JBUON 2017; 22(6): 1463-1470
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

p21 does, but p53 does not predict pathological response to preoperative chemoradiotherapy in locally advanced rectal cancer

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Summary

Purpose: *Preoperative chemoradiotherapy (CRT) is the standard treatment option in locally advanced rectal cancer. The tumor response is assessed through tumor and nodal downstaging and the tumor regression grade. Currently, there is no method to predict a tumor response to CRT. We aimed to evaluate whether p21 and p53 expressions could be a reliable predictors of pathological response to CRT.*

Methods: *Fifty patients with locally advanced rectal cancer were treated with preoperative radiotherapy combined with mitomycin C and capecitabine. p21 and p53 immumohistochemical staining was performed on pretreatment biopsies and the results were compared with tumor regression according to grading systems by Dworak (TRG grades) and by Wheeler (RCRG grades).*

Results: *Testing RCRG grades in relation to p21 expression showed statistically significant difference (p=0.021). RCRG 3 (poor response) was more frequent in the group of patients with low p21. According to Dworak, grade 4 (complete regression) was more frequent in the group of patients with positive p21 expression (p=0.032). Significant difference in p21 expression in grade 4 group compared with all other grade groups was also found (p=0.007). Patients with immune expression of p21 had significantly higher percentage of complete regression in comparison to the patients with low expression of p21. We haven't found any correlation between p53 expression and histopathological (HP) as well as regression grades.*

Conclusion: *According to both grading systems, our results suggest that p53 expression does not, but p21 expression does predict pathological response to preoperative CRT.*

Key words: *immunohistochemistry, p21, p53, preoperative chemoradiotherapy, rectal cancer*

Introduction

CRT is widely accepted as a standard treatment option in locally advanced rectal cancer. Studies have shown that CRT results in better local control (i.e., a reduction of local recurrence rate by 20–50%), increased histopathological complete regression rate (pCR) by 10–30%, increased likelihood of curative and sphincter-preserving resection, and increased long-term survival. The most commonly used neoadjuvant treatment in locally advanced rectal cancer involves radiotherapy 45–50.4 Gy with concurrent 5-fluorouracil (5-FU) administered either as bolus during the first

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Received: 29/05/2017; Accepted: 10/06/2017

and last weeks of radiotherapy or as a continuous infusion during the whole radiotherapy treatment period [1].

The tumor response to CRT is assessed through tumor and nodal downstaging and the tumor regression grade (TRG). It was shown that local recurrence and survival outcome correlate with these parameters [2,3]. pCR defined by complete absence of tumor cells is observed in 10-30% of all cases [4,5]. The remaining patients show a broad range of residual disease, from microscopic tumor foci to having no response at all [6].

Currently, there is no method to predict a tumor response to CRT, and finding one would have a great clinical utility. Pre-CRT is time-consuming, expensive and increases perioperative morbidity [7,8]. The set of possible biomarkers, able to predict a response, could save the patients from the toxicity of undergoing a treatment from which will have no benefit. Based on well-chosen set of biomarkers, these patients could receive more personalized therapy. Also, the subsequent management of the patients will be altered. In cases of complete or nearly complete responses, local excision or a sphincter-preserving operation is possible, as there would be little risk of local recurrence [9]. In these cases, further treatment may not be necessary and close observation alone may be sufficient [10].

It is still not known why the responses to CRT vary to such a high degree among patients; the clinical responses do not always correlate with histological responses. Thus, additional information provided by predictive molecular markers could be highly useful for finding those who would most likely benefit from non operative approaches. To identify factors predictive of response, research has focused primarily on histological and molecular assessment of pretreatment tumor biopsy specimens. Various molecular markers, including p53, p21, Bcl2, Bax, EGFR, MLH-1, MSH-2, Ku70, VEGF, TS and Ki-67, have all been investigated as potential predictors of the tumor response to CRT, with contradictory results [11-13].

p21 came into the spotlight as a mediator of p53 tumor suppressor activity and as an inhibitor of cell cycle progression owing to its ability to inhibit the activity of cyclin-dependent kinase (CDK)–cyclin complexes and the proliferating cell nuclear antigen (PCNA) [14].

The tumor suppressor activity of p21 arises from its role in inducing growth arrest, differentiation or senescence. Recently, it has become apparent that p21 is stimulated by many pathways that are independent of p53. p21 directly regulates gene expression and other cellular events through protein–protein interactions that are independent of CDKs and PCNA [15].

Multiple transcription factors, ubiquitin ligases, and protein kinases regulate the transcription, stability and cellular localization of p21, thereby regulating its activity [16]. Recent data suggest a tumorigenic role of p21 in certain contexts that relies on its ability to suppress apoptosis and promote the assembly of type-D cyclins with CDK4 and CDK6 [17].

Given that p21 is a tumor suppressor, but that it also behaves as an oncogene in certain cellular contexts, many studies are focused on examining its exact role in the cell and possible ability to predict response to various treatments.

The aim of this study was to evaluate whether p21 and p53 expressions could be reliable predictors of pathological response to preoperative CRT.

Methods

Fifty patients with locally advanced rectal cancer (T3/T4, N0/N+, M0) were treated with preoperative combined treatment. The total irradiation dose of 45 Gy was delivered in combination with concomitant chemotherapy (mitomycin C and capecitabine). Five to eight weeks after finishing CRT, all the patients underwent surgery.

The following eligibility criteria were applied: histologically confirmed adenocarcinoma of the rectum, locally advanced stage II (T3/4, N0M0) and stage III (T3/4, N1/2, M0), up to 16 cm from the anal verge; minimum age 18 years; no earlier specific tumor treatment; Eastern Cooperative Oncology Group (ECOG) performance status of 2 or below; adequate hematologic function (absolute white blood cell count (WBC) > 3.5 x 10^9/L, absolute neutrophil count > 1.5 x 10^9/L, platelets > 100 x 10^9/L, hemoglobin > 10 g/dL), liver function (bilirubin < 2.0 mg/dl, AST, ALT, AP, gamma GT ≤ 3X ULN) and renal function (serum creatinine < 1.5 mg/dL, creatinine clearance > 50 mL/min); able to understand and willing to comply with the study protocol and treatment plan.

Patient were not eligible for the study if they had: prior pelvic irradiation or chemotherapy; secondary malignancies, except basal cell carcinoma of the skin or cervical carcinoma *in situ*; unstable cardiac disease or myocardial infarction within the past 6 months prior to beginning of the study; neurological or mental disorders; active or uncontrollable infection or sepsis; active disseminated intravascular coagulation disorder; inflammatory bowel disease, malabsorption syndrome, synchronous colic or rectal tumors and any other severe diseases precluding administration of chemotherapy and irradiation. Pregnant or breastfeeding women were also not eligible.

All patients signed written informed consent and the study was approved by the Ethics Committee of the Institute for Oncology and Radiology of Serbia, and was

performed in accordance with the ethical standards laid down by the Declaration of Helsinki.

Study procedures

After patient signed written informed consent and eligibility was confirmed to be in accordance with inclusion/exclusion criteria the study treatment was applied. Capecitabine was administered at 825 mg/m^2, twice daily, on days 1-35. Mitomycin C, 7 mg/m^2 was administered on D1 and D29 as 2-hr infusion in 500 ml 5% glucose.

Radiotherapy started on day 1, after mitomycin administration. The total irradiation dose was 45 Gy delivered in conventional fractionation. The daily dose was 1.8 Gy at the reference point according to ICRU 50/62, once per day and 5 times per week, in 25 fractions over a period of 5 weeks, until a total reference dose of 45 Gy was reached. Radiotherapy was delivered with high energy photons (15,18 MeV) with linear accelerator. Patients were operated, 5-8 weeks after finishing the preoperative combined treatment. After surgery, patients received adjuvant treatment according to histopathology findings and tumor board recommendations.

Diagnostic workup

At baseline, the following exams were performed: Digital rectal examination, total colonoscopy with biopsy of the rectal tumor and determination of the distance between the lower edge of the tumor and the anocutaneous line, pelvic MRI, abdominal CT scan, transrectal ultrasonography and chest X-ray. All mentioned exams were repeated 5-6 weeks after preoperative treatment, before surgery. RECIST criteria were used for evaluation of tumor response. Complete blood counts, serum biochemistry including liver function tests, assessment of clinical symptoms and toxicities were done at baseline and weekly during CRT. In cases of grade 3 or 4 hematological toxicity, peripheral blood count was performed every day until recovery from the nadir.

Histopathological examination

Postoperative standardized HP examination of the specimen with regard to histological type and differentiation, tumor spread (ypTNM, R classification) and quality of the total mesorectal excision was carried out. Histological assessment of tumor regression was performed in accordance with two tumor regression grading methods. The first one, established by Dworak (TRG grades) [18] was defined as follows: grade 0 = no regression; grade 1 = minimal regression (defined as a dominant tumor mass with obvious fibrosis and vasculopathy); grade 2 = moderate regression (defined as fibrotic changes dominant with few tumor cell nests easy to locate); grade 3 = good regression (defined as very few isolated tumor cells, hard to find under the microscope in predominantly fibrotic tissues and pools of mucus); grade 4 = complete regression (defined as no tumor cells, only fibrotic tissue). Responses were thus defined as complete (Grade 4), major (Grade 3 and 2), or minor (Grade 1).

Rectal cancer regression grade system proposed by Wheeler et al. (RCRG grades) [19] was defined as follows: grade 1-sterilization or microscopic cancerous foci with no macroscopis disease; grade 2-marked fibrosis, but macroscopic disease present; grade 3-little or no fibrosis with abundant macroscopic disease.

Immunohistochemistry

Immunohistochemistry was performed using the standard avidin-biotin immunoperoxidase method with primary mouse anti-human monoclonal antibodies: p53 (Clone Doc7, code No. M7001, DAKO, working dilution 1:50) and p21^{RAS} (Clone NCC-RAS-001, code No. M0637, DAKO, working solution 1:100), according to prescribed immunostaining protocol. Sections from selected blocks of formalin-fixed paraffin-embedded tissue samples were cut at 4 μm, deparaffinized and rehydrated. For antigen retrieval, deparaffinized sections were pretreated by microwaving in Dako Target Retrieval Solution, High pH (code No. S3308) for p53 and Dako Target Retrieval Solution, code No. S 1700 for p21^{RAS} for 21 min at 800 W. After cooling, sections were immersed in distilled water containing 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Sections were then incubated in a humid chamber for 60 min at room temperature with the primary antibodies. After rinsing with PBS, slides were incubated with secondary antibody followed by streptavidin-biotin–peroxidase complex, both for 30 min at room temperature with a PBS wash between each step (DAKO LSAB™+/HRP kit, code No. K 0679). The reaction was developed with 3–5'diaminobenzidine as a chromogen (Liquid DAB+ Substrate Chromogen System, code No. K3468, DAKO), counterstained with Mayer's hematoxylin and mounted. Negative controls, in which N-universal negative control replaced the primary antibody, were run with each batch of stain and sections known to stain strongly positive for p53 and p21 were included with each run as positive controls. Quality control was done as proposed by UK NEQAS (UK National External Quality Assessment for Immunocytochemistry).

Assessment of nuclear immunostainings of p21 and p53, shown by distinct brown nuclear staining, was quantified after manual counting of a least 1000 malignant cells and expressed as number of positive nuclei on 10 high power fields (HPF). The cut off value of 10% nuclear immunopositivity was estimated with regard to the extent as well as to the intensity of nuclear immunostaining, i.e. tumor samples were regarded as positive if strong nuclear p53 or p21 immunoexpression was identified in at least 10% of all malignant cells on the examined tissue section. The results of immunostainings were semiquantitatively scored and estimated as: negative (0), focally positive (1+) with less than 10% strong nuclear stainings, moderately positive (2+) in cases with 10-49% and extensively positive (3+) in cases with 50% or more of cells showing strong nuclear immunostaining.

Statistics

Statistical analysis was done with the statistical package R (version 3.1.1 (2014-07-10) -- "Sock it to Me"

Copyright (C) 2014 The R Foundation for Statistical Computing; Platform: x86_64-w64-mingw32/x64 (64-bit); downloaded: October 22,2014.). For data summarizing, the methods of descriptive statistics were used (frequencies, percents, mean, median, standard deviation [SD], and range). For testing the differences between parameters, the Pearson x^2 test and Fisher exact test were used. The statistical significance level was set at $p<0.05$.

Results

From October 2006 to April 2008, a total of 50 patients were enrolled. The median follow-up was 28 months (range 6-29). The median age of the study population was 58 years, and all patients had a baseline ECOG status of 0 or 1. The disease stage at diagnosis was T3 in 38 patients (76%) and T4 in 12 patients (24%). Tumors were localized in the distal (36 patients), middle third (13 patients) and proximal part of the rectum (1 patient). The standardized HP examination of the resected tumor specimens included the assessment of morphology (histological type, grade, lymphatic and vascular invasion) are shown in Table 1. Patient characteristics according to the grading methods established by Dworak (TRG grades) and Wheeler (RCRG grades), are shown in Table 1.

All HP tumor grades, as well as joined grades (HP grade 1 vs HP grades 2+3+4; HP grades 1+2 vs HP grades 3+4) were correlated with p21 expression but not with p53 expression (Table 2). Frequencies of HP grades were statisticaly different between patients with low and patients high p21 expression. Higher p21 expression was correlated with lower HP grade, i.e. better differentiated tumors (Table 2, Figure 1).

The frequency of p21 and p53 expression compared with Dworak (TRG) and Wheeler (RCRG)

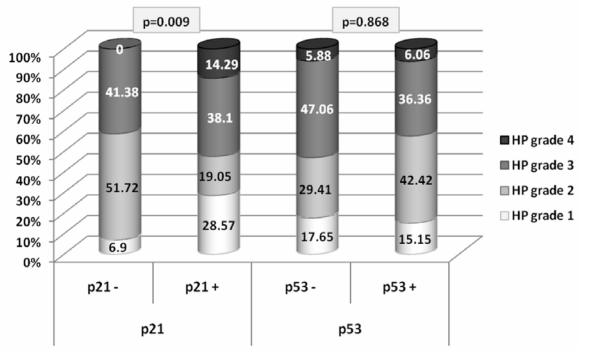

Figure 1. Histopathological tumor grades according p21 and p53 expression.

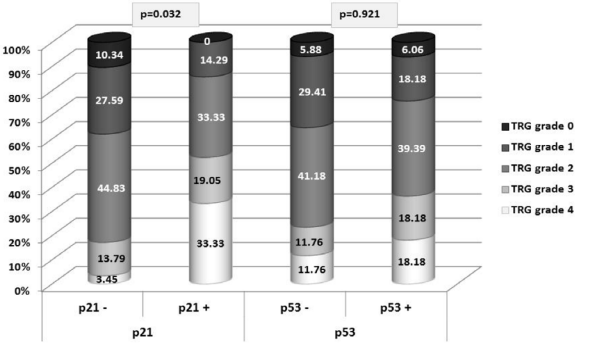

Figure 2. TRG tumor regression grades according p21 and p53 expression.

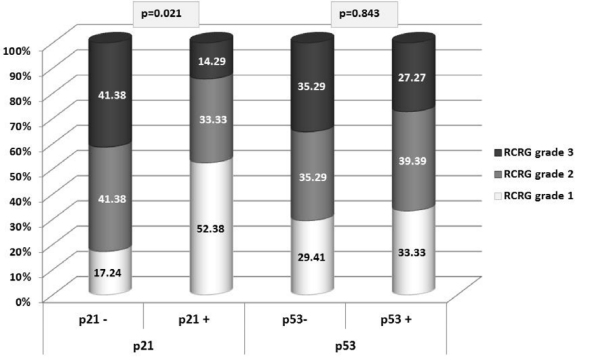

Figure 3. RCRG tumor regression grades according p21 and p53 expression.

Table 1. Histopathological characteristics and tumor regression according to Dworak and Wheeler grading method

HP characteristics	*n (%)*	*Tumor regression*	n (%)
HP grade			
Grade 1	8 (16)	Dworak grading method	
Grade 2	19 (38)	(TRG criteria)	
Grade 3	20 (40)	TRG Grade 4	8 (16)
Grade 4	3 (6)	TRG Grade 3	8 (16)
Lymphatic invasion		TRG Grade 2	20 (40)
Present	25 (50)	TRG Grade 1	11 (22)
Absent	25 (50)	TRG Grade 0	3 (6)
Vascular invasion			
Present	39 (70)	Wheeler grading method	
Absent	11 (22)	(RCRG criteria)	
Mucin		RCRG Grade 1	16 (32)
Present	31 (62)	RCRG Grade 2	19 (38)
Absent	19 (38)	RCRG Grade 3	15 (30)

Table 2. Histopathological tumor grades and regression grades according p21 and p53 expression

Characteristics	*p21 expression*			*p53 expression*		
	p21 -	*p21 +*	*p value*	*p53 -*	*p53 +*	*p value*
Histopathological tumor grade	*n (%)*	*n (%)*		*n (%)*	*n (%)*	
HP grades						
HP grade 1	2 (6.9)	6 (28.57)		3 (17.65)	5(15.15)	
HP grade 2	15 (51.72)	4 (19.05)	*p=0.009	5 (29.41)	14 (42.42)	*p=0.868
HP grade 3	12 (41.38)	8 (38.1)		8 (47.06)	12 (36.36)	
HP grade 4	0 (0.00)	3 (14.29)		1 (5.88)	2 (6.06)	
HP categories						
HP grade 1	2 (6.9)	6 (28.57)	*p=0.056	3 (17.65)	5 (15.15)	*p=1
HP grades 2+3+4	27 (93.1)	15 (71.43)		14 (82.35)	28 (84.85)	
HP categories						
HP grades 1+2	17 (58.62)	10 (47.62)	#x^2=0.59	8 (47.06)	19 (57.58)	#x^2=0.50
HP grades 3+4	12 (41.38)	11 (52.38)	p=0.441	9 (52.94)	14(42.42)	p=0.480
Tumor regression grades	*n (%)*	*n (%)*		*n (%)*	*n (%)*	
TRG (Dworak)						
TRG grade 4	1 (3.45)	7 (33.33)		2 (11.76)	6 (18.18)	
TRG grade 3	4 (13.79)	4 (19.05)	*p=0.032	2 (11.76)	6 (18.18)	*p=0.921
TRG grade 2	13 (44.83)	7 (33.33)		7 (41.18)	13 (39.39)	
TRG grade 1	8 (27.59)	3 (14.29)		5 (29.41)	6 (18.18)	
TRG grade 0	3 (10.34)	0 (0.00)		1 (5.88)	2 (6.06)	
TRG categories						
TRG grade 4	1(3.45)	7 (33.33)	*p=0.007	2 (11.76)	6 (18.18)	*p=0.699
TRG grades 0+1+2+3	28 (96.55)	14 (66.67)		15 (88.24)	27 (81.82)	
RCRG (Wheeler)						
RCRG grade 1	5 (17.24)	11 (52.38)	*p=0.021	5 (29. 41)	11 (33.33)	#x^2=0.34
RCRG grade 2	12 (41.38)	7 (33.33)		6 (35.29)	13 (39.39)	p=0.843
RCRG grade 3	12 (41.38)	3(14.29)		6 (35.29)	9 (27.27)	
Total	29 (100)	21 (100)	-	17 (100)	33 (100)	-

*Fisher's exact test, #Pearson's x^2 test

tumor regression grades and categories are shown in Table 2.

TRG grade 4 was more frequent in the group of patients with positive p21 expression (Table 2). Statistically significant difference in p21 expreesion in TRG grade 4 group compared with all other TRG grades groups was also found (Table 2; Figure 2).

Analysis of RCRG grades in relation to expression of p21 showed statistically significant differences, especially in the groups of patients with RCRG grade 1 and grade 3 (Table 2; Figure 3). More than half of the patients with complete regression (RCRG 1) belonged to the high p21 expression group.

There was no statistically significant difference in the frequency of TRG categories (tested separately, or as joined ones – Table 2, Figure 2) neither of RCRG categories (Figure 3) in relation to p53 expression (Table 2).

Discussion

Regarding HP grades it was found that grade 1, i.e. complete clinical regression, was statistically less frequent in the group of patients with sparse p21 expression (2/29 patients, 6.9%) comparing with the group with immunoexpression of p21, (6/21 patients, 28.57%) (p=0.009).

Also, there was a statistically significant difference in the analysis of regression grades. Testing RCRG grades in relation to p21 expression, showed statistically significant difference (p=0.021). RCRG 1 was recorded in 11/21 (52.38%) in the subgroup of patients with positive expression of p21 as compared with a subset of patients with negative expression of p21 noted in 5/29 (17.24%). Poor response to therapy (RCRG 3) was significantly more frequent in the group of patients with negative expression of p21, which was registered in 12/29 (41.38%) patients compared

to the positive expression of p21 found in 3/21 (14.29%).

The frequency of p21+ and p21- expression was also compared with 5 stage regression grades according to Dworak (TRG). Grade 4 (complete regression) was more frequent in the group of patient with positive p21 expression (p21- vs p21+: 1/29 (3.45%) vs 7/21 (33.33%). Statistically significant difference in p21 expression in Grade 4 group compared with all other grades groups was also found. In other words, patients with strong expression of p21 had statistically significantly higher percentage of complete regression in comparison to patients without expression of p21.

Charara et al. conducted a study on 57 patients treated with preoperative radiotherapy and concomitant chemotherapy in combination of 5-FU and CPT-11. A comparison of the complete clinical response to the conducted therapy showed no correlation between the patients who had a positive expression of p21 as determined in 14/30 patients, compared to patients with negative p21 recorded in 2/11 (p=0.09). However, a statistically significant difference in complete HP response between patients with positive expression of p21 noticed in 12/30 patients (40%) and patients with p21 negative expression, was found [20].

Bertolini et al. in their study, conducted on 91 patients, found no correlation between p21 expression and histologically verified response to therapy. Patients with high expression of p21 in the initial biopsy had disease free survival (DFS) of 57% at 4 years, while patients with low expression of p21 had a 4-year DFS of 79% and this difference was statistically significant (p=0.036). Multivariate analysis showed that patients with the increased expression of p21 had a 6.8 times greater risk of relapse than patients with low expression of p21. Overall survival at 4 years was also statistically poorer in patients with a high expression of p21 [21].

Rau et al. studied the expression levels of p21 in 66 patients in biopsy samples before CRT based on 5FU- LV, and surgery samples taken after treatment. It has been found that low levels of expression of the p21 were correlated with poor response to preoperative treatment [22].

Negri et al. examined the expression of molecular markers in 57 patients, of which 38 were treated with preoperative radiotherapy only, and 18 patients had chemotherapy and concomitant 5FU- oxaliplatin. They found no correlation between the response to the conducted treatment and the expression levels of p21 [23].

In the group of 112 patients who received 5-FU based preoperative CRT and surgery, Sim et al. looked at the expression of K67, p53, p21, CD133, CD166 and some other markers, in relation to tumor regression grades and DFS. Unlike our results, they showed that high p21 expression at the pretreatment biopsy was significantly associated with non-pCR [24].

In a recently published paper, the authors evaluated the expression levels of 12 candidate biomarkers and their correlation with pathologic response to pre-CRT using tissue microarrays and immohistochemistry, assessed through HP staging and tumor regression grade [25]. It was shown that expression of p53, VEGF, p21 and Ki67 correlated with pCR. Similar to our findings, patients with high expression of p21 had a higher pCR rate, as had also the patients with low p53 expression.

In our study no correlation between p53 expression and HP as well regression grades was found. p53 is probably the most studied molecular marker in tumor cells. Usually, the presence of wild type p53 is correlated with sensitivity to radiation or chemotherapy, while mutated p53 indicates possible radio and chemoresistance [26]. It has been previously shown that presence of nuclear p53 detected by immunohistochemistry could predict resistance to preoperative CRT [27]. Of note, other studies presented contradictory results. One of them showed that absence of p53 in pretreatment tumor biopsies is a predictive factor for complete tumor regression [28], whereas Esposito et al. found positive correlation between strong expression of p53 and better response to preoperative CRT [29].

As it is known that p21, depending on the cellular context, acts as a tumor suppressor or as an oncogene, its role was studied in many papers. Lu et al. showed that wild type p21 inhibits apoptosis in the presence of DNA damage caused by chemotherapeutic agents or radiation [30]. *In vitro* data support this by finding that loss of wild type p21 or the presence of mutated p21 can sensitize human colorectal carcinoma cells to radiation [31]. Results obtained in our study suggest that p53 expression does not, but p21 expression does predict tumor response to preoperative CRT.

Conclusion

It became clear that only a profound understanding of the biology of rectal cancer will enable the selection of patients who are more likely to respond to preoperative CRT. In our setting, patients with strong expression of p21 had statistically significant higher percentage of com-

plete regression, indicating that p21 acts as a tumor suppressor, probably through a p53 independent mechanism. Given the complexity of the molecular changes in rectal cancer, it is obvious that single-marker approach is certainly not sufficient, and that the future lies in the many-sided determination of multiple markers in order to better define the group of patients with the best response.

Acknowledgements

This work was supported by a grant from Ministry of Education and Science of the Republic of Serbia (Grant III41026 and 41033).

Conflict of interests

The authors declare no conflict of interests.

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