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ORIGINAL PAPER

Synaptophysin expression as prognostic factor for survival in colorectal carcinomas

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Abstract

The aim of this study is to assess the status of synapses in normal colorectal tissue compared to neoplastic colorectal tissue, and to correlate this status with survival in patients with colorectal neoplasia. Our study included 61 patients diagnosed with colorectal adenocarcinoma, representing the study group, and 53 patients diagnosed with benign conditions, that required a resection of a colorectal segment, representing the control group. We performed the immunohistochemical staining by using anti-synaptophysin antibody, which identifies synaptic vesicles and, so, we managed to analyze the expression of synapses in colorectal adenocarcinoma. Regarding both the signal area and integrated optical density (IOD) of the synaptophysin, the univariate analysis with a *log*-rank (Mantel–Cox) test indicated that patients with a low level of synaptophysin had a better overall survival rate than those with a high-level synaptophysin. Also, we noticed that tumor size, tumor invasion and lymph node metastasis were significantly associated with the overall survival rate, whereas the other clinicopathological features were not. In conclusion, the status of synaptic vesicles evaluated *via* synaptophysin expression in patients with colorectal cancer positively correlates with the survival rate and it can play a role in the neoplastic therapy process.

Keywords: colorectal adenocarcinoma, synaptophysin, overall survival rate.

Introduction

Although before 1900 colorectal cancer (CRC) was relatively unknown, at present it is the third most diagnosed type of cancer and the fourth most common cause of cancer death worldwide, with a dramatic increase in incidence rates with the economic development of the last century, but also with the adoption of a sedentary lifestyle and Western diet [1–3].

CRC develops from the colorectal's epithelial cells and, in early stages does not produce symptoms, while in more advanced stages it can cause symptoms such as fatigue, bloody stools, persistent abdominal discomfort (*e.g.*, pain, gas and abdominal cramps) or changes in bowel habits (*e.g.*, constipation and diarrhea) [3–5]. Despite the undeniable achievements of modern medicine, in terms of diagnosis or therapy of this type of cancer, the pathogenesis, evolution and prognosis of CRC are still not fully understood [6–8]. This situation generated numerous studies for deciphering other molecular and cellular mechanisms underlying this pathology in order to identify new therapeutic methods and also to optimize those that already exist [9–13].

The aim of this study is to assess the status of synapses in normal colorectal tissue compared to neoplastic colorectal tissue, and to correlate this status with survival in patients with colorectal neoplasia.

Patients, Materials and Methods

Patients

This study included a total number of 61 patients diagnosed with primary colorectal cancer, who underwent a curative colectomy in the 1st Surgery Clinic of the Emergency County Hospital, Craiova, Romania, between 2012 and 2013. Patients were initially diagnosed in the Clinic of Gastroenterology of the same Hospital, then they were surgically treated and none of them received chemotherapy or radiotherapy before surgery. Colorectal tissue specimens harvested from 53 patients who required a resection of a colonic segment for benign affections were used as controls.

The study was conducted in accordance with the rules and principles of the Ethics Committee of the University of Medicine and Pharmacy of Craiova, it was approved by the Ethics Committee and it complied with all the rules of international forums governing scientific research. All patients included in the study signed an informed consent.

Immunohistochemistry

Paraffin-embedded tissues were obtained from the archives of the Department of Pathology, and they were first stained for routine diagnostic confirmation using Hematoxylin and Eosin (HE).

The immunohistochemical study was performed on seriate sections from the same tissue blocks in the Research Center for Microscopic Morphology and Immunology of the University of Medicine and Pharmacy of Craiova. Briefly, the sections underwent antigen retrieval by microwaving in citrate buffer pH 6, for 20 minutes, at 650 W, then the endogenous peroxidase was quenched with 0.1% H_2O_2 and the unspecific antigen binding sites were blocked in 3% skimmed milk in 1 M phosphate-buffered saline (PBS). Next, the primary antibody was added on the slides (Synaptophysin, 1:200, Novus Biologicals, UK) for 18 hours, at 4°C, which identifies synaptic vesicles, allowing us thus to analyze the expression of synapses in colorectal adenocarcinoma. The second day, the signal was amplified with a species specific polymeric secondary (Nichirei-Bioscience, Tokyo, Japan), the color was developed with 3,3'-diaminobenzidine (DAB) (Dako) and the slides were coverslipped in DPX (Sigma-Aldrich, St. Louis, MO, USA) after Hematoxylin counterstaining. Negative controls were obtained by omitting the primary antibody.

Image processing

For capturing and quantifying the immunohistochemical expression of the antigens, we used a Nikon 90i microscope (Elta 90 Medical Research, Bucharest, Romania), equipped with the Nuance FX multispectral camera and software (Perkin Elmer, Hopkinton, MA, USA), and the Image ProPlus AMS 7 software (Media Cybernetics, Rockville, MD, USA). Spectra of DAB were separated from the slides using the multispectral camera, and then the intensity/area of the signal was quantified as the integrated optical densities (IODs) in Image ProPlus.

Statistical analysis

Data exported from Image ProPlus was plotted in Microsoft Office Excel and was analyzed by using GraphPad Software (version 6, GraphPad Software, La Jolla, CA, USA). All data was expressed as mean ± standard deviation (SD). In order to illustrate potential relationships between synaptic expression and clinicopathological features, we used the Student's *t*-test for comparing the means of two groups and one-way analysis of variance (ANOVA) with Bonferroni's *post-hoc* correction for comparing the means of more than two groups. We used the Kaplan–Meier curves to evaluate patient survival and for analyzing the prognostic factors, we used the *log*-rank (Mantel–Cox) test. $P<0.05$ was considered statistically significant.

Results

Histopathological characterization

Our study included 61 patients diagnosed with colorectal adenocarcinoma and the tissue fragments that were selected from their surgical resection represented the study group, and 53 patients diagnosed with benign conditions that required a resection of a colorectal segment, of which tissue fragments, representing the control group, were selected. As mentioned above in the "Patients, Materials and Methods" chapter, firstly, the histopathological diagnosis of colorectal adenocarcinoma was established and then both pathological and normal tissue samples were immunomarked with synaptophysin and the expression of this immunomarker was studied by analyzing both the area and the optical density for the corresponding color channel. Positive staining of synapthophysin was observed in nervous ganglia of the Meissner's (Figure 1) and of the Auerbach's (Figure 2) plexuses, but also in other well organized multiaxial nerve threads, with a diameter over 20 μm, that could not be included in Meissner's or Auerbach's nervous plexuses (Figure 3). Positive staining was also observed in numerous nerve threads having a diameter of less than 20 μm, disposed in ganglion plexuses, which are intratumorally disorganized (Figure 4).

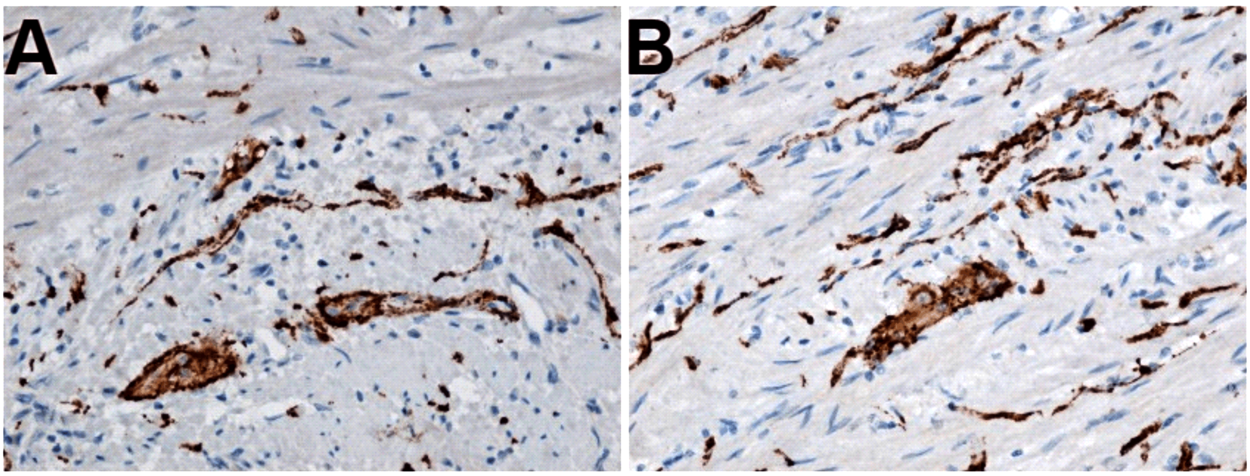

Figure 1 – ***Example of images showing synaptic vesicles in Meissner's nervous plexus (brown color), labeled with synaptophysin (×200): (A) Cross section; (B) Longitudinal section.***

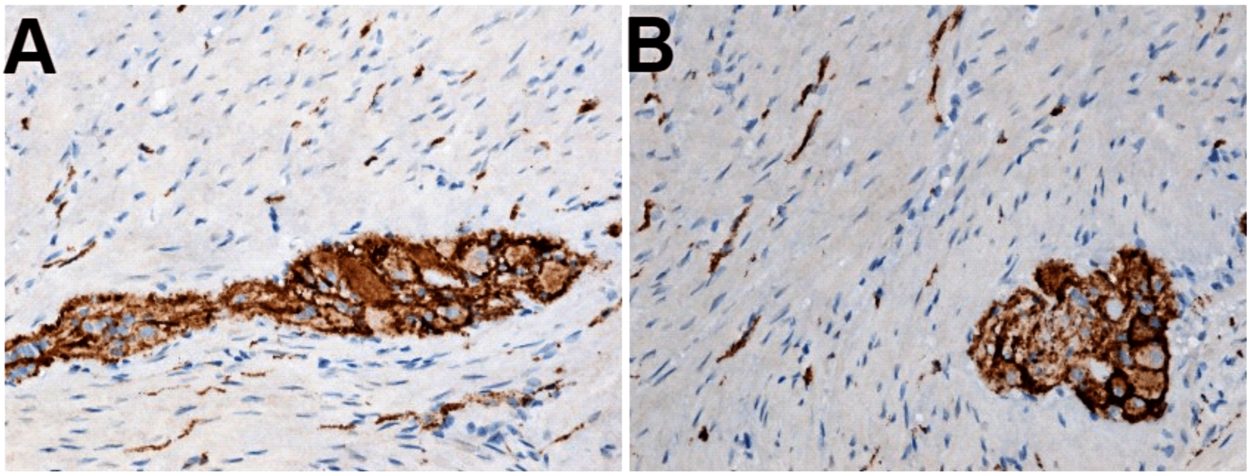

Figure 2 – ***Example of images showing the synaptic vesicles in Auerbach's nervous plexus (brown color) labeled with synaptophysin (×200): (A) Longitudinal section; (B) Cross section.***

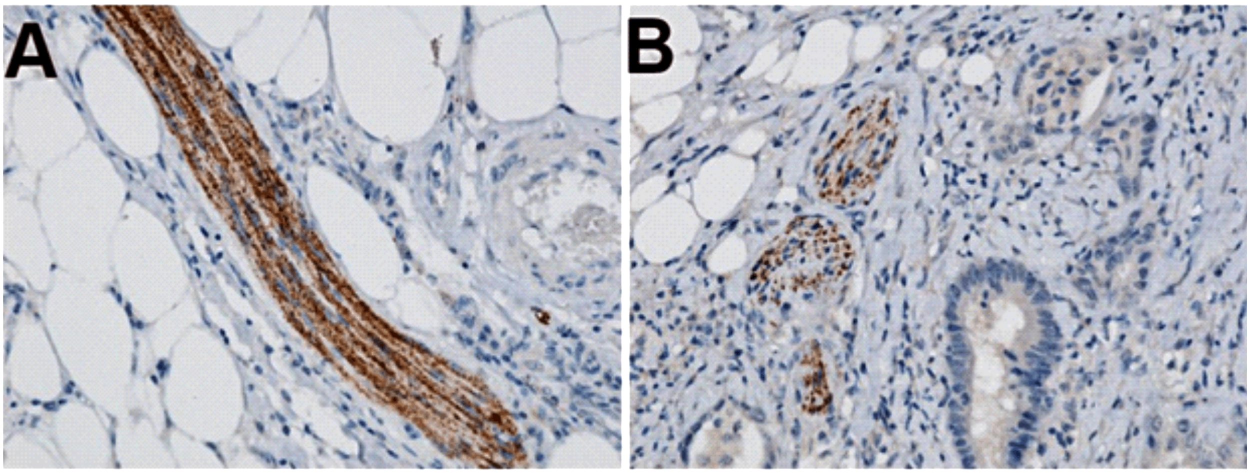

Figure 3 – ***Example of images showing synaptic vesicles in multiaxonal nerve threads having a diameter of more than 20 µm (brown color), labeled with synaptophysin (×200): (A) Longitudinal section; (B) Cross section.***

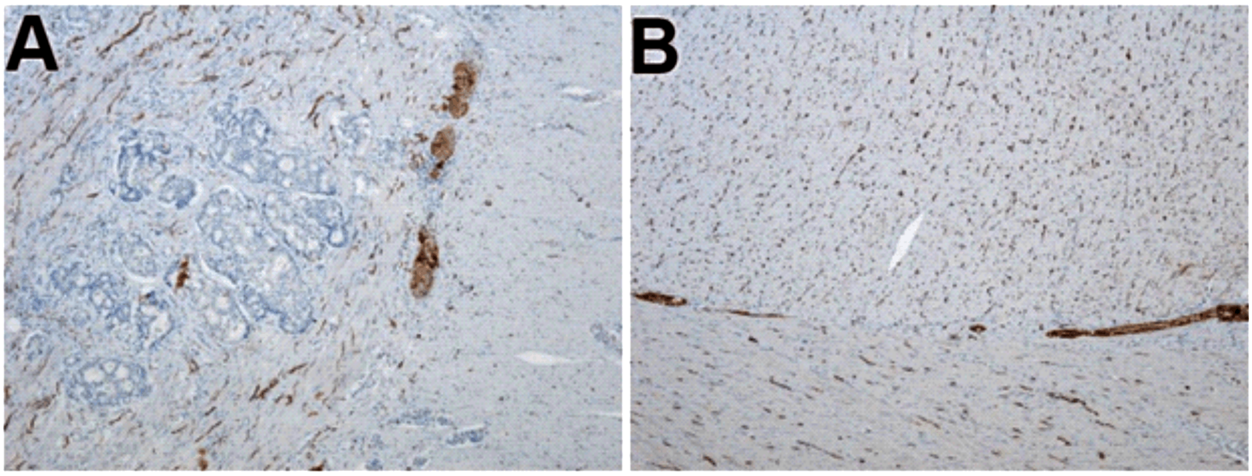

Figure 4 – ***(A) Evidence of synaptic vesicles in the muscular layer with tumor invasion in the longitudinal layer, destruction of the nerve threads in the aganglionic plexus, labeled with synaptophysin (×40); (B) Normal muscular layer, highlighting both the lining plexus (Auerbach) and the anganglionic plexus of the longitudinal and circular layers, labeled with synaptophysin (×40).***

Expression of synaptophysin in normal colorectal tissue and in different gradings of colorectal adenocarcinoma

Synaptophysin's expression was analyzed by determining the area and the integrated optical density (IOD) of the signal, both in tissue samples belonging to patients diagnosed with benign conditions of the colon, that represented the control group, but also in tissue samples belonging to patients with colorectal adenocarcinoma in different tumor differentiation gradings. In normal colorectal tissue (N), the highest values were recorded for both synaptophysin's signal area (10 162±3206 μm^2) and

IOD (1 163 006±322 122), with a gradual decrease of these parameters from normal colorectal tissue to patients with well-differentiated colorectal adenocarcinoma (G1) where values of 7443±2590 μm^2 for the area and 876 552±272 883 for IOD were recorded, to patients with moderately differentiated colorectal adenocarcinoma (G2) (6087±1795 μm^2 for the area and 763 485±214 312 for IOD) and finally to patients with poorly differentiated colorectal adenocarcinoma (G3), where values of 4872±2018 μm^2 for the area and 584 876±247 113 for IOD were recorded (Figures 5 and 6). Therefore, by using the one-way ANOVA followed by Bonferroni's *post-hoc* test, statistically significant differences between normal colorectal tissue and G1, G2, G3 and also between G1 and G3 were observed for both synaptophysin's area (Table 1) and IOD (Table 2), while between G1 *vs.* G2 and G2 *vs.* G3 we noticed no statistically significant differences.

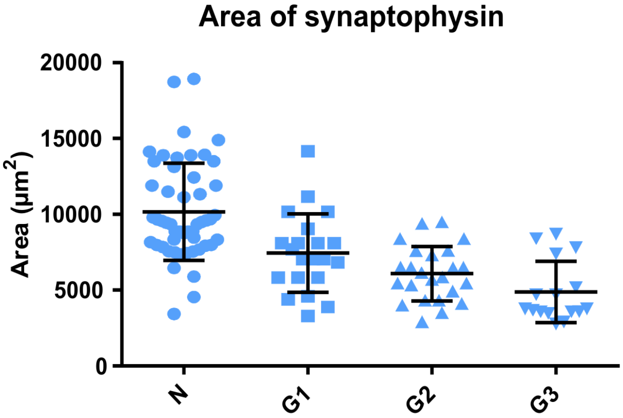

Figure 5 – ***Area of the synaptophysin expression in normal colorectal mucosa (N) and in different colorectal adenocarcinoma's tumor grading (G1: Well differentiated; G2: Moderately differentiated; G3: Poorly differentiated).***

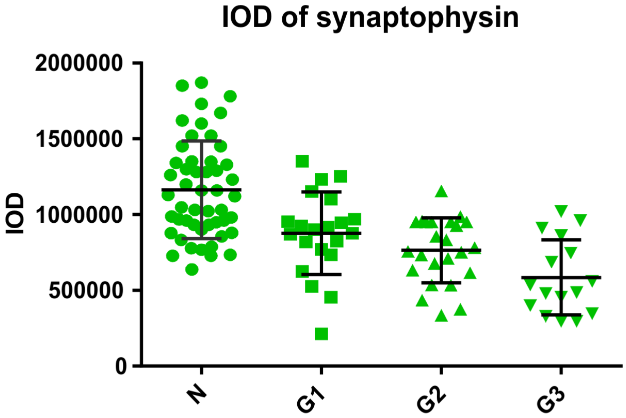

Figure 6 – ***Integrated optical density (IOD) of the synaptophysin expression in normal colorectal mucosa (N) and in different colorectal adenocarcinoma's tumor grading (G1: Well differentiated; G2: Moderately differentiated; G3: Poorly differentiated).***

Table 1 – ***The results of the ANOVA test followed by Bonferroni's post-hoc test between the expression of the synaptophysin in normal colic mucosa and in different gradings of colorectal adenocarcinoma for the area***

Ordinary one-way ANOVA depending on the synaptophysin area			
Bonferroni's multiple comparisons test	***Mean difference***	***95% CI of difference***	***Adjusted p-value***
N *vs.* G1	2719	842.4 to 4596	0.0010
N *vs.* G2	4075	2283 to 5867	<0.0001
N *vs.* G3	5289	3215 to 7364	<0.0001
G1 *vs.* G2	1356	-807.2 to 3518	0.5697
G1 *vs.* G3	2570	168.3 to 4972	0.0291
G2 *vs.* G3	1215	-1122 to 3551	0.9909

ANOVA: Analysis of variance; CI: Confidence interval; N: Normal colorectal mucosa; G1: Well-differentiated colorectal adenocarcinoma; G2: Moderately differentiated colorectal adenocarcinoma; G3: Poorly differentiated colorectal adenocarcinoma.

Table 2 – ***The results of the ANOVA test followed by Bonferroni's post-hoc test between the expression of the synaptophysin in normal colic mucosa and in different gradings of colorectal adenocarcinoma for IOD***

Ordinary one-way ANOVA depending on the synaptophysin IOD			
Bonferroni's multiple comparisons test	***Mean difference***	***95% CI of difference***	***Adjusted p-value***
N *vs.* G1	286447	88 893 to 484 000	0.0010
N *vs.* G2	399514	210 856 to 588 172	<0.0001
N *vs.* G3	578123	359 912 to 796 334	<0.0001
G1 *vs.* G2	113067	-113 941 to 340 075	>0.9999
G1 *vs.* G3	291676	39 571 to 543 781	0.0144
G2 *vs.* G3	178609	-66 587 to 423 806	0.3169

ANOVA: Analysis of variance; IOD: Integrated optical density; CI: Confidence interval; N: Normal colorectal mucosa; G1: Well-differentiated colorectal adenocarcinoma; G2: Moderately differentiated colorectal adenocarcinoma; G3: Poorly differentiated colorectal adenocarcinoma.

Univariate analyses of prognostic variables

In our study, the 61 patients with colorectal cancer were divided by using the median value of the synaptophysin's area (6154.449 μm^2) and the median value of the synaptophysin's IOD (769 312.8) into a low synaptophysin group (32/61 for area and 30/61 for IOD) and a high synaptophysin group (29/61 for area and 31/61 for IOD). Regarding the signal area of synaptophysin, the univariate analysis with a *log*-rank (Mantel–Cox) test indicated that patients with a low synaptophysin area had a better overall survival rate than those with a high synaptophysin area (75% *vs.* 17.24%, $p<0.0001$; Figure 7). Regarding the IOD signal of synaptophysin, the univariate analysis with a *log*-rank (Mantel–Cox) test indicated that patients with low synaptophysin's IOD had a better overall survival rate than those with high synaptophysin's IOD (70% *vs.* 16.12%, $p<0.0001$; Figure 8).

Analyzing overall survival rates at five years, based on tumor grading, we noticed that patients with well-differentiated colorectal adenocarcinoma had a global survival at five years of 66.66%, patients with moderately differentiated colorectal adenocarcinoma 50%, while patients with poorly differentiated adenocarcinoma had a global survival of only 37.5% (Figure 9). Finally, we noticed that tumor size, tumor invasion and lymph node metastasis were significantly associated with the overall survival rate, whereas the other clinicopathological features were not (Table 3).

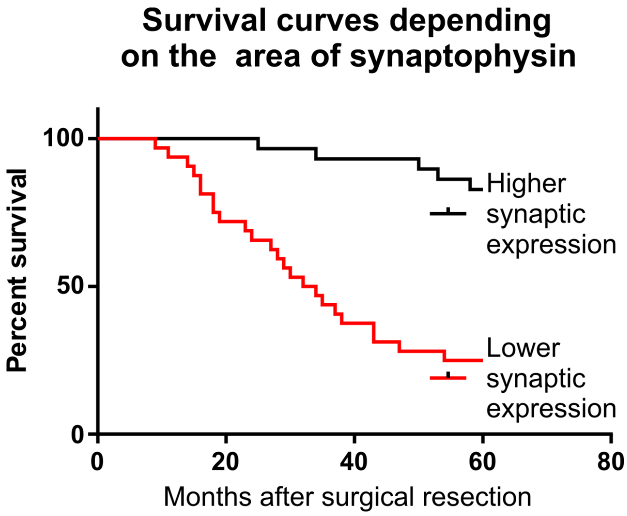

Figure 7 – ***Overall survival curves of the patients in the high and low synaptophysin area groups. Patients with high area value of synaptophysin had a significantly better overall survival rate than those with low area value of synaptophysin.***

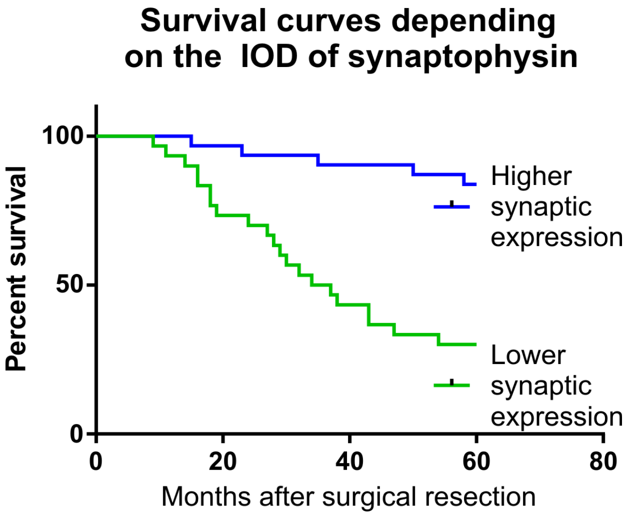

Figure 8 – ***Overall survival curves of the patients in the high and low synaptophysin IOD groups. Patients with high IOD value of synaptophysin had a significantly better overall survival rate than those with low IOD value of synaptophysin. IOD: Integrated optical density.***

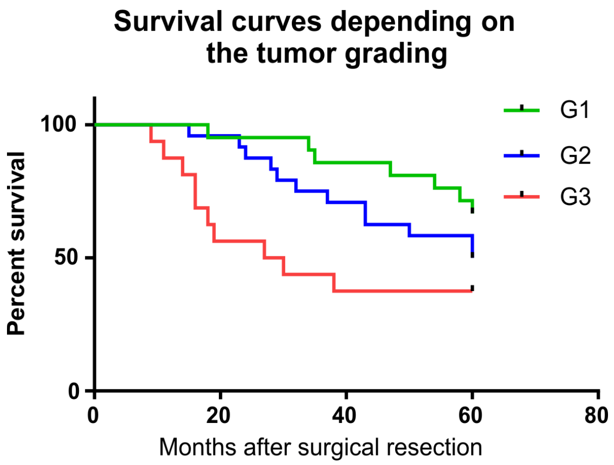

Figure 9 – ***Kaplan–Meier curves of the patients depending of the tumor grading. Survival rate of patients at five years decreases from patients with well-differentiated colorectal adenocarcinoma (G1) to patients with moderately (G2) and respectively poorly differentiated colorectal adenocarcinoma (G3).***

Table 3 – ***Log-rank (Mantel–Cox) test between the clinicopathological features and the overall survival of 61 patients with colorectal adenocarcinoma***

Clinicopathological features		***n***	**Five-year survival rate [%]**	***P*-value***
Gender	Males	39	51.83	0.230
	Females	22	48.76	
Age at diagnosis [years]	<60	19	46.19	0.370
	≥60	43	48.09	
Tumor size [cm]	<5	34	78.32	0.000[1]
	≥5	27	18.56	
Site of primary tumor	Distal	43	49.07	0.430
	Proximal	18	56.82	
Gross appearance	Exophytic	29	52.24	0.260
	Infiltrative	32	55.37	
Tumor invasion	T_{1-2}	25	86.78	0.000[1]
	T_{3-4}	36	26.88	
Lymph node metastasis	N_{0-1}	28	86.67	0.000[1]
	$N_{\geq 2}$	33	28.19	

n: No. of cases; **Log*-rank (Mantel–Cox) test; [1]*P*<0.05, statistically significant.

Discussion

Nowadays, it is well known that neoplastic cells appear as a consequence of the interaction between genetic and epigenetic mutations of normal cells. However, many studies emphasized the tumorigenic influence of the tumor microenvironment [9].

From the tumor microenvironment, we chose to analyze synapse status by evaluating the expression of synaptophysin (Syn), which is present in small synaptic vesicles involved in synaptic transmission [14].

Due to this analysis, a connection between the functional integrity of the enteric nervous system and colorectal carcinogenesis was created. The enteric nervous system and its components are the most important part of the tumor microenvironment in colorectal neoplasm [15]. In colorectal cancer, it has been shown that total nerve tissue density proportionally increases with the tumor grading, but this increase is not due to the traditional components of the enteric nervous system, Meissner's and Auerbach's plexuses, but to the multiaxonal nerve threads, which do not have the same histological features with the those mentioned above [12]. On the other hand, in a recent study, it was demonstrated that the density of enteric glial cells from the enteric nervous system inversely varies with tumor grading, this observation highlighting the role played by enteric glial cells in the control of colorectal neoplasm cell proliferation [13].

If in the above-mentioned studies, we were able to observe the morphological changes of the nervous system, in our study we attempted, by using a morphological description of the synapse expression, to functionally analyze the relationship between the nervous system and the cells of the colorectal neoplasm. As it is known, both the central and peripheral nervous system modulate the functions of the whole organism both through a direct connection at synaptic level (classical or non-classical), as well as humoral modulations [16]. Influence of the nervous system on neoplastic cells is also accomplished by these two methods, on the one hand by the release of catecholamines from the sympathetic terminals in the proximity of neoplastic cells, and on the other by the catecholamines in the blood circulation from the adrenal gland [17]. It still remains unclear whether the level of catecholamine in the plasma plays an important role in carcinogenesis, or the highest role in this process is due to locally released catecholamines [17].

Now, many studies have highlighted that the mediators of the sympathetic nervous system act *via* multiple pathways for neoplastic initiation, progression and metastasis. Influences of the nervous system on the neoplastic process were firstly suggested by clinical observations between psychological stress and cancer progression [17–19]. However, there are many studies that have shown the effect on neoplastic cell biology by the nervous system mediators. Thus, DNA damage repair can be inhibited by beta-adrenergic signaling, and an example in this way is the DNA damage accumulation at the moment of chronic stimulation with catecholamines *via* ARRB1 (β-arrestin-1) and p53-dependent mechanism [20]. Sympathetic stimulation may also trigger certain oncogenes such as HER2 and Src [21, 22]. Also, *via* this mechanism, many pathways involved in tumor growth and metastasis are also modulated. Thus, beta-adrenergic stimulation may cause the expression of certain growth and survival factors, such as vascular endothelial growth factor (VEGF), interleukin (IL)-6 and IL-8, which are also associated with resistance to tyrosine kinase inhibitors [23–26].

It must be mentioned that these observations between the nervous system influences on the neoplastic process, found in the literature, conducted studies on the effects of the therapeutic blockage of these influences on certain types of cancers. Because in nervous system's signalizing in the neoplastic process catecholamines are often involved *via* beta-adrenergic receptors expressed by neoplastic cells, there are studies performed on experimental animal models of human cancers and also epidemiological studies on the effects of blocking sympathetic stimulation on the neoplastic process. Thus, experimentally, beta-antagonists have been shown to inhibit the progression of breast [27, 28], prostate [29], ovarian [30], pancreatic [31], lung [32] and colorectal cancer [33, 34]. Also, in epidemiological studies, the protective effects of beta-blockers in breast [35, 36], pancreatic [37], hepatocellular [38], prostate cancer [39], malignant melanoma [40], as well as multiple myeloma [41] were highlighted.

However, all these observations still remain indirect in terms of the influence of the nervous system on the neoplastic process, and further studies are needed to elucidate influences. Moreover, according to the studies mentioned above, the status of synaptic vesicles in colorectal tumors should be increased, but according to our study, patients with high synaptophysin expression had a significantly better overall survival rate than those with low synaptophysin expression.

Conclusions

The status of synaptic vesicles evaluated *via* synaptophysin expression in patients with colorectal cancer positively correlates with the survival rate and it can play a role in the neoplastic therapy process.

Conflict of interests

The authors declare that they have no conflict of interests.

Author contribution

Diana Rodica Tudoraşcu and Edme Roxana Mustafa equally contributed to the manuscript.

Acknowledgments

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