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Expression of M3 muscarinic acetylcholine receptors in gastric cancer

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Abstract

Introduction: Gastric cancer represents a real public health problem as far as incidence, aggressiveness and unfavorable prognosis are concerned. The autonomous nervous system might be one of the major factors involved in the onset, progression, and metastasis, both sympathetically and parasympathetically. The increased activation of the M3 muscarinic acetylcholine receptors (mAChRs) triggers pro-oncogenic mechanisms, especially at a gastric level, through the activation of the Hippo signaling pathway and the increase of the nerve growth factor. *Patients, Materials and Methods*: In this study, biopsy or postoperative gastric resection pieces have been evaluated by histopathological (HP) and immunohistochemical (IHC) examination in a group of 77 gastric patients and 23 patients without an oncological diagnosis. To quantify the IHC signal, also considering the HP aspect, light microscopy images were obtained. *Results*: The M3 mAChR expression analysis has been correlated with the different gastric adenocarcinoma differentiation degrees (G1–G3). M3 mAChR presence has been observed also in the non-malignant gastric tissue, but it was significantly increased in the tumor tissue. The highest receptor expression was recorded in patients with a poorly-differentiated (G3) adenocarcinoma, these expressions decreasing with the increase of the differentiation degree towards moderately-differentiated (G2) and well-differentiated (G1). *Conclusions*: Surgical or pharmacological parasympathetic activity inhibition could decrease the development and progression of gastric tumors and could improve the gastric cancer patient's prognosis.

Keywords: gastric cancer, M3 muscarinic acetylcholine receptor, tumor grade, dysautonomia, parasympathetic nervous system.

Introduction

Gastric cancer (or stomach cancer) continues to be one of the main public health problems due to its aggressiveness and unfavorable prognosis. According to the *International Agency for Research on Cancer* (IARC), in 2020, gastric cancer ranks 5th in terms of incidence (1 089 103 cases), representing 5.6% of the total cancer cases, and 4th in terms of mortality (768 793 deaths), meaning 7.7% of the total deaths caused by cancer. The gastric cancer cumulative risk (determined for people without any gastric cancer risk factors, between 0–74 years of age) is 1.87% for men, 0.79% for women and 1.31% for both genders. In terms of deaths caused by gastric cancer, the cumulative risk is 1.29% for men, 0.55% for women and 0.9% for both genders. In terms of gender incidence, gastric cancer is more frequent in men, ranking 4th after lung cancer, prostate cancer, and colorectal cancer (15.8% of the total cancer cases), compared to women, where gastric cancer ranks 7th (7% of the total cancer cases) [1].

In Romania, gastric cancer has an incidence of 3970 cases and ranks 7th with 4% of the total oncological cases, with a cumulative risk of 1.16%. In terms of mortality, this cancer type ranks 5th with 3246 deaths (6% of oncological deaths) and a cumulative risk of 0.9%. Gender difference is significant, incidence in men being 14.9% compared to 5% in women [2]. The significantly increased difference between the number of gastric cancer cases in men, compared to women, is corelated with age and the increased socio-economic status [3].

The main lifestyle factors involved are increased alcohol and tobacco consumption for men, the gastric cancer risk being approximately 59% for smokers and 50% for heavy drinkers [4, 5]. The biological factors involved in the significant difference in gastric cancer gender incidence are the sex hormones and metabolism. The level decrease of estrogens [6], dehydroepiandrosterone [7] or the level increase of the sex hormone-binding globulin (SHBG), following the deficit or resistance to androgens [8], are correlated with an increased gastric cancer risk. Sex hormones have a major role in the autonomous nervous system modulation through the synthesis and metabolism of neurotransmitters [9].

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The autonomous nervous system acts at the stomach level through a complex sympathetic and parasympathetic neuron network, which ensures the homeostasis of gastric motility and secretion, but also of the local blood flow. The sympathetic system and adrenergic signaling activation are involved in the progression and metastasis of gastric cancer, especially through inflammatory mechanisms, immunity, angiogenesis, and cellular apoptosis [10]. These disturbances are accentuated in the presence of chronic stress, through the increased serum levels of catecholamines and cortisol [11]. Also, the unfavorable evolution and the increased death rate in gastric cancer are correlated with high serum levels of catecholamine metabolites, metanephrine and normetanephrine, as well as with the increase in the number of norepinephrine transporter [12].

The parasympathetic nervous system is involved in molecular mechanisms that can inhibit or stimulate tumor growth, depending on the type of the muscarinic acetylcholine receptor (mAChR) involved. It has been proved that all the five subtypes of mAChR (M1–M5) are involved in the gastric homeostasis, even though each and every receptor class's role has not been established clearly [13–15]. mAChR activity is increased in several gastrointestinal system cancer types, localized at the stomach, pancreas, and colon level.

The mechanisms through which cholinergic transmission can inhibit oncogenesis are triggered by M1 mAChR signaling. These receptors decrease the epidermal growth factor receptor (EGFR) and mitogen-activated protein kinases (MAPKs) level and inhibit the phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt) and mechanistic target of rapamycin (mTOR) intracellular signaling pathways.

On the other hand, the increased activation of M3 mAChR causes the triggering of some prooncogenic mechanisms, especially at gastric level, through the Hippo signaling pathway activation and nerve growth factor (NGF) increase [16–18]. The Hippo signaling, accomplished especially through the transcriptional co-activator with PDZ-binding motif (TAZ) and Yes-associated protein (YAP) factors, communicates with Wingless-Int (Wnt)/β-catenin signaling. YAP activation is associated with an unfavorable gastric cancer evolution [19] and tumor growth in hepatic cancer in 50% of cases, while TAZ overestimation appears in 80% of the breast cancer patients. The increase in YAP and TAZ signaling pathways is involved also in the chemotherapy therapeutic resistance in several cancer types [20]. Both molecular mechanisms involved in the oncological pathogenesis can be modulated through M3 mAChR activation [16]. In this context, the parasympathetic activity inhibition could decrease gastric tumor development and might improve the gastric cancer patients' prognosis.

Aim

The aim of this study has been of evaluating the parasympathetic nervous system involvement in the evolution of gastric cancer. M3 mAChR expressions have been measured in both gastric cancer patients and patients without an oncological diagnosis, through histopathological (HP) and immunohistochemical (IHC) methods. Receptor densities have been analyzed according to tumor differentiation degree, considering that this HP factor is corelated with oncological progression. The understanding of the mechanisms through which the autonomous nervous system parasympathetic pathway is involved in gastric cancer can bring benefits concerning the diagnosis, treatment, and prognosis of gastric cancer.

Patients, Materials and Methods

Patients

The present study included 77 gastric cancer patients at the Emergency County Hospital of Craiova, Romania. After computed tomography (CT) examination, surgery was performed. HP diagnosis methods were different, depending on the stage of the oncological disease. Stages I–III patients had gastrectomy surgery and were diagnosed based on the resection piece, and stage IV patients were diagnosed based on the upper gastrointestinal endoscopy (UGE) biopsy. The patients included in the study had the gastric adenocarcinoma diagnosis with different differentiation degrees and did not benefit from chemotherapy. A number of 23 patients with gastric cancer suspicion were included in the study, but the diagnosis was invalidated through UGE biopsy. The present study was done in conformity with the rules and principles of the Ethics Committee of the University of Medicine and Pharmacy of Craiova, being approved by the Ethics Committee. All the patients were informed regarding this study and signed the informed consent upon enrollment.

HP assessment

The biopsy or surgical resection tumor fragments were fixed in 10% neutral buffered formalin, subsequently being included in paraffin. The paraffin blocks were sectioned using the HM 355S microtome, with a thickness of 5 µm. The sections obtained were applied both on simple slides and on slides with poly-L-lysine, and left for 24 hours in the thermostat at 37°C. For the slides' staining, both classical and IHC stainings were used. For the classical stainings, Masson's trichrome (MT) and Hematoxylin–Eosin (HE) kits were used. The process began with removing the paraffin from the slides in three successive xylene baths, each lasting 15 minutes. Tissue dehydration was done in alcohol baths with decreasing concentrations from 100%, to 96%, 90% and 70%, five minutes each. Subsequently, the slides were rehydrated in three distilled water baths, five minutes each.

Staining was done differently, depending on the highlighted structure: the nuclei were marked with the help of Hematoxylin, the cytoplasm with the help of Eosin and for the collagen fibers MT staining was applied. After staining, the slides were passed through alcohol baths with increasing concentrations from 70% to 100%, five minutes each, and then were cleared in three xylene baths, for five minutes each. In the end, the slides were fixed with Canada balsam and analyzed with the microscope and photographed with the help of the Nikon Elipse 55i camera.

IHC assessment

For the IHC evaluation, the same steps from the paraffin removal, dehydration and rehydration protocol were done. After these steps, the antigenic unmasking was done with the help of the pH 6 citrate solution, for seven cycles, each cycle lasting three minutes, in the microwave at 650 W. Subsequently, the slides were left to cool, washed in distilled water and introduced in 3% hydrogen peroxide solution. Through this procedure, the inactivation of the

endogenous peroxidase took place. In the next phase, the slides were washed in 1% phosphate-buffered saline (PBS) solution, then introduced in powdered milk solution for 30 minutes, after which the main anti-M3 mAChR antibody NLS5259 was applied (1:10 dilution; Novus Biological, Abingdon, UK).

Further, the slides were placed in wet rooms in the refrigerator for 18 hours at 4°C. After cooling, the slides were taken out at room temperature for 30 minutes, washed in PBS and the secondary antibody was applied for an hour. After this step, the slides were washed again in PBS and the developing with 3,3'-Diaminobenzidine (DAB) was done, which made the highlighting of the specific receptor in shades of brown possible. The nuclei were marked with Hematoxylin, the slides were dehydrated with increasing concentration alcohol solutions of 70%, 90% and 96% and then introduced in xylene for clearing. The slide fixation was done with the help of Canada balsam. The tissues were marked off with the help of the hydrophobic marker.

Image processing was done by quantifying the IHC signal, also considering the HP aspect. Light microscopy images were done using a motorized Nikon Eclipse 90i microscope. An optic microscopy image was initially obtained, followed by a mixed image (the Hematoxylin and DAB color spectra were separately overlapped onto this image).

Results

The analyzed group included a number of 77 patients diagnosed with gastric cancer, which were evaluated in terms of demographics and specific oncological pathological characteristics (Table 1). Most of the patients were male (64.94%) over the age of 60 years old (53.25%). From the oncological point of view, the most cases had a tumor size less than 5 cm (54.55%), localized in the gastric body or pyloric area (84.42%), serous or neighboring organs tumor invasion T_{3-4} (59.74%), lymph node metastases in more than seven regional lymph nodes N_{2-3} (57.14%). According to the tumor–node–metastasis (TNM) staging system, 58.44% of the patients were diagnosed in the late stages III–IV.

In this study, a number of 23 patients were included, which represented the control group. From these patients, which had an oncological diagnosis suspicion, a biopsy was taken by UGE. HP examination did not highlight oncological lesions and the gastric adenocarcinoma oncological diagnosis was invalidated (Figure 1A). In terms of differentiation degree, 29 (37.66%) patients showed the well-differentiated (G1) adenocarcinoma type (Figure 1B), 26 (33.77%) of them had a moderately-differentiated (G2) degree (Figure 1C), and 22 (28.57%) patients were diagnosed with poorly-differentiated (G3) gastric adenocarcinoma (Figure 1D).

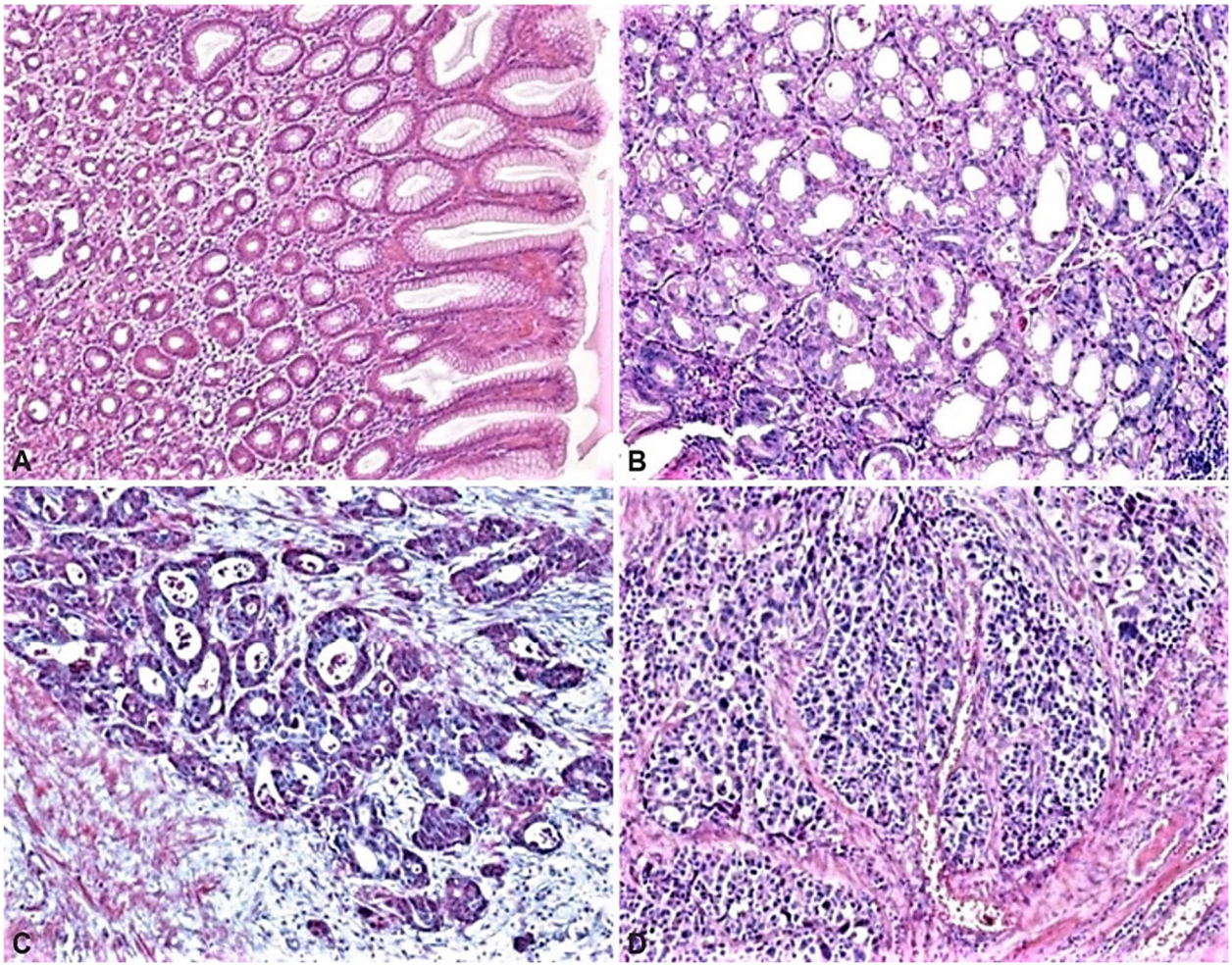

Figure 1 – ***Histopathological aspects of the biological samples from the patients included in the study: (A) Non-malignant aspect of gastric mucosa; (B) Well-differentiated (G1) gastric adenocarcinoma; (C) Moderately-differentiated (G2) gastric adenocarcinoma; (D) Poorly-differentiated (G3) gastric adenocarcinoma. Hematoxylin–Eosin (HE) staining: (A–D) ×200.***

Table 1 – ***Demographic and oncological characteristics of the gastric adenocarcinoma patients' group***

Demographic and oncological features		*n*	Percentage
Gender	Male	50	64.94%
	Female	27	35.06%
Age group [years]	<60	36	46.75%
	≥60	41	53.25%
Tumor size [cm]	<5	42	54.55%
	≥5	35	45.45%
Histology	Adenocarcinoma	77	100%
Location	Cardia	12	15.58%
	Gastric body or pyloric area	65	84.42%
Tumor invasion	$T_{1–2}$	31	40.26%
	$T_{3–4}$	46	59.74%
Lymph node metastasis	$N_{0–1}$	33	42.86%
	$N_{≥2}$	44	57.14%
TNM stage	$T_{I–II}$	32	41.56%
	$T_{III–IV}$	45	58.44%
Tumor grade of differentiation	G1	29	37.66%
	G2	26	33.77%
	G3	22	28.57%

n: No. of cases; TNM: Tumor–node–metastasis.

After corelating the HP and IHC results, a very low expression of M3 mAChR was observed in the patients with a non-malignant gastric mucous (Figure 2A). The fact that the M3 mAChR expression registered the highest density in poorly-differentiated (G3) tumors, which decreased progressively from moderately-differentiated (G2) degree to well-differentiated (G1) degree, was highlighted in the gastric adenocarcinoma patients (Figure 2, B–D).

For the gastric cancer patients' staging, CT imaging played an important role, which offered relevant information concerning the stage of the disease. In gastric adenocarcinoma patients, liver (Figure 3A) and lung (Figure 3B) metastases were highlighted, or tumor invasion in the liver, gallbladder, and pancreas (Figure 4, A–C). Imaging evaluation was also useful in the comparative evaluation of the tumor before chemotherapy (Figure 5A) and after treatment (Figure 5B).

Depending on the result, the specific treatment was adjusted according to the current stage of the oncological disease. In the early stages I–III, patients were referred towards surgery for gastrectomy, and those in advanced stage IV followed a specific oncological treatment, depending on the HP and IHC exam. The gastric adenocarcinoma macroscopic assessment was done with UGE for advanced stage inoperable tumors (Figure 6A), or after surgery from the resection piece, for operable early-stage tumors (Figure 6B).

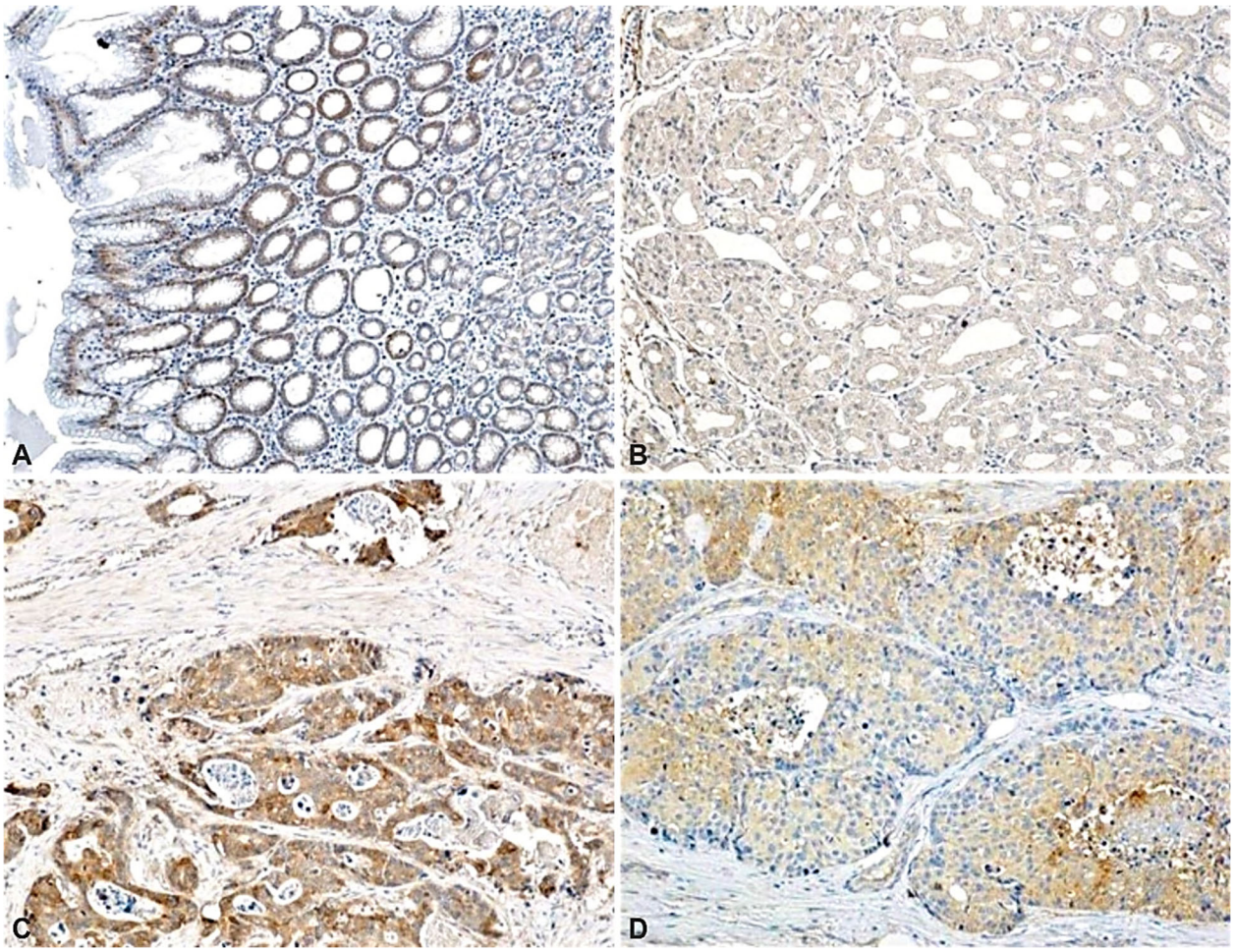

Figure 2 – ***Aspects of M3 mAChR immunohistochemical expression depending on the tumor differentiation degree: (A) Non-malignant aspect of gastric mucosa; (B) Well-differentiated (G1) gastric adenocarcinoma; (C) Moderately-differentiated (G2) gastric adenocarcinoma; (D) Poorly-differentiated (G3) gastric adenocarcinoma. Anti-M3 mAChR antibody immunomarking: (A–D) ×200. M3 mAChR: M3 muscarinic acetylcholine receptor.***

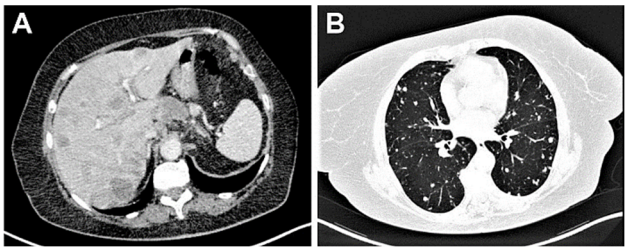

Figure 3 – *CT images highlighting metastatic lesions in a gastric adenocarcinoma patient: (A) Liver metastatic lesions; (B) Bilateral lung metastatic lesions. CT: Computed tomography.*

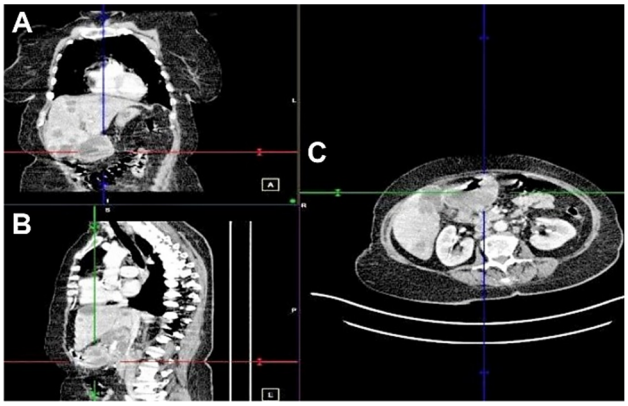

Figure 4 – *(A–C) CT image of a gastric level tissue lesion, in the pyloric antrum, with a heterogenous structure and iodophilia, with the loss of the left and right hepatic lobe, gallbladder and cephalic pancreas region cleavage space, with perilesional fat infiltration.*

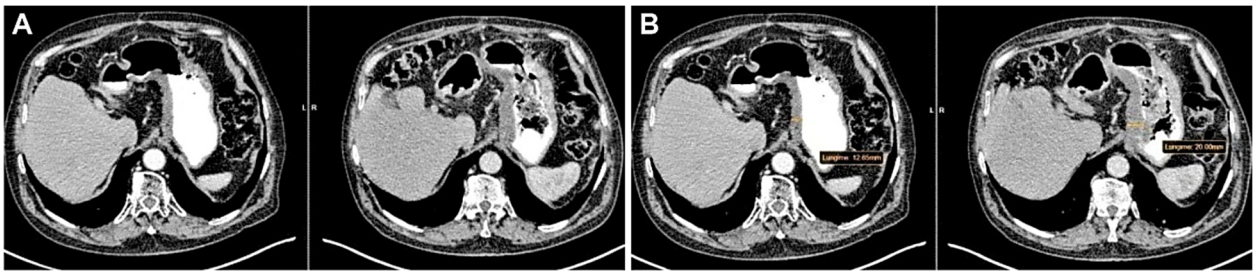

Figure 5 – *(A) Gastric adenocarcinoma CT image which highlights parietal tissue thickening, iodophile, at the small gastric curvature level with the interest of the esophageal–gastric junction, before chemotherapy; (B) Gastric adenocarcinoma CT image after chemotherapy (therapeutic response assessment).*

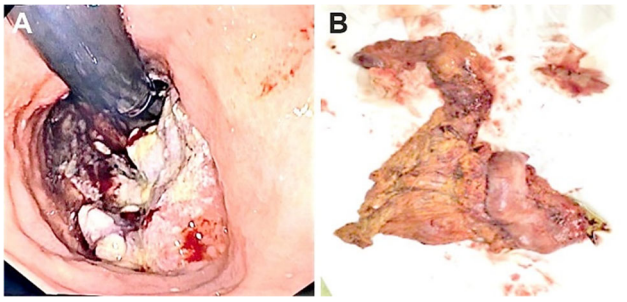

Figure 6 – *Gastric adenocarcinoma macroscopic post-operative piece: (A) Gastric adenocarcinoma macroscopic aspects; (B) UGE gastric adenocarcinoma. UGE: Upper gastrointestinal endoscopy.*

Discussions

The autonomic nervous system is perhaps the most important pathway in regulating the normal functioning of the body and is incriminated in oncological pathology, by involvement in the process of tumor development and progression, especially in gastric and pancreatic cancer, but also by promoting the spread of tumor cells with metastases [21]. The pro-oncogenic mechanisms by which the neurotransmitters of the autonomic system and the adrenergic and cholinergic receptors act, are complex and partially understood. Homeostasis of involuntary functions, at the cardiovascular, respiratory, and digestive levels, is maintained by the balance between the sympathetic and parasympathetic nervous systems. The neurotransmitters that modulate autonomic activity are norepinephrine, which acts on the postsynaptic level of sympathetic neurons, and ACh involved in the presynaptic signaling of sympathetic and parasympathetic neurons and postsynaptic signaling of parasympathetic neurons.

M3 mAChR, specific to the activity of the parasympathetic autonomic nervous system, have affinity for G proteins from the Gq/11 group and are involved at the peripheral level in regulating internal secretory processes and smooth muscle activity, and at the central level in cognitive memory and learning functions such as and in the normal development of the hypophysis gland [22].

This study showed that M3 mAChR expression was much better represented in poorly-differentiated (G3) gastric adenocarcinoma. The density of receptors decreased with the degree of differentiation, being very low in well-differentiated (G1) tumors and in normal tissue. These results suggest that increased parasympathetic activity causes a negative evolution in gastric cancer correlated with the

degree of tumor differentiation. Due to the low parasympathetic activity in normal tissue, we can say that the activation of this autonomic nerve pathway is a risk factor for the development and evolution of gastric cancer. The functioning of the stomach and especially the proliferation of epithelial gastric cells are predominantly controlled by the parasympathetic nervous system.

A particular component of the vegetative nervous system is the enteric nervous system, with specific action on the digestive system, consisting of two nerve plexuses located at the myenteric (Auerbach) and submucosal (Meissner) level, along the entire digestive tract, except the oral cavity. and the pharynx. The activity of this nervous network can be modulated by the postganglionic neurons of the sympathetic system and by the preganglionic neurons of the parasympathetic system, but it can also act independently of the activity of the central nervous system. In addition to regulating motility and gastrointestinal secretion, the enteric system is also involved in homeostasis of blood flow at this level [23]. Hyperactivity of ACh causes high levels of NGF, which favors the declassification of the gastric tumor process via the enteric nerves [17].

On the other hand, NGF hyperactivity is also involved in the process of axonogenesis which subsequently triggers a "vicious circle" by exaggerated increase in ACh. This mechanism can explain the tumor development in which the YAP and Wnt signaling pathways are involved, *via* M3 mAChR, as well as cancer pain, especially in gastric, prostate, and pancreatic cancer [24, 25].

Increased density of M3 mAChR, by activating the Wnt pathway, is associated with the degree of staging of gastric cancer. Discontinuation of parasympathetic activity in the gastric mucosa, surgically or pharmacologically, decreased tumor growth. Similar to other digestive tract cancers, the inflammatory factor plays an essential role in the evolution and prognosis of the lesion [26]. Interestingly, the inflammatory process was simultaneously reduced by inhibiting interleukin (IL)-1β and cellular signaling [27]. IL-1β can trigger gastric, inflammatory, dysplastic, and neoplastic processes, *via* nuclear factor-kappa B (NF-κB) [28]. Increased IL-1β activity is also implicated in shortening the survival of pancreatic cancer patients [29]. IL-8 also causes an unfavorably prognosis in gastric cancer [30], and negatively influences the evolution of pancreatic tumors [31], can be used as a biological marker of the evolution of these types of cancer. In gastric cancer, IL-1β, IL-8, and tumor necrosis factor-alpha (TNF-α) may be independent risk factors or associated with other factors, such as *Helicobacter pylori* infection or alcohol and tobacco consumption [32].

The oncogenic process triggered by sympathetic or parasympathetic dysautonomia can also be explained by the infiltration of the tumor by nerve fibers. Between the two structures a bidirectional influence is realized, the nervous hyperactivity determining the development of the tumor, during the tumor can generate a process of axonogenesis, which increases the risk of tumor progression and dissemination [33]. On the other hand, dysautonomia induced by stress or depression is associated with cancer recurrence and a negative prognosis [34].

The increase in the density of parasympathetic nerve endings, M3 mAChR and vesicular ACh transporter (VAChT) was also correlated with progression, unfavorable prognosis and vascular metastasis in liver cancer [35]. In prostate cancer, M3 mAChR overexpression causes castration-resistant prostate cancer (CRPC) by YAP pathway, high levels of IL-8 are associated with angiogenesis and metastases [36], while depression is a high-risk factor for unfavorable outcome [37].

In a study on animal model, the density of M3 mAChR was significantly increased in gastric malignant *versus* non-malignant cells following IHC evaluation. Vagotomy can stop the progression of gastric cancer through multifactorial mechanisms, including apoptosis, axonogenesis, MAPK and Wnt pathway [38].

Another important aspect related to the nerve–tumor binomial is the possibility of perineural invasion (PNI). This metastatic pathway, through the sheath of Schwann, may be unique or associated with the blood and lymphatic pathways. The incidence of PNI is variable in different tumor locations: 70–100% in pancreatic ductal adenocarcinoma, 6.8–75.6% in gastric carcinoma, 56–88% in biliary tract tumor, 12.4–83.6% in prostate cancer and 5.2–90% in head and neck cancer [39]. In gastric cancer, PNI metastasis is associated with a negative prognosis, regardless of the size, location and invasion of the tumor or the sex and age of patients [40]. It is necessary to monitor the risk of recurrence because it is higher in patients with gastric cancer with PNI, recurrence occurring rapidly after surgery, decreasing the survival period [41].

The neuron–tumor connection can influence tumor progression and invasion through neurotransmitters released in the perineural space. ACh, by M3 mAChR, promotes tumor proliferation and metastasis *via* Wnt pathway and leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) overexpression [42, 43]. Increased expression of LGR5 decreases overall survival in patients with gastric adenocarcinoma, especially in poorly-differentiated tumors [44].

The influence of nerve endings on tumor cells is achieved by releasing various molecules with multiple roles: neurotrophic, such as NGF and brain-derived neurotrophic factor (BDNF), axonal pathfinding modeling, or neurotransmission, especially for the autonomic nervous system (ACh, epinephrine, norepinephrine) [45].

Another important role in the development and progression of gastric cancer is played by the brain–gut axis. Parasympathetic dysautonomia and ACh imbalance induce the onset of the mechanisms of gastric oncogenesis, progression, and metastasis, by activating the Wnt pathway, increasing NGF, triggering inflammatory processes and stimulating cancer cell stemness. The increase in NGF is also correlated with the intensity of cancer pain [46, 47]. On the other hand, the Wnt/β-catenin pathway is involved in gastric cancer and resistance to chemotherapy [48] (Figure 7).

All types of mAChRs are expressed on T- and B-lymphocytes, which explains the major involvement of the cholinergic system in modulation of the immune response. A mouse knockout study showed that suppressing the activity of these cholinergic receptors causes a decrease in TNF-α and IL-6 [49]. Activation of M3 and M5 mAChRs in the cellular immune system also induces the increase of IL-2

and TNF-α [50]. The proliferation of gastric cancer is stimulated by M3 mAChR and by activating EGFR signaling pathway. EGFR inhibition suppresses the phosphorylation processes of extracellular signal-regulated kinase (ERK) and Akt, involved in the proliferation of gastric tumor cells [51].

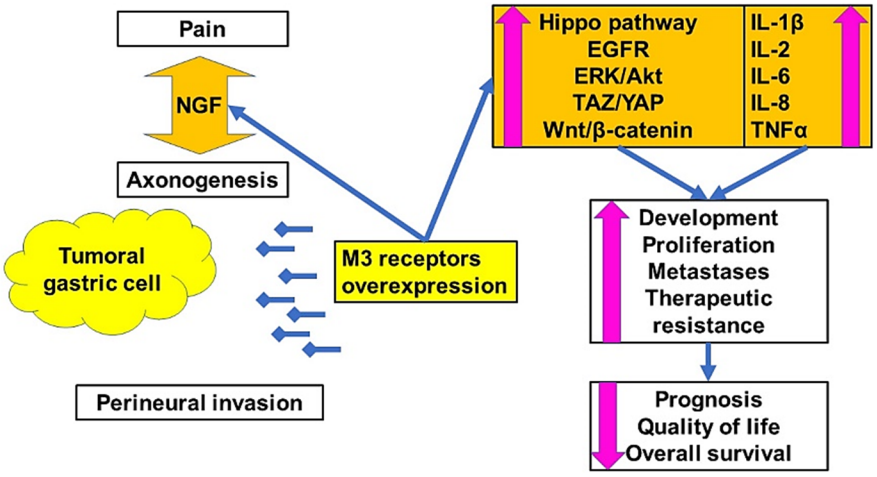

Figure 7 – ***Mechanisms involved in gastric cancer by overexpression of M3 receptors. Akt: Protein kinase B; EGFR: Epidermal growth factor receptor; ERK: Extracellular signal-regulated kinase; IL: Interleukin; NGF: Nerve growth factor; TAZ: Transcriptional co-activator with PDZ-binding motif; TNFα: Tumor necrosis factor alpha; Wnt: Wingless-Int; YAP: Yes-associated protein.***

The involvement of the parasympathetic nervous system and the overexpression of M3 mAChR in gastric cancer is correlated with the staging of the disease, tumor cell proliferation, unfavorable evolution, and metastasis. In this context, suppression of ACh activity by vagotomy or M3 mAChR antagonists or antisense therapy may bring therapeutic benefits to patients with gastric cancer [52, 53]. This approach is based on HP and IHC assessment of M3 receptors correlated with imaging examinations, such as CT and UGE, for a correct and early staging of the tumor and the tumor grade of differentiation.

In the preoperative stage, the imaging examination provides important information for the diagnosis of gastric cancer by assessing the profoundness of the gastric tumor (UGE) and the possibility of classifying the disease in one of the TNM stages, following assessment of loco-regional invasion or metastasis (CT) [54, 55]. Correlation of imaging results with HP examination increases the accuracy rate of staging, given that UGE may be underrated the grade of invasion at the level of the four layers of the gastric wall, while CT evaluation may overrate local invasion, lymph nodes or metastases [56]. In this way, it is possible to make a correct therapeutic approach, depending on the stage, as well as an evaluation of the prognosis. Preoperative evaluation UGE is a first-line method for establishing the early stage of gastric cancer, depending on which type of endoscopic surgery can be decided. CT scan used to stage gastric cancer can also be performed to assess the response to treatment [57].

On the other hand, muscarinic receptors are involved in increasing vascular endothelial growth factor (VEGF) activity, with a high risk of angiogenesis [58]. Evaluation of VEGF by IHC examination in gastric and esophageal cancer has shown the importance of this factor in tumor grade of differentiation as well as in the clinical stage of the disease. VEGF expression may occur in approximately 67% of patients with gastric carcinoma and is increased in advanced stages [59]. Increased VEGF activity has been linked to lymph node metastases and a negative prognosis in gastric cancer [60]. Another important biomarker in the clinical staging of gastric cancer may be EGFR. Increased EGFR activity by IHC assessment may alert the oncologist to the risk of recurrence, metastasis, and decreased survival [61].

From an imaging point of view, the examination of the tumor by CT texture analysis is useful for highlighting, describing, and assessing the grade of aggression of the lesions, both before and after treatment, thus determining the level of therapeutic response. The results of this type of evaluation were associated with HP factors, such as angiogenesis, hypoxia, EGFR, Kirsten rat sarcoma virus (KRAS) and the tumor grade of differentiation. In gastric cancer, CT texture has been used to assess the evolution of patients, divided into good or poor [62]. CT texture analysis can be a useful imaging marker in highlighting the tumor grade of differentiation, the depth of vascular invasion, and the level of some IHC markers present in gastric cancer [E-cadherin, Ki67, VEGF receptor 2 (VEGFR2)] [63].

In this context, the results of imaging, HP and IHC evaluations provide better accuracy in determining the degree of tumor differentiation, the staging of the disease and the prognosis of gastric cancer. In this way, personalized therapeutic strategies can be initiated, which will reduce the risk of recurrence and metastasis and increase the survival of the disease. One of the important therapeutic approaches is to inhibit the activity of M3 mAChR surgically or pharmacologically, due to the ability of these receptors to stimulate oncogenic molecular mechanisms. Early, multi-disciplinary assessment of clinical, biological, histological, and imaging markers can diagnose early-stage gastric cancer and initiate personalized treatment as soon as possible to increase the life expectancy of gastric cancer patients.

Conclusions

Hyperactivity of the parasympathetic autonomic nervous system, *via* mAChRs, especially M3, is correlated with the development, evolution, and dissemination of gastric cancer, through multiple molecular mechanisms. The unfavorable prognosis is associated with M3 mAChR overexpression, cancer stage and degree of tumor differentiation. The measurement of mAChRs expression must be correlated with the evaluation of the immune status by possible biological markers (IL-1β, IL-2, IL-6, IL-8, TNF-α), as

well as with the risk of therapeutic resistance. Subsequent research, based on the role of M3, may lead to the identification of surgical or pharmacological methods to suppress muscarinic hyperactivity. This approach can benefit the prognosis and quality of life of patients with gastric cancer.

Conflict of interests

The authors declare that they have no conflict of interests.

Authors' contribution

Alina Maria Mehedinţeanu and Cecil Sorin Mirea equally contributed to this article.

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