencies did not differ statistically when controls and epileptic patients were compared. There was a trend to suggest an increased presentation of wild type and S3 (G to A transition, 1000 bp product) carriers in the patient population. In contrast, carriers of the S1 (C to T transition, 1000 bp product) and S2 mutation (G to A transition, 400 bp product) were less frequent amongst epileptic patients. The individual genotypes are shown in Table 2. Homozygote wild type carriers were more frequent in the patient population, but within this group there was a reduction in the allelic frequencies of the genotypes S1/wt and S1/S2. Nevertheless, neither of these changes were of statistical significance. The S1/S3 and S2/S3 mutations were more frequent amongst epileptic patients, but this represents only two and one out of 93 cases, respectively. However, there was a statistically significant increase in the allelic frequency of the S2/wt genotype, i.e. 17 out of 93 cases were carriers of the G to A transition ($P < 0.05$, 95% CI 11.0–27.7). Based on a risk assessment it is highly probable that epileptic patients have a 2.4-fold increased risk in carrying this mutation. A further statistical increase was found with the S3/wt genotype. This mutation is rare amongst Caucasians and indeed only one individual out of 243 mapped to this genotype (95% CI 0.01–2.3), compared with three individuals out of 93 (95% CI 0.7–9.1). Based on the data shown in Table 2 it is highly probable that epileptic patients have an 8-fold increased risk in carrying this mutation. In contrast, homozygote S2 carriers were more frequent with controls, i.e. 23 out of 243 individuals ($P < 0.01$, 95% CI 6.1–13.9) mapped to this mutation compared with one out of 93 (95% CI 0.0–5.9) wheras the allelic frequencies of S1 homozygotes were literally identical amongst both populations.

Table 1. Comparison of polymorphic pNAT allelic frequencies in controls and epileptic patients

Allele	Control group N = 243	Epileptics N = 93
wt	22.3%	29%
S 1	48.9%	45%
S 2	27%	22%
S 3	1.7%	4%

wt = fast NAT allele; S = slow NAT allele; S1 allele (C to T transition, detected by PCR amplification with Primer 3 and 4 and digestion with KpnI); S 2 allele (G to A transition, detected by amplification with Primers 1 and 2 and digestion with TaqI); S 3 allele (G to A transition, detected by amplification with Primers 3 and 4 and digestion with BamHI). Further details are given in the Materials and Methods.

It is of interest that the S2/S2 + S3/wt genotype was not detected amongst epileptics and neither controls nor epileptic patients were carriers of the homozygote S3 genotype, as shown in Table 2.

The distribution of the CYP2D6 alleles in all epileptic patients were as follows: 64 individuals were homozygote carriers (EM) of the wild type DNA or in other words extensive metabolizers, compared with 27 individuals that were heterozygote carriers (HEM) for the G to A transition, but are still extensive metabolizers and nine individuals were poor metabolizers (PM) as they were homozygote carriers of the G to A transition. Expressed on a

Table 2. pNAT genotypes in controls and epileptic patients

Genotypes	Controls N = 243	95% CI	Epileptics N = 93	95% CI	Odds ratio (95% confidence limits)	χ^2
wt/wt	5.4%	2.9–9.0	8.6%	3.8–16.3	1.665 (0.671–4.130)	NS
S 1/wt	23.0%	17.9–28.9	19.4%	11.9–28.9	0.801 (0.442–1.453)	NS
S 2/wt	8.6%	5.4–12.9	18.3%	11.0–27.7	2.365 (1.202–4.653)	$P < 0.05$
S 1/S 1	22.2%	17.2–28.0	22.6%	14.6–32.4	1.021 (0.575–1.811)	NS
S 2/S 2	9.5%	6.1–13.9	1.0%	0.0–5.9	0.104 (0.020–0.549)	$P < 0.01$
S 2/S 2 + S 3/wt	4.1%	2.0–7.4	ND	0.0–3.9	0.000*	NS
S 1/S 2	23.9%	18.7–29.7	22.6%	14.6–32.4	0.930 (0.526–1.644)	NS
S 3/wt	0.4%	0.01–2.3	3.2%	0.7–9.1	8.067 (1.176–55.337)	$P < 0.05$
S 1/S 3	2.5%	0.9–5.3	3.2%	0.7–9.1	1.317 (0.323–5.366)	NS
S 2/S 3	0.4%	0.01–2.3	1.0%	0.0–5.9	2.630 (0.180–38.466)	NS
S 3/S 3	ND	0.0–1.5	ND	0.0–3.9		NS

wt = fast NAT allele; S = slow NAT allels; S1 allel (C to T transition, detected by PCR amplification with Primer 3 and 4 and digestion with KpnI); S 2 allel (G to A transition, detected by amplification with Primers 1 and 2 and digestion with TaqI); S 3 allel (G to A transition, detected by amplification with Primers 3 and 4 and digestion with BamHI). ND = not detected; NS = not significant. Further details are given in the Materials and Methods.

* The confidence bounds for the percentages were computed according to Pearson–Clopper, whereas confidence limits for the odds ratio were computed assuming asymptotic log normality odds ratios. To avoid undefined results, some estimates are not computed.

percentage basis the allelic frequencies were 64.6% EMs (95% CI 54.40–73.99%), 26.3% HEMs (95% CI 17.93–36.07%) and 9.1% (95% CI 4.24–16.56%) PMs. This distribution of genotypes was compared with the frequency of CYP2D6 alleles determined in N = 720 controls as reported by Wolf *et al.* [8]. There was a significant increase of PMs amongst epileptic patients ($\chi^2 = 4.01$, $P < 0.05$), i.e. 9.1 vs 4.3% in the patient and control population, respectively. An estimate of the odds ratio suggests up to a 5-fold increased risk with a mean odds ratio of 2.2 (95% CI 1.043–4.734) for epileptic patients in carrying this mutation. It should be pointed out that even the lower 95% CI corresponds directly to a statistically significant increased risk, even though it is very modest indeed. The distribution of EMs was 64.6 and 66.8% whereas the distribution of HEM was 26.3 and 29.6%, respectively, for epileptic patients and controls.

The increased representation of PMs in the epileptic patient population provides further evidence for a link between the debrisoquine hydroxylase gene polymorphism and CNS disorder and therefore warrants further study. However, it must be pointed out that the number of patients entering the study was small and the possibility of a population and/or geographical variation in gene frequency can not be excluded [8–15].

In the present study genotypes linked to the pNAT and CYP2D6 genes were determined. The results indicate significant differences in the allelic frequencies of the pNAT gene and most noticeably in the S2/wt, S2/S2 and S3/wt genotypes. As drug safety and pharmacokinetic parameters will be altered in slow acetylator phenotypes the observed differences will be important in the development of antiepileptica which undergo metabolic acetylation via pNAT. The molecular mechanisms leading up to these changes are only known in part and await further studies to rationalise the present findings. In particular, we do not know to what extent epileptic disease and polymorphic *N*-acetylation is causally related. Gene mapping and linkage analysis has shown a group of idiopathic generalized epilepsies, i.e. juvenile myoclonic epilepsy (JME), epilepsy with absences and epilepsy with generalized tonic-clonic seizures (GTCS) mapped to chromosome 6p [16, 17] and, therefore, are under genetic control. The human pNAT maps to chromosome 8. As pNAT catalyses the acetylation of a wide range of substrates it remains a priority task to determine the extent to which genotypic alteration in the allelic frequencies of the pNAT gene are causally related to inherited epileptic disease.

A further point of considerable importance is the concomitant determination of acetylator pheno- and genotype. For instance, Hickmann and Sim [3] report a comparison of 22 unrelated Caucasian individuals which had undergone DNA analysis of pNAT and were phenotyped with sulphamethazine as substrate. The authors found that only carriers of the S1/S1 and S1/S2 alleles had compromised

acetylation activity [3]. This suggests that other genotypes were less important in bringing about a reduction in sulphamethazine acetylation. For other drugs such a relationship needs to be established as the results obtained with sulphamethazine do not allow a universal interpretation. For instance, recent advances in the development of anticonvulsant drugs have led to the discovery of two unrelated groups of chemicals, namely dialkyl- and arylalkylamines of 4-aminobenzamides such as 4-amino-*N*-(2,6-dimethylphenyl) benzamide [18, 19], as well as a group of 3-substituted 1,2 benzisoxazole, i.e. the 1,2-benzisoxazole-3-methanesulfonamide (zonisamide) [20]. These drugs are effective in animal models and have been evaluated in patients with refractory partial and generalized tonic-clonic seizures [21, 22]. Drug disposition and pharmacokinetic studies have shown N-acetylation to be the major route of metabolic disposal. However, we don't know whether the genotypes responsible for slow acetylation of the anticonvulsant drugs 4-amino-*N*-(2,6-imethylphenyl)benzamide [18, 19] or 1,2-benzisoxazole-3-methanesulphonamide (zonisamide) [20] are identical to those responsible for the slow acetylation of sulphamethazine.

It is self evident that further studies are needed to establish such a relationship. In conclusion, the present study indicates significant differences in the allelic frequencies of the pNAT and CYP2D6 genes when epileptic patients are compared with a control population. Our results warrant further studies to ascertain the role of pNAT, CYP2D6 and of other monooxygeneases in the pathogenesis of CNS disorders.

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