

DNA OXIDATION IN PATIENTS WITH METASTASTIC COLORECTAL CANCER: CLINICAL SIGNIFICANCE OF 8-HYDROXY-DEOXYGUANOSINE AS AN INDEPENDENT PROGNOSTIC FACTOR

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Prognosis of metastatic colorectal cancer (mCRC) patients nowadays is an important subject in the field of oncology. R0-resection of colon with primary tumor and liver metastasis remains the only treatment which significantly improves survival rate. However, recent experimental data show that surgical trauma can indirectly stimulate tumor growth due to mitochondrial dysfunction and unregulated superoxide radical ($O_2^{\cdot-}$) generation. **Aim:** To study the clinical significance of 8-oxo-2'-deoxyguanosine (8-oxodGu) marker, to assess the oncological effects of warm ischemia of liver parenchyma on disease prognosis in patients with mCRC. **Material and Methods:** 69 urine 24-hour volume tests of patients with mCRC and 17 healthy individuals were studied. Urine 8-oxodGu level was measured using spectrophotometric method with pre-solid phase DNA extraction. The energy system and hepatocyte detoxification system state, levels of $O_2^{\cdot-}$ in tumor tissue were determined using the method of electron paramagnetic resonance (EPR) and SpinTraps technology at room temperature. Experiments were carried out on a computerized EPR spectrometer RE-1307. EPR spectra were recorded at temperature of liquid nitrogen (-196°C) in paramagnetically pure quartz dewar on a computerized spectrometer PE-1307 with resonator H011. Error of the method of spectrum integration and spread of spectrum reproduction of one sample was not more than 3%. **Results:** The average level of marker in healthy individuals was $0.244 \pm 0.063 \text{ nM/kg} \cdot \text{day}$, whereas before the resection and on day 3 after the R0-resection of liver in mCRC patients was $3.42 \pm 0.18 \text{ nM/kg} \cdot \text{day}$ and $2.12 \pm 0.08 \text{ nM/kg} \cdot \text{day}$ ($p < 0.05$), respectively. On day 3 after the liver resection due to its metastatic lesions with a total duration of warm ischemia period $< 30 \text{ min}$ and $> 30 \text{ min}$ have had marker at level $2.108 \pm 0.13 \text{ nM/kg} \cdot \text{day}$ and $2.9883 \pm 0.159 \text{ nM/kg} \cdot \text{day}$ ($p < 0.0001$), respectively. The volume of metastatic tissue significantly and directly correlated with the level of urine 8-oxodGu ($R^2 = 0.54$, 95% CI 0.037–0.0991, $p < 0.0001$), also duration of surgical intervention ($\geq 300 \text{ min}$) and duration of worm liver ischemia ($> 30 \text{ min}$) during the surgery significantly increased urine level of 8-oxodGu ($R^2 = 0.54$, 95% CI 0.001–0.004, $p < 0.001$). **Conclusions:** Warm liver ischemia ($> 30 \text{ min}$), long-term surgical intervention ($\geq 300 \text{ min}$) and metastatic tissue volume ($\geq 12 \text{ cm}^3$) in liver parenchyma in mCRC patients significantly increase urine 8-oxodGu levels. R0-resection of liver metastases in mCRC patients decreases urine 8-oxodGu levels already on day 3 after the surgery. 8-oxodGu is a new factor of oncological prognosis in patients with mCRC.

Key Words: colorectal cancer, 8-hydroxy-deoxyguanosine, superoxide radicals, metastases, liver resection.

In more than 30% of patients with colorectal cancer with metastatic lesion of liver, during the first year after the liver resection repeated metastatic lesions diagnosed, and its recurrent metastatic lesion occurs since the time of first resection [1] in 50–60% patients. Local and systemic control of the disease course in metastatic colorectal cancer (mCRC) patients is a key point of modern oncology; and individualized prediction of the disease course in mCRC patients is the subject of active research. Recently, prognosis of such patients depended predominantly on clinical, radiological, pathological, and molecular criteria, but this approach did not demonstrate sufficient clinical efficacy [2].

Ischemia-reperfusion (I/R) effect in liver usually recorded in case of large as well as advanced is liver

resections using Pringle maneuver (PM) and/or total blood supply blockage in organ [3]. It's proven that liver oxygenation is significantly reduced during the organ mobilization/retraction and its preparation for the latter transection of parenchyma, which leads to additional hepatocytes damage and death [4]. Liver with signs of fibrosis, steatosis due to toxic effect of chemotherapy (CTX) is much more susceptible to damage caused by I/R [5]. On animal models shown that damage due to PM and I/R during the liver resection leads to hepatocytes dysfunction, an increase of anti-inflammatory cytokines levels, enhanced activity of metastatic metalloproteinases, which in turn may stimulate the progression of micrometastases of mCRC carcinoma [6]. Surgical trauma creates favorable conditions for the growth of uncontrolled concentrations of superoxide radicals ($O_2^{\cdot-}$) which in turn creates a new redox state in surgery-treated organ and organism in general, which is a key factor in local relapse and distant metastases after the tumor resection performed [7].

Recently, number of centers focused on current problem studying substantially expanded. In particular, in the study by Yamashita *et al.* [3] an independent predictor of oncological prognosis for patients with

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Abbreviations used: 8-oxodGu – 8-oxo-2'-deoxyguanosine; 8-oxoG – 8-oxoguanine; ALF – acute liver failure; CT – computer tomography; CTX – chemotherapy; EPR – electron paramagnetic resonance; I/R – ischemia-reperfusion; mCRC – colorectal cancer with liver metastasis; PM – Pringle maneuver.

mCRC the degree of ischemia that arose as a result of a blood supply violation in liver parenchyma during the first 30 days after the resection (liver stroke) was assumed. Authors received a direct-proportional dependence between non-recurrent survival rate and stroke severity in resection area according to CT data. This approach and scale of ischemia described by authors as an evidence proving that level of liver ischemia after the surgery can be a significant predictive factor of mCRC patients survival rate since the time of surgical intervention. These findings were confirmed by our results as well.

It is known that O_2^- causes DNA oxidation leading to formation a number of oxidative bases and nucleotides in DNA. Such lesions become a cause of mutations and carcinogenesis initiation in healthy people or in malignant neoplasms progression, including those with CRC. 8-oxo-2'-deoxyguanosine (8-oxodGu) is the most frequently reported form of oxidative bases in DNA or in a nuclear pool. It has dangerous effects because of capacity to form pairs with adenine and/or cytosine in DNA molecule [8].

8-oxoguanine (8-oxoG) is able to accumulate in both nuclear and mitochondrial DNA. That is why the latter considered a highly informative marker of malignant neoplasms formation [8]. Among the guanine residues the nucleic acids variation is possible, hence the modification of 8-oxoguanine to deoxyguanosine (dG) may have place; in C-8 site dG molecule is most effectively oxidised by ascorbic acid generating 8-oxodGu.

Oxidative damage of nuclear and mitochondrial DNA, hypermethylation of gene promoters is a major event at all stages of carcinogenesis. Prolonged exposure of intestinal mucosa to O_2^- initiates chronic inflammation and dysplasia. Disorders caused by O_2^- include oxidative-induced mutations in genome, its functional instability and consequently cell proliferation dysregulation [9–11]. The integral quantitative analysis of urine 8-oxodGu provides an opportunity to non-invasive evaluation of degree of DNA oxidation and redox condition of tissue after surgery.

The aim of our study is to evaluate the clinical significance of 8-oxodGu marker, to assess the oncological effects of warm ischemia of liver parenchyma on disease prognosis in patients with mCRC.

MATERIAL AND METHODS

The study involved 69 CRC patients with metachronous metastatic liver disease (pT1–4N0–2M0 colon cancer and pT1–4aN0–2M0 rectal cancer) treated in National Cancer Institute from March 2015 till June 2018. All the patients provided written informed consent to participate in the research. The study was approved by Ethics Committee of National Cancer Institute and performed according to the guidelines of Declaration of Helsinki. 36 men and 33 women included in the study. Average age of patients was 61.4 ± 2.3 years. 28 patients were diagnosed rectal cancer, while 41 — colon cancer. Based on degree

of tumor differentiation patients distributed as follows: G_{1–2} tumors found in 23 patients, G_{3–4} tumors — in 46 patients (Table 1).

In each clinical case, multidisciplinary approach was used where surgeons, oncologists, chemotherapists and radiologists took part. In all cases, the diagnosis of metastatic liver damage was confirmed using cytology/histology techniques and fine-focal biopsy of the pathological liver sites. Routine computer tomography (CT) with intravenous contrast of thoracic, abdominal and pelvic cavity was applied; and in complicated cases (suspicion of canceromatosis or bilobar lesions) the examination was supplemented by magnetic resonance imaging with intravenous contrast. Positron-emission tomography was used only in case of metastatic involvement of other organs/sites.

Table 1. Group characteristics for participating patients

Characteristics	Value
Age	61.4 ± 2.3
Body mass index	26.5 ± 5.3
Sex (male/female)	36/33
Metastatic liver injury (synchronous/metachronous)	14/55
Primary tumor location (rectum/colon)	25/44
Metastatic injury of other sites at the moment of liver resection (lungs/abdomen cavity)	4/5
Number of resected metastases	3.41 ± 1.3 (4–7)
Volume of metastatic tissue in liver, cm ³	9.2 ± 7.3 (1.0–43.2)
Type of liver resection (major/minor)	45/24
Neoadjuvant CTX (yes/no)	17/52
Patient condition (ASA scale):	
I–II	47
III	22
Warm ischemia duration in time of the PM, min	19.7 ± 20.5
Liver parenchyma transection duration, min	228.9 ± 118.2
PM applied (yes/no)	51/18

Surgical technique included the implementation of radical resection with the maximum possible preservation of parenchyma and ensuring adequate retreat from the tumor edge (4–10 mm). Each operation was accompanied by intraoperative ultrasound examination in order to mark sites in relation to major hepatic veins and Glissonian pedicles and also to detect small metastases. Major liver resections referred to removal of 3 or more liver segments (class Couinaud). The technique of ischemia assumed the use of classic and selective PM (20 min — ischemia, and 5 min — reperfusion) [12]. Parenchyma transection performed using the “crashclamp” method. Resected parenchyma hemostasis performed by prolene 4.0, 5.0 flashing, bipolar forceps, clipping of LT200, LT300.

Patients received adjuvant CTX according to international standards FOLFOX-6/FOLFIRI/XELOX (4–6 courses). Patients treated with CTX and with signs of with disease progression did not undergo liver resection. Functional liver capacity assessed using Child — Turcotte — Pugh score and MELD score. The manifestations of CTX toxicity was documented according to STCAE 5.0 criteria. The degree of acute liver failure (ALF) in postoperative period was determined by International Study Group of Liver Surgery (ISGLS) classification.

Urine sampling was done 24-hours before and on day 3 after the surgery. Then 20 ml of 24-hour vol-

ume test urine filtered through a solid-phase extraction column. Measurement of 8-oxodGu level in eluate was carried out using spectrophotometric method [13]. The energy system and hepatocyte detoxification system state, O_2^- generation speed in tumor tissue, hepatocyte mitochondria and tissue-associated neutrophils was determined using method of electron paramagnetic resonance (EPR) and technology of spin traps (SpinTraps) at room temperature. Measurements were carried out on a computerized EPR spectrometer RE-1307. EPR spectra were recorded at liquid nitrogen temperature (-196°C) in a paramagnetically pure quartz dewar on a computerized spectrometer PE-1307 with resonator H0₁₁. Following parameters were used: power of microwave source level 40 mW, modulation frequency 100 kHz, amplitude 10 Gaussian, the receiver's constant time $\tau = 0.3$ s. As a standard of intensity a specially oriented sample of a single crystal Al₂O₃ with specific concentration of Cr³⁺ ions were used. Method of double integration evaluated the concentration of molecules by comparing the signals intensity in EPR spectra to standard intensity. Error of spectrum integration method and spread of spectrum reproduction of one sample was within 3%.

Statistical analysis of the results was performed using IBM SPSS Statistics (Version 20.0, Armonk, NY, USA). T-test was used to evaluate the difference of urine 8-oxodGu between healthy individuals and mCRC patients. Concentration on urine 8-oxodGu at different disease stages was compared using one-way ANOVA test. Logistic regression model designed to determine the relation between the urine 8-oxodGu concentrations in patients with CRC and other factors i.e. body mass index, presence of ischemic liver tissue, presence of distant metastases, volume of metastatic tissue in liver, stage of process, age, sex, degree of tumor differentiation. Statistical significance was assumed when $p < 0.05$.

RESULTS AND DISCUSSION

We have analyzed urine 8-oxodGu levels in mCRC patients before and after the liver metachronous metastases removal (Fig. 1). Our data show that metastatic tissue removal leads to normalization of oxidative DNA damage. Thus, the average level of the marker in healthy individuals urine is 0.244 ± 0.063 nM/kg · day, whereas on day 3 after the R0-resection of liver due to its metastatic lesion 3.42 ± 0.18 and 2.12 ± 0.08 nM/kg · day ($p < 0.05$).

Fig. 2 shows the results of urine 8-oxodGu levels in mCRC on day 3 after the surgery with different warm liver ischemia duration during the PM. Mean value of urine 8-oxodGu in healthy individuals is 0.244 ± 0.063 nM/kg · day. In patients with mCRC on 3 day after the liver resection due to its metastatic lesion with a total duration of warm ischemia < 30 min and > 30 min — 2.108 ± 0.13 nM/kg · day and 2.9883 ± 0.159 nM/kg · day ($p < 0.0001$), respectively. Obviously, urine level of 8-oxodGu is significantly increased in patients with mCRC and depends on warm ischemia duration caused by PM (Fig. 2).

We have studied a number of markers and factors that could negatively affect the level of oxidative DNA damage and subsequently the oncologic prognosis in our cohort of mCRC patients (Table 1). It was shown that statistically significant effect registered with volume of metastatic tissue in liver ($p = 0.037$), duration of warm ischemia during the liver parenchyma transection ($p = 0.001$) and duration of surgery ($p = 0.006$).

Statistical analysis of studied factor dependence on oxidative DNA damage (8-oxodGu) level show that duration of liver parenchyma transection, warm ischemia during and volume of metastatic tissue in liver are independent factors that adversely affect the level of studied marker (Fig. 3). In particular, the volume of metastatic tissue (V_{mts}) significantly and proportionally increases the level of urine 8-oxodGu in surgery-treated patients ($R^2 = 0.54$; 95% CI 0.037–0.0991 at $p < 0.0001$) (Fig. 3, a). Surgery time ($t_{\text{resection}}$) and duration of warm ischemia during the surgical manipulations (t_{ischemia}) significantly increased level of urine 8-oxodGu in surgery-treated patients ($R^2 = 0.54$; 95% CI 0.001–0.004 at $p < 0.001$) (Fig. 3, b, c).

Linear regression model equation

We have created a linear regression equation for 8-oxodGu marker levels = $1.253 + 0.483 \cdot V_{\text{mts}} + 0.342 \cdot t_{\text{resection}} + 0.352 \cdot t_{\text{ischemia}}$.

Using EPR method under conditions of low temperature stabilization ($T = 77$ K) the study of ischemic liver tissue samples and liver tissue samples dissected at a distance of 5 cm from the tumor node (postoperative material) was done. In EPR spectra of healthy liver tissue following spectra were detected: EPR signals $g = 1.94$, which characterize the state of mitochondrial electron transport chain (ETC) (iron-sulfur proteins of Complex I, NADN-dehydrogenase 1a); EPR signal $g = 2.00$ — level of flavo-, ubisemiquinone, a main electron transfer in ETC; EPR signal $g = 2.03$ — level of NOFeS-protein complexes; EPR signal $g = 2.25$ and $g = 2.42$ — activity level of cytochrome P-450 (CYP) in redox cycle of hepatocyte detoxification system; ERPR signal $g = 2.65$ — free iron level; EPR signal with $g = 4.25$ — level of lactoferrin, transferrin functioning in oxidative metabolism in mitochondria (Fig. 4). Qualitative and quantitative changes in energy and detoxification systems of ischemic liver tissue mitochondria were detected.

In conditionally healthy liver tissue at a distance of 5 cm from the metastasis, the value of cytochrome P-450 activity was detected at level 0.44 ± 0.08 a.u. while in healthy tissue — 1.48 ± 0.13 a.u. In ischemic tissue activity of cytochrome P-450 was detected at level 0.11 ± 0.07 a.u., which is an evidence of significant decrease of detoxification system effectiveness (Fig. 4). In mitochondria of these cells reduced activity of Complex 1 (NADH-ubiquinone oxidoreductase) to value 0.31 ± 0.06 a.u. was detected, while in control group 1.51 ± 0.10 a.u. and 0.59 ± 0.09 a.u. in conditionally healthy liver tissue. Decreased activity of the electron transport complexes in ETC occurs due to NO complexes with FeS proteins ($g = 2.03$)

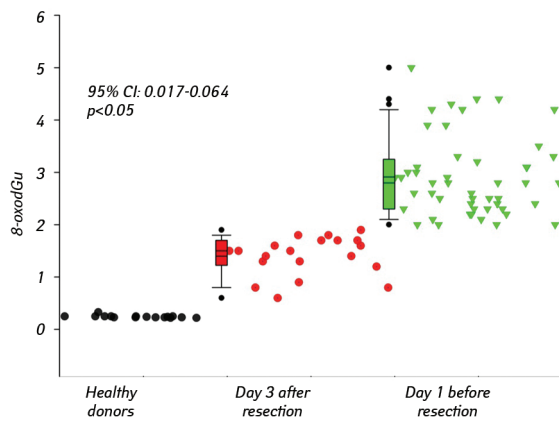


Fig. 1. Urine 8-oxodGu levels (nM/kg · day) in patients: normal — healthy individuals (n = 17); day 1 before resection (n = 30)/day 3 after the liver resection (n = 30) in mCRC patients

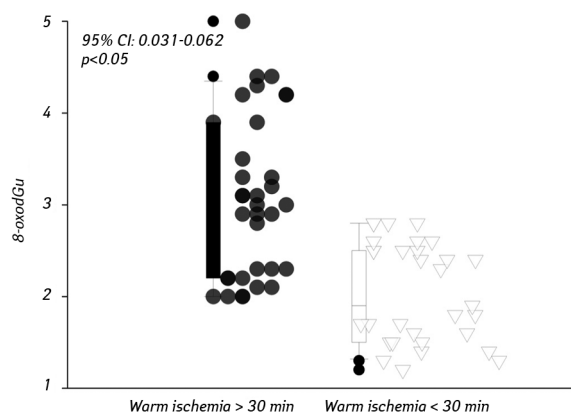


Fig. 2. Urine 8-oxodGu levels in mCRC patients: ischemia < 30 min in mCRC patients (on day 3 after R0-resection of liver) whom PM applied with a total duration of warm ischemia < 30 min; ischemia > 30 min in mCRC patients (on day 3 after R0-resection of liver) whom PM applied with a total duration of warm ischemia > 30 min

formation, which levels are increased; and this refers to formation of triplet structure in EPR. The latest is typical for mitochondria of malignant tumors (Fig. 5, ESR spectrum 2). In liver tissue at a distance of 5 cm from the metastatic node, complexes NO-FeS-proteins in ETC detected at level 0.25 ± 0.07 a.u. These changes in hepatocytes ETC functioning in ischemic liver tissue cause an increase in mitochondrial O_2^- generation rate up to 0.81 ± 0.09 nM/g tissue · min. Mitochondria of conditionally healthy liver tissue generate O_2^- at level

0.73 ± 0.07 nM/g tissue · min. Ischemic liver cells generate O_2^- at level 1.42 ± 0.15 nM/g tissue · min, conditionally healthy tissue cells produce O_2^- at level 0.96 ± 0.12 nM/g tissue · min (Fig. 4). Our results suggest the contribution of other producers of oxygen radicals, in particular NOX neutrophils, which infiltrate affected tissues. In liver tissue, under ischemia, a decrease of molybdenum-containing enzymes activity — xanthine and aldehyde oxidase ($g = 1.97$) was found, which leads to toxic products of purines and aldehydes destruction accumulation.

Detected changes in ischemic tissue mitochondria functioning cause a violation of energy metabolism (decrease ATP levels), enhanced anaerobic glucose metabolism, lactate accumulation. An ATP synthesis reduction leads to acute cellular swelling (edema) — one of the earliest manifestations of ischemic injury, decreased intracellular levels of Na^+ and increased intracellular Ca^{2+} concentration, which facilitates the activation of proapoptotic pathways. Restoration the oxygen tissues supply as a result of reperfusion causes unregulated O_2^- generation in hepatocyte mitochondria and NOX of leukocytes and platelets, inducing the opening of mitochondrial pores and cell apoptosis [15–19].

Liver ischemia is a risk factor in case of its transplantation and resection [20], and the growth of O_2^- levels in hepatocyte and tissue mitochondria is a hallmark not only of ischemic tissue, but also of many diseases (neurodegenerative, cardiovascular, chronic inflammation), those associated with enhanced O_2^- generation [21]. However, O_2^- is known to be a key factor in cell apoptosis induction, in particular in hepatocytes, which in turn plays an important role in ALF development after resection [18, 22]. In addition, persistent oxidative stress contributes to pathological progression of liver fibrosis and increases the risk of surgery-treated organ functional failure.

Our results and the results of other authors suggest that human mCRC tumors, tumor cell lines have significantly higher levels of 8-oxodGu than normal tissues or normal cell lines [23–25]. The key point is that the growth and development of malignant neoplasms is accompanied with steadily increase of O_2^- generation level produced as a result of mitochondrial ETC dysfunction [26–28]. Oxidative modification of DNA can lead to cytotoxic effects that are fundamental in pathogenesis of many diseases, including neu-

Table 2. Results of linear regression of analyzed factors in mCRC patients

Model	Non-standard coefficients		Standard coefficients	t	Significance	95.0% CI lower limit
	B	Standard error	Beta			
Constant	1.235	0.172		7.167	0.000	0.891
V_{mts}	0.064	0.013	0.486	4.738	0.000	0.037
$t_{resection}$	0.003	0.001	0.345	3.369	0.001	0.001
Age	0.023	–0.034	0.071	0.042	0.930	1.076
T	0.443	0.300	0.150	0.089	0.659	1.517
N	0.638	0.414	0.111	0.066	0.461	2.170
G	–0.071	–0.373	–0.323	–0.201	0.834	1.199
PM	0.786	0.453	0.308	0.190	0.721	1.387
BMI	0.016	–0.020	–0.121	–0.072	0.900	1.111
$t_{ischemia}$	0.017	0.005	0.352	3.277	0.002	0.006

Note: BMI – body mass index; PM – yes/no; G – degree of tumor differentiation; N – status of regional lymph nodes; T – status of primary tumor, $t_{ischemia}$ – duration of warm ischemia during the transection of liver parenchyma; $t_{resection}$ – duration of liver surgery, min; V_{mts} – volume of metastatic tissue based on CT-volumetry results, cm^3 .

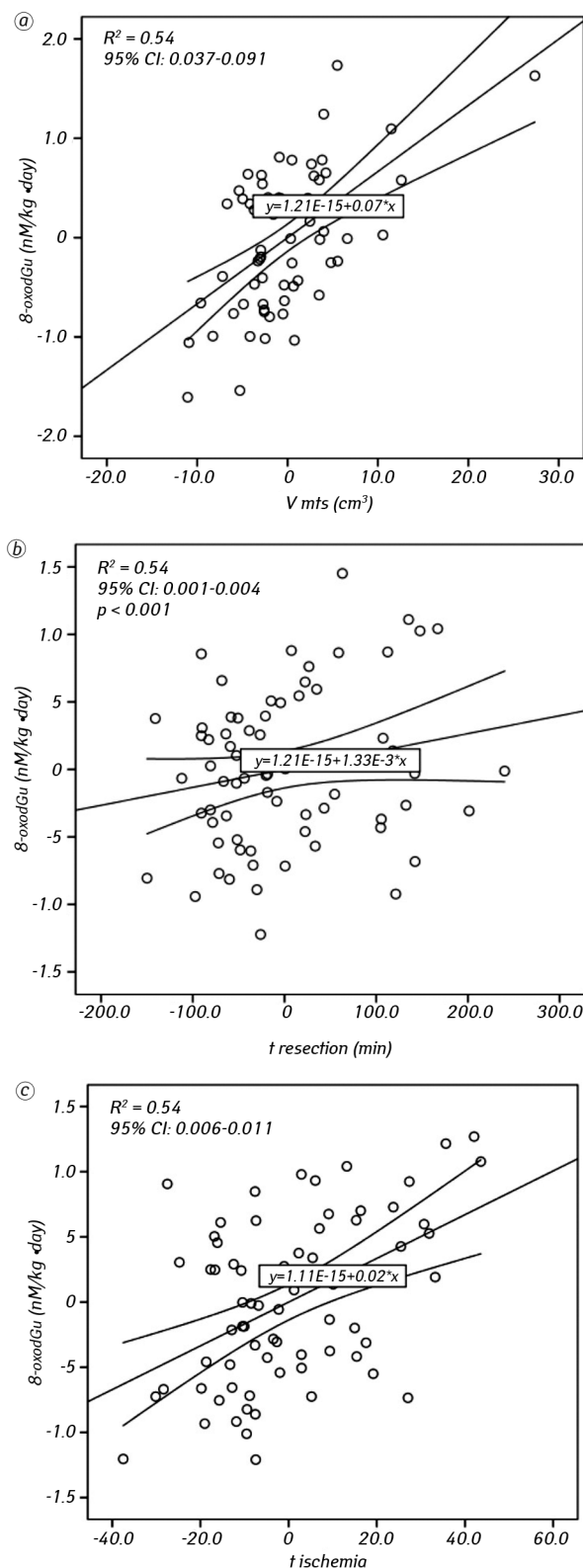


Fig. 3. Graphical representation of linear regression model showing: *a* — effect of volume of metastatic liver tissue (V_{mts}); *b* — duration of liver parenchyma transection ($t_{resection}$) and *c* — duration of warm ischemia during PM ($t_{ischaemia}$) on urine 8-oxodGu levels in mCRC patients

rodegenerative, cardiovascular, chronic inflammatory diseases and cancer [29–32]. 8-oxodGu formation leads to transcription violations due to switch G:C → T:A. Moreover, formation of 8-oxodGu may cause decreased microsatellites formation and ac-

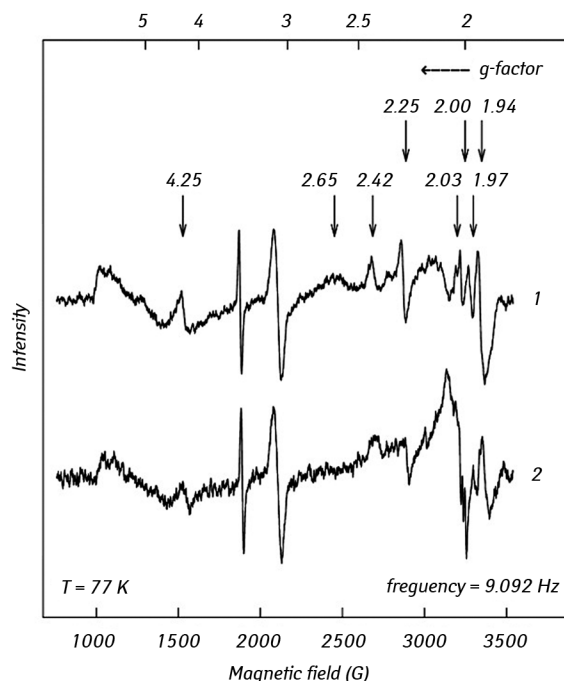


Fig. 4. ESR spectra of tissue: 1 — conditionally healthy liver; 2 — ischemic liver

celerate the telomere reduction [23, 33]. Our results indicate a significant increase of urine 8-oxodGu levels in mCRC patients. This can be an informative biomarker for evaluation of metastatic organ damage in mCRC patients.

CONCLUSIONS

Thermal ischemia of liver during the PM (≥ 40 min), long-term surgery (≥ 300 min), and metastatic tissue volume ($\geq 12\text{ cm}^3$) in liver parenchyma in patients with mCRC cause damage of mitochondrial ETC (formation of NO-FeS-proteins), resulting in an increase of O_2^- generation rate in mitochondria and tissue, and urine 8-oxodGu levels in these patients. R0-resection of metastases in liver in mCRC patients leads to decrease of urine 8-oxodGu already at day 3 after the surgery. The level of urine 8-oxodGu in mCRC patients correlates with the volume of resection, duration of ischemia, volume of post-ischemic liver tissue, volume of tumor tissue in liver and the rate of O_2^- generation in tumor tissue and degree of tumor differentiation. Urine 8-oxodGu levels are significantly higher in patients with long-term metastases as compared to those in patients who did not have metastases. Levels of urine 8-oxodGu, level of intraoperative and postoperative ischemia of liver parenchyma are new factors of oncological prognosis in patients with rectal forms of mCRC that affect the liver.

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