Prevalence of Cytochrome P450 2D6 Polymorphism in Healthy Volunteers from Maharashtra and Its Genotype: Phenotype Association in Schizophrenic Patients

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Abstract

Risperidone is metabolized by cytochrome P4502D6 (CYP2D6) to its active metabolite, 9-hydroxyrisperidone. CYP2D6 genes encoding this enzyme are polymorphically expressed and most of the variants result in decreased substrate metabolism. The influence of CYP2D6*10 polymorphism on risperidone and 9-hydroxyrisperidone plasma levels, metabolic ratio and dose adjusted active moiety in Indian Schizophrenic patients were assessed. Also, the prevalence of CYP2D6*10 in normal healthy volunteers residing in Maharashtra were determined. The study was initiated following Institutional Ethics Committee approval and written informed consent from participants. Seventy two schizophrenic patients and One hundred and eleven healthy volunteers were genotyped for CYP2D6*10 by PCR-RFLP method. Risperidone and 9-hydroxyrisperidonelevels were estimated in patients by HPLC. Clinical assessment of patients was done using PANSS score. Genotype frequencies were calculated using Hardy-Weinberg equilibrium. Differences in (corrected dose) C/Ds plasma drug, Metabolic Ratio, PANSS score between different genotypes was analyzed using one way ANOVA for normally distributed data and Kruskal-Wallis test in case of non-normally distributed. Pearson's correlation was used to examine the relationship between drug levels and dosage. Frequency of T allele observed was 16% in controls and 19% in schizophrenic patients. Poor Metabolizers had significantly higher levels of risperidone C/D and Metabolic Ratio than homozygous Extensive Metabolizers and Intermediate metabolizers. No statistically significant correlation was found between PANSS score and plasma levels. A strong correlation was however observed between the drug levels and administered dose. Our findings suggest that CYP2D6 may be a useful determinant of risperidone & 9-OH-risperidone drug levels and their Metabolic Ratio.

Keywords: CYP2D6; Genotype-Phenotype Correlation; Indian Population; Risperidone; 9-OH Risperidone; Schizophrenia.

Introdution

Risperidone is one of the second generation antipsychotic drugs with potent antagonistic properties for dopamine D2 and serotonin 5-HT2 receptors as compared to typical antipsychotic drugs [1,2]. It is effective in treating both positive and negative symptoms of schizophrenia and has a lower potential to cause extra pyramidal symptoms(EPS) [3,4]. Risperidone is well absorbed in the liver and is metabolized primarily by the cytochrome P450 especially CYP2D6 enzyme to an active metabolite 9-hydroxyrisperidone (9-OH-risperidone) [5,6]. A 9-OH-risperidoneis approximately equipotent with the parent drug in dopamine receptor affinity. Because of their similar pharmacological characteristics, the entire active moiety i.e. the combined effect of plasma levels of risperidone + 9-OH-risperidone [7] is regarded to contribute to the overall antipsychotic effect [8]. The oral bioavailability of risperidone varies from extensive metabolizers (EM) 66% to poor

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metabolizers (PM) 82% [9].

CYP2D6 is the most important polymorphic enzyme active in the metabolism of drugs. This is the only CYP which is not inducible and therefore genetic variation contributes largely to the inter-individual variation in the enzyme activity [10]. Studying polymorphic drug metabolism in a population identifies the proportion of individuals differing in ability to metabolize certain drugs and who therefore are likely to react differently or adversely to drugs. This difference in metabolism can lead to severe toxicity in case of PMs or therapeutic failure in case of ultrarapid metabolizers (UMs) by altering ratio of the blood concentrations of the drug and its pharmacologically active metabolite [11].

The SNP arising due to replacement of Cytosine by Thymine at 188 position in exon 1 of the CYP2D6 gene produces CYP2D6*10 allele.Several studies carried out in different ethnic populations have indicated that the CYP2D6*10 alleles strongly affect the metabolic ratio of risperidone to 9-hydroxyrisperidone. As a result, high incidence of a mutant CYP2D6*10 allele causes an unstable enzyme with a decreased catalytic activity [12-17]. Genotype data, seen alongside phenotype data, may be useful to predict phenotype from genotype within a population. However there is a sparse data correlating the genetic variants of CYP2D6*10 genes and risperidone levels in the Indian population.

Considering this fact, the present study was planned to determine the allele frequency of CYP2D6*10 frequency in healthy Indian volunteers residing in Maharashtra for more than 3 generations and the correlation of genotype-phenotype relationship in Schizophrenic patients.

Materials and Methods

The present study was initiated after obtaining permission from the Institutional Ethics Committee of our hospital. The study was conducted in accordance with the principles of Good Clinical Practice that has its principles in the Declaration of Helsinki.

Study Participants

Study participants comprised of 72 patients with schizophrenia (41 men and 31 women) who fulfilled the DSM IV criteria for Schizophrenia & attending the outpatient Psychiatry department of our hospital. All the patients who had received stable dose of risperidone orally for a period of at least one month with or/and benzodiazepines and/or anticholinergic

agents as concomitant medications were considered for the study. The dosage for each patient was made based on clinical evaluation and severity. The second group of participants who served as the control group comprised of unrelated healthy volunteers of either sex, age between 18 to 65 years. Participants suffering from any major uncontrolled illnesses, alcohol abuse, excessive smoking was excluded from the study. All the patients and the controls included in the study belonged to the same ethnic group and residing in Maharashtra for more than three generations.

Following written informed consent to participate, a detailed clinical history was taken from each individual and relevant physical examination including demographic data was recorded. 10 ml blood was collected from the patients aseptically, of which 5ml was dispensed in clot activator tube for the estimation of risperidone and 9-OH risperidone and 5ml of blood was dispensed in an EDTA tube for genotyping. 5ml of blood was collected in EDTA tube for the genotyping from the volunteers.

Clinical Assessment

The severity of psychopathology of the patients was assessed by an experienced psychiatrist using the Positive and Negative Syndrome Scale (PANSS) (Kay *et al*, 1987) [18].

*Genotyping Method of CYP2D6*10 using PCR-RFLP*

Genomic DNA was isolated from EDTA tube using a phenol chloroform method. The CYP2D6*10 allele, which contains the C188T mutation in exon1, was identified by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) on a Thermal Cycler, (Techne-512, UK). The targeted DNA was amplified using specific primers and digested with specific restriction enzymes as depicted in Table 1. PCR was carried out in 20 μl reaction mixture containing 1 μl of DNA (~100ng), 10pM of each primer, 9 μl of (2X) PCR Master mix (Fermentas, Thermo Fisher, UK) and 0.25 U of Taq DNA polymerase (Fermentas, Thermo Fisher, UK) and nuclease free water to make up volume of 20 μl reaction under the thermal cyclic condition as mentioned in Table 1. The amplified PCR products were digested using the HphI restriction enzymes according to manufacturer's kit instructions (Fermentas, Thermo Fisher, UK). The digested PCR products were separated by 3% agarose gel electrophoresis containing Etbr to visualise the DNA fragments on Gel Documentation System (Syne gene). Genotypes were classified based on the size of the respective DNA fragments sizes as mentioned in Table

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1. Approximately 10% of the samples were repeated randomly to check the reproducibility of genotyping methods.

Analysis of Plasma Concentrations of Risperidone and 9-Hydroxyrisperidone using HPLC

Trough plasma concentrations of risperidone and 9-OH risperidone were measured using the high performance liquid chromatography (HPLC) method with UV detector (Thermo Scientific,USA). Separation was done on a C18 column using 20mM ammonium acetate buffer (pH5.5): acetonitrile (55:45 v/v) the mobile phase pumped at a flow rate of 1.0 ml/min at 30°C. The eluted compounds, 9-OH risperidone at 4.5 mins, risperidone at 5.4 mins and tolperisone (as internal standard) at 6.9 mins were detected at 278 nm. The Metabolic ratio (MR) of the risperidone: 9-OH risperidone concentrations (R: 9-OHR ratio) and the sum of the risperidone and 9-hydroxyrisperidone concentrations divided by the dose (C: D ratio) were the main parameters used to interpret plasma risperidone and 9-OH risperidone levels. The standards risperidone, 9-OH risperidone and tolperisone were procured from Sigma Aldrich,USA and all other reagents used for the analysis were HPLC grade and procured from Merck, India.

Statistical Analysis

Data were analyzed using Graph pad Instat software (Version 3.06, GraphPad Software, Inc., USA). Overall Allele and genotype frequencies were calculated by the counting method and were tested for Hardy-Weinberg equilibrium. Chi-square test was used to determine significant differences in the allelic frequencies. Differences in C/Ds plasma drug, MR between different genotypes were analyzed using one way ANOVA for normally distributed data and Kruskal-Wallis test for in case of not normally distributed data followed by post hoc comparisons. Differences in psychopathology (measured by PANSS) were examined using one-way ANOVA. Pearson's correlation was also used to examine the relationship between concentration of risperidone and its metabolite with dose of the drug. A p-value value below0.05 was considered statistically significant throughout the population comparisons.

Results

A total of 183 Indian participants, of which 111 healthy controls (53 men and 58 women) with median age of 36years and 72 patients with schizophrenia (41 men and 31 women) were genotyped for

Table 1: PCR-RFLP Conditions for CYP2D6*10 analysis

Allele	Primers	Thermocycling conditions	Enzyme and Incubation time	Pattern after gel electrophoresis (base pair)
*CYP2D6*10*	5'GTGCTGAGAGTGTCCTGCC 3'	94°C/8 min 56°C/2 min	*HphI*	wt/wt: 263,62
	5'CACCCACCATCCATGTTTGC 3'	30 x 72°C/ 20s 94°C/ 30s	37°C for 3 hours	wt/mt:263,183,80,62
		56°C/20s 72°C/5min 25°C/∞		mt/mt:183,80,62

Table 2: Demographic data of the study participants

Parameters	Control (n=111)	Schizophrenic patients (n=72)
Age, years	36 (19 – 62)	33 (18-66)
Sex (M:F)	53:58	41:31
BMI (kg/m²)	23.63 ± 1.05	25.14 ± 1.56

BMI: Body Mass Index, M: Male, F: Female
Results are expressed as Mean ± SD for normally distributed data and Median (Range) for not normally distributed data.

Table 3: Genotypic frequency of CYP2D6*10 in controls and patients

CYP2D6*10 allele	Control N, (%)	Schizophrenic patients N, (%)
CC (*1/*1)	81 (72.97)	48 (66.67)
CT (*1/*10)	25 (22.52)	20 (27.78)
TT (*10/*10)	05 (4.50)	04 (5.56)
Chi square value	2.56	0.82
P value	0.28	0.63
Allele frequency		
C	0.84	0.81
T	0.16	0.19

Table 4: Steady-state plasma concentrations of risperidone, 9-hydroxyrisperidone, active moiety and metabolic ratio in Schizophrenic patient as per C100T genotypes

Genotype (C100T)	*1/*1 (CC)	*1/*10 (CT)	*10/*10(TT)
No. of samples (N=72)	48	20	04
Risperidone dose, mg	4.0 (2.0 – 8.0)	5.0 (3.0 – 8.0)	6.0 (3.0 – 8.0)
Risperidone C/D	9.79 ± 2.80	11.88 ± 3.60	15.54 ± 7.19**
(ng/ml/mg)	(8.97 – 10.60)	(10.19 – 19.60)	(4.09 -26.99)
9- OH risperidone C/D	12.65 ± 4.02	13.34 ± 3.94	12.15 ± 4.13
(ng/ml/mg)	(11.49 – 13.82)	(11.49 – 15.18)	(5.59 – 18.72)
Active Moiety C/D (ng/ml/mg)	22.44 ± 6.55	25.21 ± 7.31	27.69 ± 11.28
	(20.54 – 24.35)	(21.80 – 28.63)	(9.76 – 45.63)
Metabolic Ratio	0.78	0.92[@@]	1.25[@@@]
(risperidone : 9 hydroxyrisperidone)	(0.53 – 0.97)	(0.59 – 1.07)	(1.06 – 1.45)
Total PANSS Score	38.33 ± 6.98	36.25 ± 1.71	37.5 ± 4.65
	(31.01 - 45.66)	(33.53 – 38.97)	(30.09 – 44.91)

C/D: corrected dose, PANSS: Positive and Negative Syndrome Scale
Results are expressed as Mean ± SD (95% Confidence Interval) for normally distributed data and as Median (Range) for not normally distributed data, **$p<0.01$ as compared to CC using ANOVA and [@@]$p<0.01$[@@@]$p<0.001$ as compared to CC using Kruskal Wallis test.

CYP2D6*10 polymorphism. A summary of the demographic details has been depicted in Table 2. The overall genotypic and allelic frequency of the polymorphisms of CYP2D6*10 in controls and patients are summarized in Tables 3. The conformity of the genotype frequency distribution to Hardy-Weinberg proportion was examined using X^2 test. The frequency of mutant T allele was 16% for control and 19% for patients. No allele frequencies deviated from Hardy-Weinberg equilibrium.

Correlation between Plasma Concentration and CYP2D6 Activity and its Clinical Response

Risperidone was prescribed to patients at a median dose of 4 mg (range 2-8 mg). The daily dose of risperidone, the measured concentration of risperidone and 9-OH-risperidonewere corrected for the dose (ng/ml per mg) and are mentioned in Table 4. Amongst 72 schizophrenic patients, 4 patients were genotyped as the CYP2D6*10 PMs (CYP2D6 *10/*10), 20 patients as inter-mediate metabolizers (IMs) and

remaining 48 patients as extensive metabolizer (EMs). PMs had significantly highest levels of risperidone C/D and active moiety C/D than homozygous EMs and IMs. However the increase in active moiety was not statistically significant. Furthermore, there were statistical differences in the concentration of the metabolic ratio (risperidone/9-hydroxyrisperidone) among the three genotypes. Amongst all genotypes, the metabolic ratio was highest in PMs, followed by IMs and then the homozygous EMs. Also, no statistically significant difference was found when comparing genotype groups and PANSS scores. Likewise, no statistically significant correlations were found between the PANSS scores and plasma concentrations. However, decrease in symptom severity was documented in majority of our patients. Plasma concentrations of Risperidone and 9-OH risperidone indicated a strong relationship with the administered dose with a correlation of r = 0.55 and r = 0.62; p<0.0001respectively as shown in Figure 1.

r = 0.55; p=0.0001
95% CI (0.0913 to 0.6890)

r = 0.62; p=0.0001
95% CI (0.2048 to 0.7453)
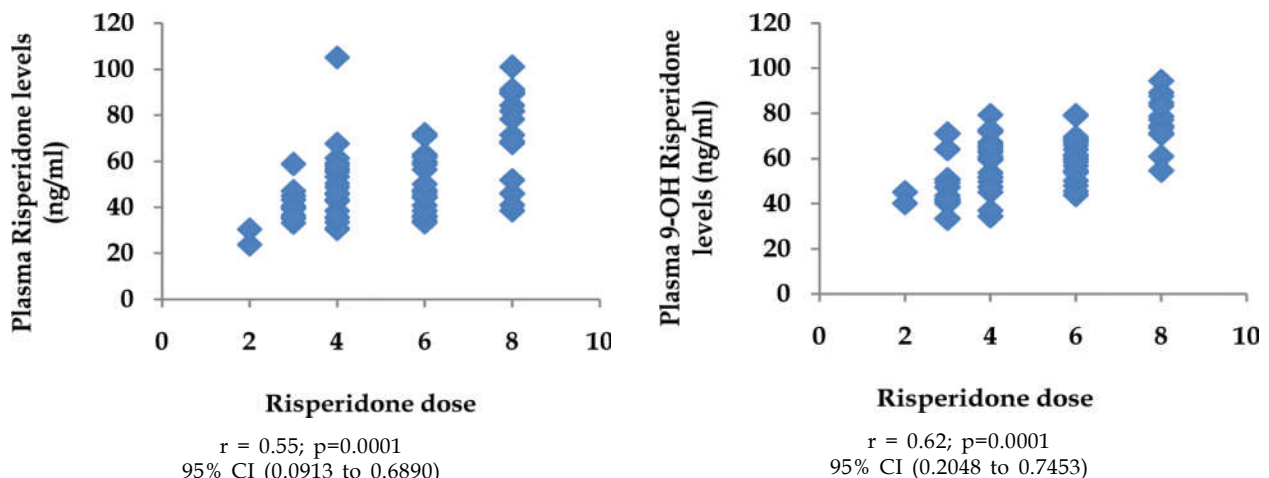
Fig. 1: Correlation between Risperidone and 9-OH risperidone and administered dose in Schizophrenic patients

Discussion

CYP2D6 gene is a highly polymorphic gene with more than 70 variant alleles has been reported till date. Most of these variants occur at rare frequencies and may not be of clinical significance. The CYP2D6*10 polymorphism is the most frequent gene mutations observed in Asian populations [19].

There are numerous reports of an association between the C100T polymorphisms and Metabolic Ratio [20-22] and little is known about the potential existence of an association in Indian schizophrenic patients. The association between the genotype and phenotype of CYP2D6 drug-metabolizing enzyme has not been well studied in Indian schizophrenic patients.

In the present study, we first analyzed the prevalence of CYP2D6*10 gene polymorphisms in healthy volunteers residing in Maharashtra. We also correlated the phenotype (serum levels of risperidone & its active metabolite) with the genotype (SNP) in Indian schizophrenic patients. The prevalence of the PM phenotype is slightly higher among Asians in the Indian subcontinent than in the Asian populations of Southeastern and Eastern Asia, with reported frequencies of 1.8–4.8% [23]. CYP2D6*10 may be present in as much as 50% of Asians and is responsible for diminished enzyme activity in IMs [24]. The percentage of PM of 2D6*10 in our population group was 4.50% and 5.56% in healthy controls and schizophrenic patients respectively. However, the percentage of IM was 22.5% and 27.8% in the controls and schizophrenic patients respectively. Also, the allele frequency observed in the healthy volunteers from Maharashtra was 16% and is in accordance with the reported data available in Indian population which is around 14.9-26.1% [25].

We suggest that the metabolic ratio of risperidone/9-hydroxyrisperidone is affected by the CYP2D6*10 allele in Indian schizophrenic patients. This may be the first study of the relationship between CYP2D6 genotypes and the metabolism of risperidone in Indian schizophrenic patients. It has been reported that the CYP2D6 genotype strongly affects plasma levels of risperidone and the metabolic ratio in different ethnic populations [12-17]. Amongst the various genotypes of CYP2D6*10, PM are of highest significance for the clinicians as these participants are at risk for adverse reactions and may also have toxic levels. As predicted, the PM in the present study exhibited higher dose corrected risperidone levels and lower levels of 9-OH risperidone levels. As a result highest level of Metabolic Ratio was observed in PM followed by IM in comparison to EM which confirms our results and further supports the suggestion that the incidence of allele*10 is likely to contribute to the decreased CYP2D6 activity. There was significant difference in the active moiety among the genotypes of CYP2D6 suggesting the clinical importance of the polymorphism. Our findings are thus, in lieu with reported literature. Also, no statistically significant difference was found when comparing genotype groups and PANSS scores. Therefore, our findings suggest that CYP2D6 may be a useful determinant of risperidone plasma concentrations. Knowledge of the drug metabolizer genotype in relation to treatment response, when conveyed to patients, would help prevent adverse events or therapy failure when administered CYP2D6 substrates. This will be particularly relevant in case of patients who are carriers of the variant allele/s.

Further comprehensive genetic and phenotypic studies of cytochrome CYP2D6 are warranted among a larger group of Indian populations.

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