Genetic Polymorphism Of Cytochrome P450 -2d6*4 In Cannabis Smokers

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Citation

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Abstract

Genetic variation in genes involved in drug metabolism may account for variations in responses to these drugs and may affect receptor adaptation, toxicity, altered drug effects and cross tolerance. Most drugs are metabolized by cytochrome P450 (CYP 450) system with metabolizing enzyme, CYP 2D6, responsible for the largest contribution. In this work the role of genetic factors in cannabis dependence (hashish smokers) was evaluated by CYP 2D6*4 genotyping (restricted fragment length polymorphism [RFLP]) to investigate whether there is specific genotype associated with cannabis dependency pattern. Results showed that 50% of the cannabis smokers have the heterozygous genotype of cytochrome P450-2D6*4, 27.8% have the homozygous allele and 22.2% have the wild type allele. Also, there is significant correlation between the genetic configuration and the cannabis dependency pattern as presented by the starting age of cannabis use, the regularity of its use and the personal and family norms about its use. It is concluded that there may be some genetic factors that determine the body response to cannabis dependence that need to be more specifically studied. This can be a marker for the risk of becoming dependent and / or the prediction of suitable effective treatment of dependence and also, prevention of relapse after withdrawal.

INTRODUCTION

Sometimes people experiment with drug abuse, but not all of them become addicted. Among those who become addicted, some eventually are able to stop taking drugs while others are not, despite the damage to their health and their personal lives. What causes these differences? Certainly environmental factors – such as drug availability and peer groups- play a major role, but increasingly, scientists found that genetic factors are also very important and many research efforts are applied on the effects of genetics, to locate genes associated with substance abuse and addiction ([1]).

The importance of cytochrome P450 isoforms in the metabolism of foreign compounds has been identified ([2]). These enzymes are expressed from multiple genes and exhibit complex patterns of differential and overlapping substrate selectivity ([3]). The human cytochrome P450 (CYP) monooxygenases are a diverse group of enzymes encoded by 57 genes ([4]). The majority of CYP genes in mammalian genomes encode enzymes that oxidize structurally diverse substrates and provide an overlapping capacity to oxidize both foreign and endogenous compounds ([5]).

CYP2D6 gene, a member of the CYP superfamily, encodes a phase I enzyme that is highly polymorphic with more than 91 allele variants identified so far. The most common CYP2D6 variants are variants *3, *4 and *5, resulting in a decreased or absence of enzyme activity, and leading to poor metabolizer phenotypes (PM) ([6]).

A tremendous variation in CYP2D6 (CYP2D6; debrisoquine hydroxylase) enzyme activity has been shown among individuals, mainly because of genetic polymorphism of the CYP2D6 gene. Historically speaking, the population has been divided into two phenotypes ([7]): extensive metabolizers (EMs), who exhibit CYP2D6 activity,and PMs, who lack CYP2D6 activity. In addition, ultrarapid metabolizers have been identified who carry multiple copies of active CYP2D6 genes ([8]). Recently, new polymorphisms were described in connection with intermediate and ultrarapid CYP2D6 metabolism. These may allow a much desired prediction of metabolic activity within the extensive metabolizer group. The functional consequences are still being discussed with few data available for clinical patients ([9]).

By knowing the P450 genetics and biology, a lot can be understood with respect to basis for metabolic activation and the relative risk for toxic or carcinogenic effects of xenobiotics. This is true for the difference between ethnic

groups and individuals with respect to exposure to xenobiotics in relation to: (i) dietary habits; (ii) environmental exposure; and (iii) expression of particular isoforms of cytochrome P450 ([10]). Dosage requirements for several commonly used drugs that have a narrow therapeutic range can differ more than 20-fold dependent on the genotype or the enzyme expression status ([11]).Several examples exist where subjects carrying certain alleles suffer from a lack of drug efficacy due to ultrarapid metabolism or, alternatively, adverse effects from the drug treatment due to the presence of defective alleles ([10]).

The aim of this research is to detect if there is specific cytochrome P450-2D6*4 genotype is associated with cannabis addiction; that may be initial step in determining genes associated with the addicting behavior aiming for future specific genotherapy for those patients.

SUBJECTS AND METHODS

The study included 46 volunteers; 18 of them confessed using cannabis (Hashish smokers) since different periods of life (test group) and the other 28 never used cannabis before (control group). All individuals were males aged 20 – 29 years old. All participants agreed to give blood and urine samples and also agreed to be tested for cannabis. The test group answered a questionnaire about their education, occupation, their family norms about hashish smoking and also about their starting age of cannabis use, pattern of its use (regular or irregular) and trials of abstinence.

Blood (3 ml) and urine (1 ml) samples were collected from all participants in the study.

Urine samples were screened for cannabis by enzyme immunoassay technique using Viva (Vitalab, Dade Behring, manufactured by vital scientific) with cannabinoids assay kits for qualitative analysis of cannabinoids in human urine.

EDTA anticoagulated blood samples were taken from all individuals by venipuncture and stored at -30ºC until used for DNA extraction and genotyping of cytochrome P450 -2D6*4 gene using conventional PCR followed by restriction enzyme (Mva1) digestion .

Genotyping of cytochrome P450-2D6*4 using PCR-RFLP technique

DNA extraction: DNA was extracted from EDTA anticogulated blood using QIA amp DNA mini kit for DNA isolation and purification from whole blood (Schur et al.,2001).

Polymerase Chain Reaction: Conventional method of PCR amplification was used for CYP2D6*4 using Ready Mix (RED-Taq-PCR Reaction Mix) (purchased from Sigma-Aldrich,Saint Louis,USA) according to the method described by Schur et al. ([12]). The 2 primers (forward and reverse primers) were purchased from QIAGEN Operon (Germany).The sequence of the two primers was designed by Schur et al.([12]) are shown as follow: The forward primer: -5'-GCCTTCGCCAACCACTCCG-3' . The reverse primer: -5'-AAATCCTGCTCTTCCGAGGC-3'.

The following mixture was prepared for each sample :25µl of RED-Taq PCR reaction Mix(1X), 1µl of forward primer,1µl of reverse primer,2µl of template DNA and 21 µl of double distelled water.This mix was put in a thin wall PCR microcentrifuge tube and gently centrifuged to collect all components to the bottom of the tube .Then ,50µl mineral oil was added to the top of each tube to prevent evaporation.Amplification was performed on a thermal cycler(Minicycler PTC-150) using the following program:- denaturation at 95ºC for 4 minutes, 30 cycles of: 94°C for 1 minute then ,60ºC for 1 minute then 72ºC for 1.5 minutes and final 72ºC for10 minutes. The product of PCR amplification were subjected to agarose gel electrophoresis using 1.8 % agarose gel,containing 10 mµg ethedium bromide (purchased from Sigma-Aldrich, St.Louis, MO) for one hour at 100 volts (355 base pair PCR fragment was indicative of the presence of the gene).

Restriction analysis: The Cyp2D6*4 restriction used the following condition: The reaction mixture was prepared as follow: 0.2µl (2 units) of the restriction endonuclease Mva-1(Roche Molecular Biochemicals), 10µl of amplified PCR products, 2.0µl of appropriate buffer (50 mM tris HCL, 100mM NaCl, 10 mM EDTA, 1mM dithiothreitol, pH 7.5 at 37ºC) and 7.8 µl of sterile glass distelled water. Then the reaction mixture was incubated in 37ºC water bath for 4 hours. Lastly, the reaction mixture was stored at -20ºC overnight .Then subjected to agarose gel electrophoresis using 3% agarose gel,containing 6 µg ethedium bromide for 2 hours at 100 volts

Statistical analyses: Data were compared by using Chi square (x2) for qualitative data (frequency and proportion). These data were run on an IBM compatible personal computer by using Statistical Package for Social Scientists (SPPS) for windows 11 (SPSS Inc., Chicago, IL , USA).

RESULTS

The results from this study showed that 19 individuals of the

control group (n=28) have the wild allele of CYP2D6*4 (67.8%), 4 have the Heterozygous allele (14.3%) and 5 have the Homozygous allele (17.9%) while 9 of the cannabis dependent (n=18) have the heterozygous allele (50%), 5 have the homozygous allele (27.8%) and 4 have the wild allele (22.2%) with significant difference between test and control group (X2= 10.005 and P 0.007) (Table 1).

All tested individuals were cigarette smokers males. All cannabis users accept it as harmless habit apart from the financial load. The entire control group had a cannabis negative screen while the entire test group had positive cannabis screen.

The numbers of individuals included in the study may be small, but these are the only available cases. It was so difficult to find a cannabis smoker who doesn't abuse another drug.

Half of the test group is educated to the secondary school level or diploma, 22.2% are colleagues, 16.7 % are educated to the primary school level, 5.6% have the preparatory education only and 5.6 % are illiterate. Half of them are workers, 16.7% are clerk, 11.1% are student and 22.2% are unemployed (Table 2).

Ten of the cannabis users had positive family norms about cannabis use (55.6%) and only 8 had negative family norms (44.4%). 14 of them (77.8%) had a positive peer group adapting the same ideas about cannabis use. All users with the wild allele have negative family norms, 40% have the homogenous allele and 22.2% have the heterogenous allele while 50% of the users with positive peer group concepts have wild type and 50% have the heterogenous allele (Table 2).

Most of users (66.7 %) started cannabis use at 15 – 20 years of age, most of individuals with the heterozygous (88.9 %) or homozygous allele (40 %) started their use at that age. Also, most of individuals with the heterozygous (88.9 %) or homozygous allele (60 %) used cannabis regularly without abstinence trials (Table 2).

Figure 1

Figure 1: The product of restriction enzyme digestion of the product of amplification of 2D6*4 gene.

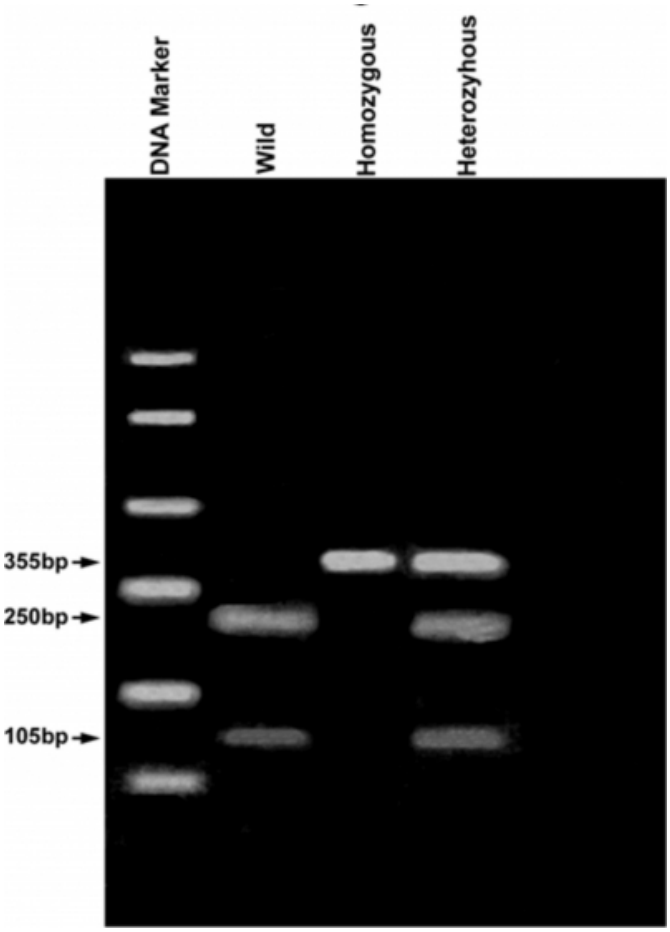

Figure 2

Table 1: Genotyping of cytochrome P450-2D6*4 in the different studied individuals.

	Wild type	Heterozygous	Homozygous
The control group (n = 28)	19/28	4/28	5/28
	67.8%	14.3%	17.9%
The cannabis users (n = 18)	4/18	9/18	5/18
	22.2%	50%	27.8%
X^2	10.005		
P	0.007		

Significant at $p < 0.05$.

Figure 3

Table 2: Characteristics of the cannabis users

Parameters	Total (n= 18)	Wild (n= 4)	Heterozygous (n= 9)	Homozygous (n= 5)	significance
Education: Illiterate:	1/18 (5.6%)	---	1/9 (11.1%)	---	$X^2 = 6.522$
Ist school:	3/18 (16.7%)	---	1/9 (11.1%)	2/5 (40%)	P 0.589
Prep. school:	1/18 (5.6%)	---	5/9 (55.6%)	---	
2nd school or diploma:	918 (50%)	2/4 (50%)	1/9 (11.1%)	2/5 (40%)	
Colleague:	4/18 (22.2%)	2/4 (50%)	1/9 (11.1%)	1/5 (20%)	
Occupation: Worker:	9/18 (50%)	2/4 (50%)	4/9 (44.4%)	3/5 (60%)	$X^2 = 2.297$
Clerk:	3/18 (16.7%)	---	2/9 (22.2%)	1/5 (20%)	P 0.890
Student:	2/18 (11.1%)	1/4 (25%)	1/9 (11.1%)	---	
Unemployed:	4/18 (22.2%)	1/4 (25%)	2/9 (22.2%)	1/5 (20%)	
Family norms about addiction Positive:	10/18 (55.6%)	---	7/9 (77.8%)	3/5 (60%)	$X^2 = 6.84$
Negative:	8/18 (44.4%)	4/4 (100%)	2/9 (22.2%)	2/5 (40%)	P 0.15
Peer group: Positive:	14/18 (77.8%)	2/4 (50%)	9/9 (100%)	3/5 (60%)	$X^2 = 5.271$
Negative:	4/18 (22.2%)	2/4 (50%)	---	2/5 (40%)	P 0.072
start age of cannabis use: < 15 years:	2/18 (11.1%)	--	--	2/5 (40%)	$X^2 = 8.467$
15-<20 years:	12/18 (66.7%)	2/4 (50%)	8/9 (88.9%)	2/5 (40%)	P 0.076
20-<25 years:	4/18 (22.2%)	2/4 (50%)	1/9 (11.1%)	1/5 (20%)	
Period of cannabis use: 5-<7 years:	6/18 (33.3%)	4/6 (66.7%)	1/6 (16.7%)	1/6 (16.7%)	$X^2 = 10.4$
7-<10 years:	6/18 (33.3%)	---	4/6 (66.7%)	2/6 (33.3%)	P 0.034
>10 years:	6/18 (33.3%)	---	4/6 (66.7%)	2/6 (33.3%)	
Pattern of use: Reg:	13/18 (77.2%)	2/4 (50%)	8/9 (88.9%)	3/5 (60%)	$X^2 = 2.6$
Irregular:	5/18 (22.8%)	2/4 (50%)	1/9(11.1%)	2/5 (40%)	P 0.272
Abstinence trial: +ve	5/18 (22.8%)	2/4 (50%)	1/9(11.1%)	2/5 (40%)	$X^2 = 2.6$
-ve	13/18 (77.2%)	2/4 (50%)	8/9 (88.9%)	3/5 (60%)	P 0.272

Significant at $p < 0.05$.

DISCUSSION

Human cytochrome P450-2D6 enzymes play a key role in the metabolism of drugs and environmental chemicals ([13]). The number of known P450 enzymes exceeds 1000, while the endogenous substrates of most of them remain unknown ([14]). Cytochromes P450 are the key enzymes for activating and inactivating many drugs. Therefore, individual expression levels of cytochromes P450 may play a crucial role in drug safety and drug efficacy ([15]). Over 90% of drugs are metabolized by the cytochrome P450 (CYP) family of liver isozymes. The most important enzymes are CYP1A2, 3A4, 2C9/19,2D6 and 2E1. Although CYP2D6 accounts for less than 2% of the total CYP liver enzymes content, it mediated metabolism in almost 25% of drugs ([16]).

CYP2D6 maps to human chromosome 22 by Southern analysis of DNA from somatic cell hybrid. Also, it is present in liver, peripheral blood leukocytes and other tissues ([17]). Schur et al. ([12]) studied CYP2D6*4 using restriction fragment length polymorphism (RFLP) (conventional PCR followed by restriction enzyme analysis using Mva1) and found that normal human CYP2D6*4 appear as two bands at 105 & 250 bp on agarose gel electrophoresis and referred as wild type determination. They described the appearance of an undigested 355 base pair PCR fragment after using RFLP was indicative of the presence of mutation of CYP2D6*4 gene and called homozygous mutation. A mixture of the three fragments was indicative of the heterozygous mutation ([18]).

Results of the present study showed that there is interindividual differences in the genotyping of cytochrome P450-2D6*4 in the control group; 19 have the wild allele (67.8%), 4 have the heterozygous allele (14.3%) and 5 have the homozygous allele (17.9%). Similar individual differences are also detected beside the interethnic differences in the Asian ([19]) and in black African populations ([20]).

The CYP2D6 is one of the major forms of importance for drug metabolism. The function and importance of those are still incompletely known ([21]). The mutations in the CYP genes can cause enzyme products with abolished, reduced, altered or increased enzyme activity ([22]). Mutations in substrate recognition sites (SRS) can cause the synthesis of enzymes with an altered substrate specificity ([23]). Increased activity is seen in subjects carrying multiple copies of an active P450 gene as described for CYP2D6 ([8]) and CYP2A6 ([24]).

Results of the present study showed that there is significant difference between the genotype distribution in the control group (sample of general population) and the test group (cannabis users), 50% of the cannabis users have the heterozygous genotype of cytochrome P450-2D6*4, 27.8% have the homozygous allele and 22.2% have the wild allele. Also, most of individuals with the heterozygous (88.9 %) or homozygous allele (60 %) used cannabis regularly without abstinence trials So, the heterozygous and the homozygous alleles may be related to the cannabis dependence.

The genetic basis of addiction have been previously studied in families, twins and adoption studies which explored that alcoholism ([25] and [26]) and smoking ([27] and [28]) are highly genetically co-morbid. The CYP2D6-dependent conversion of codeine to morphine has been shown to be required for the analgesic effect of codeine ([29]). Also, the contributions of cytochromes P4502B (P4502B) and cytochrome P4503A (P4503A) to the bioactivation of cocaine in hepatocytes isolated from Sprague-Dawley rats has been studied ([30]). Also, the change in nortryptilline concentration was attributed to a mutant allele compared with the wild type and this was recommended to be used to guide therapy dosage ([9]).

Kendler and Prescott ([31]) postulated that genetic risk factors have a moderate impact on the probability of ever using cannabis and a strong impact on the liability to heavy use, abuse, and, probably, dependence. By contrast, the family and social environment substantially influences risk of ever using cannabis but plays little role in the probability of developing heavy cannabis use or abuse. More recently, Kreek et al. ([32]) have reviewed number of genes that appear to be involved in the vulnerability to and the treatment of heroin and/or cocaine addiction. The evidence for the role of few of these genes and the influence of their variants remain tenuous and will require replication, whereas the role of others appears to be fairly well established.

Both the genetic and the shared environmental effects on risk for the use and misuse of six classes of illicit substances (cannabis, cocaine, hallucinogens, sedatives, stimulants, and opiates) were largely or entirely nonspecific in their effect ([33]). The present study of the personal charachteristic of the test group (hashish smokers) revealed that half of them are educated to the secondary school level or diploma, half of them are workers, 55.6% have positive family norms about cannabis use and 77.8% have a positive peer group adapting the same ideas about cannabis use. Beside, all users with the wild allele have negative family norms, 40% have the homogenous allele and 22.2% have the heterogenous allele while 50% of the users with positive peer group concepts have wild type and 50% have the heterogenous allele. These data are indication about the contribution of both the genetic factors and the environmental factors for the compulsive behaviour of cannabis dependence.

Associations between early cannabis use and later drug use and abuse/dependence cannot solely be explained by common predisposing genetic or shared environmental factors. The association may arise from the effects of the peer and social context within which cannabis is used and obtained ([34]).

Genetic risk factors have a moderate impact on the probability of ever using cannabis and a strong impact on the liability to heavy use, abuse, and, probably, dependence. By contrast, the family and social environment substantially influences risk of ever using cannabis but plays little role in the probability of developing heavy cannabis use or abuse ([31]).

Kreek ([35]) had hypothesized that three domains of factors contribute to the development and persistence of addictions: inherited or genetic differences in individual physiology, alterations in physiology induced by drugs or alcohol, and environmental or developmental factors.

Certainly cannabis use is related to environmental factors (availability of drug, peer effects, family norms) but there are some genetic factors that determine the body response to it that need to be more specifically studied. This can be a marker for the risk of becoming dependent and / or the prediction of suitable effective treatment of dependence and also, prevention of relapse after withdrawal.

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