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ORIGINAL ARTICLE

Genetic profiling of CYP3A4 variants influencing carbamazepine metabolism and their gender-specific association among patients with epilepsy in Khyber Pakhtunkhwa

Ayesha Jamil[1], Muhammad Saleh Faisal[2]*, Niaz Ali[3], Gulmakay Zaman[4], Rizwan Faisal[5] and Syed Hasnain Ali Shah[4]

[1]Department of Pharmacology, Khyber Girls Medical College, Peshawar, Pakistan, [2]Department of Pharmacology, Khyber Medical College, Peshawar, Pakistan, [3]Department of Pharmacology, Khyber Medical University, Peshawar, Pakistan, [4]Kabir Medical College, Peshawar, Pakistan, Department of Pharmacology, [5]Rehman Medical College Peshawar Pakistan.

ABSTRACT

Background: Cytochrome P450 plays a pivotal role in metabolizing drugs, hormones, and vitamins with genetic variability influencing enzymatic activity. Genetic consortiums categorize variables such as CYP3A4-rs2242480 and rs2740574 as third-level evidence, indicating the need for further research into their health implications and pharmacogenetic relevance in carbamazepine metabolism. This study explores the distribution patterns of selected genes and their correlation with gender in our study population, aiming to advance personalized treatment approaches.

Methods: Using Sanger sequencing, Pashtun patients diagnosed with epilepsy and treated with carbamazepine monotherapy were genotyped for *CYP3A4*-rs2242480 and rs2740574 polymorphisms. The genetic variations were identified through Finch TV and analyzed for gender correlations.

Results: Minor Allele Frequency (MAF) of *CYP3A4*-rs2242480 and rs2740574 in 223 Pashtun patients with epilepsy was 27.8% and 1.7%, respectively. The genotype frequency of *CYP3A4*-rs2242480 was as follows: CC at 53.8%, with females 35% & males 65%; CT at 36.7%, with females 36.5% & males 63.4%; and TT at 9.4%, with females 47.6% & males 52.3%. For *CYP3A4*-rs2740574, the genotype frequencies were 0% CC, 3.6% CT, and 96.4% TT. Among individuals with the CT genotype, the distribution was evenly split between males (50%) and females (50%). For those carrying the TT genotype, females comprised 36.2% of the group, while males made up 63.7%.

Conclusion: The study uncovered genotype distributions of carbamazepine-metabolizing enzymes in the previously unexplored Pashtun population, revealing significant differences in *CYP3A4*-rs2740574 and rs2242480 frequencies compared to global ethnic groups. Also, no significant association was observed between genotype distribution patterns across genders.

Keywords: Cytochrome P-450 CYP3A4, Carbamazepine, Epilepsy, Genetic Polymorphism, Polymerase Chain Reaction.

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CORRESPONDING AUTHOR

Muhammad Saleh Faisal
Department of Pharmacology,
Khyber Medical College, Peshawar, Pakistan
drsalehfaisal@gmail.com

Introduction

Pharmacogenetic differences are a crucial aspect to be considered in the management of diseases. Genetic differences between individuals or populations impact the way drugs operate. It is a fundamental aspect of personalized medicine and the cytochrome P450 (CYP) family is important in this process (1, 2). The CYP3A is well-known in phase I metabolism which is encoded by a *CYP3A* gene family consisting of *CYP3A4, CYP3A5, CYP3A7,* and *CYP3A43* (3). CYP3A4 is found in high quantities in the liver and small intestine and serves an integral part in the oral bioavailability and clearance of different drugs (4). Several drugs including carbamazepine are metabolized by CYP3A4 enzymes (5, 6).

Carbamazepine (CBZ) is an economically effective medication often employed for epilepsy. However, it is reported to exhibit high interpatient heterogeneity in clinical response and unexpected adverse effects in 30–50% of patients. Drug-resistant epilepsy affects up to 40% of patients (7). Its maintenance dosages for responders range from 200 to 2000 mg/day when administered alone, reflecting significant interpatient fluctuation (8, 9). CYP3A4 genes regulate the CYP3A4 enzymes, involved in the metabolism of carbamazepine. The *CYP3A4* gene resides on chromosomes 7q21.3–q22.1 and comprises 27,592 base pairs and 13 exons. It has about 30 single-nucleotide polymorphisms (SNPs). The promoter region contains the dominant SNP, -392A>G (rs2740574), also known as *CYP3A4*1B* which impacts the gene transcription and activity (10). *CYP3A4*1G* (rs2242480), located in intron 10 of the *CYP3A4* gene, has been linked to the pharmacokinetics and responses of drugs metabolized by CYP3A4. This variant is situated between 10.5 and 11.4kb in the *CYP3A4* gene, within a 900-base pair region known as the constitutive liver enhancer module (CLEM4). This region is crucial for regulating CYP3A4 enzyme expression (11).

Drug treatment for epilepsy is unpredictable, has unwanted effects, and calls for patient dosage adjustment. Genetic differences shape this variability (12, 13). Many CBZ metabolism-related SNPs including *CYP3A4*-rs2242480 and rs2740574 have been assigned third level evidence by genetic consortia like PharmGKB and CPIC guidelines, indicating genotypes and health outcomes are understudied (14). More racially broadened research is warranted as further investigations may yield novel leads. Learning how these SNPs affect carbamazepine plasma levels can enhance healthcare by tailoring the drug therapy.

Several researchers have investigated these SNPs in global ethnic groups, such as African Americans, European Americans, Han Chinese, Koreans, Indians, and Japanese (15). However, genetic studies involving carbamazepine metabolism in the Pakistani population are scarce. The current research aimed to fill this gap by examining the genotype distribution of *CYP3A4*-rs2242480 and rs2740574 and their correlation with gender in Pashtun patients with epilepsy, potentially revealing novel insights for personalized medicine in this region.

Methods

Following approval from the Advanced Studies and Ethics Board of Khyber Medical University, Peshawar (letter number Dir./KMU-EB/PS/000807), 223 patients were recruited from the government Lady Reading Hospital between October 2020 and April 2022. The required sample size was calculated using the Open Epi tool, based on a 7.5% margin of error, a 95% confidence interval, and 80% power. Additionally, the alpha project from dbSNP, NCBI was consulted to ensure the sample size was adequate for capturing genetic variations relevant to this study (https://www.ncbi.nlm.nih.gov/snp/rs2242480#frequency_tab). The participants were informed about the study objectives and consent was acquired**.** The study included anti-epileptic drug-naive patients with epilepsy, comprising both genders and ranging in age from 5 to 70 years, who were considered suitable candidates for carbamazepine therapy. Patients who had co-morbidities, impaired renal or hepatic profiles, were pregnant, or taking CBZ-interacting drugs were excluded.

The salting-out technique was employed to manually extract DNA from blood samples of study subjects (16). Prior to extraction, all plastic ware and solutions were sterilized, while chloroform and ethanol were kept at -20°C. The procedure involved mixing 300 µl of blood with 600 µl of cell lysis buffer (CLB), followed by vortexing and centrifugation. The supernatant was removed, and the pellet was repeatedly suspended in CLB until clear. After drying the pellet at 45^0C, it was dissolved in 400 µl nucleic lysis buffer (NLB), mixed with NaCl 150 µl and pre-chilled chloroform 600 µl, and centrifuged to form two layers. The upper layer was transferred to a new tube, where chilled ethanol 900 µl was added and centrifuged at maximum speed for 5 minutes to precipitate the DNA. The DNA was washed with 70% ethanol, resuspended in TE buffer, and stored at -20^0C. Finally, the DNA quality and quantity were verified using a nano-drop spectrophotometer and gel electrophoresis as shown in Figure 1.

Figure 1: Gel Electrophoresis of extracted DNA

The Gene JET™ purification kit was used to purify the extracted DNA followed by amplification of targeted SNPs of *CYP3A4* using a gradient Thermo Cycler. Primers were designed through UCSC genome database as shown in Table 1.

Table 1: Primers designed for target SNPs

Gene (SNP)	Primer	Sequence
CYP3A4 (rs2242480)	Forward	AGGGGACATCACACACCACT
	Reverse	TGTCTTTCCTCTCCTTTCAGCTC
CYP3A4 (rs2740574)	Forward	GTAGGTGTGGCTTGTTGGGA
	Reverse	AGGAGCCTGGACAGTTACTC

Before amplifying target SNPs, PCR assays were optimized using healthy control DNA. The targeted DNA region was amplified several times by PCR following optimization. The PCR products were later submitted to a 2% Agarose gel. Table 2 illustrates a list of essential compounds used for PCR, along with their respective concentrations.

Table 2: Reagents used for PCR amplification

Reagents	Strengths
Forward primer	1µl
Reverse primer	1µl
DNA	3µl
Master mix	15µl
Dist. H_2O	10µl
Total	30µl

Sequencing of the targeted genes was performed by Tsingke Xinye Biotechnology, China using Seq Studio TM (Applied Biosystems, USA). The manufacturer's instructions were followed to sequence the amplified products of PCR for both the reverse and forward primers using the BDT vs.3.1 master mix. The sequenced products were purified using the Big Dye Xterminator™ kit. After the samples were loaded and a medium run was selected for capillary electrophoresis, the sequence analysis was conducted using Finch TV. The analysis required the SNP flanking regions of *CYP3A4*-rs2242480 and rs2740574. This application streamlines the analysis of sequencing data, including SNPs and other regional variations. Visual inspection was used to confirm single nucleotide polymorphisms (17). Moreover, the minor allele frequencies (MAF) were also calculated by dividing the number of times the allele of interest observed in a population by the total number of copies of all the alleles at that genetic locus in the population.

The data was analyzed using SPSS version 22.0. Categorical parameters were represented using frequencies and percentages while the association between variables and groups was assessed employing Fisher's exact and Chi-square tests. The level of significance was established using an analysis of variance (ANOVA). Significant p-values were regarded as those that were less than 0.05. Based on the observed allele frequencies, the expected genotype frequencies under Hardy-Weinberg equilibrium were calculated. The chi-square test was used to assess the significance of deviations from the expected frequencies.

Results

Demographics and distribution pattern of *CYP3A4* genotypes

The study involved 223 Pashtun individuals with epilepsy who were prescribed carbamazepine as their sole treatment. The study population demonstrated a higher proportion of males, with 63.2% being male and 36.8% female. The average age of participants was 30.7 years. Additionally, 27.8% of the individuals reported a positive family history of epilepsy. The targeted genetic polymorphisms and genotypes were analyzed by Finch T.V as shown in Figures 2 & 3.

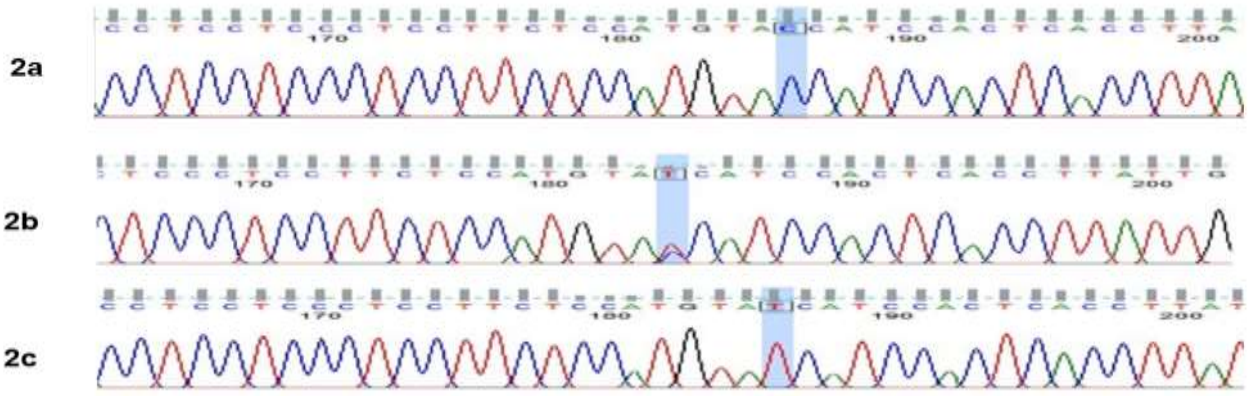

Figure 2: *CYP3A4*-rs2242480 (a). Wild genotype (b). Heterozygous variant (c). Homozygous-mutant-variant

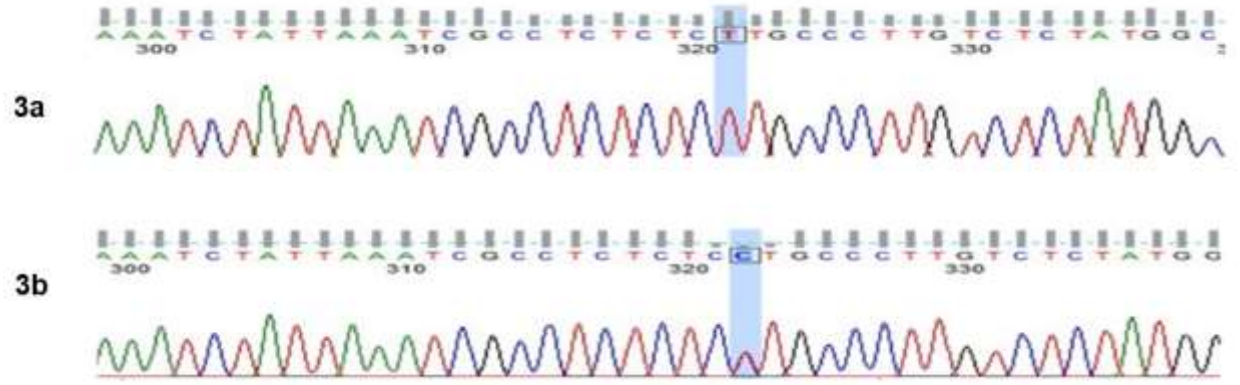

Figure 3: *CYP3A4*-rs2740574 (a). Homozygous-mutant-variant (b). Heterozygous variant

For *CYP3A4*-rs2242480, the most common genotype observed was CC (wild type), accounting for 53.8% of the sample. The heterozygous TC variant followed at 36.7%, while the homozygous mutant TT genotype was least frequent, with a prevalence of 9.4%. Regarding *CYP3A4*-rs2740574, the homozygous mutant TT was the predominant genotype, comprising 96.4% of the sample. The heterozygous CT variant was less prevalent, accounting for 3.6% while none of the individuals carried the wild genotype CC as illustrated in Table 3. It also depicts the MAFs for *CYP3A4*-rs2242480 and rs2740574 in our study sample which were observed to be 27.8% and 1.7%, respectively. Since each individual has two alleles, therefore, total number alleles were 446. These frequencies were found consistent with the Hardy-Weinberg equilibrium. The findings highlight distinct genotype distributions of *CYP3A4* variants among Pashtun epilepsy patients treated with carbamazepine, underscoring the expected genetic diversity in drug metabolism in this population.

Table 3: Genotype and allele frequency of *CYP3A4*

***CYP3A4* - rs2242480**			
Genotype	**Count**	**C allele**	**T allele**
CC	120	240	0
TC	82	82	82
TT	21	0	42
MAF			
C	332/446 (0.7219)		
T (MAF)	124/446 (0.278)		
***CYP3A4* - rs2740574**			
Genotype	**Count**	**C allele**	**T allele**
CC	0	0	0
TC	8	8	8
TT	215	0	430
MAF			
C (MAF)	8/446 (0.0179)		
T	438/446 (0.982)		

*MAF: Minor Allele Frequency

Association between distribution pattern of *CYP3A4* genotypes and gender:

Figure 4 provides a detailed breakdown of the genotype distribution of *CYP3A4* across different genders in the study population. In case of *CYP3A4*-rs2242480, the data revealed that out of 120 patients with the CC genotype, 35% were female and 65% were male. Among the 82 carriers of the heterozygous TC genotype, 36.5% were female and 63.4% were male. There were 21 carriers of the TT homozygous mutant genotype, with 47.6% being female and 52.3% male. Likewise, regarding *CYP3A4*-rs2740574, 8 patients carried the TC genotype, with an equal representation of 50% female and 50% male. Among the 215 cases of homozygous mutant TT genotype carriers, 36.2% were female and 63.7% were male.

The genotype-gender associations for *CYP3A4*-rs2242480 and rs2740574 were analyzed using statistical tests. Both the chi-square test ($p = 0.542$) for rs2242480 and Fisher's exact test ($p = 0.470$) for rs2740574 indicated no significant correlation between the distribution pattern of genotypes across various genders in the studied population.

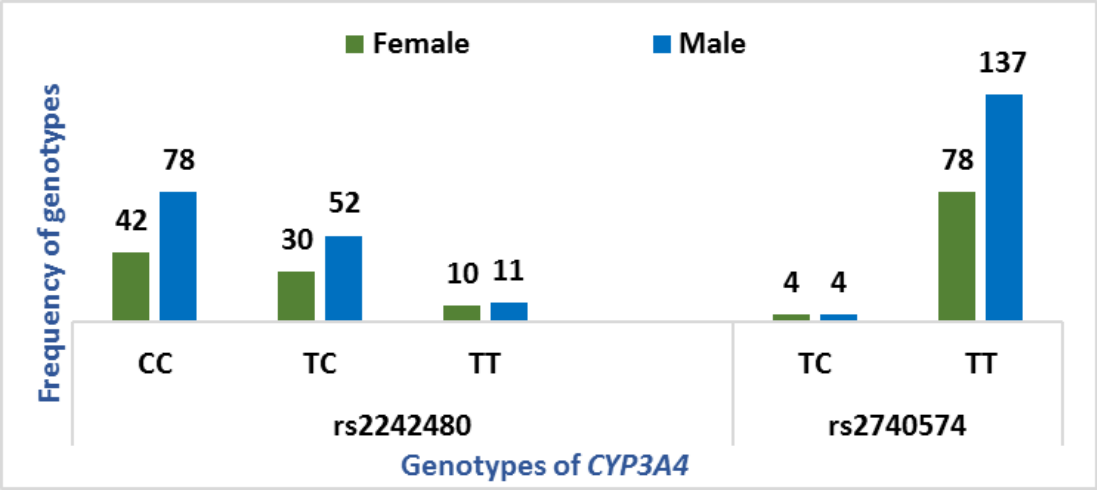

Figure 4: Gender-based distribution pattern of *CYP3A4* genotypes

Discussion

Precision medicine enhance the diagnosis and improve the treatment options by investigating how genetic differences affect individual responses to drugs (15). It is speculated that CYP3A4 is accountable for the metabolism of more than 50% of frequently administered drugs. The expression levels of the protein CYP3A4 in the liver may fluctuate up to 90-fold. These enzymes play a role in the metabolism of many pharmaceuticals, such as tacrolimus, sirolimus, methadone, and carbamazepine (18).

The minor allele frequency of *CYP3A4*-rs2242480 in our study sample was found to be 27.8%. In contrast, JS et al. reported a MAF of 21.9% for rs2242480 among Korean and African American populations, which is lower than what we observed. In a similar study, the MAF was reported to be comparable, with frequencies of 27.1% in the Han population and 26% in the Japanese population. However, frequency reported among European-Americans were found to be 8.3% (15), much lower than that reported in our study sample. Later, G. Qi et al. in 2021 reported MAF of *CYPRA4*-rs2242480 in Han population as 25.3%. In the same study, they reported MAF of 18.6% among Tibetan, 19.8% among Mongolian and 17.9% in Uyghur population, much lesser than reported in our sample (19). Xia B et al. reported the genotype frequencies of rs224280 in the Chinese population as 62.5% for CC, 34.2% for CT, and 3.2% for TT. In comparison to our sample, indicating a lower frequency of the CC genotype, a comparable frequency of the CT genotype, and a higher frequency of the TT genotype (20).

The MAF of *CYP3A4*-rs2740574 reported in our study sample of patients with epilepsy was calculated to be 1.7%. Olga et al., reported the MAF of the aforementioned gene as 4.0%, 0.5%, and 0.9% in the Russian, Tatar, and Bashkir populations, respectively (21). The MAF of *CYP3A4*-rs2740574 was reported only 4% among European population and 82% among Africans by Garsa et al (22). The genotypic and allelic frequency of *CYP3A4*-rs2740574 exhibit stark contrasts between the Malian and Pashtun populations. According to Kassogue et al., the CC genotype frequency was 57%, contrasting with its complete absence in our study sample, while CT was reported at 35%, significantly different from our sample's 3.6%. Conversely, our study found the TT

genotype to be 96.4%, whereas Kassogue et al. reported it as 7% (23). Espindola et al. in his study on Mexican children documented the highest frequency of TT genotype, but lower than that encountered in our sample population, followed by CT. A total of 3% of the patients were carriers of CC which was absent in our study sample. The observed allele frequencies were 90% for the T allele and 10% for the C allele (24). Rojo et al. found that in a Chilean population, the genotype frequencies were 88.9% for CC, 10.9% for CT, and 0.72% for TT, which are notably higher for CC and CT and significantly lower for TT compared to those reported in our study sample (25).

In gender-specific studies, Kassogue et al. reported a higher frequency of the CC variant of *CYP3A4*-rs2740574 among females, a genotype not encountered in our sample. The CT variant was found to be more prevalent among males in the Malian population, whereas our study sample showed an equal distribution between genders. The TT variant was reported at a similar frequency among both genders in the Malian population, but it was observed at a significantly higher frequency among males in our study sample (23). This discrepancy between findings highlights the importance of considering population-specific factors when evaluating pharmacogenetic data. Gender-related differences in CYP3A4 activity might be more pronounced in other ethnic or geographical groups, indicating the need for broader research to understand these variations fully.

Current study identified notable genetic differences in *CYP3A4* polymorphisms between the Pashtun population and other global groups. These genetic variations can influence carbamazepine metabolism, potentially affecting its efficacy and safety. As a result, standard dosing guidelines might not be appropriate for Pashtun patients. Therefore, large-scale population-based studies examining the association between these genetic variants and carbamazepine pharmacokinetics are needed to refine these strategies and enhance treatment precision.

Conclusion

The study investigated the genetic profile of the sample population for *CYP3A4*-rs2242480 & rs2740574. Our findings revealed striking differences from other ethnic groups offering novel insights into the distribution of genotypes related to carbamazepine-metabolizing enzymes in the Pashtun population. These findings will also advance pharmacogenetic research on other drugs metabolized by these genes, such as antiretrovirals, antimalarials, and anti-epileptics, thereby improving genotype-phenotype correlations. Additionally, no significant association was observed between genotype distribution patterns across genders.

Conflict of Interest: The authors declare no conflict of interest.

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CONTRIBUTION OF AUTHORS	
AUTHOR	**CONTRIBUTION**
Ayesha Jamil	ABC
Muhammad Saleh Faisal	BC
Niaz Ali	AC
Gulmakay Zaman	C
Rizwan Faisal	C
Syed Hasnain Ali Shah	C

KEY FOR CONTRIBUTION OF AUTHORS:

A. Conception/Study/Designing/Planning
B. Active Participation in Active Methodology
C. Interpretation/ Analysis and Discussion