Original Paper

The Evaluation of Beta-2-Adrenoreceptors' Expression in Normal Peritumoral Tissue in Patients with Colorectal Adenocarcinoma

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ABSTRACT: Introduction: The aim of this study is to evaluate the expression of beta-2 adrenergic receptors in the normal peritumoral tissue at the colorectal level, just after the front tumor invasion, in patients with colorectal cancer. Methods: In this study we analyzed normal peritumoral tissues belonging to 56 patients, who were diagnosed with colorectal adenocarcinoma. These tissues were fixed in paraformaldehyde and paraffinembedded. The immunohistochemical study was done on seriate slides following the hematoxylin and eosin staining, after diagnostic and grading. Correlations were made between beta-2-adrenoreceptors' expression in the normal peritumoral tissue and the clinical and histopathological parameters of the patients with colorectal cancer. Results: There are positive correlations between the expression of beta-2-adrenoreceptors and feminine gender, age group under 50 years, tumor size under 5cm, tumor invasion T3-4 and tumor metastasis in regional lymph nodes N≥2. By analyzing the expression of beta-2-adrenoreceptors in peritumoral tissue depending on tumor grading one can notice that there are positive correlations between beta-2-adrenoreceptors' expression and poorly differentiated colorectal adenocarcinoma. Conclusions: Positive correlation between this type of receptors in normal glandular epithelium, in the vicinity of tumor invasion front of colorectal neoplasm, and certain clinicopathological features suggests the involvement of tumor microenvironment, which expresses them, in the pathogenesis of this neoplasm.

KEYWORDS: *cancer colorectal, beta-2-adrenoreceptors, clinicopathological features*

Introduction

Colorectal cancer is a frequent neoplasia, with a high incidence and prevalence worldwide. In men and also in women is the third most common type of cancer [1]. Worldwide, the incidence in both sexes is almost the same, male gender being a bit more affected by this type of cancer [2]. For both sexes, the higher incidence is recorded in Australia/New Zealand-approximately 44.8 men and 32.2 women at 100000 inhabitants-and the smallest incidence is recorded in Africa-approximately 4.5 men and 3.8 women at 100000 inhabitants-one can notice a variation of approximately 10 times in what the incidence of this disease is concerned [3].

Many factors may be involved both in the initiation and the progression of colorectal cancer and also in its evolution and prognostic. So, colorectal carcinogenesis can be initiated and sustained by many factors such as genetic factors including chromosomal instability and microsatellite instability [4], but also factors as the intestinal flora [5], cyclooxygenase 2 [6], inflammation [7], hormonal and environmental factors [8]. Beta-adrenergic system may also be involved in colorectal carcinogenesis. It contains catecholamines and their receptors [9]. Stress hormones together with adrenaline and noradrenaline have many physiological functions in the human body, but they are also involved in pathological conditions including cancer [10].

The aim of this study is to evaluate the expression of beta-2 adrenergic receptors in the normal peritumoral tissue at the colorectal level, just after the front tumor invasion, and also to make correlations between the expression of this type of receptors in the normal peritumoral tissue and the clinical and histopathological

parameters of the patients with colorectal cancer.

Material and Method

In this study we analyzed normal peritomoral tissue from 56 patients who had undergone surgical resection for primary colorectal cancer in the Ist Surgery Clinic of the County Hospital of Craiova. This study was approved by the Ethics Committee of the University of Medicine and Pharmacy of Craiova having the following registration number 53/19.05.2016. All patients included in the study provided written acceptance and informed consent.

The histological and immunohistochemical study was performed in the Center for Microscopic Morphology and Immunology from the University of Medicine and pharmacy of Craiova, in collaboration with The Department of Pathology from the County Hospital of Craiova.

The immunohistochemical study was done on seriate slides following the hematoxylin and eosin staining, after diagnostic and grading were made by a senior pathologist. Biological material was selected by using a HM350 microtome equipped with a system of transferring the sections on water bath (STS, microM). The histological sections were taken on poly-L-lysine covered slides and they were dried in a thermostat at 37°C for 24 hours. Then, they were deparaffinated and rehydrated in grading alcohol series. For the antigen retrieval, the slides were boiled in a sodium citrate, pH 6 buffer, for 21 minutes (seven cycles of three minutes each) in a microwave oven at 600W. When the slides cooled down after the boiling process, they were washed with tap water and distilled water for 15 minutes. Then the blocking of the endogenous peroxidase was done by incubating the histological sections in 3% water peroxide, for 30 minutes at room temperature, then they were washed in distilled water for 10 minutes and in phosphate-buffered saline (PBS) solution, for 5 minutes. The next step was blocking non-specific sites by using 2% skimmed milk for 30 minutes. The sections were then incubated with the rabbit anti-human primary antibodies for 18 hours (overnight) in a fridge at 4 °C.

In our study, we used the following primary antibody: rabbit anti-beta-2 Adrenergic R/ADRB2 Antibody (Novus Biologicals, UK, 1:100 dilution).

The next day, we applied the secondary biotinylated goat anti-rabbit antibody for 30 minutes at room temperature, then we washed in PBS solution (three baths, five minutes each), and in the end the HRP (Horseradish Peroxidase) Streptavidin for 30 minutes, at room temperature; finally, the slides were washed in PBS 3×5 minutes. The signal was detected using 3.3'-Diaminobenzidine (DAB) (Dako, Glostrup, Denmark) and the reaction was stopped in PBS. By using Mayer's Hematoxylin we counterstained the sections; we dehydrated them in alcohol, cleared them in xylene and mounted on slides with DPX (Fluka).

The slides were viewed on a Nikon 55i (Apidrag, Bucharest, Romania), microscope equipped with a 5Mp color cooled CCD camera and the Image ProPlus AMS software (Media Cybernetics, Rockville, MD, USA). After image grabbing, DAB stained regions of interest (ROI) were defined based on their RGB profile in a constant manner, the ROIs were analyzed for their total area and signal intensity (measured as integrated optical density, IOD).

The data obtained with Image ProPlus AMS software were exported in Microsoft Office Excel 2010 (Microsoft Corporation, Redmond, Washington, USA) and were analyzed by using SPSS software (IBM SPSS Statistics, Version 20.0). For statistical analysis ANOVA test (ANOVA-analysis of variance) and student t-test were used. The analysis of the variance was calculated in order to observe with greater finesse the effect that the independent variable has over the dependent variable, which allows in the same time the analysis of the data, that is divided in more than two groups. For better safety of the results we used post-hoc Bonferroni test, with a level of statistical significance for $p<0.05$.

Results

The expression of beta-2-adrenoreceptors was evaluated in all 56 peritumoral normal tissue samples at the colorectal level, immediately after the invasion front in patients who were diagnosed with primary colorectal cancer.

In Fig.1-3 colorectal carcinoma's different tumor gradings are being illustrated.

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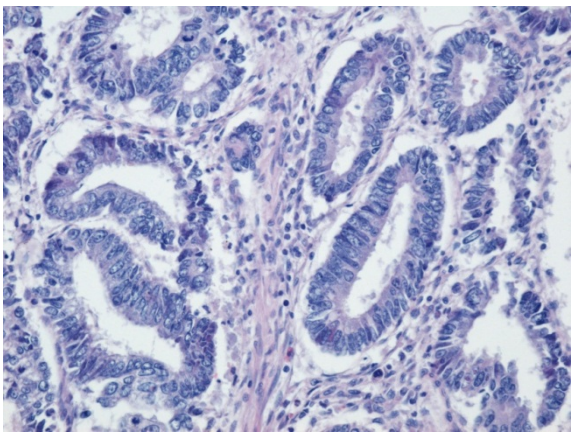

Fig. 1. Well-differentiate adenocarcinoma, HE staining, magnification x 200

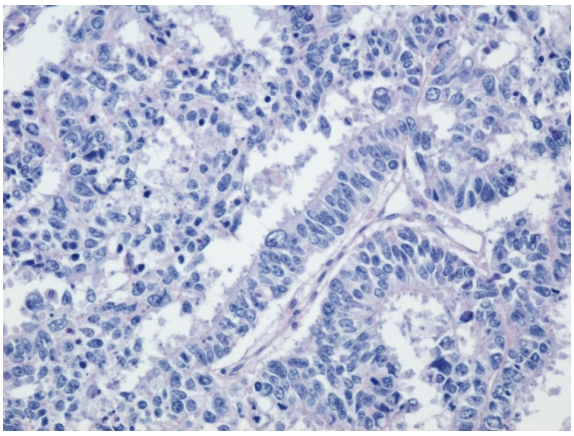

Fig. 2. Moderately-differentiated adenocarcinoma, HE staining, magnification x 200

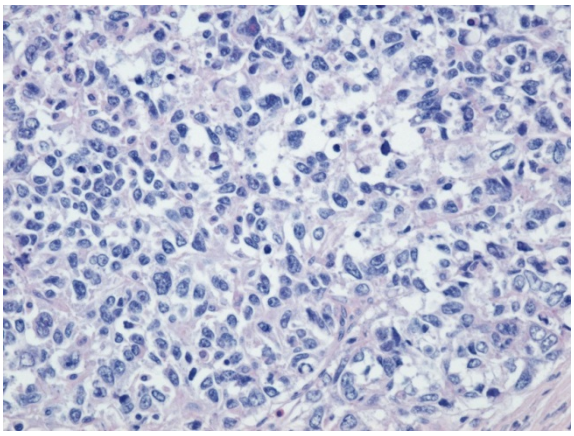

Fig. 3. Poorly-differentiated adenocarcinoma, HE staining, magnification x 200

The relationship between the expression of beta-2-adrenoreceptors and demographic and clinicopathological features of the patients is illustrated in table 1 for the signal's area, calculated with Image Pro Plus soft.

Table 1. Correlation between beta-2-adrenoreceptors' expression in peritumoral tissue in different tumor gradings of colorectal adenocarcinoma and clinicopathological features

Clinicopathological features		*n.*	*P-value*
Gender	Male	37	0.042*
	Female	19	
Age group	<50	14	0.017*
	≥50	42	
Tumor size	<5	21	0.045*
	≥5	35	
CRC location	Right, transverse and left colon	26	0.102
	Sigmoid and rectum	30	
Tumor invasion	T_{1-2}	15	0.029*
	T_{3-4}	41	
Lymph node metastasis	N_{0-1}	30	0.038*
	$N_{\geq 2}$	26	
*. P< 0.05, statistically significant			

Fig.4-6 are sections from the normal peritumoral tissue at colorectal level, just after the tumor's invasion front, with differences in what the expression of beta-2-adrenoreceptors is concerned (brown color).

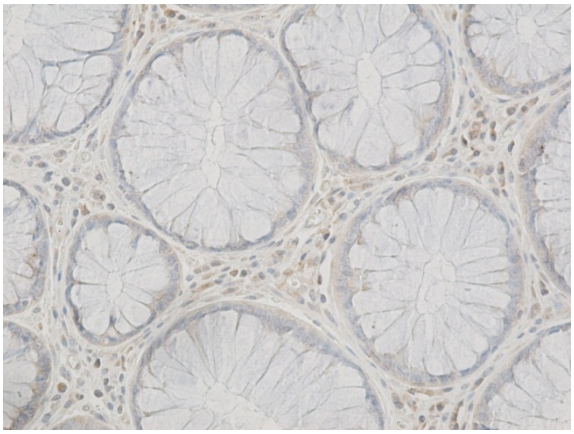

Fig. 4. Peritumoral tissue in well-differentiate adenocarcinoma, immunomarkin for beta-2-adrenoreceptors, magnification x 200

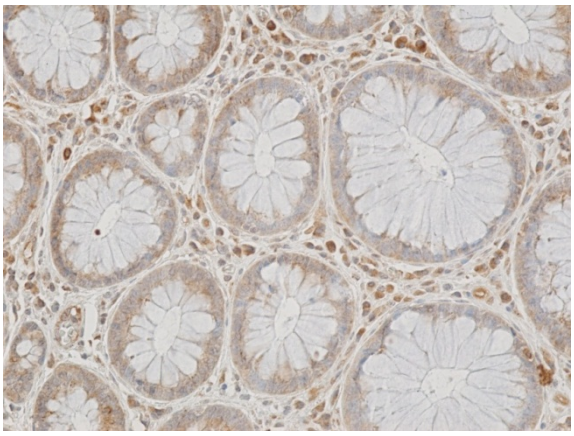

Fig. 5. Peritumoral tissue in moderately-differentiated adenocarcinoma, immunomarkin for beta-2-adrenoreceptors, magnification x 200

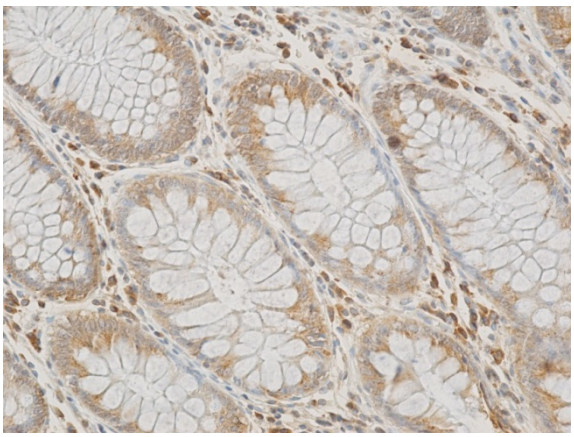

Fig. 6. Peritumoral tissue in poorly-differentiated adenocarcinoma, immunomarkin for beta-2-adrenoreceptors, magnification x 200

We observed that there are positive correlations between beta-2-adrenoreceptors' expression and feminine gender, age group under 50 years, tumor size under 5cm, tumor invasion T3-4 and tumor metastasis in regional lymph nodes N≥2. In what tumor location is concerned there are no statistically significant differences. All these results are presented in Fig.7-12.

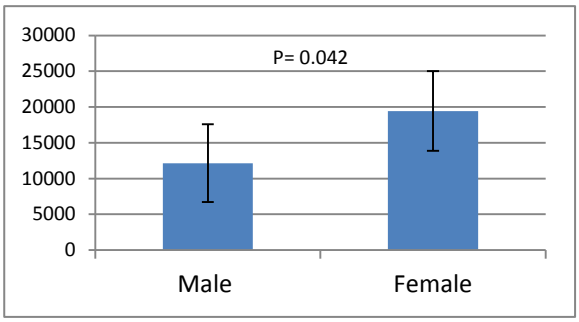

Fig. 7. Mean, standard deviaton and p-value of beta-2-adrenoreceptors' expression in peritumoral tissue dependind on gender

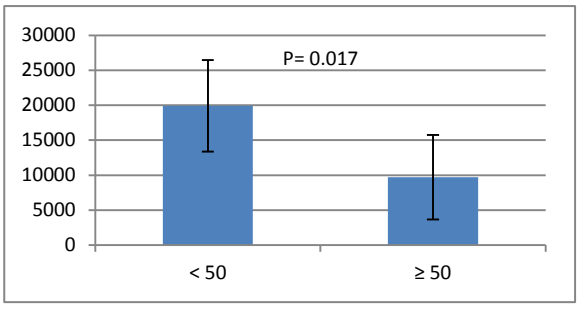

Fig. 8. Mean, standard deviaton and p-value of beta-2-adrenoreceptors' expression in peritumoral tissue dependind on age group

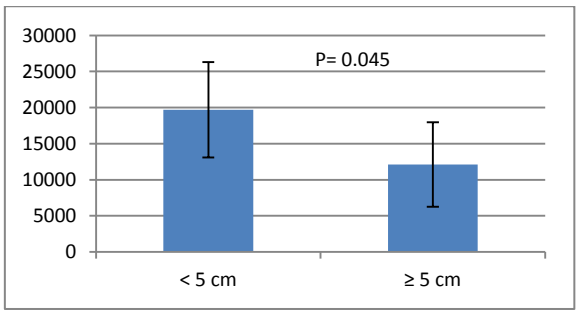

Fig. 9. Mean, standard deviaton and p-value of beta-2-adrenoreceptors' expression in peritumoral tissue dependind on tumor size

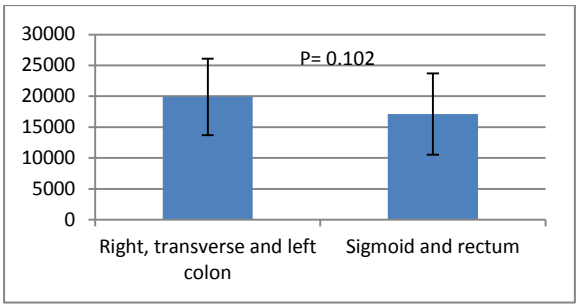

Fig. 10. Mean, standard deviaton and p-value of beta-2-adrenoreceptors' expression in peritumoral tissue dependind on CRC location

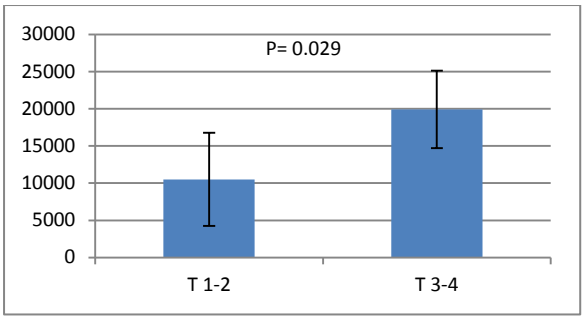

Fig. 11. Mean, standard deviaton and p-value of beta-2-adrenoreceptors' expression in peritumoral tissue dependind on tumor invasion

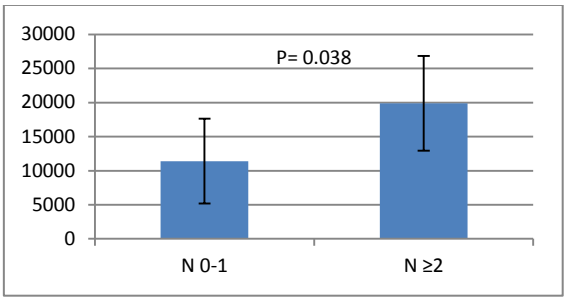

Fig. 12. Mean, standard deviaton and p-value of beta-2-adrenoreceptors' expression in peritumoral tissue dependind on lymph node metastasis

By analyzing the expression of beta-2-adrenoreceptors in peritumoral tissue depending on tumor grading, one can notice that

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there are positive correlations between beta-2-adrenoreceptors' expression and poorly differentiated colorectal adenocarcinoma (p=0.001) (Table2, Fig.13).

Table 2. The results of the ANOVA test followed by post-hoc Bonferroni between the expression of the beta-2-adrenoreceptors in normal colic mucosa and different gradings of colorectal adenocarcinoma

Correlation between Beta-2-adrenoreceptors' expression in peritumoral tissue and tumor differentiation **Pairwise Comparisons** Dependent Variable Area of B2A		
Peritumoral tissue	Peritumoral tissue	Sig. *
Well d.	Moderately differentiated Poorly differentiated	.072 .001
Moderately d.	Well differentiated Poorly differentiated	.072 .244
Poorly d.	Well differentiated Moderately differentiated	.001 .244
*. Adjustment for multiple comparisons: Bonferroni.		

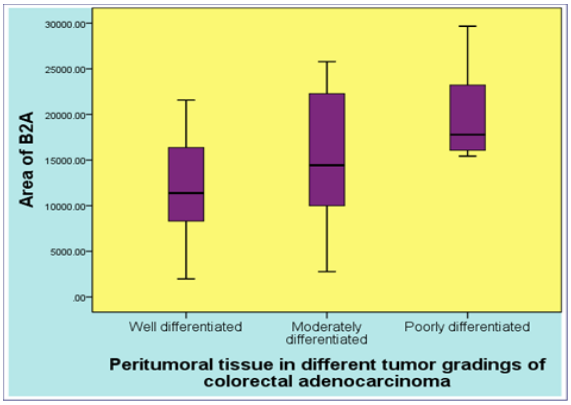

Fig. 13. Boxplot graph for the expression of the beta-2-adrenoreceptors in normal colic mucosa depending on different gradings of colorectal adenocarcinoma: the minimum, first quartile, median, third quartile and maximum value

Discussions

Like other neoplastic processes, the neoplastic process from the colorectal level is not an isolated phenomenon being in a continuous interaction with the elements belonging to the microenvironment where it develops, and also with cellular signalizing molecules released by the human body. An example is the interaction between neurotransmitters and their receptors, which in physiological conditions have well-determined roles, and also their involvement in the initiation, progression and metastasis of many types of cancers. Thus, beta-adrenergic receptors, whose expression we analyzed in this study, were the subject of many studies in different types of cancer: colorectal [11,12,13,14], ovary [15,16,17,18], breast [19], pancreas [20], prostate [21] and lung [22].

In tumor biology, the study of beta-adrenergic receptors was determined, on one hand, by epidemiological studies, which showed a positive correlation between stress and cancers' progression and incidence, and on the other hand, by cancers' decreasing progression when antagonists for these receptors, such as beta blockers, were used [23,24].

In physiological conditions, beta-adrenergic receptors are liked with heterotrimeric guanine nucleotide-binding protein (G proteins). G proteins are composed of three subunits: Gα, Gβ and Gγ (25), which are grouped in several families. Thus, Gα subunit includes: Gαs, Gαi/o, Gαq/11 si Gα12/13. All these subunits of G protein together with their families are involved in many intracellular signalizing pathways. The interaction of a ligand, such as norepinefrine or adrenaline, with beta-2-adrenoreceptors linked with this type of proteins, causes the binding between a molecule of guanosine triphosphate (GTP) and a G protein's α subunit, and, in this way, the signal's transduction takes place [26].

The ligands for beta-2-adrenoreceptors finally cause the activation of adenylate cyclase (AC), which converts adenosine triphosphate (ATP) in 3',5'-cyclic adenosine mono phosphate (cAMP). cAMP causes the activation of protein kinase A (PKA), which also mediates multiple intracellular signalization pathways [9]. Phosphorylation, induced by PAK, of the transcription factors such as cAMP response element-binding protein (CREB), activating transcription factor (ATF), transcription factors, which have the ability to bind DNA to "GATA" sequence (GATA) and signal transducer and activator of transcription 3 (STAT3), determine the expression of some proteins, which are involved both in cellular motility via cytoskeletal dynamics, and in cellular resistance to apoptosis [23]. PKA may also, via transcription factors, activate focal adhesion kinase (FAK), with the same result described above [23]. Another intracellular signalizing pathway, caused by the activation of beta-2-adrenoreceptors, is cAMP/ PKA/ phosphatidylinositol-4,5-biphosphate 3-kinase (PI3K)/ protein kinase B (AKT)/ mammalian target of rapamycin (mTOR)/ ribosomal protein S6 kinase beta-1 (p70S6K)/ hypoxia-inducible factor 1-alpha (HIF1α), which can cause the synthesis of vascular endothelial growth factor

(VEGF), that stimulates angiogenesis and so contributes to tumor progression and metastasis [9]. But the stimulation of beta-2-adrenergic receptors may lead to the activation of AC/cAMP/PKA pathway, separately of G proteins linked to receptors, by mitogen-activated protein kinase' stimulation (MAPK), leading to the release of pro-angiogenic factors such as VEGF, membrane-type 1 matrix metalloproteinase (MT1-MMP), MMP-2 and MMP9, which are involved in tumor proliferation and metastasis [9].

This data highlights the involvement of the nervous system's neurotransmitters, adrenaline and noradrenaline, via beta-2-adrenoreceptors, in the pathogenesis of colorectal neoplasm.

Conclusions

It is not known yet if the stimulation of beta-2-adrenoreceptors from the colonic glandular epithelial cells, may lead to the onset of new neoplastic cells, or it may favor the progression and metastasis of the preexisting neoplastic cells. In our study, the positive correlation of this type of receptors in the normal glandular epithelium, in the vicinity of tumor invasion front of colorectal neoplasm, on one hand with tumor grading, and on the other hand with tumor size, invasion and metastasis in regional lymph nodes, suggests the involvement of tumor microenvironment, which expresses them, in colorectal neoplasm pathogenesis. Further studies are necessary in order to confirm these things, to evaluate the opportunity of using beta-blockers in the treatment of colorectal cancer, and in order to develop new molecular targeted therapies, which must interfere and block intracellular signalizing pathways caused by the activation of beta-2-adrenoreceptors.

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All authors had equal contribution.

Conflict of interests

The authors declare that they have no conflict of interests.

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