PhD Thesis
ABSTRACT

NEURO-NEOPLASTIC INTERRELATIONSHIPS IN COLORECTAL ADENOCARCINOMA

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Summary

INTRODUCTION

Colorectal cancer still remains one of the major public health problems, being a frequent neoplasm whose incidence and prevalence have a global upward direction.

I chose this theme because, on one hand, colorectal cancer is still one of the main cancers both as incidence, morbidity and mortality among people from all over the word, and on the other hand, it is desired to replace the classic pattern of disease with the pattern of biopsychosocial disease that includes a more complex approach of both pathophysiological determinism of the diseases and diagnostic and therapeutic algorithm. The interaction between neoplastic cells, vascular endothelium's cells, extracellular matrix, immune system's cells and also other elements, such as nervous elements, was carefully studied recently in order to develop new molecular targeted therapies (example: antiangiogenetic therapies). However, the mechanisms involved in colorectal neoplasm's pathogenesis and evolution are still far from being fully understood.

This study has two main parts. In the first part I analyzed the literature especially regarding the risk factors for colorectal cancer, the main mechanisms involved in colorectal carcinogenesis, intracellular signalizing pathways also involved in colorectal carcinogenesis, and ,also, regarding some data about the enteric nervous system of the digestive tube, and data about certain receptors for the neurotransmitters, such as beta 2 adrenoreceptors for catecholamines, muscarinic M3 receptors for acetylcholine, and TrkA receptors for neurotrophins.

In the second part I actually did the study, and I studied, on one hand, the expression of the receptors, that were mentioned above, in tumor epithelium, and on the other hand, I analyzed the enteric nervous system's changes in colorectal adenocarcinoma. I also established correlations between clinicopathological features and the results obtained in the study, and finally I ended this thesis with discussions and conclusions.

Current state of knowledge

According to the World Health Organization, in 2012, worldwide, colorectal cancer (CCR) represented about 10% of all cancer cases in men, being on the second place for this category, and 9.2% of all cancer cases in women, being on the third place [1].

On January 1st 2016 in The United States of America were about 1.4 million people with CCR, 95% of the survivors patients with CCR were over 60 years (1 391 440), while about 5% of the patients (60 610) were under 60 years old [2].

Moreover, in 2016 a number of 134 490 new CCR cases were diagnosed, the average age at diagnosis was 66 years in men and 70 years in women [2].

Colorectal cancer has many risk factors, many of them overlapping over a genetic predisposition [3]. What is more, CCR represents a multistage carcinogenesis pattern characterized by the occurance of the successive genetic alterations, responsible for the transformation of a normal colic cell in a cancerous one [3]. Many studies of the genetic, somatic and constitutional alterations allowed the identification of numerous colorectal carcinogenesis pathways [3].

Colorectal carcinogenesis is a complex process where the transition normal colic epithelium-adenoma-carcinoma with invasive phenotype is due especially to the successive genetic   alterations that take place in germinal cellular lines, alterations that may be inherited or de novo appeared. Colorectal carcinogenesis has three important mechanisms: chromosomal instability (about 85% of the CCR cases), hypermethylation of the cytosines from CpG islands (about 25% of the CCR cases)   and microsatellites' instability (about 15% of CCR cases) [4-8].

Recent studies confirmed that genetic alteration contributes to colorectal carcinogenesis mainly by five intracellular signalizing pathways. These are: TGF-β, Wnt/APC/β-catenina, p53, RAS/RAF/MAPK and PI3K/AKT/mTOR [9].

Conventionally adenocarcinomas are characterized by forming glands, thus adenocarcinoma is well differentiated if >95% of the tumor contains glands, moderately differentiated if glands are found in a percentage of 50-95%, and poorly differentiated is glands are in a percentage lower than 50%. Moderately differentiated adenocarcinoma is mostly diagnosed (about 70%), while poorly differentiated adenocarcinoma is diagnosed in a percentage of about 20%, and the well differentiated one in a percentage of 10% [10].

Beta adrenoreceptors are coupled with G proteins. Their activation initiates plenty of intracellular signalizing pathways, which include adenylate cyclase, 3',5'-cyclic monophosphate adenylyl-cAMP, A-protein kinase – PKA, the activation of the arachidonic acid cascade, and also other pathways which may be involved in colorectal carcinogenesis [11, 12].

Firstly discovered by Dale in 1914, muscarinic receptors were proved to be involved in carcinogenesis at different levels [13-16].

In the last 20 years, nervous growth factor (NGF) and its receptors, TrkA and p75NTR, were studied in many cancers including colorectal cancer, and the studies suggested that these may be new molecular targets in anticancer therapies [17].

Original contributions

Purpose and objectives of the research

The present paper makes an analysis of the pathophysiological substrate of the colorectal carcinogenesis at the level of neuro-neoplastic interrelationships. So, I have established the following objectives:

- ❖ Making an analytical, prospective, descriptive, observational study for a period of two years, in order to identify some changes of the enteric nervous system and, also, of some receptors of this system's neurotransmitters, which are expressed by the cells of the colorectal neoplasm, changes that may be correlated with different aspects of colorectal carcinogenesis pathogenesis;
- ❖ Identification and definition of morphological parameters which characterize the enteric nervous system, in order to establish some correlations with the clinicopathological features of colorectal neoplasm;
- ❖ Evaluation of the expression of beta-2 adrenoreceptors for catecholamines , M3 muscarinic receptors for acetylcholine and TrkA receptors for neurotrophins in colorectal adenocarcinoma, and establishing correlations with clinicopathological features of the patients included in the study.

Material and methods

I made a prospective analytical observational study from October 2014 to December 2016 including 60 patients. The patients were consecutively included in the study in order to avoid the bias.

Patients were initially diagnosed in the Ist Medical Clinic- Gastroenterology of the Emergency County Hospital of Craiova, using diagnostic criteria, having a suspicion of malignant tumor situated in one of the colorectal segments. Then, patients suffered a potentially curative surgical treatment in the Ist Surgical Clinic of the hospital mentioned above, and from the surgically removed piece, tumor tissue fragments were taken, which represented the objective of this study.

The biological material used for the immunohistochemical studies from this paper, after the histopathological diagnosis was performed in the Pathology Laboratory, was processed and analyzed by using immunohistochemical techniques in the Center for studies of Microscopic Morphology and Immunology of the University of Medicine and Pharmacy of Craiova.

After images' acquisition, regions of interest were defined (ROI-regions of interest) on the sections colored with DAB, constantly based on the same RGB color profile, and these regions of interest (ROI) were used in order to calculate signal area and the integrated optical density (IOD – integrate optical density) by using Image-Pro Plus AMS software. Then, data were exported and graphically represented in 2010 Excel Microsoft Office ((Microsoft Corporation, Redmond, Washington, USA) and analyzed by using SPSS software (IBM SPSS Statistics, Version 20.0).

RESULTS

The expression of B2A receptors

For the first time in literature, by using unmixed multi-spectral microscopy technique, we made the characterization of the morphological expression of B2A receptors.

Besides a poorly diffused cytoplasmic reaction in normal colonic mucosa, B2A receptors presented a granular pattern in the enterocytes' cytoplasm, above the

nuclei, towards the coverage luminal epithelium. In goblet cells the signal was more frequently localized under the nuclei. Stromal cells also showed a granular pattern in the cytoplasmic compartment (Figure 1.A-D).
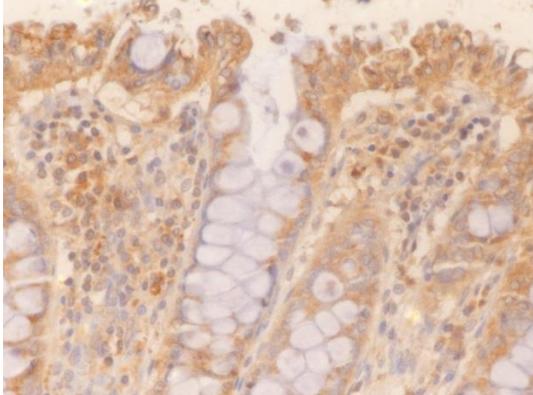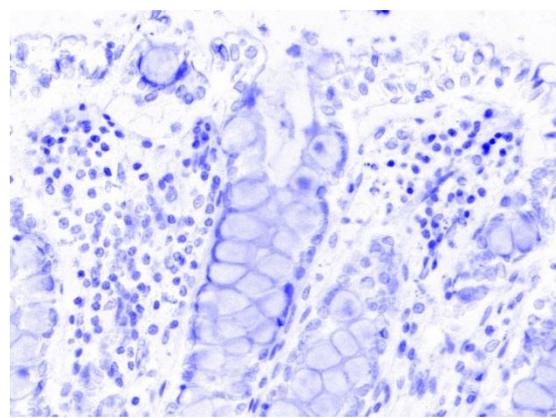

Figure 1.A. Example of spectral unmixed for seriate images of a slide. A-Immunostained with DAB for B2A receptors, RGB color profile, *X*400.
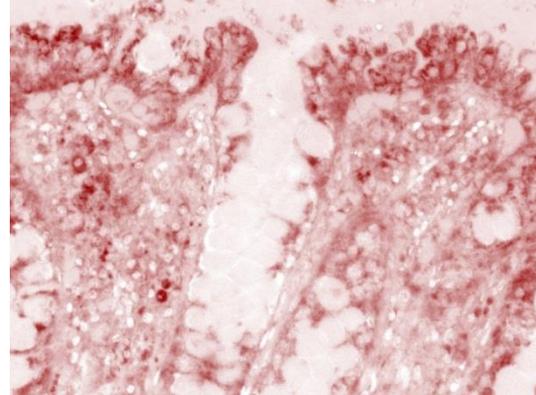
Figure 1.C. Unmixed image, pure DAB signal for B2A receptors in brown, *X*400.
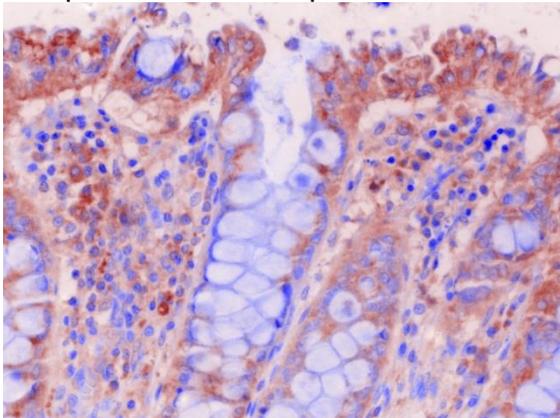
Figure 1.B. Mixed image, overlayed blue and brown colors, hematoxylin-eosin contrast image, *X*400.

Figure 1.D. Example of unmixed spectral seriate images of a slide. D-unmixed image, pure signal hematoxylin-eosin in blue, *X*400.

In well differentiated adenocarcinomas, most of the signal was still localized on the luminal part of the tumor cells, although a granular pattern of the signal in the basal pole of the cells was identified.

In moderately differentiated adenocarcinomas, the signal seemed to lose the granular aspect, becoming more diffuse and more intense in the epithelial cells.

In poorly differentiated adenocarcinomas, the signal's intensity was higher in tumor cells' cytoplasm and on a generally intense background very intense signal bridges around the nucleus could be identified.

In what the expression of B2A receptors, from the total tissue belonging to each patient included in the study, is concerned, I observed a gradual growth of both the area and the IOD from the normal tissue to different degrees of differentiation of colorectal adenocarcinoma, G1, G2, and G3.

I noticed a statistically significant difference when analyzing ANOVA variance followed by Bonfferoni post-hoc test between the signal's expression in the normal colic tissue and the signal's expression in G2, G3 (*p=0.000*) and also between G1 and G2, G3 (*p=0.000*) both for the area and IOD of B2A.

Statistically significant differences were not noticed between the average area and IOD for B2A receptors from the stroma of the normal colic mucosa and colorectal adenocarcinoma's different degrees of differentiation.

Both the area and IOD of B2A receptors were statistically significant with tumor size, tumor invasion and metastases in regional lymph nodes, while in what patients' gender, tumor's location and macroscopic aspect statistically significant correlations were not highlighted. In what patients' age is concerned, there was no statistically significant difference for B2A area between the group of patients aged up 60 years and the group of patients aged over this cut-off, while for IOD of B2A there was a significantly statistical difference (*p=0.018*).

The expression of M3 muscarinic receptors

This type of receptors for acetylcholine was expressed both in the normal colic tissue and in the different degrees of tumor differentiation. A growth of both the area and IOD for this type of receptors with the tumor grading was observed.

The expression of TrkA receptors for neurotrophines

This type of receptors for neurotrophines was expressed both in the normal colic tissue and in different tumor degrees of differentiation, but with a significant reduction of the signal in the poorly differentiated tissue.

Changes of the enteric nervous system in colorectal adenocarcinoma

I analyzed the submucosal plexus Meissner, the myenteric plxus Auerbach and the nervous intratumoral plexuses, as well as the multiaxonal nervous fibers larger than 20 μm, which could not be included neither in the Auerbach plexus nor in the Meissner plexus for the S100 immunomarker's study.

Total nervous tissue's density expressed by percentage area had the smallest values in G1 tumors (0.129±0.052%) followed by normal colic tissue (0.184±0.041%), G2 (0.355±0.131%) and G3 (0.264±0.172%).
Percentage area of the Auerbach plexus had a decrease between the normal colic tissue (0.136±0.039%) and G1 (0.067±0.043%), G2 (0.094±0.078%) and G3 (0.241±0.146%).

However, the relative area of the Meissner plexus had the same decrease as the Auerbach plexus, from the maximum value recorded in the normal colic mucosa (0.034±0.017%), to G1 (0.009±0.005%), G2 (0.012±0.013%), and G3 (0.002±0.003%).

Conclusions

1. Colorectal adenocarcinoma still remains one of the main public health problems, being a frequent neoplasm whose incidence and prevalence have a global upward direction worldwide.
2. Mechanisms involved in the pathogenesis and the evolution of colorectal neoplasm are still far from being fully understood.
3. In the pathogenesis and the evolution of colorectal neoplasm, nervous elements found in the tumor microenvironment seem to have an important role.
4. The density of the total nervous elements is higher in moderately and poorly differentiated colorectal adenocarcinomas compared with those well differentiated and normal colic mucosa.
5. Together with the decrease of the tumor differentiation or with the increasing of the tumor grading I noticed a decrease of the percentage area both for the Auerbach and Meissner plexuses, and, on the other hand, I noticed an increase of the percentage area of other nervous elements, which could not be included neither in the Auerbach nor in the Meissner plexuses.
6. B2A receptors were present in stromal cells and also in the nerves and nerve ganglia.
7. For the first time in literature, using unmixed multi-spectral microscopy technique, I characterized the morphological expression of B2A receptors in colorectal adenocarcinoma.
8. In what the expression of B2A receptors in the total tissue from each patient is concerned, I noticed a gradual increase of both the area and IOD from the normal tissue to different differentiation degrees of the colorectal adenocarcinoma, G1, G2 and G3.
9. The expression of B2A receptors only in the glandular epithelium had a gradual increase like the expression of B2A receptors in the total tissue from the normal colic tissue to G1, G2 and G3.
10. On average there was no difference between the B2a receptors' signal from the stroma of different tumor stages and the control group.
11. Both the area of B2A receptors and IOD were statistically significant correlated with tumor size, tumor invasion and metastases in the regional lymphatic nodes, while, in what patients' gender, tumor location and macroscopic aspect are concerned, statistically significant differences were not noticed.
12. In what patients' age is concerned, there was no statistically significant difference for the area of B2A in the group of patients aged under 60 years and the group of patients aged over this cut-off, while for IOD of B2A there was a statistically significant difference.
13. I noticed an inverse moderate to strong correlation between the expression of B2A receptors in tumor epithelium and the density of Auerbach and Meissner plexuses with the tumor grading.
14. Although nervous elements are components of the stroma, there was no significant correlation between the stromal or total expression of B2A in the above mentioned plexuses.
15. I observed a positive correlation between the expression of B2A receptors in normal peritumoral tissue in women, aged over 50 years, tumor size over 5 cm,

tumor invasion T_{3-4} and regional lymph metastasis $N_{\geq 2}$. In what tumor location is concerned, there is no statistically significant difference.

16. By analyzing the expression of B2A receptors in the peritumoral tissue depending on the tumor differentiation degree, I noticed a statistically significant difference between the expression of B2A receptors from G1, G2 and their expression in G3.

17. M3 receptors for acetylcholine and TrkA receptors for neurotrophins were expressed both in the normal colic tissue and ,also, in different tumor differentiation degrees, in biological samples from three patients analyzed in this paper.

18. The expression of M3 receptors increased together with tumor grading.

19. TrkA receptors had an increase of their expression only in G1 and G2 in comparison with normal tissue, while in poorly differentiated tumor tissue a significant reduction of the signal was recorded.

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