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TUMOR-ASSOCIATED REDOX STATE IN METASTATIC COLORECTAL CANCER

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The high incidence of recurrence and metastasizing in colorectal cancer (CRC) poses the challenge for the improvement in long-term treatment outcome. *Aim*: To determine the major indicators of redox-formative molecules in the tissue of metastatic CRC (mCRC), stages T2–4N0–2M0G2–3, namely the rate of superoxide radical (SR) generation, nitric oxide (NO) content, the activity of matrix metalloproteinases (MMP), lactoferrin (LF) content, and "free" iron and their association with some clinical and pathological characteristics of the patients. *Materials and Methods*: mCRC samples from 51 patients were analyzed (stage II, 31 patients; stage III, 20 patients). The LF and "free" iron were assessed by electron paramagnetic resonance (EPR) at the temperature of 77 °K. The rate of SR and NO generation was determined with spin traps (TEMPO-H, diethyl dithiocarbamate). The activity of MMP-2 and -9 was measured by gelatin zymography using SDS-polyacrylamide gel electrophoresis. Ki-67 expression was analyzed by immunofluorescence technique. *Results*: In tumors with metastases into the regional lymph nodes (N1–2 category), SR generation rate was 2.2-fold higher than in the tumors categorized as N0. In G3 mCRC, SR generation rate was 1.7-fold higher than in G2-tumors ($p < 0.05$). The rate of SR generation correlated inversely with differentiation grade of the tumor ($r = -0.61$; $p < 0.05$). MMP-2 and -9 activities in mCRC tissue correlated with SR generation rate and NO level ($r = 0.44 \div 0.53$, $p < 0.05$). The direct correlation between LF content and the stage of the disease ($r = 0.42$) and "free" iron content ($r = 0.61$) was demonstrated while the correlation between LF content and tumor differentiation grade was inverse ($r = -0.57$; $p < 0.05$). *Conclusions*: The altered tumor-associated redox state in mCRC tissue contributes to the increased cell proliferation and formation of aggressive phenotype of the tumor. The assays for the content of redox-formative components in mCRC may be used as additional prognostic markers of the course of the disease in CRC patients.

***Key Words*: metastatic colorectal cancer, superoxide radicals, NO, matrix metalloproteinase-2, -9, lactoferrin, "free" iron, EPR, spin traps.**

In Ukraine, the colorectal cancer (CRC) is the fourth most commonly occurring cancer in females and the fifth most commonly occurring cancer in males, being the second and the fourth leading cause of cancer-related deaths in females and males, respectively [1, 2]. The high incidence of recurrence and metastasizing poses the challenge for the improvement in long-term treatment outcome [3, 4]. Therefore, the further progress in CRC treatment should rely on the deep insight into the etiology and pathogenesis as well as the search for informative prognostic and predictive markers.

Imbalanced concentrations of superoxide radicals (SR) and nitric oxide (NO) change the redox state and redox-depending signaling underlying cancer progression and formation of treatment resistance [5, 6]. In addition, the disordered iron metabolism and impaired functions of iron-containing proteins in CRC tissue may contribute to the formation of the more aggressive phenotype of the tumor [7]. Since cellular and extracellular redox homeostasis should be considered as the important regulator of the tumor and metastatic microenvironment, the indicators specifying this homeostasis may be used as the additional CRC prognostic markers.

The aim of the study was to determine the major indicators of redox-formative molecules in the tissue of metastatic CRC (mCRC), stages T2–4N0–2M0G2–3, namely the rate of SR generation, NO content, the activity of matrix metalloproteinases (MMP), lactoferrin (LF) content, and "free" iron analyzing them in association with some clinical and pathological characteristics of CRC patients.

MATERIALS AND METHODS

In total, mCRC samples from 51 patients (28 males, 23 females; mean age 63 ± 1.7; stage II in 31 patients and stage III in 20 patients) with tumors categorized as T2–4N0–2M0G2–3 were analyzed. In all patients, mCRC was histologically verified as rectal adenocarcinoma of different grade (G2–G3). The patients were treated in the clinics of the National Cancer Institute (Kyiv, Ukraine). The diagnosis, the stage of the disease and the presence of the metastases have been verified according to the requirements of the evidence-based medicine taking into account the results of the clinical, instrumental and histopathological examinations. The samples of tumors were obtained upon biopsy or surgery. The LF and "free" iron were assessed by electron paramagnetic resonance (EPR) at the temperature of 77 °K. As the intensity standard, the oriented ruby monocrystal with the known number of paramagnetic centers was used. The rate of SR and NO generation

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Abbreviations used: CRC – colorectal cancer; EPR – electron paramagnetic resonance; LF – lactoferrin; mCRC – metastatic CRC; MIM – morphologically intact mucosa; MMP – matrix metalloproteinase; NO – nitric oxide; SR – superoxide radicals; TF – transferrin.

was determined with spin traps (TEMPO-H, diethyl dithiocarbamate; Sigma, USA) [8]. The data obtained with CRC samples were compared with those in the samples of the morphologically intact mucosa (MIM). All patients provided their informed consent allowing for the use of the clinical samples for research purposes.

The activity of MMP-2 and -9 in tumor samples was measured by gelatin zymography using SDS-polyacrylamide gel electrophoresis. The gelatinase activity was expressed in arbitrary units (a.u.) referred to the activity of 1 μg of enzyme per 1 g of the tissue [9].

The expression of Ki-67 in biopsy CRC samples and MIM was analyzed by immunofluorescence with anti-Ki-67 antibody (clone MIB-1, Santa Cruz, CA, USA). The secondary antibody was labeled with Alexa Fluor 546 (Invitrogen, USA). The nuclei were visualized with 4,6-diamidino-2-phenylindole (DAPI). The specimens were studied using confocal scanning microscopy. Zeiss LSM 510 microscope was equipped with 32-channel automatic polychromatic META-detector (GaAsP) and oil-immersion 40x/1.4NA objective.

The data were statistically processed using the applied licensed programs GraphPadPrism 6 and Origin 7.0. The results were presented as the mean ± standard deviation (M ± SD). Spearman correlation coefficient was calculated. The significance of the difference was assessed based on Student *t*-test. The difference was considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The impact of an altered oxygen metabolism in mitochondria and changes in iron-containing proteins such as LF and transferrin (TF) to redox state of cancer cells is a matter of common knowledge. LF as a multifunctional protein of TF family controlling the bioavailability of iron is involved in the regulation of some intracellular processes, in particular, cell proliferation [10].

The analysis of the content of redox-formative components in the tissues of CRC and MIM represented in the Table demonstrates that LF content varied depending on tumor differentiation grade. In poorly differentiated tumors (G3), LF content was significantly higher as compared with that in moderately differentiated tumors (G2) while in MIM tissue LF could not be detected. The LF content correlated inversely with tumor differentiation grade ($r = –0.51$; $p < 0.05$) and correlated directly with the stage of the disease ($r = 0.42$; $p < 0.05$). It is also worth noting that in MIM tissue the signal corresponding to TF with $g = 4.3$ was registered at the level of $0.6 \cdot 10^{15} \pm 0.3 \cdot 10^{15}$ spins/g of tissue.

The increased content of "free" iron with high prooxidant potential may be a trigger of the intense generation of SR leading to DNA mutations [10, 12]. Since the SR and ferrous ions are involved in the control of many important pathways, the change in their intracellular content in malignant cells may facilitate tumor progression due to the increased proliferation [13].

We have shown that in moderately and poorly differentiated CRC the "free" iron content was 2- and 7.4-fold higher, respectively, as compared with that in MIM (see the Table). Therefore, "free" iron content in G3 tumors was thrice as high as in G2 tumors ($p < 0.05$).

Moreover, as demonstrated by the data in the Table, SR generation rate in mCRC tissue increased significantly as compared with MIM (3- and 5.5-fold in moderately and poorly differentiated CRC, respectively). SR generation rate in mCRC tissue also correlated inversely with differentiation grade of the tumor ($r = –0.61$; $p < 0.05$).

In mCRC tissue, the "free" iron content correlated directly with LF content ($r = 0.61$; $p < 0.05$) and SR generation rate ($r = 0.67$; $p < 0.05$).

Fig. 1 presents the images of immunofluorescent study of Ki-67 expression in mCRC and MIM tissues. The average Ki-67 expression level in mCRC of stages II–III was 49.08 ± 3.18 a.u. *vs* 35.2 ± 2.41 a.u. in MIM (see Table). Moreover, the poorly differentiated CRC were characterized by more pronounced Ki-67 expression as compared with moderately differentiated CRC ($p < 0.001$). When Ki-67 expression data were singled out for T2–4 CRC without metastases in regional lymph nodes, the average level of Ki-67 expression in such tumors was 1.4-fold less than in tumors categorized as T2–4N1–2M0.

The data on the rate of SR generation and NO level in mCRC tissue presented in Fig. 2 demonstrated that in tumors with metastases into the regional lymph nodes (N1–2 category) SR generation rate was 2.2-fold higher than in the tumors categorized as N0. The same tendency holds true when NO level in tumors with metastasis into the regional lymph nodes was compared with that without metastasis ($p < 0.05$). The rate of SR generation and NO level correlated positively with CRC regional metastasizing ($r = 0.63$ and 0.69, correspondingly; $p < 0.05$). These indices in CRC with remote metastases are also superior to that in tumors categorized as M0.

The activity of MMP-2 and -9 in mCRC tissue in the context of the disease stage and metastasizing was also studied and the possible correlations between MPP activity and SR generation rate and NO levels were analyzed. The concentration of the active forms of MMP-2 in mCRC tissues

Table. Content of redox-formative components in mCRC and MIM tissues

Component	MIM	G2 mCRC	G3 mCRC
LF, spins/g of tissue	–	$1.2 \cdot 10^{15} \pm 1.4 \cdot 10^{15}$	$28.4 \cdot 10^{15} \pm 3.3 \cdot 10^{15}$
"Free" iron, spins/g of tissue	$1.3 \cdot 10^{17} \pm 0.5 \cdot 10^{17}$	$2.7 \cdot 10^{17} \pm 1.5 \cdot 10^{17}$	$9.6 \cdot 10^{17} \pm 2.3 \cdot 10^{17}$
SR, nmol/g of tissue·min	0.27 ± 0.06	0.85 ± 0.17	1.50 ± 0.22
Ki-67, a.u.	35.2 ± 2.41	37.01 ± 2.74	53.61 ± 4.38

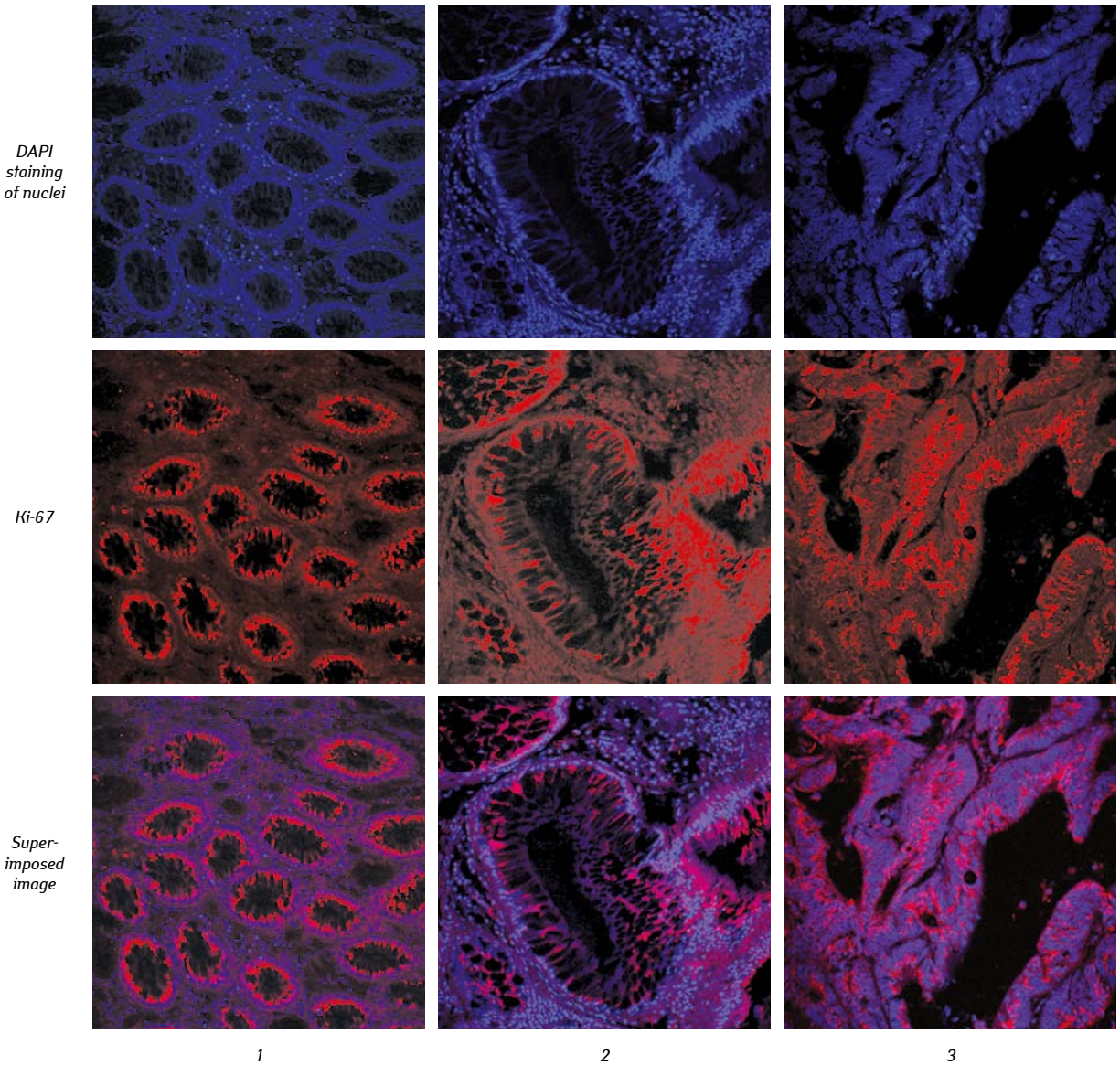

Fig. 1. Ki-67 expression in cells of MIM tissue (1), moderately differentiated CRC (2) and poorly differentiated CRC (3). × 200

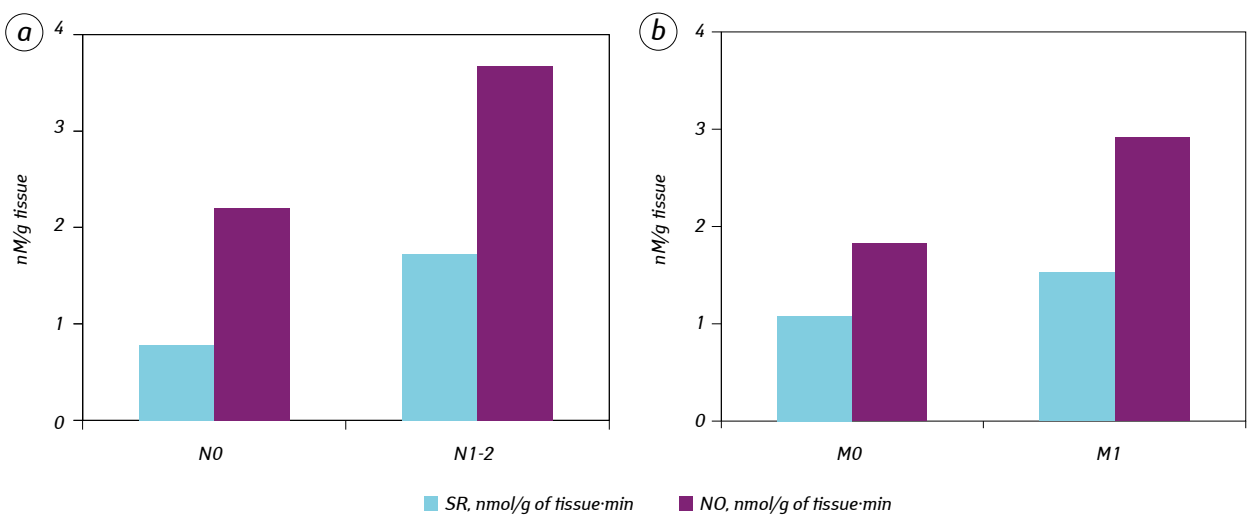

Fig. 2. Rate of SR generation and NO level in mCRC related to regional (*a*) and remote (*b*) metastasizing

ranged from 0.1 to 52.8 μg/g of tissue (mean value 8.2 ± 4.9 μg/g of tissue). For MMP-9, the corresponding values were 0.05–28.8 μg/g of tissue (mean value 8.3 ± 5.9 μg/g of tissue). The activities of both MMP-2 and -9 did not vary with the gender and age of the patients, and T category. Nevertheless, in G3 tu-

mors, concentration of the active MMP-2 was 6 times higher than in G2 tumors ($p < 0.05$). MMP-2 activity in tumors of patients with stage III of the disease was higher than in tumors of patients with stage II (13.7 ± 3.5 a.u. *vs* 7.4 ± 3.3 a.u.; $p < 0.05$) (Fig. 3). For MMP-9, the difference between activities in tumors of patients with stage III and stage II was not statistically significant.

MMP-2 and -9 activities in mCRC tissue correlated with SR generation rate and NO level (r = 0.44 ÷ 0.53, $p < 0.05$). The data on the activity of MMP-2 and -9 in CRC tissue were also analyzed depending on the presence of regional and distant metastases (Fig. 4). While the difference between gelatinase activities in CRC categorized as N1–2 and N0 was not statistically significant, MMP-2 activity in M0 tumors was substantially superior to that in M1 tumors. Such activation of latent forms of MMP-2 in cases when metastasizing has not been yet clinically detected suggests the pronounced increase in the destruction of extracellular matrix at that stage. These findings are in line with the concept implying the ability of tumors to initiate the advantageous microenvironment in premetastatic niches [14, 15].

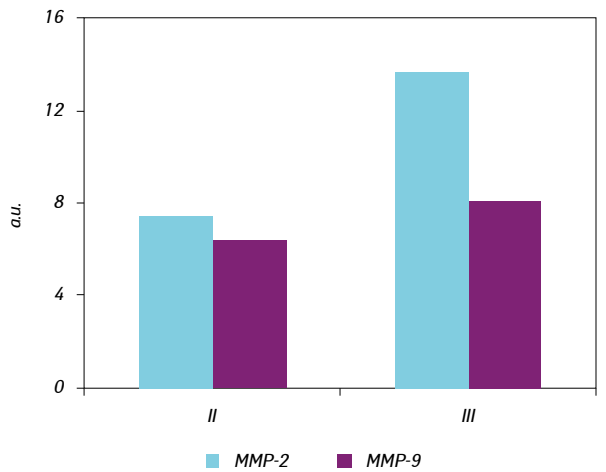

Fig. 3. MMP-2 and -9 activities in mCVRC tissues of patients with stages II and III of the disease

To sum up, LF and "free" iron content, Ki-67 expression, and SR generation rate in mCRC grade II–III have been shown to increase in comparison with MIM tissue. The positive correlation of the above indices with tumor differentiation grade as well as the direct correlation with LF content and the stage of the disease (r = 0.42) and "free" iron content (r = 0.61) have been also ascertained. Therefore, all this evidence directly implicates the association between iron metabolism and intensity of cancer cell proliferation. The favorable course of the disease in the patients with mCRC seems to be associated with the decreased LF and "free" iron content and reduced Ki-67 expression. Conversely, tumor progression is related to the increase of the indices.

MMP-2 and -9 activity correlated positively with the regional metastasizing (r = 0.45 and 0.37, respectively; $p < 0.05$) similarly to SR generation rate and NO levels. Nevertheless, the correlation between MMP-2 and -9 activity and remote metastasizing is inverse. Such "downregulation" of MMP may be explained by disintegration of signaling and regulatory pathways affecting the genomic and post-synthetic levels of MMP formation and functioning in the setting of high SR generation rate and augmentation of the oxidative processes that is characteristic of the secondary metastasizing [16].

Our findings are consistent with the current concepts on reprogrammed metabolism in mitochondria, high hypoxia level, and impaired redox system in cancer cells. We have demonstrated the shortfall in the control of SR generation and NO production in CRC. On the other hand, metal-containing proteins such as LF and TF are of no less importance in outlining redox state in cancer. The oxidative modifications of LF and TF impair their iron-binding and iron-transporting functions with accompanying accumulation of "free" iron in the tissues and the blood of the patients [17–19]. Such factors play important role in the acquisition of the aggressive phonotype with uncontrolled proliferation, migration and invasion of cancer cells and correlate with the progression of the disease. Therefore, the indices studied above in mCRR patients may be considered

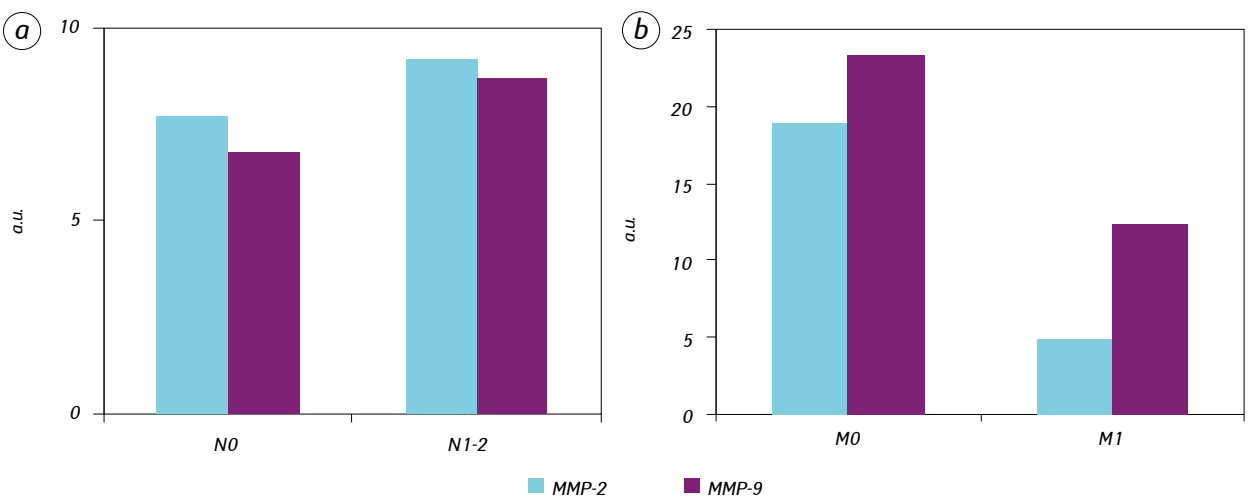

Fig. 4. MMP-2 and -9 activities in mCRC tissues depending on the presence of regional (*a*) and distant (*b*) metastases

as the supplementary objective criteria in predicting the course and the outcome of the disease.

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