Original Paper

Subtle Immunoreactivity Differences in the Fractal Patterns of Membrane E-Cadherin in Gastric Adenocarcinoma

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ABSTRACT: Gastric cancer continues to be a significant malignancy worldwide, accounting for approximately one million new cases in 2020. Scientists are focusing on the cancerous cells' plasma membrane (PM) as a potential therapeutic target in cancer because it functions as the cell's interface with its environment through a variety of mechanisms. The capacity of membrane shape and its structures to influence biological processes frequently occurs through the regulation of enzymes or preferential protein binding to membranes via membrane shape changes. We aimed here to assess the morphological irregularities of the cellular membranes in gastric adenocarcinoma tumors, and to find any putative differences from normal gastric mucosae epithelial cells. We analyzed the pattern of E-cadherin at the level of the cell membrane using the fractal dimension (FD) analysis on fluorescence immunohistochemistry samples labeled with E-cadherin in gastric well/moderate and solid gastric adenocarcinoma from patients without any associated chemotherapeutic treatment or radiotherapy. Images were binarized based on a fixed threshold of the E-cadherin fluorescence channel, and then the FD of the binarized image outlines has been calculated in order to assess the ruggedness of the cellular membranes. Overall assessment of the FD revealed that the subtle membrane variations were evident enough to deem a statistically significant difference and the complexity of the membrane roughness was clearly higher for adenocarcinoma cases. We intended to evaluate if separating adenocarcinoma cases as low grade (G1 and G2) and high grade (G3 and solid), FD analysis could still differentiate membrane patterns and check if the available clinical parameters like age, gender, tumor location, lymph ganglia involved might correlate with FD values for adenocarcinoma patients. Altogether, the morphological analysis of a simple marker for the cell membrane can identify and distinguish tumor cells. Although there was a limited correlation between this analysis and the main clinical and pathological indicators of the disease, it will be very useful in the future for automatic computer-assisted diagnosis on slides, as well as for evaluating cellular adhesion and inter-cellular trafficking in cancer cells.

KEYWORDS: *Gastric adenocarcinoma, E-cadherin, fractal analysis.*

Introduction

According to GLOBOCAN 2020 recent data, stomach cancer continues to be a significant malignancy worldwide, accounting for approximately one million new cases in 2020 and an approximated 769,000 deaths (one in every 13 deaths worldwide), currently ranked fifth for incidence and fourth for death worldwide.

Men have rates that are double those of women [1].

Currently, scientists are focusing on the cancerous cells' plasma membrane (PM) as a potential therapeutic target in cancer [2].

The cytoplasmic transport of molecules is determined and regulated by the cell membrane (CM), a biological barrier [3].

The CM is an essential part of every cell because it functions as the cell's interface with its environment through a variety of mechanisms, including transport, endocytosis, signaling, and receptors [2,4].

A potent tool for treating cancer is the potential for manipulating its structure and function, both alone and in association with chemotherapeutic drugs and physical membrane alteration techniques [5].

In order to succeed in changing its basic function and be able to acquire an invasive phenotype, the malignant cell adapts both its cell membrane and cytoplasm.

The surface of malignant cells exhibits antigens specific for the tumor, that occur with oncogenic transformation, caused by a change in the cell's genetic program.

10.12865/CHSJ.48.03.08

The location of receptors in malignant cells changes, altering cell agglutination behavior.

Specific surface proteases on the cell surface determine the agglutination capacity of cells.

Tumor cells obtain metabolic independence as a result of eliminating contact inhibition, thus favoring both cell proliferation and migration [6].

Tensional homeostasis in the PM may be an innate mechanical property that keeps epithelial cells in a non-motile condition, with processes that, if restored in tumor cell environments, could serve as an effective metastasis suppressor [7].

Various modifications let cancer cells withstand chemotherapy and immune system reactions.

The altered cell membrane composition as a result of the abnormal lipid metabolism represents one of the most important characteristics of malignant cells [8].

Healthy tissue homeostasis is totally dependent on cell-to-cell adhesion but also cell-to-ECM (extracellular matrix) interactions [9].

Numerous studies have shown that the E-cadherin adhesion pathway is disrupted in cancer cells in multiple ways and that these defective E-cadherin functions lead to the release of malignant cells from the initial lesion and cell dedifferentiation [10].

With a broad variety of applications, soluble E-cadherin has the potential to be utilized as a biomarker for the earlier detection of gastric cancer as well as an independent and relevant factor to predict the long-term survival of patients who have cancer [11].

As a result of reduced cell-surface expression of E-cadherin brought on by the failure of E-cadherin trafficking, cancer cells might separate and spread widely.

Multiple malignancies have been linked to E-cadherin erroneous mutations, especially mutations in the cytoplasmic area that interacts with β-catenin [12].

We aimed here to assess the morphological irregularities of the cellular membranes in gastric adenocarcinoma tumors, and to find any putative differences from normal gastric mucosae epithelial cells.

Materials and Method

We have included here pathological specimens harvested from N=16 patients who were subjected to surgery for primary gastric cancer, performed in the Surgery Clinics of the Emergency County Hospital of Craiova, Romania, diagnosed with well/moderate and poor differentiated/solid tubular gastric adenocarcinoma, and without any associated chemotherapeutic treatment or radiotherapy, between 09.2018 and 12.2018 (average age=62.06±11.70 years).

For control gastric mucosae, we have included tissue fragments taken from N=8 patients who died of non-digestive pathologies (average age=65.5±8.33 years).

Initial hematoxylin and eosin slides have been revisited and most representative areas have been chosen from each specimen for this study.

All pathology cases have been classified as either low grade adenocarcinoma (tubular well and moderately differentiated tumors), or high-grade tumors (solid, poorly differentiated adenocarcinoma).

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the University of Medicine and Pharmacy of Craiova, Romania (no 10/17.01.2019).

Selected best representing paraffin tissue blocks were cut as 4μm-thick sections utilizing a rotary microtome and processed for fluorescence immunohistochemistry for visualizing E-cadherin.

Briefly, the sections were deparaffinized in xylene, rehydrated in decreasing ethanol series, processed for antigen retrieval by microwaving in 0.1M citrate buffer pH6 for 20 minutes, incubated for 30 minutes in a 3% skimmed milk solution prepared in PBS for blocking unspecific antibody binding sites.

The anti-E-cadherin primary antibody (mouse, Dako, Glostrup-Denmark, diluted as 1:50) was incubated onto the slides at 4°C for 18h, next day the slides were thoroughly washed in PBS, and the signal detected with a goat anti-mouse Alexa Fluor 594 labelled secondary antibody (Thermo Fisher Scientific, Waltham, MA USA), diluted as 1:300, for 2 hours at room temperature.

Slides were coversliped with an anti-fading medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vectashield, Vector Laboratories, Burlingame, CA, United States) and archived for analysis.

Negative controls were obtained by omitting the primary antibodies.

Random high-resolution images have been captured with a Nikon 90i motorized microscope (Nikon Europe BV, Amsterdam, the Netherlands) equipped with a plan apochromat high numerical aperture immersion objective (40x, NA=0.95), a high-resolution low noise 16Mp DS-Ri Nikon

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CMOS camera (7.3×7.3μm pixel-size), a high resolution Prior motorized stage, a LED fluorescence lamp, and custom-made narrow-band fluorescence filters (Chroma Technology).

Images have been centered on capturing gastric mucosa epithelia for control tissue, and tumor epithelia for pathology cases.

All images have been converted to non-compressed tiff files (4908×3264 pixels or 137.48×91.43μm) and further processed for image analysis.

A fixed rectangle of 14.00×8.00μm has been overlaid on each image repeatedly 6 times, randomly, on epithelial areas, and each time selected areas were cropped out resulting 6 smaller images of the same size filled with the pattern of E-cadherin signal (Adobe Photoshop, Adobe Systems Incorporated).

Afterwards, images were imported in the Image ProPlus AMS software (Media Cybernetics, Bethesda, MD, USA), were binarized based on a fixed threshold of the E-cadherin fluorescence channel, and then the Fractal Dimension of the binarized image outlines has been calculated in order to assess the ruggedness of the cellular membranes.

Average values have been calculated for each 40x image, then for each patient, and finally for either control/low-grade, or high-grade adenocarcinoma.

Data were exported and plotted in Microsoft Office Excel 2010 (Microsoft Corporation, Redmond, Washington, United States), and were analyzed using the GraphPad Prism 9.2 software (GraphPad Software LLC).

In order to assess statistical differences, we used the student t-test for comparing the means of two groups and a one-way ANOVA (ANOVA-analysis of variance) with Tuckey post hoc analysis in order to compare the means of controls and the two pathological groups.

Data were reported as mean±standard deviation of the means (SD).

In all cases, $p < 0.05$ was used to indicate statistical significance.

Results

The main clinical and pathological features of the patients involved in the study are presented in Table 1.

Table 1 Patients included in the study.

Patient no.	Degree of differentiation	Age	Gender	Localization	Lymph node stage
1	Low grade	72	F	Cardia	N1
2	Low grade	51	M	Body	N2
3	Low grade	54	M	Body	N1
4	Low grade	78	F	Body	N3a
5	Low grade	76	M	Pyloric	N1
6	Low grade	69	M	Body	N2
7	Low grade	65	F	Cardia	N3a
8	Low grade	55	M	Body	N2
9	Low grade	69	M	Cardia	N2
10	High grade	69	M	Body	N1
11	High grade	52	F	Body	N1
12	High grade	65	M	Body	N2
13	High grade	52	F	Body	N1
14	High grade	44	F	Body	N3b
15	High grade	44	M	Pyloric	N2
16	High grade	78	M	Body	N1
17	High grade	79	M	Body	N2
18	High grade	60	M	Body	N0
19	Control	67	F	-	-
20	Control	59	M	-	-
21	Control	78	M	-	-
22	Control	65	F	-	-
23	Control	68	M	-	-
24	Control	58	F	-	-
25	Control	54	M	-	-
26	Control	75	F	-	-

After confirming once more the histopathology and tumor grading, we have processed the E-cadherin-stained slides (Figure 1).

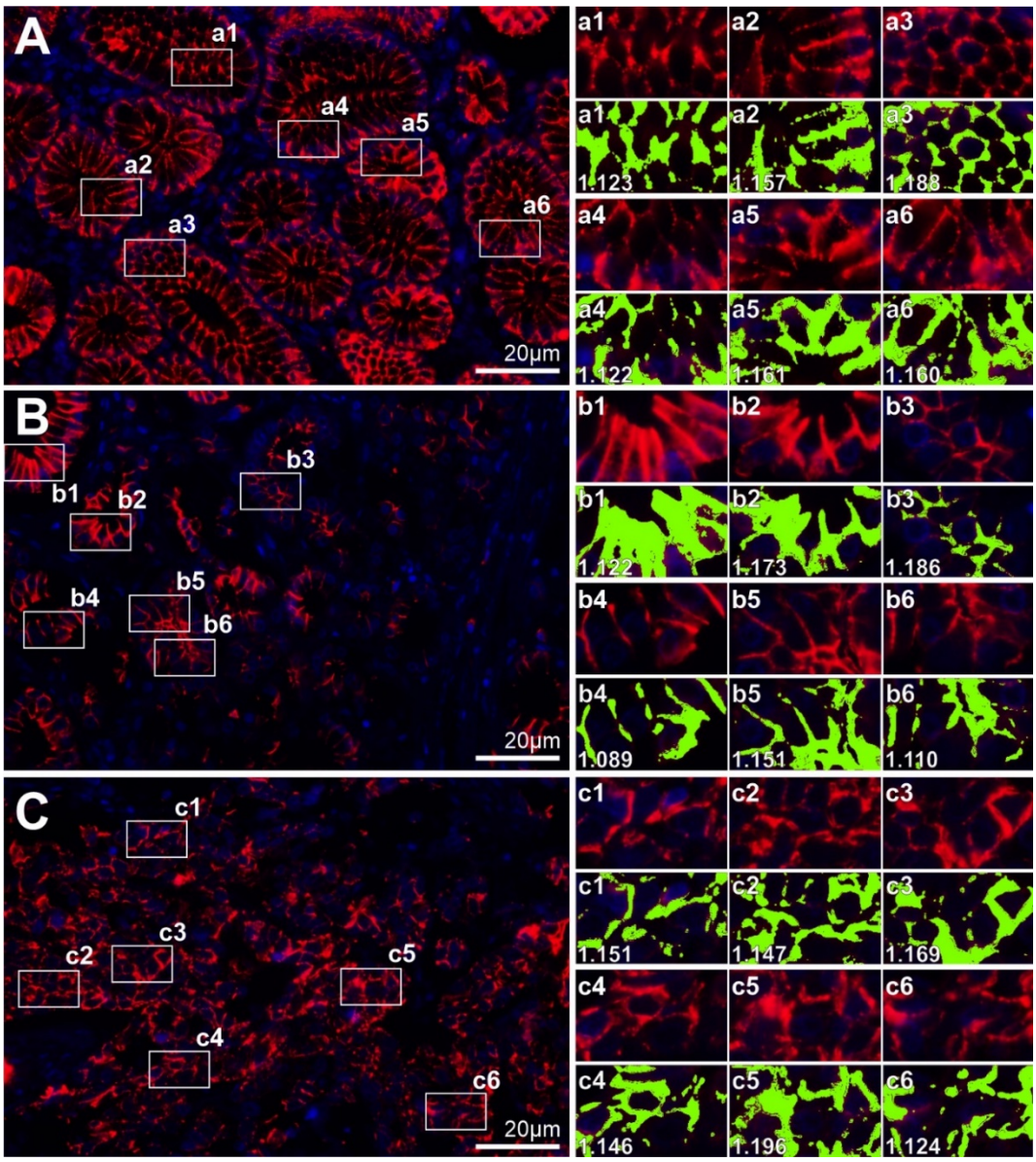

Figure 1. Exemplary images showing fractal analysis on epithelia based on a constant area rectangle for control gastric mucosa (A), low grade (B) and high grade (C) gastric adenocarcinomas. Image insets are presented separately as original enlarged images and binarized images (1-6) with Fractal Dimension calculated. Immunofluorescence for E-cadherin.

Thus, we have started with more than 200 high-resolution 40×images, and cropping fixed epithelial areas lead to more than 600 images that were processed for FD analysis.

Overall assessment of the FD revealed that the subtle membrane variations were evident enough to deem a statistically significant difference between control cases and cancer patients [t(22)=2.428, p=0.0119] (Figure 2A).

Besides the fact the complexity of the membrane roughness was clearly higher for adenocarcinoma cases, the data also showed that in normal gastric mucosae there is quite a high variability of the FD, probably reflected in the presence of the normal different type of cells that comprise the structure of the gastric pits, elements that are lost in adenocarcinoma.

Next, we intended to evaluate if separating adenocarcinoma cases as low grade (G2) and high grade (G3 and solid), FD analysis could still differentiate membrane patterns.

An analysis between control, low-grade, and high-grade adenocarcinoma revealed that there is no overall difference between these three categories, although high grade tumors showed a clear tendency for lower FD values compared to low grade tumors [F(2,21)=3.330, p=0.0554] (Figure 2B).

Again, it seems that the higher variability of control cases deemed these differences non-significant.

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Gender-dependence analysis also did not reveal any differences between males and females in what it regards the E-cadherin FD values [t(14)=0.845, p=0.206], although it revealed that although there were more males enrolled in the study, there was a tendency for higher FD variability here compared to female patients (Figure 2C).

We have next tried to see if the available clinical parameters might correlate with FD values for adenocarcinoma patients.

Thus, we have first looked at the age-FD relationship, and found a week indirect correlation between the two parameters [r(14)=-0.243, p=0.041] (Figure 2D).

Statistical analysis revealed thus that increasing age of the patients leads to lower FD complexity in the tumor membrane structures.

If we separated the analysis for low grade adenocarcinoma, the correlation became much stronger [r(9)=-0.5113, p=0.006] (Figure 2E), but if we looked at only high grade tumors, there was no correlation [r(7)=-0.048, p=0.127] (data not showed).

We could conclude that age is an important denominator for the morphology of the tumor cells' membrane morphology, but only for low grade cases, while, as expected, higher tumor grading would also lead to more altered morphology.

Also, statistical analysis revealed no difference between FD values for tumors located at the level of the cardia compared to the body of the stomach [t(11)=1.358, p=0.100], and although the average values seemed to be lower for the stomach body, this region also harbored higher variability between cases (Figure 2F).

Even when we narrowed down this analysis only for low grade tumors, the difference also did not attend statistical significance, although the data tended to show that the tumors located in the body region of the stomach would show lower E-cadherin FD values compared to cardia tumors [t(6)=1.747, p=0.0656],

Finally, a correlation analysis with the number of lymph ganglia involved, showed no relationship between the lymphatic extension of the disease and the profile of the tumor cells membranes.

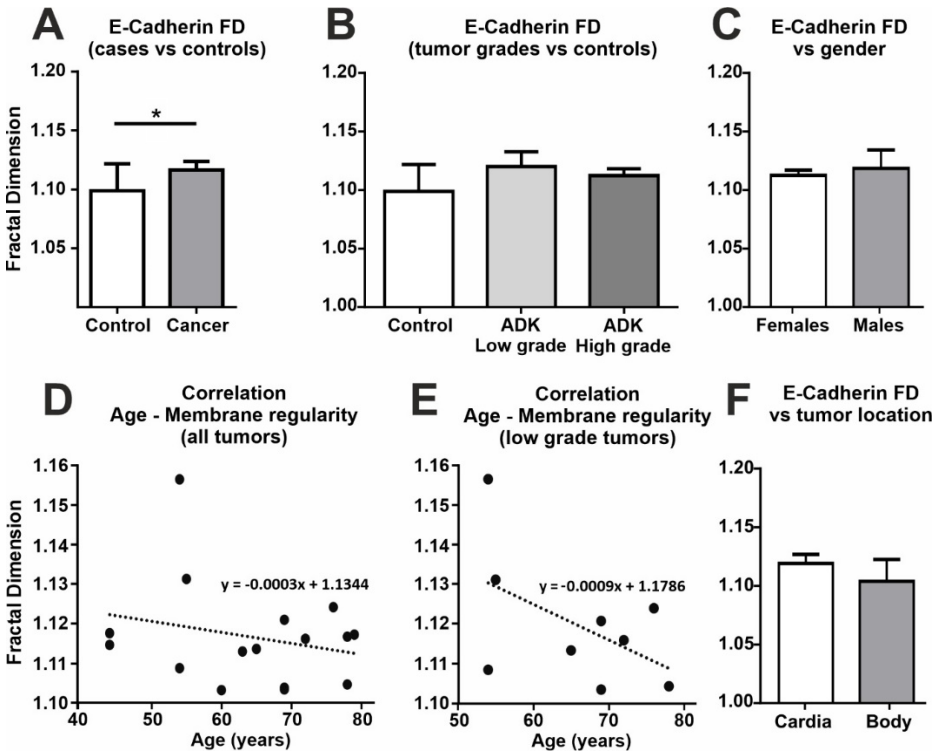

Figure 2. Fractal dimension for E-cadherin (FD) can differentiate between control mucosae and gastric tumors (A), while stratified tumors as low grade and high grade cannot be separated (B). Males tend to have a higher FD value for developed gastric tumors (C), with a moderate correlation between FD and age for all tumor cases (D), and with a strong correlation when considering only low-grade tumors (E). Location of the tumor seems to induce small variations in the calculated FD values (F). *, statistically significance, p<0.05. All error bars represent standard deviation.

Discussions

Cancer cells have a higher degree of fractality than non-malignant cells due to the anarchic growth of tumors, which creates highly irregular convexities of different sizes on the cell membrane.

It is possible to determine the progression of cancer in a cell using a mathematical method based on the fractal dimension in conjunction with sophisticated image recognition [13].

Additionally, as the rate of cancer cell divisions rises, which is frequently linked to the progression of neoplasia, cancer cells diverge from the ideal fractal (multi-fractality is reaching zero) [14].

Some research findings suggest that chaotic behavior happens to coincide with the cancer conversion of the immortalization stage of oncogenesis and that further cancer transition recovers the determinism of mechanisms responsible for cell surface configuration due to the relationship between fractal behavior and "chaos" [15].

Moreover, by using a computer-aided/automated quantitative approach of the FD detection of available commercially tissue microarrays samples by optical transmission imaging, it could be possible to distinguish correctly the various stages of colon, prostate, and breast cancer cases.

In comparison to currently used approaches, Klein K. and Co. demonstrate a high accuracy in recognizing cancerous cells with a failure chance of only 3% [13].

In gastric cancer, our data revealed that normal gastric mucosae also have a high level of variability in the FD, which is likely due to the presence of different types of cells that make up the structure of the gastric pits, elements that are absent in adenocarcinomas.

Through architectural reorganization, cell shape is known to impact a number of gene regulatory pathways, acting as a significant independent factor influencing cell commitment to quiescence, death, or proliferation.

Breast cancer cells can undergo drastic changes in shape that are reflected by fractal measurements when exposed to an experimental morphogenetic field [16].

The epithelium in GC tissue displays improved cell-ECM adhesion and diminished cell-cell adhesion.

Studies have proven that the loss of E-cadherin expression plays a significant role in the initiation and spread of gastric cancer [11].

One of the characteristics of carcinogenesis, along with tumor invasion and metastasis, is the modification of intercellular integrity [17].

The loss of E-cadherin expression on the CM weakens or splits cell-cell interactions and inhibits the activity of transcription factors, leading to EMT (epithelial-to-mesenchymal transition) [18].

In this disease, on immunohistochemistry, E-cadherin displayed granular-diffuse or vesicular-like patterns both beneath the membrane and, in fact, throughout the cytoplasm, in addition to a strict lateral and occasionally basally situated membrane pattern [19].

In a previous study, we demonstrated that E-cadherin colocalizes with the intracytoplasmic vesicle system for both control and tumor tissue, using LAMP1 (a lysosomal marker) and Giantin (a Golgi marker) by applying high-resolution deconvolution fluorescence imaging [19].

There are studies that focus on the distribution of paranuclear E-cadherin in adenocarcinomas, and they reported that E-cadherin appears paranuclear, punctate to vesicular in 18% (16/87) of intestinal-type adenocarcinomas [20] and others who report that E-cadherin is found in the lateral membranes of different epithelial cells in a scattered dot-like pattern [21].

Because E-cadherin is a crucial component of adherens junctions, which are essential for cell adhesion and the maintenance of the epithelial phenotype of cells [22] and decrease of E-cadherin expression is strongly linked to the initiation and progression of gastric cancer [11], in this study, we wanted to investigate in detail the pattern of E-cadherin at the level of the cell membrane using analysis of the fractal dimension on fluorescence immunohistochemistry samples labeled with E-cadherin in gastric adenocarcinoma.

Evaluation of the FD indicated that there was a statistically significant difference between control cases and cancer patients due to the membrane alterations.

In terms of E-cadherin FD values, gender-dependence analysis similarly did not show any differences between males and females, but it did show that even though there were more male patients included, there was a tendency for larger FD variability in this population compared to female patients.

Biological tissues have a self-similar organization with spatial variation in their mass density distribution.

The fractal dimension (FD) can be used to investigate and express this self-similar structure.

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The fractal dimension of an item is a number that quantifies how similar the structure remains as the length scale varies and is linked to the structural porosity of tissue sections.

It is now understood that the fractal dimension of tissue alters as cancer progresses [23-25].

Furthermore, our statistical analysis showed that FD complexity in cancer membrane structures decreases as patient age increases.

The fractal dimension (FD) of variate tissue and cellular-related morphological features varies during carcinogenesis, allowing for the diagnosis of cancer.

A higher FD or decreased goodness-of-fit of its regression line in certain malignant tumors signals more aggressive behavior and a poorer prognosis [26].

The FD of a tissue changes when cancer progresses because of the greater production and rearrangement of intracellular components such as DNA, RNA, lipids, and the ECM, which results in an increase in mass density and tissue reconfiguration.

Because cancer development alters the fractal dimension of a tissue, a quantitative diagnostic test based on these alterations can be theoretically devised.

Regarding our study, high grade tumors demonstrated a definite propensity for lower FD values when compared to low grade tumors, an examination of control, low-grade, and high-grade adenocarcinoma found that there is no overall difference between these three categories.

Once more, it appears that the greater variability of the control instances regarded these changes to be insignificant.

Tumor vascularization has long been thought to be more irregular and chaotic than normal vascularization.

Now that angiogenesis has been established as a fundamental process in tumor growth and a therapeutic target, there is a growing desire to comprehend the origins and effects of the aberrant vascular architectures identified in malignancies.

Fractals have the potential to be helpful measurements of these complicated systems [27].

This tool can be applied to other components of the cell.

Research on the neoplastic nuclei of melanomas as well as other carcinomas, like laryngeal carcinoma [28], oral squamous cell carcinoma [29], multiple myeloma [30], revealed an increase in nuclear fractal dimension (NFD) when compared to normal tissue, implying that increased nuclear complexity predicts increasingly aggressive invasion patterns [31], being used as a biomarker.

The study's primary limitation is the small number of patients who were available, who had no prior chemotherapeutic treatments or radiotherapy that would have affected the metabolism of tumor cells, and who provided pathology samples.

Also, due to the high inconsistency in E-cadherin staining, we did not include here signet-ring cell carcinoma cases.

In conclusion, the morphological analysis of a simple marker for the cell membrane can identify and separate tumor cells, and although this analysis showed a limited correlation with the main clinical and pathological denominators of the disease, it will be extremely helpful in the future for automatic computer-assisted diagnosis on slides, but also for assessing cellular adhesion and inter-cellular trafficking in cancer cells.

Acknowledgements

This work was supported by a grant of Ministry of Research and Innovation, CNCSUEFISCDI, PN-III-P4-ID-PCCF2016-0158 (THERRES), within PN III.

Conflicts of Interest

The authors declare no conflict of interest.

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