Relationships Between Glial Enteric Cells, Beta-cell Signaling and Tumor Proliferative Activity in Patients with Colorectal Neoplasia

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The aim of our study was to evaluate the expression of glial enteric cells at different stages of differentiation of colorectal neoplasms and to correlate these changes with the tumor proliferation index and with the sympathetic influences evaluated by the expression of beta-2 adrenoreceptors. Given that nowadays colorectal neoplasm is a major public health problem and that the molecular mechanisms responsible for malignant transformation are not fully yet elucidated, more studies are needed in order to establish other intracellular signaling pathways in such a neoplasm. By this study we concluded that the proportional decrease in the density of glial enteric cells in colorectal cancer with the degree of tumor differentiation and also their inverse correlation with the tumor proliferation index and with the expression of the adrenergic beta-2 adrenoreceptors can be considered a negative prognostic factor in this type of cancer.

Keywords: enteric glial cells, beta-adrenergic signaling, proliferative tumor activity

Despite the progress from the recent years, both in the etiopathogenesis and evolution of colorectal neoplasm, band also in the diagnostic and therapeutic methods, this type of cancer continues to be the third most common type of cancer diagnosed in the world, and also the fourth leading cause of death worldwide [1].

Worldwide, in 2012 there were over 1.3 million new cases of colorectal cancer (9.7% of all neoplasms, excluding other skin cancers besides melanoma) and about 690 000 deaths (8.5% of all deaths of cancer, excluding other skin cancers, besides melanoma) [1].

In Romania, according to the World Health Organization, in 2012, colorectal cancer recorded an incidence of 10,256 cases for both genders (about 13% of all cancers regardless patient gender in this country) occupies the second place after lung cancer, which recorded 11,644 cases [2,3].

The molecular mechanisms responsible for the occurrence of this type of neoplasm are multiple and blocking the intra/intercellular signaling pathways had beneficial results, negatively influencing the process of colorectal tumorigenesis, which is the basis for the emergence of targeted molecular therapies [4-8].

However, this type of neoplasm creates major public health problems, both in terms of morbidity and mortality, so things are not yet fully understood and it is still necessary to conduct research in order to discover new therapeutic targets and to reduce the negative statistical indicators for this disease.

Experimental part

The aim of the study

The present study proposed full and detailed evaluation of glial enteric cell remodeling, of beta-adrenergic signaling and of tumor progression assessment in colorectal cancer in order to identify possible therapeutic and prognostic targets.

Material and methods

The study, a prospective analytical, descriptive observational one, was conducted on a total number of 69 patients diagnosed with colorectal adenocarcinoma, who were selected over a two year period (2016-2017). To avoid the bias in this study we included patients consecutively.

The analyzed cases were from patients who were hospitalized in the Emergency County Hospital of Craiova, Romania, where suspicion of malignant tumor formation at the colorectal level after clinical and laboratory investigations was raised. Subsequently, these patients underwent surgical resection of a colorectal segment in the Surgery Clinic of the same hospital. The biological material, taken in the surgical procedure, was immediately put in 10% formalin fixative solution and then sent to the Pathological Anatomy Laboratory of the Emergency County Hospital of Craiova, where it was firstmacroscopically examined, then subjected to different processing techniques in order to be ready for the microscopic analysis. Fragments of histological material were further processed in the *Center for Microscopic and Immunological Morphology Studies* of the University of Medicine and Pharmacy of Craiova, where the immunohistochemical study was carried out.

A single group of patients diagnosed with colorectal adenocarcinoma was established, and it was then divided into subgroups, depending on the different clinical and pathological features of the patients included in the study.

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All authors made equal contribution to the paper, to that of first authors.

The subgroups were taken into consideration at the time of statistical tests and they were compared with a control group consisting of patients with benign disease, that needed a resection of a colon fragment.

The study was conducted in accordance with the rules and principles of the Ethics Committee of the University of Medicine and Pharmacy of Craiova, approved by it and complied with all the provisions of the international forums regulating the scientific research, namely the Helsinki Declaration issued by the International Medical Association (WMA - World Medical Association). Each patient enrolled in the study agreed, by signing the informed consent and acceptance form for the biological material to be taken for the study and also for the use of clinical and laboratory data from the Medical Observatory Sheet.

Immediately after surgical resection, the biological material was introduced into a 10% formalin fixative solution, quantity depending on the size of the colorectal resection pieces, for 24-48 h. After successive washes in order to remove the fixative solution, the biological material was included in paraffin. After obtaining the tissue blocks, serial sections of 3-5 µm thickness were made by using a high precision HM355S automatic rotating microtome equipped with the original section transfer system on a cold water bath and they were then transferred to a bath of hot water at 40°C to be stretched and uniformed. The sections obtained were then picked and plated onto poly-L-lysine blades (a compound that greatly increased tissue adhesion to the blade), they were placed in an incubator at 60°C and held for 24 h. The tissue samples, used in our study, were first stained by using hematoxylin-eosin (HE) staining technique, which produces the pink cytoplasm, nuclei with nucleoli in blue and blue-violet and pale pink collagen fibers, while the fibers of elastin and of reticulin do not stain. The immunohistochemical study was performed on the same surgical resection pieces included in paraffin by methods mentioned above. For the immunohistochemical (IHC) study, the following antibodies were used: anti-GFAP (1:50 dilution, Dako) for highlighting enteric glial cells; anti-B2A (1: 100 dilution, Dako) for highlighting receptors for adrenaline and noradrenaline; Ki-67 (1: 200 dilution, Dako) to assess the degree of tumor proliferation. Both for glial enteric cells evaluation and for adrenergic beta-2 receptors evaluation, we used the ImagePro Plus imaging software. In order to assess the degree of tumor proliferation, the tumor proliferation index was quantified by ki-67 immunomarker.

The data were assessed using Student's t-test, ANOVA (analysis of variance) with Bonferroni'sposthoc correction and Pearson's correlation coefficient. The data were reported as mean ± standard deviation (SD). In all cases, $p<0.05$ was used to indicate statistical significance. Moreover, p-values <0.05, <0.01 and <0.001 representing significant differences were signalized with *, **, and ***.

Results and discussions

Regarding the glial enteric cells area (fig. 1), in the different stages of colorectal cancer tumor differentiation, we noticed that the area of the color sign for these nerve elements is higher in the control group(0.004502 ± 0.00156 %/mm^2) and in well-differentiated colorectal tumors (G1) where a percentage area of this type of cells was recorded at 0.004451 ± 0.001487 %/mm^2, while in moderately differentiated colorectal tumors (G2) a percentage area of 0.004051 ± 0.00223 %/mm^{2}was recorder, with a significant decrease of this parameter at 0.00144 ± 0.000905 %/mm^{2}in poorly differentiated colorectal tumors (G3) (fig. 2).

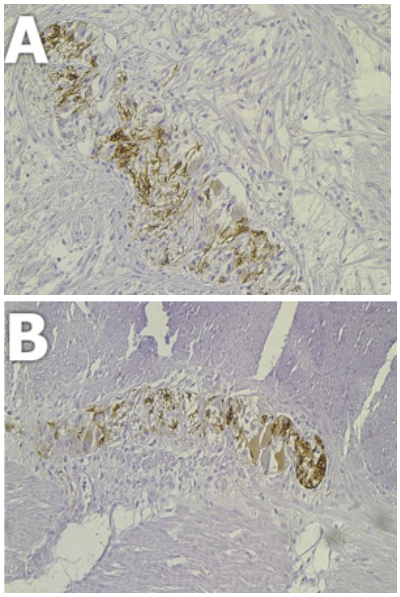

Fig. 1.Glial enteric cells in the Auerbach nervous plexus. There is a brownish color that does not mark the entire nerve ganglion but has an irregular distribution of the glial enteric cells that fold on the bodies and extensions of the enteric neurons. Immunomarking GFAP, 20X.

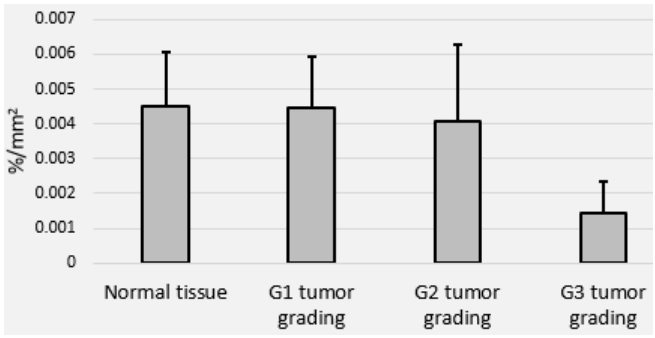

Fig. 2.Glial enteric cells area in normal tissue and different stages of colorectal cancer

Also, by evaluating glial enteric cells by dividing in nerve plexus for all patients included in our study, we noticed that the percentage area of the glial cells found in the Meissner plexus was 0.00016 ± 0.000096 %/mm^2, the percentage area of the glial cells found in theAuerbach plexus was 0.001988 ± 0.001433 %/mm^2, and the percentage of glial cells in other multiaxonal bundles of nerves with a diameter greater than 20 ìm was 0.001123 ± 0.000704 %/mm^2, the percentage area of glial cells in the Auerbach plexus being higher than the percentage areas of the other two categories (fig. 3).

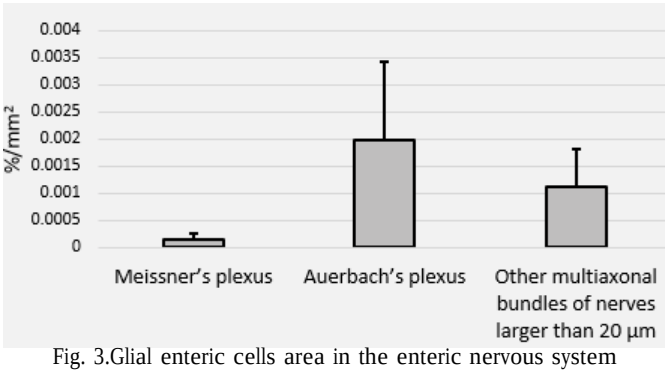

Fig. 3.Glial enteric cells area in the enteric nervous system

When evaluating with the ANOVA, with post-hoc Bonferroni correction, we noticed that there was a statistically significant difference between the density of glial enteric cells in well-differentiated tumors and poorly differentiated tumors (p = 0.005 **) and also between density glial enteric cells in moderately differentiated tumors and in poorly differentiated tumors (p = 0.041 *), whereas between tissue samples from the control group and well-differentiated and moderately differentiated tumorsstatistically significant differences of this type of cells were not recorded.

To assess the relationship between the sympathetic nervous system and the glial enteric cells in colorectal tumors, we evaluated the area and optical integrated density by using beta-2adrenoceptors expressed by colorectal tumor cells. Images with the expression of beta-2 adrenoreceptor are shown in figure 4. For beta-2 adrenoreceptors expression in normal tissue we determined an area of 5607.3 ± 2901.7 ìm^2 and an IOD of 859887.2 ± 458559.6, in well differentiated colorectal andenocarcinoma an area of 11381.2 ± 5203.7 µm^2 and an IOD of 1699676.3 ± 8952623.4, in the moderately differentiated colorectal adenocarcinoma an area of 26782.1 ± 11206.3 µm^2 and an IOD of 4312267.2 ± 1842105.3 and in the poorly differentiated colorectal adenocarcinoma an area of 36926.7 ± 9611.7 µm^2 and an IOD of 5482651.1 ± 1731096.2 (fig. 5 and 6).

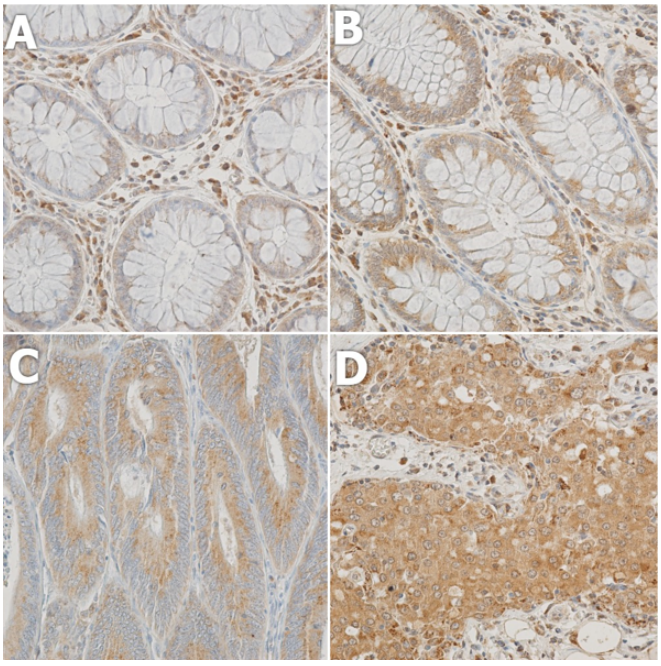

Fig. 4. Images with the expression of beta-2 adrenoreceptor in normal tissue (A), G1 tumor grading (B), G2 tumor grading (C) and G3 tumor grading (D), 20X

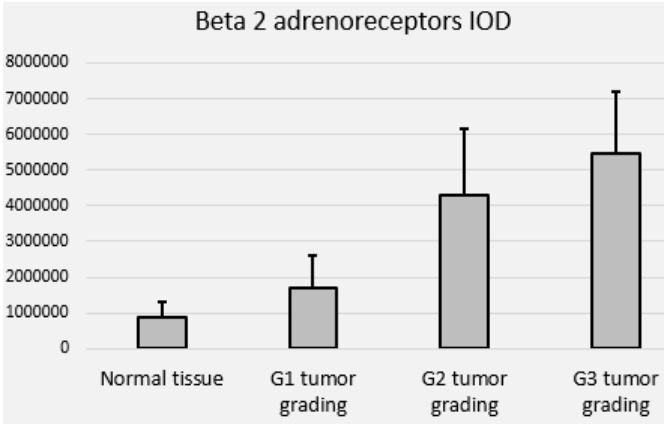

Fig. 5.Beta 2 adrenoreceptors area in normal tissue and in different stages of colorectal cancer

The proliferative activity of colorectal carcinoma for the patients included in our study was assessed by using the Ki67 monoclonal antibody, for each patient the tumor proliferation index expressed as a percentage was calculated (fig. 7). We observed that for patients diagnosed with well-differentiated colorectal adenocarcinoma (G1), the tumor proliferation index was 27.64 ± 12.82%, whereas in patients with moderately differentiated

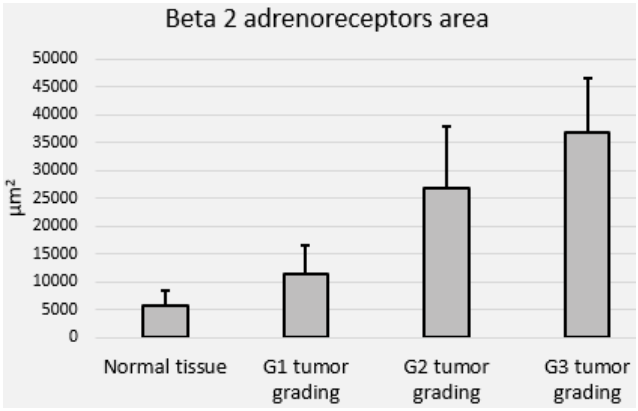

Fig. 6.Beta 2 adrenoreceptors IOD in normal tissue and in different stages of colorectal cancer

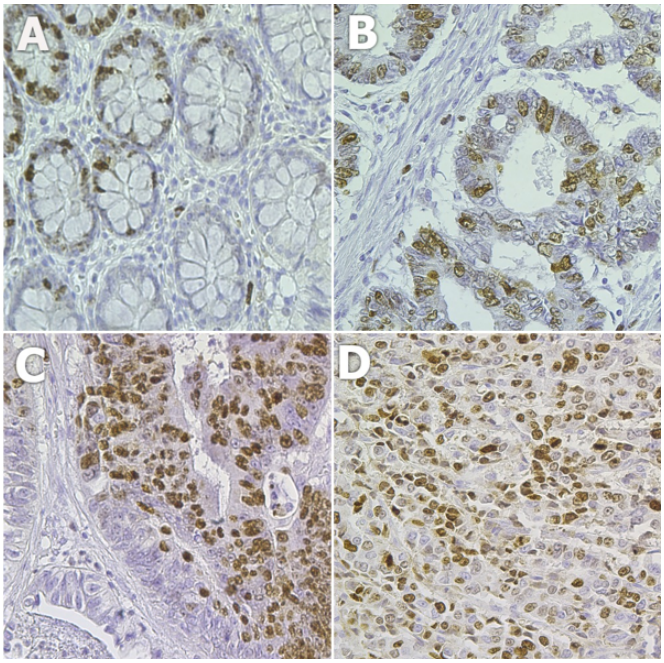

Fig. 7. Images with the expression of Ki-67 in normal tissue (A), G1 tumor grading (B), G2 tumor grading (C) and G3 tumor grading (D), 20X

colorectal adenocarcinoma, the tumor proliferation index scored a rate of 45.63 ± 15.34%, and in the case of patients diagnosed with poorly differentiated colorectal adenocarcinoma, the tumor proliferation index had the highest rate of 47.15 ± 27.32% (fig. 8). Comparing with the ANOVA test followed by the post-hoc Bonferroni correction, the average of the tumor proliferation index at the different stages of colorectal cancer differentiation, we noticed that there was a statistically significant

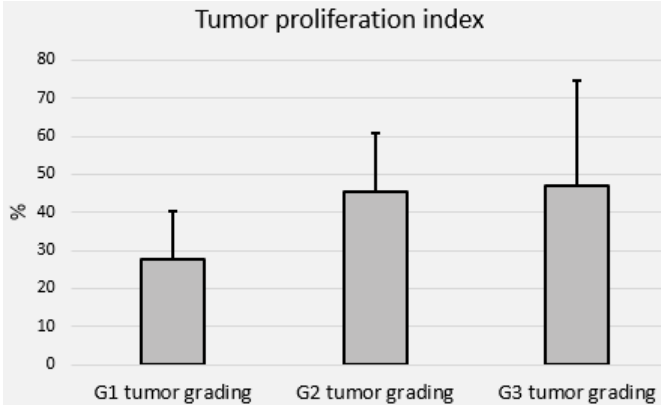

Fig. 8.Tumor proliferation index (assessed with Ki-67 immunomarker) in different stages of colorectal cancer.

difference between the tumor proliferation index in well differentiated tumors and in moderately differentiated ones (p = 0.036 *) and between the tumor proliferation index in well-differentiated tumors and in poorly differentiated tumors (p = 0.006 **), while between the tumor proliferation index in moderately differentiated tumors and in poorly differentiated tumors statistically significant differences were not observed.

Regarding the correlations between the glial enteric cells area in the whole group of patients included in the study and the tumor proliferation index, there was a global inverse correlation at the limit (r = -0.438), whereas between the glial enteric cells area of the whole group of patients enrolled in the study and the beta-2 adrenergic receptor expression, there was a high overall inverse correlation (r = -0.715).

The aim of our study was to evaluate the expression of glial enteric cells in different stages of differentiation of colorectal neoplasms and to correlate these changes with the tumor proliferation index and with the sympathetic influences evaluated by the expression of beta-2 adrenoreceptors. Taking into account that colorectal neoplasm is a major public health problem and that the molecular mechanisms responsible for malignant transformation are not fully elucidated, more studies are needed in order to discover other intracellular signaling pathways in such a neoplasm [9-13].

Glial enteric cells, which have long been considered as only supporting cells in the enteric nervous system, are involved in maintaining the integrity of the intestinal barrier and play an antiproliferative role [14, 15]. In recent decades, the role of glial enteric cells in health or illness has been reconsidered, as many studies showed that this type of cells is an important link between epithelial cells of the digestive tube, enteric neurons, inflammatory cells and enteroendocrine cells [16].

Previously, changes in the enteric nervous systemin colorectal adenocarcinomawere reported in the sense of decreasingenteric tissue density, both of the Auerbach plexus and the Meissner plexus, but on the other hand an increase in the expression of beta-2 adrenergic receptors with the decrease of tumor differentiation was noticed [4-7, 17,18].

Beta adrenergic receptors are part of the G protein coupled receptor and their activation by adrenaline and norepinephrine causes multiple intracellular signaling pathways including cyclic cAMP 3‘, 5’ adenyl monophosphate, activation of arachidonic acid cascade, adenylate cyclase, protein kinase A - PKA, but also other pathways that may be involved in colorectal tumorigenesis [19, 20]. Adrenergic beta receptors are of three types β1-AR, β2-AR and β3-AR, whereas alpha adrenergic receptors are of 6 α1A, α1B, α1D, α2A, á2B, α2C types [21]. Binding of norepinephrine or epinephrine to beta adrenergic receptors activates G_a from G protein, which in turn induces adenylylatedcyclase to synthesize cyclic 3‘, 5’ adenyl monophosphate-cAMP [143]. The latter may cause various intracellular processes, can initiate the phosphorylation of a wide variety of cellular structures, cellular cytoskeletal proteins, ionic channels, and other effector enzymes, activation that eventually leads to tumor initiation, progression and metastasis [22, 23].

Conclusions

As a final conclusion, we can say that the decrease of the density of glial enteric cells in colorectal cancer proportional with the degree of tumor differentiation and also their inverse correlation with the tumor proliferation index and the expression of adrenergic beta-2 adrenoreceptors can be considered a negative prognostic factor in this type of neoplasm.

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