# Characteristics of Redox-Metabolism in Patients with Metastatic Colorectal Cancer: Clinical Significance

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## ABSTRACT

Colorectal cancer (CRC) is a heterogeneous disease with a high mortality rate, poor prognosis, high percentage of relapses and metastases. Search for the reliable indicators of CRC development and progression is still necessary for the improvement of long-term results of treatment. Objective of this work was to investigate generation rate of superoxide radicals (SR), nitric oxide (NO) levels, matrix metalloproteinase (MMP) activity, lactofferin (Lf) content, amount of "free" iron, Ki-67 expression levels in tissue of tumors in patients with metastasis of II-III stages (T2-4N0-2M0-1G2-3).

Tumor tissue samples from 51 CRC patients diagnosed with metastatic CRC (mCRC) were investigated. 28 patients had stage II disease (T2-4N0M0G2-3) while 23 – stage III (T2-4N1-2M0-1G2-3). In 100% of cases, tumors of the histological type were verified as intestinal adenocarcinomas of different degrees of differentiation (G2-G3). Levels of Lf, "free" iron were determined by conventional X-band electron paramagnetic resonance (EPR) at T = 77 K. SR generation rate and NO levels in mCRC tissues were determined by spin-trapping EPR (TEMPO-H and diethyldithiocarbamate, Fe-DETC; Sigma, USA). The activity of matrix metalloproteinases MMP-2 and -9 was determined by the method of zymography in a polyacrylamide gel. The determination of the Ki-67 expression level was performed by immunofluorescence.

It is shown that for tumors of patients with regional lesions of the lymph nodes (category N1-2), SR generation rate is 2.8 times higher than those in tumors of patients without regional metastases (category N0). In G3-tumors the SR generation rate is 1.7 times higher as compared to G2-tumors ($p < 0.05$). An inverse correlation is found between the SR generation rate and the level of differentiation ($r = -0.61$; $p < 0.05$). The activity of MMP-2 and MMP-9 correlates with the SR generation rate and NO level ($r = 0.44 \div 0.53$, $p < 0.05$). A direct correlation was found between the level of Lf in mCRC tissue and the stage of the disease ($r = 0.42$), the level of "free" iron ($r = 0.61$) and the inverse correlation with the degree of differentiation of tumors ($r = -0.57$; $p < 0.05$) was established.

The determined tumor redox metabolism parameters in patients with mCRC can be used to predict the course of the disease.

## Introduction

Colorectal cancer (CRC) is a heterogeneous disease with high mortality and poor prognosis and is one of the most frequent and most serious malignancies in humans [1]. Malignant neoplasms of the colon are in the structure of general cancer incidence in Ukraine, the fourth place among women and the fifth among men. CRC in the structure of female cancer mortality ranks second and fourth in the structure of male mortality [2]. A high percentage of relapses and metastases require a significant improvement of treatment of metastatic CRC (mCRC) [3,4]. Therefore, a study

of the etiology and pathogenesis, the search for reliable tumor markers of the prognosis and the effectiveness of the treatment of CRC remains a priority. Unregulated levels of generation of superoxide radicals (SR) and nitric oxide (NO) alter the redox balance and redox-dependent signaling, are common signs of cancer progression and resistance to treatment [5,6]. In addition, impaired iron metabolism and the functions of iron-containing proteins in mCRC tissue can enhance proliferative activity and, therefore, also form an aggressive tumor phenotype [7]. Cellular and extracellular redox homeostasis is an important regulator of the tumor and metastatic microenvironment, its indicators can be used as additional prognostic markers in mCRC.

The purpose of this work was to investigate the rates of SR, NO generation, matrix metalloproteinase activity, levels of transferrin (TF), lactoferrin (Lf), "free" iron, expression of Ki-67 factor in the tissue of tumors of stage II – III of mCRC patients (T2-4N0-2M0G2-3) and follow their changes in connection with some clinical and pathological characteristics.

## Materials and Methods
All patients were divided into subgroups according to gender, age and disease stage (TNM), size and histological type of tumor. Tumor tissue samples were examined for 51 patients (T2-4N0-2M0-1G2-3) (28 men and 23 women, mean age $61 \pm 1.4$ years) with a diagnosis of metastatic colorectal cancer (mCRC). 28 patients had disease stage II (T2-4N0M0G2-3) and 23 were at the stage III (T2-4N1-2M0-1G2-3). In 100% of cases adenocarcinoma of the rectum of various degrees of differentiation (G2-G3) was morphologically verified. The patients were treated at the clinic of the National Cancer Institute. For all patients the diagnosis, the stage of the disease and the presence of metastases were verified in accordance with the requirements of evidence-based medicine in the course of relevant clinical and instrumental examinations, morphologically.

Samples of clinical material were obtained prior to treatment during biopsy and surgery. In samples of tumor tissue, the level of Tf, Lf, and "free" iron was determined by electron paramagnetic resonance (EPR) at $T = 77$ K [8-10]. Simultaneously with the spectra of the samples, spectra of an independent intensity standard were recorded (specially oriented single crystal of ruby with the known g-factor and number of paramagnetic centers). The intensity of the standard (reference) was taken as 1 arbitrary unit (1 a.u.) measurements. SR generation rate and NO level in CRC tissue was determined by the EPR method using spin trap technology (TEMPO-H, and diethyldithiocarbamate (Fe-DETC) from Sigma, USA were used as spin traps for SR and NO, correspondingly) [11]. Morphologically unchanged intestinal mucosa served as a control. All participants expressed their prior written consent to take part in the research. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later amendments. The investigated species were stored at liquid nitrogen temperature ($T = 77K$).

Concentrations of matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9) in samples both in active and latent forms were determined by gelatine zymography, the polyacrylamide gel electrophoresis-based method with using sodium dodecyl sulfate (SDS). MMP-2, 9 are considered to be the major MMPs involved in invasion and metastasis of cancer because of their capacity to degrade type IV collagen, an important component of basement membranes [12]. After the gel washing active forms of MMP-2 and MMP-9 were visualized in the form of discolored strips on a blue background, their localization was determined by molecular weight standards (Sigma, USA, 72 and 92 kDa, correspondingly). Proteolytic activity was estimated from the area of clear lysis bands of degraded protein on a uniformly blue background and was expressed in arbitrary units (a.u.). 1a.u. of gelatinase activity in tumor tissue is taken as the activity of 1 µg of enzyme in 1 g of tissue. TotalLab 1.01 program tool was used for the calculation and report [8].

Ki-67 is a protein widely applied in routine clinical work and has been also studied in relation to the progression of human CRC and survival [13], although its prognostic value remains controversial [14]. Determination of Ki-67 expression in the CRC biopsy material and morphologically unchanged intestinal mucosa was performed by immunofluorescence using monoclonal antibodies Anti-Ki-67 (clone MIB-1, Santa Cruz, CA, USA) and (as secondary one) fluorescent-containing Alexa Fluor 546 antibody (Invitrogen, USA). Cell nuclei were visualized using 4,6-diamino-2-phenylindole (DAPI). The studies were carried out using a Carl-Zeiss LSM 510 laser confocal scanning microscope with a 32-channel automatic polychromatic META-detector (GaAsP) and oil immersion lens 40X / 1.4NA.

Statistical processing of data was provided using variational statistics methods using programs GraphPadPrism 6 and Origin 7.0. The data are presented as mean $\pm$ SD (standard deviation). Correlation analysis was performed taking into account the Spearman correlation coefficient. The significance of differences between the indicators was assessed using Student's t-test. The statistical significance was accepted for $p < 0.05$.

## Results and Discussion
Figure 1 shows the rate of superoxide generation rate and NO level in mCRC tissues. It is seen that in tumors of patients with regional lesions of the lymph nodes (category N1-2) SR generation rate is significantly (2.8 times, $0.78 \pm 0.18$ vs. $2.20 \pm 0.43$) higher than those in tumors of patients without metastases (category N0). A similar trend is observed for NO: its level in the tissue of tumors with metastases to regional lymph nodes significantly exceeds the level in the tumor tissue of patients without metastases ($1.73 \pm 0.36$ vs. $3,67 \pm 0.28$). It is established that SR generation rate and NO level correlates positively with regional metastasis ($r = 0.63$ and $0.69$, respectively; $p < 0.05$).

A significant increase in the SR generation rate of CP generation in the mCRC tissue was revealed as compared to that in the
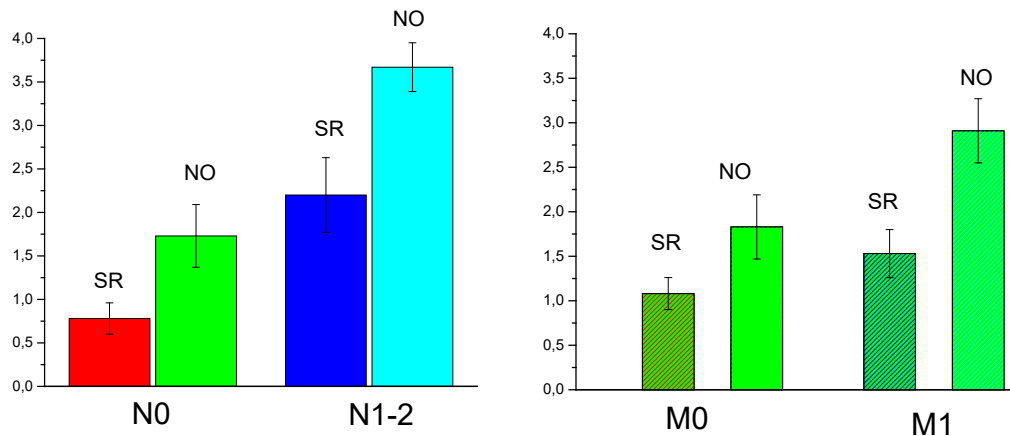
**Figure 1:** Relations between the SR generation rate and NO level with regional (left panel) and distant (right panel) CRC metastasis. Data are presented as mean ± SD for n = 28(N0); 23 (N1-2); 33(M0) and 18 (M1), correspondingly.

morphologically unchanged intestinal mucosa (Figure 2). In moderately and poorly differentiated mCRC tumors SR generation rate was measured to be 0.83 ± 0.14 and 1.40 ± 0.21 nmol / g tissue / min, respectively. In the morphologically unchanged intestinal mucosa this indicator was 0.29 ± 0.06 nmol / g of tissue / min. An inverse correlation relationship was established between the SR generation rate in mCRC tissue and the degree of differentiation (r = -0.61; p <0.05).
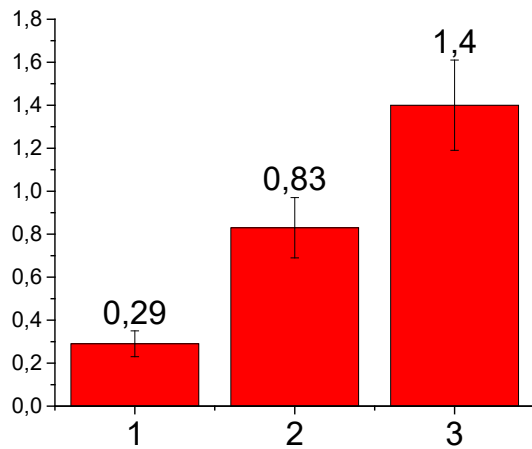


**Figure 2:** SR generation rate for 1 - morphologically unchanged intestinal mucosa (n =8); 2 - mCRC of moderate degree of differentiation (n = 24); 3 - low degree of differentiation mCRC (n = 27). Data are presented as mean ± SD.

The levels of MMP-2 and -9 activities in mCRC tissue were investigated depending on the stage of the disease and the level of metastasis. A relationship between these indicators, SR generation rates and NO levels in the tumor was found.

The concentration of active forms of MMP-2 for the whole series of 39 investigated tumor samples was in the range 0.1 ÷ 43.3 a.u., the average value was 8.7 ± 4.9 a.u. For MMP-9 variation in the range 0.05 ÷ 33.8 a.u. was observed with the average value of 8.3 ± 5.9 a.u. No correlation between the activity of both gelatinases and

gender, age of patients, T category was revealed. But in tumors with a degree of differentiation G3, the concentration of active forms of MMP-2 was almost 4 times higher than in mCRC with a degree of differentiation G2.

The activity of MMP-2 in a tumor at stage III of the disease is found to be 13.7 ± 3.5 a.u. that is significantly higher compared with stage II (7.4 ± 3.3 a.u., Figure 3). The activity of MMP-9 increases from stage II to stage III, but this difference is not statistically significant.
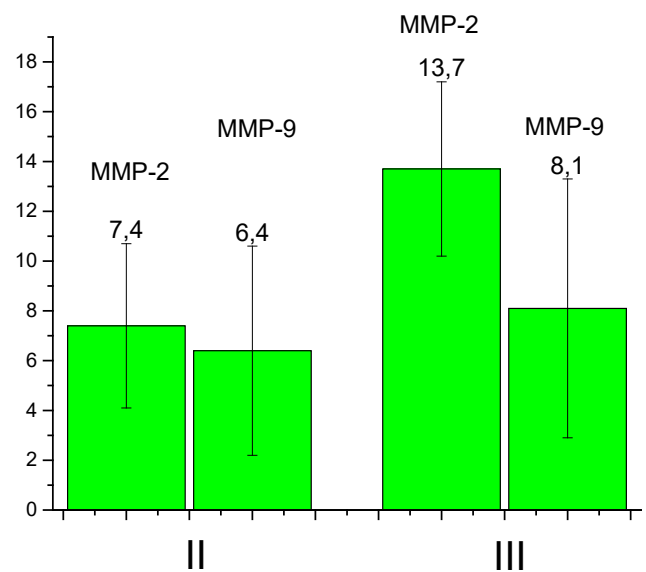


**Figure 3:** MMP-2 and 9 activities depending on the stage of the disease. Data are presented as mean ± SD for n = 28 (Stage II) and 23 (Stage III).

It was revealed that the indicators of MMP-2 and MMP-9 activity in mCRC tissue correlate with the SR generation rate and NO level (r = 0.44 ÷ 0.53, p <0.05). In addition, all the above indicators are found to be in direct positive dependence on the stage of the disease (r = 0.53 ÷ 0.68, p <0.05).

The relationship between the activity of gelatinases in mCRC and the metastatic level is analyzed. Despite the fact that the concentration of active forms of gelatinases in the tumor in N1-2 category is slightly higher than this in the N0 category, no significant difference between them was found (p> 0.05, Figure 4). Maximum activation of latent forms of MMP-2 in tumors with M0 indicates a significant increase in the destruction of the extracellular matrix at that stage of the tumor development when distant metastases are not clinically established, i.e. probably a formation and / or dissemination of tumor cells occurs. The results obtained are correlated with the data on the ability of the tumor through the corresponding signaling pathways to create a favorable microenvironment in the so-called pre-metastatic niches [15-18].

Thus, while SR generation rates and NO levels correlate positively with regional metastasis (Figure 1, left panel), gelatinase activity correlate negatively with distant metastasis (Figure 4, right panel). Such "down-regulation" of MMP activity can be explained by the fact that at ultrafast SR generation rates and in the conditions of the enhancement of oxidative processes, which can be observed at the terminal stage of the tumor process during secondary metastasis, a disintegration of signaling pathways and regulatory links may lead to a violation of the MMP activity [19,20].

It is known that under conditions of malignant growth, the redox state of tumors is largely determined by changes in oxygen metabolism in mitochondria and iron-containing proteins. "Iron is an essential element that promotes the proliferation of cells and their growth. Ions of "free" iron (labile iron pool, LIP) take part in redox reactions in cells, forming their redox state. The nature of the intensive broad EPR signal at $g = 2.2–2.4$ that often appears in the tumor affected tissues is connected with the ferritin molecules or non–ferritin (anti) ferromagnetically ordered iron–containing (nano) particles" [9,10,21,22]. Multifunctional iron-containing glycoprotein Lactoferrin belongs to the iron-binding Tf family which is produced by glandular cells and neutrophils in infected tissue and blood during the inflammatory process. Lf is one of the main components of the innate immune system, regulates bioavailability of iron in realization of cells' metabolic functions, specifically proliferation. So, "free" iron (EPR detected at $g = 2.2–2.4$) and Lf (with the EPR signal corresponding to the complexes of $Fe^{3+}$ ions at $g = 4.3$) can be attributed to the important factors of tumor growth, their microenvironment and metastasis [10,21,22].

It was established that in tumors with moderate and low degree of differentiation, the Lf content was $0.9·10^{15} ± 0.14·10^{15}$ and $32.0·10^{15} ± 3.6·10^{15}$ spins / g of tissue, respectively ($r = -0.51$; $p < 0, 05$), whereas in a morphologically unchanged intestinal mucosa this signal was not detected (Figure 5). On the other side, in morphologically unchanged intestinal mucosa a signal with $g = 4.3$ was recorded, which corresponds to the spectral characteristics of TF, but not detected in mCRC tissues. The level of TF in the morphologically unchanged intestinal mucosa was $0.6·10^{15} ± 0.3·10^{15}$ spins / g of tissue. In addition, a direct correlation was established between the LF content and the stage of the disease ($r = 0.42$; $p < 0.05$).

An increase of the level of "free" iron in the body, taking into account its high pro-oxidant potential, can lead to an increase in the rate of SR generation and DNA mutations [23,24]. In malignant cells, the processes in the regulation of which superoxide radicals and iron ions are involved, undergo significant changes, which may contribute to tumor progression by increasing proliferation [25]. In moderately and poorly differentiated tumors of the rectum the level of "free" iron was $2.8·10^{17} ± 1.1·10^{17}$ and $9.4·10^{17} ± 2.1·10^{17}$ spins / g of raw tissue, respectively (Figure 6). In morphologically unchanged intestinal mucosa this indicator did not exceed $1.1·10^{17} ± 0.4·10^{17}$ spins / g of raw tissue. Thus, the level of "free" iron in G3 tumors of the rectum was 9 times higher than that in the morphologically unchanged intestinal mucosa and three times higher than for G2 tumors ($p < 0.05$).
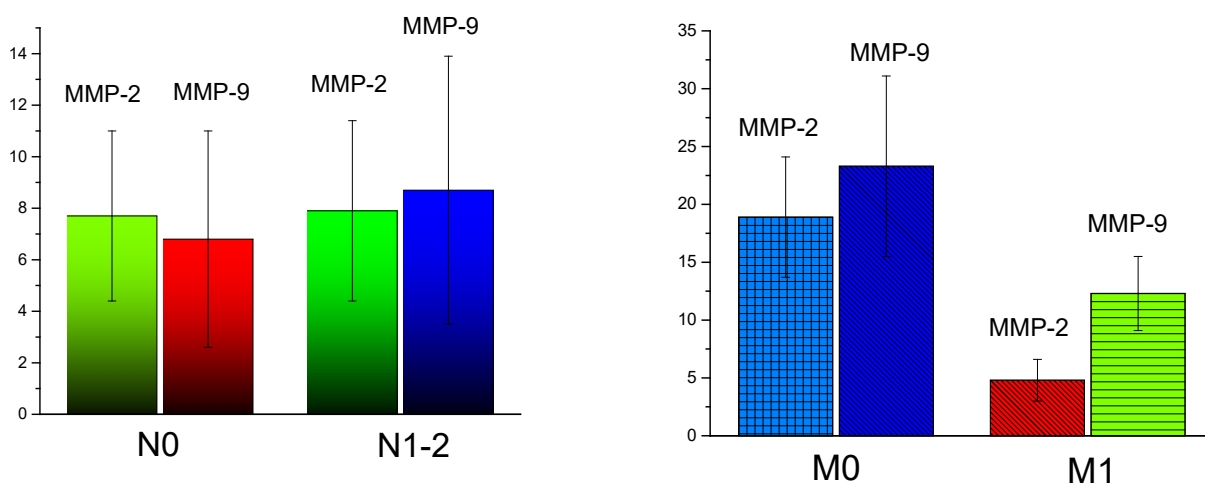


**Figure 4:** MMP-2 and 9 activities with regional (left panel) and distant (right panel) CRC metastasis. Data are presented as mean ± SD for n = 28(N0); 23 (N1-2); 33(M0) and 18 (M1), correspondingly.
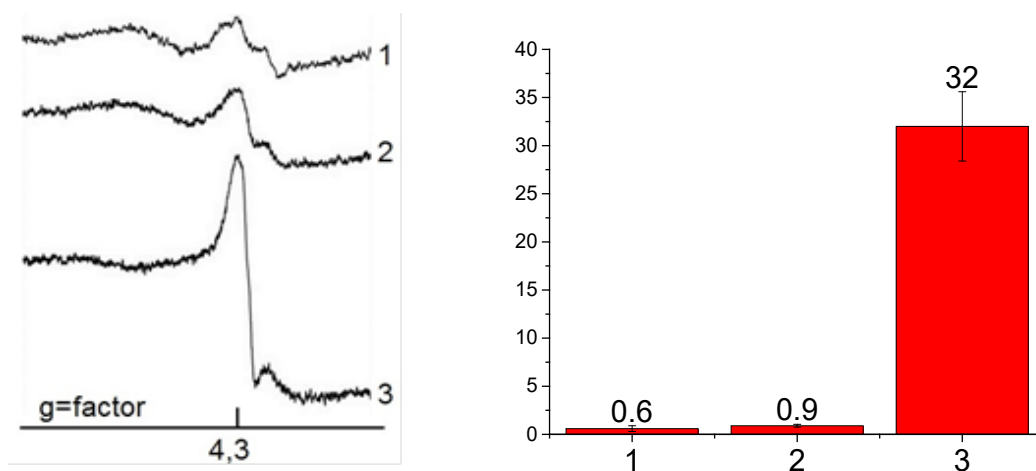
**Figure 5:** Typical EPR spectra of the iron-containing proteins (left panel) and their level (right panel): (1) TF in the morphologically unchanged intestinal mucosa (n = 8); (2) LF in the moderately differentiated mCRC (n = 24); (3) LF in the poorly differentiated mCRC (n= 27). The data on the right panel are presented as mean ± SD.
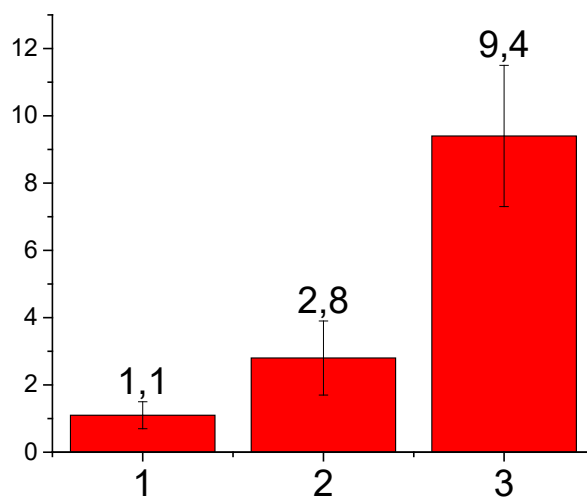


**Figure 6:** Level of "free" iron in 1 - morphologically unchanged intestinal mucosa mucosa (n = 8); 2 - mCRC of moderate degree of differentiation mucosa (n = 24); 3 - mCRC of low degree of differentiation mucosa (n = 27). Data are presented as mean ± SD.
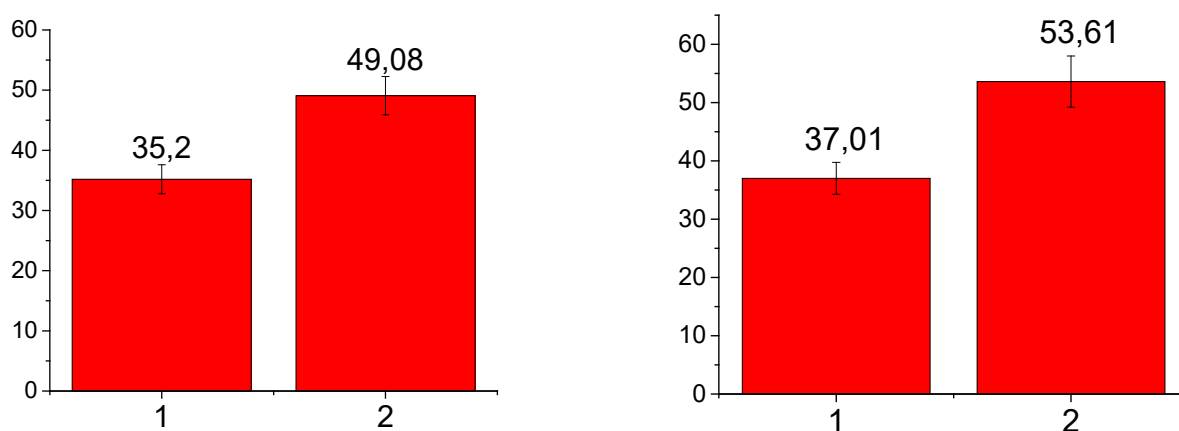


**Figure 7:** Ki-67 expression levels. Left panel: morphologically unchanged intestinal mucosa (1, n = 8) and mCRC stage II-III (2, n = 51). Right panel mCRC of moderate degree of differentiation (1, n = 24) and low degree of differentiation (2, n = 27).

A direct correlation was established between the level of "free" iron and the Lf content (r = 0.61; p <0.05), and the rate of SR generation in the tumor tissue of CRC (r = 0.67; p <0.05).

In tumor tissue of CRC stage II-III, the expression level of Ki-67 is averaged as $49.08 \pm 3.18$ a.u. against $35.2 \pm 2.41$ a.u. in morphologically unchanged intestinal mucosa (Figure 7, left panel). In tumors of the rectum with a low degree of differentiation, this indicator was significantly higher than that in moderately differentiated tumors, constituting, respectively, $53.61 \pm 4.38$ a.u. and $37.01 \pm 2.74$ a. u. ($p < 0.001$, Figure 7, right panel).

In neoplasms with the T2-4 criterion and no signs of metastasis the expression level of Ki-67 was found to be 1.4 times less than in tissues with the regional lymph nodes (T2-4N1-2M0). It follows that favorable clinical course of the disease in patients with mCRC is characterized by a decrease in Lf levels, "free" iron and Ki-67 expression while tumor progression is associated with an increase of the mentioned indicators. The results of the immunofluorescence study in mCRC tissue are shown in Figure 8.
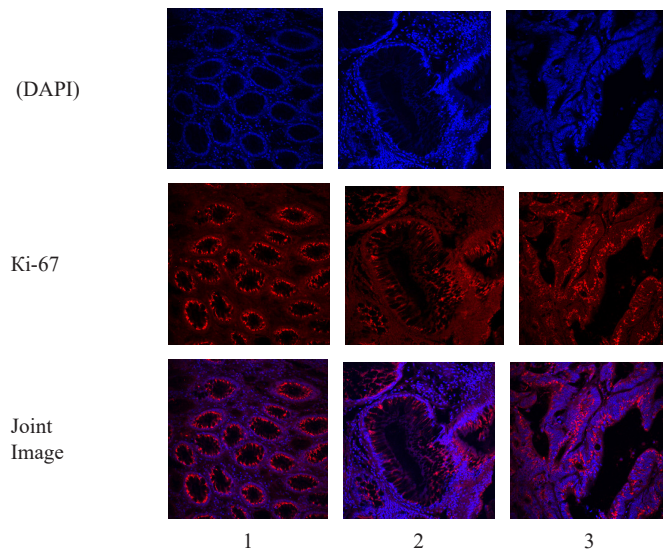


**Figure 8:** Expression of Ki-67 in morphologically unchanged intestinal mucosa (1), in mCRC tissue of moderate degree of differentiation (2) and low degree of differentiation (3). 200x magnifications.

## Conclusions
- The revealed changes in the redox metabolism in mCRC tumor (by measuring SR generation rate, levels of NO, Lf, "free" iron, Ki-67) increase the proliferation rate and form an aggressive tumor phenotype.
- MMP-2 and MMP-9 activities correlate with the rates of SR generation and NO levels.
- All the above indicators (mentioned in items 1 and 2) positively correlate with the disease stage (r = 0.53 ÷ 0.68, p <0.05).
- A positive correlation between the levels of Lf, "free" iron and Ki-67 (r = 0.57; r = 0.61; p <0.05) indicates the effect of the disturbed iron metabolism on the intensity of proliferative processes in the mCRC tumor.

Summarizing the results, our research shows that tumor cells are characterized by reprogrammed mitochondrial metabolism, high levels of cellular hypoxia, functioning of the defective redox system and unregulated levels of SR and NO molecules. On the other hand, the redox state of tumors is determined, in particular, by the peculiarities of the functioning of iron-containing proteins - Lf and Tf. Oxidation-induced modifications of Lf and Tf lead to a violation of their iron-binding and iron-transport functions, causing the accumulation of "free" iron in tissues and blood [26-28]. These factors are important in the formation of an aggressive tumor phenotype, which is manifested by unregulated proliferation, migration and invasion of tumor cells; they correlate with the course of the disease and can be used as an auxiliary objective criterion in diagnosis. The assessment of the course of the disease in patients with mCRC by determining the indicators of the redox metabolism of tumor tissue can provide an additional opportunity to monitor the effectiveness of antitumor therapy.

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