UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA
DOCTORAL SCHOOL

PhD THESIS

ABSTRACT

IMPLICATIONS OF INFLAMMATION AND REMODELING OF THE ENTERIC GLIAL CELLS IN COLORECTAL CANCER

PhD COORDINATOR:

PROF. UNIV. DR MARIA INCĂU

PhD STUDENT :

PUIU ELENA-ANCA (TÂRTEA)

CRAIOVA 2017

Content

Key words: colorectal adenocarcinoma, enteric glial cells, intratumoral leukocyte infiltrate, tumor proliferation index.

Introduction

Although important progress was made in what the evolution, the pathogenesis of the colorectal cancer and, also, the screening and the diagnostic procedures and the therapeutic methods are concerned, this type of neoplasm remains the third most frequent type of cancer diagnosed in the world and, also, the fourth main cause of death worldwide [1]. There are many molecular mechanisms underlying this neoplasm and blocking the intra/intercellular signalizing pathways had beneficial results, negatively influencing colorectal tumorigenesis, this principle underlies the emergence of molecular targeted therapies. However, this type of neoplasm creates important problems both in terms of morbidity and mortality, thus, things are not yet fully understood and, it is still necessary that more possible pathways involved in the pathogenesis of this type of neoplasm to be studied, in order to discover new therapeutic targets and, also, to reduce the negative statistical indicators of this disease.

I chose this doctoral research topic taking into account, on one hand, the epidemiological data and, on the other hand, the suspicion of involvement in colorectal carcinogenesis of some elements of the peripheral nervous system, the glial cells, together with inflammatory elements.

In accordance with the current rules and principles regarding the drafting of a doctoral thesis my paper contains two parts.

The first part contains three main chapters. In the first one, I reviewed the latest data on colorectal cancer, emphasizing, in the first place, the risk factors and the main elements of the colorectal carcinogenesis, in order to understand the mechanisms by which the enteric glial cells and the inflammatory elements affect colorectal carcinogenesis. In the last two chapters of the first parts, I analyzed the enteric glial cells and the inflammatory elements.

In the second part, I presented my own contributions to the doctoral theme approached. Thus, after I structured the aim and the objectives of the study, I presented the material and the methods that I used, exposing pathological anatomic tissue especially to the multispectral microscopy technique, utilized for the first time in analyzing the expression of the enteric glial cells in colorectal cancer. Subsequent, I realized a clinical and epidemiological study of the patients included in the study, in order to use these data for analyzing the expression of the enteric glial cells, the intratumoral leukocyte infiltrate and the tumor proliferation degree. I also established correlation between the parameters evaluated in patients enrolled in the study, then, I compared the results of my research with the results directly or indirectly referring to the approached theme, results already existing in literature. Finally, I established the conclusions derived from this study's research.

First Part. The state of knowledge

Colorectal cancer (CCR) is the third most frequently diagnosed type of cancer in the world and, also, the fourth main cause of death worldwide, in 2012 more than 1.3 million cases (9.7% of the total number of neoplasms, excluding other skin cancer, excepting melanoma) were recorded and also caused 690 000 deaths (8.5% of the total number of deaths caused by cancer, excluding other skin cancer, excepting melanoma) [1].

Understanding the interaction between the additional factors of the tumor micromedium, such as enteric glial cells or inflammatory cells, with colorectal neoplasm's cells may elucidate many of the pathways of the colorectal neoplasm's pathogenesis.

About the enteric glial cells, considered for a long period of time only playing a supporting role for the enteric neurons, now, it is well known their role in maintaining the homeostasis of the enteric neurons, in the support and the stability of the enteric nervous system from the intestinal wall, in the enteric neurotransmission and, also, it is well known that they represent an important resource for the enzymes involved in the neurotransmitters' synthesis and, nevertheless, their important role in adjusting the functions of the intestinal barrier [2-8]. The studies anteriorly mentioned suggest that the enteric glial cells, through the soluble factors that they synthetize, play an important role in maintaining the intestinal barrier and in controlling cell proliferation, so, affecting their integrity in colorectal adenocarcinoma would represent a favorable element for cell proliferation and metastasis.

Inflammation is unlikely to cause sporadic CCR, because the majority of the immune intratumoral cells are being recruited after the tumor is formed and, in this case, the inflammation does not precede, but follows colorectal tumor initiation [9]. However, after tumor initiation, tumor micromedium recruits inflammatory cells, which may cause the accumulation of additional mutations in the neoplastic cell genome thus, contributing to tumor progression [10 - 13].

Second Part. Own contributions

Aim and objectives

The current study has proposed full and detailed evaluation of the enteric glial cells remodeling and of the inflammatory elements in colorectal cancer, in order to identify possible therapeutic and prognostic targets.

The study was prospectively led and, for achieving the main goal, the following objectives were pursued:

- Creating a database containing the main epidemiological, clinical, histopathological, as well as immunohistochemical parameters of the patients included in this study;
- Identifying and defining the immunohistochemical parameters of the enteric glial cells and of the inflammatory elements, with the aim of subsequently correlating them with the clinical and pathological features of the patients enrolled in the study, in order to evaluate the enteric glial cells remodeling and the intratumoral leukocyte infiltrate;
- Evaluation of cell proliferation, of intratumoral leukocyte infiltrate and of sympathetic nervous system's influence, by analyzing the expression of B2 adrenergic receptors, in order to establish correlations with the enteric glial cells expression;
- Identifying some possible molecular and prognostic targets, with the purpose of applying early and different therapies, depending on the correlation between enteric glial cells remodeling and intratumoral leukocyte infiltrate with sympathetic influences, tumor proliferation degree and also clinical and pathological features of the patients included in the study.

Material and methods

The study was a prospective analytical, descriptive observational one, comprising a total number of 52 patients, diagnosed with colorectal adenocarcinoma, selected over a period of two years (2015-2016). In order to avoid bias, patients were consecutively included in the study.
The cases that were analyzed were from patients, who were admitted to the Gastroenterology Clinic of the Emergency County Hospital of Craiova, where the suspicion of a malignant tumor formation at the colorectal level arose after clinical and imagistic exams were performed. Subsequently, these patients underwent surgical resection of a colorectal segment, in the Surgery Clinic of the same hospital. The biological material, taken during the surgical therapy, was immediately put in formalin solution 10% and, then it was sent to the Pathological Anatomy Laboratory of the Emergency County Hospital of Craiova, where it was initially macroscopically examined, and then it was subjected to processing techniques for microscopic analysis. Fragments of the histological material were further processed in the "Center for Microscopic and Immunological Morphology Studies" of the University of the Medicine and Pharmacy of Craiova, where the immunohistochemical study was performed.

For the acquisition and computerized imaging analysis, I used the optical microscopy method and the multispectral microscopy method.

For the first time in literature, I performed the study of the enteric glial cells remodeling, as well as the study of the intratumoral leukocyte infiltrate by using the multispectral microscopic technique. This is based on the multispectral camera's capacity of transforming an image, obtained by optical microscopy, in a spectral compound and then, of obtaining separate images for each color spectrum of the spectral compound obtained by the camera, by using the soft anteriorly mentioned. This process is highlighted in the following figures, where, initially, we have an image of a lymph node of the Auerbach nervous plexus obtained by optical microscopy (**Figure 1**), then the image obtained by the spectral compound, obtained with the multispectral camera, is presented (**Figure 2**) and finally to have the non-mixed monochromatic spectral images, for each color (**Figure 3, 4**), for each image obtained by optical microscopy a spectral "library" was created.

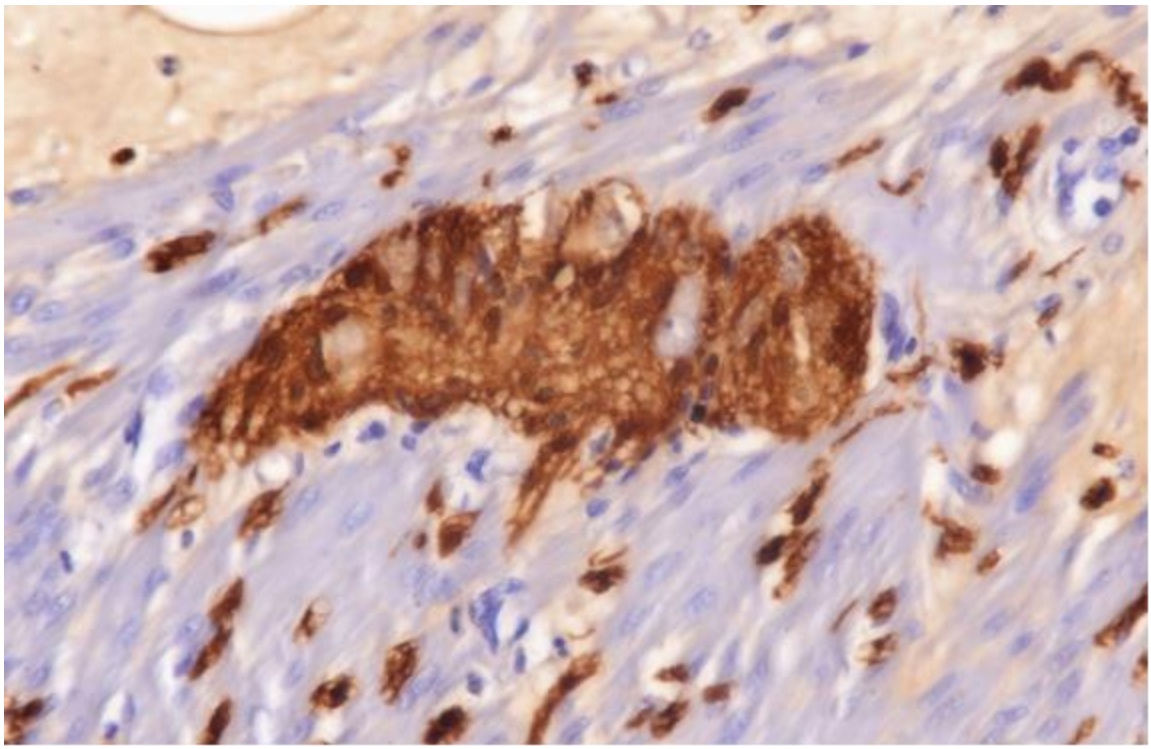

Figure 1. Image of a lymph node from the Auerbach nervous plexus, in optical microscopy, immunomarking S100, 40x.

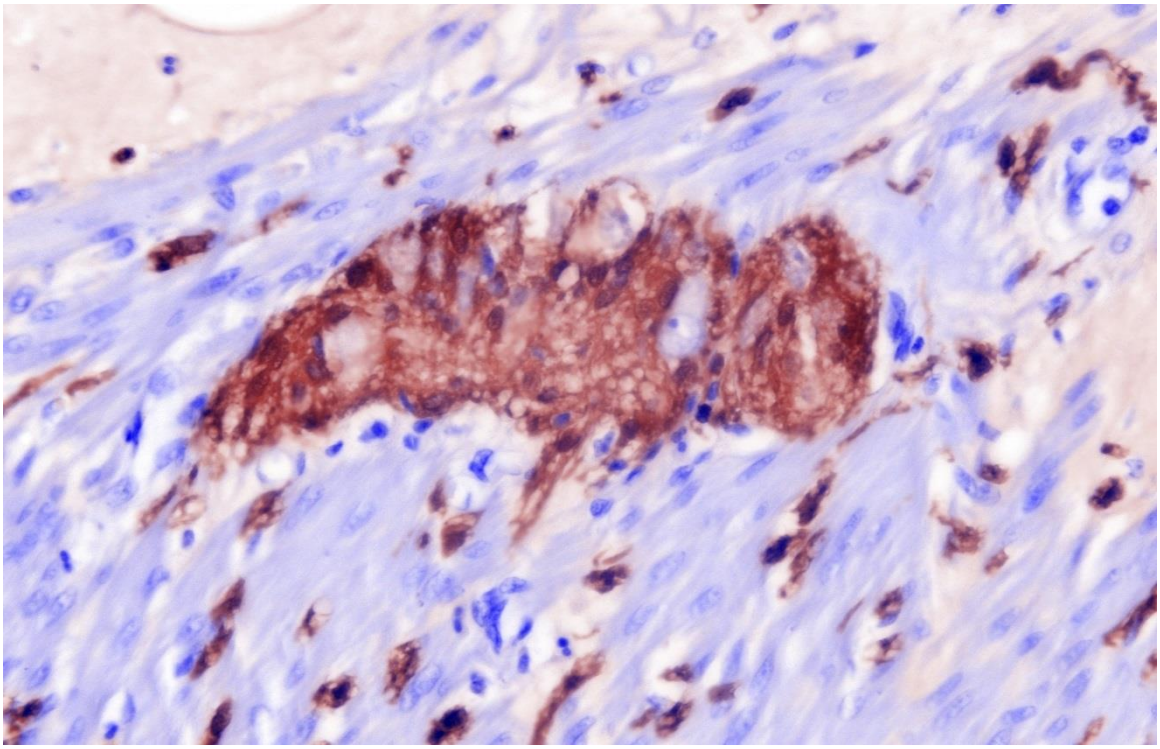

Figure 2. Spectral composite image; mixed colors can be seen: blue for the nuclei and brown for the target antigens, that identify nervous elements (S100 protein), 40x.

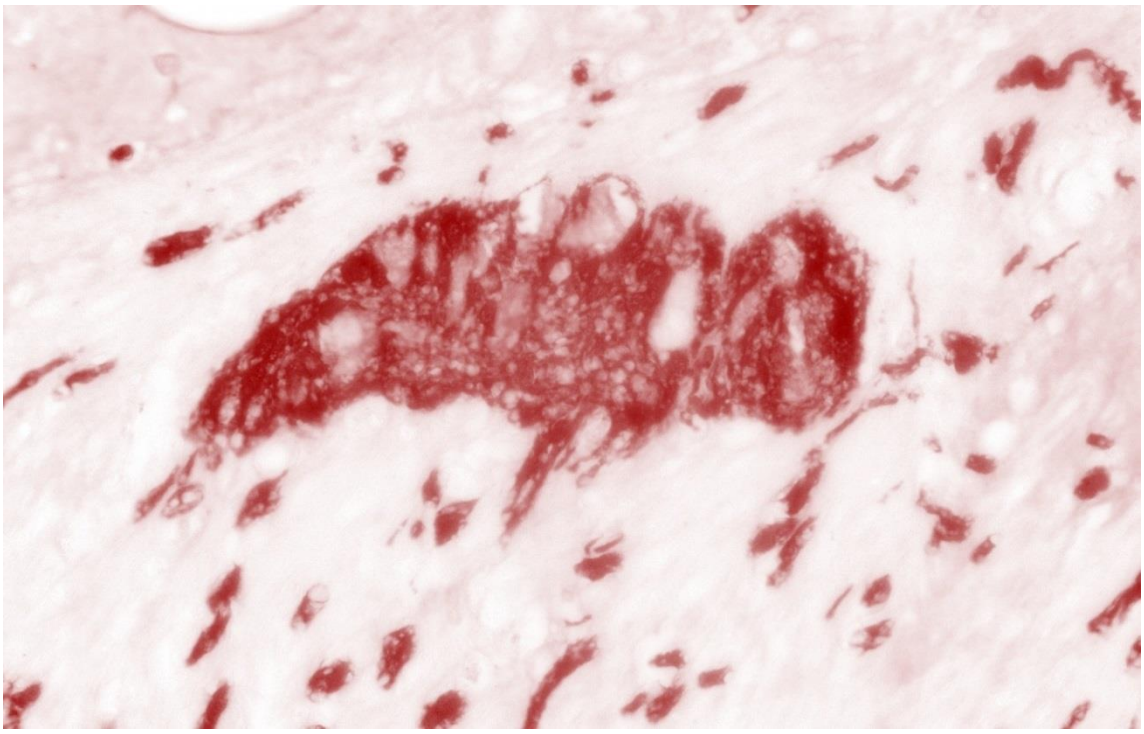

Figure 3. Non-mixed image for the brown spectral color. Only the color for the target antigens, which identify nervous elements can be observed (protein S100), 40x.

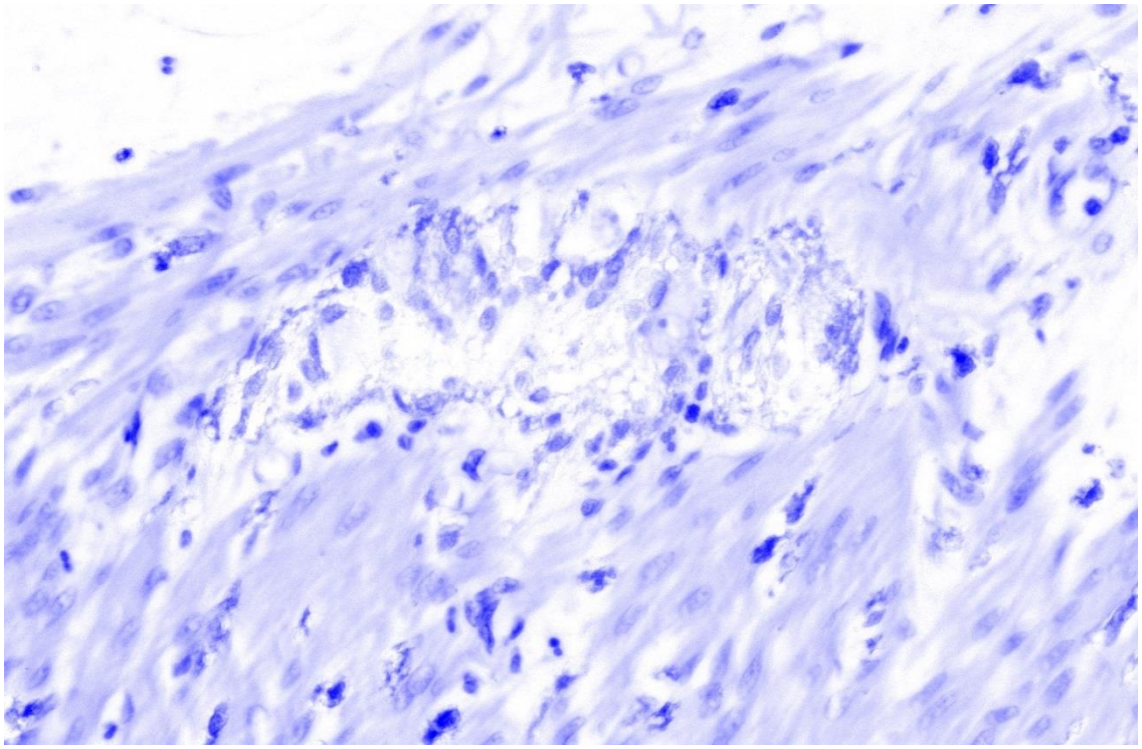

Figure 4. Non-mixed image for the blue color spectrum. Only the color for the nuclei can be observed, 40x.

The main parameters were calculated by using ImagePro Plus AMS 7 software and were represented by the area and the integrated optical density of the color signal that has been selected.

For these parameters and, also, for the proliferation index, numerical data were obtained and were analyzed by using Microsoft Office Excel 2010 (Microsoft Corporation, Redmond, Washington, USA) software and, after the graphic representation, they were exported in SPSS (IBM SPSS Statistics, Version 20.0) software, where, firstly, the mean and the standard deviation for each group were calculated and then the statistical analysis followed.

Results

Assessment of the enteric glial cells, that expressed GFAP, in the enteric nervous system

The percentage area of the total nervous tissue, calculated with S100 immunomarker, was on average 0.121296±0.079121%/mm2, while the percentage area of the enteric glial cells, calculated with GFAP immunomarker, was on average 0.003056±0.001485%/mm2, which meant a percentage of the enteric glial cells of 2.52% of the total nervous tissue.

Subdividing the area occupied by the enteric glial cells, evaluated with GFAP immunomarker, in plexuses, I observed that the percentage area of the glial cells of the Meissner nervous plexus was 0.00013±0.000101%/mm^2 , the percentage area of the glial cells of the Auerbach nervous plexus was 0.002237±0.001562%/mm^2 and, the percentage area of the glial cells of other multiaxial nerve threads with a diameter greater than 20 μm was 0.001074±0.000677%/mm^2, the percentage area of the glial cells of the Auerbach plexus being higher than the percentage areas of the other two categories.

Assessment of the intratumoral leukocyte infiltrate

The intratumoral leukocyte infiltrate was evaluated only intraepithelial, the intratumoral leukocyte infiltrate from the stroma was not taken into account. Immunohistochemically, the intratumoral leukocyte infiltrate was evaluated by using CD45 immunomarker (CLA – common leukocyte antigen), which identifies all leukocytes, calculating for this, both the signal area and the integrated optical density (IOD) in all colorectal cancer's three stages of differentiation, by using multispectral microscopy. I noticed a gradual increase of both the area and of the integrated optical density –IOD, from well differentiated colorectal adenocarcinoma (2104.296 ± 826.0828 μm^2 for area and 259863.0316 ± 106263.5552 for IOD) to moderately differentiated colorectal adenocarcinoma (4450.312 ± 1328 μm^2 for area and 523583.331 ± 151044.9749 for IOD), and respectively to poorly differentiated colorectal adenocarcinoma (7693.812 ± 1840.834 μm^2 for area and 946815.1146 ± 236017.7378 for IOD).

Assessemnt of tumor cells proliferation

The proliferative activity of the colorectal carcinoma, for the patients included in the study, was evaluated with Ki67 monoclonal antibody, calculating for each patient the tumor proliferation index, expressed as a percentage. I noticed that in patients diagnosed with well differentiated adenocarcinoma (G1), tumor proliferation activity was 25.9381 ± 13.5667 %, while in patients with moderately differentiated adenocarcinoma, tumor proliferation activity was 44.2435 ± 16.9495 %, while in patients diagnosed with poorly differentiated adenocarcinoma, tumor proliferation activity recorded the higher rate of 45.2962 ± 29.2526%.

Correlations between the parameters evaluated for the patients included in the study

In what correlations between the area of the enteric glial cells from the whole group of patients included in the study and the tumor proliferation activity a global inverse at the limit correlation was observed (r= -0.438), while, between the area of the enteric glial cells from the whole group of patients included in the study and the tumor leukocyte infiltrate a high global inverse correlation was noticed (r = -0.701). Also, between the area of the enteric glial cells from the whole group of patients included in the study and the expression of β2 adrenoreceptors a high inverse correlation was observed (r = -0.734).

Conclusions

1. Colorectal cancer is the third most frequently diagnosed type of cancer in the world and, also, the fourth main cause of death worldwide.
2. The exact nature of neoplastic colorectal transformation and, also, the tumor progression and metastasis are not yet fully understood, new research in this field is needed.
3. Enteric glial cells, through the soluble factors that they synthesize play an important role in maintaining the intestinal barrier and in controlling cell proliferation, so, their integrity is affected in colorectal adenocarcinoma, as reported in our study and this represents a favorable element for cell proliferation and metastasis.
4. The percentage of the enteric glial cells was 2.52% of the total enteric nervous tissue, for the patients included in our study.
5. Evaluating enteric glial cells in different tumor differentiation stages of colorectal cancer showed that density of these nervous elements is higher in well differentiated colorectal tumors, as opposed to moderately and poorly differentiated colorectal tumors.
6. By analyzing the intratumoral leukocyte infiltrate, we noticed a gradual increase of both the area and the integrated optical density, from well differentiated colorectal tumors to moderately and, respectively, poorly differentiated colorectal tumors.
7. It was noticed that tumor proliferation index increases on average with tumor grading.
8. In moderately differentiated colorectal tumors a high inverse correlation between enteric glial cells and tumor proliferation activity, on one hand, and also between enteric glial cells and intratumoral leukocyte infiltrate, on the other hand, was observed.
9. In poorly differentiated colorectal tumors, an inverse at limit correlation between enteric glial cells and tumor proliferation activity was noticed and a highly significant inverse correlation between enteric glial cells and intratumoral leukocyte infiltrate was observed.
10. Concerning the correlations between the area of the enteric glial cells, from the whole group of patients included in the study and the tumor proliferation activity, a global inverse at limit correlation was noticed, while between the area of the enteric glial cells from the whole group of patients included in the study and the tumor leukocyte infiltrate a highly significant inverse correlation was observed.

The final conclusion is that the decrease of the enteric glial cells in colorectal cancer with tumor differentiation and, also, their inverse variation with tumor proliferation activity and with intratumoral leukocyte infiltrate may represent a negative prognostic factor in this type of cancer. However, the role played by the enteric glial cells in colorectal cancer remains indirectly and further studies are necessary in order to sustain the results of our research.

Bibliography

[1]. Giovannucci EL, Keum N. Epidemiology of Colorectal Cancer. Chapter 12: Epidemiology of Colorectal Cancer. In: Loda M, Mucci LA, Mittelstadt ML, Hemelrijck MV, Cotter MB (eds.) Pathology and Epidemiology of Cancer. Springer International Publishing Switzerland, 2017, 391 – 409.

[2]. Gulbransen BD. Enteric glia. In: Verkhratsky A, Parpura V (eds). Colloquium series on neuroglia in biology and medicine: from physiology to disease. Morgan & Claypool Life Sciences, 2014, 1(2):1–70.

[3]. Gulbransen BD, Sharkey KA. Novel functional roles for enteric glia in the gastrointestinal tract. Nat Rev Gastroenterol Hepatol. 2012 Nov;9(11):625-32.

[4]. Matteoli G. Enteric glial cells: new players in mucosal defence against bacteria? Gut. 2011 Apr;60(4):429-30.

[5]. Neunlist M, Rolli-Derkinderen M, Latorre R, Van Landeghem L, Coron E, Derkinderen P, De Giorgio R. Enteric glial cells: recent developments and future directions. Gastroenterology. 2014 Dec;147(6):1230-7.

[6]. Van Landeghem L, Mahé MM, Teusan R, Léger J, Guisle I, Houlgatte R, Neunlist M. Regulation of intestinal epithelial cells transcriptome by enteric glial cells: impact on intestinal epithelial barrier functions. BMC Genomics. 2009;10:507.

[7]. von Boyen G, Steinkamp M. The role of enteric glia in gut inflammation. Neuron Glia Biol. 2010; 6(4):231-6.

[8]. Yu YB, Li YQ. Enteric glial cells and their role in the intestinal epithelial barrier. World J Gastroenterol. 2014; 20(32):11273-80.

[9]. Terzić J, Grivennikov S, Karin E, Karin M. Inflammation and colon cancer. Gastroenterology. 2010 Jun;138(6):2101-2114.e5.

[10]. Meira LB, Bugni JM, Green SL, Lee CW, Pang B, Borenshtein D, Rickman BH, Rogers AB, Moroski-Erkul CA, McFaline JL, Schauer DB, Dedon PC, Fox JG, Samson LD. DNA damage induced by chronic inflammation contributes to colon carcinogenesis in mice. J Clin Invest. 2008 Jul;118(7):2516-25.

[11]. Wu S, Rhee KJ, Albesiano E, Rabizadeh S, Wu X, Yen HR, Huso DL, Brancati FL, Wick E, McAllister F, Housseau F, Pardoll DM, Sears CL. A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. Nat Med. 2009 Sep;15(9):1016-22.

[12]. Janakiram NB, Rao CV. The role of inflammation in colon cancer. Adv Exp Med Biol. 2014;816:25-52.

[13]. Lasry A, Zinger A, Ben-Neriah Y. Inflammatory networks underlying colorectal cancer. Nat Immunol. 2016 Mar;17(3):230-40.