A pilot study on the role of fractal analysis in the microscopic evaluation of colorectal cancers

LILIANA STREBA\textsuperscript{1,2)}, MIRCEA CĂTĂLİN FORȚOFOX\textsuperscript{3)}, CARMEN POPA\textsuperscript{4)}, DANIELA CIOBANU\textsuperscript{3)}, CORINA LAVINIA GRUIA\textsuperscript{5)}, STELIAN ȘTEFĂNȚĂ MOGOANTĂ\textsuperscript{6)}, COSTIN TEDOR STREBA\textsuperscript{6)}

\textsuperscript{1)Research Center for Microscopic Morphology and Immunology, University of Medicine and Pharmacy of Craiova, Romania
\textsuperscript{2)Postdoctoral Fellow, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania
\textsuperscript{3)Department of Internal Medicine, University of Medicine and Pharmacy of Craiova, Romania
\textsuperscript{4)Department of Pathology, Emergency County Hospital, Craiova, Romania
\textsuperscript{5)Department of Surgery, University of Medicine and Pharmacy of Craiova, Romania
\textsuperscript{6)Research Center of Gastroenterology and Hepatology, University of Medicine and Pharmacy of Craiova, Romania

Abstract

Introduction: Colorectal cancer (CRC) represents one of the most common cancers worldwide; its growing incidence and prevalence quickly transforming it into a major health burden. Globally, survival varies from one country to another and constantly remains significantly low, despite increasing diagnostic efforts and tools. Fractal geometry and, specifically, fractal dimension (FD) are interesting tools to quantify cellular elements. In this paper, we aimed to identify and quantify by fractal analysis the elements obtained from medical images from pathological and immunohistochemical investigations of colonic biopsy fragments. Materials and Methods: We prospectively selected the study group between September 2014 and January 2015, from patients who underwent surgery for previously diagnosed CRC at the Emergency County Hospital, Craiova, Romania. We performed the histological and immunohistochemical studies by following standardized protocols. Anti-Ki67, anti-p53 and anti-VEGF-C antibodies were used for immunostaining. We performed the fractal analysis with an in-house tool and we performed statistical tests on the results. Results: We have included 41 (29 males) consecutive patients with different characteristics; after analyzing the FDs we found significant differences between adenocarcinomas and the other types of colonic cancers (p<0.001). However, we found no significant differences between most types of CRCs. We found significant statistical differences when compared well-differentiated tumors with all other stages (p<0.001). Conclusions: Fractal analysis with the calculation of FDs is a novel, interesting tool, for determining the pathologic diagnosis of CRCs and may further improve diagnostic and prognostic rates, thus improving patient care.

Keywords: colorectal carcinoma, fractal dimension, fractal analysis, diagnosis, staging.

\section*{Introduction}

Colorectal cancer (CRC) is one of the most common cancers worldwide, being the fourth cause of death by cancer after lung, stomach and liver cancer [1–3]. Globally, survival varies from one country to another, being influenced by the possibility of early diagnosis, the existence of modern methods of curative treatment and not least, the socio-economic level [3–5]. There are several factors that may influence prognosis: clinical, histopathological and biological factors related to tumor stage. Romania has seen the doubling of colorectal cancer incidence and mortality in the last 20 years [6]. Both sexes are approximately equally affected, with a male to female ratio of 1.4. The risk of disease increases after the age of 45, doubling every decade from thereafter [3]. Histologically, approximately 98% of all CRC cases consist of adenocarcinomas [1, 4].

Fractal geometry has as fundamental notion the idea that objects should be invariant with respect to scale [7–9]. The relationship between the measurement instrument and spatial distribution of the reference space underlies all methods of measuring fractal dimension (FD) [10]. In medical imaging, the most appropriate models for quantitative analysis were those proposed by Hausdorff and Kolmogorov, although several others were proposed by different authors and can be found in current literature [11–14].

\section*{Aim}

Our aims were the identification and quantification by fractal analysis of elements from medical images obtained from pathological and immunohistochemical investigations of colonic biopsy fragments.

\section*{Materials and Methods}

We prospectively selected the study group between September 2014 and January 2015, from patients who underwent surgery for previously diagnosed CRC at the Emergency County Hospital, Craiova, Romania. All 41 selected patients were adults and provided informed consent for all procedures and tissue collection after we have informed them regarding the methods and objectives of the study. We have obtained the Institutional Review Board approval prior to actively including patients and have written the protocol in accordance to the Helsinki Declaration of the Human Rights and all current national and international legislation.
Previous noninvasive imaging techniques included either magnetic resonance or computed tomography and invasive techniques such as colonoscopy with or without tissue biopsy. After reaching a positive diagnosis, they underwent curative surgery of the colon. We have collected data regarding patient age, gender, place of origin, previous medical history and exposure to risk factors.

We performed the histological and immunohistochemical studies at the Research Center for Microscopic Morphology and Immunology within the University of Medicine and Pharmacy of Craiova. The examined pieces included macroscopically identifiable tumor lesions as well as resection margins for normal tissue comparisons. Immediately after surgery, we have fixed all biological material in a fixing solution of 10% neutral formalin and after the usual paraffin technique previously described [15].

Classical staining was performed with Hematoxylin–Eosin (HE). We performed the immunohistochemical study with anti-Ki67, anti-p53 and anti-VEGF-C antibodies; we have summarized the data in Table 1.

We have paired 10 slides with intratumoral tissue as well as surrounding mucosa for each case. We digitized series of 10 images per slide by using a 5-megapixel CCD camera attached to a Nikon Eclipse 90i microscope (Apidrag, Bucharest, Romania) with apochromatic 40× and 60× objectives. We saved all images in uncompressed TIFF format to prevent loss of information. Two expert pathologists (CG and LM) supervised the procedure and provided tissue description where appropriate.

Fractal analysis was performed with an in-house tool developed in Matlab (Mathworks, USA) that was previously described elsewhere [14]; briefly, we used the fractal box counting algorithm to obtain the FDs as the regression slope of the regression line for the log-log plot of the scanning box size and the count from a box-counting scan [13, 14].

We have exported all data in Excel (Microsoft Corporation, Redmond, Washington, USA) and further analyzed with the trial version of the GraphPad Prism 5.0 software (GraphPad, USA). The ANOVA – ANalysis Of VAriance test was used to analyze the difference of mean for all study groups simultaneously, followed by post-hoc Bonferroni test for multiple comparisons. Both intra- and extratumoral regions were analyzed in our study.

### Results

We have included 41 (29 male) consecutive patients that underwent surgery with curative intent. Median age was 54 years (min. 38, max. 81) for males and 56 years (min. 41, max. 88) for females. The majority came from rural areas (25 subjects); 30 were smokers and 22 reported regular alcohol consumption; 12 had diagnosed diabetes while an additional five had high glycemia levels upon inclusion. We also found abnormal cholesterol and triglyceride levels in 22 patients (10 diabetics); all 22 patients were also overweight (18 cases) or obese (four cases with body mass indexes above 30).

We have found numerous cellular atypia, deriving both from altered architectonics as from altered growth rhythm and abnormal cellular growth. The nuclei displayed varied shapes – round, oval, with an irregular contour and often having multiple lobes. The abnormal chromatin disposition led to higher FDs inside tumors when compared with the outside regions.

Immunohistochemistry revealed an increased proliferative activity within abnormal cells, with highly positive Ki67 staining (Figure 2, a–d). We also observed positive anti-p53 staining, testifying for the abnormal cellular division, which took place within tumors (Figure 3, a–d). Angiogenesis was present within colonic tumors, as we found positive VEGF staining, especially within moderately differentiated, weakly differentiated and three undifferentiated.

During the analysis of the modified gland epithelium, we identified a heterogeneous arrangement of several types of glandular epithelia, such as normal cylindrical, often combined with the pseudostratified cylindrical and with simple cubic epithelium (Figure 1, a and c). Cellular detritus (partially lysed remains with modified malignant cells) and rich inflammatory infiltrate often occupied the inside of the glands (Figure 1, b and d).

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Fractal analysis of chromatin disposition revealed significant differences between each tumor grade ($p<0.0001$), as follows: mean FD for well-differentiated carcinomas was 1.431, for moderately differentiated tumors 1.516, for poorly differentiated 1.669 and for undifferentiated 1.741.

Statistical analysis of the differences between FDs of immunostained elements revealed interesting results. Anti-Ki67 and anti-p53 immunostaining showed significantly higher FDs in intratumoral areas compared with normal mucosa ($p<0.0001$). We found significant statistical differences when compared well-differentiated tumors with all other stages ($p<0.001$). Differentiating between

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more advanced stages proved however difficult by using FD as the only discriminating factor ($p=0.33$). We did not observe significant differences when analyzing anti-VEGF areas in different subtypes ($p=0.2$); however, early stages (well and moderately differentiated) had lower FD values compared to more advanced stages ($p<0.05$).

**Figure 1** – (A) Glandular epithelia with normal cylindrical epithelium combined with pseudostratified cylindrical and with simple cubic epithelium (HE staining, ×100); (B) Cellular detritus composed of partially lysed remains and malignant cells inside of some of the glands present within tumors (HE staining, ×400); (C and D) Interface of the in-house developed software showing how the FDs were calculated for each image.

**Figure 2** – (A and B) Positive Ki67 staining showing an increased nuclear activity (Ki67 immunostaining, ×200 and ×400); (C and D) Selecting the items of interest in each image through the software interface and separating them from the background.
Figure 3 – (A and B) Anti-p53 nuclear immunostaining showing an increased number of abnormal cellular division, which took place within tumors (anti-p53 immunostaining, ×100 and ×400); (C and D) Image segmentation with the specially designed software.

Figure 4 – (A and B) Positive VEGF staining found predominantly in well and moderately differentiated carcinomas, showing an increased angiogenesis process (anti-VEGF-C immunostaining, ×100 and ×400); (C and D) Calculation of FDs by selecting the areas of interest in each image with the specially-developed software.
Colorectal tumors are very heterogeneous in anatomical, physical or microscopic appearance [16–18]. Racial differences, genetic and dietary interactions all play a role in their behavior. All these are due to multiple genetic mutations that tumor cells embody during their transition stages [18,19]. Conventionally, carcinoma is characterized by the formation of glandular structures, microscopic aspect which is particularly important and on which the classification of the adenocarcinomas is based. This in turn plays an important role in the prognosis and treatment options of this disease [16,17,20]. When over 95% of the tumor mass is composed of glandular structures, we can consider this a well-differentiated adenocarcinoma; moderately differentiated forms contain 50 to 95% normal glandular structures, and below 50% signify poorly differentiated formations. Approximately 70% of colorectal adenocarcinomas are detected as moderately differentiated in normal practice [21,22].

Over 90% of colorectal carcinomas are adenocarcinomas originating from colorectal mucosal epithelial cells, according to some authors [19]; other publications considered over 95% of colorectal cancers as adenocarcinomas [23].

We found numerous alterations of cell metabolism, proliferation rates and vascular changes in all 41 cases. The Ki67 protein is involved in cell proliferation, expressed in the nuclei of cells throughout the cell cycle except the G0 phase [24]. It is a nuclear protein, its expression being strictly associated with cell proliferation. It is widely used in pathology as “proliferation marker” in order to evaluate the cell growth fraction of human tumors. Being a factor that brings great information on cell proliferation, Ki67 is commonly used in differential or positive diagnosis of neoplasia. During interphase, the antigen was detected exclusively within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes [25].

In our study, we observed that the p53 antibody response was very intense, over 90% of tumor cells were positive, regardless of the degree of tumor differentiation. These histological aspects confirm the hypothesis that numerous alterations in gene inactivation of tumor suppressor genes and activation of oncogenes occur during the development of colorectal cancer [25–27].

The vascular endothelial growth factor is a protein produced by certain cells stimulated by hypoxia or soluble factors developed by other cells, called cytokines, which stimulate vasculogenesis and angiogenesis. VEGF normal function is to create new blood vessels during embryonic development, new blood vessels when an injury occurs, or because of pathological blood vessel differentiation [28]. In carcinogenesis, angiogenesis is necessary to carry oxygen and nutrients to the growing neoplasm [29,30]. In our study, we observed that the majority of colorectal cancer cells in well-differentiated adenocarcinomas showed a positive reaction (moderate or intense positive) in the cytoplasm.

Currently, fractal image analysis of pathology and especially comparing the fractal dimensions of the studied elements play an increasingly important role in accurate diagnosis and staging of various diseases [31–33]. Although introduced after more than 30 years ago [7], fractal modeling of natural forms has gained importance in the last decade, mainly due to the rapid evolution of computing power of conventional computer systems [7,8]. Develop complex models designed to quantify the features of the various types of records has become a new dimension, as there are currently many applications of fractal dimension [8]. Most studies originally referred to general problems and complex natural phenomena unrelated to medicine. A new trend has emerged in recent years, when applications in biology, pathology and morphometric studies became apparent. It outlined a new approach to computer-aided morphometry and beyond [14].

Regarding histological assessment, apart from preliminary studies published by our team, we could not find any other work where fractal dimension is used as a discriminating factor between types of colorectal tumors. Combining the morphometric analysis of multiple nuclear markers and markers of angiogenesis is an absolute novelty in histological investigations for malignant colorectal tumors. Research team led by L. Goutzianis [34] used the fractal dimension of oral squamous carcinoma cell nucleus as the discriminant factor in tumor staging. There are also a number of studies that have validated methods with similar fractal calculation for staging and prognosis of malignant neoplastic disease series [13,34]. The statistical analysis performed in the current study revealed several opportunities to utilize the FD of nuclear shapes or arrangement within a particular vascular tumor. Vascular structures are suitable for this type of evaluation as well, having complex fractal shape, which is however very easy to interpret in the context of this measurement. There are now multitudes of researches that attempt to study the angiogenic models of various malignant diseases. Oczeretko et al. [35] have concluded that the fractal dimension can be a non-morphometric parameter in the evaluation of pulmonary neoplastic processes. Moreover, because the extension process of neoangiogenesis is a very important prognostic marker [4,36], we appreciate the contribution that the fractal dimension can play in the future in the histological staging and therapeutic indication within the optimal setting of colorectal tumors.

**Conclusions**

We demonstrated here that an in-house developed software might prove useful in calculating FDs of elements in histology images of CRC. Fractal analysis with the calculation of FDs is a novel, interesting tool, for determining the pathological diagnosis of CRCs and may further improve diagnostic and prognostic rates, thus improving patient care.

**Conflict of interests**

The authors declare that they have no conflict of interests.

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Author contribution
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References

Corresponding author
Costin Teodor Streba, Teaching Assistant, MD, PhD, University of Medicine and Pharmacy of Craiova, 2 Petru Rareş Street, 200349 Craiova, Romania; Phone +40722–389 906, e-mail: costinstreba@gmail.com

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