Neuro-neoplastic interrelationships in colorectal level – immunohistochemical aspect in three cases and review of the literature

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Abstract
Colorectal cancer represents a severe public health issue. Recent studies have shown the essential role played by nerves and their neurotransmitters in tumor initiation and progression. The aim of this study is to assess the expression of beta 2-adrenergic receptors (β2A) for adrenaline and noradrenaline, and the expression of M3 muscarinic receptors (M3R) for acetylcholine (neurotransmitters produced and released by sympathetic and parasympathetic afferents of the digestive tract and also by the enteric nervous system) in different tumor gradings of colorectal adenocarcinoma, and also the tropomyosin receptor kinase A (TrkA) for the nerve growth factor produced by the cells of colorectal adenocarcinoma. Beta 2-adrenergic receptors were expressed both in normal colic tissue and in the tumor tissues, from the three patients included in the study. It was observed that both area and integrated optical density (IOD) are more elevated for this type of receptor in tumor tissues than in normal colic tissue. For the M3 muscarinic receptors, similarly to beta 2-adrenergic receptors, it was observed a growth both of the area and of the IOD with the tumor grading. The presence of TrkA receptors was also observed both in the normal colic mucosa and in the tumor tissues examined, but with a significant reduction of the signal in the poorly differentiated tumor tissue. Understanding the neurobiology of cancer in this context becomes necessary for establishing much more complex and targeted molecular targeted therapies.

Keywords: colorectal cancer, beta 2-adrenergic receptors, M3 muscarinic receptors, tropomyosin receptor kinase A.

Introduction
Colorectal cancer currently remains one of the main public health problems. According to World Health Organization, in 2012, worldwide, it is the third type of cancer for men (746,000 cases, representing 10% of the total cases of cancer for men) and the second type of cancer which affects women (614,000 cases, representing 9.2% of the total cases of cancer in women) [1]. In what mortality is concerned, colorectal cancer represents the fourth cause of death from cancer in the world (694,000 deaths, 8.5% from the total deaths caused by cancer) [1]. Colon and rectum cancer statistics is the same due to the existence of a high percentage of misclassification of the two pathologies [2]. However, the pathogenesis and the evolution of colorectal cancer still remain incompletely elucidated, that is why the study of some factors, which can be correlated with the initiation and the development of colorectal cancer, is extremely important [3].
the tumor cells can also influence the nervous elements through the production of neurotrophic growth factors, which may be implicated even in the neoplastic process where they are being secreted [4].

Furthermore, a study published in 2014 by Zhao et al. showed that surgical or pharmacological denervation of the stomach by vagotomy, or local injection of botulinum toxin type A on mice models of gastric cancer, reduced, on one hand, the incidence and the progression of cancer but only in the segment where denervation was made, and on the other hand, the effects of systemic chemotherapy were increased and the survival of the animals included in the study was prolonged [5]. This study also showed that in patients with gastric cancer, the tumor stage is positively correlated with the neural density, and that pharmacological inhibition or genetic deletion of M3 muscarinic receptors suppressed gastric tumorigenesis [5].

The aim of this study is to present the expression of beta 2-adrenergic ($\beta_2$A) receptors for adrenaline and noradrenaline, and the expression of M3 muscarinic receptors (M3R) for acetylcholine in different tumor gradings of colorectal adenocarcinoma, and also the troponymosin receptor kinase A (TrkA) for the nerve growth factor produced by the cells of colorectal cancer.

Materials and Methods

We analyzed specimens from three patients who had undergone surgical resection for primary colorectal cancer in the 1st Surgery Clinic of the Emergency County Hospital, Craiova, Romania, during 2016. For control, we analyzed a segment of tissue belonging to a patient who was diagnosed with intestinal obstruction and then had a surgery that required the excision of a segment of the colon. This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the University of Medicine and Pharmacy of Craiova with the registration No. 53/19.05.2016. All patients provided written acceptance and informed consent.

The immunohistochemical study was done on seriate slides following the Hematoxylin and Eosin (HE) staining, after diagnostic and grading. Paraffin blocks were cut using a rotary microtome HM350 (Thermo Fischer Scientific, Waltham, MA, USA), which was equipped with a system of transferring the sections on a water bath (Section Transfer System) and a Peltier cooling module. The histological sections were taken on poly-L-lysine covered slides and sections (overnight) in a fridge at 4°C. In our study, we used the following primary antibodies: rabbit anti-muscarinic acetylcholine receptor M3/CHRM3 antibody (Novus Biologicals, UK, 1:20 dilution), rabbit anti-beta 2-adrenergic receptor/ADRB2 antibody (Novus Biologicals, UK, 1:100 dilution), rabbit anti-TrkA antibody (Novus Biologicals, UK, 1:20 dilution) and rabbit anti-S100-protein 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 after washing in PBS (three baths, 5 minutes each), the sections were incubated with the Streptavidin–HRP (Horseradish peroxidase) 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. At the end, the sections were counterstained in Mayer’s Hematoxylin, dehydrated in increasing alcohols, cleared in xylene and mounted with DPX (Fluka).

Slides were viewed on a Nikon 55i (Apidrag, Bucharest, Romania), microscope equipped with a 5 megapixel color cooled CCD camera and the Image ProPlus AMS 7 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, and these ROIs were utilized to calculate signal area and 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 analyzed using SPSS software (IBM SPSS Statistics, ver. 20.0). The analysis of the variance was calculated (ANOVA – analysis of variance) 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 Bonferroni post hoc test, with a level of statistical significance for $p<0.05$ and high statistical significance for $p<0.001$.

Results

All the three patients included in our study were males, as well as the control patient from which we have obtained unaffected colon tissue. A positive staining of $\beta_2$A receptors for adrenaline and noradrenaline, of M3R for acetylcholine (neurotransmitters produced and released by sympathetic and parasympathetic afferents of the digestive tract and also by the enteric nervous system, nervous elements shown in Figures 1–3) and of TrkA for the nerve growth factor produced by the cells of colorectal cancer, was observed in all tissue samples.

The first patient included in the study was 49 years old; he had a right hemicolectomy with a surgical resected piece of 23 cm diameter. The macroscopic examination revealed a tumor of 7 cm diameter, with a vegetant form. A positive staining of $\beta_2$A receptors for adrenaline and noradrenaline, of M3R for acetylcholine receptor M3/CHRM3 antibody (Novus Biologicals, UK, 1:100 dilution), rabbit anti-TrkA antibody (Novus Biologicals, UK, 1:20 dilution) and rabbit anti-S100-protein antibody (Novus Biologicals, UK, 1:100 dilution).
diagnosis was a well-differentiated adenocarcinoma (G1) with mucinous areas less than 20%, moderate polymorphic inflammatory reaction with necrotic areas and invasion of the muscular tunica (T2), without metastasis in the four nodes examined (pT2N0Mx).

The second patient included in the study was 65 years old. He had a left hemicolectomy with a surgical resected fragment with a diameter of 11 cm. The macroscopic examination revealed a tumor of 5 cm diameter, with a circumferential, stenosing aspect. Histopathological diagnosis was a moderately differentiated adenocarcinoma (G2) with the invasion of the wall into adjacent adipose tissue, with necrotic areas, important inflammatory reaction with microabscesses and with the presence of reactive lymphoid follicles with vascular invasion being present. The epiploon did not show any macroscopic involvement. The third patient included in the study was 57 years old. He had a right hemicolectomy with a surgical resected piece with a diameter of 62 cm. Macroscopic examination showed a whitish tumor of 3 cm diameter, with a circumferential, stenosing aspect. Histopathological diagnosis was of a poorly differentiated adenocarcinoma (G3), with the invasion of the whole wall and of the adipose tissue, with necrotic areas, reduced chronic inflammatory reaction, with the presence of vascular and perineural invasion, with metastasis in six of the 13 lymphatic regional nodes examined (pT3N2Mx).

Beta 2-adrenoreceptors were expressed both in normal colic tissue and in tumor tissues examined, tissues belonging to the three patients included in the study (Figures 4–7). It was observed that both signal area and integrated optical density (IOD) are more elevated for this type of receptor in tumor tissues than in normal colic tissue, observing a growth between the two performed measurements with the degree of histopathological differentiation (Figures 8 and 9). It was also observed a better signal expression of this type of receptor in the cytoplasmic regions both for normal colic cells of the mucosa and for the cells of the colorectal adenocarcinoma. The results of the ANOVA testing followed by Bonferroni post hoc analysis between the expression of the β2-adrenoreceptors in normal colic mucosa and in different gradings of colorectal adenocarcinoma are illustrated in Table 1 for area and in Table 2 for IOD.

M3 muscarinic receptors for acetylcholine were also expressed both in the normal colic tissue as well as in the tumor tissues examined (Figures 10–13). Similarly, to β2-adrenergic receptors, it was observed a growth of both the area and of the integrated optical density (IOD) for this type of receptor with the tumor grading (Figures 14 and 15). As in the first case, it was observed a distribution of the signal especially in the cytoplasmic fields. The result of the t-testing between the expression of M3 muscarinic receptors from the normal colic mucosa and from the tumors included in the study was statistically significant both for the signal area of this type of receptor, and also for the IOD. The results of the ANOVA testing followed by Bonferroni post hoc test between expression of the M3 muscarinic receptors in normal colic mucosa and in different gradings of colorectal adenocarcinoma are illustrated in Table 3 for area and in Table 4 for IOD.

As for TrkA receptors for neurotrophins, their presence was also observed both in the normal colic mucosa and in the tumor tissues examined, but with a significant reduction of the signal in the poorly differentiated tumor tissue (Figures 16–19). For this type of receptors, a growth of the two parameters that we measured was observed (area and IOD) only in the well and moderately differentiated tumor tissue compared with the normal colic tissue (Figures 20 and 21). The results of the ANOVA testing followed by Bonferroni post hoc test between expression of TrkA receptors in normal colic mucosa and in different gradings of colorectal adenocarcinoma are illustrated in Table 5 for area and in Table 6 for IOD.

Nevertheless, in the normal colic tissue the presence of the signal was observed especially in basal glands of the colic mucosa, in the cytoplasmic areas of the cells, for both M3 muscarinic receptors and TrkA receptors (Figures 22 and 23).

Table 1 – The results of the ANOVA test followed by Bonferroni post hoc test between expression of the β2-adrenoreceptors (β2A) in normal colic mucosa and in different gradings of colorectal adenocarcinoma for area

<table>
<thead>
<tr>
<th>(I) Tissue</th>
<th>(J) Tissue</th>
<th>Sig.*</th>
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</thead>
<tbody>
<tr>
<td>G1</td>
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<tr>
<td></td>
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<td>Normal tissue</td>
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</tbody>
</table>

*Adjustment for multiple comparisons: Bonferroni.

Table 2 – The results of the ANOVA test followed by Bonferroni post hoc test between expression of the β2-adrenoreceptors (β2A) in normal colic mucosa and in different gradings of colorectal adenocarcinoma for integrated optical density (IOD)

<table>
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<tr>
<th>(I) Tissue</th>
<th>(J) Tissue</th>
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<td>G2</td>
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<tr>
<td>Normal tissue</td>
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</table>

*Adjustment for multiple comparisons: Bonferroni.
Table 3 – The results of the ANOVA test followed by Bonferroni post hoc test between expression of the M3 muscarinic receptors (M3R) in normal colic mucosa and in different gradings of colorectal adenocarcinoma for area

<table>
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<th>Pairwise comparisons</th>
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<th>Sig.*</th>
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<tbody>
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*Adjustment for multiple comparisons: Bonferroni.

Table 4 – The results of the ANOVA test followed by Bonferroni post hoc test between expression of the M3 muscarinic receptors (M3R) in normal colic mucosa and in different gradings of colorectal adenocarcinoma for integrated optical density (IOD)

<table>
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<td>(J) Tissue</td>
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</tr>
<tr>
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<td>Normal tissue</td>
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*Adjustment for multiple comparisons: Bonferroni.

Table 5 – The results of the ANOVA test followed by Bonferroni post hoc test between expression of the TrkA receptors in normal colic mucosa and in different gradings of colorectal adenocarcinoma for area

<table>
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<th>Sig.*</th>
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</thead>
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<td>(J) Tissue</td>
<td></td>
</tr>
<tr>
<td>Normal tissue</td>
<td>G1</td>
<td>.001</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>G2</td>
<td>.166</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>G3</td>
<td>.086</td>
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</table>

*Adjustment for multiple comparisons: Bonferroni.

Table 6 – The results of the ANOVA test followed by Bonferroni post hoc test between expression of the TrkA receptors in normal colic mucosa and in different gradings of colorectal adenocarcinoma for integrated optical density (IOD)

<table>
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<th>Dependent variable IOD_TrkA</th>
<th>Sig.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>(I) Tissue</td>
<td>(J) Tissue</td>
<td></td>
</tr>
<tr>
<td>Normal tissue</td>
<td>G1</td>
<td>.000</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>G2</td>
<td>.133</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>G3</td>
<td>.030</td>
</tr>
</tbody>
</table>

*Adjustment for multiple comparisons: Bonferroni.

Figure 1 – Sympathetic/parasympathetic afference in adjacent adipose tissue of the colon labeled with anti-S-100 protein antibody (×200).

Figure 2 – Auerbach plexus between the longitudinal and circular layers of muscularis externa of the colon wall, labeled with anti-S-100 protein antibody (×200).
Figure 3 – Meissner’s plexus between the muscularis mucosae and the mucous membrane of the colon wall, labeled with anti-S-100 protein antibody (×200).

Figure 4 – Normal colic mucosa immunolabeled for β2-adrenoreceptors (×200).

Figure 5 – Well-differentiated adenocarcinoma immunolabeled for β2-adrenoreceptors (×200).

Figure 6 – Moderately differentiated adenocarcinoma immunolabeled for β2-adrenoreceptors (×200).

Figure 7 – Poorly differentiated adenocarcinoma immunolabeled for β2-adrenoreceptors (×200).
Figure 8 – Area of the β2-adrenoreceptors expression in normal colic mucosa and in different colorectal adenocarcinoma’s tumor grading.

Figure 9 – Integrated optical density (IOD) of the β2-adrenoreceptors expression in normal colic mucosa and in different colorectal adenocarcinoma’s tumor grading.

Figure 10 – Normal colic mucosa immunolabeled for M3 muscarinic receptors (×200).

Figure 11 – Well-differentiated adenocarcinoma immunolabeled for M3 muscarinic receptors (×200).

Figure 12 – Moderately differentiated adenocarcinoma immunolabeled for M3 muscarinic receptors (×200).

Figure 13 – Poorly differentiated adenocarcinoma immunolabeled for M3 muscarinic receptors (×200).
Figure 14 – Area of the M3 muscarinic receptors expression in normal colic mucosa and in different colorectal adenocarcinoma’s tumor grading.

Figure 15 – Integrated optical density (IOD) of the M3 muscarinic receptors expression in normal colic mucosa and in different colorectal adenocarcinoma’s tumor grading.

Figure 16 – Normal colic mucosa immunolabeled for TrkA receptors (×200).

Figure 17 – Well-differentiated adenocarcinoma immunolabeled for TrkA receptors (×200).

Figure 18 – Moderately differentiated adenocarcinoma immunolabeled for TrkA receptors (×200).

Figure 19 – Poorly differentiated adenocarcinoma immunolabeled for TrkA receptors (×200).
The results of the analysis of the β2-adrenergic receptors, M3 muscarinic receptors and TrkA receptors in colorectal cancer are sustained by many recent studies, which tried to elucidate the role played by the nervous system’s neurotransmitters via the receptors expressed by the colorectal cancer’s cells.

Some neurotransmitters of the sympathetic and parasympathetic nervous system and also of the enteric nervous system serve to stimulate the locomotor activity of the cells. Treating the cellular cultures from the colon adenocarcinoma with norepinephrine caused a rise of the locomotor activity from 25% to 65%. The administration of a nonselective β-blocker (propranolol) caused a reduction of the locomotor activity induced by norepinephrine, while administration of a β1 blocker (atenolol) influenced this process [6]. This study pointed, on one hand, the implication of norepinephrine in the neoplastic process and, on the other hand, highlighted the role of β2-adrenergic receptors in this process. β2-Adrenergic receptors are G protein-coupled receptors. Their activation initiates plenty of intracellular signalizing pathways, which include adenylate cyclase, cyclic adenosine monophosphate (cAMP), protein kinase A (PKA), cAMP-response element binding (CREB), signalizing pathways, which can boost the expression of epidermal growth factor receptor (EGFR), the activation of the arachidonic acid cascade, but also other pathways, which can be implicated in colorectal carcinogenesis [10–13]. These types of receptors have also been studied in other types of cancer such as cancers of: pancreas [14, 15], ovary [16, 17], breast [18, 19], prostate [20–22], stomach [23, 24], lung [25–27], etc. The most recent study of the β2-adrenergic receptors’ implication in colorectal carcinoma showed that these are not correlated with the tumor differentiation degrees, but they have a greater expression in the neoplastic cells than in normal colic mucosa [28]. On the other hand,
selective blocking of these receptors cause a tumor growth reduction both in vivo on HT-29 xenograft and in vitro on colorectal cancer (CRC) cell lines [28].

Another neurotransmitter, acetylcholine, was described for the first time by Dale, the one that highlighted in 1914 its roles and functions, and that together with Professor Otto Loewi took the Nobel Prize in 1936 [29]. The neurotransmitter role of acetylcholine is mediated via muscarinic and/or nicotinic receptors [30]. Although, traditionally acetylcholine had only the role of neurotransmitter, recent studies revealed also other roles. Thus, acetylcholine can be produced and released not only by neuronal cells’ terminations but also by other normal and neoplastic cells, other than neuronal [31]. Beside the fact that it can be also produced by non-neuronal cells, acetylcholine may have many roles in these cells: in epidermis’ cells, it may cause apoptosis, directed migration, it may decrease random migration [32], it is involved also in other anti-inflammatory effects [34] and it can be involved in the regulation of antibody production [35]; in endothelial cells, it causes angiogenesis [36] and the release of NO [37]. Acetylcholine may also have many roles in neoplastic cells via muscarinic and nicotinic receptors, on which it acts. Muscarinic receptors are part of the great family of receptors coupled with G proteins and they are divided in five subtypes (M1–M5) with are encoded by different genes, receptors that mediate distinct cytoplasmic signaling pathways implicated in various cellular functions, as shown above [38, 39]. This type of receptors have a similar structure made by three extracellular loops, the intracellular loops and seven trans-membrane helices [38, 40]. It has been shown that selective inhibition of M2 muscarinic receptors with Methoctramine selective antagonist causes the decrease of cellular proliferation in non-small cells in lung cancer, both in vitro and in vivo [41]. Also, in lung cancer, it was shown that the activation of M3 muscarinic receptors increases the production of interleukin-8 (IL-8) and induces the activation of EGFR, phosphoinositide 3-kinase (PI3K) and protein kinase B (PKB, Akt), having as a result the proliferation, the invasion and the metastasis of this type of cancer [42, 43]. This type of receptors were also studied in gastric cancer, where, on one hand, it was shown that the activation of M3R induced by acetylcholine causes the neoplasms’ proliferation, and the overexpression of this receptor is correlated with cancer stage and with lymph node metastasis, on the other hand, the use of gene therapy with small hairpin RNA for this receptor inhibited cell proliferation in gastric cancer [44]. Also, M3 receptors are involved in other types of cancer such as pancreatic cancer, skin cancer, breast cancer, ovarian cancer, prostate cancer and brain cancer [45]. In colorectal cancer, M3 muscarinic receptors may activate multiple ways involved in the initiation, proliferation and tumor metastasis. It is well known that EGFR mediates intracellular signaling by activating mitogen-activated protein kinase (MAPK) and PI3K signaling cascades [46]. Using human colon cancer cell lines (H508 cells), Cheng et al. showed that acetylcholine (via M3R with transactivation of EGFR) and epidermal growth factor (EGF) cause the phosphorylation of MAPK, leaving as a result the stimulation of cellular proliferation in these types of neoplastic cellular lines [47, 48]. Also, by using these cellular lines was shown that acetylcholine, by inducing gene transcription, causes the transcription of messenger RNA (mRNA) and of proteins for the extracellular matrix metalloproteinases MMP-1, MMP-7, MMP-10, resulting in cell proliferation, migration and invasion in colorectal adenocarcinoma [48]. By using in vivo models, on which genetic ablation of M3 muscarinic receptors was performed, it was observed an attenuation of the epithelial cells’ proliferation, an attenuation of the decreasing number of adenocarcinomas in mouse colon with about 65%, which suggested the implication of this type of receptors in tumor initiation and promotion [49, 50].

In 1950, Levi-Montalcini & Cohen were the first who described the nerve growth factor, the first from the list of neurotrophic factors, and for their discovery, they received in 1986 the Nobel Prize [51]. Subsequent other neurotrophins such as brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) were discovered in vertebrates. These neurotrophins have many roles in the process of development of the nervous system, roles such as differentiation, migration, proliferation, synaptic plasticity, survival and apoptosis, but they also participate in the development and functioning of other human body’s system such as cardiovascular system [52–54]. Neurotrophins act on the distinct classes of receptors: the tropomyosin receptor kinase (Trk) family, which includes TrkA, TrkB, TrkC, p75 neurotrophin receptor and sortilin [51]. These neurotrophins and their receptors, especially tropomyosin receptor kinase family are increasingly reported in some cancers such as: colorectal cancer, gastric cancer, pancreatic cancer, breast cancer, ovarian cancer and also other types of cancer [55–60]. Different types of tyrosin kinase receptors are involved in tumor progression in various types of cancer and some of them are included in the therapeutic targets in clinical practice. Normally, the stimulation of neurotrophins’ receptors activates Ras signaling pathway, which interacts with MAPK, with PI3K and with phospholipase-C (PLC) [61, 62].

All these pathways of intracellular signaling are part of the development of physiological processes controlled by neurotrophins, especially in the nervous system. Some of them may also be implicated in carcinogenesis.

In our study, the neuro-neoplastic interrelationships at the colorectal level cannot be generalized because they were studied only on the three patients, which had been diagnosed with colorectal adenocarcinoma, each one of them with a various histopathological degree of differentiation. Literature data support our study, bringing into discussion a better understanding of the colorectal neoplastic process, but plenty of questions and perspectives are being generated by the discovery of nerve involvement in colorectal cancer.
Conclusions

β2-Adrenergic receptors for adrenaline and noradrenaline, M3 muscarinic receptors for acetylcholine and TrkA receptors for neurotrophins are present in the normal colic mucosa and also in the cells of colorectal adenocarcinoma, a thing that suggests that both the nervous system, via the neurotransmitters it releases, exerts its influence on colorectal neoplasm via the above-mentioned receptors and which are expressed by colorectal adenocarcinoma cells, but also the cells of colorectal neoplasm may interact with nervous elements via the neurotrophins they secrete. On one hand, the role played by the neurons in stimulating cancer cells’ growth and metastasis is assessed. On the other hand, the understanding of the neurobiology of cancer in this context becomes necessary for establishing the much more complex molecular targeted therapies, which can interfere and can block the initiation, the development and the neoplastic metastasis process at the colorectal level. Studies with certain therapies, such as β-adrenergic blockers, used for treating some cardiovascular disease, can be performed in order to demonstrate their efficiency also on the survival of the patients with colorectal neoplasm. New antineurogenic strategies can also be developed and one can even use human behavior in order to control the nervous factors implicated in colorectal carcinogenesis.

Conflict of interests

The authors declare that they have no conflict of interests.

Author contribution

Cristina Florescu and Elena-Anca Târtea equally contributed to the article.

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