

**THE UNIVERSITY OF MEDICINE AND PHARMACY  
CRAIOVA  
PhD SCHOOL**

# **PhD Thesis Abstract**

**Pathogenic implications of EGF and TGF growth factors as well  
as of their receptors in gastric carcinomas**

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## INTRODUCTION

The digestive tube cancers present an increased incidence nowadays, their etiology being connected both to genetical predisposition and to environmental factors. Among these, the gastric cancer, although having a decreasing incidence, especially in the western countries, as compared to the colon and esophageal cancers, maintains an increased severity and mortality. Paralleled to the increase of esophageal cancer incidence we presently note an enhancement of the gastric cardia cancer forms frequency and a decrease of the antropyloric forms. This evolution is related first of all to the modification of the nutrition and life style in the western countries.

The elaborated study brings clear arguments on the fact that a complex immunohistochemical exploration of the tumor tissue is useful in evaluating the disease prognostic and evolution. Thus, the investigated intestinal-type gastric adenocarcinomas associated to an overexpression in the EGF factor, and in almost 50% of the cases, also to an overexpression of its HER2 and EGFR1 receptors. It seems that the future in gastric carcinogenesis research is represented by the immunohistochemical and genetic evaluation of the tumor tissue, our study representing a small step in this sense. We hope that further research will clarify many aspects remaining still unclear, aspects that will allow the prevention and increase of the antineoplastic therapy efficiency with beneficial consequences for patients' survival chances increase.

**Keywords:** gastric adenocarcinoma, epidemiology, histopathology, immunohistochemistry, growth factors, EGF, EGFR1, HER2 neu, TGF- $\beta$ 1, TGFBR1, Ki67

## STAGE OF KNOWLEDGE

**CHAPTER I – "Epidemiology and risk factors in gastric cancer"** – this chapter presents gastric cancer epidemiology worldwide and in Romania. We insist on repartition according to sex, age, and according to location (cardia and pyloric forms). We elaborate a survey of risk factors in gastric cancer, with special mentions for the influences of nutrition, helicobacter pilory infection, smoking, obesity and diseases with increased risk (operated stomach, Biermer anaemia, gastric polyposis) on gastric carcinogenesis. The role of heredity and family history in this process is also mentioned.

**CHAPTER II – "Gastric carcinogenesis"** – presents the main theoretic patterns attempting to explain gastric tumors growth (Correa pattern). A survey of the main molecular events involved in gastric carcinogenesis (p53 gene, microsatellites instability, cyclins, vascular, hepatocellular and fibroblast growth factors, etc.) was also included.

**CHAPTER III – "Gastric carcinomas classification and staging"** – presents gastric cancer macroscopic classification (early and advanced gastric cancer) and gastric cancer microscopic classification with emphasis on the OMS, Lauren and Goseki classifications.

## RESEARCH SCOPES

The present study proposes a complete and detailed evaluation of the growth factors (EGF, EGFR1 and HER2 neu, TGF- $\beta$ 1 and TGFBR1, Ki67) involvement in gastric carcinogenesis, in order to identify potential prognostic and therapeutic targets. The identification of complex mechanisms that take place at a molecular level and of the

interaction between them supplies valuable and useful information concerning tumor initiation, progression, and patients' prognostic. The present study evaluates the markers involved in all stages of gastric carcinogenesis starting from the pre-invasive stages to the invasive and metastatic disease.

For this purpose, we observed the achievement of the following objectives:

- The extension of knowledge to the clinical, endoscopic, histopathological and immunohistochemical features of gastric carcinomas related to tumor phenotype, for the carcinogenic mechanisms study;
- The establishment of a database with the recording of the main clinical, histopathological and immunohistochemical parameters for the patients taken into consideration in the study;
- The identification and definition of the endoscopic parameters characterizing gastric carcinomas, for their subsequent correlation to the histopathological and immunohistochemical data, in order to select the most reliable prognostic markers;
- The identification and definition of the histopathological parameters characterizing gastric carcinomas, in order to apply an early and differentiated therapy by means identifying the potential molecular targets;
- The identification of the mechanisms by means of which operate the different growth factors involved in the invasion and aggressiveness of gastric carcinomas and the identification of the most specific markers of prognostic in the invasive and metastatic gastric carcinomas.

## **PERSONAL RESEARCH**

**CHAPTER IV –"Material and Method"**– supplies information on the studied material and on the methods used in research.

- The studied biological material was represented by human material, comprising a number of 464 cases selected during a 5-years period (2008-2012), from patients hospitalized in the Medical and Surgery Clinics of Emergency Clinical County Hospital in Craiova. The 464 cases supplied the biological material represented by tissue fragments obtained after endoscopy followed by biopsy with diagnosis purpose, or by surgical specimens represented by one or several or portions of anatomic structures (stomach, ganglions, caul, liver, etc). Also, we used the surgery logs and the anatomo-pathological registers which, with the clinical observation sheets allowed the procurement of data such as epidemiological, clinical, macroscopic aspect of the surgery excision parts (shape, dimensions, number, consistency) and the histopathological diagnostic for the retrospectively studied cases.

- Methods used. In all investigated cases we were interested in the clinical and epidemiological data, as well as in the information obtained as a result of the endoscopy, in the histopathological and immunohistochemical parameters, data recorded in the standardized sheets and then stored in electronic databases using the Microsoft Access 2003 platform and that represented the basis for the elaboration of the patients' groups that were analyzed statistically.

The biological material was stabilized in buffered formalin 4%, then processed by the usual technique of inclusion in paraffin and colored with hematoxylin-eosin. The histopathological analysis was performed on a group of 464 gastric carcinomas confirmed microscopically and included the following evaluation criteria: analysis of gastric carcinomas type and histological degree; evaluation of invasion profoundness; lympho-ganglionar metastases; metastases in remote structures; invasion of sanguine/lymphatic vessels and perineural invasion; status of the limits of surgical resection.

For the immunohistochemical study we investigated a number of 25 cases of gastric carcinomas. We used the paraffin blocks from which were extracted the histopathological sections that were processed by the LSAB (Labelled Streptavidin-Biotin2 System) technique. We used concentrated antibodies developed in mouse or rabbit directed against man, whose main characteristics are presented in the table below (Table 1).

**Table 1. Antibodies used in gastric carcinomas study**

ANTIBODY	CLONE	ANTIGENIC EXPOSURE	DILUTION	POSITIVE CONTROL
<b>TGF-β</b>	TB21	citrate pH 6	1:1000	Hepatocellular carcinoma
<b>TGFBR1</b>	T-19	citrate pH 6	1:300	Hepatocellular carcinoma
<b>Ki67</b>	Mib-1	citrate pH 6	1:50	Invasive ductal breast carcinoma
<b>EGF</b>	-	citrate pH 6	1:200	Parotid gland
<b>EGFR</b>	-	citrate pH 6	1:100	Oral squamous carcinoma
<b>c-erbB-2</b>		citrate pH 6	1:200	Invasive ductal breast carcinoma

The statistical analysis was performed with the help of Microsoft Excel (Microsoft Office 2007) programme package, by using the test –"  $\chi^2$ " (chi- square) for the emphasis of the significant differences between the various category groups of investigated patients.

**CHAPTER V –"Findings" and CHAPTER VI –"Discussions"** present the findings within our research as compared to the recent literature data.

- From the clinico-epidemiological and endoscopic study of gastric carcinomas we note that the incidence of stomach cancer increases with age, reaching the climax at the 60-80 age decade [15], thing that we also noticed, the percentage of patients diagnosed with gastric cancer in this age decade being of 62.5%. The increased consumption of red meat for more than 5 days/week correlates with digestive cancers risks increase. In our study, the number of vegetarian patients was quite reduced (0.6%), and those with an increased consumption of red meat represented an important weigh factor (38.8%). The results being in conformity with those in the EPIC study that brought arguments on the gastric cancer risk increase for the red meat consumers [7].

Gastric atrophy and chronic gastritis associated with an increased number of patients diagnosed with gastric cancer in our study. Gastric atrophy was documented in 29.3% of the cases, and chronic gastritis in 38.4% of the cases.

The infection with *Helicobacter pylori*, a class I carcinogen, is the most important risk factor known for gastric cancer [17], especially for the non-cardia located carcinomas. We also noticed it, the percentage of the patients infected with this germ presenting non-cardia gastric cancer being 65.8% (263 cases), as compared to 18.8% (12 cases) patients with cardia gastric cancer.

From the point of view of clinical manifestations we observe a particular frequency of the dyspeptic phenomena, especially inappetence and anorexia (82.1%) and epigastric aches (52.4%). Also, especially in the cardia gastric cancer forms, heartburn (42%) and dysphagia (22%) were present in a significant proportion. Nausea and eructation were signaled as well in 43.3% of the cases. The diagnostic in the stage with hepatic secondary determinations proves

deficiencies either related to diagnostic, or to the ignorance of patients' who come to see a doctor in late stages. The results are in conformity with those mentioned in literature [18, 3]. The analysis of the endoscopic data in gastric cancer patients shows in 36.9% of the cases the location at gastric antrum level, 24.1% location at lesser curvature level, 13.8% at cardia level and 10.1% at fornix level. Locations with a more reduced percentage have been noticed at lesser curvature level, 7.1% of the cases, at stomach anterior wall level 4.1% of the cases and at stomach posterior wall level, 3.9% of the cases. An increase of the proximal forms (cardia, fornix) incidence can also be observed in our country in conformity with the present trend noticed in western countries [10, 2].

The histopathological analysis performed on a series of 464 gastric carcinomas selected during a 5-years period (2008-2012), indicated a net predominance of the intestinal-type gastric carcinomas as compared to the diffuse ones. In this sense, by using Lauren's (1965) classification, we found a number of 376 (81%) cases of intestinal-type gastric carcinomas against only 88 (19%) cases of diffuse gastric carcinomas in conformity with the recent research [13, 16; 14]. Casuistry classification in the clinical-pathological stages (pTNM categories) indicated the following incidents: stage I (A and B): that included 7.8% of the intestinal-type carcinomas and 17% of the diffuse carcinomas; stage II: represented by 33.5% of the intestinal type tumors and a more reduced proportion of diffuse type carcinomas (22.6%); stage III (A, B and C): that included most of the intestinal-type carcinomas (50.4%) and a significant number of the tumors with signet ring cells (47.2%); stage IV: represented by 13.2% of the diffuse-type tumors and 8.3% of the intestinal-type tumors.

- The immunohistochemical study aimed at the expression of TGF- $\beta$ , TGFBR1, Ki67, EGF, EGFR, c-erbB-2 in relation to the markers involved in tumor progression, as well as to some of the histopathological prognostic parameters for gastric tumors. The researched material was represented by 25 cases of gastric carcinomas. The immunohistochemical analysis of the growth factors in gastric cancer leads to a better understanding of the gastric carcinogenesis mechanisms, fact that can contribute to the development of aimed molecular therapies able to point at processes of cellular differentiation, proliferation and survival [8].

Our findings show the presence of a reactivity for both markers (TGF- $\beta$ 1 and TGFBR1) in all three investigated cases of diffuse gastric adenocarcinoma, but in that concerns immunoreactivity for TGF- $\beta$ 1 in the 25 studied cases of intestinal-type adenocarcinomas we noticed a heterogeneous pattern of the reaction, with reactivity differences depending on the tumor differentiation degree as well as on the tumor invasion profoundness [6].

In our research we found that 88% of the intestinal-type gastric adenocarcinomas were immunoreactive for EGF, and 52% of them presented an enhanced reactivity [5]. In the case of immunoreactivity to EGF we have been able to establish only one correlation, namely to the tumor differentiation degree. Concurrently, we have not been able to emphasize the existence of a correlation between the expression of EGF and that of its receptors c-erbB-2 and EGFR in the investigated cases of gastric carcinoma. On the contrary, Czyzewska and collab. found a significant correlation between the expression of c-erbB-2, EGF, and EGFR at tumor specimens level and the lympho-ganglionic metastases [4].

According to the literature data, the rate of immunoreactivity for HER2 in gastric cancer varies between very wide limits, thus, in the immunohistochemical type determinations these reached 6.8% - 34%, while the FISH-type investigations reported variations between 7.1% and 42.6% [9]. In our research, the immunoreactivity rate for HER2 was 32%, with most of the cases presenting an immunoreactivity score 2 [5].

According to the literature data, the frequency of the expression EGFR1 and/or of EGFR1 gene amplification in gastric cancer varies between 0% and 35% [1; 11; 12]. In our research, EGFR1 immunoreactivity percentage was higher, respectively 46.4% [5], approaching to the levels reported by Czyzewska and collab. (54%) or Yk and collab. (52.2%) [4; 19].



**CHAPTER VI –“Conclusions”** presents research conclusions.

- The risk factors most significantly correlated to the gastric cancer occurrence in our research were represented by the following: provenance environment (61.85% of the patients coming from the urban environment in what concerns the processed nutrition style) and diet rich in salt (42.2%) and in red meat (38.8%). Concerning the pre-existing lesions most frequently associated to gastric cancer these were gastric atrophy (29.3%), chronic gastritis (38.4%) and flat gastric dysplasia (21.1%) and that associated to gastric polyps (6.7%).
- The association with the *Helicobacter pylori* infection was significant for the non-cardia gastric cancer cases (65.8%) as opposed to the cardia gastric carcinomas (18.8%).
- The most frequent clinical manifestations were the dyspeptic phenomena, especially inappetence and anorexia (82.1%) and epigastric aches (52.4%).
- The early gastric cancer limited to mucous or sub-mucous was diagnosed in our research in 15.5% of the cases with predominance of the types 0I protrusive and 0IIa prominent superficial. The most frequent endoscopic forms of advanced gastric cancer (84.5%) are the type I Bormann - polypoid form (29.5%) and the type II Bormann – ulcerative form (29.2%).
- Histopathologically, the intestinal-type (Lauren) gastric carcinomas were predominant with a net superior percentage of 81% of the cases, with the greatest incidence in the 7<sup>th</sup> decade of life (47.6%) and predominantly affected the male sex (65.7%)
- Related to the differentiation degree, among the intestinal-type gastric carcinomas the poorly differentiated tumors were predominant (49.2%), followed by the moderately differentiated ones (in a percentage of 39.4%) and by those well-differentiated (11.4% of the cases)
- The diffuse –type gastric carcinomas (Lauren) constituted 19% of the cases, with a slight predominance in the male sex (59.1%) and with the most increased incidence in the 6<sup>th</sup> decade of life (42%); as opposed to the intestinal-type carcinoma, in this type of tumor we noticed an incidence increase in the female sex (proportion male /female 1.91/1 for the intestinal type versus 1.44/1 for the diffuse type)
- Among the morphological parameters evaluated in gastric carcinoma in our research, the muscular invasion was present in 23.2% of the intestinal-type carcinomas, being predominant in the poorly differentiated forms, the lymphatic invasion was more frequent in the diffuse-type tumors (55.9% versus 39%), while the perineural invasion was predominant in the intestinal-type carcinomas (37.7%).
- The residual tumor was identified more frequently in the diffuse type carcinomas (50% of the cases), comparatively to the intestinal-type carcinomas (27.9%) in which it associated predominantly to poorly differentiated tumors, involving predominantly the proximal resection limit in both types of tumors.
- The clinical-pathological stages (pTNM) were the following: stage I (A and B) that included 7.8% of the intestinal type carcinomas and 17% of the diffuse-type ones; stage II was represented by 33.5% of the intestinal-type tumors and a more reduced proportion of the diffuse type carcinomas (22.6%); stage III (A, B and C) that included most of the intestinal-type carcinomas (50.4%) and a significant number of the tumors with signet ring cells (47.2%); stage IV was represented by 13.2% of the diffuse-type tumors and 8.3% of the intestinal-type tumors.
- The presence of an immunoreactivity for TGF-β1 in the parietal cells of the normal gastric glands suggests the involvement of this factor in the autocrine and paracrine regulating processes of the normal gastric mucous functions;
- The reactivity of the dysplastic and metaplastic lesions to TGF-β1 and TGFBR1 suggests the intervention of these factors in the progression of the intestinal-type gastric adenocarcinomas; the immunoreactivity of the two markers increasing progressively from

normal in the dysplastic and metaplastic lesions in order to reach the maximum reactivity in the carcinomatous lesions;

- In our study approximately 60% of the carcinomatous lesions were positive for TGF- $\beta$ 1 and approximately 80% for TGFBR1, in the intestinal variant the pattern of immunoreactivity to TGF- $\beta$ 1 being heterogeneous and presenting variations with the differentiation degree and with the tumor invasion profoundness;
- The tumor expression of TGF- $\beta$ 1 was significantly higher in the tumors with a reduced differentiation degree, but concerning the immunoreactivity for TGFBR1 we were not able to find significant differences in relation to the differentiation degree and to the tumor invasion profoundness;
- Moreover, both for TGF- $\beta$ 1, and for TGFBR1 we were not able to find significant correlations either with the lympho-ganglionar invasion status or with the tumor stage. Only in the case of immunoreactivity for Ki67 we were able to establish a correlation with the tumor stage, the highest values of the proliferation index being obtained in the gastric carcinomas advanced stages.
- Immunoreactivity for TGF- $\beta$ 1 at the stromal fibroblasts level from the poorly differentiated forms of intestinal adenocarcinomas and from the diffuse carcinoma cases suggests their involvement in tumor progression;
- All these results prove the prognostic role of immunoreactivity to TGF- $\beta$ 1 in the intestinal type gastric adenocarcinomas;
- In 88% of the investigated intestinal gastric adenocarcinomas we recorded reactivity for EGF, 52% presenting an intense reaction for this marker.
- The only significant correlation emphasized in our research refers to the dependence of EGF reactivity to the tumor differentiation degree;
- HER 2 tumor expression rate on the investigated casuistry was of 32%, most of the cases being classified at the immunoreactivity score 2;
- The percentage of the cases with positivity to the EGFR1 marker on the investigated casuistry was of 46.4%, the immunoreaction pattern being dual, both cytoplasmic and membrane-related; immunoreactivity for the EGFR1 receptor did not significantly correlated to any of the investigated morphoclinical parameters;
- The investigated intestinal-type gastric carcinomas associated to an overexpression of the EGF factor, and in almost 50% of the cases also to the overexpression of its receptors HER2 and EGFR1. Such an immunoprofile suggests the intervention of certain autocrine and paracrine regulating loops, EGF growth factor – its receptors HER2 and EGFR1 in the carcinogenesis of such gastric malignancies.

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