Role of Dihydropyrimidine Dehydrogenase Genetic Polymorphism and Vitamin D Assay for 5-Fluorouracil Therapy in Upper Egypt Colorectal Cancer Patients

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Abstract

Background: 5-Fluorouracil (5-FU) is a cornerstone of advanced colorectal cancer (CRC) treatment. Pharmacogenomics variations in DNA may explain why 5-FU is safe for certain people while negatively affecting others. Examination of dihydropyrimidine dehydrogenase (DPYD) allelic variations would aid in improving dose adjustment. The impact of vitamin D on survival has led to the hypothesis that it lowers cancer mortality.

Objectives: This work aimed to assess the correlation between 5-FU response and toxicities and their association with DPYD genetic polymorphism and vitamin D levels in CRC-patients.

Patients and method: A prospective cohort study of 60 CRC patients who received 5-FU for 6 months was performed. Patient follow-up to detect clinical adverse effects was done. CBC and liver enzymes were measured before and after treatment. A genotyping study using real-time polymerase chain reaction (PCR) to detect the DPYD*2A (IVS14+1G>A) (rs 3918290) variant was performed. Vitamin D (25(OH)D) serum levels were estimated.

Results: There was a significant increase in chest pain, nausea, febrile neutropenia, and bruising among the 5-FU non-responders compared to the responders. The study showed that hemoglobin, WBCs, neutrophils, and platelet counts were significantly higher among 5-FU responders than non-responders. The decrease in blood parameters was higher 6 months post-treatment compared to pre-treatment in responder and non-responder patients. ALT and bilirubin levels were significantly lower among responders than non-responders. The genotyping study indicated the absence of the DPYD*2A variant among the study cases. There was a higher level of vitamin D among 5-FU responders than the non-responders (31.83 ± 4.35, 11.62 ± 4.68 ng/ml) respectively.

Conclusion: 5-FU efficacy and toxicity were unrelated to DPYD genetic polymorphisms among the study patients. However, there was a strong correlation between vitamin D level and 5-FU efficacy.

Keywords: Colorectal cancer; Dihydropyrimidine dehydrogenase; 5-fluorouracil; Genetic polymorphism; Vitamin D.

DOI: 10.21608/SVUIJM.2024.308049.1949

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Received: 14 August,2024.
Revised: 17 September,2024.
Accepted: 18 September,2024.
Published: 20 December, 2024

Cite this article as: Mohamed M. Elbadr, Mirna M. Mahrous, Mohamed Alorabi, Hala M. Elbadre, Rehab Mohamed , Reham A.M.Ellisy.(2024). Role of Dihydropyrimidine Dehydrogenase Genetic Polymorphism and Vitamin D Assay for 5-Fluorouracil Therapy in Upper Egypt Colorectal Cancer Patients. *SVU-International Journal of Medical Sciences*. Vol.7, Issue 2, pp: 917-935.

Introduction

Fluoropyrimidines (FP) such as 5-fluorouracil (5-FU) and capecitabine are antimetabolite anticancer drugs commonly used to treat breast, colorectal, and other gastrointestinal tract cancers **(García-Alfonso et al., 2022).** More than 80% of the active 5-FU is metabolized in the liver. The most important enzyme in its metabolism is dihydropyrimidine dehydrogenase (DPYD), which encoded with the DPYD gene **(Ockeloen et al., 2023).** FP class is now a milestone in CRC treatment. In early and metastatic cases, 5-FU and its prodrug, capecitabine, remain the chemotherapy backbone of CRC treatment.

5-FU and capecitabine can cause gastrointestinal and hematological toxicities, that limit their use **(Bruera & Ricevuto, 2020).** 5-FU has a narrow therapeutic index and can cause severe life-threatening toxicity in 10–26% of patients **(Sharma et al., 2019).** Chest pain, angina, arrhythmias, heart failure, acute coronary syndrome, and myocardial infarction are the most frequent cardiovascular adverse effects caused by these drugs **(White et al., 2021).**

FP can cause hematological toxicity such as leukopenia, anemia, and thrombocytopenia and can lead to hospitalization **(Wintner et al., 2020).** Gastrointestinal toxicity 5-FU is also a common problem **(Sims et al., 2020).** Diarrhea, nausea, stomatitis, vomiting, and mucositis are the most common manifestations. Diarrhea may occur in up to 50 % of patients using this drug **(Universitet et al., 2022).**

Patients carrying (DPYD) variants are at a higher risk of developing severe toxicity when treated with therapeutic doses of 5-FU **(Universitet et al., 2022).** In the Caucasian population, DPYD*2A is the most frequent single nucleotide polymorphisms (SNPs) **(Battaglin et al., 2018)**. Since the detection of the DPYD*2A variant, several methods are now available for the identification of variants in the DPYD-gene in a clinical setting. Real-time polymerase chain reaction (RT-PCR) is now the most broadly used technology **(Soroka et al., 2021).**

Sufficient vitamin D status has an impact on cancer prognosis: the active hormone 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) influences signaling pathways that regulate cell proliferation, differentiation and cell survival, thus actying as an anti-proliferative agent in many tissues and slowing the growth of malignant cells **(Krebsforschungszentrum et al., 2023).** Vitamin D encourages a cytotoxic effect of 5-FU in colon carcinoma lines in humans providing a reasonable mechanistic clarification for a synergistic therapeutic association between vitamin D and 5-FU, which supports the importance of measuring the level of vitamin D in CRC patients undergoing chemotherapy **(Savoie et al., 2019).** This work aimed to assess the correlation between 5-FU response, and toxicities, and their association with DPYD genetic polymorphism, and vitamin D levels in upper Egypt CRC patients.

Patients and methods

Type of study:

A prospective cohort study that started from April 2023 to April 2024, at Shefa El Orman Hospital, Luxor, Egypt. The study protocol was approved by Shefa El Orman Hospital with assurance number FWA00025406, and its registration number in the Egyptian Ministry of Health was RHDIRB2017061502. The study also approved by the South Valley University of Medicine Ethical Committee with approval code: SVU-MED-ONM027-1-23-5-641. All patients signed a written informed consent before participating in the study.

Sample Size Calculation:

The study was carried out by 60 colorectal cancer patients according to the

Steven K Thompson equation to calculate the sample size.

$$n = \frac{N \; X \; p(1-p)}{[N-1 \; X \; (d2 \div z2] + [p(1-p)]}$$

Where N: Population size, z: Confidence level at 95%, d: Error proportion (0.05), p: Probability (50%), and we reached sample size (n) = 60.

Inclusion criteria:

The patients were more than 18 years old and had a good performance status ECOG (0-2). Patients with metastatic CRC disease planned to use one of the 5-FU regimens, had measurable disease, and had informed consent.

Exclusion criteria:

Patients without metastatic stage, had a past or current history of other malignancies rather than CRC. Pregnant patients and patients received chemotherapy before the enrollment (last 6 months).

Study design:

The study was conducted on metastatic CRC patients receiving 5-FU for 3 to 6 months. After following up of the patients, they were divided according to the antitumor effect of 5-FU into two groups:

Group 1: responder patients (45 cases), and included patients with partial or complete responses or those with a stable course. **Partial response patients:** if there was a decrease in tumor size or metastasis sites or sizes (at least a 30% reduction in the sum of diameters of target lesions, taking as reference the baseline sum diameters). **Complete response patients:** if there was a marked decrease in tumor size and metastasis sites and sizes and the disappearance of all target lesions. **Stable patients:** if there was stability in tumor size and metastasis sites and sizes (neither sufficient shrinkage to qualify for partial response nor sufficient increase to qualify for progressive disease).

Group 2: non-responder patients (15 cases), included patients with a progressive course. **Progressive disease patients:** if there was at least a 20% increase in the sum of diameters of target lesions, taking as a reference the smallest sum of diameters of target lesions at the start of the study, or the appearance of one or more new lesions (**Fig.1**).

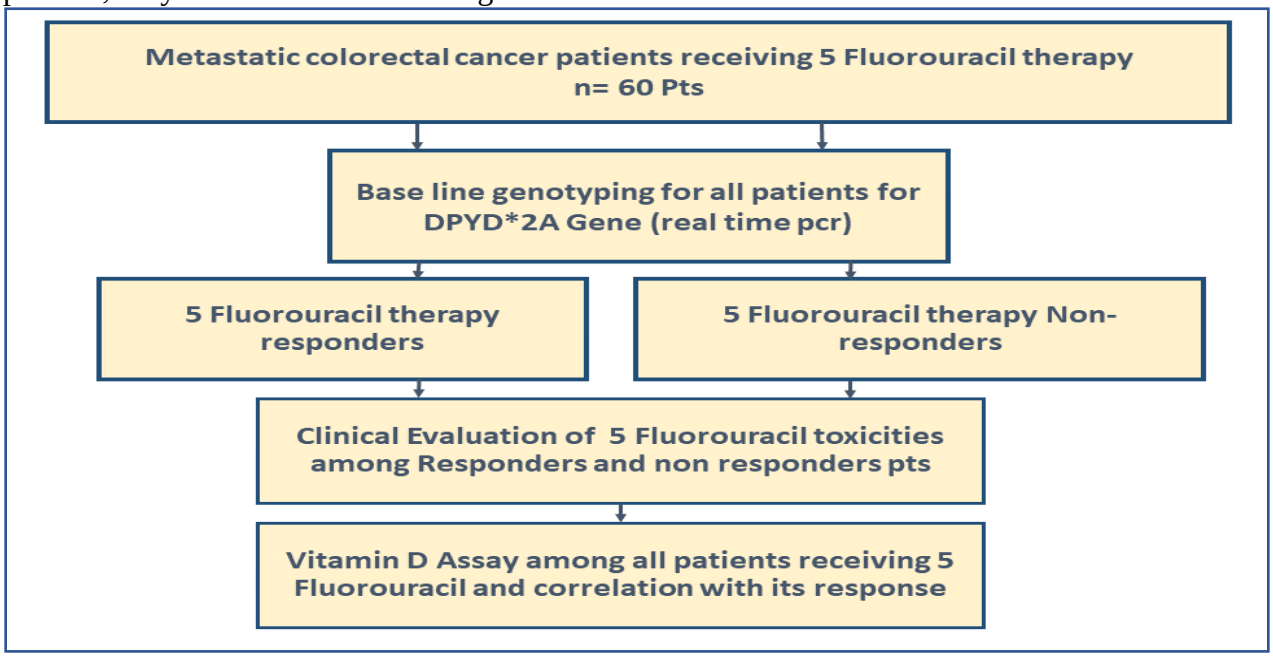

Fig. 1. Flow chart showing patients and study design

Colorectal cancer chemotherapy (5-fluorouracil regimens) used by the patients in the study:

FOLFIRI (Folinic acid, 5-FU, and irinotecan): First day**:** 5-FU 400 mg/m^2 by intravenous (IV) bolus shot, irinotecan 180 mg/m^2 by IV route, and folinic acid through a drip into the bloodstream over 2 hours. Second day: 5-FU 1400 mg/m^2 by IV infusion, irinotecan 180 mg/m^2 by IV route. Each cycle of treatment lasted 2 weeks (drugs were used on the first and second day of each cycle). Patients may had up to 12 cycles, lasted for up to six months **(Ychou et al., 2013; Glimelius et al., 2021).**

Capecitabine (Xeloda, a prodrug changed to 5-FU inside the body): 1250 mg/m^2 by the oral route twice daily from day 1 to day 14 for up to six months **(Punt et al., 2022).**

De Gramont (5-FU and leucovorin calcium): Leucovorin (200 mg/m^2) was given as a 2-hour IV infusion followed by bolus IV 5-FU (400 mg/m^2) and 22-hours IV infusion 5-FU 600 mg/m^2 for 2 consecutive days every 2 weeks for up to six months **(Glimelius et al., 2021).**

Data collection

Data collection was done from the patients or their accompanied family member as name, age, occupation, address, contact number, family history of CRC, symptoms related to the disease, history of using anticancer drugs, smoking habit, and careful medical history for chronic medical diseases (such as diabetes mellitus and hypertension). Patient physical examination and important investigations such as CBC, liver function tests, abdominal ultrasonography, CT scan, MRI, or positron emission tomography/CT scan (PET/CT scan) were done before and during the use of 5-FU therapy to detect the disease progression, the response to treatment (to know if the patient was a responder or non-responder to 5-FU therapy), and the number of organ metastases.

Reporting of 5 Fluorouracil adverse events:

Common terminology criteria for adverse events (CTCAE) created by the National Cancer Institute (NCI) were used to assess the adverse events (AEs) of chemotherapy, displaying grades 0 to 5 with unique clinical descriptions to assess the severity of AEs (grade 0 = no AE, grade 1 = mild, grade 2 = moderate, grade 3 = severe/medical intervention is needed, grade 4 = life-threatening, grade 5 = death). CTCAE classifies all adverse events into three categories: laboratory AEs, measurable AEs, and subjective AEs **(Liu et al., 2020).**

Blood samples collection and processing:

Five ml of peripheral blood was obtained from sixty CRC patients by venipuncture, divided into two tubes each of which was labeled with the patient's name and date of collection. 2 ml of blood was placed into sterile tubes containing ethylene diamine tetraacetic acid (EDTA), stored at -80˚ to be used for DNA extraction and genotyping. The other 3 ml was collected into sterile plain tubes and then centrifuged at 3000 rpm. for 10 minutes. The serum was collected into two new Eppendorf tubes and stored at -20˚for for liver enzymes and complete blood count (CBC) measurement.

Laboratory investigations (pretreatment and 6 months post treatment):

Complete blood count was estimated using a CBC analyzer (Derui BCC-3600, China), aspartate aminotransferase (AST) and alanine transaminase (ALT) were estimated using a fully automated clinical chemistry analyzer (Cobasc 311, Roche Diagnostics, Germany).

Vitamin D assay (6 months post-treatment):

Serum 25-OHD levels were determined using enzyme-linked immunosorbent assays (ELISA) in serum

samples of all subjects using the Abcam human vitamin D ELISA kit (Cat No. ab213966, Chicago, IL, USA) according to the manufacturer's guidelines using microplate ELISA reader (EMR−500, Labomed, Inc., LA, USA). Deficient vitamin D levels (10 ng/mL), insufficient (10 - 30 ng/mL), and sufficient (30-100 ng/mL) were the three vitamin D levels that were taken into consideration in the investigation **(Saeed et al., 2021).**

DNA Extraction (during treatment):

Genomic DNA was extracted from buffy coat samples using the QIA amp. DNA mini-kit with the following procedure: 20 µL QIAGEN protease (Hilden, Germany) catalog Number (51106) was added to a 1.5 mL microcentrifuge tube, then 200 µL of the sample and 200 µL of buffer AL were added with mixing for 15 seconds by pulse vortex. Incubation for 10 minutes at 56 °C was done. Ethanol (96 -100%) 200 µL was added and mixed using the vortex for 15 seconds. The mixture was applied to the QIA amp with centrifugation for 1 minute. 500 µL of buffer AW1 then was added and centrifuged at 8000 rpm for 1 minute, after which 500 µL of buffer AW2 was applied and centrifugation for 3 minutes at 14,000 rpm was performed. QIA amp mini spin column was transferred to a 1.5 mL microcentrifuge tube, and 200 µL of buffer AE was added and incubated at room temperature for 5 minutes. Then, centrifugation for 1 minute at 8000 rpm was done. A spectrophotometer (Multiskan Sky High Microplate Spectrophotometer) was used for the assessment of DNA quality and purity. The DNA aliquots were then stored at -80 °C until used for PCR **(Kim et al., 2022).**

PCR and genotyping: The DPYD gene analysis was performed using an RT-PCR instrument (Quant studio™ 3 real-time PCR System, 96-well, 0.2 mL, laptop) and the catalog number of SNP PACE® 2.0 Genotyping Master Mix low ROX 003-0005 and PACE Genotyping assay CUST-ASSAY-002, which is a homogeneous, PCR-based allele-specific technology for SNP analysis. This assay utilized two probes, one labeled with FAM dye and the other with VIC dye, for allelic specificity.

PCR setup: PCR mixtures were prepared and contained genomic DNA template, DPYD*2A (IVS14+1G>A) gene primer (see **Table.1**), dNTPs (deoxynucleotide triphosphates), PCR buffer, DNA polymerase, PCR amplification, and Mg Cl_2 (for enzyme activity).

Table 1. DPYD*2A genotyping data

DPYD*2A gene	**Rs number**	3918290	**(Abbasian et al., 2020)**
	Forward primers	(ACTCAATATCTTTACTCTTTCATCAGGAC)	
	Reverse primers	(ACATTCACCAACTTATGCCAATTCT)	
	Vic sequence probe	VIC-AGACAACATAAGTGTGATTTA-MGB	
	Fam sequence probe	FAM-AGACAACGTAAGTGTGATTTA-MGB	

Amplification was conducted in a thermal cycler under the following conditions: Enzyme activation at 94 °C for 15 minutes and template denaturation at 94 °C for 20 seconds were done. Annealing and extension at 65-57 ˚C for 60 seconds (drop 0.8 ˚C per cycle). Then repetition of the denaturation, annealing, and extension steps for 10 cycles. Denaturation at 94 ˚C for 20 seconds and annealing and extension at 57 ˚C for 60 seconds. Repeatation of the denaturation, annealing, and extension steps for 30 cycles was done. Lastly, holding at 4-10 °C was performed **(Fig. 2 and 3**).

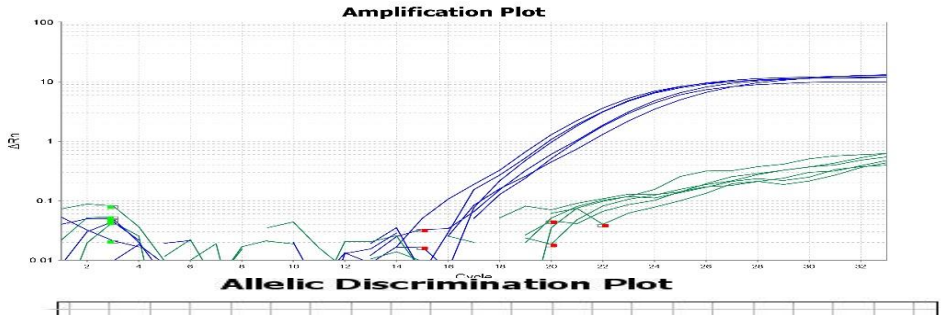

Fig.2. PCR showing the study run amplification plot of DPYD *2A gene.

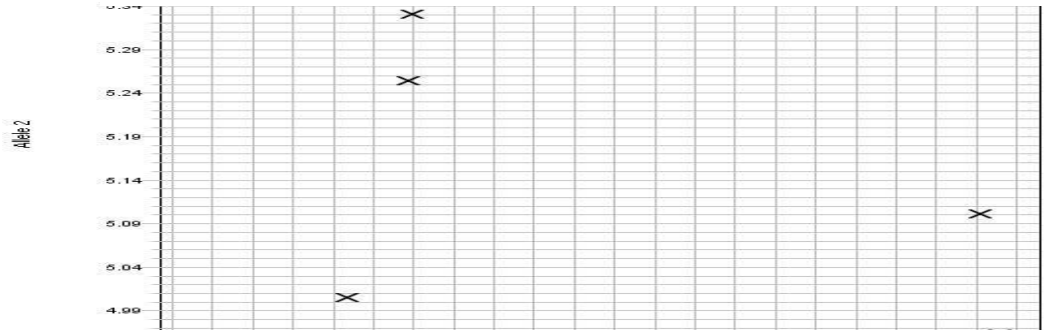

Fig.3. PCR showing the study run discrimination plot of DPYD*2A gene

Genotyping results were analyzed to identify DPYD gene mutations. Statistical analysis was performed to assess the association between DPYD polymorphisms and clinical outcomes of drug toxicities.

Statistical analysis

Data were collected, coded, revised, and entered into the Statistical Package for Social Science (IBM SPSS) version 27. Data were tested for normality using the Kolmogorov–Smirnov and Shapiro–Wilk tests. Categorical variables were presented as numbers and percentages and compared using the Chi-square test. Numerical variables with parametric distribution were presented as means and standard deviations and compared using an independent t-test for inter-group differences and a paired t-test for intra-group differences. Numerical variables with non-parametric distribution were presented as median and IQR and compared using the Mann-Hitney test for inter-group differences and the Wilcoxon signed test for intra-group differences. Binary logistic regression analysis was used to determine predictors for 5-FU response. The allowable margin of error was set at 5%, while the confidence interval (CI) was set at 95%. Consequently, the p-value <0.05 was considered significant.

Results

This cohort study involved 60 patients with metastatic CRC from the Medical Oncology Department at Shefa Al-Orman Hospital, Luxor.

Characteristics of patients (age, sex, co-morbidities, smoking, BMI, family history)

Patients age ranged from 24 to 87 years, with a mean age of 55.83 ± 12.983 years, and 65% were males. There were comorbidities in 14 of them (23.3%), these comorbidities were diabetes mellitus in 8 patients (13.3%), hypertension in 4 patients (6.7%), and associated diabetes and hypertension in 2 patients (3.3%). Family history of CRC was positive in only 8 cases (13.3%), and 11 cases (20%) were smokers. Concerning the BMI, 13.3%, 45%, 15%, and 26.7% of patients were underweight, normal, overweight, and obese, respectively.

Anticancer medication used and performance status among the studied cases

Results showed that FOLFERI was the most frequently used drug in 35 cases among 60 cases (58.3%), followed by Capecitabine in 24 cases (40%), and the least frequent was De Gramont in only 1 case (1.7%) of all the CRC patients. Concerning the performance status, most patients (75%) were symptomatic and ambulatory; with self-care, 23.3% were ambulatory more than 50% of the time and needed occasional assistance, and only 1.7 % were ambulatory less than 50% of the time and needed nursing care.

Metastatic organs sites

The study showed that 50 % of patients had liver metastasis, 33.3% had lung metastasis, 8.3% had bone metastasis, 36.7 % had lymph node metastasis, and 46.7% had metastasis in the peritoneum, so that the most common sites were the liver and peritoneum, then the lymph node and the lung while the bone was the least common site for metastasis.

Results of 5-FU toxicities among drug responders and non-responders

Cardiovascular, gastrointestinal toxicities, and constitutional symptoms:

A statistically significant difference existed between 5-FU responders and non-responders concerning chest pain, nausea, and febrile neutropenia (P-values were 0.003, 0.006, and <0.001, respectively); the non-responders had higher grades of chest pain, nausea, and febrile neutropenia than responders (**Table 2 and Fig. 4).** No statistically significant difference existed between 5-FU responders and non-responders regarding vomiting, diarrhea, and constipation (P>0.05) **(Table. 2)**

Table 2. Relation between gastrointestinal toxicity and treatment response

Symptoms		Response to 5-Fluorouracil		P-value
		Responder (N=45)	Non-responder (N=15)	
		N (%)	N (%)	
Chest Pain*	G0	32 (71.1%)	6(40.0%)	**0.003**
	G1	11 (24.4%)	3(20.0%)	
	G2	2(4.4%)	5(33.3%)	
	G3	0(0.0%)	1(6.7%)	
Nausea*	G0	22(48.9%)	2(13.3%)	**0.006**
	G1	16(35.6%)	6(40.0%)	
	G2	6(13.3%)	3(20.0%)	
	G3	1(2.2%)	4(26.7%)	
Vomiting*	G0	24(53.3%)	2(13.3%)	0.061
	G1	6(13.3%)	4(26.7%)	
	G2	8(17.8%)	5(33.3%)	
	G3	7(15.6%)	4(26.7%)	
Diarrhea*	G0	15(33.3%)	10(66.7%)	0.137
	G1	12(26.7%)	2(13.3%)	
	G2	11 (24.4%)	1(6.7%)	
	G3	7(15.6%)	2(13.3%)	
Constipation*	G0	32 (71.1%)	11(73.3%)	0.979
	G1	5(11.1%)	2(13.3%)	
	G2	4(8.9%)	1(6.7%)	
	G3	4(8.9%)	1(6.7%)	

Febrile neutropenia *	G0	44 (97.8%)	8(53.3%)	**<0.001***
	G1	1 (2.2%)	0(0.0%)	
	G2	0(0.0%)	6(40.0%)	
	G3	0(0.0%)	1(6.7%)	

*Chi-square test.

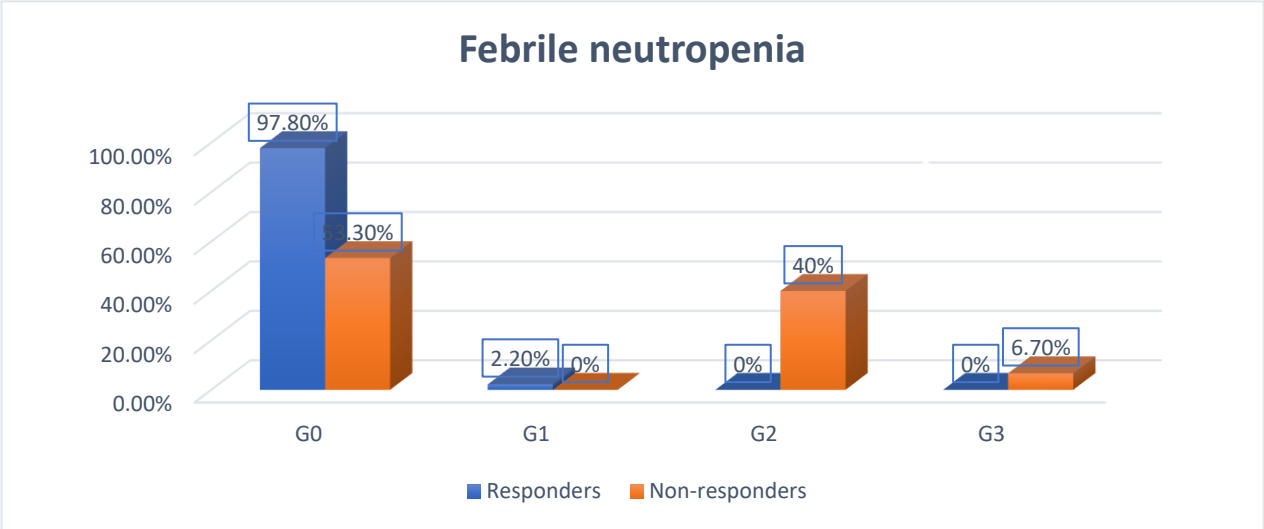

Fig. 4. Febrile neutropenia among 5-FU responders and non-responders

Neurosensory toxicities and skin manifestations:

A statistically significant difference existed between 5-FU responders and non-responders concerning bruising (P = 0.006); 33.33% of non-responders had bruising compared to 4.4% among responders. No significant difference was reported between the responders and the non-responders regarding skin rash and neurosensory symptoms, as p-values were 0.114, and 0.101, respectively **(Table 3 and Fig. 5).**

Table 3. Relation between neurological and dermatological toxicity and treatment response

Symptoms		**Response to 5-Fluorouracil**		**P-value**
		Responder (N=45)	**Non-responder (N=15)**	
		N (%)	**N (%)**	
Bruising*	G0	43 (95.6%)	10 (66.67%)	**0.006**
	G1	2 (4.4%)	3(20.0%)	
	G2	0 (0%)	2 (13.33%)	
Rash*	G0	27 (60%)	8 (53.3%)	0.114
	G1	11 (24.4%)	1 (6.7%)	
	G2	6 (13.3%)	4 (26.7%)	
	G3	1 (2.2%)	1 (6.7%)	
Neurosensory*	G0	13 (28.9%)	3 (20%)	0.101
	G1	22 (48.9%)	4 (26.7%)	
	G2	7 (15.6%)	7 (46.7%)	
	G3	3 (6.7%)	1 (6.7%)	

*Chi-square test

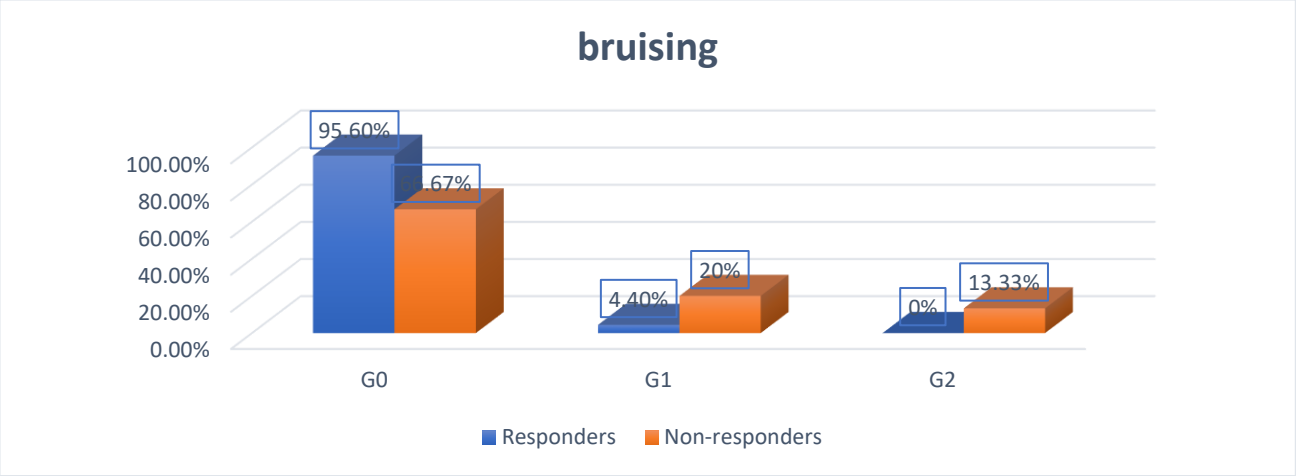

Fig. 5. Bruising grades among 5-fluorouracil responders and non-responders

Relation between treatment response and hematological and hepatic toxicities 6 months post-5-Fluorouracil treatment:

The study showed that the mean hemoglobin level, total WBCs, and neutrophil counts were significantly higher among responders to 5-FU than the non-responders (P-values were <0.001, 0.003, and <0.001, respectively). The results demonstrated also that the median platelet count was significantly higher among responders than among non-responders (P= 0.012). The mean ALT was significantly lower among responders than non-responders (P=0.035). No statistically significant difference existed between 5-fluorouracil responders and non-responders concerning AST level (P= 0.071) **(Table.4 and Figs 6 and 7).**

Table 4. Relation between treatment response and CBC and liver function tests 6 months post 5-FU treatment.

Parameters	Response to 5-Fluorouracil		P-value
	Responder (N=45)	Non-responder (N=15)	
	Mean ± SD	Mean ± SD	
Complete blood count			
• Hemoglobin (gm/dL)*	10.5± 1.19	8.3 ± 1.42	**<0.001**
• WBCs /mm^3 *	3331.1 ± 903.23	2540.00 ± 719.92	**0.003**
• Neutrophils / mm^3 *	1789.7± 560.08	1196.6 ± 334.12	**<0.001**
• Platelets / mm^3 -median (IQR)#	99000 (79500-180000)	76000 (66000-92000)	**0.012**
Liver function tests			
• AST (U/L)*	94.06 ± 29.27	110.46 ± 31.67	0.071
• ALT (U/L)*	75.75 ± 26.35	92.93 ± 27.74	**0.035**
• Bilirubin (mg/dL) median (IQR)#	1.32 (1.22-1.73)	1.90 (1.42-2.80)	**0.003**

*Student t-test; #Mann-Whitney test; **bold:** Significant.

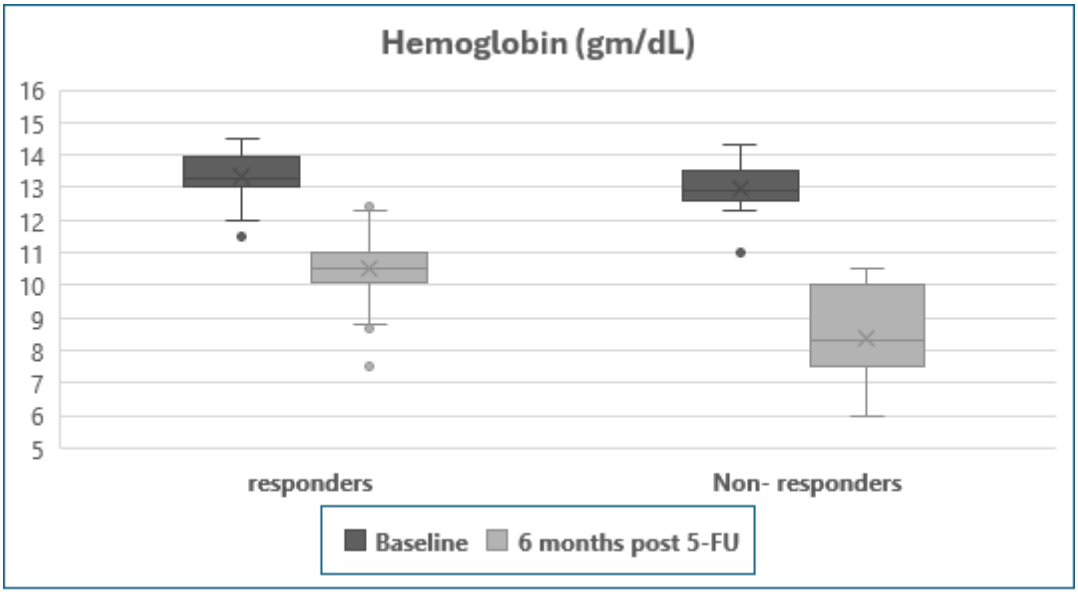

Fig. 6. Box plot showing Hemoglobin levels at baseline and 6 months post-5-FU among responders and non-responders

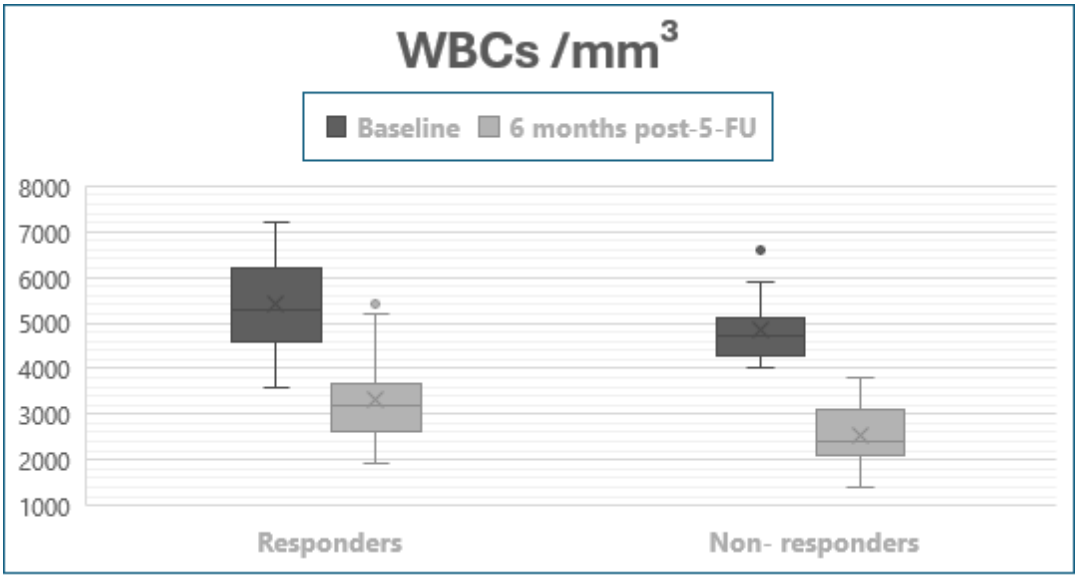

Fig.7. Box plot showing WBCs count at baseline and 6 months post-5-FU among responders and non-responders

CBC and liver function test 6 months post-5-FU compared to pre-treatment among responders to the drug:

The study results indicated that the mean hemoglobin level, total WBCs, and neutrophil counts decreased significantly 6 months post-5-FU treatment compared to the pre-treatment data in the responders (P<0.001). The median platelet count decreased significantly after 6 months of 5-FU treatment (P<0.001). The mean AST, ALT, and bilirubin levels increased significantly 6 months post-treatment in contrast to 5-FU pretreatment (P<0.001) **(Table.5).**

Table 5. CBC and liver function results in -5-FU responders pre- and 6 months post-treatment

Parameters	5-Fluorouracil (Responders)		P-value*
	Pre-treatment	6-months post-treatment	
	Mean ± SD	Mean ± SD	
Complete blood count			
• Hemoglobin (gm/dL)*	13.3 ± 0.68	10.5 ± 1.19	< **0.001**
• WBCs /mm^3 *	5435.5 ± 934.4	3331.1 ± 903.2	< **0.001**
• Neutrophils / mm^3 *	3524.4 ± 799.5	1789.7 ± 560.08	< **0.001**
• Platelets / mm^3 median (IQR) #	178000 (155000-2030000)	99000 (79500-180000)	< **0.001**
Liver function tests			
• AST (U/L)*	30.1 ± 17.4	94.0667 ± 29.27875	<**0.001**
• ALT (U/L)*	21.3 ± 11.8	75.76 ± 26.35	<**0.001**
• Bilirubin (mg/dL)*	0.85 ± 0.2	1.5 ± 0.7	<**0.001**

*Paired t-test, #Wilcoxon signed rank test; **bold:** significant

CBC and liver function tests post 5-FU compared to pre-5- FU treatment among non-responders to the drug:

In the 5-FU non-responder patients, there was a significant reduction in the mean hemoglobin level, total WBCs, and neutrophil counts 6 months post-5-FU treatment compared to the pre-treatment data (P<0.001). The median platelet count decreased significantly after 6 months of 5-FU treatment (P<0.001) **(Table.6 and Figs 8 and 9).** The mean AST, ALT, and median bilirubin level increased significantly post-treatment than pretreatment

Table 6. CBC and liver function results in-5-FU non-responders pre-and 6 months post-treatment

Parameters	5-Fluorouracil (non-responders)		P-value
	Pre-treatment	6-months post-treatment	
	Mean ± SD	Mean ± SD	
Complete blood count			
• Hemoglobin (gm/dL)*	12.98 ± 0.81	8.34 ± 1.42	< **0.001**
• WBCs /mm^3 *	4853.33 ± 707.98	2540.0 ± 719.92	< **0.001**
• Neutrophils /mm^3 *	3200 ± 662.24	1196.66 ± 334.12	< **0.001**
• Platelets /mm^3 *	159333.3 ± 26826.6	85400.0 ± 31945.9	< **0.001**
Liver function tests			
• AST (U/L)*	25.93 ± 9.71	110.46 ± 31.67	< **0.001**
• ALT (U/L)*	19.20 ± 7.79	92.93 ± 27.74	< **0.001**
• Bilirubin (mg/dl) Median (IQR)#	1.050 (0.775-1.40)	1.90 (1.42-2.80)	< **0.001**

*Paired T-test, #Wilcoxon signed rank test; **bold:** significant

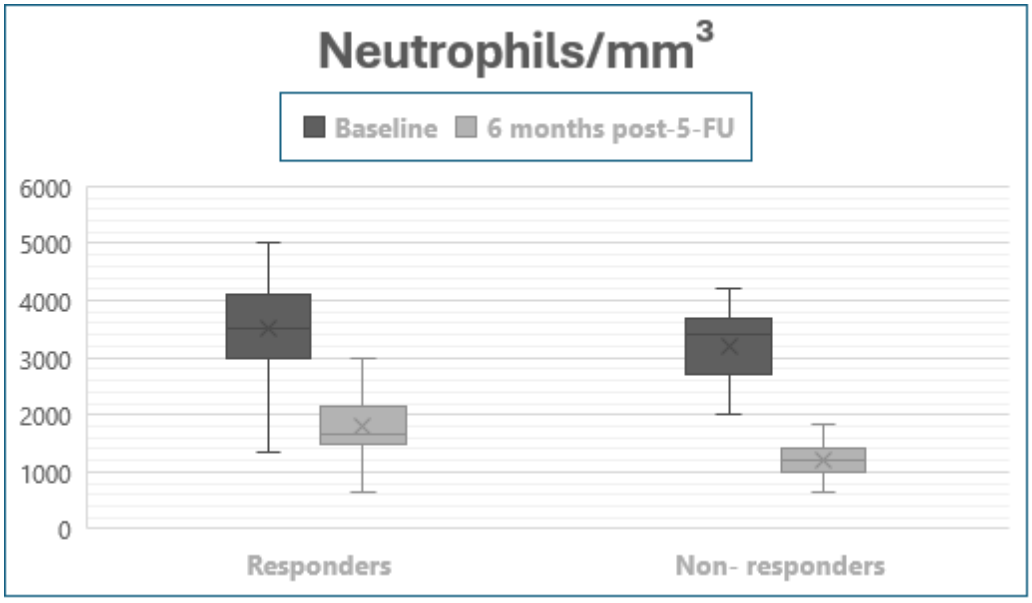

Fig.8.Box plot showing neutrophil count at baseline and 6 months post-5-FU among responders and non-responders

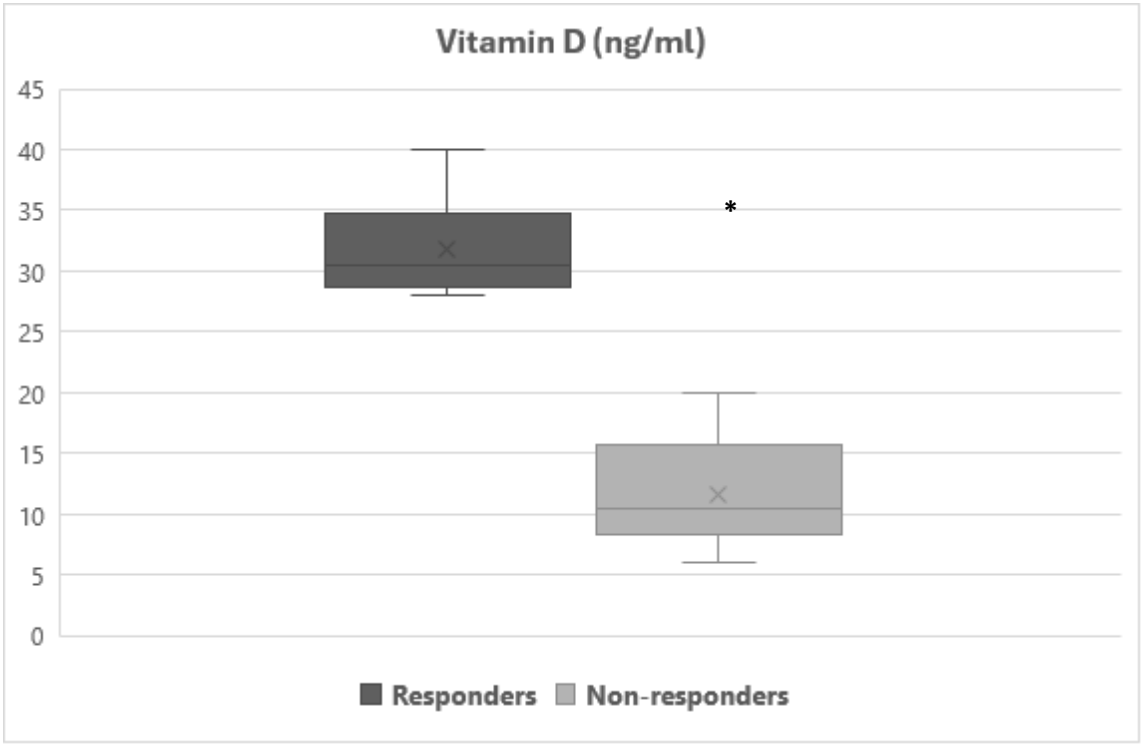

Fig. 9. Box plot showing Vitamin D level post-treatment among 5-fluorouracil responders and non-responders.

Results of DPYD gene analysis using PCR

A genotyping study using PCR indicated that there was absence of the DPYD*2A variant among the study cases; 0% DPYD*2A genetic polymorphism as all the patients were wild-type patients, so the association between DPYD*2A variant and

5-FU-associated toxicity was not significant in the included upper Egypt CRC Patients

Results of vitamin D level in 5-FU responder and non-responder patients:

The results demonstrated statistically significant higher serum vitamin D levels among 5-FU responders than non-responders; most responders had sufficient vitamin D while most non-responder patients had deficient vitamin D **(Table 7 and Fig.10).**

Table 7. Serum vitamin D levels post-treatment among 5-FU responders and non-responders

Vitamin D categories *	5-Fluorouracil Response		P-value
	Responders (N=45)	Non-responders (N=15)	
	Frequency (%)	Frequency (%)	
Deficient	3 (6.67%)	7 (46.67%)	**<0.001**
Insufficient	12 (26.67%)	6 (40%)	
Sufficient	30 (66.67%)	2 (13.33%)	
Vitamin D (ng/ml) (mean ± SD)#	31.83 ± 4.35	11.62 ± 4.68	**< 0.001**

*Chi-square test, #Student t-test **bold:** significant

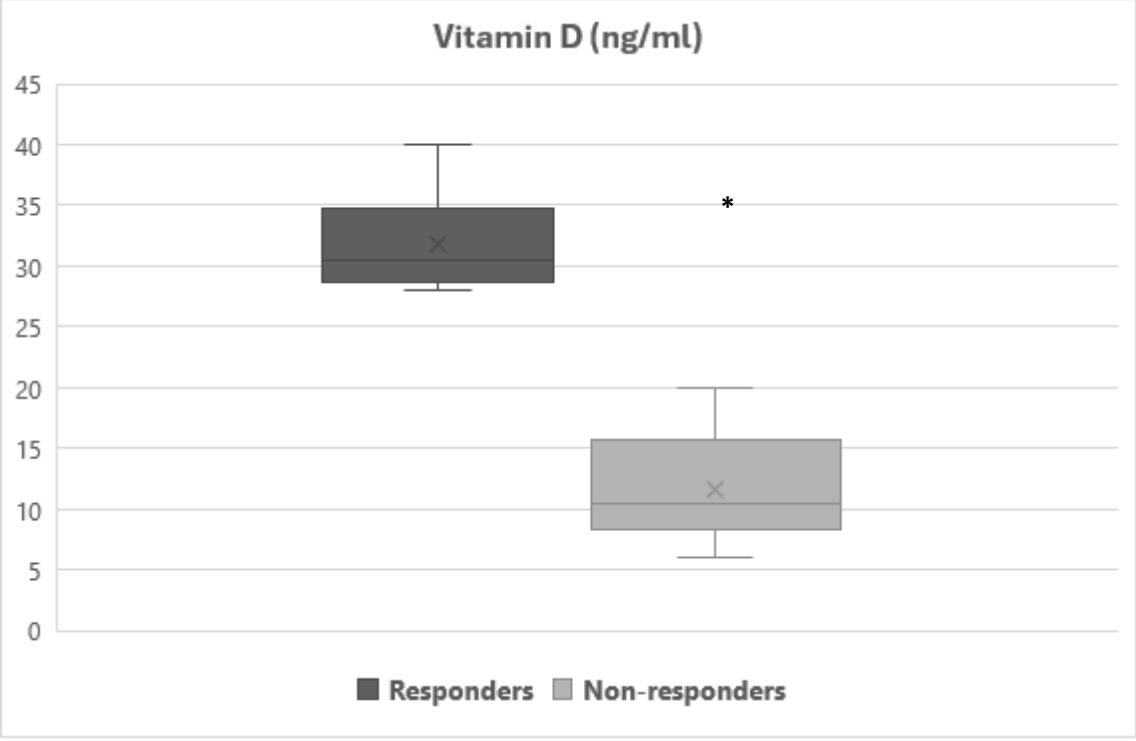

Fig. 10. Box plot showing Vitamin D levels among 5-fluorouracil responders and non-responders.

Predictors for 5-FU response

There were 4 predictors for 5-FU response; the most predictor was sufficient vitamin D level, followed by an absence of nausea, and a higher level of WBC count, and the least

predictor was a lower level of bilirubin (**Table 8).**

Table 8. Multivariable logistic regression analysis for predictors of 5-FU response

Predictor Variables		**Odds ratio (OR)**	**Confidence interval (CI)**		**P value**
			Lower	Upper	
Vitamin D	Deficient	Reference			
	Insufficient	4.667	0.878	24.796	0.071
	Sufficient	35	4.885	250.792	**<0.001**
Nausea	G3	Reference			
	G2	8	0.598	106.936	0.116
	G1	10.667	0.984	115.683	0.052
	G0	44	3.183	608.158	**0.005**
Baseline WBCs count		1.001	1.00	1.002	**0.038**
Baseline bilirubin		0.134	0.023	0.774	**0.025**

Discussion

Up to now, FP drugs class is a milestone in colorectal cancer treatment, in early and metastatic disease. 5-FU and its pro-drug capecitabine remain the chemotherapy backbone for the treatment of this type of cancer **(Bruera and Ricevuto, 2020).** 5-FU inhibits cancer cell replication and RNA by inhibiting thymidylate synthase. Although 5-FU-based regimens effectively cause cytotoxic effects in cancerous cells, they are also recognized to induce bone marrow suppression, inhibition of immunity, intestinal mucositis, and diarrhea **(Lo et al., 2023).**

As regards patients 'study data, their mean age was 55.83 years, 65% were males, and 35% were females. These results were in agreement with several studies that indicated that the mean age of patients with CRC was about 50 years and the male-to-female ratio was about 2:1 **(Liu et al., 2024).** In the present study, there were comorbidities in 23.3% of the patients; these comorbidities were diabetes mellitus in 13.3%, hypertension in 6.7%, and associated diabetes and hypertension in 3.3%. These results were in harmony with several studies that reported that diabetes mellitus and hypertension are risk factors for CRC **(Cheng et al., 2021 and Jeon et al., 2021).** Concerning the BMI, the study demonstrated that 13.3%, 45%, 15%, and 26.7% of patients were underweight, normal, overweight, and obese, respectively. The results indicated that obesity is an important risk factor for CRC, which was in agreement with **Jeon et al., 2021 and Cayún et al., 2024.**

Our study showed that FOLFERI was the most frequently used drug among CRC cases in about 58.3% which was in harmony with the reports of **Chai et al. (2024)**. The study indicated that 5-FU caused a significant increase in number of patients who developed nausea, vomiting, diarrhea, and constipation. The results were in harmony with the previous investigations, which indicated that the common side effects of 5-FU were the gastrointestinal–which can be attributed to inflammatory or ulcerative lesions of the mucosa of GIT due to damage from chemotherapy which targets active dividing cells **(Lo et al., 2023).** Common clinical toxicities of FP result from the inhibition of cells with rapid division, such as epithelial cells of GIT and bone marrow, causing diarrhea and cytopenia, respectively. Studies have indicated that chemotherapy-induced diarrhea, a common non-hematological toxicity, can be dependent on

the drug dose, schedule, and route of administration **(Sakumura et al., 2020).** The results demonstrated that 5-FU-treated patients developed neuropathy which was one of the FU complications. Capecitabine-induced neuropathy appeared to be caused by metabolites like fluor beta-alanine or 5-FU itself, which formed during drug metabolism in the liver **(Nitipir et al., 2018 and Bano and Ikram, 2019).**

The study indicated that hemoglobin levels significantly decreased 6 months post - 5-FU treatment among responders in contrast to pre-5-FU treatment. The same results were recorded with WBCs and platelets count among drug responders. The hematological abnormalities were more severe with 5-FU non-responders than responders. These results were in harmony with the reports of **Machover (1997)** who demonstrated that lecucopenia and anemia were the most common adverse effects for 5-FU. No death from hematological toxicities was reported in CRC patients receiving 5-FU **(Terzoli et al., 2004).** FP do not only affect cancer cells but can also affect normal cells. The major toxicity of this treatment on the healthy tissues with rapid proliferation, such as bone marrow cells and mucous membrane epithelial cells **(Afolabi et al., 2023).**

In the present study, the most common sites of metastatic were the liver in 50%, peritoneum in 46.7%, lymph nodes in 36.7% and the lung in 33.3%. The results were agreement with this study of **Brouwer et al. (2020)**. **Hernandez Dominguez et al. (2023)** demonstrated that the liver was the most common metastatic site for CRC followed by the lung.

The current study showed that AST, ALT, and bilirubin levels were higher in 5-FU post-treatment than pretreatment, which was in harmony with the report of **Challoob and Mohammed (2024) and da Silva et al. (2023).** Hepatotoxic features including alterations in hepatocyte structure and mild or severe elevations in serum hepatic markers, have been associated with 5-FU use. In addition, studies have suggested that oxidative stress, inflammation, and cell apoptosis are possible mechanisms of 5-FU-induced hepatotoxicity (**Anerobi et al., 2020).**

One of the main causes of 5-FU toxicity is the deficiency of the enzyme DPYD, which encoded by the DPYD gene, that is responsible for 5-FU catabolism. An impairment of DPYD enzymatic function can cause accumulation of 5-FU toxic metabolites, which have an important role in the development of adverse events **(Montella et al., 2024).** The DPYD* 2A allele is considered the most frequent SNP **(Sharma et al., 2019).** The current study, indicated that an absence in the DPYD*2A (rs3918290) variant gene among the upper Egypt CRC patients; 0% had DPYD*2A genetic polymorphism variability**.** These results were in agreement with this study by **Chan et al. (2023)** who reported that the frequency of DPYD variants is 0% in East Asian reference populations, and with the report by **Botton et al. (2022)** who noted that DPYD*2A (rs3918290) and DPYD*13 (rs55886062) had no function alleles and the assigned activity score for the Brazil population was zero. This genetic polymorphism result was in contrast with the results of **Tutillo et al. (2022)** who demonstrated that rs3918290, rs55886062, rs67376798, rs1801159, and rs17376848 variants of the DPYD gene were connected with decreased DPYD enzyme activity and responsible for increasing the risk of severe toxicity after the administration of 5-FU. The current results were also opposite to the report of **Granados et al. (2024)** who showed analysis focused on carriers of the 5 DPYD alleles [DPYD*2A (rs3918290), DPYD p.Y186C (rs115232898), DPYD p.D949V (rs67376798), DPYD HapB3 (rs56038477), and DPYD*13 (rs55886062)] that were

validated to be associated with the increase in the risk of toxicity by 5-FU.

In CRC, calcitriol (active biological form of vitamin D) can cause inhibition of proliferation as well as angiogenesis, invasion caused by cancer cells, and induction of apoptosis **(Na et al., 2022).** In the current study, the mean vitamin D level was significantly higher among 5-FU responders than non-responder patients. These results were in agreement with many studies, which indicated that there was a correlation between the prognosis of CRC patients and vitamin D status, as there was a better prognosis in patients with higher levels of serum vitamin D **(Maalmi et al., 2018).** Vitamin D can increase the expression of E-cadherin and arrest the cells in the G0/G1 cell cycle phase. It was noted that vitamin D could enhance and extend the anticancer effect of 5-FU **(Javed et al., 2020).**

Conclusion

Colorectal cancer (CRC) is a significant cause of death worldwide; the second most frequent cause. 5-Fluorouracil (5-FU) is still a widely used anticancer drug. More than 85% of the administered 5-FU is catabolized by dihydropyrimidine dehydrogenase (DPYD) in the liver. However, mutations in the DPYD gene have been found to be associated with low DPYD activity, causing severe complications. This is one of the earliest study exploring the prevalence of the DPYD*2A mutation in 60 CRC patients in Upper Egypt. The study confirmed the clinical toxicities associated with CRC treated with 5-FU. However, there was an absence of the DPYD*2A mutation, so no clear correlation between the mutations in DPYD activity and 5-FU toxicity was established in the study.

Higher levels of vitamin D have been suggested to decrease mortality in CRC patients by decreasing the 5-FU toxicity and by increasing the response to 5-FU. The study results recommend the measurement of vitamin D level in CRC patients receiving 5-FU. More studies on a larger scale of patients are required to acquire a better understanding of the 5-FU metabolism-associated genetic polymorphisms and clinical toxicities for better steps towards an individualized, safe patient treatment.

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