The role of galanin in the progression and prognosis of colorectal cancer: the unfinished story

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ABSTRACT

The paper presents a summary of immunohistochemical (IHC) and biochemical investigations on the presence of galanin (Gal), one of the neuropeptides abundant in the enteric nervous systems, and three types of its receptors (GalR1-3) in colorectal cancer (CRC) tissue and non-involved colon wall and their associations with clinical-pathological data of the CRC patients. We were the first to morphologically demonstrate the presence of endogenous Gal in CRC sections and measure its content in homogenates of tumor tissue and dissected compartments of unchanged colon wall. The prominent atrophy of myenteric plexuses displaying Gal immunoreactivity (Gal-Ir) located close to the tumor invasion was found to be accompanied by higher Gal content in the tumor-adjacent muscularis externa than in tumor-distant tissue. In further studies for the first time, we demonstrated by the IHC technique the presence of the GalR1-3 receptors in the CRC tumors and the colon mucosa and found that higher GalR3-Ir in the tumor tissue correlated with longer overall survival of CRC patients. Furthermore, we discovered that lower GalR1 expression in submucosal plexuses located near the tumor correlated with a better prognosis in patients with CRC. These findings suggest that GalR1 could be considered as a novel therapeutic target in CRC. In conclusion, our morphological investigations provided novel data documenting the involvement of Gal and its receptors in the progression of CRC and showed the usefulness of the IHC technique for the prognosis of CRC patients.

Key words: immunohistochemistry; galanin; galanin receptors; colorectal cancer; enteric nervous system; biomarkers.

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Introduction

The enteric nervous system (ENS) is composed of neurons and enteric glial cells located in submucosal and myenteric plexuses localized in the submucosa and muscularis externa, respectively, of the gastrointestinal (GI) tract wall.[1,2] The ENS neurons release not only classical neurotransmitter molecules like acetylcholine, dopamine, γ-aminobutyric acid, nitric oxide and serotonin but also many neuropeptides such as vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), calcitonin gene-related peptide (CGRP), cocaine and amphetamine-regulated transcript, galanin (Gal), galanin-like peptide 2, gastrin-releasing peptide, oxytocin, neurotensin, neuromedin U, pituitary adenylate cyclase-activating polypeptide (PACAP), somatostatin (SOM), substance P, tachykinin and other mediators and growth factors including ATP, calbindin, calretinin, enkephalin, dynorphin, endocannabinoids, glial-derived neurotrophic factor and nerve growth factor.[2-4] The ENS controls such basic functions of the GI tract as motility, secretion, intestinal permeability, local blood flow, osmolality, nociception, chemical and mechanical sensation, regulation of mucosal immunity and many others, both in physiology and pathology.[2-4]

Colorectal cancer (CRC) is the second and third most frequent cancer in males and females worldwide, respectively, and is associated with high mortality and morbidity rates.[5] Despite much progress in the basic and clinical sciences, the elucidation of factors responsive for the colorectal carcinogenesis and CRC progression still presents an enormous challenge to the research community. During the past 20 years, it became clear that the ENS participates in the progression of CRC.[6,7] We have previously observed that myenteric and submucosal plexuses located in the proximity of CRC tumor undergo atrophy[8,9] what suggested the interactions between cancer tissue and tumor-adjacent part of the large intestine wall. The initial immunofluorescence investigations of the occurrence of ENS neurons displaying various neuropeptides (CGRP, Gal, NPY, PACAP, SOM, substance P, VIP) in the CRC tissue and the wall of the large intestine revealed differences in their localization and frequency in the submucosa and muscularis externa.[8,10,11] Additionally, it was found that the frequency of the immunoreactive neurons seemed to depend on the neurons' distance from the tumor mass. Therefore, we decided to study the distribution of the immunoreactivity for Gal (Gal-Ir), a neuropeptide with important role in the functions of the GI tract,[12,13] as well as for Gal receptors (GalR1-3) in the CRC tissue and colon wall in the proximity and at distance from primary tumor mass.

Galaninergic neurons are widely spread in mammals, mainly in the central nervous system and ENS, but also in many other organs and tissues. In the GI tract, Gal is involved in the direct and indirect modulation of intestinal motility, secretion, and fluids' flow.[13] Moreover, Gal seems to play a role in various malignant cancer types including CRC.[14] Therefore, we decided to investigate by immunohistochemistry whether the CRC tissue contains Gal-Ir cells since the expression of the *GAL* mRNA had been described in previous studies.[15,16] Since the Gal-Ir neurons are present both in the myenteric and submucosal plexuses[17,18] we decided to assess the content of Gal in the two major separable compartments of the large intestine wall, *i.e.*, mucosa with submucosa and muscularis externa (Figure 1). Because we had obtained intriguing results (see below), in the next logical step we evaluated the distribution and immunoreactivity of the three known types of GalRs. All the obtained morphological and biochemical results had been correlated with clinical-pathological characteristics and CRC patients' overall survival.

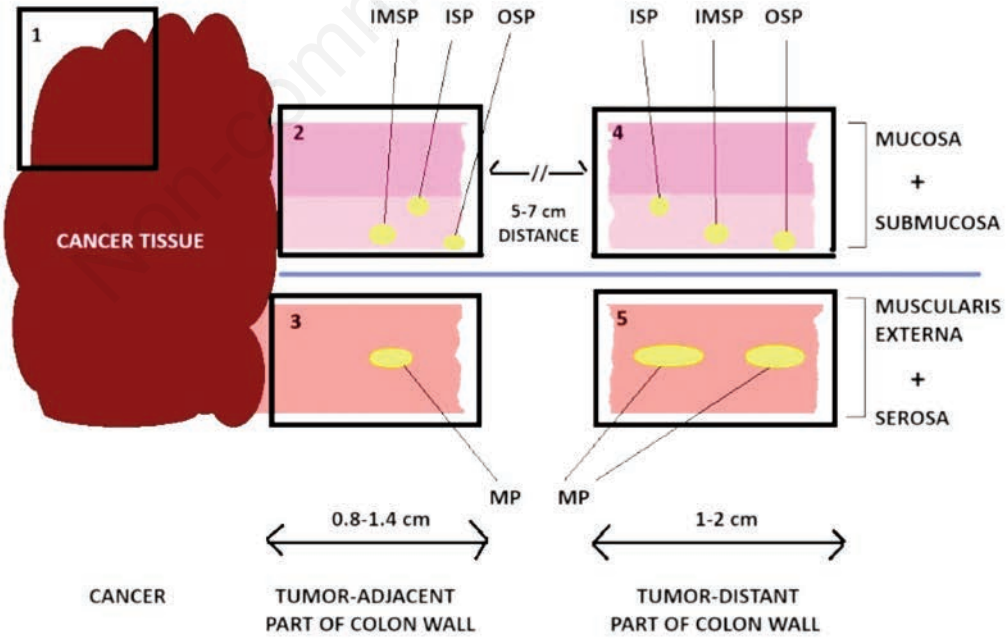

Figure 1. The design of the study. Two major compartments of large intestine wall, the mucosa with submucosa (2 and 4) and muscularis externa (3 and 5) that were localized closely to (2 and 3) or distantly from (4 and 5) colorectal cancer (CRC) tissue (1) were separated (central blue line) immediately after tumor resection within the morphologically unchanged margins of the 'healthy' tissue by an experienced surgeon (JG). The tissue samples were frozen in liquid nitrogen for biochemical measurements and fixed in 4% formaldehyde for morphological investigations. IMSP, intermediate submucosal plexus; ISP, inner submucosal plexus; MP, myenteric plexus; OSP, outer submucosal plexus.

Concentrations of galanin in serum and two separated compartments of the large intestine wall of CRC patients

We reported for the first time that the serum concentration of Gal was by 2.4-fold higher in 68 CRC patients than in the 39 healthy volunteers ($p<0.001$).[19] Although there are various sources of Gal in the body we decided to determine Gal content in homogenates of 22 samples of cancer tissue and corresponding samples of separated mucosa with submucosa or muscularis externa that were located close and distantly to the CRC tumor mass (Figure 2, based on[19]).

The measurements of Gal content in tissue homogenates revealed Gal presence in CRC tissue and the studied compartments of the colon wall. Specifically, we found that the Gal content in the mucosa and submucosa located in the proximity of the tumor was lower than in the CRC tissue and mucosa and submucosa located distantly from the tumor (Figure 2). However, the Gal content in the muscularis externa obtained in tumor-adjacent tissue was higher than in the tissue located distantly from the tumor (Figure 2). The differences in the Gal content between the studied compartments suggested that the CRC tissue may affect the galaninergic system in the large intestine wall.

Immunohistochemistry revealed suppression of galaninergic neurons in the myenteric plexuses in the vicinity of the tumor

According to previous studies[8,10,11] we had assumed that the immunohistochemical (IHC) technique would reflect the presence of Gal-Ir mainly in the submucosal and myenteric plexuses of the ENS, respectively. However, we also found strong Gal-Ir in the CRC cells, epithelium of intestinal crypts and mucosa stromal cells (Figure 2). Since submucosal plexuses occupy a markedly smaller area in the human colon than the myenteric plexuses[1] we decided to check by morphometry if and how the proximity to the CRC tissue affected the area occupied by Gal-Ir neurons. We found that the mean area of Gal-Ir myenteric plexuses in the intestinal wall proximal to the CRC tumor was 50% smaller than that in the distant section of the colon wall (Figure 2). Thus, the histomorphometric study of Gal-Ir in myenteric plexuses revealed that the proximity of the CRC tumor is associated with the atrophy of Gal-Ir neurons in the myenteric plexuses. However, the strong intensity of Gal staining and the high content of Gal in the muscularis externa in the vicinity of CRC tissue (Figure 2) suggested that proximity of CRC tissue could induce a compensatory increase of the Gal production in the myenteric plexuses neurons.

The immunoexpression of Gal receptors in the CRC tissue and in the adjacent and distantly located muscularis externa, submucosa and mucosa

Since our previous study suggested interactions between the CRC tissue and Gal expression in myenteric plexuses we decided to determine the localization of the three known types of GalRs in the tumor tissue and the wall of tumor-adjacent and distant parts of the large intestine. The immunoreactivities of GalRs, detected by

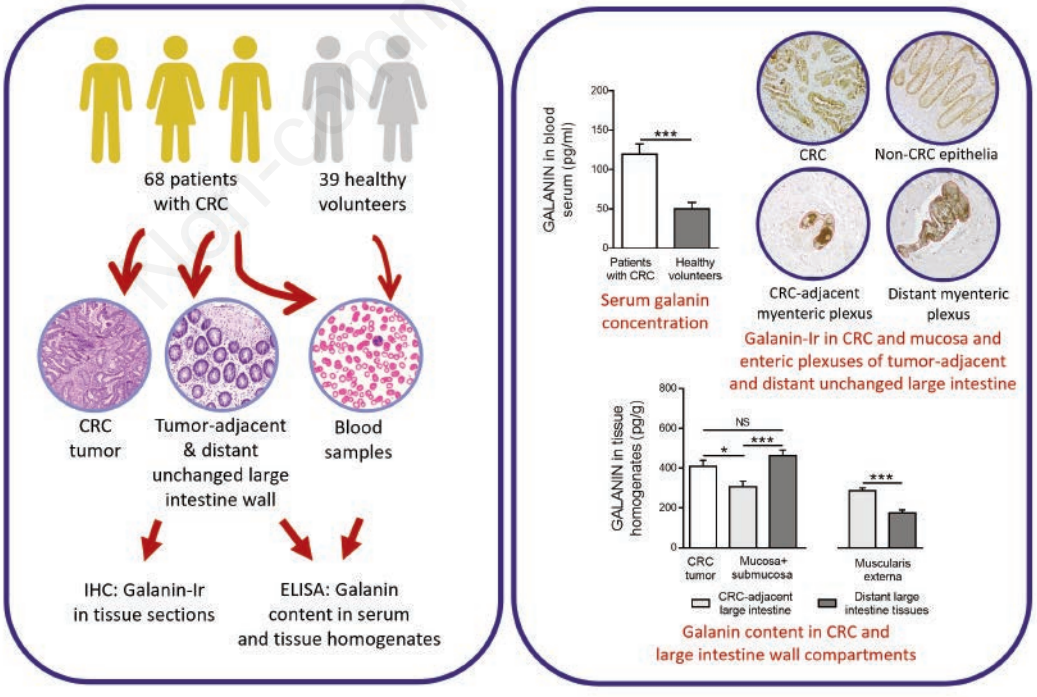

Figure 2. The design and major findings of the study of serum galanin (Gal) concentration in CRC patients and healthy volunteers, and Gal content in the tissue homogenates of CRC tissue and two separate compartments of large intestine wall located closely or distantly from tumor mass. Gal serum and tissue contents were determined by ELISA. Histograms were modified based on Kwiatkowski *et al.* Oncol Lett 2016;12:3323-9 (with permission). Data represent mean ± SEM; *$p<0.05$; ***$p<0.001$.

immunohistochemistry and immunofluorescence[20,21] were found in the plasma membrane and cytoplasm of the CRC cells. Moreover, the intensity of the GalR1 and GalR3 immunostaining in the CRC cells was clearly higher than in the epithelial cells of the tumor-adjacent mucosa, whereas that of GalR2 was similar in the tumor tissue and non-tumor mucosa (Figure 3, based on[20]). The immunoreactivity of GalRs was also found in the plasma membrane and cytoplasm of mucosal stromal cells. The analysis of IHC and clinical-pathological data of the studied CRC patients revealed that relative immunoreactivity of GalR3 (tumor cells *vs* epithelial cells), but not GalR1 and GalR2, was associated with shorter overall survival of patients with CRC ($p<0.0079$) and correlated with the larger tumor size and its metastases to regional lymph nodes.[20] Thus, the GalR3 immunoreactivity in the tumor tissue can be regarded as a prognostic factor in CRC patients. Moreover, we decided to assess the prognostic utility of the GalRs immunoexpression in the submucosal and myenteric plexuses located in the vicinity and distally from the CRC tumor invasion. Therefore, we compared relative GalRs-Ir (in plexuses close to CRC invasion *vs* plexuses distant from tumor mass) and classified CRC patients into two groups described as i) 'down-regulated or no change' (GalR immunoreactivity ≤1); and ii) 'up-regulated' (relative GalR immunoreactivity >1) to obtain survival curves according to the Kaplan-Meier method. We found that immunoreactivities of all three studied GalRs were similar in myenteric plexuses located closely to and distantly from the tumor invasion (Figure 4, based on[21]) and were not associated with the overall survival of the CRC patients.[21] However, it was not the case when clinical-pathological data and GalRs immunoexpression in the submucosal plexuses were evaluated. The statistical analysis revealed that relative immunoreactivity of GalR1 (GalR1-Ir in submucosal plexuses close to CRC tissue *vs* plexuses distant from the tumor) correlated with the overall survival of CRC patients (Figure 4). The increased immunoexpression of GalR1 in submucosal plexuses close to cancer invasion correlated with the higher tumor grading (Figure 4). Thus, the lower GalR1 expression in submucosal plexuses in the vicinity to the CRC tumor correlated with better prognosis. Such relationships were not found when the immunoreactivities of GalR2 and GalR3 were assessed.[21]

In summary, our investigations showed that Gal and GalRs might be involved in the progression of the CRC. However, the ways by which this neuropeptide and its receptors act during CRC development could not be easily explained on the basis of our morphological studies of the human clinical material. Nevertheless, the *in vitro* data concerning the action of Gal on gastrointestinal cancer cell lines as well as studies of human GI cancers provide a framework to consider possible mechanisms of interactions between CRC cells and Gal and its receptors.

Discussion

The present paper summarizes our studies on the occurrence of the Gal-Ir cells and GalRs in the tumor tissue and adjacent large intestine wall of CRC patients and their association with the clinical-pathological parameters. Our studies suggest that Gal may play a role in the progression of CRC and that the IHC evaluation of GalRs may prove useful for the CRC prognosis and possible treatment.

Kim *et al.*[22] were the first to show that the serum Gal concentration in CRC patients was 1.6-fold higher as compared to healthy subjects and that it correlated with tumor size. In our primary study we confirmed their findings, and by homogenizing tumor and two compartments of non-involved colon wall documented that at least part of serum Gal level increase was caused

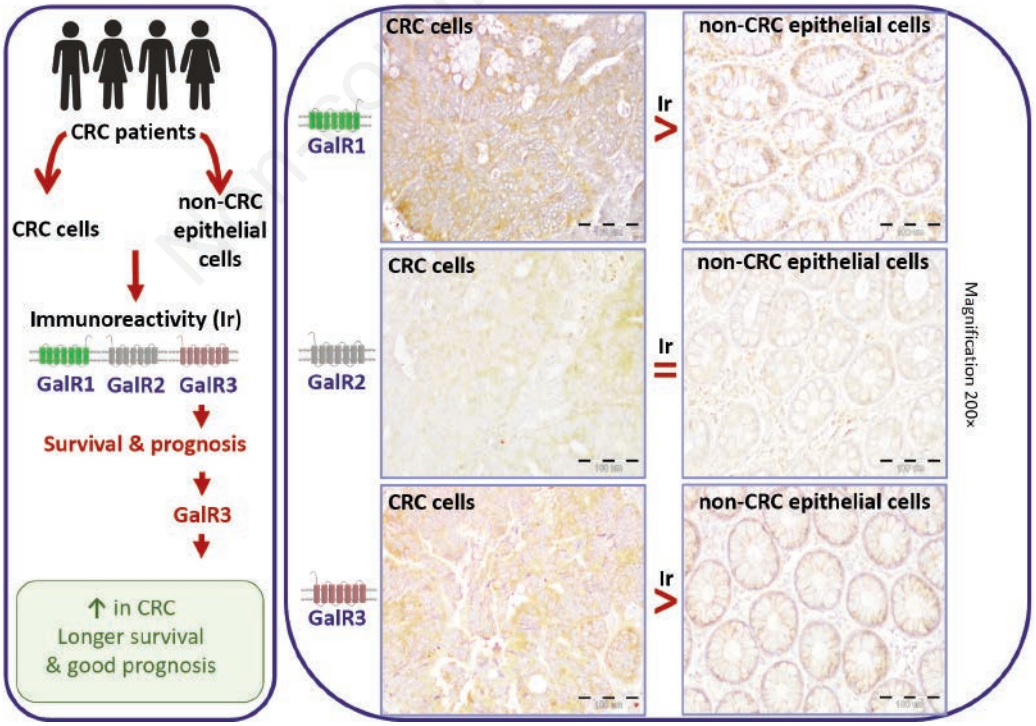

Figure 3. The immunohistochemical expression of galanin receptors (GalR1, GalR2 and GalR3) in colorectal cancer cells compared with their immunoreactivity in the epithelial cells of the unchanged mucosa (based on[20]).

by high Gal content in the CRC tumor tissue and the tumor-adjacent muscularis externa.[19] Despite statistical significance of this increase, it is doubtful if Gal serum levels could be used as CRC biomarker since the only 2.4-fold difference between healthy subjects and CRC patients could not be high enough to set a cut-off value. However, our morphological study provided evidence that probably mutual interactions exist between CRC tissue and adjacent myenteric plexuses, since the evident atrophy of these plexuses was accompanied by increased content of Gal protein in tumor-adjacent muscularis externa.

Apart from the data on Gal protein and mRNA expression in other types of cancers,[13,14] our IHC study was the first (apart from Western blot detection of Gal in two CRC tumors[22]) to show endogenous Gal in the CRC tissue of a large group of patients.[19] Previously, increased *GAL* mRNA, but not protein, levels, were detected in the tumor tissue of CRC patients and colon cancer cell lines.[16,22] Recently, Talaat *et al.*[23] demonstrated by IHC varied intensity of Gal-Ir in CRC tumors and found a trend of negative correlation with TNM staging of the tumors (p=0.054, Pearson correlation -0.215).

We confirmed earlier human studies that demonstrated Gal immunoreactivity in the myenteric and submucosal plexuses in the uninvolved sigmoid colon regions remote from the CRC tumor.[1,17,18] Moreover, we found prominent expression of Gal not only in the epithelial and goblet cells of intestinal crypts but also in mucosa stromal cells which was also demonstrated, however, with lower staining intensity, by Talaat *et al.*[23] in the mucosa covering tumor mass. The previously reported lack of Gal-Ir in the human colon mucosa epithelial cells could be possibly attributed to the use of in-house produced primary antibodies that were not standardized.[17] At the gene expression level, increased levels of *GAL* mRNA in CRC were associated with tumor recurrence.[16] It is well known that Gal affects cell function by interacting with three types of its receptors that belong to a large family of G protein-coupled receptors.[24] Our use of the immunofluorescence and IHC techniques made it possible to demonstrate, for the first time, the presence of GalR1-3 proteins in the CRC tissue and the epithelium of morphologically unchanged large intestine.[20] Brunner *et al.*[25] evaluated by IHC the presence of GalRs in the mucosa of inflammatory bowel disease patients and mice with induced colitis. Contrary to our results these authors did not observe immunoreactivity of GalRs in epithelial and mucosa stromal cells in the colon biopsies of nine healthy young subjects, however, they found in the inflamed mucosa immunoreactivity of all three GalRs in lymphocytes and GalR2-Ir and GalR3-Ir in approximately 50% of granulocytes. Since we had used the same antibodies against GalR1 and GalR3 receptors, the discrepancy between their and our results might had been caused by either different age of the studied subjects (19 and 67 years, respectively) or/and the interactions of the mucosal cells with the neighboring CRC tissue. However, it has to be mentioned that we confirmed our IHC results by detecting the presence of GalRs in the morphologically unchanged part of colon wall of five CRC patients using Western blot method.[20]

Moreover, we found that the intensity of GalR1 and GalR3 immunoreactivities in CRC tumor cells was higher than in the epithelial cells of the unchanged mucosa. The longer overall survival of the CRC patients with higher GalR3-Ir suggests that GalR3 immunoreactivity may be considered as morphological prognostic factor in CRC.[20] Although the IHC method does not allow to determine the amount of GalR3 in the CRC tissue, the intensity of the staining reaction to some extent reflects the number of the detected antigens and may prove useful for the prognosis. However, the mechanisms of the correlation between GalR3-Ir and longer overall survival of CRC patients need further studies since

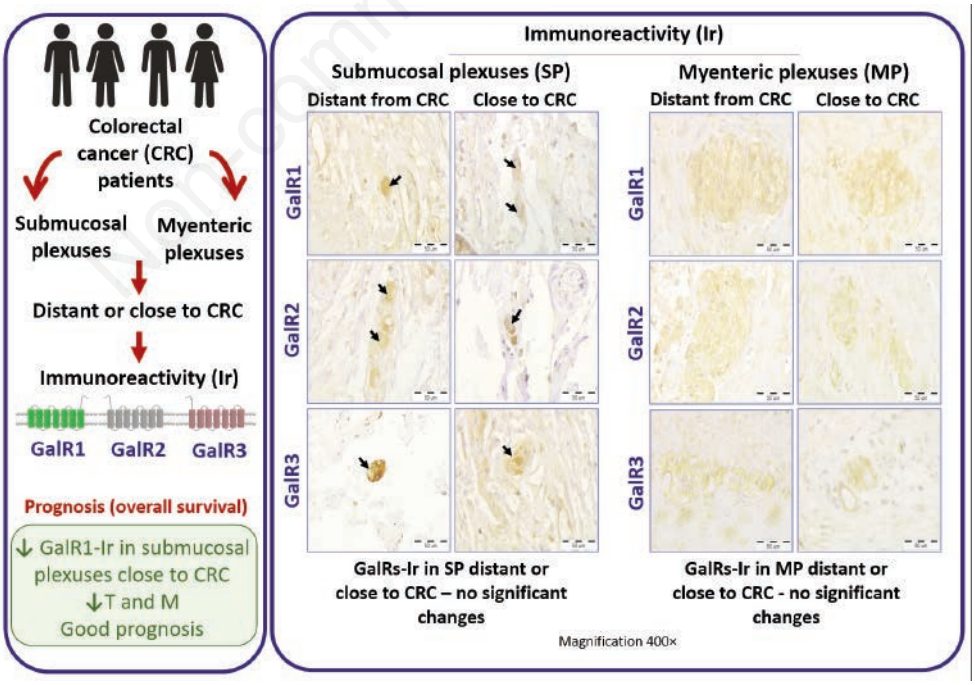

Figure 4. The immunoreactivity of three types of galanin receptors (GalRs) in the submucosal (SP) and myenteric (MP) plexuses located closely to and distantly from the colon cancer tissue and their association with the prognosis of the CRC patients. Arrows indicate localization of GalR3-Ir within neurons of the submucosal plexuses (based on[21]). T and M refer to the TNM tumor grading: T, tumor size; N, lymph node involvement; M, metastasis.

the GalR3 signaling has been less investigated than that of GalR1 and GalR2.[24,26] It was shown that GalR3 activates a PTX-sensitive $G_{i/o}$-type G protein what results in decreased adenylate cyclase activity and lower cytosolic cAMP concentration.[27,28]

Finally, we analyzed the associations of the immunoreactivity of GalRs in the ENS plexuses with the clinical-pathological data of the CRC patients. Although there were no differences in the intensity of GalRs expression in the tumor-adjacent and tumor-distant myenteric and submucosal plexuses, the finding of the correlation between higher GalR1-Ir in the submucosal plexuses near the CRC invasion and shorter survival of the CRC patients suggests that GalR1 expression in this localization can be a predictive factor for CRC progression.[21]

Our morphological studies on the expression of the GalRs provide evidence that protein expression should be an ultimate parameter of gene expression studies. Thus far, Benya *et al.*[29] identified the presence of GalR1 mRNA in the cultures of normal human colon epithelial cells (obtained at non-emergency colon biopsies) while Stevenson *et al.*[15] reported the presence of the GalR1 mRNA in eight cases of CRC liver metastases and demonstrated that the GalR1/Gal silencing induces apoptosis in HCT116 CRC cells. These reports and results of our studies suggest that Gal and its receptors may play a role in CRC progression. In contrast to the expanding knowledge on Gal and its receptors in either promotion or inhibition of cell proliferation in various types of cancers[13,14,30-33] their role in CRC tumorigenesis has been only sparsely investigated[34] despite clinical importance of this malignant tumor.

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