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## IMMUNOHISTOCHEMICAL AND GENETIC PROGNOSTIC FACTORS OF NEOADJUVANT CHEMORADIO THERAPY EFFICACY IN PERSONALIZED TREATMENT OF LOCAL ADVANCED RECTAL CANCER

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### ABSTRACT

**The aim:** The aim of our study was to define the factors that can robustly predict a response to neoadjuvant chemoradiotherapy (NCRT) in patients with local advanced rectal cancer (LARC) and prognosis factors of progression free survival (PFS) using molecular (8-oxodGu), immunohistochemical (Ki-67) and genetic (GSTP1 and MTHFR genes polymorphism) markers.

**Materials and methods:** *GSTP1* and *MTHFR* polymorphisms were studied by real-time PCR on tumour material from 110 patients with LARC. Ki-67 protein expression was assessed using rabbit monoclonal antibodies to Ki-67 (Dako, Denmark) on EnVision™ FLEX detection system (Dako, Denmark). 8-oxodGu level in eluate was measured by spectrophotometry.

**Results:** Patients from both groups showed significant pathomorphological response to NCRT. It is robust correlation between 8-oxodGu levels in patients' blood and their response to CRT (mrTRG scale) in MG was determined. Oxaliplatin-containing chemotherapy promotes statistically significant decrease of 8-oxodGu levels. With the decrease of Ki-67 protein expression level the probability of tumour relapse increases. It is determined that critical value of Ki-67 protein expression level makes less than 27 and tumour relapse probability in this case makes 50%. Tumour relapse risk in patients with *GSTP1* and *MTHFR* polymorphism is 12.3 and 16.3 times higher than in patients who do not carry such polymorphism, respectively. Combination of *GSTP1*, *MTHFR* polymorphisms and Ki-67 protein expression factors determines prognostic probability of tumour relapse within 51-99%.

**Conclusions:** 8-oxodGu level can serve as independent prognostic factor of NCRT efficacy in patients with LARC. Combination of *GSTP1*, *MTHFR* genes polymorphism with Ki-67 protein expression decrease enables monitoring and robust prognosis of LARC relapse.

**KEY WORDS:** neoadjuvant chemoradiotherapy, rectal cancer, prognosis factors.

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### INTRODUCTION

Modern tactics for localized rectal cancer treatment is strictly standardized and regulated by International Guidelines (NCCN Guidelines v.2. 2017) [1]. In contrast to localized forms, for LARC that features high probability of relapse and inner organs systemic lesion there still is no finally formulated treatment and diagnostic standard and until very recently even definition by itself was not formulated [2].

To reach proper resectability levels, NCRT is the only solution it was supported by 100% of the experts and certified as the form of Consensus (2013). This procedure enables "stage downgrade" – transformation of locally spread form into localized one, significantly improves postponed treatment results and decreases the number of local relapses. Tumour regression degree after NCRT directly correlates to survival rates and local relapse frequency [3].

Tumour reaction on CRT can vary from complete pathological regression (pCR) to full resistance (according to different authors, regression value makes from 3 to 54 %) [4]. Tumour regression degree after NCRT directly

correlates to survival rates and local relapses frequency. Based on randomized clinical trials, it is proved that preliminary course of radiotherapy both fluorouracil and with oxaliplatin promotes tumour regression. Such effect enables definitive organ preserving surgery and leads to local relapses decrease in postsurgical period. NCRT is integral part of combined LARC treatment and can induct partial, significant and total regression of primary tumour with long lasting PFS [5, 6].

Nowadays, the main goal of LARC NCRT is to decrease the relapse development risk in true pelvis by means of maximal reduction of primary tumour size and tumour process stage downgrade. To meet this goal, modified regime of neoadjuvant chemotherapy (NCT) – replacement of fluoropyrimidine monochemotherapy to oxaliplatin polychemotherapy, looks as a very promising strategy. Yet, growing NCRT interruption frequency resulting from toxicity increase remains to be an issue. Positive impact on general ten-years survival was showed only in one of four multi-institutional randomized research published as for today [7-10]. Nevertheless, in recently published scientific

reports there is no any data about any of chemotherapy agents' advantages. The results of STAR-01, ACCORD 12/0405-Prodige-2, NSAPB P-04, PETACC-6 randomized studies did not demonstrate significant improvement in pathological complete locoregional response and increase in survival rates connected to oxaliplatin addition to the treatment regimen. Moreover, in patients treated with oxaliplatin toxicity increase of 3-4 grade was observed [11-33].

Opposed to this, German CAO/ARO/AIO-04 studies involving integration of oxaliplatin into fluoropyrimidine-based NRT of rectal cancer demonstrated higher pathological complete response (pCR – 17% versus 13%;  $p = 0.038$ ), higher 3-years PFS (75.9% versus 71.2%;  $p = 0.03$ ) and no increase of general toxicity in oxaliplatin group [14, 15].

Though, there is no clear data about efficacy of NCRT with multimodal oxaliplatin including treatment in patients with LARC. According to ESMO and NICE recommendations, improved treatment results can be expected in field of personalized approach that considers immediate and postponed relapse risks. The problem of choosing right NCRT should be further investigated taking into account clinical, radiological, pathomorphological data and prognostic markers.

Other very important and insufficiently studied aspects are immunohistochemical and genetic peculiarities of tumour as prognostic factor in RC.

Level of malignancy maturation depends on intensity of tissue and cellular atypism and mitotic activity level. To assess cell proliferation in tumor, series of markers are commonly used. Conventional cell proliferation marker Ki-67 is one of them. This protein is expressed in proliferating cells during G1 phase and is absent in G0 resting cells [16]. This makes Ki-67 clinically significant proliferation marker for prognosis of several cancer types [17-21].

Clinical trials of Z. Pap et al. showed that in adenoma of the large intestine the Ki-67 expression level directly correlates with seriousness of dysplastic changes [22]. Several studies showed that significant Ki-67 expression is mainly connected to lower overall survival rates [23-27], while other studies highlight that high Ki-67 expression correlates with improvement of general survival and better oncological prognosis [28-30].

It is well known that gene polymorphisms participate in colorectal cancer pathogenesis and many chemotherapeutic drugs metabolism. Individual peculiarities of glutathione S-transferase (*GSTP1*) and methylene-tetra-hydro-folate-reductase (*MTHFR*) enzymatic activity mediated by gene polymorphism can predict rectal cancer and development of the resistance to NCT (oxaliplatin, 5-fluorouracil, irinotecan). *GSTP1* and *MTHFR* polymorphism detection in patients with RC can be efficient for predicting tumor cell response to chemotherapy and toxicity effects of NCT [31-33].

It is very important that *MTHFR* participates in anti-neoplastic agent metabolism (methotrexat, fluorouracil). 5-fluorouracil acts via fluorodeoxyuridine monophosphate and inhibits thymidilate synthase [34]. Drop of *MTHFR*

fermentative activity leads to increase of methylenetetrahydrofolate level and thereby enhances 5-fluorouracil cytotoxicity. *MTHFR* gene polymorphism and efficacy of 5-fluorouracil therapy were estimated in experimental research and clinical trials [35, 36].

It is known that chemical cancerogens such as polycyclic aromatic hydrocarbons and heterocyclic aromatic amines are connected to RC; aromatic amines and some of cytostatics such as platinum products, anthracyclines and steroid hormones are the substrates for *GSTP1* enzyme. Substitution of adenine for guanine in position 313 of the 5<sup>th</sup> exon in *GSTP1* gene leads to isoleucine substitution for valine in position 104 and reduction of its affinity to electrophilic substances [37].

Moreover, polymorphic variants of *GSTP1* and *MTHFR* are the risk factor for gastrointestinal and cardiovascular toxicity that can be associated with development of resistance to chemotherapy. Genotyping of *MTHFR* C677T polymorphism enables personalization of chemotherapy agents.

In a number of scientific studies, much attention is paid to tumor microenvironment that is one of the key progression factors of tumor resistance to chemotherapy [38]. In addition, further investigations of superoxide radicals and 8-oxoguanine influence as potential markers of RC development are very promising [39-41].

8-oxodGu present in DNA in the absence of reparation can cause cell cycle arrest and apoptosis. Most scientific research indicate the role of oxidative stress in pathogenesis of colon cancer. Chang et al. report that 8-oxodGu content in blood serum can serve as a sensitive biomarker in patients with rectal adenocarcinoma [42] and is considered as a high-informative marker of tumorigenesis [43] and important marker of tumor response to treatment [44].

As a result, this review of combined LARC treatment based on immunohistochemical and genetic prognosis factors implies many complicated unresolved issues that should be addressed in future scientific studies.

## THE AIM

Our aim was to define prognostic factors of NCRT efficacy in patients with LARC, namely the prognostic factors of PFS using molecular (8-oxodGu), immunohistochemical (Ki-67) and genetic (*GSTP1* and *MTHFR* genes polymorphism) markers.

## MATERIAL AND METHODS

This research is based on the retrospective data analysis from 110 patients, who underwent combined treatment of LARC in Oncocolonoproctology Department of National Cancer Institute from 2016 to 2019 years.

The diagnostic algorithm included estimation of overall patient's status according to ECOG scale, fibrocolonoscopy with biopsy and morphological verification, magnetic resonance imaging (MRI) of thoracic, abdominal and pelvic organs with intravenous contrast, laboratory tests and

electrocardiography. Local staging was conducted using Philips Intera 1,5 T MRI scanner according to MERCURY protocol [45]. All patients confirmed by MRI to be CRM+ were included into the research. It means tumor invasion, metastatic transformation of lymph nodes or extranodal tumor deposits, tumor infiltration of mesorectal soft tissue to a distance of not less than 1 cm from the edge. Accuracy of tumor topography in relation to mesorectal fascia played a critical role in choosing treatment plan, namely administration of NCRT and was the most important reference point for total mesorectal excision (TME). Estimation of tumor regression grade according to MRI is based on mrTRG and RECIST 1.1 (Response Evaluation Criteria in Solid Tumours) criteria.

All patients with LARC considering NCRT method were randomized at a ratio of 1:1 with respect to cTNM-pTNM (T3-4 N0-2 M0, CRM+) index.

Patients from MG underwent radiotherapy with total radiation dose of 50.4 Gy (28 sessions 1.8 Gy each) and polychemotherapy according to CAPOX regimen with oxaliplatin in non-adjuvant regime: capecitabine 200 mg/m<sup>2</sup> in two equal doses, one in the morning and one in the evening, peroral in 30 minutes after meals from day 1 to day 14; oxaliplatin 130 mg/m<sup>2</sup> intravenous on day one of the cycle. The interval between cycles made 21 days.

Patients from CG underwent radiotherapy with total radiation dose of 50.4 Gy (28 sessions 1.8 Gy each) and monochemotherapy based on fluoropyrimidines in non-adjuvant regime: capecitabine 200 mg/m<sup>2</sup> twice a day. The interval between cycles made 5 days. In general, every patient underwent two cycles of chemotherapy. Toxicity effects of NCRT were evaluated according to CTC-NCIC scale (version 4.03, 2010). Eight days after NCRT course its efficacy in all patients was evaluated using MRI according to the protocol mentioned above.

Eight weeks after NCRT course, all patients underwent TME. Histopathological estimation of TME quality was conducted according to P. Quirke method. Post-surgical complications were estimated according to Clavien-Dindo-Strasberg classification within 1-30 days period starting from the day of surgery. All observations are in compliance with Ethics Committee requirements.

To study *GSTP1* and *MTHFR* genes polymorphism, genomic DNA was extracted from paraffin embedded tumor material using method of NC adsorption on silica-membrane (PureLink Genomic DNA Kits, Invitrogen, USA). DNA concentration was measured using NanoDrop-1000 spectrophotometer (Thermo Scientific, USA). Prior to amplification, DNA concentration was from 2 to 8 ng/μL. *GSTP1* and *MTHFR* genes polymorphisms were studied by allele-specific real-time PCR on 7300/7500 Real-Time PCR Systems (Applied Biosystems, USA). To study *A313G*, *GSTP1* and *C677T* *MTHFR* single nucleotide polymorphisms TaqMan Probes of MGB-type were used. TaqMan Probes and primer sequences were selected using Primer Express® Software v3.0 (Applied Biosystems, USA) and synthesized by Applied Biosystems, USA. PCR reaction conditions were as following: 50 °C for 2 min, 95 °C

for 10 min, followed by 45 cycles at 92 °C for 30 s and 60 °C for 1 min.

The levels and accumulation rates of guanine DNA oxidative damage – 8-oxo-dGuo in neutrophilic blood granulocytes of LARC patients were measured 1 day before surgery [46]. For this purpose, 5 mL of patient's blood from median cubital vein were collected into centrifuge tube with 1 mL of Trilon B. Neutrophils were separated from according to H.T. Lee [42]. The 8-oxo-dGuo was evaluated in eluate on spectrophotometer.

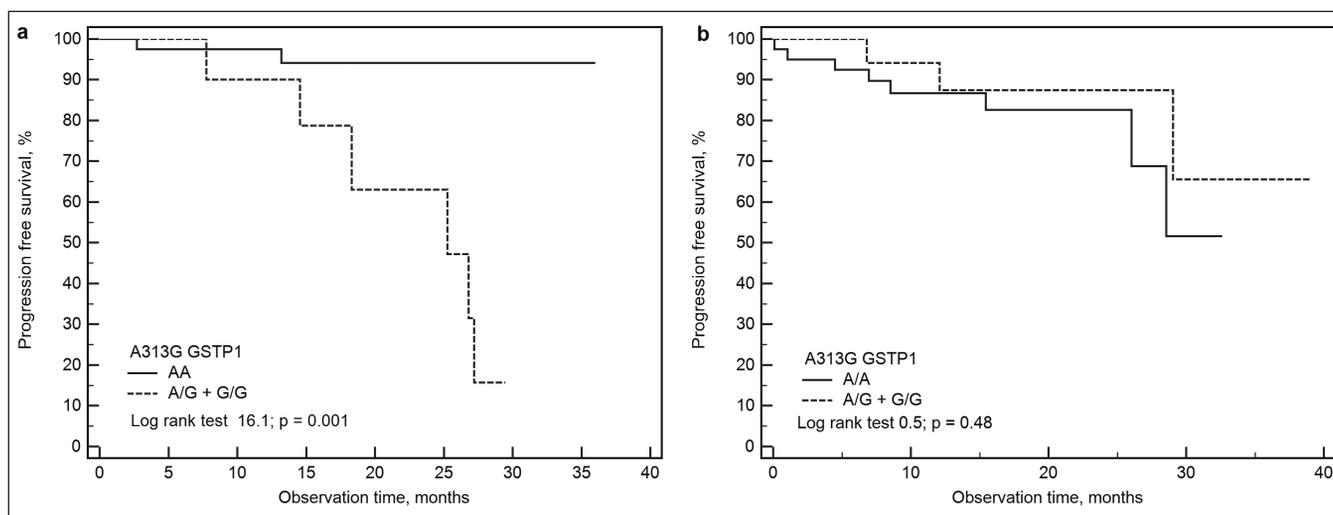
To study Ki-67 expression, obtained material was fixed in buffered 10% formaldehyde, pH 7.4, and embedded in paraffin using Histo-5 (Milestone, Italy). Histological slices 5 μm in depth were obtained using Microm HM325 (Thermo Scientific, USA). The slices were stained with haematoxylin and eosin and general tumor estimation was conducted. Immunohistochemistry was performed using rabbit monoclonal antibodies to Ki-67 (Dako, Denmark) on EnVision™ FLEX detection system (Dako, Denmark). For antigen retrieval, citric buffer with pH 6.0 was used. Primary antibodies were incubated at room temperature for 30 min., secondary antibodies – for 20 min. at room temperature. After this, slices were additionally stained with haematoxylin Gill. As a positive control, specimens with determined positive reaction were used. The same procedure without primary antibodies served as a negative control. Quantitative microscopic assessment of NCRT efficacy was done according to I. Miller and S. Payene (2003).

Statistical analysis included descriptive statistics (means as  $M \pm m$  or median –  $Me$ , minimal value –  $min$ , maximal value –  $max$ ). Comparison of two independent groups based on one feature was conducted using Mann-Whitney test; comparative assessment of the groups based on variable fraction proportion expressed in nominal or ordinate scale was taken using  $\chi^2$  Pearson test. Two variables correlation analysis was based on Spearman's rank correlation coefficient. Logistic regression was used for prognostic marker estimation in LARC treatment efficacy. Null-hypothesis of variables equality was rejected when  $p < 0.05$ . Data analysis was carried out in IBM SPSS Statistics 22.

## RESULTS AND DISCUSSION

Fifty seven patients were randomized into MG and fifty three to CG. Average age of the patients in MG makes  $59.3 \pm 11.4$  years, in CG –  $62.5 \pm 10.2$  years. There were no statistically significant differences in gender, age and anthropometric parameters (body-weight index and total body area) between patients. According to the pathology report, all tumors were adenocarcinomas on varied differentiation stages. Full scale NCRT was conducted in all patients. As a result, the groups were representative.

After the analysis of regression value after NCRT according to MRI (mrTRG and RECIST 1.1 scales), it was determined that tumor response to NCRT was higher in MG – 34 patients (59.7%) and 37 (64.9%) than in CG – 25 (47.2%) and 26 (49.1 %), respectively, however, this difference was statistically insignificant ( $p < 0.576$ ;  $p < 0.329$ ).



**Fig. 1.** The effect of A313G GSTP1 gene polymorphism on 3-year PFS rate in LARC patients after combined treatment. a – patients received capecitabine as neoadjuvant chemotherapy; b – patients received CAPOX as neoadjuvant chemotherapy.

Significant difference was observed in conversion level of CRM+ to CRM: in MG – 39 (68.4%) and 24 (45.3%) ( $p < 0.05$ ).

The estimation of morphometric and topographic anatomy features of tumor regression depending on NCRT scheme showed that in MG the distance between dental line and lower edge of the tumor significantly increased and made  $1.1 \pm 0.1$  cm versus  $0.7 \pm 0.1$  cm in CG ( $p < 0.005$ ). The length of the tumor decreased by  $28.1 \pm 2.1\%$  versus  $20.4 \pm 2.7\%$  ( $p = 0.024$ ), respectively. Thus, positive effect of NCRT on tumor pathogenesis treatment conducted according to the CAPOX scheme and on the quality of TME was proved.

To make oxaliplatin-based NCT more personalized, the level of 8-oxodGu in neutrophils from human blood of LARC patients in both groups was measured. Physiological level of 8-oxo-dGuo makes  $0.23 \pm 0.04$  nmol/mLxmin [19].

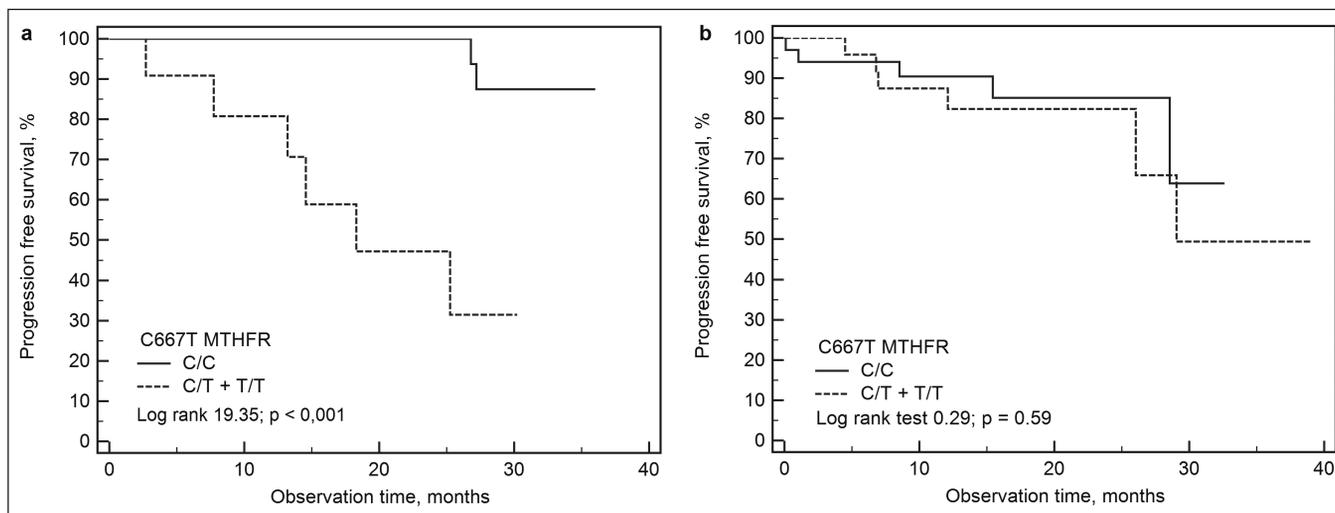
The statistical analysis of 8-oxodGu level dependent on chemotherapy scheme prescribed, revealed that application of oxaliplatin-based NCT as a part of comprehensive treatment of LARC patients aids the significant decrease of studied marker levels: ( $R^2 = 0.465$ ; 95% CI: 0.004 – 0.016,  $p < 0.0001$ ). Before NCRT 8-oxodGu level in MG was  $3.07 \pm 0.08$  nmol/mLxmin, in CG –  $2.94 \pm 0.06$  nmol/mLxmin, after it –  $1.96 \pm 0.04$  nmol/mLxmin and  $2.72 \pm 0.04$  nmol/mLxmin ( $p < 0.001$ ), respectively. Thus, we can conclude, that 8-oxodGu level can be considered as an independent prognostic marker of NCRT efficacy in LARC patients.

Based on logistic regression analysis, the concentration border level of 8-oxodGu in blood is 2.9 nmol/mLxmin. Based on the data supplied above, lowering of 8-oxodGu concentration is recommended before NCRT according to CAPOX scheme when its level is  $\geq 2.9$  nmol/mLxmin.

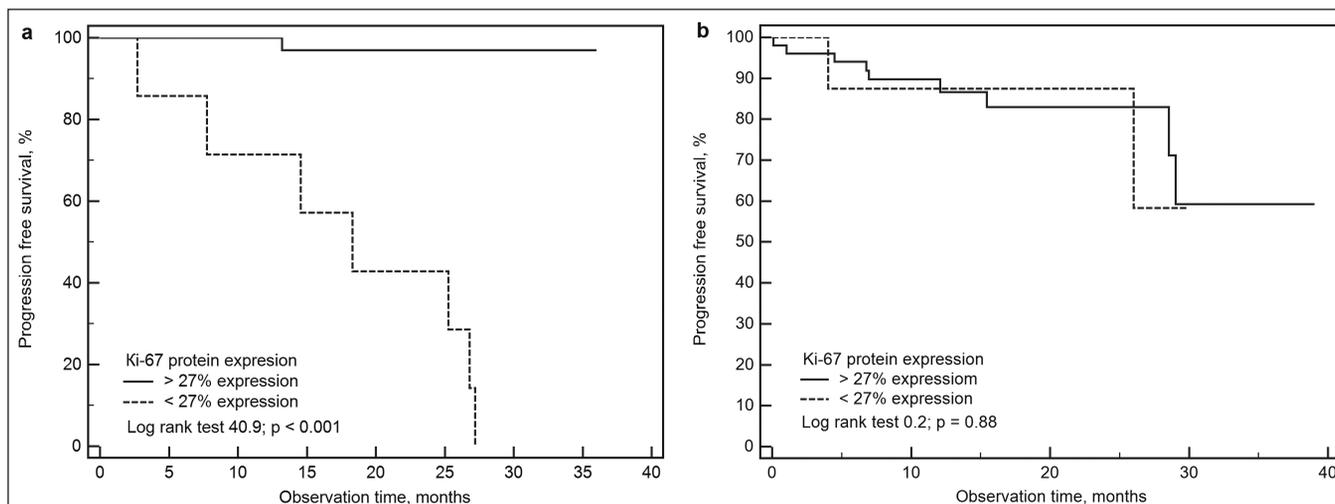
All patients in both groups underwent surgery 8 weeks after the NCRT. Surgery extent was determined depending on localization and primary tumor expansion rate. All surgical procedures were R0-resections. In MG 37

patients (65%) and in CG 26 patients (47%) underwent laparoscopic surgery. There was no statistically significant difference between groups in terms of surgical intervention structure ( $p = 0.196$ ). According to visual estimation, TME performance in both groups was satisfactory. Positional level of tumor location related to anus ( $r = 0.431$  in MG and  $r = 0.417$  in CG) positively correlated ( $p < 0.05$ ) to distal border of rectum resection ( $r = 0.510$  and  $r = 0.532$ ), respectively, and inversely correlates to malignancy stage after NCRT ( $r = -0.390$  and  $r = -0.370$ ) involving circumferential resection margin (CRM+) ( $r = -0.514$  and  $r = -0.522$ ). Quality of mesorectumectomy was evaluated after Quirke P et al. However, there was no significant difference between groups in terms of differences in surgical intervention method.

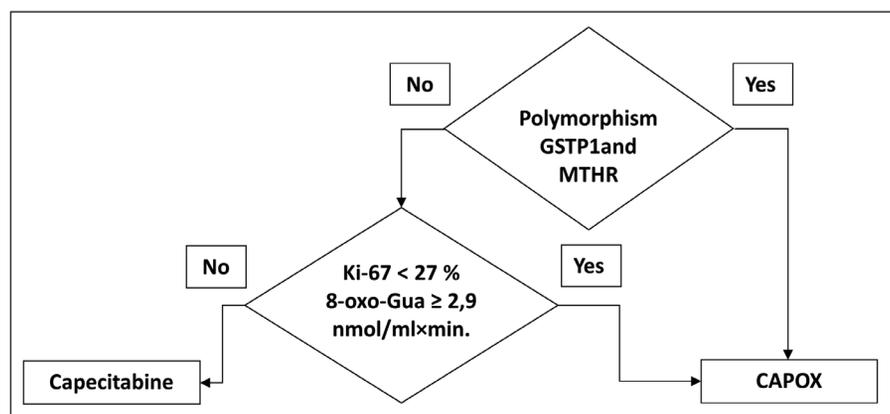
In postsurgical period, surgical material was investigated in terms of morphology. Therapeutic pathomorphosis level was evaluated according to parenchymatous tumor tissue percentage, fibrosis and necrosis levels, considering NCRT conducted (CAPOX regimen and capecitabine-based NCRT). Therapeutic pathomorphosis level in MG was 42.0 %, 58.0 %, 31.2 %, 51.6 %; in CG: 56.9 %, 43.1 %, 34.3 %, 39.2 %, respectively. Evaluation of prescribed NCRT regimen did not confirm higher efficacy of them in terms of stronger pathological response. In this context we talk about polychemotherapy according to CAPOX scheme versus capecitabine-based monochemotherapy. The fact that we did not observe any significant statistical difference in cumulative frequency of PFS between MG and CG ( $73.5 \pm 8.5\%$  versus  $70.2 \pm 2.5\%$ ,  $p = 0.522$ ) and correlative general survival ( $87 \pm 7.0\%$  and  $96 \pm 2.7\%$ ) proves this. However, according to morphometrical data, there is a statistically significant difference in locoregional therapy response, namely a decrease of parenchymatous tissue and stroma volume 56.9 % and 43.1% in CG resulting from CAPOX scheme NCRT compared to 42 % i 58% in MG ( $p < 0.001$ ). This findings lead to the conclusion that none of above described chemotherapy agents has advantages over the



**Fig. 2.** The effect of C667T MTHFR gene polymorphism on 3-year PFS rate in LARC patients after combined treatment. a – patients received capecitabine as neoadjuvant chemotherapy; b – patients received CAPOX as neoadjuvant chemotherapy.



**Fig. 3.** The effect of Ki-67 protein expression on 3-year PFS rate in LARC patients after combined treatment. a – patients received capecitabine as neoadjuvant chemotherapy; b – patients received CAPOX as neoadjuvant chemotherapy.



**Fig. 4.** The personalized algorithm of NCRT application in patients with LARC.

others. A series of international randomized studies listed in introduction came to the same output.

At the same time, to define prognostic factors of rectal cancer course we measured Ki-67 expression levels. The single-factor logistic regression analysis showed that probability of tumor relapse increases when Ki-67 expression level drops. The critical

value of Ki-67 expression makes <27% where the probability of tumor relapse chance makes 50%. According to the literature, Ki-67 expression level is a prognostic immunohistochemical biomarker that can predict survival rates decrease in patients with rectal cancer [57]. Comparison of Ki-67 expression level and PFS revealed statistically significant difference in terms of

**Table 1.** Recurrence prognostic model in patients with local advanced RC ( $\beta$  coefficient value and probability levels for independent prognostic factors)

Value	$\beta^*$	Vald.**	P	GSTP1	MTHFR	Ki-67 <27%	Recurrence prognostic probability, (%)
GSTP 1	3,036	4,293	0,038	+	+	+	= 99
MTHFR	3,169	4,270	0,039	+	+	-	≥ 62
Ki-67	-0,141	4,520	0,034	+	-	-	≥ 73
Constant	1,397	0,330	0,565	-	+	+	≤ 75
				-	+	-	≤ 51
				-	-	+	≥ 51

\* –  $\beta$  coefficient in logistic regression; \*\* – significance criterion for  $\beta$  coefficient.

PFS rate in CG. Patients with Ki-67 expression >27% have 27.5 times higher relapse risk compared to patients with Ki-67 expression <27%. In MG, such statistically significant difference was not observed (Fig.1).

Detection of polymorphism in *GSTP1* and *MTHFR* genes in LARC patients is a new innovative research direction. Three-year PFS in patients from CG that carry *GSTP1* and *MTHFR* polymorphism is significantly higher. According to logistic regression analysis, relapse risk in patients who carries *GSTP1* and *MTHFR* polymorphism is 12.3 and 16.3 times higher compared to patients without it, respectively. In CG three-year PFS decreases significantly in patients with A313G *GSTP1* and C667T *MTHFR* polymorphisms compared to the patients without these mutations ( $p < 0.001$ ). In MG, there was no statistically significant difference in relapse-free survival level in patients with or without *GSTP1* and *MTHFR* polymorphism (Fig.2, Fig.3).

Based on such factors as *GSTP1* and *MTHFR* gene polymorphism and Ki-67 expression level, we developed a mathematical model of relapse in LARC patients (table I). Based on multifactorial logistic regression analysis next formula was generated (Formula 1):

$$p = \frac{1}{1 + e^{-z}} \quad (1)$$

where  $Z = c + \beta_{f1} \times GSTP1 + \beta_{f2} \times MTHFR - \beta_{f3} \times Ki-67$ ;  $e = 2.72$ ;  $Z$  – likelihood of relapse;  $c = 1.397$  (constant);  $\beta_{f1}$  – coefficient for *GSTP1* factor;  $\beta_{f2}$  – coefficient for *MTHFR* factor;  $\beta_{f3}$  – coefficient for Ki-67 factor.

It helps estimate relapse risk patient-specifically within 3 years after treatment. Prognostic probability of relapse can be evaluated within 51–99% depending on *GSTP1*, *MTHFR* and Ki-67 factors combination.

As a result, personalized algorithm of NCRT selection was developed (Fig.4). In case of *GSTP1* and *MTHFR* polymorphism or their combination as significant prognosis factors, CAPOX scheme NCRT is recommended. If polymorphisms in these genes are absent, Ki-67 (< 27 %) and 8-oxoGu ( $\geq 2,9$  nmol/mLxmin) expression levels should be considered prior to NCRT prescription.

## CONCLUSIONS

The analysis of many scientific publications proves that LARC diagnostics and treatment is an essential and very

complicated issue in modern clinical oncology. Based on our own knowledge and practical experience in optimization of combined modality LARC treatment considering prognostic factors (8-oxoGu, Ki-67, A313G *GSTP1* and C667T *MTHFR*) we came to clear understanding of personalized NCRT schemes. The algorithm developed from multifactorial system allows to predict and monitoring LARC relapse development. Consequently, it will improve immediate and postponed results of treatment in patients with this complicated heterogenetic oncological disease.

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*The authors have no financial conflicts of interest.*

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