RESEARCH ARTICLE

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Pharmacogenetic Study of the Dihydropyridine Dehydrogenase Gene in Jordanian Patients with Colorectal Cancer

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Abstract

Introduction: Several studies have shown an association between 5-fluorouracil toxicity and variations in the dihydropyrimidine dehydrogenase (DPYD) gene. **Objectives:** This cross-sectional study aims to elucidate the association between genetic variations in the DPYD gene and 5-fluorouracil toxicity among Jordanians with colorectal cancer (CRC). **Methods:** 80 CRC Patients were recruited to screen for mutations in the DPYD gene using the Sanger sequencing technique. Sequencing results were analyzed using Mutation Surveyor software, and mutational effects were predicted by the Mutation Tester bioinformatics tool. **Results:** Three reported variants (c.85T>C, c.1740+40A>G, c.1740+39C>T) and one novel (g.97515583_97515584insA) variant were identified in this study. Results showed a significant association between these variants and toxicity to 5-Fluorouracil with P-values 0.002, 0.005, 0.019, 0.017, respectively. However, there was no significant association between variants and cancer free survival. **Conclusion:** The present study identified several variants in the DPYD gene among Jordanians with colorectal cancer, which are associated with toxicity to 5-Fluorouracil treatment.

Keywords: DPYD- Colorectal cancer- 5-Fluorouracil- Pharmacogenetics

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Introduction

Colorectal cancer is the second most common cause of cancer death resulting in 862000 deaths in 2018 (Bray et al., 2018). In Jordan, the second most prevalent type of cancer is colon cancer, with a prevalence of 11% of all registered cancer cases (Abdel-Razeq et al, 2015). Colorectal cancer which usually starts as noncancerous growth on the inner wall of colon and rectum is called polyps, then starts to be developed and metastasized (Cappell, 2005). Most of the colorectal cancer types (95%) are adenocarcinoma (Aparicio et al., 2014). The factors which cause colorectal cancer are genetic and environmental factors. Environmental factors like age, family history, obesity, meat-based diet and smoking. Genetic factors as tumor suppressor genes mutations, chromosomal instability, CpG island hypo or hypermethylation (Haggar and Boushey, 2009).

The treatment option for CRC depends on several factors including the stage of cancer, the side effects of treatment, and patient's health. This option includes surgery, radiation, chemotherapy such as 5-fluorouracil, and targeted therapy (Van Cutsem et al., 2014). 5-Fluorouracil is the standard chemotherapy to treat colorectal cancer which reduces relapse and prolongs survival rate (Sargent et al., 2009). 5-Fluorouracil is a pyrimidine analog that inhibits nucleoside metabolism. However, the response rate for 5-fluorouracil is only 10-15 % in advanced CRC patients and is accompanied by the increased toxicity (Pardini et al., 2011). Several factors that increase the systemic toxicity of 5-fluorouracil include age, sex and genetic variation in DPYD gene by decreasing elimination or increasing 5-fluorouracil activity in healthy tissue (Amstutz,et al., 2009).

The DPD protein encoded by the DPYD gene is the rate-limiting enzyme in uracil and thymidine catabolism. DPYD gene is located on chromosome 1, its cytogenetics address is (1p21.3) and it contains 23 exons spanning about 950 kb. There are different types of genetic variations in the DPYD gene, such as exon skipping, insertion-deletion, frame shift, and missense mutations (Yamaguchi et al., 2001). Several studies have highlighted the association between variations in DPYD gene and sever 5-fluorouracil related toxicity. Some individuals died from 5-fluorouracil treatment due to severe toxicity. It is significantly correlated with low DPD activity in liver (Maring et al., 2002)

The most common mutation in DPYD gene is a splice site mutation, which leads to skipping of 165bp of exon 14 (DPYD*2A) causing DPYD deficiency, a rare disorder inherited in an autosomal recessive manner (Saif, et al., 2007). The frequency of DPYD variant in general population is ~0.1-1% (Deenen et al., 2016). People with this deficiency have a susceptibility of developing life-

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threatening toxic reaction when treated with 5-fluorouracil because it doesn't break down efficiently and build up toxic molecules (fluoropyrimidine) which lead to severe inflammation, hemorrhage, neutropenia, and hand-foot syndrome (Diaz et al., 2004).

Other mutations that showed an association with 5-fluorouracil toxicity was (1236G>A) in Swiss population (Amstutz et al, 2009), (DPYD*9B) and (DPYD*7) in British population (Loganayagam et al., 2010), missense mutation at uracil binding site (1845G > T) in Portuguese population (Salgueiro et al., 2004). In contrary, a study on Korean patients with colorectal cancer showed no link between DPYD variants and 5-fluorouracil toxicity (Cho et al, 2007).

For this reason, identification of the relationship between polymorphisms in the DPYD gene and 5-fluorouracil toxicity among Jordanian patients with colorectal cancer is important and useful to predict the efficacy of treatment and decrease adverse drug reaction. The goal of this study was to genotype the common and novel single nucleotide polymorphisms (SNPs) in the DPYD gene to determine the association between these SNPs and toxicity resulting from 5-fluorouracil chemotherapy and to determine the effect of these SNPs on cancer-free survival.

Materials and Methods

The study subjects

Eighty colorectal cancer patients were recruited during the period between January 2016 and February 2017 from King Abdulla University Hospital. From each patient 5ml of blood were collected. The study was approved in advance by the Institution Review Board at King Abdulla University Hospital and informed written consents were obtained from all participants.

Genomic DNA extraction

Genomic DNA was extracted from blood samples using the QIAamp DNA Kit (Qiagen, USA) according to the manufacturer's instructions. The quality and quantity of the extracted DNA were determined by agarose gel electrophoresis and DNA concentration was determined using NanoDrop 2000 (Thermo Scientific, USA). Samples were stored at -80°C, until used.

Polymerase chain reaction (PCR) procedure

Four exons of DPYD gene with hotspot mutations as well as exon-intron boundaries were amplified using Polymerase Chain Reaction (PCR). The primers were designed using the Primer-BLAST. Amplification of target sequences was done using conventional PCR (veriti™ Thermal Cycler from Applied Biosystems). PCR was carried out in a reaction volume of 25µl containing 12.5µl 2X commercial Master Mix that contains (0.05U/µl Taq DNA polymerase, reaction buffer, 4mM MgCl2, 0.4mM of each dNTP). Then, 2 µl of each forward and reverse primer (10uM) were added. Finally, 6.5 µl of nuclease free water was added to bring the final total volume to 25µls. The primers list and sequences for each SNP are shown in (Supplementary, Table S1).

Gel electrophoresis

Five microliters from each PCR product were loaded onto 2% agarose gel. In addition, 5 µL of 100 bp DNA ladder (Promega, USA) were loaded onto the same gel. The electrophoresis was run at 150 volts for 45 minutes. The running TBE buffer was diluted to 1X and the bands were visualized under UV light in Gel Doc™XR system (Bio-Rad, USA) using Red Safe stain (iNtRON, South Korea). The PCR product size was determined by comparing it to known bands of the DNA ladder.

PCR Product purification

PCR products were purified from impurities like nucleotides using MEGAquick-spin plus Fragment DNA Purification Kit (iNtRON, South Korea) according to the manufacturer's instructions. Briefly, 20µl of PCR product was added to 100µl of a binding buffer. After that, 750µl of wash buffer was added to remove impurities. Lastly,20µl of elution buffer was added to elute the DNA. DNA concentration was measured using NanoDrop. The final concentration of purified DNA was within 15-30 ng/ul.

Cycler sequencing and cleaning

Sequencing of common and novel SNPs in the DPYD gene was carried out on an ABI Prism 3130/3130xL in the Genomics Sequencing Laboratory at Princess Haya Biotechnology Center. The DNA sequencing was carried out on all PCR-amplified fragments representing the different gene exons. All SNPs were sequenced by reverse or forward primers. The reaction mixture was consisting of 4 µLs of 5X sequencing buffer, 5 µL of nuclease-free water, and 1 µL of Ready Reaction Mix, 2 µL of the forward primer of the same concentration as that used in regular PCR (10uM), 4 µLs of purified DNA. The sequencing reaction was performed under the following conditions for a total of 25 cycles; an initial step 96°C for 10 seconds followed by 60°C for 4 minutes. The excess (Dye DeoxyTM) terminator was removed from the DNA sequencing reaction using ZR DNA Sequencing Clean-Up Kit (ZymoResearch, USA). Finally, the cleaned samples were loaded on an ABI 3130/3130xL genetic analyzer (Applied Biosystems, USA), after denaturing at 96°C for 5 minutes and placing the samples on ice for 10 minutes. The sequencing products were separated by capillary electrophoresis equipped with a laser beam used to detect fluorescent molecules.

Sequencing data analysis

Sequence analysis were carried out using Mutation Surveyor software available at (https://softgenetics.com/requestTrial.php).

Bioinformatics and Computational Analysis of Variants

Several bioinformatics tools were used in the study. For example, primer design was carried out using Primer-BLAST online software(https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and the presence of SNPs in primer binding sites was checked by SNPCheck (https://genetools.org/SNPCheck/snpcheck.htm). The pathogenicity for each variant was predicted by mutation taster online software (http://www.mutationtaster.org/).

Moreover, PolyPhen-2 software was used for prediction of variant effect on the protein (http://provean.jcvi.org/index.php),(genetics.bwh.harvard.edu/pph2). Each novel variant was mapped and named based on an integrated genome viewer (http://software.broadinstitute.org/software/igv/) according to the Human Genome Variation Society Guidelines (http://www.hgvs.org/mutnomen).

Statistical analysis

SPSS software was used for statistical analysis. Particularly, the Chi-square and Kaplan-Meier tests were used. Statistical analysis with a p-value less than 0.05 was considered statistically significant.

Results

Characteristics of the study participants

The Eighty Jordanian patients with colorectal cancer were recruited to study the relationship between toxicity to 5-fluorouracil and genetic variation on DPYD gene. The 80 CRC patients were 42 (52.5%) males and 38 (47.5%) females. Age mean for males was 49.9±2.0 and 47.9±1.9 years for females.

Several parameters were examined in the study group. For instance, toxicity to 5-fluorouracil, dose limiting toxicity to 5-fluorouracil and survival period after diagnosis with CRC. Out of 80 CRC patients 44

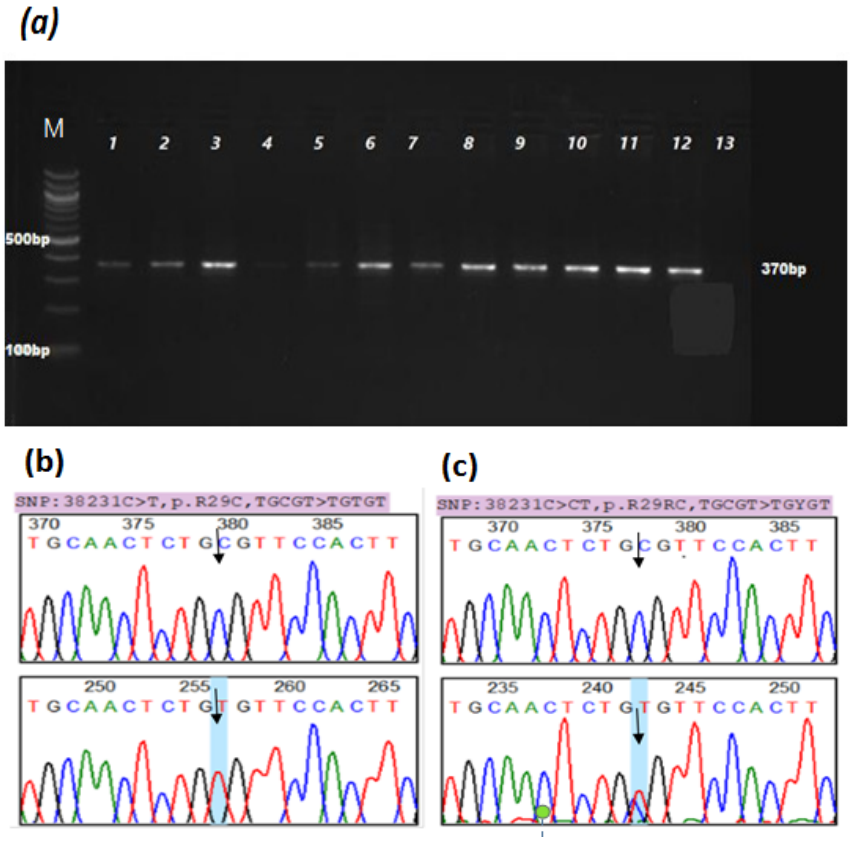

Figure 1. Mutational Analysis in Exon 2 of the DPYD Gene. (a) 2% agarose gel electrophoresis of PCR amplified exon 2 (370 bp). Lanes from 1-12 are representative CRC patient's samples, lane 13 represents negative control and lane M represents 100 bp DNA ladder. (b) Representative partial chromatogram for exon 2 showing rs1801265 (c.85T>C) variant. Wild type sequence (TGCGT)in the upper inset and homozygous variant sequence (TGTGT) in the lower in set. (c) Representative partial chromatogram for exon 2 showing wild type sequence (TGCGT) in the upper inset and heterozygous (c.85T>C) variant sequence (TGYGT) in the lower inset.

Table 1. Genotype and Allele Frequency of DPYD Mutations Found in Jordanians Patient With CRC (N= 80). wt, Wild type; vt, variant.

Exon/Intron Number	Identified Variant	Nucleotide (wt/vt)	Genotype Frequency (wt/wt%) (wt/vt%) (vt/vt%)	Allele Frequency (wt: vt) N (%)
2	c.85T>C	C: T	16(20.0) 18(22.5) 46(57.5)	C: 50 (31.3) T: 110 (68.7)
14	-	-	-	-
22	-	-	-	-
13	c.1740+40A>G	A: G	18 (22.5) 30 (37.5) 32 (40.0)	A: 66 (41.3) G: 94 (58.7)
	c.1740+39C>T	C: T	62 (77.5) 15 (18.8) 3 (3.8)	C: 139(86.9) T: 21(13.1)
	g.97515583_97515584insA	NoinsA: insA	70 (87.5) 10 (12.5) 0 (0)	NoinsA: 150 (93.7) insA:10(6.3)

Table 2. The Association between rs1801265 (c.85T>C) and Toxicity to 5-fluorouracil (N=80)

			rs1801265 (c.85T>C)			Total	p-value
			CC	CT	TT		
Toxicity	Yes	Count	5.0	14.0	35.0	54.0	0.002
		% of Total	6.3	17.5	43.8	67.5	
	No	Count	11.0	4.0	11.0	26.0	
		% of Total	13.8	5.0	13.8	32.5	
Total		Count	16.0	18.0	46.0	80.0	
		% of Total	20	22.5	57.5	100.0	

P-value< 0.05; Odd Ratio, 0.128 and 95% Confidence Interval: 0.035-0.465

(55.0%) had toxicity after treatment with 5-fluorouracil. Dose limiting toxicity to 5-fluorouracil included several symptoms with different frequency, for instance hypertension, hemorrhage, neutropenia, and neurotoxicity (Supplementary, Table S2). Survival period from one month to more than one year (Supplementary, Table S3).

Mutational analysis

Four exons in the DPYD gene were amplified in 320 reactions using specific forward and reverse primers that cover the four exons and exon-intron boundaries. Four variants were found among Jordanian patients with CRC, three of them were reported previously (rs1801265, and rs2811178, rs2786783) and one novel variant (g.97515583_97515584insA). The most common germline variant identified in this study was rs1801265, found in 64 (80.0%) subjects, followed by rs2811178 found in 62 (77.5%) (Table 1).

Exon 4 and 22 were amplified in 80 subjects with CRC to examine the rs67376798 and rs3918290; respectively. PCR technique was used and yielded 499 bp, 392 bp; respectively. PCR products were purified using the iNtRON Research PCR purification kit (iNtron, South Korea). Purified DNA was amplified by cycle sequencing using forward or reverse primer. Finally, sequencing products were cleaned using the Zymo Research PCR purification kit (Zymo Research, South Korea). To our surprise, none of the above-mentioned SNPs was found in the study subjects. However, one reported variant has been found in Exon 2 of the DPYD

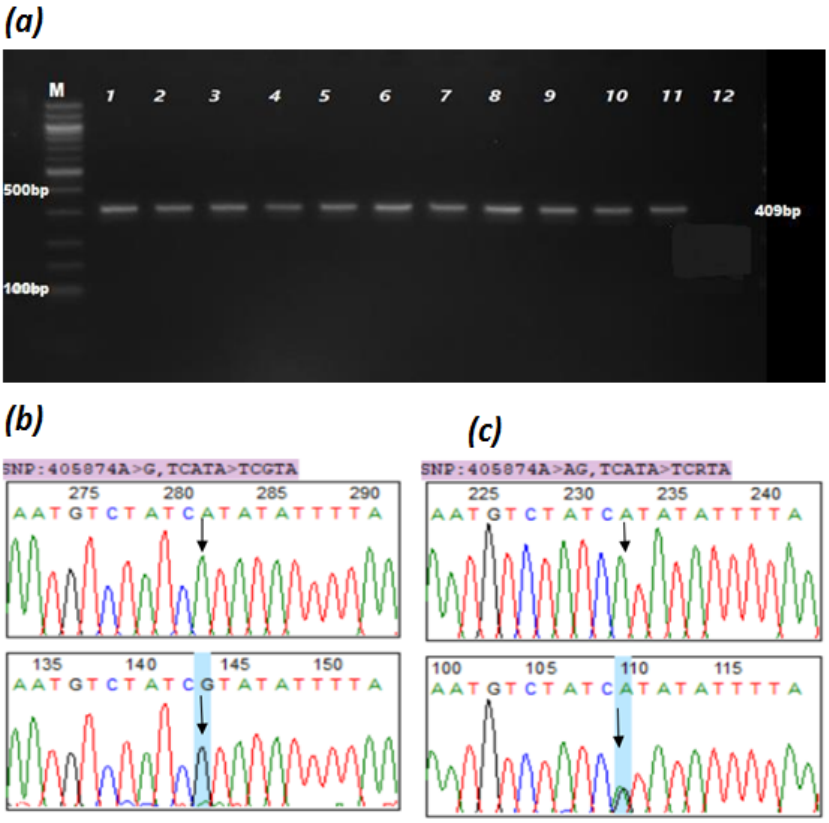

Figure 2. Mutational Analysis in Exon 13, Intron 13 and Exon-Intron Boundaries of the DPYD Gene. (a) 2% agarose gel electrophoresis of PCR-amplified product (409 bp). Lanes from 1-11 are representative CRC patient's samples, lane 12 represents negative control and lane M represents 100 bp DNA ladder. (b) Representative partial chromatogram showing rs2811178(c.1740+40A>G) variant. Wild type sequence (TCATA) in the upper inset and homozygous variant sequence (TCGTA) in the lower inset. (c) Representative partial chromatogram showing wild type sequence (TCATA) in the upper inset and heterozygous (c.85T>C) variant sequence (TCRTA) in the low inset.

Table 3. The Association between rs2811178 (c.1740+40A>G) and Toxicity to 5-fluorouracil (N=80)

			rs2811178(c.1740+40A>G)			Total	p- value
			AA	AG	GG		
Toxicity	Yes	Count	4.0	21.0	18.0	44	0.005
		% of Total	5.0	26.3	23.8	57.3	
	No	Count	14.0	9..0	13.0	36	
		% of Total	17.5	11.3	16.3	45	
Total		Count	18.0	30.0	32.0	80	
		% of Total	22.5	37.5	40.0	100	

P-value< 0.05; Odd Ratio, 0.157 and 95% Confidence Interval: 0.046-0.536

gene which is rs1801265 (c.85T>C). c.85T>C, found in 46 (57.5%) patients as a homozygous pattern and in 18 (22.5%) patients as a heterozygous pattern (Table 1 and Figure 1). c.85T>C is a missense mutation that lead to substitution of Cysteine with Arginine (Cys29Arg). This might affects protein features (Supplementary, Table S5). A significant relation was found between c.85T>C and 5-fluorouracil toxicity (p=0.002) (Table 2). However, no association was found between c.85T>C and cancer free survival (p-value= 0.432) (Figure 5). In addition, the most dose-limiting toxicity symptom to 5-fluorouracil was neutropenia observed in 32(51.6%) subjects (Supplementary, Table S4).

Moreover, Exon 13, intron 13 and exon-intron boundaries of DPYD gene (409 bp amplicon) were sequenced using Sanger sequencing technique. Three variants have been observed, two of them were reported previously and one is novel. Rs2811178 (c.1740+40A>G), located in intron 13 was observed in 30 (37.5%) patients with colorectal cancer as heterozygous, and in 32 (40.0%) as homozygous (Table 1 and Figure 2). Statistical analysis showed a significant association between rs2811178 variant and 5-fluorouracil toxicity (p = 0.005) (Table 3). Rs2811178 altered DNA sequence that may be disease causing as predicted by Mutation Taster software (Supplementary, Table S5). There was no significant association between rs2811178 variant and cancer-free survival (p=0.577) (Figure 5). In addition, the most common dose-limiting toxicity symptom to 5-fluorouracil was neutropenia, observed in 27 (54.0%)

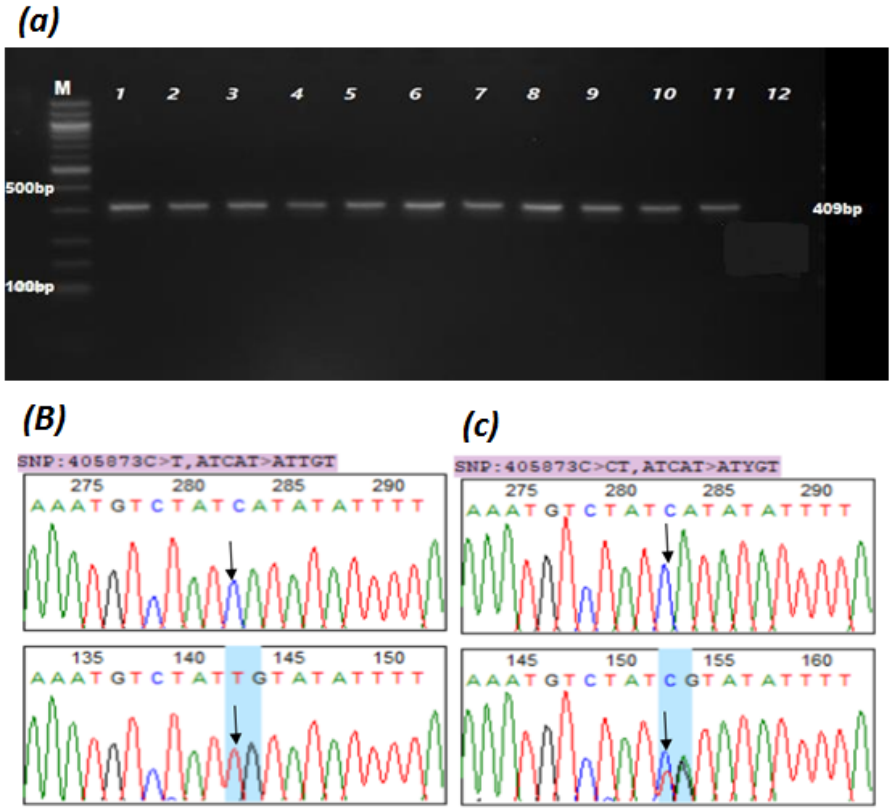

Figure 3. Mutational Analysis in Exon 13, Intron 13 and Exon-Intron Boundaries of the DPYD Gene. (a) 2% agarose gel electrophoresis of PCR amplified product (409 bp). Lanes from 1-11 are representative CRC patient's samples, lane 12 represents negative control and lane M represents 100 bp DNA ladder. (b) Representative partial chromatogram showing rs2786783 (c.1740+39C>T) variant. Wild type sequence (ATCAT) in the upper and homozygous variant sequence (ATTGT) in the lower. (c) Representative partial chromatogram showing wild type sequence (ATCAT) in the upper inset and heterozygous (c.85T>C) variant sequence (ATYGT) in the lower inset.

Table 4. The Association between rs2786783 (c.1740+39C>T) and Toxicity to 5-fluorouracil (N=80)

			rs2786783 (c.1740+39C>T)			Total	p- value
			CC	CT	TT		
Toxicity	Yes	Count	29.0	13.0	2.0	44	0.019
		% of Total	36.3	16.3	2.5	55	
	No	Count	33.0	2.0	1.0	32	
		% of Total	41.3	2.5	1.3	47	
Total		Count	62.0	15.0	3.0	80	
		% of Total	77.5	18.8	3.8	100	

P-value< 0.05; Odd Ratio, 0.157 and 95% Confidence Interval: 0.046-0.53

subjects (Supplementary, Table S4).

Rs2786783 (c.1740+39C>T), an intron variant, was found as a heterozygous in 15 patients (18.8%) and in 3 (3.8 %) as homozygous (Table 1 and Figure 3). A significant association was found between c.1740+39C> and 5-fluorouracil toxicity (p = 0.019) (Table 4). However, no significant association was found between rs2786783 and cancer-free survival (p=0.849) (Figure 5). In addition, neutropenia was the most common dose-limiting toxicity symptom to 5-fluorouracil observed in 9 (69.2%) subjects (Supplementary, Table S4).

A novel insertion variant (g.97515583_97515584insA)

Table 5. The Association between g.97515583_97515584insA and Toxicity to 5-fluorouracil (N=80)

			g.97515583_97515584insA		Total	p-value
			No insertion	Insertion A		
Toxicity	Yes	Count	35.0	9.0	44	0.017
		% of Total	43.8	11.3	55	
	No	Count	35.0	1.0	36	
		% of Total	43.8	1.3	45	
Total		Count	70.0	10.0	80	
		% of Total	87.5	12.5	100	

P-value< 0.05; Odd Ratio, 0.157 and 95% Confidence Interval: 0.046-0.53

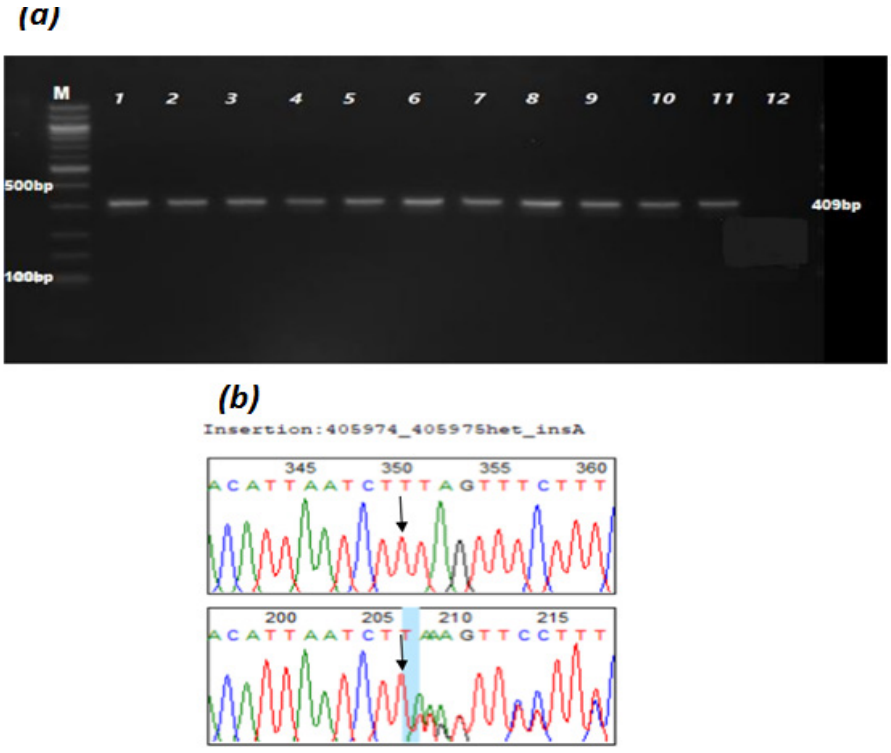

Figure 4. Mutational Analysis in Exon 13, Intron 13 and Exon-Intron Boundaries of the DPYD Gene. (a) 2% agarose gel electrophoresis of PCR amplified product (409 bp). Lanes from 1-11 are representative CRC patient's samples, lane 12 represents negative control and lane M represents 100 bp DNA ladder. (b) Representative partial chromatogram showing (g.97515583_97515584insA) variant. Wild type sequence (TCTTT) in the upper inset and insertion variant sequence (TCTTA) in the lower in set.

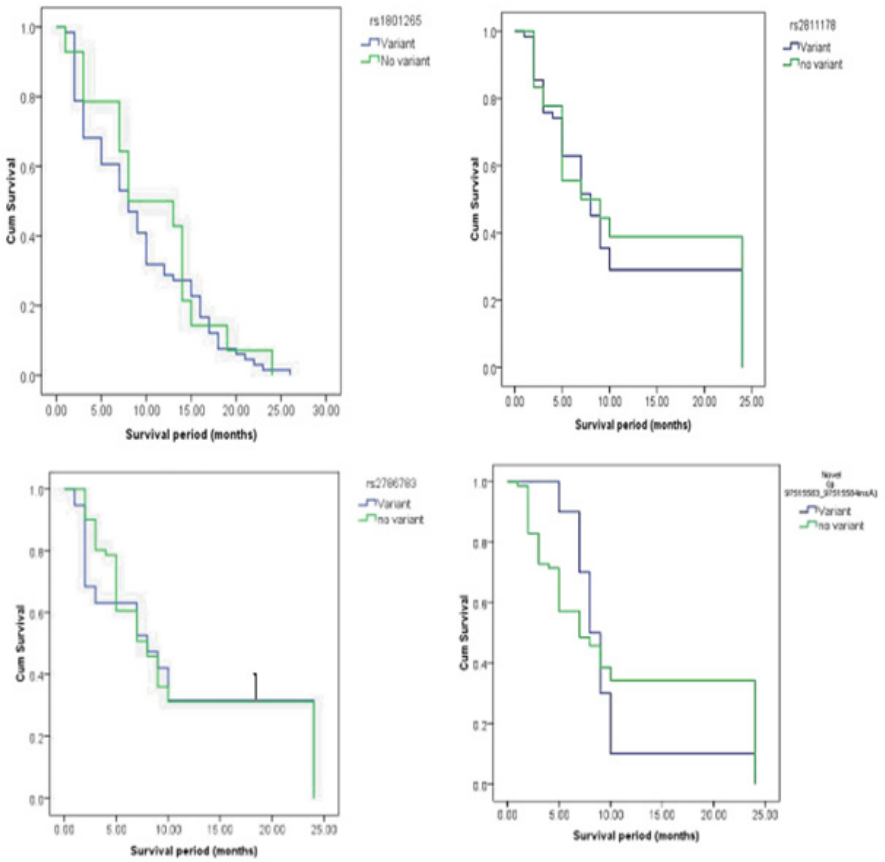

Figure 5. Kaplan-Meier Plots Demonstrating the Correlation DPYD Variants and the Survival Period in CRC Patients.

was identified in 10 (12.5%) Jordanians patients with CRC, this variant result from an insertion of adenine at position 97515584 (Table 1 and Figure 4), which might chang amino acid sequence (frameshift variant) and this may affect protein features (Supplementary, Table S5). A significant association was found between the (g.97515583_97515584insA) variant and 5-fluorouracil toxicity (p= 0.017) (Table 5). However, no association was observed with cancer-free survival (p=0.550) (Figure 5). Moreover, neutropenia was the most common dose-limiting toxicity symptom to 5-fluorouracil which observe in 8(72.2%) (Supplementary, Table S4).

Discussion

Variants in the DPYD gene have a strong impact on the catabolism of pyrimidine nucleotides and 5-FU chemotherapy agent (Yen and McLeod, 2007). Therefore, these variants should be reported and examined so it can be used as a useful tool to predict 5-FU treatment outcomes and prophesy patients who might show toxicity to 5-FU therapy.

In this study, we were interested in studying four variants in DPYD gene c.1905+1G> C, c.2846A>T, c.1679T>G and c.61C>T. Unfortunately, none of them were found because they have low frequency (<0.01). The number of participating patients (80) was the limiting factor and can explain the absence of these rare variants. Despite this, we found four other variants, three of them were reported previously; c.85T>C, c.1740+40A>G, and c.1740+39C>T and one novel variant g.97515583_97515584insA. This in contrast to what has been found in the Portuguese population, in which c.1905+1G> C is the most common variant associated with 5- FU toxicity with a prevalence of 2.7% (Salgueiro et al., 2004) , but it is rare in Japanese, British, Dutch and Afro-American patients (Wei et al, 1996). In contrary, an Italian study which included the non-synonymous variant c.2846A> T, showed a strong association with partial or complete loss of enzymatic activity of DPD and severe toxicity to 5-FU (Del Re et al., 2019).

The c.85T>C missense mutation that lead to substitution of Cysteine with Arginine (Cys29Arg), reduces dihydropyridine dehydrogenase activity (Moh'd Khushman et al., 2018). It has been found in the Japanese, Taiwanese and Korean patients with allele frequencies of 3.7%, 2.2%, and 2.5%; respectively (Maekawa et al., 2007; Ruzzo et al., 2017). In contrast, some studies suggested that 85T>C mutation is a common polymorphism and is not associated with 5-Fu toxicity (Collie-Duguid et al, 2000; Seck et al., 2005). However, our study showed a strong association between c.85T>C mutation and 5-Fu toxicity.

Also, our results showed that neutropenia is the most dose-limiting toxicity symptom to 5-fluorouracil. This is in agreement with previous studies, which suggest that 5-FU is responsible for severe gastrointestinal toxicity, increased risk of grade IV neutropenia in addition to increasing mortality rates in patients who have a partial or complete DPD deficiency (Mugada et al, 2017 ;Van

Kuilenburg et al, 2002). Previous studies showed that the rs72549310 (c.61C>T) variant in exon 2, which changes amino acid sequence from Arginine to a stop codon leads to production of nonfunctional protein. In addition, individuals with c.61C>T mutation had DPD deficiency which is associated with severe 5-Fu toxicity (Offer et al., 2014).

The novel variant g.97515583_97515584insA in intron 13 was found as heterozygous in 10 (12.5%) patients. It is a frameshift, which may affect protein. Moreover, two variants were reported in exon 13; rs55886062 (c.1679T>A) and rs1057516968 (c.1681C>T). Rs55886062 changes the amino acid sequence at 560 positions from Isoleucine to Asparagine which leads to reduced DPD activity. While rs1057516968 is a stop gain mutation that result in premature termination of translation which produces nonfunctional DPD protein (Kuilenburg et al., 2016). Previous studies showed a significant association between c.1679T>A, c.1681C>T variants and sever 5fu toxicity (Grade ≥3 toxicity) (Meulendijks et al., 2015; van Kuilenburg et al., 2016).

None of the identified variants was associated with cancer-free survival. This is in agreement with studies that showed DPD was not a good predictor for survival (Kornmann et al., 2002 ; Ikeguchi et al, 2002).However, other studies in CRC patients found that patients with low DPD levels tended to survive longer than patients with high DPD level (Kornmann et al., 2003).

In Conclusion; variations in the DPYD gene are prevalent among Jordanian colorectal cancer patients. Particularly c.85T>C and c.1740+40A> G, which are significantly associated with 5-Fluorouracil toxicity. Furthermore, neutropenia was the most common dose-limiting toxicity symptom and is associated with the four identified variants. More functional studies are required to determine the impact of these variants on DPD activity. These variants are potentially useful predictive markers of patients' responses to 5-FU chemotherapy in Jordanian patients.

Author Contribution Statement

Nusaiba A. Almashagbah: Data collection; Methodology; Investigation; critical review of the manuscript. Amjad Mahasneh: Conceptualization; Investigation; Supervision; Project administration; critical review of the manuscript. Khaldon G. Bodoor; Investigation; supervision, critical review of the manuscript.

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Scientific Approval

This paper is part of an approved Master thesis for Nusaiba A. Almashagbah who was a graduate student and obtained her degree in 2019.

Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest

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