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CRAIOVA
DOCTORAL SCHOOL**



DOCTORAL THESIS

– ABSTRACT –

**CLINICAL, HISTOLOGICAL AND
IMMUNOHISTOCHEMICAL STUDY OF
COLORECTAL CARCINOMA**

**PhD Advisor,
Prof. Univ. Dr. LAURENȚIU MOGOANTĂ**

**PhD Candidate,
LILIANA NIȚĂ-ȘTEFĂNESCU (STREBA)**

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TABLE OF CONTENTS

INTRODUCTION

STATE OF KNOWLEDGE

1. Anatomy and physiology of the large bowel

1.1 Topographic and descriptive anatomical data of the large bowel

1.2 Histological structure of the large bowel

1.3 Vascularization and innervation of the large bowel

2. Colorectal cancer

2.1 Epidemiology

2.2 Etiopathogeny

2.3 Morphopathology

2.4 Stadialization of colorectal cancer

2.5 Colorectal cancer diagnosis

2.6 Evolution, complications and prognostic factors for the colorectal cancer

3. Therapeutic options in colorectal cancer

3.1 CRC prophylaxis

3.2 Stage-based therapeutic strategies

3.3 Surgical treatment

3.4 Adjuvant chemotherapy

PERSONAL CONTRIBUTIONS

Objectives

4. Clinical and statistical study of colorectal carcinomas

5. Histological study of colorectal carcinomas

6. Immunohistochemical study of colorectal carcinomas

7. Endomicroscopic study of colorectal carcinomas

FINAL CONCLUSIONS

KEYWORDS

Colorectal cancer, morphometric analysis, fractal image analysis, immunohistochemistry, confocal laser endomicroscopy.

STATE OF KNOWLEDGE

EPIDEMIOLOGY OF COLORECTAL CANCER

Colorectal cancer (CRC) represents 15% of worldwide malignancies and is the third cause of cancer in men (10% of total) and the second cause of cancer in women after breast cancer (9.4% of total). Every year there are registered around one million new cases and 500 000 deaths caused by CRC worldwide. Incidence rates are similar between men and women with cancer of the large bowel and higher for males with rectal cancer, but the overall incidence of CRC is higher in males (*sex ratio* M:F = 1.4/1) [1]. In Romania, according to GLOBOCAN estimated data for 2008, CRC is the second leading cause of morbidity through cancer in men (after lung cancer) and in women (after breast cancer). The number of new cases in 2008 was 8696 (22.8/100,000 population), out of which 4554 men and 4142 women [2].

ETIOPATHOGENY OF COLORECTAL CANCER

Although the etiology is not known, CRC is considered a multifactorial disease, an important role being attributed to the impact of environmental factors on a genetically prone land. Hereditary predisposition is considered an important factor in colorectal carcinogenesis, although 80% of colorectal neoplasms occur in the absence of a family history of CRC [3]. A hypercaloric diet, high in fat and low in dietary fiber is positively correlated with the CRC occurrence. Obesity, Western diet and lack of physical activity are common risk factors for both type 2 diabetes mellitus and for CRC [4]. Ulcerative colitis and Crohn's disease are also considered risk factors for CRC [5].

MORFOPATHOLOGY

CRC is more commonly located in the sigmoid and rectum. Macroscopic appearance of CRC is influenced by evolutionary phase in the moment of discovery, several aspects being described: polypoid form, ulcero-vegetant, ulcerative and infiltrative form. The two major histological types of CRC tumors consist of epithelial and mesenchymal tumors [6]. For uniformity and consistency in reporting, internationally accepted and used classification is that proposed by the WHO: adenocarcinoma, medullary carcinoma, colloid adenocarcinoma, „signet ring" squamous cell carcinoma, epidermoid carcinoma, adenosquamos, small cell carcinoma, undifferentiated carcinoma and other types [7].

THERAPEUTIC OPTIONS IN COLORECTAL CANCER

Primary prevention mostly refers to identify risk groups (hereditary intestinal syndromes associated with increased risk for CRC, inflammatory bowel disease, genital cancers, radiation therapy, hormonal dysfunction etc.) and monitor them differently. Surgery is the first choice treatment for most cases of CRC. Type of intervention depends on the evolutionary stage of the disease, the clinical condition of the patient, presence of complications and comorbidities. Adjuvant radiation therapy can be used in inoperable cancer of the rectum or for positive circumferential edges, perforation in the area of the tumor or in case of high risk for local recurrence. Adjuvant chemotherapy has seen many advances in the last 10 years, being used within well standardized protocols in accordance with the stage of the disease [8].

FRACTAL ANALISYS OF MEDICAL IMAGES

For qualitative and quantitative analysis of any form of medical image is first necessary to acquire it in digital format. Medical image registration techniques are either based on the calculation of areas, or based on the analysis of specific graphics. Fractal analysis of images cannot be accurately included in a single category, as the fractal dimension provides data on areas such existing structures in an image, and the form and nature of each element investigated in the analysis [9]. The most accurate method of morphometric measurement of a series of shots that would cover the entire area of interest is, at this moment, fractal analysis. It can be easily adapted to many types of files, is fast and has a high degree of reproducibility [10].

CONFOCAL LASER ENDOMICROSCOPY

Confocal laser endomicroscopy (CLE) enables *in vivo* microscopic study of gastrointestinal mucosa during endoscopy, being recently introduced as a new method for direct examination of the intestinal mucosa [11]. The main advantage of this technique is to obtain real-time pathological images with details of cellular and vascular structures without the need for biopsies, which make possible an immediate diagnosis for various injuries and tissue types. Clinical research has gained new opportunities due to endomicroscopy applications expansion to functional and molecular examinations, not just limited to mere description of the morphological appearance of the gastrointestinal mucosa [12].

PERSONAL CONTRIBUTIONS

CLINICO-STATISTICAL STUDY OF COLORECTAL CANCER

PACIENTS AND METHODS

In this prospective study conducted from January 2011 - May 2013 were initially included all patients suspected to have a malignant tumor of one of the segments of the colon. Patients were selected from those admitted to the Medical Clinic - Gastroenterology and Medical Clinic II of the Emergency County Hospital of Craiova and patients who presented in the ambulatory of Research Center in Gastroenterology and Hepatology (CCGH) Craiova, University of Medicine and Pharmacy (UMF) of Craiova. Histological and immunohistochemical study was performed at the Center for the Study of Microscopic Morphology and Immunology of the UMF Craiova. Thus, there were initially included a total of 289 patients with suspected malignant neoplasm of the colon . Of these, 120 patients were excluded because the diagnosis was imagistically invalidated (115 patients) or refused to sign the informed consent (5 patients).

The study group ultimately comprised 169 patients from who were sampled tumor tissue fragments either by colonoscopy with biopsy (96 patients) or surgical resection of the tumor (73 patients). A total of 17 patients were also investigated using an international novel endoscopic method – confocal laser endomicroscopy. Ten patients underwent direct endomicroscopy using a dedicated laser endomicroscop, the remaining seven being investigated by the endomicroscopic method introducing miniprobos within the working channels of conventional endoscopes.

To assess risk factors, we used the Student t test to compare continuous variables means, together with Fisher's exact test and chi-square for proportions. We calculated the relative risk (RR), in a confidence interval (CI) of 95% to determine the risk of developing malignant tumors in patients with risk factors previously mentioned. Statistical significance was achieved for a value of $P < 0.05$.

RESULTS

Subjects' ages were between 29 and 91 years, with a mean age of 62.04 ± 13.13 years. There predominated subjects aged over 50 years ($n = 138$, or 81.7% of the entire group). We could observe that men were predominantly affected ($n = 98$, male:female ratio was 1.38:1). The average age for men was $60,86 \text{ years} \pm 12,86$ and for women $63.66 \text{ years} \pm 13.4$. We could

not observe significant differences in terms of age distribution according to gender (Student t test , $P = 0.17$). We further studied group of patients distribution according to area of origin, noting an almost equal distribution between urban and rural patients (81 urban patients *versus* 88 rural patients, urban:rural ratio 1,09:1). Thus, patients from urban areas had a mean age of 58 years \pm 11, while the rural patients had a mean age of 66 years \pm 14 (Student t test , $P < 0.0001$).

We noticed a bad diet in 76 cases. A total of 73 patients (22 female, 51 male) recognized in excess alcohol consumption (over 4 g alcohol per day, at least four days a week). Calculating the body mass index (BMI) we observed that a total of 68 patients (40% of study group) suffered from obesity ($BMI > 30$), 79 patients (including 66 with problems of diet and 51 obese patients) also had diabetes mellitus II (41 men and 38 women). Various family factors and familial polyposis disorders were found in a total of 56 patients (35 men, 21 women).

We observed a statistically significant increased risk in patients with poor diet ($RR=6.83$, $CI_{95\%} = 3.16$ to 11.21), in obese patients ($RR=4.11$, $CI_{95\%} = 2.72$ to 8.12), in patients with excessive alcohol consumption ($RR=6.11$, $CI_{95\%}=3.84$ to 10.96) and in smoking patients ($RR=5.97$, $CI_{95\%}= 1.38$ to $7,92$).

As regarding histological type, a total of 134 cases showed colonic adenocarcinoma (79.2% of all patients, 78 men and 56 women), 28 colloid carcinoma (16.56%, 16 men and 12 women), 6 patients "signet ring" cell carcinoma (3 male and 3 female) and one man with squamous cell carcinoma. We identified a total of 88 well-differentiated carcinomas (52% of the study group, 48 men and 40 women), 49 moderately differentiated carcinomas (29%, 29 men and 20 women), 25 poorly differentiated carcinomas (14.8%, 16 men and 9 women) and only 7 undifferentiated carcinomas (4.1%, 5 men and 2 women).

Most tumors were found in the sigmoid colon (66 cases, 39%), followed by those located in the descending colon (41 cases, 24.3%), transverse (28 cases, 16.5%), cecal tumors (18 cases, 10.7%) and the ascending colon (16 cases, 9.5%).

We identified 34 patients (20.11% of study group) with lymph node metastases (28 men and 6 women). Remote metastasis in various major organs was also seen for a total of 19 patients (11.24% in the studied group). In numerical order, we identified 11 liver metastases (6 men, 5 women), 4 lung (three men and one woman), two bone (both male patients) and two kidney metastases.

HISTOLOGICAL STUDY OF COLORECTAL CARCINOMA

MATERIALS AND METHODS

Histopathological material was collected through direct endoscopic obtained biopsy and after curative intended surgery performed in the Emergency County Hospital of Craiova. The examination included the adjacent mesenteric lymph nodes as a necessary step in formulating the diagnosis stage of tumor lesion. Biologic material (colorectal resection pieces) was introduced immediately after resection in 10% neutral formalin fixing solution and were then processed for conventional histological techniques including paraffin techniques that allowed us to carry out series of 3-5 μ thickness sections. We used two standard considered stainings for a correct and complete anatomopathological diagnosis of colorectal cancer, namely staining with hematoxylin-eosin (HE) and light green trichromic staining after Goldner-Szeckelly method.

Images were acquired with a Nikon Eclipse 90i microscope and a 5 megapixel camera with CCD sensor. In the study of nuclear chromatin arrangement, image segmentation followed appropriately blue color channel. Binary images thus obtained were further analyzed using Image J software with FracLac plug-in. Fractal dimensions (DF) were calculated according to the counting algorithm through approximation in boxes (*fractal box-counting algorythm*) as the slope of the regression line obtained from log-log representation of the scan box size and number obtained through a box-count scan. A second analysis of nuclear chromatin arrangement tracked shape changes and internal disposal, in this case using commercial software Image Pro Plus 7.0. Binarized images were segmented using a Sobel filter, then a clear delineation filter of each element to individually segment grouped nuclei. In short, there were thus obtained representative individual images for each nuclear variation, being possible to calculate again the fractal dimension, this time expressing the complexity of the nuclear form, not only its modified volume.

RESULTS

Well differentiated adenocarcinomas accounted for most encountered forms; they contained glandular structures enclosed by tall columnar cells. Glands were arranged in a random way compared to normal epithelial structures, occupying both mucosa and submucosa, sometimes encountering invasion of the muscular tunic. Glandular structures modified by the neoplastic process were sinuous, with various sizes and shapes in different areas of the tumor. Glandular lumeni were either large, filled with the above described debris, sometimes reduced

in size, up to the complete absence in some cases. We have noticed the presence of many atypical cells in all types of adenocarcinomas, regardless of glands shapes.

These features have focused our further fractal analysis on the shape and size of tumor cell nuclei, which have been fully correlated with the degree of tumor differentiation, but also with the histologic type.

We followed the disposal of nuclear chromatin in standard HE staining, thus quantifying shape and size aberrations of malignant cells. After analyzing the data, we observed low variability of values, with a very low standard deviation, most of the values being consistently higher for images recorded in tumor areas. There could be observed, however, significant differences between different degrees of differentiation ($p < 0.001$). We identified significant differences between the three distinct DF - adenocarcinomas without excess mucus secretion, excessive extracellular mucus secretion carcinomas and increased intracellular mucus secretion carcinomas (ANOVA test with 2 degrees of freedom, $P < 0.0001$). Bonferroni post-test showed us that these differences remained valid regardless of the considered type. Disposal of nuclear chromatin revealed major structural differences in case of malignant modified nuclei, with net increased average DF for adenocarcinomas but only moderately increased for "signet ring" cell carcinomas due to the particular shape that their nuclei gets through intracellular accumulation of mucus secretion. Statistical analysis of the data by ANOVA test showed significant difference of the averages for each range of values ($p < 0.0001$).

DF of the cell nuclei belonging to the inflammatory infiltrate had a median value 1.151 (minimum 1.101, maximum 1.189), normal ones 1.202 (minimum 1.215, maximum 1.231), while the malignant 1.302 (minimum 1.256, maximum 1.378). Statistical analysis revealed significant differences between the mean values of tumoral nuclei DF, compared with those of their own cells or those of inflammatory infiltrate (ANOVA test, $p < 0.0001$). We obtained a series of DF values corresponding the contour complexity of these proliferative elements, not so significant for the occupied area, but especially for the degree of entropy present in the image.

IMMUNOHISTOCHEMICAL STUDY OF COLORECTAL CARCINOMA

MATERIALS AND METHODS

Of all the selected blocks, included in paraffin, were cut 4 μm thick sections using a microtome equipped with a special transfer system of sections. To start immunohistochemistry sequences, sections were first dewaxed in three successive baths of xylene and then rehydrated

by washing in decreasing concentrations alcohols. Immunohistochemistry technique itself comprised a standard algorithm, with some variations depending on the used antibodies. Invariably, first step of the immunohistochemistry technique was the antigenic recovery by microwave boiling in a specific solution chosen according to the antibody. There was added on the slide one of the primary used antibodies: Anti-p53, Anti-Ki67, PCNA și Anti-VEGF-C. This was followed by the addition of the secondary detection system, namely a primary anti-antibody - secondary antibody complex, bound to a polymer with multiple molecules of peroxidase.

We took a series of 100 images for each studied case. A red-green-blue signature was created and all images were automatically segmented, obtained masks being then saved as binary images containing texture information of each original image. Automated scripts allowed visualization of the segmentation process.

RESULTS

Cell proliferation study

Ki-67 protein is involved in cell proliferation; the antigen was detected only in the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. In poorly differentiated carcinomas rate of Ki67 positive cells appeared irregular. Also, polyploid cells with monstrous nuclei had an intensely positive reaction to Ki67. Thus, we could observe such a large variability of the intergroup values, with high standard deviation due to the large DF range through different stages of differentiation. Next, using ANOVA test, we studied differences between malignant cells nuclei DF of different histological types of colonic carcinomas, immunomarked with Anti-Ki67 antibody. We found significant differences between the DF of the three studied types (ANOVA test with 2 degrees of freedom, $P < 0.0001$).

Assessment of TP53 gene alteration

In our study we observed that p53 antibody response was very high, over 90% of tumor cells were positive in both colon tumor and the lymph node metastasis, regardless of tumor differentiation. DF cell nuclei have been similar to those of anti Ki67 immunostaining study, concluding that in this case there are significant differences both between carcinoma types, as between the four degrees of differentiation specific to adenocarcinomas. We also conducted a study of anti-p53 antibody immunomarked nuclear forms, establishing differences through fractal analysis. Thus, we observed a difference in size and shape between histological studied types, and the increasing complexity of forms with decreasing degree of differentiation.

Vascular endothelial growth factor (VEGF)

In our study we observed that the majority of neoplastic cells in well-differentiated colorectal adenocarcinomas showed a positive reaction (moderately or intensely positive) in the cytoplasm. We also noticed that moderately or poorly differentiated adenocarcinomas showed little or no response to VEGF-c. We did not observe significant differences between DF of immunomarked areas in subgroup analysis by type of colorectal carcinoma, or the degree of differentiation of adenocarcinomas included in the study.

ENDOMICROSCOPIC STUDY OF COLORECTAL CANCER

MATERIALS AND METHODS

Lower endoscopy was performed using Olympus Exera and Pentax systems. All colonoscopies were performed until the ileocecal valve or until evidence of tumor formation, where it could not be exceeded by the colonoscope. Confocal laser endomicroscopy was performed using the was performed using Pentax ISC-1000 (EC-3870 CIFK) dedicated system in a total of 10 patients and a system based on endomicroscopic miniprobes produced by Mauna Kea Technologies (Cellvizio system). CLE examination was performed in vivo, to obtain a real time histopathological diagnosis, after intravenous administration of 10% fluorescein, 5 ml. CLE scan was done after identifying a region of interest in videoendoscopy by easy aspiration of the mucosa, to keep close contact with confocal microscope lens. Images saved during CLE examinations were transferred to a processing offline unit. We used at least 5 pictures for each case, in absence of caused by motion artifacts or interposition of foreign particles, which were processed using image analysis program Image J public domain and extension for image fractal analysis FracLac . The technique was similar to that used in the studies described above, the only major difference being that there was need for selecting a color channel, the images already being represented in black and white.

RESULTS

Endomicroscopic appearance was conclusive for a poorly differentiated adenocarcinoma, unable to identify normal glandular formed structures. Areas presented glands with elongated lumen, with pseudostratified epithelium or aberrant cell shapes and abundant mucus secretion. We did not observe well-defined blood vessels and cellular debris were abundant in most optical investigated fields. Lakes of sodium fluorescein showed vascular ruptures in tumoral territory, with irregular invasion of malignant cells. Malignant cell nuclei were enlarged, and the ratio between nucleus and cytoplasm bounded by intercellular areas.

We measured DF on a significant lot of minimum 10 images per case investigated by eCLE, resulting in a total of 184, or 272 values. After analyzing data on glandular structures, we observed low variability of values, with a very low standard deviation, most of the values being consistently higher for images recorded in tumor areas. Mann-Whitney statistics showed also here significant differences between mean values assigned DF for tumor areas and those resulted in areas of normal mucosa ($p < 0.0001$). We observed differences in the distribution of means for both tested endomicroscopic systems.

CONCLUSIONS

- Colorectal cancer is a major public health problem worldwide, mainly in industrialized countries. We can talk about a high-risk familial populational group, by existence of multiple genetic disorders which determine premalignant lesions for colon cancer. There are many external risk factors associated with CRC carcinogenesis, the most important being related to diet, excessive alcohol consumption or smoking. An accurate and fast diagnosis can provide greater chances of patients with CRC.

- Image fractal analysis by calculating DF is an extremely useful tool in the characterization and classification of colorectal carcinoma types. Analysis of nuclear chromatin arrangement through DF calculation method can accurately quantify the size of the nuclei of malignant cells found both in different types and in all degrees of differentiation of colorectal carcinomas we studied.

- Studying malignant cells nuclei form through fractal image analysis adds important information in morphometric classification of the main three types of colorectal carcinomas. There is possible a complex characterisation of tumoral stroma morphology by quantitative image quantifying based on fractal analysis.

- Lymph nodes extension study allowed us to identify three main types of cells, based on the differences between the DF of their nuclei: normal cells, inflammatory infiltrate cells and cancer cells.

- Immunohistochemical study is of major importance in determining the exact diagnosis of colorectal carcinomas. Immunohistochemistry provides valuable information on the processes of aberrant nuclear division occurring in colon malignancies, using Ki67 and p53 nuclear markers. Fractal image analysis by calculating DF is an extremely useful tool in the characterization and classification of the nuclei immunomarked by these two antibodies.

- Neoangiogenesis processes play a crucial role in the development of colorectal carcinoma, immunohistochemical study with anti-VEGF antibody proving the importance of neoformation vascularization. A complex characterization of newly formed vessels is possible by quantifying the exact spatial arrangement of VEGF receptor immunostaining.

- Confocal laser endomicroscopy represents a cutting-edge method that combines endoscopic techniques with histologic ones and can provide rapid results without direct mucosal biopsy.

- There are currently miniaturized systems, such as pCLE Cellvizio system, that can be used with existing equipment and will have extensive applications in all future endoscopic or laparoscopic investigations. Both dedicated systems and those based on miniprobes can deliver crystal clear images and conclusive for all colonic pathology.

- Fractal image analysis can be successfully applied to morphometric characterization of normal colonic mucosa structures, but also to differentiate malignant areas. We showed statistical differences between the mean values of fractal dimensions of colonic mucosal glands compared to the average of the DF values corresponding to tumoral modified areas.

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