PhD THESIS

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ROLE OF EPITHELIAL – MESENCHYMAL TRANSITION IN GASTRIC CANCER PROGRESSION AND METASTASIS

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2019
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INTRODUCTION

Gastric cancer (GC) represents a major public health problem worldwide, especially in developing countries, both by diagnosing a high number of cases annually and by the difficulty of early detection of this neoplasia, thus being an important cause of mortality worldwide.

Gastric carcinogenesis is a complex multifactorial, multistage process, with differences in production mechanisms depending on the two histopathological types, intestinal and diffuse, but with the involvement of the epithelio-mesenchymal transition (EMT) process in both forms, both in initiation, as well as in progression and metastasis. EMT is a biological process by which epithelial cells lose their polarity and cell-cell contact and acquire a mesenchymal phenotype [112]. The process is characterized by loss of epithelial markers and overexpression of mesenchymal ones, thus having a key role in the invasion and metastatic process [152]. This process involves the molecular reprogramming of cells, by decreasing the expression of E-cadherin, which maintains cell-cell adhesion and reorganization of the cytoskeleton, with the increase of N-cadherin expression under the action of transcription factors, such as SNAIL, SLUG, TWIST1 and ZEB1.

Key words: gastric cancer, epithelial – mesenchymal transition, AE1/AE3, Vimentin, E-cadherin, P-cadherin, SNAIL, SLUG, TWIST, Fibronectin.

STAGE OF KNOWLEDGE

CHAPTER I. Epidemiology and risk factors of gastric carcinomas

GC continues to be a major health problem globally as the latest statistics show that in 2018 there were 1,033,701 new cases worldwide (5.7% of all new cancer cases) [70], placing it on the 5th place after lung, breast, prostate and colon cancer, although in the last 70 years, the incidence, prevalence and mortality of GC has decreased dramatically worldwide [45]. In terms of mortality, gastric cancer is the third leading cause of cancer death for both sexes worldwide [70].

Gastric cancer is a multifactorial disease which associates both environmental and genetic factors, each having an important role in its etiology. Risk factors associated with cardiac cancer but not found in the etiology of non-cardial cancer include obesity and gastroesophageal reflux.
On the other hand, risk factors for non-cardial cancer exclusively include infection with Helicobacter Pylori, low socio-economic status, and dietary factors such as reduced fruit and vegetable consumption and excessive consumption of smoked and salty food [118].

CHAPTER II. Gastric carcinogenesis

The two histological types according to Lauren classification, must be regarded as two different entities, with different etiology: the predominance of environmental factors in the intestinal type and the individual genetic profile in the diffuse type [225]. Professor Pelayo Correa proposed in 1975 a model for the development of intestinal type gastric cancer according to which the precursor lesions of the malignancy develop in sequential stages starting from chronic atrophic gastritis, later passing through the stages of intestinal metaplasia and dysplasia, offering the basics in understanding the role of various environmental and constitutional factors in gastric carcinogenesis [41]. Another model of gastric carcinogenesis was proposed by Sipponen et al., which describes the differences between the pathogenesis of intestinal and diffuse gastric cancers, the major role of genetic factors in the appearance of the diffuse form being observed [217].

CHAPTER III. Epithelial – mesenchymal transition and gastric carcinomas

More and more evidence has been presented to support the theory that, during the EMT process, epithelial cells do not only acquire mesenchymal phenotype but also a stem cell phenotype, a feature called "stemness". It appears that these induced CSCs-TEMs would be major contributors to gastric carcinogenesis. By immunohistochemical methods, Ryu et al. noted the presence of EMT-specific proteins (Snail1, ZEB1, vimentin, β-catenin) as well as the marker CSCs CD44 in 276 cases of primitive gastric carcinomas and in 54 lymph node metastases, proving that CD44 is significantly associated with the expression of Snail1, ZEB1 and E-cadherin [199]. EMT process is not only involved in gastric carcinogenesis but also in progression and metastasis, giving tumor cells invasive and metastatic properties, thus preventing aging and apoptosis [184]. E-cadherin mutations play a major role in initiating gastric carcinogenesis and inducing the specific characteristics of gastric cell malignancy. The process of EMT in gastric cancer has as triggers various transcription factors that subsequently activate a complex network of signaling pathways that interact as well, actively regulating the process of EMT.
PERSONAL CONTRIBUTIONS

PURPOSE AND OBJECTIFS OF THE STUDY

The present study aims to evaluate the involvement of EMT in gastric carcinogenesis, in order to identify new prognostic factors and possible therapeutic targets. The identification of the relationships between the expression of the various markers analyzed with the tumor type or stage, can outline new criteria for stratification of gastric carcinomas in order to apply personalized therapies.

CHAPTER IV. Material and methods

The study was conducted retrospectively and prospectively, over a period of 3 years (2017 - 2019), included 95 cases of gastric carcinomas and followed the analysis of the clinical, epidemiological, imagistical, histopathological and immunohistochemical parameters of patients with such tumors.

The clinical and epidemiological study provided data on: year of diagnosis, sex, age, patients’ origin, risk factors, time period from onset of symptoms to diagnosis, frequency of clinical manifestations, topography, shape and size of lesions.

The histopathological study of the analyzed gastric carcinomas aimed to identify the main histopathological parameters related to the prognosis: the histological type and degree of tumor differentiation, depth of invasion, lymph nodes status, the presence/absence of distant metastases, the presence/absence of the tumor, tumoral stage, the presence/absence of vascular and perineural invasion.

The immunohistochemical study aimed to evaluate the expression of some markers involved in EMT in GC, such as AE1/AE3, Vimentin, E-cadherin, P-cadherin, SNAIL, SLUG, TWIST, Fibronectin.

Results were subjected to statistical analysis and statistical tests were used to evaluate the differences between the immunoreactivity scores obtained for each case, considering a significant difference at $p < 0.05$ level.

CHAPTER V. Results

The clinical-epidemiological study showed that the highest incidence of gastric carcinomas
was observed after the age of 50, when 86 cases were registered, which represented 90.5% of the investigated cases. The maximum incidence was registered in the VIIth decade of life, over half were male (69.4%) and the risk factors correlated with the occurrence of GC were: environmental origin (54.7% urban area), diet rich in salt (54.7%), in red meat (45.2%) and smoker status (91.3%); Among the preexisting lesions associated with gastric cancer, the most commonly involved were: chronic gastritis (38.9%), gastric atrophy (23.1%) and gastric polyps associated with high-grade dysplasia (13.6%).

Helicobacter pylori infection was significantly associated with non-cardial gastric carcinomas (67.3%). Of the clinical manifestations, the most frequent were the lack of appetite and selective anorexia (87.3%), as well as epigastric pain (54.7%). Endoscopic analysis revealed the presence of early gastric carcinoma in a small number of cases (24.2%), with the predominance of protrusive type I (11.5%) and excavated type III (5.2%); the most common forms of advanced gastric carcinoma (75.7%) were Borman I (32.6%) and Borman II (27.3%) type.

Histopathological analysis revealed the predominance of low-grade tubular gastric carcinomas (35.7%), the majority in advanced stages of the disease (II and III), regardless of the histopathological subtype; thus, we found 8.4% carcinomas corresponding to stage I, 45.2% corresponding to stage II, 42.1% corresponding to stage III, 4.2% corresponding to stage IV. The lympho-vascular invasion was detected in 31.5% cases, including all histological subtypes, but with a higher incidence in the case of tubular (17.8%) and discohesive (7.3%) GC and the perineural invasion was detected in 30.5% cases, also including all the histological subtypes except for the mixed mucinous and discohesive carcinomas, but with a higher incidence among the low grade tubular (9.4%) and discohesive (7.3%) gastric carcinoma.

Immunoreaction for AE1 / AE3 was identified in all investigated cases (100%), in the epithelial component of tumors, regardless of tumor grade or stage, at cytoplasmic and membranous level.

Immunoreaction for vimentin was identified in 18 of the investigated cases (36%), present only in discohesive and mixed carcinomas, regardless of tumor stage. Immunostaining was identified in the cytoplasm of the tumor cells, as well as in the stromal elements. The mark intensity had high values for stage II, III and IV tumors.

Immunoreaction for E-cadherin was identified in 31 of the investigated cases (62%), in both high- and low-grade gastric carcinomas, for stage I, II and III tumors. Immunostaining was
identified at the apical membrane and/or cytoplasmic level. For the whole analyzed group the intensity of E-cadherin reactions for positive cases was moderate or increased.

Immunoreaction for P-cadherin was identified with variable incidence in 29 of the investigated cases (58%), in both high and low gastric carcinomas. Immunostaining was identified at the membrane level or at the cytoplasmic and membrane level. Analysis of P-cadherin expression regarding percentage and marking intensity for the investigated tumors, indicated composite scores with both low and high values for the analyzed gastric carcinomas, depending on the tumor grade and stage.

Immunostaining for Twist was identified in 27 of the investigated cases (54%), in both high and low grade gastric carcinomas, regardless of the tumor stage. Immunostaining was identified at the cytoplasmic or nuclear level. From the percentage point of view and the marking intensity, we identified predominantly high values in relation to the tumor grade and stage. The intensity of Twist reactions was variable, with a number of cells marked between 35-90% and a mean value of 56.2 ± 17.5

Immunostaining for Snail was identified in 21 of the investigated cases (42%), both in high and low gastric carcinomas, regardless of the tumor stage. Immunostaining was identified only at cytoplasmic and nuclear focal level. The intensity of the reactions for the analyzed group was variable, with a number of labeled cells between 25-80%, with an average value of 53.8 ± 17.3

Immunostaining for Slug was identified in 13 of the investigated cases (26%), in both high and low grade gastric carcinomas. The positivity of the reaction was identified only in the tubular carcinomas of stages I, II and III. Slug immunostaining had low or moderate intensity, with 15-55% marked cells and a mean value of 33.3 ± 11.6.

Fibronectin immunoexpression was identified as vimentin in both epithelial and stromal components. The stromal component was positive in all 50 cases investigated. In the epithelial component of tumors, fibronectin positivity was identified in 17 of the investigated cases (34%). Tumor cell immunostaining was quantified in the cytoplasm. For the whole group, the intensity of the markings was variable, with a number of marked cells between 20-70% and a mean value of 53.5 ± 15.5.

CHAPTER VI. Discussions

Multiple international studies have shown that the incidence of gastric cancer increases
with age, reaching the maximum between 50 and 70 years [45] and worldwide it is accepted that the incidence of gastric cancer is twice as high in men compared to women [118], facts observed in the present study also. Numerous studies showed that increased salt intake is associated with increased risk of gastric cancer. For example, D'Elia observed in a meta-analysis performed on 268,718 patients the relationship between salt consumption and gastric cancer incidence [53]. There are more and more arguments in favor of increased consumption of red meat and the development of gastric cancer. Bertuccio et al. demonstrated a two-fold increased risk for the development of gastric neoplasia in patients with diet based on red meat compared to high fiber diet [17].

Helicobacter Pylori infection plays a predominant role in gastric carcinogenesis, especially in non-cardial location, with a 5.9-fold higher risk of developing non-cardial neoplasia compared to cardial neoplasia [84]. In a study that evaluated the correlation between chronic gastritis topography in Helicobacter Pylori positive patients and the risk of gastric cancer, the risk reduction was found in the following order: pangastritis with atrophy in the body - predominantly bodily gastritis - pangastritis without bodily atrophy - gastritis without bodily atrophy predominantly antral, thus suggesting that not only the degree of atrophy is important but also the topography of gastritis [101].

The incidence of early gastric cancer in our study was quite small, 24.2% of which type I and type III were predominant, with incidences of 11.5% and 5.2% respectively.

Early gastric cancer was defined more than 30 years ago in Japan, where there is a high incidence of gastric neoplasms, which has led to the need for effective screening programs to detect gastric tumors as quickly as possible. In Japan, between 30% and 50% of gastric cancer cases are detected in early stages, and in the US between 8% and 25% [35].

Regarding the histopathological subtype and the differentiation degree, the results of our study are in agreement with those of the literature: tubular adenocarcinoma is the most common histological type of gastric cancer, followed by discohesive carcinoma found in 18% of all gastric carcinomas, papillary adenocarcinoma in 6% -11% and the lowest incidence belongs to mucinous carcinoma in 2-6% of all malignant gastric tumors [131]. In a recent study conducted in China, of the 240 cases of early gastric cancer, limited to the mucosa and submucosa, the only histological types encountered were tubular and papillary carcinoma [284], similar to the results of our study. Increased tendency of lymphatic, vascular and perineural invasion directly proportional to the
decrease of the differentiation degree observed in our study was also noted by Li P. et al. Who observed a positive correlation between the presence of lympho-vascular invasion and the degree of differentiation, parietal invasion, lymph node invasion, distant metastases and TNM stage [133].

In several studies, the genesis of gastric carcinoma is closely related to EMT. Among specific changes of EMT process are the expression of some genes and corresponding proteins, including cytokeratin (CK) and vimentin, as well as adhesion molecules. Also, in the EMT process the loss of E-cadherin, a major component of the adhesion junctions, occurs concomitantly with the acquisition of mesenchymal markers such as vimentin, N-cadherin and fibronectin. In addition, TGF-β1, Twist, Snail, Slug and vimentin levels are overexpressed in patients with early gastric dysplasia or cancer, while E-cadherin levels are low [36]. Transcriptional repression of E-cadherin is mediated mainly by transcription factors related to the Snail family (SNAIL1), homeobox-2 (ZEB2) and the helix-base-helix-base (TWIST) family [184]. Snail expression is significantly correlated with the expression of ZEB2, TWIST (Twist1 and Twist2) and N-cadherin (CDH2), which also interact with each other. As major E-cadherin repressors and EMT inducers, Snail and Slug play a critical role in the invasion and metastasis of many human cancers [182], [173]. A more recent study investigating the relationship between serum fibronectin levels and prognosis indicated its utility only as a diagnosis marker in gastric cancer patients [232].

**CHAPTER VII. Conclusions**

The present study, which included 95 cases of GC, showed the following conclusions:

- The clinico-epidemiological analysis showed a constant increase every year, with the highest incidence after the age of 50 (maximum incidence in the 7th decade of life - 43.1%), regarding especially the male sex (69.4%), with a male / female ratio of 2/1;
- The risk factors of gastric carcinoma are related to the origin environment (54.7% urban area, in relation to processed foods), salt-rich diet (54.7%), red meat diet (45.2%) and smoker status (91.3 %). Among the preexisting lesions associated with gastric cancer, the most commonly involved were: chronic gastritis (38.9%), gastric atrophy (23.1%) and gastric polyps associated with high-grade dysplasia (13.6%);
- Helicobacter pylori infection was significantly associated with non-cardial gastric carcinoma cases (67.3%);
- Of the clinical manifestations, the most frequent ones were the lack of appetite and selective anorexia (87.3%), as well as epigastric pain (54.7%);
- Endoscopic analysis revealed the presence of early gastric carcinoma in a small number of cases (24.2%), with the predominance of protrusive type I (11.5%) and excavated type III (5.2%); the most common forms of advanced gastric carcinoma (75.7%) were type Borman I (32.6%) and Borman II (27.3%);
- Histopathological analysis for the 95 cases analyzed revealed the predominance of low-grade tubular gastric carcinomas (35.7%);
- Tumors classification according to pTNM staging system showed that most cases of gastric carcinoma analyzed in our study were found in advanced disease stages (II and III), regardless of the histopathological subtype; thus, we found 8.4% carcinomas corresponding to stage I, 45.2% corresponding to stage II, 42.1% corresponding to stage III, 4.2% corresponding to stage IV.
- The lymphovascular invasion was detected in 31.5% cases, including all the histological subtypes, but with a higher incidence in the cases of tubular gastric carcinomas (17.8%) and discohesive carcinomas (7.3%);
- Perineural invasion was detected in 30.5% cases, also including all histological subtypes except for mixt mucinous and discohesive carcinoma, but with a higher incidence among low grade (9.4%) and discohesive (7.3%) gastric carcinoma;
- Regarding the cases of tubular and papillary carcinoma, we found that the lymphovascular and perineural invasions were mainly associated with low degree of differentiation;
- Immunohistochemical analysis regarding the AE1 / AE3 immunoexpression indicated a decrease in the number of cells marked cells along with the stage increase and in tubular and mixed carcinomas, while the expression of Vimentina was associated with high stage and degree and with discohesive and mixed carcinomas;
- The decrease of AE1 / AE3 expression in the tubular lesions and the presence of Vimentin in the discohesive and mixed ones, as well as the association of these patents with the advanced stage and high degree support the involvement of EMT in gastric carcinogenesis;
- The absence of AE1 / AE3 expression relationship with Vimentina may suggest different patterns of involvement in gastric EMT of the two mechanisms, either by disturbing the expression of epithelial markers, or by overexpression of Vimentina, or both, depending on the tumor type and tumor progression;
Low or absent immunoexpression of cadherins was associated with advanced stage, high grade and discohesive and mixed carcinomas, with positive linear correlation between E-cadherin and P-cadherin;

Vimentin immunoexpression was negatively correlated with cadherin markers, which supports the association of loss of intercellular adhesion with the acquisition of the mesenchymal phenotype in gastric carcinomas;

Twist markings showed high scores associated with advanced stages, high grade and discohesive and mixed carcinomas. Twist immunoexpression was cytoplasmic in case of stage I tumors, cytoplasmic and nuclear in stages II-III and only nuclear in stage III, an aspect that indicates the nuclear translocation of the protein during tumor progression;

Snail immunoexpression indicated the association of high scores with stages II-IV, high grade and discohesive carcinomas. Nuclear markings were present focally only in cases of stages II-IV tumors;

Slug reactions showed positivity in association with tumor stage I, low grade and tubular type gastric carcinomas;

In this study, high grade carcinomas in the advanced stages were associated with Twist and Snail markings while low grade lesions in the early stages were associated with Slug markings. While Twist expression was associated with mixed and discohesive carcinomas, Snail markings were more commonly associated with discohesive carcinomas and Slug markers with tubular carcinomas.

High scores of Fibronectin immunoexpression have been associated with advanced stage and high-grade carcinomas, most of which were discohesive type;

The study indicates different patterns of the molecular mechanisms involved in gastric EMT both in terms of loss of the epithelial phenotype, as well as the acquisition of the mesenchymal one and in relation to the involvement of transcription factors, while the loss of intercellular adhesion is invariably present.

The results obtained in this study can be used both for understanding the gastric EMT mechanisms, as well as for addressing potential therapeutic targets and improving the stratification criteria of patients for personalized therapy.
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