



MINISTERUL EDUCAȚIEI ȘI CERCETĂRII
Universitatea de Medicină și Farmacie din Craiova
ȘCOALA DOCTORALĂ

SUMMARY OF THE DOCTORAL THESIS
CHANGES OF THE AUTONOMIC NERVOUS
SYSTEM IN MALIGNANT GASTRIC TUMORS

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2022

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ABBREVIATIONS USED IN THE TEXT

ACC - American College of Cardiology

AHA - American Heart Association

AJCC - American Joint Committee on Cancer

DNA - Deoxyribonucleic acid

EBV - Epstein-Barr Virus

ECG - Electrocardiography

EDTA - Ethylenediaminetetraacetic acid

EGD - Esophagogastroduodenoscopy OR Upper GI Endoscopy

GC - Gastric Cancer

HE - Hematoxylin-eosin stain

MRI - Magnetic resonance imaging

MT - Masson's trichrome stain

mTOR - Mechanistic Target of Rapamycin

MUC4 - Mucin 4 gene

NET - Norepinephrine Transporter

VACHT - Vesicular acetylcholine transporter

VEGF - Vascular endothelial growth factor

VEGFR - Vascular endothelial growth factor receptor

1. INTRODUCTION

Gastric cancer is a malignant disease that currently represents a real public health issue due to the fact that this cancer type, despite the availability of multiple early diagnosis methods, but also of advanced treatment options, continues to be one of the most aggressive oncological diseases. This multifactor pathology is characterized both by the rapid evolution and the high number of deaths among patients with this diagnosis.

A major risk factor in this pathology is considered to be stress, which through the disturbance of the central vegetative nervous system, both sympathetic and parasympathetic, can cause the triggering of the gastric carcinogenesis process.

The involvement of the autonomic nervous system in the cardiac function of the gastric cancer patients included in the study was assessed through the study of the heart rate variability (HRV), during Holter electrocardiographic monitoring (ECG). By a histopathological (HP) and immunohistochemical (IHC) point of view, the sympathetic nervous system receptors activation was highlighted through the assessment of the norepinephrine transporter (NET), but also the parasympathetic nervous system ones, represented by the M3 muscarinic receptor, depending on the different histopathological differentiation degrees of gastric adenocarcinoma. The third assessment done in this study was biological, through the dosing of plasma metanephrines and normetanephrines from the gastric cancer patients' serum.

2. CURRENT STATE OF KNOWLEDGE

2.1. EPIDEMIOLOGY AND INCIDENCE OF GASTRIC CANCER

Gastric cancer is a malign disease with a major impact on public health worldwide. According to the International Cancer Research Agency – Globocan, in 2020, over a million new cases were reported and an increased death toll, of approximately 769.000 deaths per year, was recorded. Based on these statistics, the gastric oncological pathology ranks fifth regarding incidence and fourth regarding lethality of the total cancer cases diagnosed annually [1].

Gastric cancer is a multifactor disease, determined by various risk factors, both genetic and environmental [2], of these the most important being the *Helicobacter pylori* infection, diet and lifestyle, precancerous lesions.

2.2. DIAGNOSTIC PRINCIPLES IN GASTRIC CANCER

The gastric cancer patient's symptomatology, especially in early stages, is absent most of the times. When manifested, the clinical picture is influenced by the tumor topography, complications that appear with the progression of the disease and of course the presence of the paraneoplastic syndromes that accompany this disease.

Establishing the diagnosis of certainty is a mandatory element in oncological pathology, it being the one that guides the doctor first of all towards a therapeutic strategy, but also towards determining the prognosis and disease progression.

The paraclinical diagnosis means regarding gastric cancer are complex, this disease having upper GI tract endoscopy techniques, which allow the

highlighting of the primary tumor, but also the tissue biopsy for the subsequent determination of the histopathological diagnosis of certainty. Imaging examinations such as MRI, CT, PET-CT, abdominal ultrasound are extremely useful for establishing the true extension of the disease [3, 4].

Tumor markers are not sensible and specific enough to establish the gastric cancer diagnosis [2], being most of the times used in monitoring the oncological treatment. The main markers tracked in gastric cancer are: Carcinoembryonic antigen CEA, Carbohydrate antigen CA19-9 and Carbohydrate antigen CA72-4.

The most frequent histopathological diagnosis type for gastric cancer is the adenocarcinoma, which represents approximately 95% of the total gastric tumors [5]. The most common location if found in the antro-pyloric region (approximately 50-60% of cases), followed by the small curvature region (20%), the rest of the tumors being locate in the gastric body, the gatro-tuberous region and the large curvature [4].

The system used for the staging of cancer is the TNM system (tumor, node, metastasis) which, according to the AJCC (American Joint Committee on Cancer) staging, has the following meanings: T assesses tumor invasion or, more exactly, describes the primary tumor, N characterizes the state of the lymph nodes, and M means the presence or absence of distant metastases. This eighth edition of the staging system, developed in 2018, is presently the most used and accepted cancer assessment and staging method [6].

2.3. THE VEGETATIVE NERVOUS SYSTEM AND GASTRIC CANCER

2.3.1. The sympathetic nervous system, the norepinephrine transporter, the serum metanephrine and normetanephrine

A component of the vegetative nervous system, namely the sympathetic nervous system, is responsible for the sympathetic-adrenergic response or the fight reaction. At the adrenal glands' level, more specifically at the medulla level (central region), similar hormones called catecholamines are secreted, among which are adrenaline (epinephrine) and noradrenaline (norepinephrine). These are released in the blood stream in both physical and emotional stress periods, leading to the appearance of changes both at an endocrine and physiological level. The free serum metanephrines and normetanephrines are biologic inactive metabolites of adrenaline and noradrenaline [7].

The norepinephrine transporter (NET) is a monoamine transporter which has the main role of cellular norepinephrine intake, but also of extracellular dopamine transport. The intake process of these two mediators is important for the regulation of their serum concentrations, reason for which this mechanism is used as a therapeutic target in the case of some antidepressant medication such as reboxetine, duloxetine and desipramine [8].

2.3.2. The parasympathetic nervous system and the M3 muscarinic receptor

The parasympathetic nervous system activity, exercised through the main receptor of this system, namely the M3 muscarinic receptor, has been associated with the development, progression and dissemination of gastric cancer.

The sympathetic nervous system is involved in the 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 of the 5 subtypes of mAChR (M1-M5) are involved in the gastric homeostasis, even if the role of each receptor class has not been clearly established [9, 10]. The mAChR activity is increased in a couple of the gastrointestinal system cancer types, localized in the stomach, the pancreas and at the colon level [11].

2.4. THE HEART RATE VARIABILITY RATE IN GASTRIC CANCER

By definition, the heart rate variability (HRV) is a parameter used to measure the sinus rhythm variations in time. The variations reflect the regulatory effects of the hormonal factors and the nervous system on the sinoatrial node. The assessment of this indicator is useful through the simplicity and reliability of the quantitative evaluation of the variations and the autonomic nervous system status [12].

Based on current specialty research, the heart rate variability assessment can be used in the early diagnosis of gastric cancer, prognosis evaluation and treatment planning, or as an objective for intervening in disease prevention or slowing of progression [13].

3. PERSONAL CONTRIBUTIONS

3.1. WORK HYPOTHESIS AND GENERAL OBJECTIVES

The main purpose of this study was that of highlighting the possible correlations between the vegetative nervous system activity and gastric cancer. Based on the results obtained, an improvement of the gastric cancer diagnostic means can be subsequently tried, as well as identifying new therapeutic targets that allow the development of superior treatment strategies. The starting point was represented by the fact that gastric tumors are an important public health problem, because their development is most of the times unfortunate, even if presently there are modern diagnosis and treatment means.

The 4 major objectives of the study, concerning the activity and the influence of the vegetative nervous system, consisted of: 1. the measurement of the serum values of the serum catecholamines' metabolites (serum metanephrine and normetanephrine); 2. the immunohistochemical identification of the norepinephrine transporter (NET) density; 3. the assessment of the M3 muscarinic receptor density in the patients' neoplastic tissue depending on the cellular differentiation degree; 4. the identification of the heart rate variability through Holter monitoring of the cancer patient for 24 hours. All of these parameters have been corelated with the stage of the disease, but also with the cellular differentiation degree of the gastric adenocarcinoma.

3.1.1. MATERIAL AND METHODS

Study I. Correlations between clinicopathological aspects and the vegetative nervous system in gastric cancer

Within this study, which was the starting point of our research, the cases of four patients were analyzed, of which three were diagnosed with gastric adenocarcinoma and one patient was diagnosed with gastric lymphoma. The diagnosis was made at the Emergency Clinical County Hospital in Craiova, between November 2018 and April 2019. Patient inclusion in the study was realized following the written consent of the patient and in accordance with the Ethics Committee rules, without the patient diagnostic and treatment procedures being affected.

The diagnosis was established following the upper GI endoscopy. The samples resulted from the biopsy were histopathologically processed, but also immunohistochemically, and afterwards interpreted from an anatomopathological point of view at the Pathological Anatomy Department of the Emergency Clinical County Hospital in Craiova.

Study II. The assessment of epinephrine and norepinephrine in gastric cancer

This study was an observational one, in which 91 patients diagnosed with gastric cancer with various tumor differentiation degrees were included. The diagnosis was made following surgery or upper GI endoscopy, at the Emergency Clinical County Hospital in Craiova, between November 2017 and October 2018. Also, a control group was used, in which 200 cancer free patients were selected, from the same age group and the same gender as the cancer patients. The primary

objective of this study was mortality by any cause in the first two years after enlistment in the study.

To assess the predominance of the sympathetic or parasympathetic nervous system, the patients were monitored using a TLC5000 Holter ECG (Contec Medical Systems, Qinhuangdao, Hebei Province, China), along with an anterior analysis of the heart rate variability regarding both frequency and the domain of time. Ten patch type electrodes were applied to the patients' thorax, to which 12 ECG cables were connected. The important parameters in our study were the minimum, average and maximum heart rate, not only over the course of 24 hours, but also during the day and night.

The main antibodies used in this research were the norepinephrine transporter monoclonal antibodies (CL3063)/NBP2-62704 (1:20 dilution; Novus Biological, Abingdon, UK). In the end, the signal was identified via 3,3'-diaminobenzidine (DAB) (Dako, Glostrup, Denmark).

To quantify the target immunohistochemical signal and taking into account the histopathological aspect, the light microscopy images were obtained using a motorized Nikon Eclipse 90i microscope (Apidrag, Bucharest, Romania).

The blood samples were collected from the patients, in food rest, who were informed to avoid alcohol and caffeine for 24 hours prior to collection.

The serum metanephrines and normetanephrines were quantified through the enzyme-linked immunosorbent assay (ELISA), and the values were determined after a precipitation stage. Their reference values were the following: metanephrines: <65pg/mL and normetanephrines: <196pg/mL, with detection limits of 5 and 10pg/mL respectively.

The statistical data was obtained with the use of the Image-Pro Plus AMS 7 image graphic analysis software and were exported in Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA) and analyzed using GraphPad Prism 8 (San Diego, CA, USA). All of the results are reported as average and

standard deviations. To compare the means of the two groups we used the Student's t-test.

Study III. The acetylcholines muscarinic M3 receptors' expression in gastric cancer

The present study included a number of 77 gastric cancer patients from the Emergency Clinical County Hospital in Craiova. Following the CT examination to assess the true extension of the disease, surgery was performed.

The histopathological (HP) diagnosis methods were different, depending on the stage of the oncological disease. For the patients with stage I-III disease, gastrectomy was performed and the diagnosis was made based on the resection piece, and the stage IV patients were diagnosed based on the biopsy obtained through upper GI endoscopy/Esophagogastroduodenoscopy (EGD).

Staining was done differently depending on the highlighted structure: the nuclei were marked with the help of hematoxylin-eosin (HE), the cytoplasm with the help of eosin, and for the collagen fibers MT staining was used. After staining, the slides were passed through alcohol baths of increasing concentrations, from 70% to 100%, for 5 minutes each, and after that were clarified in three xylene baths, for 5 minutes each. In the end, the slides were fixed Canada balsam, analyzed with the microscope and photographed with the help of the Nikon Eclipse 55i camera.

3.2. RESULTS

Study I. Correlations between clinicopathological aspects and the vegetative nervous system in gastric cancer

This study's purpose was the initiation of base research. Following the results of this study, we decided an even more elaborate and thorough assessment of the impact that the vegetative nervous system has on the occurrence and evolution of gastric cancer. Another aim was the statistical analysis of age, disease location, gender, neoplastic differentiation degree and serum values of the metanephrines and normetanephrines, subsequently correlations between these parameters being achieved.

Within this study we analyzed the case of four patients with ages between 51 and 82 years old, with an age average of 69,75 years (regarding gender, three of them were men and one woman). Three of them were diagnosed with gastric adenocarcinoma and one of them with gastric lymphoma.

Study II. Assessment of epinephrine and norepinephrine in gastric cancer

Assessment of the implication of serum epinephrine and norepinephrine values in gastric cancer was done on a group of 91 patients with gastric cancer, depending on the tumor differentiation degree. Of these, 34 patients were diagnosed with well-differentiated adenocarcinoma (G1), representing 37%, 29 patients with moderately-differentiated adenocarcinoma (G2), meaning 32%, and a number of 28 patients (31%) were diagnosed with poorly-differentiated adenocarcinoma (G3).

Study III. M3 muscarinic receptors' expression in gastric cancer

The analyzed group included a number of 77 patients diagnosed with gastric cancer that were assessed concerning demographic and specific oncological pathological characteristics.

Depending on the result, specific treatment was adjusted according to the current stage of the oncological disease. In early stages I-III, patients were guided towards surgery for gastrectomy and those in advanced stage IV followed a specific oncological treatment depending on the histopathological (HP) and immunohistochemical (IHC) exams. Macroscopic assessment of the gastric adenocarcinoma was done via EGD for inoperable advanced stage tumors, or after surgery from the resection piece for tumors in early stages.

4. DISCUSSIONS

Over the course of the last decades there have been numerous studies that have shown the fact that the sympathetic nervous system activation leads to the release of catecholaminic neurotransmitters from the adrenal glands in the blood stream via the sympathetic-adrenomedullary system axis. However, the adrenal glands' role regarding the malign tumors' control is not yet fully understood, fact that has determined us to thoroughly study the effect that the sympathetic nervous system has in the development of cancer through the local release of adrenergic neurotransmitters in peripheral tissues [14].

Social or stress factors can predict the effects on health among cancer patients [15], fact proven by the recent epidemiological data which has shown that social and physiological stress can be associated with the onset of cancer [16].

Recently, following thorough research, it has been proven that individuals sufferin from chronic stress had increased neutrophils, monocytes and lymphocytes values, caused by the hematopoietic proliferation activation in the bone marrow, associated with the increase of adrenergic neurotransmitters' expression [17].

The gastric cancer patients is a patient subject to a severe stress form. The experiences generated by the stress of the disease are founded on the threat represented by the oncological disease – gastric cancer. The cancer diagnosis is associated with a disabling disease, with death, with mutilation after surgeries.

Underlining the fact that the nervous system plays an important role in the malign process through the interaction between the cancer cell and the nerve, which is explained not only through the fact that in gastric cancer the neurotransmitters lead to the progression of cancer, but also through the fact that in the tumor microenvironment the nervous elements are constantly recruited by the cancer cell, fact that leads to the start and progression of malignity [18].

The aim of this paper is to try and improve the gastric cancer early diagnostic means and also the possibility of uncovering new targeted therapies for this aggressive oncological disease.

The free serum metanephrines and normetanephrines are metabolites of catecholamines, the latter being considered a hormone that is released in the blood stream especially during periods of intense physical or emotional stress, depression or anxiety, causing both physiological alterations as well as endocrine [19], involved in the occurrence, growth and metastasis of tumors [20, 21].

We have shown in this study that the most increased metanephrines and normetanephrines serum values were in the case of poorly-differentiated gastric adenocarcinoma patients. These varied depending on the differentiation degree and increased values were found in the case of patients with tumor location in the gastric body or pyloric area and with an adenocarcinoma histopathological aspect, but also in the case of patients that had metastases in the regional lymph nodes or distant metastases.

It is well known that the sympathetic nervous system influences heart activity, causing an increased heart rate, as was shown in the case of our patients. Most of the gastric cancer patients from our study had heart rates increased directly proportional with the free metanephrines and normetanephrines serum

levels. Shi et. al recently reported that the gastric cancer severity in diagnosed patients can also be predicted by disruptions in the dynamic nonlinear patterns of the heart rate variability (HRV) [22].

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

5. CONCLUSSIONS

- Gastric cancer is an oncological disease whose diagnosis is delayed because of unspecific symptoms that do not lead to a fast diagnosis. The initial symptoms of this type of disease are quite vague in the beginning and very easily mistaken for other less serious diseases. This is the reason for which confirming such a diagnosis generates such an increased stress level, assimilated by the patient as an emotional shock.
- The sympathetic nervous system activity predominance in gastric cancer patients, through increased heart rates, increased free serum metanephrines and normetanephrines and an increased norepinephrine transporter expression in the cancer cells, is a negative prognosis factor for these patients. These observations can highlight future prognosis or therapeutic targets.
- Subsequent research, based on the M3 role, can lead to identifying surgical or pharmacological means of suppressing the muscarinic hyperactivity. This approach can benefit the gastric cancer patients' prognosis and quality of life.

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