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Combined analysis of *KRAS* mutation and $p16^{INK4a}$ and $p14^{ARF}$ methylation status in locally advanced rectal carcinoma treated with preoperative chemoradiotherapy

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Abstract: Current management of locally advanced rectal carcinoma (LARC) involves preoperative chemoradiotherapy (preCRT) before surgery. Despite improved local control rate, the response to preCRT of individual patients is variable and may reflect heterogeneous biological properties among tumors of the same clinical stage. Identifying novel molecular parameters with predictive and/or prognostic value is of great clinical importance for a personalized therapeutic approach. In this study, *KRAS* mutation status was analyzed by direct sequencing, while methylation-specific polymerase chain reaction (MSP) was used to examine $p16^{INK4a}$ and $p14^{ARF}$ gene methylation status in pretreatment tumor biopsies of 60 patients with LARC. The examined molecular changes of *KRAS*, $p16^{INK4a}$ and $p14^{ARF}$ genes were mutually independent ($p16^{INK4a}$/*KRAS*, P=0.272; $p14^{ARF}$/*KRAS*, P=0.923; $p16^{INK4a}$/$p14^{ARF}$, P=0.715). However, the simultaneous presence of $p14^{ARF}$ methylation and *KRAS* mutation was associated with a more frequent appearance of local recurrences and distant metastasis (P=0.027). Moreover, patients with the simultaneous presence of $p16^{INK4a}$ and $p14^{ARF}$ methylation and *KRAS* mutation had significantly shorter overall survival (P=0.011). The obtained results strongly suggest that combined analyses of examined genetic and epigenetic molecular alterations could contribute to the identification of LARC patient subgroups with more aggressive tumor behavior and worse disease outcome.

Keywords: rectal carcinoma; *KRAS*; $p16^{INK4a}$; $p14^{ARF}$; gene methylation

INTRODUCTION

Rectal carcinoma (RC) represents approximately 30% of cases of colorectal carcinomas (CRC), and is considered a distinct clinical entity in terms of etiology and treatment strategy [1]. In the case of locally advanced rectal carcinomas (LARC), the standard treatment consists of preoperative chemoradiotherapy (preCRT) followed by radical surgery [2]. Despite a reduced local recurrence rate, the response of individual tumors to this multimodal treatment is variable and ranges from complete regression to complete resistance [3]. The observed variability of the tumor response indirectly reflects the molecular heterogeneity of tumors with similar clinicopathological characteristics. The identification of molecular predictive markers in pretreatment tumor biopsies may have clinical relevance in early patient stratification and therapy optimization.

It is widely accepted that different molecular events are responsible for cancer development at different positions throughout the colon and rectum, but the general molecular pattern of CRC carcinogenesis includes both genetic and epigenetic alterations [4]. One of the most common molecular alterations in CRC is somatic mutations of the *KRAS* proto-oncogene, a membrane-bound G protein with kinase activity that plays an important role in cell growth and proliferation. *KRAS* mutation occurs in about 30-40% of CRC as a point mutation mostly located at codons 12 and 13 of exon 2 of the *KRAS* gene [5]. The most investigated epigenetic modification in colorectal cancer is the aberrant DNA methylation of CpG islands within promoter regions associated with gene silencing [6]. Genes $p16^{INK4a}$ and $p14^{ARF}$ are located in the INK4a/ARF locus, which is commonly deleted or methylated in many tumor types. The protein prod-

ucts of these two genes are tumor suppressor proteins $P16^{INK4a}$ and $P14^{ARF}$, which act as key negative regulators of the cell cycle [7]. $P16^{INK4a}$ is a cyclin-dependent kinase inhibitor that prevents retinoblastoma (Rb) phosphorylation and thereby induces G1 phase arrest, while $P14^{ARF}$ indirectly facilitates tumor protein P53 (p53)-mediated cell-cycle arrest and apoptosis by its interaction with the mouse double minute 2 (MDM2) homolog also known as E3 ubiquitin-protein ligase protein [8].

KRAS mutations, as well as the inactivation of $p16^{INK4a}$ and $p14^{ARF}$ genes by promoter hypermethylation, have been frequently reported as early events in colorectal neoplasia [9,10]. However, a correlation between the mutation/methylation statuses of these three genes and clinical outcomes in patients with LARC is not fully established. The results of previous CRC studies have shown that the presence of a mutation in the *KRAS* gene may indirectly induce the methylation of the INK4a/ARF locus [11]. Moreover, literature data indicate that oncogenic activation of the *KRAS* gene induces protective mechanisms in tumor cells, such as oncogene-induced senescence via the $P16^{INK4a}$/pRb pathway [12], and oncogene-induced cell growth arrest and initiation of apoptosis via the $P14^{ARF}$/p53 pathway [13]. Considering these facts, in this study, we examined epigenetic changes – $p16^{INK4a}$ and $p14^{ARF}$ promoter methylation and genetic changes – *KRAS* mutations, in pre-treatment tumor biopsies of patients at locally advanced stages. The obtained results may be of clinical importance for the identification of subgroups of LARC patients with a distinct pattern of response to preCRT, as well as for the course and outcome of the disease.

MATERIALS AND METHODS

Ethics statement, patients and tumor samples

This retrospective study included 60 patients (38 male, 22 female; median age 65 years, range 49-82 years) with locally advanced rectal cancer (clinical stage cT3b, cN0-2 with positive circumferential margin (CRM) and cT4N02). All patients were diagnosed and treated with preoperative chemoradiotherapy at the Oncology Institute of Vojvodina, Sremska Kamenica, Serbia, during 2006-2010 according to the National Guidelines of the Ministry of Health of Serbia for Diagnosis, Therapy and Management. Written consent of patients, according to the Declaration of Helsinki, was obtained. The study was approved by the Ethical Committee of the Oncology Institute of Vojvodina, Sremska Kamenica, and conforms to legal standards. Data with all clinicopathological parameters, response to the treatment, overall survival, as well as the immunohistochemical detection of EGFR, VEGF, Bcl-2 and Ki67 expression were taken from medical documentation of the above institution.

The location of the tumor was determined by MRI in the lower (≤7 cm from the anal verge), upper (>7 cm from the anal verge) or middle rectum (< and > than 7 cm from the anal verge). Biopsy specimens were collected during a colonoscopy before preoperative treatment, which consisted of a total irradiation dose of 50.4 Gy in 28 fractions of 1.8 Gy with concomitant application of 5-fluorouracil (425 mg/m2) and leucovorin (25 mg/m2). Eight to ten weeks after the completion of preCRT, standardized total mesorectal excision (TME) surgery was performed. Pathological grading of primary tumor regression in post-treatment specimens was performed semi-quantitatively by determining the number of residual tumor cells compared with the extent of fibrosis. The response to preCRT was classified as positive when complete or partial remission (CR/PR) was detected, or as negative in the case of the presence of stable or progressive disease (SD/PD).

Immunoreactivity in the preoperative biopsy specimens was semi-quantitatively evaluated by determining the percent of stained tumor cells. Tumor samples were considered to have a high level of expression when at least 10% of tumor cells expressed vascular endothelial growth factor (VEGF), B-cell lymphoma 2 (Bcl-2) apoptosis regulator or antigen KI-67 (Ki-67) proteins, respectively. Samples were considered to have high epidermal growth factor receptor (EGFR) expression if >25% of the tumor cells demonstrated membranous staining of any intensity. According to our previous reports [14,15], alterations of the examined genes within the same group of patients were as follows: 35% *KRAS* mutations, 43.3% $p16^{INK4a}$ and 39.6% $p14^{ARF}$ methylation detected within the same group of patients.

DNA extraction and mutation analysis of the *KRAS* gene

The mutation status of *KRAS* was evaluated on diagnostic tumor biopsies obtained as formalin-fixed, paraffin-embedded tumor tissues (FFPE), as described earlier [14]. Briefly, genomic DNA was isolated from deparaffinized tumor specimens using standard proteinase K, phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. Before automated sequencing, exon 2 of the *KRAS* gene containing codons 12 and 13 was amplified with primer sets listed in Supplementary Table S1. Amplification was carried out in an Applied Biosystems 2720 temperature cycler by initial denaturation at 95°C for 3 min, followed by 40 cycles (30s at 94°C, 30s at the annealing temperature specific for each reaction, and 30 s at 72°C, followed by final extension for 10 min at 72°C). Cycle sequencing reactions were performed with 2 μL of each purified product using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the same set of primers. Sequenced products were further processed with an automated ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Methylation analysis of the $p16^{INK4a}$ and $p14^{ARF}$ genes

The DNA methylation status in the promoter CpG islands of the $p16^{INK4a}$ and $p14^{ARF}$ genes was determined by methylation-specific PCR (MSP), as previously described [15]. Initially, sodium bisulfite conversion of genomic DNA (100-500 ng) was performed using an EZ DNA Methylation-Lightning™ kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. For the MSP reaction, 1 μ of 10 μL bisulfite-modified DNA was used. Amplification was carried out in an Applied Biosystems 2720 temperature cycler for 40 cycles (45 s at 95°C, 45 s at the annealing temperature specific to each primer set and 60 s at 72°C, followed by final extension for 4 min at 72°C). Primer sequences and annealing temperatures used for each MSP reaction are listed in Supplementary Table S1. DNA from peripheral blood lymphocytes from a healthy donor was used as a negative control for the methylated alleles. The same leukocyte DNA was methylated *in vitro* using CpG methyltransferase (M.SssI) (New England BioLabs, Ipswich, MA, USA) to generate completely methylated DNA at all CpG sites and used as a positive control for all genes. PCR products were separated by electrophoresis on 6% acrylamide gels, stained with silver nitrate and sodium carbonate.

Statistical analysis

Contingency tables were analyzed using the χ2-test or Fisher's exact two-tailed test when the expected frequencies were lower than five. Continuous variables were compared with the Student's t-test. Overall survival distributions were estimated by the Kaplan-Meier method and differences were evaluated by the log-rank test. In all tests, a P-value less than 0.05 was considered statistically significant. All statistical analyses were performed using the Sigma Plot 14.0 licensed statistical analysis software package.

RESULTS

Mutation status of the *KRAS* gene and the methylation status of $p16^{INK4a}$ and $p14^{ARF}$ genes in LARC

Analysis of *KRAS* mutation and $p16^{INK4a}$ methylation status was successfully performed in all 60 cases, while the $p14^{ARF}$ methylation status was determined in 58/60 patients. Of the total number of samples analyzed for methylation, 36.2% (21/58) had a mutant in the *KRAS* gene. Simultaneous alterations of *KRAS* and $p16^{INK4a}$ genes were detected in 11.7% (7/60) of tumors, while the simultaneous occurrence of *KRAS* mutation and $p14^{ARF}$ methylation was detected in 15.5% (9/58) of the patients. Simultaneous *KRAS*, $p16^{INK4a}$ and $p14^{ARF}$ alterations were present in 4 out of 58 cases (6.9%). Considered separately, alterations in the examined genes seemed to occur independently, as estimated by the χ2-test ($p16^{INK4a}$ vs *KRAS*, P=0.272; $p14^{ARF}$ vs *KRAS*, P=0.923; $p16^{INK4a}$ vs $p14^{ARF}$, P=0.715). In further analyses, we examined the relationship between the simultaneously altered mutational status of the *KRAS* gene and the methylation status of the $p16^{INK4a}$ and $p14^{ARF}$ genes with the clinicopathological and immunohistochemical parameters of patients with LARC.

Simultaneous presence of *KRAS* mutation and $p16^{INK4a}$ methylation in LARC patients

Correlations between the simultaneous presence of *KRAS* mutation and $p16^{INK4a}$ methylation with clinicopathological and immunohistochemical parameters are summarized in Table 1. In a subgroup of patients with parallel *KRAS* mutation and $p16^{INK4a}$ methylation ($KRAS_{mut}/p16^{INK4a}{}_{meth}$), the tumors were localized in the upper and middle rectum (15.6% and 14.3% vs 0%, respectively), and high Ki-67 protein expression (16.3% vs 0%) was observed, but the detected differences were not statistically significant (P>0.05 in all examined cases). The results related to the correlation of the simultaneous presence of *KRAS* mutation and $p16^{INK4a}$ methylation with response to therapy, course and outcome of the disease are presented in Table 2. In the $KRAS_{mut}/p16^{INK4a}{}_{meth}$ group of LARC patients, a death outcome was a more common event according to the simultaneous examination of *KRAS*/16^{INK4a} gene alterations (23.1% vs 8.7%), but the observed difference was not statistically significant (P=0.173). At the same time, concurrent alterations in *KRAS* and $p16^{INK4a}$ genes did not significantly affect the response to the preCRT, or the occurrence of local recurrences and/or metastases.

Table 1. Simultaneous presence of *KRAS* mutation and $p16^{INK4a}$ methylation in LARC patients – association with clinical-pathological and immunohistochemical parameters

Clinicopathological and immunohistochemical parameters	Mutation status of *KRAS*	Methylation status of $p16^{INK4a}$	P*
	$KRAS_{mut}/p16^{INK4a}{}_{meth}$	$KRAS_{mut}/p16^{INK4a}{}_{unmeth}$ $KRAS_{wt}/p16^{INK4a}{}_{meth}$ $KRAS_{wt}/p16^{INK4a}{}_{unmeth}$	
Age (yr. median)	67 ± 8.307	63.943 ± 8.520	0.375#
Gender			
Male	5/38 (13.2%)	33/38 (86.8%)	1.000
Female	2/22 (9.1%)	20/22 (90.9%)	
Tumor localization[a]			
Down (≤ 7 cm)	0/13 (0%)	13/13 (100%)	0.174
Upper (> 7 cm)	2/14 (14.3%)	12/14 (85.7%)	
Middle (<7> cm)	5/32 (15.6%)	27/32 (84.4%)	
TNM stage before surgery			
TNM II	3/21 (14.3%)	18/21 (85.7%)	0.687
TNM III	4/39 (10.3%)	35/39 (89.7%)	
EGFR expression[b]			
Low (< 25 %)	3/41 (7.3%)	38/41 (92.7%)	0.178
High (≥ 25 %)	4/17 (23.5%)	13/17 (76.5%)	
VEGF expression[b]			
Low (< 10 %)	3/30 (10 %)	27/30 (90%)	0.701
High (≥ 10 %)	4/28 (14.3%)	24/28 (85.7%)	
Bcl-2 expression[b]			
Low (< 10 %)	4/40 (10%)	36/40 (90%)	0.665
High (≥ 10 %)	3/18 (16.7%)	15/18 (83.3%)	
Ki-67 expression[b]			
Low (< 10 %)	0/15 (0%)	15/15 (100%)	0.173
High (≥ 10 %)	7/43 (16.3%)	36/43 (83.7%)	

mut – mutated; wt – wild type; meth – methylated; unmeth – unmethylated
[a] Data are missing for one patient for the given parameters; [b] data are missing for two patients for the given parameters; # P-value revealed by Student's t-test; * All P-values except for the patient's age were revealed by the χ2-test or Fisher's exact two-tailed test.

Simultaneous presence of *KRAS* mutation and $p14^{ARF}$ methylation in LARC patients

The results of the association of the simultaneous occurrence of *KRAS* mutation and $p14^{ARF}$ methylation with clinicopathological and immunohistochemical parameters are summarized in Table 3. Concurrent mutation of the *KRAS* gene and $p14^{ARF}$ promoter methylation was more frequently detected in the group of LARC patients with mid-rectum localized tumor (23.3% vs 7.7% and 7.1%, respectively), as well as in samples with high VEGF protein expression (22.2% vs 6.9%); however, the observed differences were not statistically significant (P>0.05). Results related to the correlation of the simultaneous presence of *KRAS* mutation and $p14^{ARF}$ methylation with response to therapy, course and outcome of the disease are presented in Table 4. Regarding the course of the disease, local recurrence and/or metastasis were significantly more frequently detected in LARC patients with concomitant *KRAS* mutation and $p14^{ARF}$ methylation ($KRAS_{mut}/p14^{ARF}{}_{meth}$) than in the other three groups (29.2 % vs 6.1%, P=0.027). Such associations were not observed as regards the response to the preCRT and death.

Survival analysis

Follow-up data were available for 53/60 patients, and the median follow-up period was 23 months (range 2-101 months). The combined analysis of alterations in the *KRAS*, $p16^{INKa}$ and $p14^{ARF}$ genes showed that *KRAS* mutation was concomitant with either $p16^{INK4a}$ or $p14^{ARF}$ methylation and was not significantly as-

Table 2. Simultaneous presence of *KRAS* mutation and $p16^{INK4a}$ methylation in LARC patients – association with response to therapy and disease outcome

Clinicopathological parameters	Mutation status of *KRAS*	Methylation status of $p16^{INK4a}$	P*
	$KRAS_{mut}/p16^{INK4a}{}_{meth}$	$KRAS_{mut}/p16^{INK4a}{}_{unmeth}$ $KRAS_{wt}/p16^{INK4a}{}_{meth}$ $KRAS_{wt}/p16^{INK4a}{}_{unmeth}$	
Response to preCRT			
PR/CR (positive)	3/30 (10%)	27/30 (90%)	1.000
SD/PD (negative)	4/30 (13.3%)	26/30 (86.7%)	
Local recurrence/metastasis[a]			
Yes	4/24 (16.7%)	20/24 (83.3%)	0.427
No	3/35 (8.6%)	32/35 (91.4%)	
Death outcome[a]			
Yes	3/13 (23.1%)	10/13 (76.9%)	0.173
No	4/46 (8.7%)	42/46 (91.3%)	

mut – mutated; wt – wild type; meth – methylated; unmeth – unmethylated; PR – partial remission; CR – complete remission; SD – stable disease; PD – progressive disease
[a] Data are missing for one patient for the given parameters; * All P-values were revealed by the χ2-test or Fisher's exact two-tailed test

Table 3. Simultaneous presence of *KRAS* mutation and $p14^{ARF}$methylation in LARC patients – association with clinicopathological and immunohistochemical parameters

Clinicopathological and immunohistochemical parameters	Mutation status of *KRAS*	Methylation status of $p14^{ARF}$	P*
	$KRAS_{mut}/p14^{ARa}{}_{meth}$	$KRAS_{mut}/p14^{ARF}{}_{unmeth}$ $KRAS_{wt}/p14^{ARF}{}_{meth}$ $KRAS_{wt}/p14^{ARF}{}_{unmeth}$	
Age (yr. median)	68.111 ± 9.778	63.735 ± 8.286	0.162#
Gender			
Male	7/37 (18.9%)	30/37 (81.1%)	0.465
Female	2/21 (9.5%)	19/21 (90.5%)	
Tumor localization[a]			
Down (≤ 7 cm)	1/13 (7.7%)	12/13 (92.3%)	0.234
Upper (> 7 cm)	1/14 (7.1%)	13/14 (91.9%)	
Middle (<7> cm)	7/30 (23.3%)	23/30 (76.7%)	
TNM stage before surgery			
TNM II	4/21 (19.1%)	17/21 (80.9%)	0.710
TNM III	5/37 (13.5%)	32/37 (86.5%)	
EGFR expression[b]			
Low (< 25 %)	5/40 (12.5%)	35/40 (87.5%)	0.676
High (≥ 25 %)	3/16 (18.8%)	13/16 (81.2%)	
VEGF expression[b]			
Low (< 10 %)	2/29 (6.9%)	27/29 (93.1%)	0.137
High (≥ 10 %)	6/27 (22.2%)	21/27 (77.7%)	
BCL-2 expression[b]			
Low (< 10 %)	6/39 (15.4%)	33/39 (84.6%)	1.000
High (≥ 10 %)	2/17 (11.8%)	15/17 (88.2%)	
Ki-67 expression[b]			
Low (< 10 %)	1/15 (6.7%)	14/15 (93.3%)	0.428
High (≥ 10 %)	7/41 (17.1%)	34/41 (82.9%)	

mut – mutated; wt – wild type; meth – methylated; unmeth – unmethylated
[a] Data are missing for one patient for the given parameter; [b] data are missing for two patients for the given parameters; # P-value revealed by Student's t-test; * all P-values except for the patient's age were revealed by the χ2-test or Fisher's exact two-tailed test.

sociated with overall survival (P=0.241, P=0.190, respectively) (Fig. 1A and B). A subgroup of LARC patients with the simultaneous presence of *KRAS* mutation, $p16^{INK4a}$ methylation and $p14^{ARF}$ methylation ($KRAS_{mut}/p16^{INK4a}{}_{meth}/p14^{ARF}{}_{meth}$) exhibited a significantly shorter overall survival compared to the other patient subgroups (P=0.011) (Fig. 1C). Since this subgroup consisted of only 4 patients, further statistical analysis of the correlation with the clinicopathological immunohistochemical response to CRT and disease course was not possible. However, it was observed that 3/4 (75%) of these subjects had an unfavorable response to preCRT, local recurrences and/or metastases of the disease, increased VEGF protein expression, and in all four cases (4/4, 100%) the tumor was localized in the middle rectum.

DISCUSSION

The use of preoperative chemoradiotherapy has significantly contributed to improved local control of the disease in terms of less frequent recurrences after primary surgical treatment [16]. Although the combined use of preCRT and surgery has been standardized, the pathological complete response (pCR), as the best outcome, is achieved in only about 20% of patients [17]. Despite established clinical prognostic factors in LARC, more reliable molecular factors must be identified for treatment response prediction and disease outcome prognosis. Since inactivation of the INK4a/ARF locus was observed in 70% of CRC cases together with a mutation in the *KRAS* gene [18-21], we examined the interrelationship between these epigenetic and genetic changes. Although no direct relationship was observed between the methylation status of the $p16^{INK4a}$ gene, the methylation status of the $p14^{ARF}$ gene and the mutation status of the *KRAS* gene, two significant results of the combined genetic/epigenetic analysis were noted.

Table 4. Simultaneous presence of *KRAS* mutation and $p14^{ARF}$ methylation in LARC patients – association with response to therapy and disease outcome

Clinicopathological parameters	Mutation status of *KRAS* gene	Methylation status of $p14^{ARF}$	P*
	$KRAS_{mut}/p14^{ARF}{}_{meth}$	$KRAS_{mut}/p14^{ARF}{}_{unmeth}$ $KRAS_{wt}/p14^{ARF}{}_{meth}$ $KRAS_{wt}/p14^{ARF}{}_{unmeth}$	
Response to preCRT			
PR/CR (positive)	3/29 (10.3%)	26/29 (89.7%)	0.470
SD/PD (negative)	6/29 (20.7%)	23/29 (79.3%)	
Local recurrence/metastasis[a]			
Yes	7/24 (29.2%)	17/24 (70.8%)	**0.027**
No	2/33 (6.1%)	31/33 (93.9%)	
Death outcome[a]			
Yes	3/13 (23.1%)	10/13 (76.9%)	0.412
No	6/44 (13.6%)	38/44 (86.4%)	

PR – partial remission; CR – complete remission; SD – stable disease; PD – progressive disease

[a] Data are missing for one patient for the given parameters

*All P-values were revealed by the χ2-test or Fisher's exact two-tailed test.

Firstly, in a subgroup of patients with concomitant *KRAS* mutation and $p14^{ARF}$ methylation, local recurrences and/or metastases were significantly more often detected (P=0.027). Previous studies have shown that the presence of *KRAS* mutations in the early stages of RC is associated with the occurrence of distant recurrences in the later stages of the disease [22]. Mutation of this gene in LARC is common, but the mutational status of the *KRAS* gene is not enough to predict more aggressive tumor behavior, and other parameters are needed to indicate a potential risk of recurrence [23]. The effect of the simultaneous presence of *KRAS* mutation and $p14^{ARF}$ methylation can be explained by the well-known function of the $p14^{ARF}$ gene protein product in suppressing cell growth in response to oncogene activation [13]. The obtained result indicates the potential cooperation of these two genes in the development of a more aggressive form of tumor [24]. On the one hand, the activation of the *KRAS* oncogene as an initial event leads to the uncontrolled proliferation of tumor cells and further growth of a potentially invasive tumor. On the other hand, inactivation of the $p14^{ARF}$ gene via promoter methylation indirectly affects tumor-suppressor mechanisms such as cell cycle arrest and induction of apoptosis, which allows for further tumor propagation and local tissue invasion. This model of oncogene-induced carcinogenesis of pancreatic duct adenocarcinoma was shown in mice in which mutation of the *KRAS* gene in combination with inactivation of the INK4a/ARF locus led to the early formation of premalignant lesions that rapidly propagate to invasive and metastatic tumors [25]. It should be noted that in our study, *KRAS* gene mutations and $p14^{ARF}$ methylation were more often detected in the group of patients with tumors localized in the middle rectum than in other rectum segments (23.3% vs 7.1% vs 7.1%), as well as in a higher percentage in samples with high VEGF protein expression than in LARC patients with low VEGF expression (22% vs 6.9%). Although the observed differences were not statistically significant, it was shown that the mid-rectum

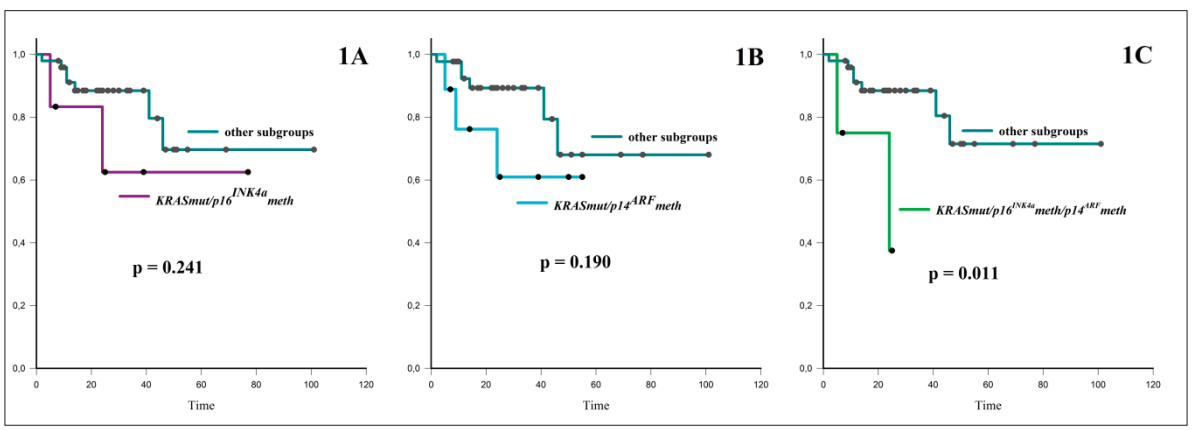

Fig. 1. Overall survival among LARC patients according to the simultaneous presence of *KRAS* mutation and $p16^{INK4a}$ and/or $p14^{ARF}$ methylation. **A**, **B** – No significant difference in the overall survival between patients with concurrent KRAS mutation and $p16^{INK4a}/p14^{ARF}$ methylation, respectively. **C** – The group of patients with the parallel occurrence of *KRAS* mutation, $p16^{INK4a}$ methylation and $p14^{ARF}$ methylation showed significantly shorter survival compared to LARC patients.

tumor position (up to 10 cm from the anocutaneous line), as well as increased expression of VEGF factor as a mediator of angiogenesis, may be associated with a higher likelihood of recurrence and metastasis development [26,27]. The mutation status of the *KRAS* gene and the methylation status of the $p14^{ARF}$ gene define a subgroup of LARC patients that exhibits more aggressive behavior, which should be considered in further studies on a larger number of samples.

Combined analyses revealed a subgroup of patients with a concomitant mutation in the *KRAS* gene and methylation of $p16^{INK4a}$ and $p14^{ARF}$ genes with significantly shorter overall survival. Although this subgroup included a small number of samples (only 4), the result could have a potential prognostic significance in patients with LARC. Rapid tumor development and shorter survival were also observed in mice in which different tumor types were experimentally induced by oncogenic activation of the *KRAS* gene and inactivation of the INK4a/ARF locus [25,28,29]. A study conducted on CRC cell lines revealed a molecular mechanism, a signaling protein, that mediates methylation inactivation of the INK4a/ARF locus in response to the oncogenic activation of the *KRAS* gene [11]. The ZNF304 protein functions as a transcriptional repressor and recruits a repressive protein complex, including DNA methyltransferase 1 (DNMT1), that induces methylation and suppresses the expression of the entire INK4a/ARF locus. It was shown that this regulatory protein can also induce methylation inactivation of other tumor suppressor genes responsible for the CIMP (CpG island methylator phenotype) in CRC. For a better understanding of the prognostic role of the alterations in *KRAS*, $p16^{INK4a}$ and $p14^{ARF}$ gene expression in rectal carcinoma, analysis of a larger number of samples and a longer follow-up period after treatment are necessary.

CONCLUSION

Combined analysis of the mutational status of the *KRAS* gene and the methylation status of the $p16^{INK4a}$ and $p14^{ARF}$ genes identified two subgroups of patients with potential clinical significance for the prognosis of patients with LARC. The obtained results indicate the need for further larger prospective studies with a longer follow-up period. Standardization of the use of existing biomarkers in combination with the examination of new potential genetic and epigenetic molecular parameters is an approach that could lead to the development of personalized LARC therapy.

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Author contribution: Bojana Kožik: conducting the experiments and statistical analysis, writing the manuscript; Milena Krajnović: conception and design of the study, data interpretation and revision of the manuscript; Nikola Kokanov: conducting the experiments; Snežana Jovanović-Ćupić: revision of the manuscript; Ana Božović: data analysis; Lidija Todorović: data analysis and revision of the manuscript; Vesna Mandušić: revision of the manuscript. All authors read and approved the final manuscript.

Conflict of interest disclosure: The authors declare that they have no conflict of interest.

Data availability: All data underlying the reported findings have been provided as part of the submitted article and are available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Kozik%20et%20al_7519_Data%20Report.pdf

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Supplementary Data

The Supplementary Material is available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Kozik%20et%20al_7519_Supplementary%20Material.pdf