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Altered arginine metabolism in colon cancer: a sign of increased proliferative potential of tumor-adjacent tissue

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Abstract: Colorectal cancer (CRC) is one of the most frequent forms of malignant tumors in the human population. The literature data about the role of arginine metabolism in CRC point out its double-faced role. In three tissue specimens of 50 patients who underwent surgical resection for colon adenocarcinoma (tumor, adjacent and healthy tissues more than 10 cm from the tumor border – at the incision margin) taken during surgery, polyamines and the concentration of NO_2+NO_3 and arginase activity were determined. Polyamine levels and arginase activity were significantly increased in cancer and adjacent tissue specimens compared to healthy ones, while the level of NO_2+NO_3 was significantly lower in cancer compared to both adjacent and healthy tissues. The high polyamine content in the adjacent colonic mucosa indicates a high proliferative potential of tumor-adjacent tissue. Although we found individual correlations indicating the possible prognostic value of arginase, the performed statistical analysis did not show a predictive significance of arginase activity in the examined tissue specimens for five-year survival of the patients. Nevertheless, the obtained results provide the rationale for further studies of arginine metabolism in tissue specimens after surgery in patients with CRC, which could be useful in the evaluation of the risk for tumor growth, recurrence, metastases and survival after surgical intervention.

Keywords: colon cancer; arginine; arginase; polyamines; nitric oxide

INTRODUCTION

One of the most frequent forms of malignant tumors in the human population and the leading cause of death caused by cancer is colorectal cancer (CRC) [1]. CRC is the second most common cancer in men and the third most common in women. Effective treatment of CRC is based on surgery; unfortunately, CRC metastatic disease and cancer recurrences are not treated as effectively. As molecular mechanisms involved in the pathogenesis of colorectal cancer could be related to prognosis and the clinical outcome of CRC, it is of clinical importance to study them.

Cancer is multifactorial disease. There are many biochemical alterations found in cancer tissue [2], but one of the most consistent is a change in intracellular polyamine content. Intracellular polyamine homeostasis is lost during the process of cancer development [3], so cancer cells have much higher intracellular polyamine concentration than the equivalent normal tissue. In some studies, colorectal cancer tissue had about a 4-fold higher polyamine content of colonic mucosa compared to patients with non-malignant diseases [4]. Increased polyamine levels are reported to be associated with enhanced malignant potential [5].

Polyamine metabolism depends on the levels of its precursor amino acids, L-arginine and ornithine, formed from arginine by the enzyme arginase. Studies using human colon cancer cell lines have generated evidence that the key enzyme of polyamine biosynthesis, ornithine decarboxylase (ODC), is upregulated during carcinogenesis [6]. The hypothesis that arginase may

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regulate arginine availability for polyamine synthesis is supported by observations that arginase activity is often co-induced with ODC [7] and that cells deficient in arginase cannot proliferate in a serum-free medium unless ornithine or polyamines are provided. Also, sufficient arginase activity can limit the availability of arginine for other pathways, such as nitric oxide (NO) synthesis, and it has been proved that arginase and nitric oxide synthase (NOS) can compete for arginine in certain conditions [8]. Thus, based on literature data, there is no doubt about polyamine usefulness for monitoring the progression of the disease and patients' response to therapy, as well as the importance of polyamines and their metabolism-related molecules in hyperproliferation and cancer development.

L-arginine is an essential amino acid for patients with catabolic conditions [9] since it is involved in various cellular events of cancer metabolism, such as polyamine, nitric oxide, agmatine, glutamate and nucleotide synthesis. Literature data prove the important role of arginine and its metabolites in the development and progression of CRC. But although there are numerous studies of arginine metabolism in cancer [10], literature data about the role of arginine metabolism in CRC is still sparse and contradictory, indicating a double-faced role of arginine in carcinogenesis [11]. Besides polyamine synthesis, arginine could also serve as the substrate of NOS in the synthesis of NO. It has been shown that NO plays an important role in an organism's defense against different pathogens, as well as the immune response during carcinogenesis. Cancer is the endpoint of a multistep process that includes three fundamental components: initiation, promotion and progression. Literature data report conflicting roles of NO with quite opposite effects in these three phases of carcinogenesis [12,13]. The role of NO in tumor biology is extremely complex. Considering carcinogenesis, NO exerts different effects, including modulation of transcription factors, DNA damage, inhibition of DNA repair enzymes [14], modulation of oncogene expression, inhibition of apoptosis and induction of angiogenesis [15,16]. There is no doubt that the biological effect of NO is the result of the equilibrium between several factors, including its concentration, temporal expression, cell source and target cell.

An approach in cancer research is that reprogramming metabolic pathways, including arginine metabolism, could be important for carcinogenesis and the survival of patients [17]. Finding cancer therapeutics that can target specific metabolic pathways is very promising. The interactions of NO and polyamine metabolism, considering they originate from the same substrate, L-arginine, and their roles in carcinogenesis are a very intriguing area of research. The focus of our study were the changes in polyamine and NO production and arginase activity in cancer and in adjacent and healthy tissue taken during surgery and their possible prognostic value in colon cancer patients.

MATERIALS AND METHODS

Ethics statement

The study was performed in compliance with the Declaration of Helsinki. The Ethics Committee of the Medical Faculty University of Niš approved the study protocol (Decision No. 12-12123-14 dated December 2nd, 2015). All participants agreed to participate in the study, and informed consent was signed by both healthy subjects and by the patients or their legal guardians.

Patients

The research was performed as the prospective study at the Clinic of Surgery, Department of Colorectal Surgery, University Clinical Centre Niš and the Center for Biomedicine, Faculty of Medicine University of Niš. The study included 50 patients (37 male and 13 female) who had undergone colon resection for CRC adenocarcinoma at the Clinic of Surgery, Department of Colorectal Surgery, Clinical Centre Niš. The average age of the patients was 68.0±10.1, and there was no statistically significant difference in gender distribution (Table 1). None of the patients received any preoperative cancer therapy. Clinical studies included

Table 1. Gender distribution in the patients with CRC.

	Number	Percentage	Age X±SD	Age 95% CI for mean
Male	37	74.0	67.9±11.6	63.1-72.7
Female	13	26.0	68.1±5.90	64.3-71.9
Total	50	100.0	68.0±10.1	64.6-71.3

NS for all parameters

analyses of the CRC clinical stage in the patients, local recurrence incidence, occurrence of metastases, three- and five-year survival.

During surgery, three tissue specimens were taken for further studies (tumor tissue, a specimen of tumor surrounding tissue/adjacent tissue, and a normal colonic mucosa specimen (healthy tissue more than 10 cm from tumor border, at the incision margin). Immediately after surgery, the tissue specimens were washed in cold saline and kept at -20°C, cut into small pieces and homogenized using an Ultra Turrax IKA T18 basic homogenizer with a Teflon pestle (IKA-Werke GmbH & Co.KG, Staufen, Germany). The homogenates were frozen at -20°C until biochemical analysis.

Biochemical analysis

In the tissue samples obtained from the patients, the following biochemical parameters were determined: polyamine content (putrescine, spermidine and spermine), NO [NO_2^- (nitrite) +NO_3^- (nitrate) concentration] and arginase activity. Polyamine (putrescine, spermidine and spermine) concentrations were determined by high-performance liquid chromatography (HPLC) as described [18], using precolumn derivatization with 9-fluorenylmethyl chloroformate. In the presence of oxygen, NO is rapidly oxidized to nitrite and nitrate. Therefore, the concentration of nitrate and nitrite was used as the marker of nitric oxide synthase activity and endogenous NO production in biological systems. Tissue nitrate plus nitrite concentration was determined as described [16,19], based on the Griess reaction. Arginase activity was measured as described [20], based on spectrophotometric determination of released ornithine by the Chinard reaction, while tissue proteins were determined according to the method of Lowry et al. [21].

Statistical analysis

The polyamine and NO levels, as well as arginase activity, were expressed as the mean±standard deviation (SD). There was a normality assumption for all studied parameters. The statistically significant differences in values between colon cancer tumor, adjacent and normal tissues were determined by Student's t-test for two independent samples. A P-value < 0.05 was considered statistically significant.

RESULTS

Clinical data

After surgery, local recurrences were registered in 10 (24.4%) patients, occurring in 23.3% of male and 27.3% of female patients. The performed statistical analysis did not show a significant difference in incidence related to gender (Table 2).

The incidence of CRC metastases in the examined patients in relation to gender is presented in Table 3. Although the incidence of CRC metastases was registered in a higher percentage in men than in women, the performed statistical analysis did not show a significant difference in the frequency of metastasis related to the gender of the examined patients (Table 3).

The three- and five-year survival rates of the CRC-operated patients were also monitored. Three-year survival was registered in 87.8% of the patients of both sexes (86.2% for men, 91.6% for women). The analysis did not show a significant difference in survival between genders. During the five-year follow-up period,

Table 2. Cancer recurrence incidence in CRC patients.

			Male	Female	Total
Recurrence	No	Number	23	8	31
		% (within a gender)	76.7%	72.7%	75.6%
	Yes	Number	7	3	10
		% (within a gender)	23.3%	27.3%	24.4%
	Total	Number	30	11	41
		% (within a gender)	100.0%	100.0%	100.0%

NS for all parameters

Table 3. Occurrence of metastases in CRC patients

			Male	Female	Total
Metastases	No	Number	25	10	35
		% (within a gender)	83.3%	90.9%	85.4%
	Yes	Number	5	1	6
		% (within a gender)	16.7%	9.1%	14.6%
	Total	Number	30	11	41
		% (within a gender)	100.0%	100.0%	100.0%

NS for all parameters

26.9% of patients died, while 73.1% had a five-year survival. Five-year survival was registered in 63.3% of male and 100% of female patients, with significance of this difference in survival (P<0.05). In the group of patients with a T1 tumor stage, five-year survival was 100%, while in those with T4 stage it was only 33%.

Results of biochemical analyses

The total polyamine content in cancer tissue specimens was significantly higher compared to both adjacent and healthy tissues (Fig. 1A). It is important to emphasize that there was a high polyamine concentration in cancer-adjacent tissue, indicating the high proliferative potential of this tissue, which supports the rationale for excising this part of the colon during surgery. Preliminary results of the polyamine study have been reported [22], but not for the whole study group of patients. The high SD, especially in adjacent tissue samples, points out the great diversity of values, so it was of great importance to compare each patient's individual results with the clinical outcome, since they were followed for five years in an attempt to establish important correlations. The concentrations of the main polyamines (putrescine, spermidine and spermine) showed the most prominent increase, especially of spermine, in tumor tissue, but also a statistically significant increase in adjacent tissue (Fig. 1B).

We previously reported that in a sample of 50 patients, NO synthesis was reduced in tumor and adjacent tissue compared to healthy tissues [23]. The concentration of NO_2^- + NO_3^- was significantly lower compared to both adjacent and healthy tissue.

Correlation analysis

The correlation analysis, which was not presented in the abovementioned article, showed a tendency of positive correlation between the NO content only to putrescine in adjacent tissue, and a tendency of negative correlation in tumor tissue, which were not statistically significant (Fig. 2A). There was no correlation for either spermidine or spermine. Arginase activity was significantly increased in tumor tissue in comparison to both adjacent and healthy tissue (Fig. 1C). Correlation analysis showed a positive correlation of arginase activity and nitric oxide content, which was statistically significant only in healthy tissue (P<0.05) (Fig. 2B).

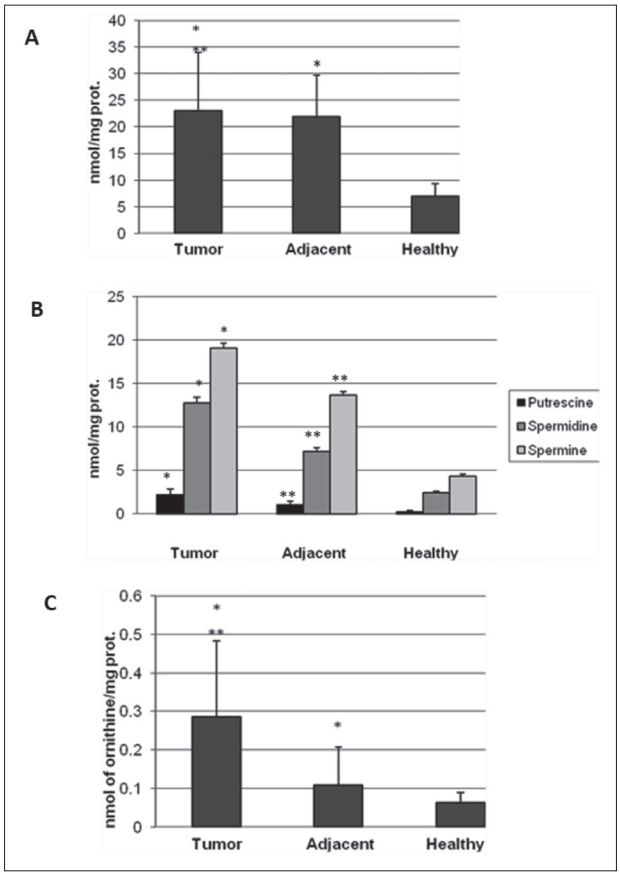

Fig. 1. Polyamine concentrations and arginase activity in colon cancer, adjacent and healthy tissues. Three tissue specimens, tumor tissue, tissue surrounding the tumor/adjacent tissue and normal colonic mucosa specimen (healthy tissue more than 10 cm from the tumor border at the incision margin) were taken during surgery. **A** – Polyamines were determined by HPLC and the concentrations were expressed in mg per tissue proteins. Total polyamines represent the sum of all three main polyamines in each of the three tissue specimens. *P<0.001 vs. healthy, ** P<0.001 vs. adjacent and healthy. **B** – Concentrations of polyamines (putrescine, spermidine, spermine) in colon cancer, adjacent and healthy tissues. The concentrations of each polyamine in tumor tissue was compared with the concentrations in adjacent and healthy tissues. *P<0.001 vs. adjacent and healthy; **P<0.001 vs. healthy. **C** – Arginase activity in colon cancer, adjacent and healthy tissues. Arginase activity measurement was based on the spectrophotometric determination of released ornithine and expressed as nmols of ornithine per mg of proteins. *P< 0.001 vs. adjacent; **P<0.001 vs. healthy

DISCUSSION

The examined clinical parameters of patients in our study after surgery were similar to recent reports [24]. Although the early diagnosis and treatment of CRC are in constant progress, the results of CRC patient treatment regarding postoperative recurrence and

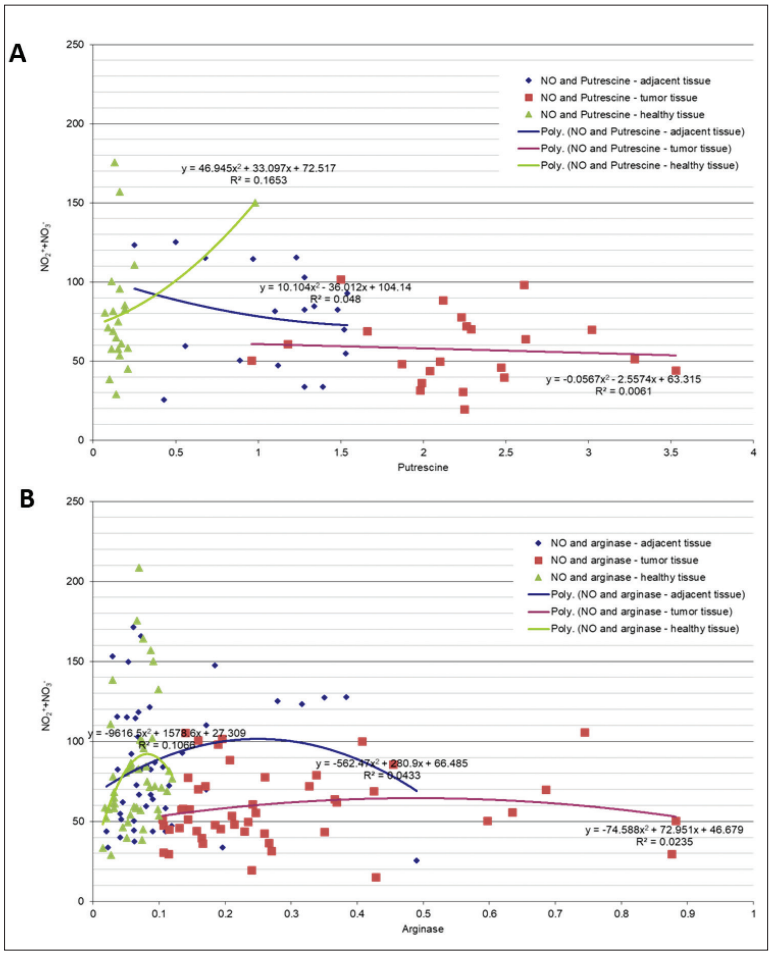

Fig. 2. Correlation analyses between studied parameters. **A** – Correlation analysis between Putrescine and NO concentrations. **B** – Correlation analysis between arginase activity and NO concentrations. P<0.05.

metastases, are unsatisfactory. It is obvious that the individual patient approach in the prognosis and treatment of the disease is promising. Therefore, searching for new biomarkers is an imperative for reducing unwanted disease outcomes and increasing patient survival.

In the human body, arginine is mostly derived from diet, and a smaller part is synthesized in the kidneys. There is evidence that some animal and human tumors require arginine for growth [25,26]. Since human tumors, unlike normal cells that can synthesize arginine from citrulline through the citrulline cycle, are not able to synthesize arginine, they require dietary arginine for growth. Based on the cancer cell requirement for arginine, the possibility of cancer starvation by arginine depletion was suggested [27]. In contrast to these studies, in some animal tumor models arginine supplementation augmented both specific and nonspecific antitumor mechanisms, retarded tumor growth and prolonged survival [28]. Summarizing the results of several studies, it was concluded [29] that arginine supplementation during the initiation stage of carcinogenesis decreased tumor production and crypt cell hyperproliferation, but stimulated tumor growth during the promotion stage. However, there are conflicting results about arginine and arginine metabolite roles in carcinogenesis.

Arginine is the substrate for two metabolically important enzymes, arginase and NOS. Ornithine, formed by the action of arginase, is used for the synthesis of polyamines, polycations that are known to modulate the proliferation of normal and tumor cells [30]. It is thought that exogenous arginine plays a more significant role in modulating polyamine than NO levels. It was shown [31] that dietary arginine supplementation enhances colon tumor incidence and adenoma grade in mice, probably by enhancing polyamine synthesis. Depletion of arginine by arginase, the enzyme that catalyzes the conversion of arginine to ornithine, limits arginine availability for NO production. The result of increased arginase activity is that less substrate is available for the production of cytostatic NO, while more arginine is metabolized to pro-proliferative polyamines [32], with which our results are in agreement [23].

Literature data report variable polyamine profiles in different types of human cancers. The significant increase of polyamines established at the site of cancer compared to non-neoplastic mucosa and adjacent colonic mucosa, suggests that polyamines can serve as oncological markers in early detection of colon cancer [33]. The increase of the main polyamines in our investigation, especially spermine, correlates with the findings of this and other authors [33,34]. It has

been known that the mechanism by which cancer cells enhance their metastatic potential is their response to hypoxia. In solid tumors such as CRC, there are scattered regions with compromised oxygen delivery due to structural abnormalities of tumor microvessels and disturbed microcirculation. Through the suppression of adhesion molecule expression, hypoxia attenuates cancer cell adhesion, promoting cancer cell migration and initiating cancer metastasis and invasion. It was reported [35] that spermine accelerated hypoxia-initiated cancer cell migration, so high spermine levels in tumor and especially cancer adjacent tissue specimens of our patients could indicate higher tumor growth and a metastatic potential.

Although a significant correlation of the polyamine content with Duke's stage, tumor site or size was not found [4], in most of our patients (but not all) there were positive correlations between the polyamine levels and the severity of the disease, highlighting the importance of analyzing the results for each individual patient. The high polyamine content in adjacent colonic mucosa, indicating a high proliferative potential of surrounding tissue, provides the rationale for polyamine determination in postoperative material in the assessment of disease prognosis, which is in agreement with other reports [10].

There is some evidence that arginase has important roles in cancer immunobiology [36,37]. However, the literature data are contradictory and the mechanisms underlying arginase activity in cancer tissue and tumor clinical characteristics are unclear [38]. Previous data have reported increased arginase activity in different cancer tissues [32,39], which was also confirmed by our results. Elevated arginase activity in our patients correlates with high concentrations of polyamines necessary for cell proliferation, intensive tumor growth and enhanced collagen synthesis, which is important for angiogenesis. Also, by reducing arginine availability, highly significantly increased arginase activity in colon cancer tissue compared to both adjacent and healthy tissues could explain the decrease in NO in tumor tissue.

Recently it was reported [40] that high arginase expression was significantly associated with advanced CRC and metastases; the authors hypothesized that this could serve as a prognostic biomarker in patients with advanced-stage CRC. Furthermore, it was suggested that patients who had high arginase levels had shorter overall and disease-free survival. In our study, statistical analysis did not reveal a predictive significance of arginase activity in the tumor and surrounding healthy tissues on the five-year survival of patients, although analyses of individual patients' results showed a correlation between high arginase activity and tumor recurrences, as well as overall survival. This discrepancy could be explained by the number of patients used in the study or by race differences. Therefore, regarding the potential prognostic value of arginase in CRC, we agree with [41] that more studies are needed to provide the scientific foundation for future clinical development of the results of research related to the prognostic role of arginine metabolism in CRC carcinogenesis.

CONCLUSION

Increased polyamine levels in colon cancer and adjacent tissue specimens after surgery compared to healthy individuals, and increased arginase activity in cancer tissue compared to adjacent and healthy tissue in patients with colorectal carcinoma points to the increased proliferative potential of tissue surrounding the cancer. Although in this study we did not observe a statistically significant predictive value of arginase on the five-year survival of patients, the obtained results provide a rationale for future research on larger sample of CRC patients that would focus on arginine metabolic pathway changes in cancer and in adjacent and healthy tissue specimens, on blood and urine polyamine levels and blood arginase activity, and the relation to the occurrence of metastases and tumor recurrence, as well as patient survival. This information could be useful in the risk assessment for tumor growth and metastases after surgery and prediction of patient survival.

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Author contributions: BB designed the experiment, conduct the investigation, performed the experiment, analyzed the data, wrote the original draft, reviewed the draft of the paper. GS wrote and edited the paper and reviewed the drafts. AV managed the clinical material in the laboratory, performed the experiments and wrote

the paper. GK contributed to the analysis tools and reviewed the drafts. MN performed the investigation, analyzed data and wrote the paper. BĐ performed the statistical analysis of the data, prepared the tables and figures and wrote the paper. JB performed the experiments, contributed to the analysis and wrote the paper. IS designed the experiments, contributed to the reagents/materials/analysis tools, wrote the paper and reviewed the drafts. All authors read and agreed to the final version of the manuscript.

Conflicts of interest disclosure: The authors declare no potential conflict of interest with respect to the research, authorship and/or publication of this article.

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/Brankovic%20et%20al_7824_Data%20Report.pdf

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