Dihydropyrimidine dehydrogenase level and the redox status in patients with colorectal cancer are prognostic for adverse effects of fluoropyrimidines

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ABSTRACT
Drug resistance and toxicity are the most widespread limitations in the pursuit of sufficient antitumor effectiveness and successful control of oncological diseases, including colorectal cancer (CRC). Herein the objective was to investigate some hematological side effects of the chemotherapeutic regimen FOLFOX-4, their relation to dihydropyrimidine dehydrogenase (DPD) levels and the associated alterations in CRC patients’ plasma free-radical scavenging properties and extent of oxidative molecular damage. Thirty-eight patients with histologically confirmed CRC diagnoses, assigned to chemotherapy with the FOLFOX-4 regimen, were recruited. The diagnostic methods included a complete physical examination, blood routine test and general biochemistry. The DPD levels were assayed. The patients’ plasma free-radical scavenging properties and extent of molecular oxidative damage were determined by spectrophotometry and enhanced chemiluminescence. The clinico-pathological and demographics characteristics of the patients were in agreement with the reports from retrospective cohort studies. The FOLFOX-4 regimen induced a decrease in plasma free-radical scavenging properties and increased extent of lipid peroxidation. White blood cells, granulocytes and lymphocytes decreased significantly after the first cycle of the therapy. The patients’ DPD level decreased statistically significantly in the case of severe reduction (more than 25%) of white blood cells and granulocyte counts. The obtained data are in agreement with already known facts concerning the side effects of the FOLFOX-4 regimen and associated changes in redox homeostasis. Genetic predisposition to effectively metabolize and tolerate the applied therapy, i.e. DPD levels, could modulate some aspects of the observed changes in the aforementioned parameters.

Introduction
Colorectal cancer (CRC) is among the major causes of morbidity and mortality worldwide. It is the third most commonly diagnosed type of cancer throughout the world and is the second most common cause of cancer-related death [1–3]. During the past two decades, a stable trend toward increased disease incidence has been observed among individuals of both sexes and all age groups [4]. Typical of the disease are its multifactorial, pathophysiological mechanisms and genetic heterogeneity.

Several factors have been proven to play an important role in CRC development [5]. Some of them, like age and hereditary factors, cannot be controlled. The role of genetic factors in the development of CRC is estimated at 28% for men and 45% for women. Approximately 25% of CRC cases have family history without an obvious genetic link arising from acquired genomic aberrations [6]. Many research groups have proven the substantial role of environmental and lifestyle risk factors, which could be pointed out as modifiable risk factors, i.e. diet rich in animal fat, high meat consumption, low consumption of fruits and...
vegetables, excess body weight, lack of physical activity [7].

The poor outcome and increased mortality associated with the disease are mainly due to late diagnosis of colorectal cancer. The early stages of the disease are asymptomatic or with mild symptoms. Most patients are diagnosed long after the first onset of clinical symptoms, when the disease is in Stage III or Stage IV. At these stages symptoms vary with tumor location but patients typically observe changes in bowel habits, abnormal pain, rectal bleeding, anemia, loss of weight and intestinal obstruction [8].

The treatment of the disease is complex. It could include surgery, radiation therapy chemotherapy, target therapy and immunotherapy, which could be applied separately or in combinations, depending on the stage of the disease [9]. Current chemotherapy regimens in CRC treatment involve a single drug, like fluoropyrimidine (5-FU), and multi-agent regimens that include one or more drugs, such as oxaliplatin, irinotecan and capcitabine. Drugs with a target mechanism of action have shown good therapeutic effects at molecular levels, but despite that, new therapeutic approaches are unlikely to replace the classic cytotoxic drugs in the foreseeable future [10].

The present study aimed at investigating some hematological side effects of the chemotherapeutic regimen FOLFOX-4, their relation with dihydropyrimidine dehydrogenase levels and the associated alterations in CRC patients’ plasma free-radical scavenging properties and extent of oxidative molecular damage.

The FOLFOX-4 regimen is a combination chemotherapy comprised of oxaliplatin, folinic acid and infusion of 5-fluorouracil (5-FU) [11]. 5-FU, a pyrimidine analogue irreversibly inhibiting the enzyme thymidylate synthase, has been for more than 40 years the most effective single agent first line in CRC therapy. It acts as an antimetabolite following intracellular conversion to the active deoxyribonucleotide [12]. The enzyme dihydropyrimidine dehydrogenase (DPD) is essential for the effective catabolism and clearance of 5-FU. Leucovorin has proven its utility in modulating 5-FU effectiveness regarding the tumor response rate. Oxaliplatin is attractive due to its activity against cisplatin-resistant colonic cancer cells and its capability to induce a decrease in DPD activity, which slows down the 5-FU catabolism pathway [13–15].

Systemic administration of both 5-FU and oxaliplatin is characterized by a dose-toxicity relationship and a narrow therapeutic index [16, 17]. The listed drugs have been known to be capable of altering patients’ oxidative status, which could also influence the extent of the observed side effects of the treatment and the patients’ main disease [18, 19].

Subjects and methods

Ethics statement

The study design was approved by the Research Ethics Committee at Medical University of Sofia (KENIMUS). All patients signed informed consent forms for participation in the study.

Patients

The study was carried out in the Department of Oncology at the SofiaMed University Hospital. A total of 38 CRC patients were recruited to the study between September 2019 and March 2020, admitted at the Department of Medical Oncology for chemotherapy. During the initial evaluation, detailed anamnesis was assessed based on accompanying medical records, study proforma, including clinical manifestation of the disease, personal history and information concerning patients’ objective condition. The patients’ disease stage was clarified according to the TNM classification system and their ECOG performance status was determined. The patients underwent a complete physical examination including ECG, lung and heart X-ray, abdominal ultrasound, blood routine test and general biochemistry. Plasma DPD levels were assessed using Human DPD Elisa Kit, allowing detection and quantification of DPD levels expressed as concentration [ng/mL] via quantitative enzyme immunoassay technique.

Eligibility

The inclusion criteria were as follows: Aged > 18 with no upper age limit; histological verification, obtained from the endoscopy procedure histological report; adequate hematological parameters, suggesting proper functioning of bone marrow; performance status of 0, 1 or 2 according to the Eastern Cooperative Oncology Group; ability to give written consent; minimum life expectancy of 3 months. The exclusion criteria included: another primary cancer; a history of treatment with some other chemotherapeutic regimen; serious medical comorbidity, not currently controlled and/or able to compromise the ability to tolerate the present therapy.

Study design

Combination chemotherapy comprised of Oxaliplatin, Folinic acid and 5-Fluorouracil (FOLFOX-4) was used. In brief, Leucovorin was administrated at a dose of 200 mg/kg (as a 2-hour infusion) followed by bolus 5-FU 400 mg/m², for two consecutive days (day 1 and
day 2). Oxaliplatin was administrated only on day 1 at a dose of 85 mg/m² in a 2-hour infusion. The treatment was discontinued in cases of unacceptable toxicity, indications of disease progression, patients’ decision to decline further treatment and application of the approved maximum of 12 cycles. Blood samples for the complete blood count, biochemical tests and antioxidant status were collected from each of the 38 patients by venous puncture.

**Free-radical scavenging properties and oxidative molecular damage estimation**

Spectrophotometric and chemiluminescent model systems were applied for this part of the experimental work.

**Thiobarbituric acid-reactive substances assay**

A standard procedure based on the thiobarbituric reaction under conditions of ferrous iron induced oxidative molecular damage, was used for the determination of the lipid oxidation products in patients’ plasma [20]. Malondialdehyde (MDA) is a secondary end-product of the oxidation of polyunsaturated fatty acids and, due to this, is considered a useful marker of general lipid peroxidation. Each sample is comprised of 0.1 mL blood plasma and FeCl₂ at a final concentration of 0.1 mmol/L added to 1 mL of phosphate buffer with pH 7.4. Three independent, parallel samples were prepared for each patient. All samples were incubated for 30 min at 37°C. After the incubation, 2.5 mL of 0.5% thiobarbituric acid and 0.5 mL of 2.8% trichloroactic acid were added to the reaction mixture. Samples were vigorously shaken and incubated at 100°C in a water bath for 20 min, followed by centrifugation with a Janetzky K23 centrifuge at 3000 rpm for 20 min. The samples’ absorbance data were recorded at 532 nm and 600 nm. TBARS were expressed as malondialdehyde equivalents MDA nmol/L per milliliter plasma using extinction coefficient 1.56 × 10⁵ L/mol cm.

**Chemiluminescent investigation of the free-radical scavenging properties**

The free-radical scavenging properties of the CRC patients’ plasma were determined via enhanced chemiluminescence (CL) using an LKB 1251 luminometer (BioOrbit, Turku, Finland). This is a proven rapid method for the determination of antioxidant properties, possessing a wide linear dynamic range and low limits of detection [21–23]. The chosen assays include evaluation of the antioxidative properties using luminol-dependent CL in a system of KO₂-generated superoxide anion radical and luminol-dependent CL in a system of NaOCl-generated hypochlorite. The luminometer was set at 37°C, and the ‘flash assay’ option of the MultiUSE program was used during the CL response detection. The working solution of the chemiluminescent reagent -amino-2,3-dihydro-1,4-phthalazinedione (luminol) was prepared by dissolving the substance in a small amount of NaOH (0.01 mol/L). As a next step, the newly-prepared solution was further diluted with 50 mmol/L phosphate buffer until reaching a final concentration of 1 mmol/L. The pH was adjusted to 7.4 using 0.01 mol/L HCl. The CL response was determined based on the area under the chemiluminescent curve. The ratio between the CL response in the presence (sample) and in the absence of the tested patients’ plasma (control) was termed chemiluminescent scavenging index (CL-SI) and was used as a marker for plasma scavenging capacity. CL-SI is calculated as follows:

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\text{CL-SI} = \frac{I_{\text{sample}}}{I_{\text{control}}} \times 100
\]

**Luminol-dependent CL in a system of NaOCl-generated hypochlorite** – the assay was performed using 1-mL samples of phosphate saline buffer (50 mmol/L, pH 7.4) containing 0.1 mmol/L luminol, 0.06 mmol/L NaOCl and patients’ plasma (or buffer for controls). The CL response was registered for 1 min every 50 milliseconds after the addition of NaOCl.

**Luminol-dependent CL in a system of KO₂-generated O₂** – the assay was performed using 1-mL samples of PBS (50 mmol/L, pH 7.4) containing 0.1 mmol/L luminol. Two sets of samples were prepared – controls, where plasma has been omitted, and samples containing patients’ plasma. The CL response was registered for 1 min every 50 milliseconds immediately after the addition of KO₂ (20 µL solution dissolved in DMSO) due to the fast release of superoxide.

**Data analysis**

Demographics, as well as some hematological/biochemical parameters are illustrated using histograms and distribution plots. D’Agostino and Pearson omnibus normality test was chosen for analyzing the actual data distribution. For the related samples’ data analysis, the paired student’s T-test or the Wilcoxon signed rank test were applied. The statistical analysis of independent groups of samples was performed by way of the unpaired T-test and non-parametric analysis including the Mann-Whitney U-test and the Kruskal-Wallis test depending on the distribution normality. The analysis
of data having normal distribution has been performed using parametric tests. For data with proven abnormal distribution or in the case of small groups’ size we applied the non-parametric Mann-Whiney and Kruskal-Wallis H tests. Differences were considered statistically significant at the $p < 0.05$ level.

**Results**

**Clinico-pathological and demographic description of the enrolled patients**

The baseline demographics and medical characteristics of the patients included in the study are presented in Table 1. Initially, the number of patients was 40. Thirty-eight patients were enrolled as follows: 19 (50%) men and 19 (50%) women. Two patients (men) were excluded due to their refusal of treatment between the first and second cycle of the chemotherapeutic treatment. The mean and median age were, respectively 63.6 and 68 years (range: 43 $\rightarrow$ 80). Of the 38 CRC patients, 16 (42.1%) were rectal cancer patients and 22 (57.8%) colon cancer patients. In 5 (13.2%) the initial tumor had been localized in the caecum, in 4 (10.5%) in the ascending colon, in 2 (5.3%) in the transversal colon, in 1 (2.6%) in the descending colon; 9 (23.7%) had sigmoid colon cancer, 2 (5.3%) had recto-sigmoidal position and 1 (2.6%) had an initial tumor in the hepatic flexure. Patients’ distribution according to the TNM system (I, II, III and IV stage) was, respectively, 0 (0%), 8 (21.1%), 13 (34.2%) and 17 (44.7%).

Most of the patients had ECOG of 1 (79%) indicating a relatively good performance status. None of them were fully active and 8 (21%) were capable of self-care but unable to perform any work activities.

Table 2 represents data for some basic hematological and biochemical parameters used routinely by physicians to monitor the response in patients undergoing cancer therapy. From the presented data it is evident that the applied regimen induced some changes in the patients’ red blood cell count, hemoglobin and hematocrit levels, but no statistically significant reduction was observed. A similar situation was observed with the parameters concerning the liver and kidney function. No significant increase in the ALAT, ASAT, total bilirubin and creatinine was observed. The applied treatment resulted in a statistically significant decrease in white blood cells, granulocytes and platelets.

The DPD enzyme levels detected in the patients’ blood plasma are presented in Figure 1. Individual DPD levels in the tested group varied from 0.052 ng/mL to 1.20 ng/mL. The mean and median values of the data set were, respectively 0.46 ng/mL and 0.41 ng/mL. We evaluated the influence of FOLFOX-4 infusion and the patients’ DPD individual levels on the extent of modulation of the most altered parameters (white blood cell count, granulocyte count, platelets count). For this purpose we used the initially determined level of these parameters as a baseline and recalculated the percentage of the change after the infusion. The effect of DPD level on the observed individuals’ decrease in these parameters was estimated (Figure 2). The patients were divided into two groups: Group I (patients with a decrease in the parameters’ level of more than 25% of its initial value) and Group II (patients with a decrease in the parameter level of less than 25% of its initial value or with a slight increase).

Regarding the white blood cell counts, the mean DPD level in Group I was 0.30 ng/mL and in Group II, 0.52 ng/mL. For the granulocyte count the values were, respectively 0.32 ng/mL and 0.53 ng/mL. In both cases, there was a statistical difference between the mean DPD level in Group I and Group II (Figure 3). No statistically significant difference was observed when comparing these two groups regarding the platelet parameter. A DPD level of 0.45 ng/mL was observed in Group I compared to 0.47 ng/mL in Group II.

**Anti-radical properties and extent of molecular oxidative damage**

The effect of the applied chemotherapeutic regimen on patients’ antioxidant defenses as well as their levels...
The results from the chemiluminescent model systems revealed a decrease in the chemiluminescent signal, corresponding to a lower CL-SI index (Figure 4A and B). This effect is due to the plasma components possessing scavenging activity against reactive oxygen species (ROS) introduced into the system. The extent of the decrease was more noticeable in the system containing hypochlorite (CL-SI_{max} < 51%), compared to the one with superoxide anion radicals (CL-SI_{min} > 58%).

Table 2. Comparison of mean values of measured parameters (complete blood count and some basic biochemical parameters) before and after the administration of oxaliplatin/fluoropyrimidine chemotherapy regimen.

| Parameter                      | Before ChT (Mean ± SEM) | After ChT (Mean ± SEM) | P value (Wilcoxon test) |
|--------------------------------|-------------------------|------------------------|-------------------------|
| WBC × 10^9/L                   | 6.90 ± 0.38             | 5.86 ± 0.29            | p = 0.020               |
| Lymphocyte × 10^9/L            | 1.58 ± 0.09             | 1.52 ± 0.09            | p > 0.05                |
| Granulocyte × 10^9/L           | 4.70 ± 0.30             | 3.89 ± 0.23            | p = 0.014               |
| Monocyte × 10^9/L              | 0.48 ± 0.04             | 0.43 ± 0.02            | p > 0.05                |
| RBC × 10^12/L                  | 4.19 ± 0.08             | 4.35 ± 0.12            | p > 0.05                |
| Hg (g/dL)                      | 121.2 ± 2.41            | 125 ± 2.96             | p > 0.05                |
| HCT %                          | 36.99 ± 0.61            | 38.26 ± 0.90           | p > 0.05                |
| PLT × 10^9/L                   | 273.8 ± 16.94           | 220.1 ± 14.15          | p = 0.0004              |
| ALAT (U/L)                     | 30.91 ± 5.23            | 27.15 ± 3.99           | p > 0.05                |
| ASAT (U/L)                     | 35.97 ± 4.78            | 33.63 ± 3.59           | p > 0.05                |
| Total bilirubin (µmol/L)       | 12.25 ± 1.48            | 12.47 ± 0.99           | p > 0.05                |
| Creatinine (µmol/L)            | 78.13 ± 4.25            | 76.76 ± 6.67           | p > 0.05                |
| Glucose (mmol/L)               | 6.28 ± 0.24             | 6.31 ± 0.24            | p > 0.05                |

Figure 1. Distribution of dihydropyrimidine dehydrogenase level: scatter plots (A) with mean value and SD; and patient frequency distribution (B) according to their plasma level of DPD expressed as ng/mL.

Figure 2. Possible effect of DPD on some basic blood cell counts: White blood cell counts (A); Granulocyte counts (B); platelet counts (C).

Figure 3. Summary presentation of DPD effect on white blood cell count and granulocyte count. Data are mean values ± SEM.

Figure 4. Effect of oxidative stress markers is presented.
Figure 4. Antioxidant scavenging capacity properties of CRC patients' plasma detected using: luminol-dependent CL in a system of KO$_2$-generated superoxide anion radical (A); luminol-dependent CL in a system of NaOCl-generated hypochlorite (B); TBA-RS assay (C).

Table 3. Distribution of patients' plasma free-radical scavenging properties and extent of lipid peroxidation, divided into groups according to the DPD level.

|                        | Before ChT | After ChT | p-value |
|------------------------|------------|-----------|---------|
| **Luminol-dependent CL in a system of KO$_2$-generated superoxide anion radical** |            |           |         |
| CL-SI, %               |            |           |         |
| Group A                | 94.28 ± 10.42 | 103.4 ± 7.39 | > 0.05  |
| Group B                | 83.15 ± 11.81 | 92.54 ± 13.08 | 0.0026  |
| Group C                | 80.43 ± 10.38 | 89.16 ± 9.108 | 0.0061  |
| **Luminol-dependent CL in a system of NaOCl-generated hypochlorite** |            |           |         |
| CL-SI, %               |            |           |         |
| Group A                | 28.67 ± 4.984 | 35.65 ± 2.783 | 0.044   |
| Group B                | 32.71 ± 5.686 | 37.14 ± 6.409 | 0.014   |
| Group C                | 31.29 ± 6.850 | 38.58 ± 5.908 | 0.003   |
| **Thiobarbituric acid reactive substances – Lipid peroxidation assay** |            |           |         |
| MDA, nmol/L            |            |           |         |
| Group A                | 0.75 ± 0.5331 | 0.95 ± 0.2752 | > 0.05  |
| Group B                | 1.48 ± 0.9660 | 1.79 ± 0.9392 | 0.013   |
| Group C                | 1.26 ± 1.1060 | 1.68 ± 1.6801 | 0.024   |

Group A – patients with DPD below 0.15 ng/mL; Group B – patients with DPD varying from 0.15 to 0.39 ng/mL; Group C – patients with DPD higher than 0.39 ng/mL. Data are presented as mean values ± SD.

The relationships between some systemic inflammatory response markers and the measured parameters concerning the free-radical scavenging properties and the extent of lipid peroxidation were explored (Figure 5). We chose the platelets lymphocyte ratio (PLR) and lymphocyte-to-monocyte ratio (LMR), which have been recognized and substantiated to influence cancer progression [25]. Spearman test demonstrated a satisfactory extent of correlation between the utilized systemic inflammatory response markers and the observed generation of MDA products. The generation of MDA products correlated negatively with PLR and positively with LMR.

Discussion

The patients recruited in the study are a representative group due to the fact that their clinic-pathological and
demographics characteristics are in agreement with the existing information from retrospective population-based cohort studies. A similar number of patients from both sexes were enrolled in our study. That is in accordance with the tendency for a slightly elevated incidence rate in men compared to women [26]. The age of the women included was significantly more advanced (mean age 66.55 f vs. 62.15 m). The patients' age distribution in the four presented groups (Table 1) was in accordance with the observed tendencies of increased likelihood of disease development with the increase of age (more than 65% of the patients were in the age range of 60 - 80) and the disturbing trend of disease 'rejuvenation' (more than 1/10 of the patients being under 50 years old) [27]. The classification, based on the initial onco-localization, denoted location of the initial tumors mainly in the rectum and in the sigmoid colon, which is in agreement with data cited in scientific literature [6]. Most patients were diagnosed with advanced regional and metastatic stages (III and IV) of the disease, which is the tendency worldwide [28]. The observed distribution of the DPD level of Bulgarian patients with CRC diagnosis shows some similarity with the data from previous studies in other countries performed using different methods for enzyme estimation: (1) large range of variation of the DPD level and (2) small interval around the mean value, where none of the patients recruited in the study had DPD levels measured [29].

The toxicity of fluoropyrimidines is well known to be associated with neutropenia and thrombocytopenia. Many research groups have suggested a relation between DPD enzyme concentration/activity and some toxic effects associated with the treatment [30, 31]. Our experiment demonstrated that lower DPD values were associated with a decrease in the white blood cell count and the granulocyte count. The threshold of the decrease in both parameters, compared to their initial value, was 25%. The patients having a more than 25% decrease in these two parameters had a mean DPD level less than 60% of that of the patients where only moderate changes had been observed (Figure 3). The only trend toward a decrease in the DPD level without statistical significance was observed in severe cases of a drop in the platelet counts.

It is a well-known fact that CRC is associated with changes in the redox homeostasis in patients [32], alterations in plasma and tissue levels of specific markers of oxidative stress [33–35], as well as changes in enzymatic and non-enzymatic antioxidants' concentrations and activities [36–38]. In our previous work on the plasma antioxidant capacity in CRC patients, we demonstrated that the FOLFOX-4 regimen and the patients' DPD level had a different effect, depending on the indicator used in the system. No statistically significant changes in the plasma antioxidant capacity, using the ABTS assay and decreased ferric reducing ability, were observed [24]. The application of the more sensitive and specialized chemiluminescent systems supported the weakened antioxidant barrier observed in the FRAP assay. The present research demonstrated a decrease in the blood plasma's capacity to eliminate the hypochlorite and superoxide anion radical after the infusion. The differences in the CL signals and plasma reactivity against the used ROS could be attributed to the higher reactivity of hypochlorite ions compared to some other ROS. The observed effect of deterioration after the regimen could be attributed to many factors, such as ROS overproduction associated with some of the mechanisms of actions of the applied chemotherapeutic treatment and the subsequent exhaustion of antioxidant pools in the recruited patients. Such effects have

![Figure 5. Scatter plot showing correlation analysis of lipid peroxidation extent and some inflammatory response markers in 38 patients with CRC: platelets lymphocyte ratio (A); lymphocyte-to-monocyte ratio (B). Spearman's approach was used to estimate the correlation. The corresponding correlation coefficient and p-values are indicated in the scatter plot.](image-url)
often been observed by many research groups during cancer treatment [39, 40]. The elevation in the MDA products, indicating intensification of lipid peroxidation and increased sensitivity to oxidative molecular damage, is another evidence for the decrease in radical-trapping blood plasma capacity.

Conclusions

The results of the present investigation are in accordance with the already known facts concerning the side effects of the FOLFOX-4 regiment and the associated changes in redox homeostasis. Increased oxidative damage and a decline in antioxidant capacity were observed after the administration of the chemotherapeutic regimen. Genetic predisposition to effectively metabolize and tolerate the applied therapy, i.e. the level of DPD, also modulates some aspects of the observed changes in the mentioned hematological and redox parameters. The fact that the associated oxidative changes were observed not locally in the tumor area but in the plasma indicates that the patients had been exposed to systemic oxidative stress. Other contributing factors could include a possible systemic circulation of malignant cells and overproduction by activated, circulating immune cells.

Disclosure statement

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Author statement

All persons meeting the authorship criteria are listed as authors. The authors certify that they have participated sufficiently in the work to take public responsibility for the content. Each author certifies that material or similar material to that presented in this article has not been and will not be published in any other publication.

Data availability

The data that support the findings of this study are available from the corresponding author, N. H-A, upon reasonable request.

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