International Journal of Hospital Research 2020, 9(1)
http://ijhr.iums.ac.ir
Research Article

A Evaluation of distribution of genetic polymorphism CYP2D6*10 in tramadol poisoning with seizure: A Case-Control study

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Abstract

Background and Objective: Tramadol overdose is inappropriately prevalent in Iran and is one of the most common causes of hospital admissions in recent years. Tramadol is both a codeine family and a weak opioid receptor agonist (AG) that is used at regular doses as an analgesic and may lead to seizures. The aim of this study was to investigate the relationship between physiological effects of high dose tramadol administration and cytochrome c enzyme gene polymorphism. CYP2D6 (p450) was surveyed in patients with tramadol intoxication referred to the poisoning ward of Razi Hospital, Ghaemshahr city, Mazandaran province.

Method: This case-control study was performed on 121 patients admitted for poisoning by Tramadol only in 2016. These patients did not have any previous underlying diseases such as cardiac and renal complications. Sixty of the patients had seizures due to tramadol. At first, the demographic data of patients were collected via a questionnaire and from each patient 5cc blood was taken. After preparing the blood samples, RFLP-PCR was used to evaluate the CYP2D6 gene polymorphism (rs1065852).

Results: The mean age of patients was 25 years. 78% of them were smokers. P<0.05 was considered as the level of significance. Serum tramadol concentration was higher in those with and without convulsion. There was no significant difference between the two groups in terms of genotype CC. Therefore, it can be concluded that tramadol-induced seizures are not related to the locus of techno-nucleotide polymorphism (rs1065852).

Conclusion:These healthy young people were probably poisoned due to their size and arbitrary consumption. Therefore, more comprehensive laws and restrictions on the distribution and consumption of tramadol in the community should be formulated.

Keywords: Overdose, Tramadol, Genetic polymorphism, CYP2D6*10

Background and Objectives

Addiction is a growing epidemic. Besides, one of the most worrying forms of addiction in the world is the hidden addiction or the addiction to the arbitrary use of drugs that are likely addictive1. In Iranian society, drug addiction is unprecedented outbreak, with the onset of addiction under 15 years[1,2]. Tramadol is one of the self-medications that have been used in the past few years (3). Tramadol as an opioid-like analgesic used by young people and especially students was first introduced in 1970. In Germany it has been suggested for relieving post-operative pain and for controlling chronic pain[3]. Research in the United States has shown that one in seven people taking tramadol is dependent on the drug[4]. Other side effects of tramadol tablets include psychosis and cerebral dysfunction, personality traits, inability to make decisions, imbalances in walking and dry mouth, as well as seizures, aggressive and uncontrollable behaviors[5]. These have led the World Health Organization's Task Force on Substance Abuse to put the tramadol on the international list of specific drugs[6].

Tramadol hydrochloride is a synthetic analogue of phenylpiperidine codeine used in acute and chronic moderate to severe pain due to osteoarthritis, musculoskeletal pain, diabetic neuropathy, neuropathic pain, and preoperative pain[7].

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| Tramadol exhibits opioid agonist properties and activates the spinal cord system[8]. Tramadol has little potential compared to opioids for respiratory suppression and gastrointestinal side effects and therefore plays an important role in the management of pain in acute and chronic conditions[9]. In terms of central neurotransmitter effects, tramadol is a receptor agonist (mu), and tends to be 10-fold lower than codeine for the receptor, but the active metabolite of tramadol, desmethyl tramadol-0, is 200-fold greater than tramadol[10]. Various convulsive effects of monotherapy and multi-drug use have been observed in human studies at both medium and high doses[10].

Sequence analysis to find mutations and polymorphisms of the CYP2D6*10 gene located on chromosome 22, thus, we need sequencing analysis of the genome information. The Genbank can be used to search for the desired sequences (www.ncbi.nlm.nih.gov/web/search/index.html). In Iran, such studies have not been performed despite a large number of tramadol poisoned patients. In this study, we aimed to investigate the relationship between the 450M cytochrome gene polymorphism in tramadol poisoned patients with seizures.

Materials and methods:

Study population:

This research was a case-control prospective study based on the genetic polymorphism CYP2D6*10. The study population was patients admitted to the hospital due to tramadol poisoning. Inclusion criteria for the case group were the confirmed tramadol poisoning with at least a seizure episode; while the control group did not experience a seizure. Available sample selection underwent till case and control groups were matched based on age and gender. Exclusion criteria were the non-satisfaction with the study. All participants fulfilled the informed consent form and if they had not enough consciousness to cooperate their family was asked for the informed consent. Then 5 cc blood sample was taken from all subjects.

DNA Extraction:

DNA was extracted from blood samples by salting-out method and after extraction, the quality and quantity of DNA extracted were assessed by spectrophotometer. Then, DNA samples were stored at -20 °C (20).

Genotyping of Patients:

Genotyping was conducted using Polymerase Chain Reaction by Enzymatic Digestion (Figs 1 & 2).

PCR-restriction fragment length polymorphism (RFLP)

Duplication was performed using a dedicated primer pair and a 344bp fragment was generated and primers were designed using Gene Runner software. The sequence of primers is listed in Table 1.

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Fig 1: The position of the polymorphism nucleotide rs1055852 in the NCBI database

Fig 2: Nucleotide polymorphism position CYP2D6 *10 (rs1055852) in the NCBI database, which is a mutation in the nucleotide change of the missense mutation type.

Tab 1: The specifications of the primer used and the restricted enzyme

The location of gene polymorphism	Primers sequence	Tm	Restriction enzyme acting	PCR product (bp) allele
CYP2D6*10 (rs1065852) (C>T)	Forward: 5-CAGTCAACACAGCAGGTTCAC-3 Reverse: 5-GCAGTATGGTGTGTTCTGGAAG-3	63	HphI GGTGA(8/7)^	C: 344 bp T: 70+274 bp

Polymerase Chain Reaction (PCR) in 25 µl volume of 1 µl genomic DNA (100 ng / ml), 5.12 µl Master Mix PCR and 1 µl of each primer which was finally distilled in volume until it reached 25 microliters. PCR reaction time: 94 °C for 5 minutes, then 35 cycles including 94 °C for 30 seconds to double-strand DNA, 63 °C for 30 seconds to bind primer to DNA and 72 °C for 40 seconds to elongate. After 36 cycles, the reaction mixture was kept at 72 °C for 10 minutes until the final elongation was performed. Then, to evaluate the quality of the PCR product, each sample was evaluated by 2% agarose gel electrophoresis[10].

BLAST Results

The primer sequences designed from the NCBI database are shown in Table 2.

The primer design was performed with the Gene Runner software and performed in the NCBI primer blast database. The desired SNP and the cleavage site of the enzyme are specified in the sequence and the primers were sequenced (the final result was as follows [CAGTCAACACAGCAGGTTCACTCACAG

CAGAGGGCAAAGGCCATCATCAGCTCC
CTTTATAAGGGAAGGGTCACGCGCTCG
GTGTGCTGAGAGTGTCCTGCCTGGTCCT
CTGTGCCTGGTGGGGTGGGGGTGCCAG
GTGTGTCCAGAGGAGCCCATTTGGTAG
TGAGGCAGGTATGGGGCTAGAAGCACT

GGTGCCCCTGGCCGTGATAGTGGCCAT
CTTCCTGCTCCTGGTGGACCTGATGCAC
CGGCGCCAACGCTGGGCTGCACGCTAC(
C/T)CACCAGGCCCCCTGCCACTGCCCG
GGCTGGGCAACCTGCTGCATGTGGACT
TCCAGAACACACCATACTGC]).

Table 2. Primer sequences designed in the NCBI database

Product length	Primers sequence	Features
344	Forward primer 1 CAGTCAACACAGCAGGTTCAC 21 Template 42130964..... 42130944 Reverse primer 1 GCAGTATGGTGTGTTCTGGAAG 22 Template 42130621..... 42130642	cytochrome P450 2D6 isoform X3 cytochrome P450 2D6 isoform X1

Statistical analysis:

To interpret the results obtained in this study statistical analysis using SPSS software version 21 and statistical tests of Chi-square (X2), and odds ratio (OR) were provided and parameters such as Confidence Interval (CI) were also determined. P values under 0.05 were considered significant.

Results:

A total of 121 tramadol users (non-seizure group = 61 and seizure group = 60) were admitted to Razi Hospital of Ghaemshahr, diagnosed by a specialist physician. Demographic characteristics and clinical findings were collected by a questionnaire.

The age range of patients in the two groups in this study was 13 to 35 years. There were no significant differences between the two groups in terms of age and gender (P>0.05).

Component Duplication and Genotyping:

PCR was performed using specific primers to amplify fragments in the CYP2D6*10 gene. All DNA extracted from both groups produced a single-stranded PCR product with no other non-specific bands. Genotyping and CYP2D6 Allele Screening, PCR- RFLP was performed using the Hphl restriction enzyme, then PCR RFLP's products were analyzed by agarose gel electrophoresis. (Figure 3)

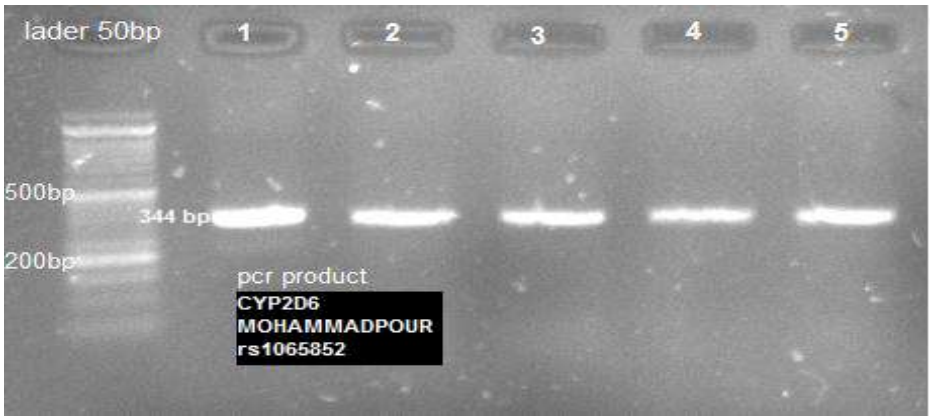

Fig 3: RFLP results for the rs1065852 polymorphism of the CYP2D6*10 gene.

The PCR product of the CYP2D6 gene shows a fragment size of 344 bp. A 50hp marker sequence was amplified for fragment detection. After enzymatic digestion (Hphil) we expected fragments of 344, 274, and 70 bp to be visible, indicating normal homozygous (CC), homozygous mutant (TT), and heterozygous mutants (TCs). But all individuals in the two groups showed genotype (CC).

CYP2D6 Gene Sequencing:

Ten samples were sent for sequencing. The Figure 5 and 6 shows the CC genotype as non-mutant homozygous and is considered healthy, and BLAST nucleotide results show that the sequenced sequence corresponds to the nucleotide sequence in the NCBI database.

Figure 4. The BLAST nucleotide results show that the sequenced sequence corresponds to the nucleotide sequence in the NCBI database.

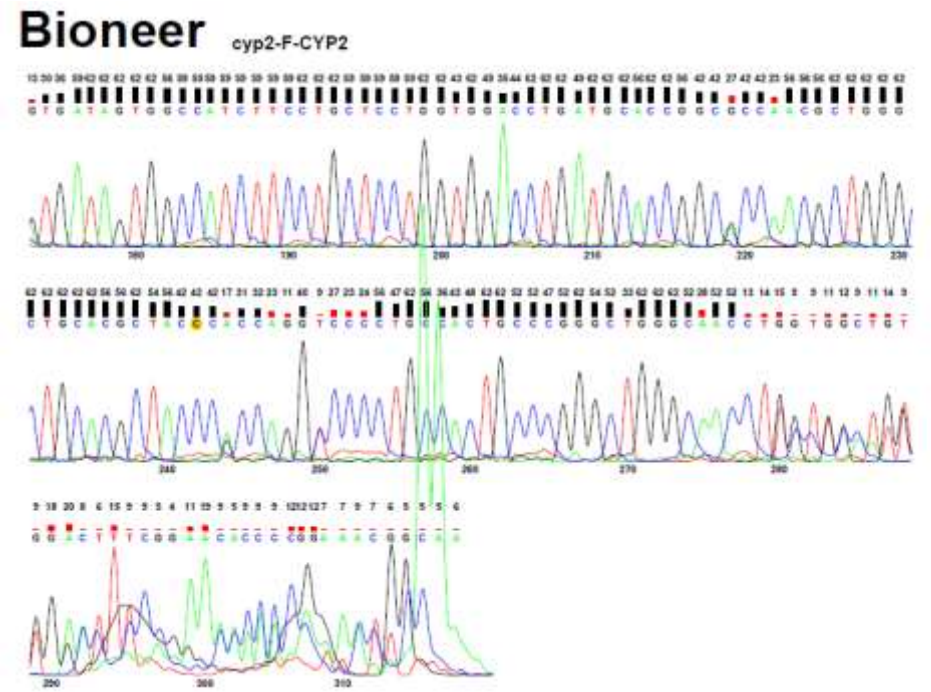

Figure 5. Sequencing results of the CYP2D6 gene performed by Bioneer Korea. And the desired nucleotide in yellow is displayed in the sequence above.

Results of these sequences in the NCBI database:
CYP2D6 gene sequence obtained from the NCBI database (Fig 6).
rs1065852 in CYP2D6
5-
GCGCCAACGCTGGGCTGCACGCTAC
[C/T]CACCAGGCCCCCTGCCACTGCC
CGG-3>cyp2-F-CYP2
5AATAAGGCAAGCATCATCAGCTCC
TTTATAAGGGAAGGGTCACGCGCTC
GGTGTGCTGAGAGTGTCCTGCCTGG
TCCTCTGTGCCTGGTGGGGTGGGGG
TGCCAGGTGTGTCCAGAGGAGCCCA
TTTGGTAGTGAGGCAGGTATGGGGC
TAGAAGCACTGGTGCCCCTGGCCGT
GATAGTGGCCATCTTCCTGCTCCTG
GTGGACCTGATGCACCGGCGCCAAC
GCTGGGCTGCACGCTACCCACCAGG
TCCCCTGCCACTGCCCGGGCTGGGC
AACCTGGTGGCTGTGGACTTTCGGA
ACACCCCGGAAACGGCAA-3

Score	Expect	Identities	Gaps	Strand
497 bits(269)	4e-145	285/293(97%)	1/293(0%)	Plus/Plus

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Query  12   CATCATCAGCT-CCTTTATAAGGGAAGGGTCACGCGCTCGGTGTGCTGAGAGTGTCCTGC  70
Sbjct  270  CATCATCAGCTCCCTTTATAAGGGAAGGGTCACGCGCTCGGTGTGCTGAGAGTGTCCTGC  329

Query  71   CTGGTCCTCTGTGCCTGGTGGGGTGGGGGTGCCAGGTGTGTCCAGAGGAGCCCATTTGGT  130
Sbjct  330  CTGGTCCTCTGTGCCTGGTGGGGTGGGGGTGCCAGGTGTGTCCAGAGGAGCCCATTTGGT  389

Query  131  AGTGAGGCAGGTATGGGGCTAGAAGCACTGGTGCCCCTGGCCGTGATAGTGGCCATCTTC  190
Sbjct  390  AGTGAGGCAGGTATGGGGCTAGAAGCACTGGTGCCCCTGGCCGTGATAGTGGCCATCTTC  449

Query  191  CTGCTCCTGGTGGACCTGATGCACCGGCGCCAACGCTGGGCTGCACGCTACCCACCAGGT  250
Sbjct  450  CTGCTCCTGGTGGACCTGATGCACCGGCGCCAACGCTGGGCTGCACGCTACYCACCAGGC  509

Query  251  CCCCTGCCACTGCCCGGGCTGGGCAACCTGGTGGCTGTGGACTTTCGGAACAC  303
Sbjct  510  CCCCTGCCACTGCCCGGGCTGGGCAACCTGCTGCATGTGGACTTCCAGAACAC  562
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Figure 6. Results of these two CYP2D6 genes that were sequenced by the PCR product which indicates their conformity and the desired toluene is indicated in blue

Outcome comparison

CYP2D6 Gene Genotyping Analysis Results showed that among 61 non-seizure individuals, 61 (100%) had CC genotype, 0 (0%) had CT genotype and 0 (0%) had TT genotype. Among 60 patients with seizure, 60 (100%) had CC genotype, 0 (0%) had CT genotype and 0 (0%) had TT genotype. It can be said that there is no significant difference in the distribution of polymorphism between the two groups of patients and the control group.

Discussion

In a study of tramadol abuse, Mohammadpour et al. Showed that a total of 121 people (non-seizure group = 61 and seizure group = 60) of tramadol users who had been hospitalized for one year had seizures. Tramadol induced is not associated with age, sex, or dose in this population. Biochemical parameters including urea, creatinine, and troponin, creatine phosphokinase myocardial band (CPK-MB) were determined using spectrophotometry in cases that tramadol

administration with seizures can lead to cardiac and renal complications in patients[15]. At least 11 tramadol metabolites have been identified, of which only one (0-dimethyl tramadol, m1) has pharmacological activity and is more dependent on the receptor than the main drug. The production of this metabolite is dependent on the isoenzyme CYP2D6 of the cytochrome P450 enzyme system and hepatic impairment results in reduced metabolism of the major constituents and active metabolites of the drug[11,12].

The half-life of 1-dimethyl tramadol is approximately 9 hours long. Tramadol is widely metabolized in the liver and is largely eliminated by renal excretion. One of the dangerous side effects of tramadol is seizure. Seizures are not dose-dependent with tramadol administration[15]. One of the most common causes of seizure is the sum of neuronal defects grouped as epilepsy. The seizure is one of the most common disorders of the central nervous system. Tramadol is not included in the list of OTC drugs in Iran. However, due to its properties, its use among Iranian youth is increasing.

Opioid abuse is common in Iran and its pattern is rapidly changing[21,22]. In recent years, drug addicts have begun to use newer, cheaper, and more accessible compounds like tramadol[21-23].

Although Tramadol is not an OTC drug, some consumers illegally obtain it through untrusted drug dealers and retailers. The control of tramadol poisoning varies due to its various adverse effects[24]. Tramadol is a synthetic opioid prescribed for its soothing effects especially after surgery[24], renal colic, and premature ejaculation[25]. However, it is abused like narcotics to induce euphoria and improve sexual activity[25,26]. Tramadol works by opioid and non-opioid receptors[27].

Mechanisms of action include inhibition of serotonin reuptake, stimulation of serotonin release, and inhibition of norepinephrine reuptake[28]. Activation of the opioid and serotonin pathways leads to its adverse effects[28]. In cases of intoxication, tramadol may lead to a variety of adverse effects, particularly on the central nervous system, leading to a lack of consciousness, convulsions, and serotonin syndrome. Tramadol can also induce increased creatine phosphate kinase (CPK) and acute renal failure[31].

Tramadol may cause constipation, dizziness, shortness of breath, while the most severe side effects of tramadol overdose include severe seizures, rhabdomyolysis, and renal failure[32-34]. These serious complications are likely to occur in tramadol poisoning, in a way that seizures can induce rhabdomyolysis and kidney failure is often the result of rhabdomyolysis[31,32]. Seizures have been reported to occur in one-third of overdoses of tramadol[32,34,35]. Rhabdomyolysis is probably caused by multiple seizures[31,33].

In the present study, all patients (121 patients) who were hospitalized with tramadol intoxication during one year were evaluated. 60 people with tramadol poisoning who suffered from seizures and 61 people with tramadol poisoning who did not have seizures.

In this study, the mean age of patients with tramadol intoxication was 25 years, which was in accordance with the findings of Javanivik[37] with a mean age of 22 years. This result may indicate the prevalence of tramadol use among young people. In the present study, the rate of poisoning as a suicide attempt was 36%, which is consistent with the results of Markwood et al.[36]. In their study, 55% of patients were female, and 4.37% used tramadol for suicide. In the present study, 15 patients (4.38%) attempted suicide using tramadol. This indicates that tramadol consumption is higher in developing countries, especially Iran. A 2016 study by Farnsah et al., conducted a CYP2D6 sequence to detect lower tramadol metabolism in post-mortem blood samples using tramadol. This study showed that tramadol

concentration and its antinociceptive effect are dependent on CYP2D6 enzyme activity and it is well known that some genetic polymorphisms are responsible for variation in expression of this enzyme and response to specific drugs. The presence of enzymatic inhibitors significantly affects the degree of metabolism, and Sanger sequencing can be successfully used to detect genetic polymorphisms in CYP2D6 in post-mortem blood samples[39]. The reason for the discrepancy was that all the subjects in the present study were those without any heart or kidney disease who attempted to take tramadol without a doctor's prescription.

A 2013 study by Prissner 6 et al. on the cytochrome P450 enzyme polymorphism and their role in the individual treatment of individuals showed that cytochrome P450 (CYP) plays a key role in drug metabolism and they have over 2000 known types of mutations, some of their single nucleotide polymorphisms (SNPs) have been shown to have a major effect on the activity of CYPs[40], which was not consistent with the present study and lack of alignment due to tramadol therapeutic use.

Since drugs and chemicals are readily available in Iran, there is an accidental and deliberate use and abuse of drugs. In a study conducted over three years at Imam Reza Hospital in Mashhad, deliberate abuse of specific drugs and substances was very common and was about 54.4%[41].

In Sioux Hogan et al. study on the role of CYP2D6 genetic polymorphism on the pharmacokinetics and pharmacodynamics of tramadol 138 patients were enrolled all of whom received 100 mg of intravenous tramadol as the first analgesic after surgery. Their blood samples were collected at specific time points and serum concentration of tramadol was measured by HPLC. Patients were also genotyped for CYP2D6. As the result, CYP2D6 activity probably plays an important role in determining the pharmacokinetics of tramadol and predicting its side effects and that genotyping is probably an important tool in determining the required dose of tramadol. The disadvantages of this study was its small sample size[42].

There are few studies regarding the association between tramadol levels and the CYP2D6 gene rs1065852 polymorphism. Many studies have evaluated the association of this polymorphism with various cancers, which is an enzyme involved in metabolizing drugs in that disease. Only a study by Taskin et al[38] . in the 2016 study examined CYP2D6*10, *4, *3, *2 polymorphisms in the Turkish population among 200 people who responded with variable drug use. Genotypes of CYP2D6 polymorphisms are crucial for determining the genotype-phenotype relationship indicate clinical efficacy in the use of antidepressants, neurolytic, antiarrhythmic, antihypertensive, and morphine which shows poor metabolism and adverse drug reaction. This was not in line with our study results probably because of racial and ethnic differences.

The authors propose further investigations in different individuals by race, geographical area, and with larger sample size, to determine the type of whole-gene mutation by sequencing, to investigate the effect of other receptors and pathways on tramadol-induced seizures.

Limitations

An important weakness of the current work was the lack of cooperation between patients and trained interviewers.

Conclusion

In spite of a large number of tramadol poisoned patients in Iran, we lack studies to identify the factors associated with seizure-related toxicity with tramadol. In this study, we investigated the relationship between cytochrome P450 gene polymorphism in tramadol poisoned patients with seizures.

The results of this study indicate that the polymorphism (rs1065852) of the

CYP2D6 gene is probably not associated with seizures in patients and screening of this CYP polymorphism may not be useful in the prognosis, and prevention of disease progression. The use of appropriate therapeutic strategies to reduce seizures and improving quality of life in patients with tramadol is helpful. It is noteworthy that this result is limited to the population under study and further studies are needed to confirm the association of CYP2D6 polymorphisms with consumer patients.

Conflict of Interest

The authors declare that they have no competing interests.

Acknowledgments

The data provided in this study was extracted from the Ph.D. thesis of Abbas Mohammadpour. This work was financially supported by Medical Biotechnology Research Center, Ashkezar Branch, Islamic Azad University, Ashkezar Yazd, Iran

Research ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Please cite this article as:

Mohammadpour A, Shokrzadeh M, Farahmand B, Dehghani M. Evaluation of distribution of genetic polymorphism CYP2D6*10 in tramadol poisoning with seizure: A Case-Control study. Int J Hosp Res. 2020;9(1)