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SHORT COMMUNICATION

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CYTOCHROME P450 CONTENT IN PRIMARY TUMORS AND LIVER METASTASES OF PATIENTS WITH METASTATIC COLORECTAL CANCER

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***Aim*: To determine the content of low-spin form of cytochrome P450 in primary tumors and liver metastases of the patients with metastatic colorectal cancer (mCRC) and and assess its prognostic significance. *Materials and Methods*: The levels of the oxidized and low-spin forms of cytochrome P450 in the tissues of patients with mCRC were studied by electron paramagnetic resonance. To detect CYP 1A2 and CYP 1B1 isoforms, Western blot analysis was used. The activity of metalloprotreinases was studied by gelatine zymography. *Results*: In the liver metastases and tissues adjacent to metastasis, the levels of low-spin forms of cytochrome P450 are lower than in the samples of conventionally normal liver tissue. Western blot analysis revealed that low-spin form of cytochrome P450 in primary tumors and liver metastases detected by electron paramagnetic resonance is attributed largely to CYP 1B1 isoform. The content of low-spin form of cytochrome P450 inversely correlated with the the activity of gelatinases (matrix metalloproteinase-2 and -9). The survival of patients with high levels of the low-spin form of cytochrome P450 in tumor tissues was higher than that of patients with low levels of the enzyme (105 months *vs* 61 months). *Conclusion*: In the primary tumors and liver metastases of patients with mCRC, the content of the low-spin form of cytochrome P450 decreased, which correlated inversely with patient's survival.**

***Key Words*: colorectal cancer, cytochrome P450, metalloproteinase, liver metastases, survival.**

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Colorectal cancer (CRC) is one of the most common cancers in Ukraine, and environmental factors and genetic susceptibility make an important contribution to the increase in its incidence rate. In spite of screening programs and novel chemotherapeutics, the medium 5-year survival of CRC patients remains not more than 55% [1].

P450 cytochromes are a family of enzymes that play a central role in the oxidative metabolism of a wide range of xenobiotics and biologically active endogenous compounds [2–4]. Cytochromes CYP 1A2 and CYP 1B1 are the main representatives of the cytochrome P450 family, which play an important role in the carcinogenesis of the female reproductive system and colon tissues in which they accumulate [5–9]. In fact, CYPs are key enzymes in the initiation of cancer and are relevant to the treatment outcomes since they mediate the metabolic activation of carcinogens and the inactivation or activation of chemotherapeutic drugs [6, 7]. The study of the activity of cytochrome P450 is important to identify potential factors that contribute to the development of metastatic CRC (mCRC).

Given that cytochrome P450 metabolizes endogenous and exogenous molecules, including drugs, monitoring the activity of these enzymes is important to analyze their state in the tumor in order to control antitumor therapy. The search for the novel biomarkers might be advantageous for improving the screening and diagnosis as well as survival in CRC.

The aim of the study was to determine the content of low-spin form of cytochrome P450 in tumors and metastases of the patients with CRC and assess its prognostic significance.

Materials and Methods. The samples of liver tissue and metastases of patients with mCRC have been analyzed: 121 samples of primary tumor tissue, 91 samples of liver metastases, 91 samples of liver tissue adjacent to metastasis and 10 samples of liver and intestinal tissue taken at a distance of 3 cm from metastasis and primary tumor, respectively. The patients treated in the National Cancer Institute (Kyiv, Ukraine) in 2015–2019 were enrolled into the study. All patients gave their informed consent on the use of the clinical samples for research purposes.

The levels of the oxidized and low-spin forms of cytochrome P450 were studied by electron paramagnetic resonance (EPR). To determine the levels of the oxidized and low-spin forms of cytochrome P450, the samples of tissues (1 g) were prepared in a special mold. EPR spectra were obtained at –196 °C on a PE-1307 computerized spectrometer with an H_{011} resonator. The SHF power was 40 mW, the modulation frequency — 100 kHz, the amplitude — 10 Gauss, and the receiver time constant τ = 0.3 s. A specially oriented sample of an Al_2O_3 single crystal with a specified concentration of Cr^{3+} ions was used as an intensity standard. Using the double integration method, the concentration of molecules was estimated by compar-

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Abbreviations used: CRC – colorectal cancer; EPR – electron paramagnetic resonance; mCRC – metastatic colorectal cancer; MMP – matrix metalloproteinase.

ing the signal intensities in the EPR spectra with the standard intensity. The error in the method of integrating the spectra and the variation in reproducibility of the spectra of each specified sample was ⩽ 2%.

For Western blot analysis, the samples of tissues were extracted with 0.01 M Tris lysis buffer containing 7 M urea, 2 M thiourea, 1 mM ethylenediaminetetraacetic acid 10% glycerol and sonicated for 1 min on ice. The samples containing the equal amounts of protein were loaded into the wells of SDS-PAGE (12.5%) and the gel was ran at 100 V in 27.5 mM Tris buffer containing 1.4% glycin and 0.1% SDS. Following electrophoresis, the proteins were transferred onto polyvinylidene fluoride membrane. Immunoblotting was performed according to the standard protocols with antibodies against CYP 1A2 (CYP 1A2 Antibody (3B8C1):sc-53614), CYP 1B1 (CYP 1B1 Antibody (G-4):sc374228) i glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz Biotechnology, USA) at 1:1000 dilution in PBS/0.5% Tween-20. The membrane was incubated with primary antibodies overnight, washed 4 times with PBS/0.5% Tween-20 and incubated with goat anti-mouse IgG conjugated with horseradish peroxidase (sc-2005 Santa Cruz Biotechnology, USA). The expression of GAPDH was used as the housekeeping gene control to confirm equal loading of the samples. The gels were scanned by HP Scanjet 5590 and quantified with the aid of TotalLab 1.01.

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. 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.). 1 a.u. of gelatinase activity in tumor tissue is taken as the activity of 1 μg of enzyme per 1 g of tissue. TotalLab 1.01 program tool was used for the calculation.

Statistical analysis was performed using free statistical packages Datamash and R (https://www.gnu.org/software/datamash/, https://www.r-project.org/). The data are presented as M ± SE. P values < 0.05 were considered statistically significant. The survival of patients was analyzed by Kaplan — Meier method.

Results and Discussion. The reduced levels of oxidized (EPR signal with g = 2.42) and low-spin forms of cytochrome P450 (EPR signal with g = 2.25) were revealed not only in liver metastases but also in the liver tissue contacting with the metastatic focus as compared with the liver tissue at the distance of 3 cm from the metastasis. Here, the level of the low-spin form and oxidized forms of cytochrome P450 was determined at the level of 0.71 ± 0.16 a.u., in the liver tissue contacting with metastases — 0.49 ± 0.08 a.u., in metastasis — 0.16 ± 0.05 a.u. (Fig. 1). In normal tissue of the colon and liver, the level of the low-spin form of cytochrome P450 was 0.58 ± 0.06 a.u. and 0.89 ± 0.12 a.u., respectively (data not shown). Analyzing the distribution of the patients according to the levels of the low-spin form of cytochrome P450, we have found that in 73% of patients its content in the primary tumors and liver metastases was relatively low (0.15 ± 0.07 a.u.) while in the rest of patients it was higher (0.27 ± 0.05 a.u.). Nevertheless, these values were much lower than those characteristic of the corresponding normal tissue.

Since EPR signals of low-spin forms of CYP 1A2 and CYP 1B1 overlap, we analyzed several tumor samples by Western blot analysis to discriminate between these isoforms. In fact, we detected low levels of CYP 1A2 expression in the primary tumor, metastatic liver tissue, and liver tissue contacting with metastases (Fig. 2, *A*). Contrary to CYP 1A2, the protein levels of CYP 1B1 were significantly elevated in all investigated samples (Fig. 2, *B*). Therefore, the signals of low-spin forms of cytochrome seem to represent predominantly CYP 1B1 isoform. Recently, considerable attention has been paid to CYP 1B1, as a factor that affects the development and treatment of tumors [8]. Indeed, we showed high level of this isoform of cytochrome P450, which indicates a high level of metabolic activity in primary tumor of mCRC, liver metastases and the liver tissue contacting with metastasis. High levels of CYP 1B1, which can catalyze not only the activation of several antitumor drugs, but also the inactivation of widely used taxanes, paclitaxel and docetaxel, alkaloids, must be taken into account, since this could affect sensitivity to chemotherapeutics.

It was also shown that the activity of low-spin form of the cytochrome correlated inversely with the activity of gelatinases (MMP-2, MMP-9) ($p < 0.05$) (Fig. 3, *a, b*).

According to several studies, an increase of CYP activity enhances the expression of genes that promote metastasis [9]. In addition, oxygen radicals generated by cytochrome P450 can promote metastasis via MMP activation, enhance tumor growth, and angiogenesis [10].

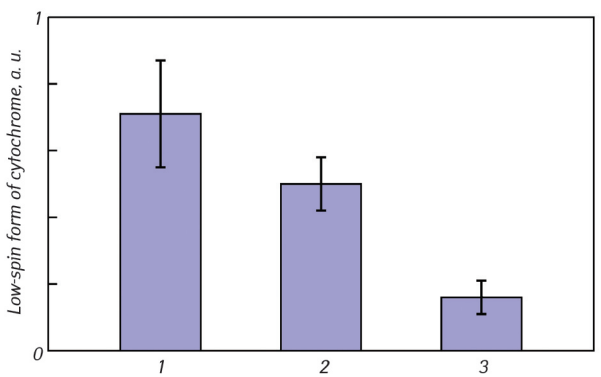

Fig. 1. Activity of the low-spin form of cytochrome P450: 1 — in the liver tissue at a distance of 3 cm from metastasis); 2 — in the liver tissue contacting with metastasis; 3 — in the liver metastases of mCRC

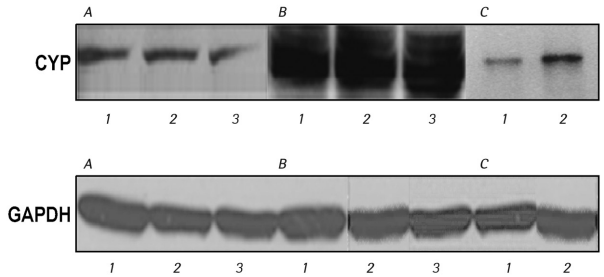

Fig. 2. Expression levels (Western blot) of CYP 1A2 (A) and CYP 1B1 (B): 1 — in liver tissue adjacent to metastatic focus; 2 — in CRC tumor tissue; 3 — in metastatic focus. The lanes marked C correspond to expression levels of CYP 1B1 (1) and CYP 1A2 (2) in the liver tissue at the distance of 3 cm from metastatic focus

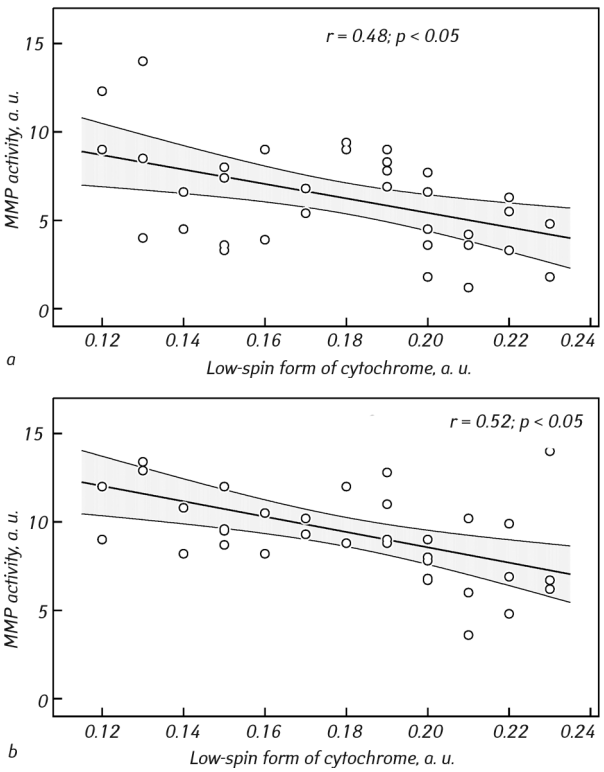

Fig. 3. The correlation between the levels of cytochrome and MMP-2 (*a*) or MMP-9 (*b*) activity in the tumor tissue of patients with mCRC

A significant difference was found in the survival of patients in tumors of which moderate levels of the low-spin form of cytochrome were recorded in comparison with those patients whose tumors showed low content of this enzyme (105 months *vs* 61 months (Fig. 4)).

Considering the chemoprevention of tumors as the promising line of research in oncology, it is interesting to note that several phytochemicals possessing anti-inflammatory and antiangiogenic activities have been shown to be the regulators of cytochrome P450 activity, in particular CYP 1B1 and CYP 1A1/2. Protumorous effects of CYP 1B1 through the metabolic bioactivation of xenobiotics and steroid hormones into the carcinogenic substances should also be taken into account.

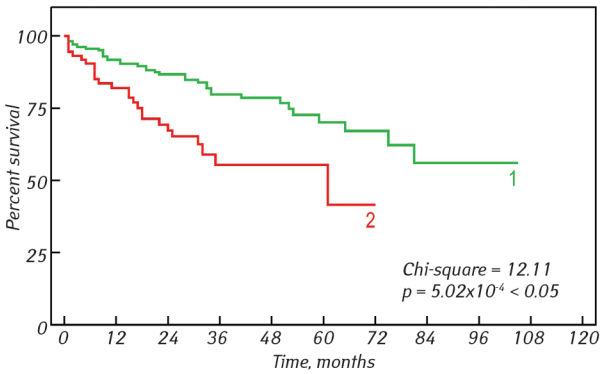

Fig. 4. Kaplan — Meier diagrams of the survival of patients with low (1) or high (2) content of low-spin form of cytochrome P450 in liver metastases of mCRC

To sum up, low levels of the low-spin cytochrome P450, CYP 1A2, and high levels of CYP 1B1, compared with the conventionally normal liver and colon tissue, were found in the primary CRC, liver metastases, and the liver tissue contacting with metastases. The strategies directed onto the specific inhibition of CYP 1B1 could be advantageous in clinical approaches for chemoprevention and angioprevention.

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