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Original Article

The frequency of DPYD*2A and MTHFR C677T single nucleotide polymorphisms related to Capecitabine toxicity in Colorectal Cancer patients from Sulaymaniyah, Iraq

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ABSTRACT

Purpose

Colorectal cancer (CRC) is the second leading cause of cancer-related mortality worldwide. Capecitabine, an oral prodrug which primarily used in CRC chemotherapy. Variations in the enzymes dihydropyrimidine dehydrogenase *DPYD* (DPYD*2A) and methylenetetrahydrofolate reductase *MTHFR* (MTHFR C677T) genes can result in severe capecitabine-related toxicity in CRC patients. The main purpose of this study was to analyse the frequency of these two important polymorphisms in the Iraqi population.

Methods

Blood samples were collected from 170 individuals for MTHFR genotyping, and from 422 individuals for DPYD*2A genotyping, all from the Sulaymaniyah population in Iraq. Genomic DNA was isolated from whole blood, and the tetra-ARMS PCR assays were developed to detect MTHFR C677T and DPYD*2A variants.

Results

The genotype distribution for MTHFR C677T among the 170 participants was as follows: C*/C*: 52. 4%, C*/T*: 37%, and T*/T*: 10. 6%. The corresponding allele frequencies were C*: 70. 9% and T*: 29. 1%. In contrast, all 422 samples tested for DPYD*2A were homozygous wild genotype, with a mutant allele frequency of 0.0%. These findings were validated by DNA sequencing for a fraction of the samples.

Conclusion

The high prevalence of the MTHFR C677T polymorphism in the Sulaymaniyah population of Iraq, highlighting the necessity of its genetic screening before initiating capecitabine therapy in CRC patients. Although the DPYD*2A variant was not observed, pre-treatment genotyping remains advisable due to its potential clinical impact. Furthermore, the tetra-ARMS PCR represents a rapid and cost-effective method for SNP genotyping.

Keywords: Capecitabine, Colorectal Cancer, Tetra-ARMS PCR, DPYD, MTHFR, Sulaymaniyah, Iraq.

1 Introduction

Colorectal cancer (CRC) is the second most prevalent cancer in women and the third in men, and it represents the second leading cause of cancer-related deaths globally [1]. Each year, over 2 million people are diagnosed with CRC, and approximately one million die from the disease [2]. Treatment typically involves chemotherapy, radiation, and surgery, depending on the patient's circumstances [3, 4].

Capecitabine (Xeloda), an oral prodrug of 5-fluorouracil (5-FU), a fluoropyrimidine (FP) adjuvant, is widely used in the treatment of CRC [5]. Capecitabine offers comparable efficacy to intravenous 5-FU regimens while providing superior convenience and safety for patients [6]. The drug undergoes three phases of metabolism to become the active agent, 5-FU: First, carboxylesterase 1 and 2 (CES1/2) convert capecitabine into 5´-

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deoxy-5-fluorocytidine (5´-DFCR), which is then converted to 5´-deoxy-5-fluorouridine (5´-DFUR) by cytidine deaminase (CDA), and finally, thymidine phosphorylase (TP) converts 5´-DFUR into 5-FU. TP, expressed significantly in the tumor tissues, is essential for this conversion [7]. 5-FU functions as an antimetabolite, exerting cytotoxic effects through inhibition of thymidylate synthase (TS), a key enzyme involved in DNA synthesis. Additionally, it is incorporated into DNA and RNA as a pyrimidine analogue, disrupting their synthesis [8].

Despite its widespread use, the safety and efficacy of capecitabine therapy exhibit considerable inter-individual variability [9]. The observed variability may result from a combination of clinical and demographic determinants, such as underlying genetic differences [10]. Genetic variations impact the function of proteins necessary for capecitabine's pharmacokinetics (PK) and pharmacodynamics (PD) [11]. More than 50% of patients undergoing capecitabine treatment develop some form of toxicity [12]. About 10-30% of these patients experience severe, life-threatening adverse effects [13]. The most common side effects related to capecitabine chemotherapy are Hand-foot syndrome (HFS), neutropenia, diarrhea, nausea, abdominal pain, and stomatitis [14, 15]. Key genes involved in capecitabine metabolism include dihydropyrimidine dehydrogenase (DPYD), Methylenetetrahydrofolate reductase (MTHFR), Cytidine deaminase (CDA), Thymidylate synthase (TYMS), ATP-Binding Cassette Sub-Family B1 (ABCB1), and Enolase superfamily member 1 (ENOSF1) [16].

Dihydropyrimidine dehydrogenase (DPD) is the primary metabolizing enzyme responsible for the metabolism of 5-FU, with over 80% of the drug being catabolized by this enzyme in the liver [17, 18]. DPD is encoded by *DPYD*, an extensive gene on chromosome 1p22 that spans 950 kb and has 23 exons with a total of 4399 nucleotides [19]. Several single-nucleotide polymorphisms (SNPs) in DPYD, such as DPYD*2A, significantly affect the enzyme's activity, leading to severe capecitabine-related toxicity [20]. Consequently, in 2020, the European Medicines Agency (EMA) recommended that the DPYD activity should be assessed before administering 5-FU or capecitabine-based chemotherapy in cancer patients [21]. One of the most studied and clearly demonstrated DPYD variants linked to 5-FU and capecitabine-related toxicity is DPYD*2A (c.1905+1 G>A/ rs 3918290) [22]. DPYD*2A is an SNP at the splice donor location of intron 14, which causes exon 14 to be skipped during pre-messenger RNA (mRNA) splicing and results in loss of DPD activity [23].

MTHFR is a key enzyme in folate metabolism, regulating intracellular folate levels and influencing DNA synthesis and methylation [24]. It also plays a critical role in the metabolism of fluoropyrimidines into their active forms. consequently, MTHFR enzymatic activity is considered a significant predictor of clinical response to capecitabine and other fluoropyrimidine-based chemotherapies [25]. The *MTHFR* gene is located on the short arm of chromosome 1 at position 1p36.3. One of the most common SNPs in this gene is C677T (rs1801133), where a cytosine-to-thymine substitution at nucleotide position 677 (in exon 4) results in an alanine-to-valine amino acid change at codon 222 [26, 27]. This substitution reduces MTHFR enzymatic activity by approximately 30% in heterozygous individuals (CT) and by up to 70% in homozygous mutants (TT) [28].

With the incidence of CRC rising in low- and middle-income countries, like Iraq [29], improving treatment outcomes and minimizing chemotherapy-related toxicities, particularly those associated with capecitabine, has become increasingly important. Given the lack of data on the prevalence of DPYD*2A and MTHFR C677T SNPs within the Iraqi population. Therefore, the present study aimed to determine the frequency of these two clinically relevant SNPs and to develop a rapid and cost-effective tetra-ARMS PCR method for their genotyping.

2 Methods

2. 1 Sample collection

Peripheral blood samples (2 mL) were collected from 170 individuals for MTHFR C677T genotyping, comprising 120 CRC patients registered at the Hiwa Cancer Hospital and 50 healthy volunteers from the Sulaymaniyah community. Informed consent was obtained from all individual participants included in the study. For DPYD*2A genotyping, a total of 422 individuals were analyzed, including 175 CRC patients and 247 healthy volunteers. Written informed consent was obtained from participants before their inclusion. Blood samples were drawn in EDTA tubes and stored refrigerated for a few days before DNA extraction.

2. 2 DNA isolation

Genomic DNA was extracted from 200 µL of whole blood using the Easy Pure® genomic DNA purification kit (TransGen Biotech, China), which utilizes silica-membrane column technology. DNA concentration and purity were assessed using a nanodrop spectrophotometer (Thermo Fisher Scientific, USA).

2. 3 Genotyping

Genotyping was performed using the tetra-ARMS PCR method for both MTHFR C677T and DPYD*2A. Primers were designed using the NCBI primer blast tool [30], which were synthesized by Macrogen (Seoul, South Korea). The PCR reactions were carried out using a thermal cycler (Bio-Rad CFX Manager, USA). Each MTHFR C677T reaction had a total volume of 40 µL, containing 3 µL of each outer primer, 1.5 µL of inner forward, 1 µL of inner reverse, 11.5 µL of DNA, and 20 µL of Hot-Start Taq Master Mix (Add Bio, South Korea). The PCR cycling conditions were as follows: an initial denaturation step at 96 °C for 4 minutes; 30 cycles of denaturation at 95°C for 40 seconds, annealing at 66°C for 25 seconds, and extension at 72 °C for 45 seconds; followed by a final extension at 72°C for 7 minutes.

Table 1: Primer sequences were used for MTHFR C677T and DPYD*2A polymorphisms.

Mutation	Primer sequence	Tm (°c)	Size (bp)
MTHFR C677T (rs1801133)	Outer forward: 5′- ATCCGGTGCCTAGAGAAAAGTC -3′	61.4	700
	Outer reverse: 5′- TCTGGGAAGAACTCAGCGAAC -3′	62.2	
	Inner forward: 5′- AGAAGGTGTCTGCGGGAGT -3′	63.9	*246*
	Inner Reverse : 5′- GCTGCGTGATGATGAAATCGG -3′	60.7	*493*
DPYD*2A c.1905+1G>A (rs 3918290)	Outer forward: 5′- GAAGATATGCTGCTTCTGCCTC -3′	59.4	671
	Outer reverse: 5′- CTGCAAAAATGTGAGAAGGGAC -3′	58.9	
	Inner forward: 5′- CTCTTGTTTTAGATGTTAAATCACACTTAC -3′	55.2	*311*
	Inner Reverse : 5′- AGGCTGACTTTCCAGACAACT -3′	60.7	*410*

For DPYD*2A genotyping, each PCR tube had a total volume of 41 µL, comprising 2 µL of outer forward primer, 3 µL of outer reverse primer, 3 µL of inner forward primer, and 1 µL of inner reverse primer, 12 µL of genomic DNA, and 20 µL of Hot-Start Taq Mastermix (Add Bio, South Korea). The PCR cycling conditions were: an initial denaturation at 96 °C for 5 minutes; 30 cycles of denaturation at 95°C for 40 seconds, annealing at 64°C for 25 seconds, and extension at 72 °C for 40 seconds; with a final extension at 72°C for 4 minutes.

2. 4 Agarose gel electrophoresis

The Tetra-ARMS PCR products were separated on a 1.5% agarose gel (TransGen Biotech, China), stained with either safe dye (GENETBIO, South Korea) or ethidium bromide (Sigma-Aldrich, USA). Electrophoresis was conducted at 100 volts for 1 hour. Gels were visualized using a SCOPE 21 gel imaging system (OPTIMA, Japan).

2. 5 Sequencing

To validate the Tetra-ARMS results, 5 samples representing each MHFR C677T genotype and 42 randomly selected DPYD*2A samples were subjected to Sanger sequencing (Macrogen, South Korea). The target regions were amplified using outer primers, and the PCR products were assessed by agarose gel electrophoresis. The sequences were analyzed using Chromas software version 2.6.6 (Technilysum, Australia).

2. 6 Statistical analysis

Genotype and allelic frequencies were analyzed for Hardy-Weinberg equilibrium (HWE) using the Gene Calculators online tool (https://www.genecalculators.net/pq-chwe-check.html), based on the chi-square test ($p > 0.05$). Additionally, allele frequencies were compared with those reported in the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/snp/).

2. 7 Ethical approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the University of Charmo, Kurdistan region of Iraq (Approval No. 5, dated 10/09/2024).

3 Results

Tetra-ARMS PCR analysis of the MTHFR C677T polymorphism revealed three distinct genotypic patterns (Figure 1). The 700 bp bands in all lanes represent the product of common outer forward and reverse primers and indicate the validity of PCR reactions. In lanes 1, 8, and 9, the additional 493 bp band (wild-type allele C) indicates homozygous wild-type (C/C) genotypes. Lanes 2, 3, 4, 6, and 7 display 700 bp (common), 493 bp (wild-type C), and 246 bp (mutant T) bands corresponding to heterozygous (C/T) genotypes. Lanes 5 and 10 exhibit only the 700 bp and 246 bp bands, indicative of homozygous mutant (T/T) genotypes.

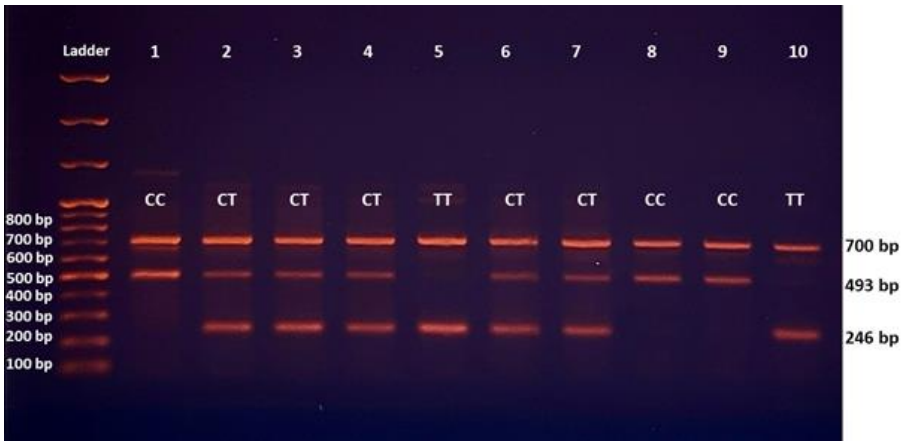

Figure 1: Agarose gel electrophoresis of MTHFR C677T (rs1801133). Ladder: 100 bp molecular weight marker; one subject is represented by each lane. Lanes 1, 8, and 9 are wild-type (C/C), lanes 2,3,4,6, and 7 are heterozygous (C/T), and lanes 5 and 10 are homo- mutant (T/T) subjects.

For DPYD*2A genotyping, as shown in Figure 2, all lanes display a 671 bp band (common band) and a 311 bp band (wild-type allele C), signifying homozygous wild-type (C/C) genotypes. No mutant alleles were detected in any of the samples analysed.

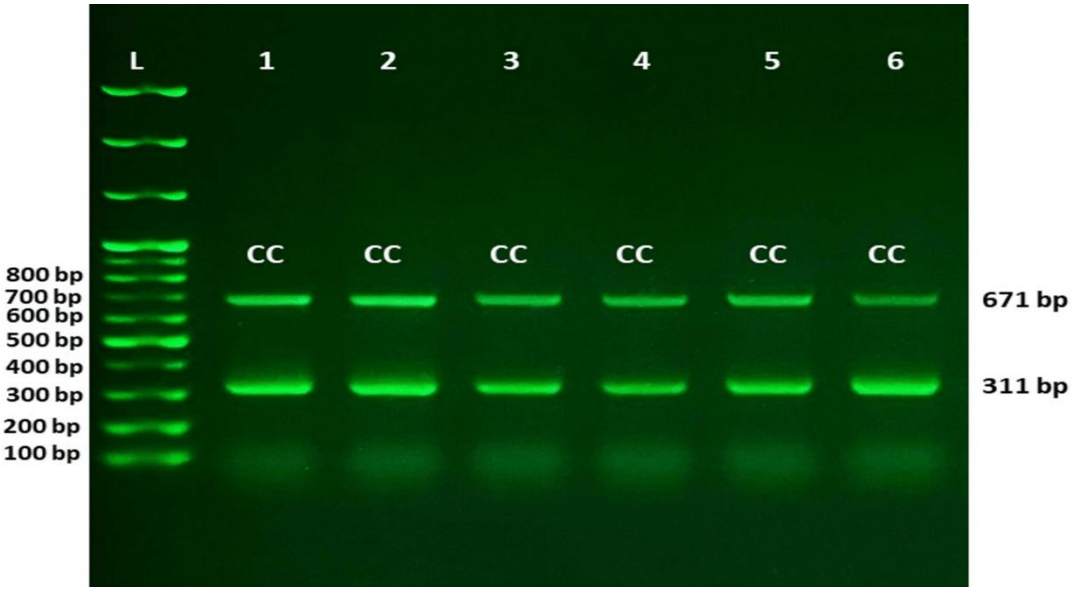

Figure 2: Agarose gel electrophoresis of DPYD*2A (rs3918290). Ladder: 100 bp molecular weight marker; each lane represents one subject. All lanes (1-6) are wild-type (C/C).

The Tetra-ARMS PCR results for MTHFR C677T and DPYD*2A genotyping were validated by direct sequencing of the PCR products and showed concordant results. The genotypic distribution and allele frequencies for MTHFR C677T among the 170 samples were as follows: 89 individuals (52.4%) were homozygous wild-type (C/C), 63 individuals (37%) were heterozygous (C/T), and 18 individuals (10.6%) were homozygous mutant (T/T). The allele frequencies were 70.9% for the C allele and 29.1% for the T allele, as shown in Table 2. In the case of DPYD*2A, all 422 analyzed samples were homozygous wild-type (C/C), with no mutant alleles detected. Consequently, the frequency of the mutant allele (A) in this study population was zero.

Table 2: Genotypic distribution and allele frequency of MTHFR C677T (rs 1801133) and DPYD*2A (rs 3918290).

variant	No.	Genotype frequency (%)			Allele frequency(%)		HWE
		C*/C*	***C*/T****	***T*/T****	**C***	**T***	
MTHFR C677T	170	89(52.4%)	63(37%)	18(10.6%)	241(70.9%)	99(29.1%)	1.776
		C*/C*	**C*/A***	**A*/A***	**C***	**A***	
DPYD*2A	422	**422(100%)**	**0.0(0.0%)**	**0.0(0.0%)**	**844(100%)**	**0.0(0.0%)**	0.00

HWE: Hardy-Weinberg equilibrium.

4 Discussion

A more individualized or precision-based approach is rapidly replacing the conventional treatment strategy for CRC. Molecular biomarkers have become increasingly significant for selecting targeted therapies [31]. Identifying Patients' genetic variations can assist in choosing the appropriate chemotherapy agent and dosage [32, 33]. Consequently, selecting the right medication based on an individual's genetic profile is a crucial first step toward personalized medicine. Since SNPs in genes related to drug metabolism increase the risk of adverse drug reactions (ADRs), early identification of these variants can help tailor treatment plans [34].

This study represents the first investigation into the genotype distribution and allele frequencies of MTHFR C677T and DPYD*2A polymorphisms within the Kurdish population of Iraq. Analysis of 170 individuals revealed that 52.4% were homozygous wild-type (C/C), 37% were heterozygous (C/T), and 10.6% were homozygous mutant (T/T) for the MTHFR C677T polymorphism. The frequency of the wild-type allele (C) was 70.9%, while the frequency of the mutant allele (T) was 29.1%.

Table 3: Compare the genotype distribution and allele frequency of the MTHFR C677T (rs1801133) variant obtained from this study with those reported in other populations. (C*: wild allele, T*: mutant allele).

Population	NO.	Genotype frequency (%)			Allele frequency (%)		Reference
		C/C**	*C*/T**	*T*/T**	C*	T*	
Kurdistan- Iraq	170	52.4%	37%	10.6%	70.9%	29.1%	This study
Iraq	50	70%	16%	14%	78%	22%	[35]
Iran	151	72%	21%	7%	83%	17%	[36]
Turkey	100	52%	38%	10%	71%	29%	[37]
Italy	113	35.4%	48.7%	15.9%	59.7%	40.3%	[38]
African	16554	78%	20%	2%	88%	12%	NCBI
Asian	3984	46%	41%	13%	66%	34%	NCBI
European	332384	43%	45%	12%	65%	35%	NCBI
Latin American	7238	30%	49%	21%	55%	45%	NCBI
Global	388968	44.5%	43.5%	12%	66%	34%	NCBI

For DPYD*2A genotyping, we initially tested samples from 170 CRC patients and healthy individuals from the Sulaymaniyah population, and found no mutant alleles; all tested individuals were wild-type. To ensure comprehensive analysis, we then genotyped an additional 55 CRC samples and 197 healthy volunteers from the same population. However, none of these 422 samples exhibited a mutant allele for DPYD*2A, indicating that this SNP is absent in the studied population.

Table 4: Compare the allele frequency of DPYD*2A, obtained in this study, with those reported in other populations. (C*: wild allele, T*: mutant allele)

Population	No.of samples	Allele frequency(%)		Reference
		C*	T*	
Kurdistan-Iraq	422	100%	0.0%	This study
Iran	83	100%	0.0%	[39]
Iran	121	99.2%	0.8%	[40]
Turkey	250	100%	0.0%	[41]
Turkey	200	99.25%	0.75%	[42]
Egypt	239	100%	0.0%	[43]
European	282358	99.5%	0.5%	NCBI
African	13264	99.9%	0.01%	NCBI
Asian	3982	100%	0.0%	NCBI
Latin America	3120	99.9%	0.01%	NCBI
Global	326508	99.6%	0.4%	NCBI

According to data available from the NCBI database, the global allele frequency of the MTHFR C677T variant is approximately 34%, as shown in Table 3. This value is relatively higher than the frequency observed in our study (29.1%). A study conducted in Iraq, involving a small sample size of only 50 healthy individuals, reported a 22% frequency of this SNP [35]. Additionally, a study in Iran (with 151 samples) reported a muatant allele frequency of 17% for MTHFR C677T [36], which is lower than our result, while a study in Turkey (with 100 samples) found an allele frequencies of 29%, which aligns with our findings [37]. The highest allele frequency for this SNP, as recorded in the NCBI, is 45%, observed in the Latin American population. Furthermore, a study involving 113 individuals from Italy reported a frequency of 40.3% for the MTHFR C677T variant [36]. The lowest reported frequency in the NCBI database is 12%, corresponding to the African population, while the allele frequency among Europeans is 35%, which is slightly higher than the frequency observed in the Kurdish population in this study.

Regarding DPYD*2A genotyping, all 422 samples in our study were wild-type, with no mutant alleles detected. As shown in Table 4, studies conducted in various populations and the NCBI database indicate that this variant is extremely rare. Two studies conducted in Iran with 83 and 123 samples reported frequencies of 0.0% and 0.8%, respectively, for this SNP [37, 38]. In Turkey, studies involving 250 and 200 individuals reported frequencies of 0.0% and 0.75%, respectively [39, 40]. Furthermore, a study conducted on 239 volunteers from the Egyptian population found a mutant allele frequency of 0.0% for DPYD*2A [41]. According to the NCBI database, the frequencies of this SNP in the European, African, and Asian populations are 0.5%, 0.01%, and 0.0%, respectively. The global mutant allele frequency for

DPYD*2A is reported as 0.4%. These align with our results, indicating that DPYD*2A is a rare SNP across most populations globally.

The Tetra-ARMS PCR method employed in this study offers a rapid, cost-effective, and reliable approach for genotyping SNPs such as MTHFR C677T and DPYD*2A. This technique allows for the simultaneous amplification of both alleles in a single PCR reaction, facilitating efficient genotype determination. Validation through Sanger sequencing confirmed the accuracy of the Tetra-ARMS PCR results.

Given the limited data on pharmacogenetic polymorphisms in the Iraqi Kurdish population, this study contributes valuable insights that may inform personalized treatment strategies. It contributes to the completion of the global genetic map, as these two polymorphisms are associated with various diseases beyond cancer pharmacokinetics.

5 Conclusions

The result of our study on the MTHFR C677T variant indicates that the frequency of this SNP in the Sulaymaniyah, Iraq population is relatively lower than the global frequency. However, the high prevalence of the mutant allele for this SNP underscores the necessity of molecular testing before starting capecitabine-based chemotherapy for cancer patients. Our findings on the DPYD*2A variant demonstrate that its frequency aligns with that observed in most other populations. Despite this, due to the severe adverse reactions associated with capecitabine in CRC patients, and in line with recommendations from the EMA, molecular testing for this variant remains critical before administering capecitabine-based chemotherapy. The tetra-ARMS PCR method developed in this study provides a rapid, cost-effective approach for genotyping these SNPs, offering a more efficient alternative to the previously used technique.

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