

# Morphometric study of uninvolved rectal mucosa 10 cm and 20 cm away from the malignant tumor

Sanja Z. Despotović<sup>1</sup>, Novica M. Milićević<sup>1</sup>, Dragoslav P. Milošević<sup>2,3</sup>,  
Nebojša Despotović<sup>2,3</sup>, Predrag Erceg<sup>2,3</sup>, Božidar Bojić<sup>4</sup>, Danijela Bojić<sup>2,4</sup>, Petar  
Svorcan<sup>2,4</sup>, Gordana Mihajlović<sup>3</sup>, Jelena Đorđević<sup>4</sup>, Ivana M. Lalić<sup>1</sup> and Živana Milićević<sup>1</sup>

<sup>1</sup>Institute of Histology and Embryology, Faculty of Medicine, University of Belgrade, Belgrade, Serbia, <sup>2</sup>Faculty of Medicine, University of Belgrade, Belgrade, Serbia, <sup>3</sup>Department of Geriatrics, Zvezdara University Clinical Center, Belgrade, Serbia and <sup>4</sup>Center for Gastroenterology and Hepatology, Zvezdara University Clinical Center, Belgrade, Serbia

**Summary.** Recently, many details of the interplay between tumor cells and tumor-associated stromal elements leading to the progression of malignant disease were elucidated. In contrast, little is known about the role of uninvolved stromal tissue in the remote surrounding of the malignant tumor. Therefore, we performed a computer-aided morphometric study of rectal mucosa in samples taken 10 cm and 20 cm away from the malignant tumor during endoscopic examination of 23 patients older than 60 years. The samples of rectal mucosa from 10 healthy persons of corresponding age subjected to diagnostic rectoscopy during active screening for asymptomatic cancer were used as control. All structural elements of the rectal mucosa were studied and the number of nucleated cells in the lamina propria per 0.1 mm<sup>2</sup> of tissue was assessed. Our study revealed a reduced number of cells in the lamina propria of the rectal mucosa 10 cm and 20 cm away from the tumor lesion in both male and female patients. The decreased mucosal height and increased crypt number were registered in female patients 10 cm away from the tumor. The connective tissue of lamina propria showed a disorderly organization: the collagen fibers were frail, loosely arranged and signs of tissue edema were present. Small blood vessels and capillaries were much more frequently seen than in healthy tissue. Our results demonstrate the complex interactions between the cancer and remote mucosal tissue of the affected organ.

**Key words:** Computer-Assisted Image Analysis, Rectum, Mucosa, Adenocarcinoma, Human

## Introduction

Although enormous advances have been made in the understanding of tumor initiation, progression and metastasis, these processes are still not fully elucidated. In recent times, much attention has focused on the interplay between tumor cells and neighboring elements constituting the tumor stroma, notably, fibroblasts and extracellular matrix (for example, Crawford and Ferrara, 2009; Bremnes et al., 2011; Ungefroren et al., 2011). The studies demonstrated extensive reciprocal influences between tumor cells and neighboring non-malignant cells. On the one hand, tumor progression and aggressiveness are promoted by the surrounding supporting tissue through cell-to-cell contacts or secreted molecules (Kalluri and Zeisberg, 2006; Grisendi et al., 2011). For example, the invasive potential of pancreatic duct adenocarcinoma (a tumor exhibiting a pronounced stromal compartment) may be enhanced by interactions with stromal fibroblasts (Maehara et al., 2001; Ijichi et al., 2011). In turn, via secretion of growth factors (for example, TGF- $\beta$ , FGF, VEGF, lysophosphatidic acid etc.), tumor cells may induce the transdifferentiation of normal fibroblasts to myofibroblasts, which produce the factors stimulating tumor progression (Desmouliere et al., 2004; Kellermann et al., 2008; Fuyuhiko et al., 2011; Grisendi et al., 2011; Mazzocca et al., 2011). In this manner, the feed-back loop closes and through the reciprocal interplay between tumor cells and stromal

cells the advancement of malignant disease is promoted.

However, despite the wealth of data regarding the interactions between tumor cells and tumor-associated stromal elements in the progression of malignant disease (Kunz-Schughart and Knuechel, 2002a, 2002b; Grisendi et al., 2011), no attention has been devoted to the studies of uninvolved stromal tissue in the remote surrounding of the tumor. This prompted us to perform a morphometric study of all elements of the rectal mucosa 10 cm and 20 cm away from the malignant tumor. We revealed significant structural alterations and reduced cellularity of the unaffected rectal mucosa not only at 10 cm, but also at a further distance 20 cm away from the adenocarcinoma of sigmoid colon. Thus, our results demonstrate the complex interactions between cancer and remote mucosal tissue of the affected organ.

## Materials and methods

### Tissue samples

Tissue samples were endoscopically collected at the Center for Gastroenterology and Hepatology, Zvezdara Clinical Center, Belgrade, Serbia during examination of patients suspected on clinical grounds to suffer from a malignant disease. According to WHO histological classification of tumors, all patients were subsequently diagnosed with adenocarcinoma. The samples of rectal mucosa were obtained from 23 patients older than 60 years (15 of male and 8 of female gender; Table 1). In the majority of cancer patients the tumors were located in sigmoid colon 25-30 cm from the external anal verge (two cancers were located in colon descendens). The biopsies were taken 10 cm and 20 cm away in caudal direction from the malignant tumor. Care was taken that a second tumor was not present at distances closer than those. The samples of rectal mucosa collected at the same institution from 10 healthy persons of the corresponding age (5 male and 5 female; Table 1) during the active endoscopy screening procedure of asymptomatic individuals possibly suffering from as yet unidentified, asymptomatic cancer, with a family history of intestinal malignancy, in which no disease involving the rectal mucosa was found, were used as control. The samples were taken from the upper third of rectum. The patients were divided into groups according to their gender. All patients were on standard mixed meals regimen and not on any particular form of diet. As only the patients with newly discovered tumor were included

in the study, they had not received any previous treatment for the malignant disease. The patients with diverticular disease of the colon, previous infectious colitis or inflammatory bowel disease were excluded from the study.

The study has been approved by the Ethics Committee of Zvezdara Clinical Center, Belgrade, Serbia (03/11/2003) and performed in accordance with ethical standards laid down in the 1964 Declaration of Helsinki. All persons involved received detailed verbal information and gave their informed consent prior to their inclusion in the study.

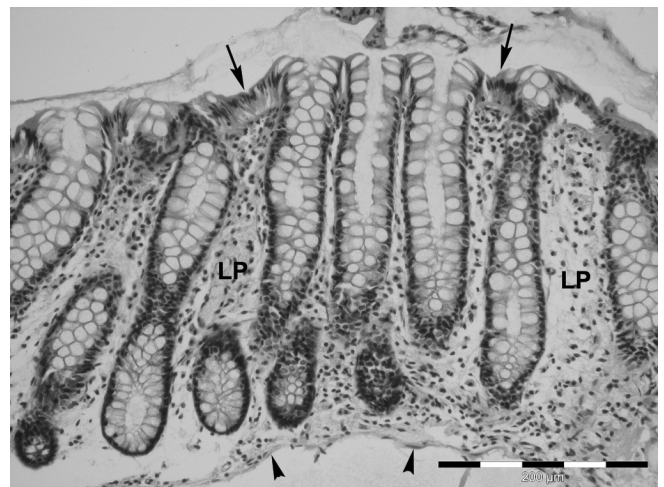
### Tissue preparation, staining and morphometric measurements

The tissue preparation and morphometric measurements were performed as described earlier (Milošević et al., 2007). Briefly, the biopsies of the rectal mucosa were fixed in 10% neutral buffered formalin, processed to Paraplast and tissue sections (3-5  $\mu\text{m}$  thick) were routinely stained with hematoxylin-eosin. Morphometric measurements were performed using a light microscope Opton Photomicroscope III (Carl Zeiss AG, Oberkochen, Germany) at magnification  $\times 100$  and  $\times 250$ . The tissue elements of rectal mucosa, hand labeled on microphotographs acquired with Olympus C3030-Z digital camera (Olympus Deutschland GmbH, Hamburg, Germany) and projected on the monitor, were measured using a computer-aided image analysis software Analysis 3.1 (Soft Imaging System GmbH, Münster, Germany). Measurements were performed on two sections of each tissue sample.

All tissue elements of the rectal mucosa were measured. The height of the rectal mucosa (in  $\mu\text{m}$ )

**Table 1.** Demographic characteristics of patients included in the study.

Patients	Number	Age in years	Gender	
			Male	Female
Cancer	23	73.6 $\pm$ 7.3	15	8
Healthy	10	75.2 $\pm$ 7.8	5	5



**Fig. 1.** Longitudinal section of the rectal mucosa, 20 cm away from the cancer. Female patient, 69 years. LP: lamina propria; arrows: surface epithelium; arrowheads: lamina muscularis mucosae. Hematoxylin-eosin staining. Bar: 200  $\mu\text{m}$ .

## Uninvolved rectal mucosa in cancer

between the lamina muscularis mucosae and surface epithelium was measured (3 measurements on each section), as well as the crypt depth (3 measurements on each section), using tissue samples with strictly longitudinally sectioned Lieberkühn crypts at x100 magnification (Fig. 1). The number of Lieberkühn crypts (per 0.1 mm<sup>2</sup> of tissue) was measured at x100 magnification. The height of the surface epithelium and parameters of the crypts (in  $\mu\text{m}$ ) were measured at x250 magnification. Only tissue samples with strictly cross-sectioned crypts (Fig. 2) were measured (6 crypts on each section) to estimate the following parameters: crypt diameter, crypt perimeter and the height of the crypt epithelium (in  $\mu\text{m}$ ). The number of nucleated cells in the lamina propria (per 0.1 mm<sup>2</sup> of tissue) was assessed on cross-sections of the rectal mucosa at x250 magnification on each section. Briefly, the connective tissue of lamina propria between the crypts was carefully encircled by the cursor and then the nucleated cells were counted by marking on the total of the specified area.

Masson trichrome technique was used for demonstration of connective tissue elements (Bradbury and Gordon, 1982).

### Statistical analysis

The statistical package SPSS for Windows 12.0 (SPSS Inc., Chicago, IL, USA) was used to calculate the means and standard deviations, as well as to indicate significant differences (Student's T-test at  $P < 0.05$ ).

### Results

The comparison of morphometric data revealed no significant differences for any structural element of the rectal mucosa between male and female healthy persons (Tables 2 and 3).

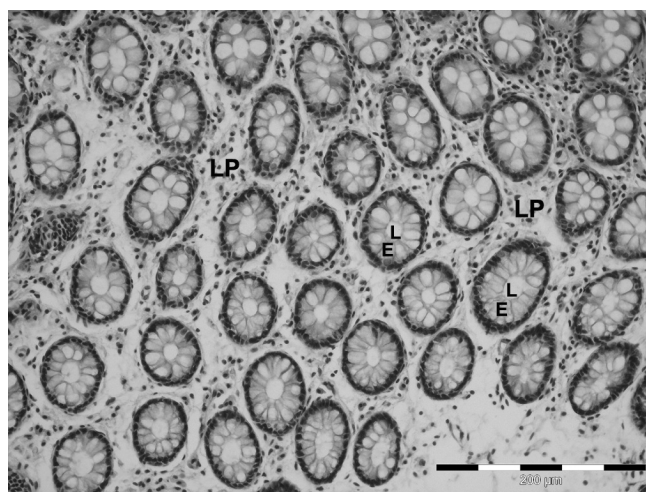
However, at the distance of 10 cm away from the tumor lesion a significant decrease of mucosal height, as well as an increase in crypt number per 0.1 mm<sup>2</sup> of rectal mucosa, was registered in female cancer patients, which could be attributed to the diminished diameter of

crypts. Such changes were not observed in male patients (Table 2).

Similar alterations of the rectal mucosa, but less prominent in comparison with the normal tissue and without statistical significance, were observed both in male and female cancer patients 20 cm away from the tumor (Table 3). No gender difference for any structural element of the rectal mucosa was observed at this distance from the tumor.

Very interestingly, 10 cm away from the adenocarcinoma, a highly significant reduction in number of cells per 0.1 mm<sup>2</sup> of lamina propria in the rectal mucosa of cancer patients was registered in comparison with healthy persons (Table 2).

Furthermore, the reduced cellularity of lamina propria of the rectal mucosa remained preserved both in male and female cancer patients compared with healthy persons at the distance of 20 cm from the tumor,



**Fig. 2.** Transverse section of the rectal mucosa, 20 cm away from the cancer. Female patient, 69 years. L: lumen of the crypt; E: crypt epithelium; LP: lamina propria. Hematoxylin-eosin staining. Bar: 200  $\mu\text{m}$ .

**Table 2.** Morphometric parameters of the rectal mucosa in healthy subjects and cancer patients 10 cm away from the tumor.

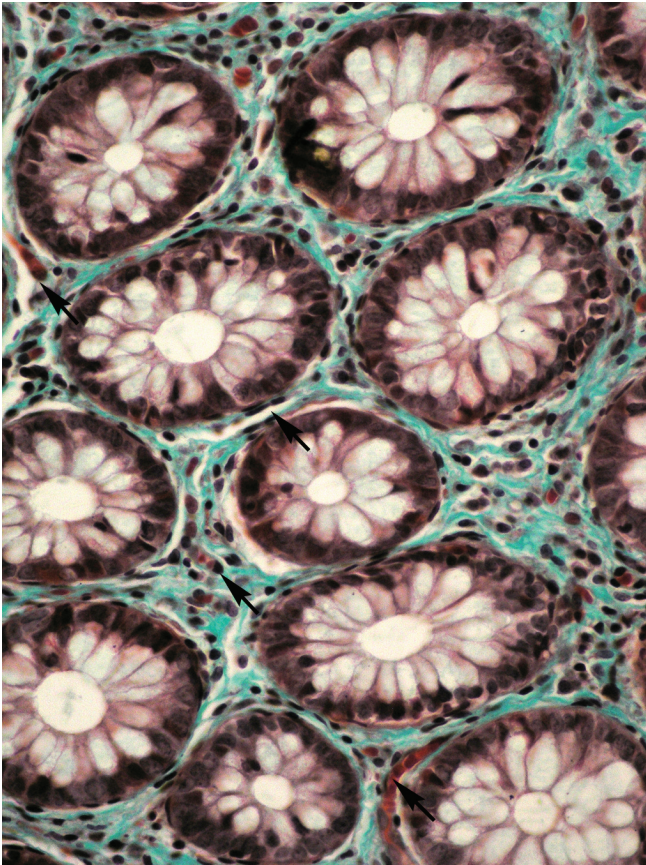
Parameter	Male		Female	
	Healthy	Cancer	Healthy	Cancer
Crypt epithelium height	26.8±1.6	26.6±2.8	27.9±2.2	22.8±4.4
Diameter of crypts	77.1±4.1	66.9±7.8	76.6±7.5	58.2±14.4
Perimeter of crypts	266.8±13.9	252.2±25.9	266.5±23.5	219.1±47.5
Surface epithelium height	37.9±4.1	39.7±7.5	46.4±10.3	42.4±12.7
Depth of crypts	269.5±12.9	262.1±65.8	399.1±66.5	250.7±58.8
Mucosal height	391.2±123.6	320.7±56.2	426.3±76.2	269.4±75.2***
Number of crypts per 0.1 mm <sup>2</sup>	10.9±5.9	16.5±9.8	10.1±2.6	18.7±5.1**
Number of cells per 0.1 mm <sup>2</sup>	1459.9±270.5	947.5±211.4*	1344.5±230.0	1044.3±125.7***

\* healthy males vs. cancer males ( $p < 0.01$ ); \*\* healthy females vs. cancer females ( $p < 0.05$ ); \*\*\* healthy females vs. cancer females ( $p < 0.01$ ); n=5

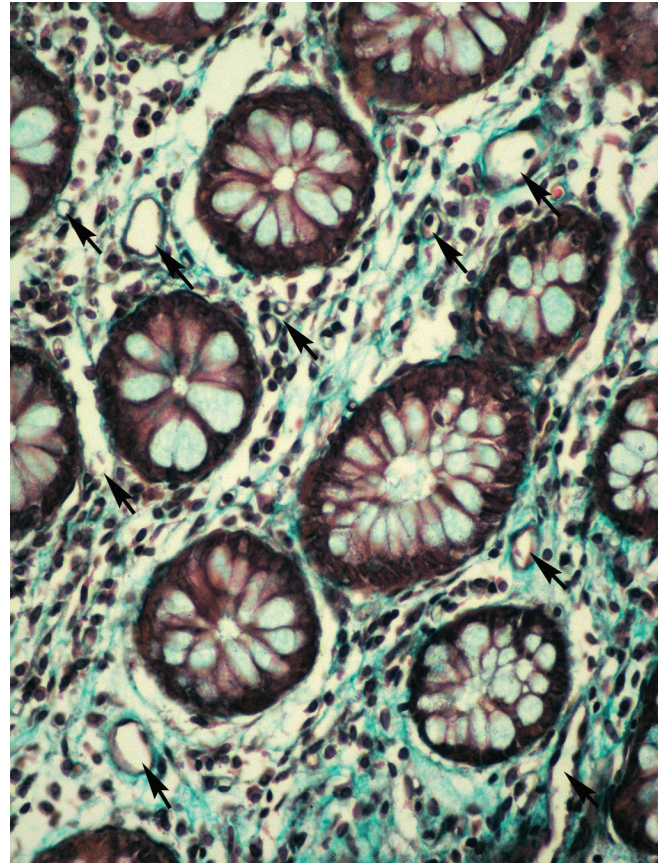
although at a lower level of significance (Table 3).

Morphological analysis of healthy mucosa demonstrated that the collagen fibers in the connective tissue of lamina propria were thick, closely appositioned and orderly organized. Smaller blood vessels and

capillaries were infrequently seen (Fig. 3). On the contrary, in lamina propria of the mucosa 10 cm away from the tumor the connective tissue showed a



**Fig. 3.** Transverse section of the rectal mucosa. Male healthy person, 66 years. Thick collagen fibers of lamina propria, closely appositioned and orderly organized. Small blood vessels and capillaries are infrequent (some indicated by arrows). Masson trichrome staining. x 400



**Fig. 4.** Transverse section of the rectal mucosa, 20 cm away from the cancer. Female patient, 79 years. Connective tissue of lamina propria shows a disorderly organization: the collagen fibers are frail, in loose apposition, notable signs of edema. Small blood vessels and capillaries are plentiful (some indicated by arrows). Masson trichrome staining. x 400

**Table 3.** Morphometric parameters of the rectal mucosa in healthy subjects and cancer patients 20 cm away from the tumor.

Parameter	Male		Female	
	Healthy	Cancer	Healthy	Cancer
Crypt epithelium height	26.8±1.6	28.7±3.5	27.9±2.2	26.4±3.7
Diameter of crypts	77.1±4.1	73.8±9.6	76.6±7.5	69.7±11.8
Perimeter of crypts	266.8±13.9	261.4±34.3	266.5±23.5	246.6±38.8
Surface epithelium height	37.9±4.1	47.1±7.7	46.4±10.3	46.6±9.8
Depth of crypts	269.5±12.9	262.8±47.8	399.1±66.5	306.3±50.6.2
Mucosal height	391.2±123.6	297.7±38.7	426.3±76.2	327.4±41.2
Number of crypts per 0.1 mm <sup>2</sup>	10.9±5.9	13.1±3.6	10.1±2.6	14.7±3.9
Number of cells per 0.1 mm <sup>2</sup>	1459.9±270.5	1084.1±130.8*	1344.5±231.0	1158.2±309.8**

\* healthy males vs. cancer males (p<0.05); \*\* healthy females vs. cancer females (p<0.05); n=5

disorderly organization: the collagen fibers were frail, loosely arranged and signs of tissue edema were present. Small blood vessels and capillaries were copious and much more frequently seen than in healthy tissue. All changes were also evident in lamina propria of the gut mucosa 20 cm away from the tumor (Fig. 4).

## Discussion

The present data regarding the morphometric parameters of the rectal mucosa in healthy individuals are in accordance with the results of our earlier study (Milošević *et al.*, 2007).

Our morphometric analysis revealed the significant structural and cellular changes of rectal mucosa in cancer patients in comparison with healthy individuals. Interestingly, a significant reduction of cellularity of the lamina propria of uninvolved rectal mucosa was observed not only in the vicinity, but also at a further distance from the malignant tumor in both sexes.

The mutual influences between the tumor cells and the intratumor stromal elements have been convincingly demonstrated in recent years (see for example, Crawford and Ferrara 2009; Bremnes *et al.*, 2011; Ungefroren *et al.*, 2011). Additionally, different genetic or biochemical changes of uninvolved mucosa distal from the cancer were found to be in many respects more similar to cancer tissue than to normal mucosa. They were believed to represent an early sign of malignancy before the morphologic changes appear (Carda-Abella *et al.*, 1982; Mandard *et al.*, 1997; Sattar *et al.*, 1999). This work is, to the best of our knowledge, the first to address the morphological and morphometric changes of remote mucosal tissue, 10 cm and 20 cm away from the tumor and shows that the uninvolved mucosal tissue is substantially altered. The decreased number of cells in the connective tissue of lamina propria may reflect the exit of fibroblastic cells into the circulation, which is known to occur under the influence of carcinoma and contributes to its metastasing capacity (Wels *et al.*, 2008; Jones *et al.*, 2013). Since lymphocytes and other inflammatory cells are common in the lamina propria of gut mucosa, their changes are of particular interest too. Therefore, the precise identification of missing cells is currently in progress in our laboratory. In addition to reduction in cell number, we registered alterations of extracellular matrix components and vascular elements of the lamina propria, as marked by the reduction of collagen fibers, signs of tissue edema and increased frequency of blood vessels. Taken together, our morphometric and morphological data suggest that general reorganization of unaffected rectal mucosa occurs in the remote surrounding of neoplasia.

However, from our results it cannot be concluded whether the observed changes of the mucosal structure and the reduction of connective tissue cellularity can be ascribed to the detrimental influence of developing cancer or, the other way round, they reflect the initial alterations providing a suitable background for tumor

formation. The fact that these changes are more prominent at smaller distance of 10 cm from the tumor, in comparison with 20 cm, deserves full attention. Further investigations are necessary to resolve this question.

Finally, although studies have shown that in general men are more likely to have colorectal tumors than women (McCashland *et al.*, 2001), our work revealed little difference between the genders. Considering that the number of patients in our study was small, further investigations are warranted.

In conclusion, our work documents the structural and cellular alterations of the uninvolved rectal mucosa distant from the malignant tumor and may represent a valuable model for further studies of mutual interactions between the cancer and stromal tissue of the affected organ.

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