Does Dihydropyrimidine Dehydrogenase Level Modify Plasma Antioxidant Capacity in Colorectal Cancer Patients Treated with Fluoropyrimidines?

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Abstract

Introduction: Colorectal cancer is the third most common cancer type worldwide. Fluoropyrimidines and their prodrug-based regimens are widely applied as primary medications. The main enzyme responsible for the rate-limiting step in pyrimidine and for the 5-fluorouracil catabolism is dihydropyrimidine dehydrogenase (DPD).

Aim: We aimed to screen DPD level and the changes of plasma antioxidant capacity of colorectal cancer patients on 5-fluorouracil regimen.

Materials and methods: Human DPD Elisa Kit based on sandwich enzyme-linked immune-sorbent assay and spectrophotometric methods (FRAP and ABTS) were used in the study.

Results: No statistically significant changes in plasma scavenging activity according to the results obtained in the ABTS system have been observed after evaluating all patients and considering DPD concentration. A decrease of the ferric reducing ability of patients’ plasma taken after the administered treatment was found. The increase of DPD level is accompanied by a decrease in the p values and therefore the statistical significance of the differences increases.

Conclusions: Based on the aforementioned observations, it could be concluded that some aspects of plasma antioxidant capacity and individuals’ antioxidant status might be involved in the pathogenesis of the disease and could be altered by the activity of some enzymes. The cancer therapy in question, by the specificity of its mechanism of action, can modify patient’s oxidative status.

Keywords

ABTS, colorectal cancer, dihydropyrimidine dehydrogenase, fluoropyrimidines, FRAP
INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer type worldwide and the fourth most common cause of death according to GLOBOCAN 2018. CRC usually develops from the glandular epithelial cells lining the large intestine, which are known to divide rapidly and have high metabolic rate. The disease affects men and women almost equally and in 70% to 80% of the cases it occurs sporadically. Survival rate highly depends on the stage, 70% for regional to 10% for patients with distant metastatic cancer.6 Due to inconspicuous early symptoms, most patients are diagnosed in the advanced stage of the disease, which is associated with lowering of the success of surgical intervention and poor prognosis. Many factors have been pointed to potentially contribute to an increased risk of colorectal cancer including obesity, high-meat, high-calorie, fat-rich diet, smoking, age, family history of CRC, and overconsumption of alcohol. The main strategies for treatment depend on the disease stage and are comprised of a combination between surgical procedure with chemotherapy and/or radiotherapy.2,3

Oxidative stress has been demonstrated as one of the risk and contributing factors of the pathophysiological mechanism of colorectal carcinogenesis. It is a well-known fact that cancer patients exhibit changes in their antioxidant status and antioxidant defense systems.4 Alterations in the quantity of both the low molecular weight substances with antioxidant effect and the enzymatic antioxidants have been observed quite often.5

Apart from this, additional increase of ROS generation in patients’ bodies might be observed as a result of the applied chemotherapeutic treatment suggesting increased oxidative stress compared to that induced by the cancer disease itself. This could be associated with the mechanism of action of the drugs and/or by its interference with some of the compensatory oxidative stress mechanisms.

Fluoropyrimidines and their prodrug-based regimens are widely applied as primary medications in the treatment of colorectal cancer. Dihydropyrimidine dehydrogenase is a ubiquitous enzyme, mostly represented in the liver. It is responsible for the rate-limiting step in pyrimidine and respectively in 5-fluorouracil (5-FU) catabolism. That makes it essential for treatment efficacy and associated toxic reactions.6 In many studies, application of regimens including fluoropyrimidines has been associated with increased reactive oxygen species generation, oxidative molecular damage and suppression of the antioxidant defense system.7,9

AIM

From this point of view obtaining additional information concerning therapy, dosage, potency for metabolization, efficacy and toxicity is an important task when discussing strategies for personalized treatment.

Herein we present results from the first pilot study concerning estimation of the DPD level of Bulgarian patients with histologically proven diagnosis of colorectal cancer receiving 5-FU. We screened their DPD levels and the changes of their plasma antioxidant capacity.

MATERIALS AND METHODS

The study was conducted within a period of six months – from July 2019 to February 2020 – in the Department of Oncology of Sofiamed University Hospital. The inclusion criteria involved patients aged between 18 and 80 years willing to give blood samples for the purpose of the study, to provide a signed voluntary informed consent to participate and to complete a structured questionnaire, including information about their smoking and eating habits. All patients with any other serious comorbidity or those who have received any other pharmaceutical products that could influence the studied parameters were excluded from the study. Medical histories based on the information provided by the patients accompanying their medical records and the patients’ objective condition were assessed. The diagnostic methods applied include full blood count and general biochemistry tests, imaging methods (lung and heart X-ray, abdominal ultrasound, computed tomography, nuclear magnetic resonance) and histological verification. Blood samples were collected from 38 patients by venous puncture using heparinized tubes at baseline and before the infusion of the drug from the next cycle of treatment.

Dihydropyrimidine dehydrogenase estimation

Human DPD Elisa Kit based on sandwich enzyme-linked immune-sorbent assay was used. This method is based on detection of the pre-coated anti-DPD antibody onto 96 well plates via biotin conjugation. On the basis of the measured absorbance at 450 nm, DPD concentration [ng/ml] was calculated.

Antioxidant capacity estimation

ABTS assay

Plasma total antioxidant capacity was estimated by the method described by Re et al.10 The method is based on decolorization of the blue-green solution of the preformed stable ABTS cation radical which has absorbance maximum at 650, 734, and 820 nm. The observed decline of the absorbance at 734 nm indicates the level of eliminated radicals and is directly related to the antioxidant capacity of the patients’ plasma. The radical solution was prepared by mixing equal parts (v/v) 14 mM ABTS stock solution and potassium persulfate 2.45 mM (final concentration) the day before the experiments. The obtained solution was stored for 16 to 18 hours in dark at 4°C. The working solution of
the radical was prepared by dissolving the stock solution until an absorbance of 0.700±0.005 at 734 nm was achieved. Two types of samples with equivalent radical concentrations were prepared: controls with no plasma and samples containing the patients’ plasma. The obtained results were presented as RSA [%] (the ratio in percentages between the subtraction of the absorbance of the sample containing plasma from that of the control sample on one hand and the absorbance of the control on the other).

$$RSA, \% = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

**FRAP assay**

The procedure was performed according to Benzie and Stain. The method is based on the reduction of the ferric-tripyridyltriazine complex ($Fe^{III}$-TPTZ) to its ferrous, blue colored form ($Fe^{II}$-TPTZ) by plasma antioxidants. The working FRAP reagent solution was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-Tripyridyl-s-triazine (TPTZ) solution (in 40 mM HCl) and 20 mM $FeCl_3\cdot6H_2O$ in a 10:1:1 ratio. Final plasma dilution in the reaction mixture was 1/34. The mixture is prepared just before use and heated to 37°C. The absorbance, exactly 4 min after starting the reaction, of the obtained blue colored product and the blank were determined at 593 nm. 1 mmol/L $FeSO_4$ was used as a standard solution. The results were expressed as millimoles of $Fe^{II}$ equivalents using $FeSO_4$ calibration curve.

**Statistical analysis**

All measurements were performed in triplicates and the obtained values were expressed as mean ± SD. The significance of the observed differences was estimated using parametric statistical methods. Data sets were compared using paired sample t-test. Levels of significance of $p<0.05$ were accepted. Data were presented as “box and whiskers” plots: each box summarizes data from 38 values corresponding to 38 patients (each individual value being mean of triplicate measurements). Each plot is bordered at the first (Q1) and third (Q3) quartile of the variable and is cut by a horizontal line corresponding to the median. Whiskers correspond to minimal and maximal value.

**RESULTS**

**Clinical characteristics**

Thirty-eight patients were included in the present study. The baseline clinical characteristics of the recruited patients with histologically confirmed diagnosis of colorectal cancer are summarized in Table 1. The 38 patients with CRC were distributed equally among women and men. The majority were in stage three and stage four (34.2% and 44.7%, respectively) and none of them was stage one. This is indicative for the presence of local (stage III) and distant (stage IV) metastasis and correlates with the observed worldwide tendency for diagnosis in the advanced stage of the disease. 81.6% of the studied patients were in medium grade.

![Table 1. Clinical and histopathological features of colorectal cancer patients](image)

| Patients (n=38) | Gender | Age |
|----------------|--------|-----|
| Male           | 19     | 64  |
| Female         | 19     | 68  |
| Mean           | 64     |     |
| Median         | 68     |     |
| Range          | 43 ↔ 80|     |

| Site | Col | Rectum | Colon | Rectum | Number |
|------|-----|--------|-------|--------|--------|
| Colon| 22  |        |       |        |        |
| Rectum| 16 |        |       |        |        |

| Tumor stage | Stage I | Stage II | Stage III | Stage IV | Number |
|-------------|---------|----------|-----------|----------|--------|
| Stage I     | 0       |          |           |          |        |
| Stage II    | 8       |          |           |          |        |
| Stage III   | 13      |          |           |          |        |
| Stage IV    | 17      |          |           |          |        |

| Histological grading | Low | Medium | High | Number |
|-----------------------|-----|--------|------|--------|
| Low                   | 5   |        |      |        |
| Medium                | 31  |        |      |        |
| High                  | 2   |        |      |        |

| DPD [ng/ml] | < 0.15 | 0.15 ↔ 0.27 | 0.27 ↔ 0.39 | 0.39 ↔ 0.51 | > 0.51 |
|-------------|--------|-------------|-------------|-------------|--------|
|             | 5      | 5           | 9           | 2 (all higher than the mean) |        |
|             | 4      | 7           | 4           | 2           |        |
|             | 4      | 7           | 4           | 2           |        |

Antioxidant capacity

In order to quantify the effect of chemotherapy on patients’ plasma antioxidant capacity we have analyzed the distribu-
tion of its radical scavenging activity (RSA, %) and ferric reducing ability before and after the first infusion in the applied treatment regimens (Fig. 1).

The observed RSA [%], determined using the ABTS assay before the administration of 5-FU was in the interval between 35.46% and 50.06% (Fig. 1A). After the application of the treatment, the lowest and highest values of RSA were, respectively, 36.82% and 48.87%. The estimated mean values from these results were 43.17% before and 43.02% after the infusion. Comparing the means of the two groups of dependent samples we see that they are statistically identical in the two related groups (before and after chemotherapy) (p=0.62).

The obtained data from the FRAP assay before 5-FU administration indicate that the ferric reducing ability expressed as mM FeII equiv. was ranging from 0.29 mM to 0.79 mM (Fig. 1B). After the drug administration the lowest and the highest values were, respectively, 0.26 mM and 0.70 mM. The estimated mean values were 0.48 mM and 0.45 mM. Comparing the means of the two groups of dependent samples this time, we observe statistically significant change (p<0.001) indicating alterations in the mM FeII equiv. after the applied treatment. The effect consists in a decrease of the plasma capability to convert ferric-tripyr-rydiltriazine complex (FeIII-TPTZ) to its ferrous form.

As a next step, we followed the effect of the applied treatment on the determined antioxidant capacity using the ABTS and FRAP assay in relation to patients' DPD level. In the three subgroups presented in Fig. 2, when applying the ABTS assay, we did not observe conversion of the witnessed lack of statistically significant effect presented in Fig. 1. The observed p values were 0.40, 0.18, and 0.60 for patients' plasma in group I, group II, and group III, respectively.

Figure 1. Distribution of patients' plasma radical scavenging activity and ferric reducing ability. Parameters' values before and after administration of the chemotherapeutic treatment, determined using ABTS (A) and FRAP (B) assays, are shown. p<0.05 was considered to indicate statistical significance.

Figure 2. Distribution of patients' plasma radical scavenging activity. Parameters values, determined using the ABTS assay, before and after the administration of chemotherapeutic treatment are shown, divided in groups according to their DPD level. A. Group I: patients with DPD below 0.15 ng/ml. B. Group II: patients with DPD in the interval 0.15 to 0.39 ng/ml. C. Group III: patients with DPD higher than 0.39 ng/ml. p<0.05 was considered to indicate statistical significance.
In the FRAP system, the effect after the applied treatment varied depending on the DPD level. In group I the mean values of the group of samples before and after were, respectively, 0.47 and 0.45 mM Fe\textsuperscript{II} equiv. and no statistically significant difference between the mean values was observed ($p=0.81$) (Fig. 3). In groups II and III, where we had higher DPD levels, we observed conversion of the effect compared to group I, i.e. statistically significant decrease of the mean plasma ferric reducing ability after the infusion of 5-FU. In group II, the plasma ferric reducing ability decreased from 0.51 mM to 0.47 mM Fe\textsuperscript{II} equiv. ($p=0.022$) and in group III from 0.46 mM to 0.43 mM Fe\textsuperscript{II} equiv. ($p=0.0021$).

**DISCUSSION**

ROS generation within normal physiological ranges takes place in all living organisms. There is substantial scientific evidence suggesting redox dyshomeostasis, altered scavenging capacity of biological fluids and increased free radical generation are phenomenologically related to different events associated with oncological disease development and treatment. Increased generation of free radicals is crucial for tumor cell proliferation, angiogenesis, cancer cell migration and metastatic processes which are essential for cancer development and disease progression. Conversely, it is also one of the basic chemotherapeutic strategies for cellular damage and induced necrosis of the malignant cells.\textsuperscript{13} This complicated dual role has made the use of methods based on the estimation of the cumulative effect of all plasma components an appropriate strategy which may provide relevant biological information when combined with patients’ individual clinical and histopathological features.

We have applied different spectrophotometric methods for estimation of plasma antioxidant capacity. Our results denoted that the determined effect on the alterations due to the conducted treatment is different.

In the system using the biologically irrelevant (not naturally occurring in the human body), ABTS radical no statistically significant difference were observed between the determined scavenging activity before and after the treatment when evaluating all patients and regarding DPD concentration. This suggests no effect after the applied treatment on patients’ plasma capability to decrease the concentration of the stable ABTS radical and, respectively, its scavenging activity.

On the contrary, according to the results obtained using the FRAP system after the applied treatment, a decrease of the ferric reducing ability of patients’ plasma was observed. When studying the effect of the observed decrease of DPD levels, it is evident that for the patients’ with DPD levels below 0.15 ng/ml, the observed difference in the plasma ferric reducing capacity before and after treatment is not significant. With the increase of DPD levels, these differences become statistically significant: the $p$ values decrease and the statistical significance of the differences increases with the rise in DPD levels.

Observing different results when estimating the antioxidant capacity of biological samples and other types of probes is not uncommon and has been described by many authors. It results from the different indicators applied in
DPD Level Modifies Plasma Antioxidant Capacity

CONCLUSIONS

Based on the aforementioned observations, it could be concluded that some aspects of plasma antioxidant capacity and individuals’ antioxidant status might be involved in the pathogenesis of the disease and could be altered by the activity of some enzymes. The cancer therapy in question, by the specificity of its mechanism of action, can modify patient’s oxidative status.

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Изменяет ли уровень дигидропиримидин дегидрогеназы антиоксидантную способность плазмы у пациентов с колоректальным раком, получавших фторпиримидины?

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Резюме

Введение: Колоректальный рак – третий по распространенности вид рака во всём мире. Фторпиримидины и схемы на основе их пролекарств широко используются в качестве основных лекарственных средств. Основным ферментом, ответственным за фактор, ограничивающий скорость пиримидина, и за катаболизм 5-фторурацила, является дигидропиримидиндегидрогеназа (ДПД).

Цель: Наша цель состояла в том, чтобы проверить уровни ДПД и изменения антиоксидантной активности в плазме у пациентов с колоректальным раком, получавших 5-фторурацил.

Материалы и методы: В исследовании использовали набор Elisa для ДПД человека, основанный на иммуноферментных анализах и спектрофотометрических методах (FRAP и ABTS).

Результаты: Не было обнаружено статистически значимых различий в активности очистки плазмы согласно результатам, полученным с помощью системы ABTS, после оценки всех пациентов и в учете концентрации ДПД. Отмечалось снижение способности снижать содержание железа в плазме у пациентов, принимавших после лечения. Повышение уровня ДПД сопровождается уменьшением значений р, поэтому статистическая значимость различий возрастает.

Заключение: На основании вышеизложенных наблюдений можно сделать вывод о том, что некоторые аспекты антиоксидантной способности плазмы и индивидуального антиоксидантного статуса могли играть роль в патогенезе заболевания и могут быть изменены активностью определённых ферментов. Обсуждаемая противоопухолевая терапия может изменять окислительный статус пациента за счет специфичности механизма её действия.

Ключевые слова

ABTS, колоректальный рак, дигидропиримидиндегидрогеназа, фторпиримидины, FRAP