UNIVERSITATEA DE MEDICINĂ ȘI FARMACIE
GRIGORE T. POPA IAȘI

Summary of the PhD Thesis

Specific morphological and molecular changes of colorectal cancer – clinical implications

Academic advisor,
Professor Dr. Carmen Elena COTRUTZ

PhD candidate,
Dr. Pavel ONOFREI

-2019-

Table of contents

This thesis is illustrated with 56 figures and 24 tables and contains 250 reference indexes. This summary selectively illustrates the textual iconography and references and it complies with the numbering and the table contents of the thesis in extensor.

Key words: ezrin, maspin, MMP-1, MMP-2, MMP-9, electron microcopy

Introduction

In the 21st century, one of the fundamental problems of medicine is the neoplastic pathology, with the diversity of locations, of the involved cell types and of the different modes of manifestation (1). CRC is ranked the third as incidence and fourth as mortality rate, of the totality of neoplasms (2). We considered it necessary to initiate a study addressing colorectal tumors compared to gastric tumors from the perspective of ultrastructural and molecular changes to highlight the specific cellular phenotype of this cancer.

Motivation and objectives of the study

Study motivation

It is known that numerous researches aimed at identifying specific molecular markers of CRC, highlight the essential role in tumorigenesis of different localization of proteins such as ezrin, maspin, metalloproteinases and their expression characteristics in different subcellular compartments with prognostic significance and for personalized therapy, for which they are considered by the WHO as potential diagnostic and prognostic biomarkers for different types of malignancies (8).

Considering that a series of morphological changes cannot be detected by photon microscopy whose resolution power is limited, as well as for a complete morphological characterization of the cellular microclimate in which the studied molecules operate, using transmission electron microscopy alongside H&E stained samples (which were used for anatomopathological diagnosis) we performed an ultrastructural study comparing colorectal cancer with gastric cancer (158).

Purpose of study

- Identification of morphological context and ultrastructural changes in CRC compared with gastric tumors and their correlation with the aspects highlighted by classical photonic microscopy, in order to highlight morphological characteristics of colorectal tumors.
- Comparative IHC study of ezrin expression in CRC and GC respectively, as a molecule involved in the

development of signaling pathways through which the proliferation and migration of tumor cells is regulated.

- Comparative immunohistochemical study of the expression of maspin in colorectal versus gastric carcinomas, a molecule involved in the malignant phenotype, in the tumor evolution and in the modulation of peritumoral angiogenesis.
- Comparative immunohistochemical study of the expression of MMP-1, MMP-2 and MMP-9 in colorectal carcinomas versus gastric carcinomas considered to be essential factors for invasiveness through the formation of tumor cell proliferation centers.

Research objectives

- the evaluation of the specific morphological context of the considered tumors as well as the highlighted comparison based on the ultrastructural aspects of the severity of the lesions and the morphological changes at their level.
- correlating the results of the ultrastructural study with those of the immunohistochemical study regarding the expression of the molecules considered.
- identifying a pattern of expression of these molecules in CRC and the existence of possible correlations between their expression and their involvement in the alteration of some signaling pathways playing and important role in the evolution of colorectal cancer
- highlighting possible correlations between the expression of the molecules considered and the efficiency of specific therapies for CRC.
- highlighting the existence of a molecular biomarkers panel in the case of CRC whose expression is

interconnected and realizes the molecular specificity of these tumors.

Materials and methods

Materials

For the present study, tumor and normal mucosal tissue fragments were collected from 172 patients aged between 49 and 75 years, which were presented for specialized consultation within the Regional Institute of Oncology Iasi, due to the symptoms specific to the neoplasms of the digestive tract and which have been diagnosed clinically, imagistic and histopathological with: moderately differentiated colorectal adenocarcinoma – 92 cases: 17 T2N0M0 cases, 54 T3N0M0 cases and 21 cases with T3-4N0-2M0-1 tumors and moderately differentiated gastric adenocarcinoma – 80 cases: 12 T2N0M0 tumors, 50 T3N0M0 cases and 18 cases with T3-4N0M0-1 tumors.

Both tumor and normal mucosa fragments, gastric and colorectal were harvested by endoscopic biopsy (colonoscopy and upper digestive endoscopy) for which the PENTAX EPK-I5000 endoscopic processor was used, with PENTAX biopsy forceps, within the Gastroenterology service of the Regional Institute of Oncology, Iasi. Prior to endoscopic biopsy, the informed consent of each patient was obtained, according to the legislation in force. The anatomopathological diagnosis was established within the Pathology and Prosecution Service of the Regional Institute of Oncology, Iasi.

Reactants:

- Immunohistochemical EnVision® Kit + Dual Link System-HRP produced by Dako, Carpinteria, CA, USA;

- ,,Anti-Ezrin antibody produced in rabbit" by SIGMA-ALDRICH® for ezrin immunohistochemical determination;
- ,,Maspin mouse monoclonal antibody C-8" by Santa Cruz Biotechnology, Inc for maspin immunohistochemical determination;
- ,,MMP-1 antibody (3B6) mouse monoclonal IgG_1" by Santa Cruz Biotechnology, Inc for MMP-1 immunohistochemical determination;
- ,,MMP-2 antibody (2C1) mouse monoclonal IgG_1" produced by Santa Cruz Biotechnology, Inc for MMP-2 immunohistochemical determination;
- ,,MMP-9 antibody (2C3) mouse monoclonal IgG_1" by Santa Cruz Biotechnology, Inc for MMP-9 immunohistochemical determination;
- ,,Liquid DAB+ Substrate Chromogen System" by Dako, Carpinteria, CA, USA;

Methods

The tissue fragments collected from the patients included in the study, a part were processed by the paraffin inclusion technique, after which they were sectioned and stained with H&E to establish the anatomopathological diagnosis, other fragments were processed by the immunohistochemical technique and a series of fragments were processed by the standard technique for transmission electron microscopy. The association between the clinicopathological variables (tissue type, tumor staging, dissemination and the presence of metastases) were analyzed using Chi-square tests and we compared the frequency of occurrence of events using the IBM SPSS version 18.0 for Windows (SPSS Inc., Chicago, IL).

Results and discussions

Morphological aspects of specific colorectal tumors tissular microenvironment compared with gastric tumors

Results

Electronomicroscopically, in the case of colorectal adenocarcinoma, a number of aspects have been objectified that come to complement those identified by the photon microscopy but at the same time indicate a higher degree of severity of the morphological changes:

- the presence of plasmalemma's discontinuities and areas with modifications of the normal membrane structure like invaginations; also was noticed the existence of both desmosomes and hemidesmosomes with normal structure in some cells, but also a series of adhesion junctions with modified structure (partial disorganization of the tonofilaments, sometimes associated in clusters with different orientations outside the structure of the cytoplasmic plates);

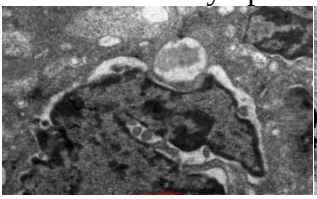

Figura 6.1.5 Nucleus aspect CRC

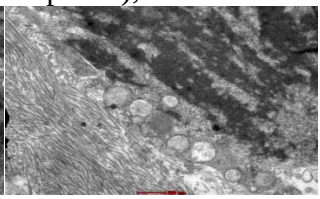

Figura 6.1.8 Organnels, ECM in CRC

- cytoplasmic lysis and perinuclear vacuolization with the disorganization of the mitochondrial structure, showing frequently aspects of advanced mitochondrial degeneration: mitochondrial ballooning, amorphous and electronodense material in the cellular matrix, partial / total disappearance of the mitochondrial cristae;

- hypertrophic nuclei, with an edentate appearance, having a strongly folded nuclear envelope and highly dilated perinuclear cisterns (Fig. 6.1.5);
- compact, electronodense nucleoli, sometimes with a disorganized structure, located peripherally towards the inner lamina and in some cells, two nucleoli paired "in eyeglasses" manner can be observed - an important characteristic aspect for advanced malignant transformation;
- it was observed the disorganization of collagen fibers at the extracellular matrix level, the presence of enlarged fibroblasts with hypertrophic nuclei (Fig. 6.1.8);

Electronomicroscopic examination of the tissue samples collected from patients diagnosed with gastric adenocarcinoma reveals morphological changes less important in the subcellular compartments than in the case of colorectal carcinoma and which complement the ones detected by photon microscopy:
- disorganization of the morphological structures of the plasmalemma and of the cell coupling areas, with a lower frequency than the ones met in colorectal cancer cases; it is also noticeable some areas where the tight junctions are heaving a disorganized architecture, with a vacuolar aspect;
- most nuclei are increased in volume and with a small amount of nuclear chromatin disposed in an irregular manner - characteristic aspect of malignant transformations; nucleoli are observed with changes in both morphological and numerical aspects;
- mitochondrial degeneration is present only at the level of some mitochondria that have the same amorphous material in the matrix as in colorectal carcinoma, only

that in this case, are also some normal mitochondrial cristae;

Discussions

Regarding the ultrastructural changes of the mitochondria mentioned above, some researchers have associated the mitochondrial morphological changes with the evolution of carcinogenesis which they have compared in some cases with the mitochondropathies (162). These aspects of mitochondrial morphological changes are related with aerobic glycolysis known as the Warburg effect, which refers to inhibition of mitochondrial respiratory chain function, impaired oxidative phosphorylation, and subsequently development of resistance to chemotherapy (164). Another important ultrastructural aspect is the changes suffered by the nucleus. The remodeling of the nuclear structure of the tumor cells, suggests a more invasive phenotype, gives the nucleus a higher capacity of deformation, favoring the tumor invasion. In the case of colorectal tumors, the existing studies associate these aspects highlighted in the present study with the modified expression of laminina A and / or C and indicate an increased risk of recurrence (170), aggressiveness of tumors and poor prognosis (171). Elliptical nuclei are identified in the mucosa of the well differentiated gastric carcinoma, whereas in the case of poor differentiated lesions spherical nuclei are observed (172).

The disorganization of the cellular junctions observed especially in the case of colorectal tumors, represents a modification of these interactions which ultimately leads not only to the detachment of the tumor cells from the neighboring ones, but also to the reorganization of the

cytoplasmic structures with changes in the dynamic properties of the cells. These morphological changes of the cell junctions also indicate changes in the signaling pathways involved in carcinogenesis. The electronomicroscopic study of the considered tumors reveals the high prevalence of important ultrastructural changes, specific to the tumor cells, while highlighting the severity of the morphological changes in the case of colorectal tumors compared to those of the gastric level in the studied cases. Some of these ultrastructural changes have been described in the literature in the few existing studies, without being analyzed comparatively between colorectal and gastric cancers and without pointing out the existence of important differences regarding ultrastructural changes.

Immunohistochemical study of ezrin expression in colorectal cancer compared with gastric cancer

Results

The tissue fragments collected from the 172 patients diagnosed anatomopathologically with colorectal and gastric adenocarcinoma were processed by IHC technique and examined in order to quantify ezrin expression using the Olympus BX40 photonic microscope.

Discussions

Ezrin, a member of the ezrin / radixin / moesin family of proteins, binds the actin (actin F) filaments of the cytoskeleton to plasmalemma molecules, thus playing a role in the control of cytoskeletal actin, in the development of intercellular signaling pathways involved

in cell proliferation, cell adhesiveness, cell adhesion, etc. and by these in tumorigenesis, development, invasion in the case of numerous malignancies with various locations (176).

Table 6.2.I Ezrin expression in colorectal cancer compared with gastric cancer

LOCALIZARE		NR. CAZURI	EXPRESIA EZRINEI		
			MEMBRANAR	CITOPLASMATIC	NUCLEAR
COLORECTAL	Țes. Normal	92	++	-	+
	Țes. Tumoral	71 (77,2%) T2N0M0 T3N0MO	68 +	68 ++	-
			3 -	3 +++	
		21(22,8%) T3-4N0-2M0-1	-	+++	-
GASTRIC	Țesut Normal	80	++	-	-
	Țesut Tumoral	62 (77,5%) T2N0M0 T3N0MO	-	55 ++	-
				7 +++	
		18 (22,5 %) T3-4N0-2M0-1	-	+++	-

Multiple studies have shown that altered ezrin expression at the cytoplasmic level compared to the predominantly membranous one in normal tissue is correlated with an unfavorable prognosis for several types of tumors, including colorectal ones, but without a completely elucidated mechanism of action and interaction.

The results of the study showed that ezrin expression differs in normal and tumor tissue; in normal colon tissue, we found that ezrin has predominantly membrane and rarely nuclear immunoreactivity, and in tumor tissue the immunoreactivity is moderately positive (++) diffuse cytoplasmic and weakly positive (+) at membrane level in most of the studied cases. The immunohistochemical study also highlighted the fact that within the same TNM stage, in 3 of the tumors there was a differentiated expression: negative (-) membranous and intensely positive (+++) at the cytoplasmic level (Table 6.2.I). These results are in agreement with the data from the

literature that similarly identified ezrin`s IR at the cell-cell junctions, thus at membrane level and a weak immunoreactivity in the basal area of the epithelium in the case of normal colonic tissue, whereas in the colonic adenocarcinoma there was an intensified cytoplasmic localization (135). Compared with the results of these studies, we found in most of the studied tumors the maintenance of a weak (+) positive IR at membrane level, important aspect for the prognosis of these tumors.

Immunohistochemical study of maspin expression in colorectal cancer compared with gastric cancer

Results

The present study evaluated maspin`s expression, a protein involved in the organization of cellular coupling areas, in cell motility, apoptosis and/or angiogenesis, which most studies indicate as a potential prognostic biomarker for colorectal cancer.

Table 6.3.I Maspin expression in colorectal tumors compared with gastric tumors

LOCALIZARE		NR. CAZURI	EXPRESIA MASPINEI		
			MEMBRANAR	CITOPLASMATIC	NUCLEAR
COLORECTAL	Tes. Normal	92	-	++	+
	Tes. Tumoral	71 (77,2%) T2N0M0 T3N0MO	65 + 6 -	65 +++ 6 ++	65 ++ 6 +
		21 (22,8%) T3-4N0-2M0-1	-	++	+
GASTRIC	Țesut Normal	80	-	++	+
	Țesut Tumoral	62 (77,5%) T2N0M0 T3N0MO	-	+	+
		18 (22,5 %) T3-4N0-2M0-1	-	12 – 6 +	+

Discussions

A specific aspect of the molecular structure of maspin is the Gα helix structure of the reactive center

(RCL). Due to this it is possible to change the conformation between closed/relaxed state, mechanism by which regulates cell migration and apoptosis, being the only serpin involved in regulating apoptosis - incomplete elucidated molecular mechanism. According to the same studies, RCL allows the binding of maspin to the membrane receptor for collagen I and fibronectin and thus promoting cell adhesion (157).

In vitro researches have shown the suppressive role of maspin on tumor invasiveness and implicitly on metastasis as well as its involvement in the initiation of apoptosis and peritumoral angiogenesis (209). Also in vitro, it was found that the maspin induces changes in the expression of the proteins associated with the actin cytoskeleton leading to a less invasive phenotype of the tumor cells, reducing the risk of metastasis.

Following these results, there was an enthusiasm of the researchers to propose maspin as a marker of tumor aggression and prognosis, which was tempered by the heterogeneous and contradictory results of in vivo and clinical studies. The study of maspin in in the tumor cells represents a challenge in the absence of a molecular pattern of its expression and functionality. The results of the present IHC study highlighted for CRC tumors studied a number of aspects that are in agreement with some studies in the literature but also showed the existence within the same staging of a heterogeneity of maspin`s localization.

Considering all these elements, it can be considered that the cytoplasmic overexpression of maspin accompanied by nuclear immunoreactivity represents an element of molecular specificity of the colorectal

carcinomas for the localized stages. At the same time, the data presented above suggest a function dependent on the subcellular localization of maspin in colorectal cancer, a useful aspect in identifying the localized tumors without adjacent tissue invasion and chemotherapy sensitive.

Immunohistochemical study of MMP-1, MMP-2 and MMP-9 expression in colorectal cancer compared with gastric cancer

Results

The present immunohistochemical study aimed to highlight and correlate the expression of metalloproteinases 1,2 and 9, most commonly indicated by the literature studies so far as being involved in the invasiveness and metastasis process of numerous types of malignancies.

Table 6.4.I MMP-1,MMP-2 and MMP-9 expression in colorectal tumors compared with gastric tumors

LOCALIZARE		NR. CAZURI	EXPRESIA MMPs		
			MMP-1	MMP-2	MMP-9
COLORECTAL	Țes. Normal	92	-	-	+
	Țes. Tumoral	71 (77,2%) T2N0M0 T3N0MO	66 ++	68 +	67 +
			5 +	3 ++	4 ++
		21 (22,8%) T3-4N0-2M0-1	19 +	20 ++	21 ++
			2 ++	1 +	
GASTRIC	Țesut Normal	80	-	-	-
	Țesut Tumoral	62 (77,5%) T2N0M0 T3N0MO	59 +	60 ++	60 ++
			3 ++	2 +++	2 +++
		18 (22,5%) T3-4N0-2M0-1	15 ++	16 +++	16 +++
			3 +	2 ++	2++

Discussions

MMPs represent a family of Zn-dependent proteolytic enzymes that can degrade any component of the extracellular matrix. I found it useful to study the immunohistochemical expression of these MMPs since most studies in the literature investigated either the expression of only one MMP (eg MMP-9, MMP-7) or MMP-2 and 9, or MMP-2,9 and 7 without correlating

with them the expression of MMP-1 in the circumstances of the outbreak of a malignant phenotype; also the expression of MMPs was not correlated with that of molecules with equally important role in tumor invasion and the organization of secondary tumors such as ezrin and maspin. In this regard, the few existing studies in the literature regarding the expression of MMP-1, also called interstitial collagenase because it can disintegrate type I, II, III, VII and X interstitial fibrillar collagen, entactin, α2macroglobulin, highlighted its importance for the prognosis and implicitly the progression of the CRC (228). For the normal colorectal and gastric tissue we highlighted the lack of MMP-1 expression, similar to most of the results from the literature studies, and for 71.2% of the colorectal tumors studied it was observed a moderately positive expression while for 22.8% weak positive immunoreactivity (Table 6.4.I) It can be considered that the results are in agreement with those of some studies in the literature, highlighting that in normal tissues MMP-1 is in its proenzyme form, thus inactive. Regarding the tumoral tissues, its expression was differentiated and clinically indicates at least in 71.2% of CRC cases an early stage of tumor development and a less aggressive phenotype. For the rest of 22.8% CRC cases, the expression MMP-1 suggested a lower degree of differentiation and implicitly a poor prognosis due to the increased possibility of short-term metastasis occurring.

In the case of gastric tumors, for the T2-3N0M0 stages, there was a weak (+) positive expression of MMP-1 and for 3 of the cases the expression was moderately positive (++), whereas the colorectal tumors

with the same TNM stage showed a majority moderately positive expression (++). For 15 of the gastric tumors with stages T3-4N0-2M0-1 the expression was moderately positive (++), with the presence of a weak positive expression (+) in 3 of the tumors with the same staging. These results indicate an increase in MMP-1 expression in advanced stages and a heterogeneity of the expression of this MMP within the same TNM stage - a similar aspect encountered in colorectal carcinomas. According to the literature data, the increase of MMP-1 expression indicates a lower degree of differentiation and a more aggressive tumor cell phenotype compared to those with weak positive expression.

The overexpression of MMP-2 and MMP-9 in the tumors considered in this study indicates the degradation of type IV collagen from the basal membrane and implicitly the disorganization of this structure, which is confirmed by the ultrastructural study performed for these tumors. These results are in agreement with the hypothesis launched by Chu et al. who believe that the level of MMP-9 highlighted by IHC is positively correlated with invasiveness, lymphatic and distant metastases, suggest a poor prognosis, and that this type of MMP may be a new prognostic marker in completing the TNM staging system. In addition, serum levels of MMP-2 and MMP-9 are considered by some authors as markers with higher diagnostic sensitivity than those used in current practice (CEA, CA19-9) (236).

Conclusions

- The comparative electro-microscopic study of the samples collected from the colorectal and gastric tumors respectively provides a series of additional

morphological aspects that could not be observed in classical photon microscopy, but also new elements, important for establishing the diagnosis, stage of these tumors, as well as for their therapeutic management.

- The ultrastructural aspects of the different cellular components observed in the present study and the highlighting of a correlation between them, the results of the molecular studies performed and the prognosis of tumors, support the hypotheses existing in the literature, which suggest the existence of a link between the ultrastructural aspects and the secondary phenomenon of the tumorigenesis process.
- The correlation of the data from the literature with the results of the immunohistochemical study indicates that the level of ezrin expression may be a predictive marker for the response to 5-FU therapy but also to the resistance to some chemotherapeutics, in the case of CRC.
- Given the roles of ezrin at the cellular level, the identified aspects can be considered a molecular reflection of the morphological changes highlighted by the ultrastructural study.
- The presence at the nuclear level of the positive immunoreactivity for maspin in the cases of colorectal tumors is correlated with the hypotheses of the literature studies indicates an early stage of tumor progression and sensitivity to chemotherapy.
- In colorectal tumors - stages III and IV, the cytoplasmic and nuclear overexpression of maspin correlates with an increased invasive phenotype of the tumor cells being a biomarker of the aggressiveness and the increased risk of ganglionic metastasis compared to the gastric tumors,

where overexpression or even lack of expression is considered potential aggression biomarker.

- Considering the localization of both molecules at the membrane, cytoplasmic and nuclear levels in the case of colorectal tumor cells, the existence of an inverse correlation between their expression according to the IHC study, their involvement in the activation and development of the same signaling pathways with role in colorectal oncogenesis as well as in the realization of the connections between the cytoskeleton and the membrane, the maintenance of the integrity of the cell coupling zones, I suggest the possible existence of a molecular partnership between ezrin and maspin in the progression of colorectal tumors.
- The results of the present IHC study on the expression of MMP-1 in colorectal and gastric carcinomas revealed an element of molecular specificity of colorectal carcinomas - the decrease of MMP-1 expression in advanced stages, therefore the existence of a negative correlation between TNM stage and its expression.
- The level of MMP-1 expression can be considered as a predictive marker for identifying, within the same TNM stage, patients with an increased risk of tumor invasiveness and recurrence development and thus the need for more aggressive chemotherapy.
- The comparative IHC study of ezrin, maspin, MMP-1, MMP-2 and MMP-9 in colorectal and gastric tumors, respectively, reveals a specific CRC molecular phenotype and indicates the possibility of functional interdependence between these molecules.

Selective references:

1. Haggar AF, Boushey RP. Colorectal cancer epidemiology: Incidence, Mortality, Survival and Risk Factors. *Clin Colon Rectal Surg* 2009; 22: 191-197.

2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin* 2016; 66: 7-30.

8. Dakubo GD. *Cancer biomarkers in body fluids*. Springer International Publishing AG, 2017; 179- 212.

158. Said AH, Raufman J-P, Xie G. The role of Matrix Metalloproteinases in colorectal cancer. *Cancers* 2014; 6: 366-375.

162. Czarnecka A, Czarnecki J, Kukwa W, et al. Molecular oncology focus - Is carcinogenesis a 'mitochondriopathy'? *J Biomed Sci* 2010; 17: 31–38.

171. Mitmaker B, Begin L.R, Hordon P.H. Nuclear shape as a prognostic discriminant in colorectal carcinoma. *Dis Colon Rectum* 1991; 34:249-59.

135. Patara M, Monteiro Santos EM, de Almeida Coudry R et al. Ezrin expression as a prognostic marker in colorectal adenocarcinoma. *Pathol Oncol. Res.* 2011; 17: 827-833.

157. Berardi R, Morgese F, Onofri A, et al. Role of maspin in cancer. *Clinical and Translational Medicine* 2013, 2:8.

209. Lockett J, Yin S, Li X et al. Tumor suppressive maspin and epithelial homeostasis. *J Cell Biochem* 2006; 97: 651-660.

228. Meteoglu I, Erdogdu IH, Tuncyurek P et al. Nuclear Factor Kappa B, Matrix Metalloproteinase-1, p53, and Ki-67 Expressions in the Primary Tumors and the Lymph Node Metastases of Colorectal Cancer Cases. *Gastroenterol Res Pract* 2015; 2015:945392