Correlation Criteria Between Extramural Invasion of Blood Vessels and Immunohistochemical Markers in the Processes of Neovasculogenesis

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Establishing the treatment strategy for patients diagnosed with colorectal cancer is based on their imaging and immunohistochemical evaluation. Depending on the stage of tumor invasion and its aggressiveness, is individualized, surgical, oncological, or both treatment. The most important factor for establishing these protocols is the impact of the tumor on intra- and perilesional colorectal vasculature and implicitly on the process of vascular neoformation in the paraneoplastic syndrome. In this regard, we studied a group of patients, colonoscopically diagnosed with colorectal cancer. The study method was to compare the expression of CD34 tumor marker with EMM (extramural vascular invasion) staging criteria. The results of our study were compared with the intraoperative aspects. In all cases studied tumor extension reached to at least intestinal muscle layer. We conclude that expression of the CD34 marker has always been increased in correlation with EMM 3-4 stages. In 2 patients with EMM score 2, the immunohistochemical marker expression was 6% above the maximum threshold. The expression of vascular endothelial proliferation marker is closely correlated with the EMM score - these values increase proportionally. The use of both criteria for establishing neovasculogenesis, significantly increases the predictability of postoperative progression and long-term survival.

Keywords: EMM, immunohistochemistry, CD34, colorectal cancer

Colorectal cancer is considered to be world number 3 as a prevalence of malignant tumors and second as mortality caused by these conditions [1]. Modern management of colorectal cancer is multidisciplinary, with a major focus on preoperative assessment. This has become possible with the advancement of medical imaging and immunohistochemical techniques. The formulation of a certain diagnosis and therapeutic protocol is a common element for aspiring multidisciplinary effort made by surgeons, oncologists and pathologists. In order to achieve preoperative staging of rectal cancer, it should be known locoregional extension of the tumor and systemic dissemination of cancer cells. Formulation of a therapeutic protocol and prognosis of colorectal cancer depend on tumor stage, tumor type, invasion of lymph nodes, as well as whether or not the sphincter apparatus and neighborhood fascia are involved.

Neoangiogenesis occurs independently of malignant transformation, involves growth factors, extracellular matrix enzymes, endothelial cell migration and proliferation, lumen formation and anastomosis with other vessels, in relation to phenotypic and tumor genetic changes [2]. Some authors have experimentally demonstrated that neoplastic cell populations release angiogenic molecules *in vivo*, before their neoplastic transformation reaches the level of formation of a solid tumor. Tumor growth and transformation of tumor cells into an angiogenic phenotype are associated with increased secretion of angiogenic molecules, such as fibroblast growth factor, FGF and endothelial proliferation factor VEGF. In addition, it has been shown that a tumor develops a pronounced angiogenic phenotype when angiogenesis inhibitors (anti-angiogenic factors) are suppressed during tumorigenesis, such as the case of thrombospondin, which normally helps keep the vessels in a non-angiogenic status [3, 4].

Initially, was used antibody measurement for CD31, CD34 markers and for growth factor VIII, but was recently used the quantification of CD105 cell endothelial cell marker expression [5, 6]. It seems, however, that this marker is not specific only to vascular endothelium [7].

Neovascularization that crosses the tumor mass develops rapidly and presents itself as a disorganized chaotic network with tortuous, thin walls; as a result, the irrigation of different tumor segments is differentiated, with deficiencies in some segments which can lead to tissue necrosis. In this context it was found that the tumor aggressiveness is much higher in the periphery than in the tumor center, in terms of microvascular density per mm2 [8]. Structural features of the vascular network in tumors [8,9] are: flattened endothelium, no differentiation between arteries and veins, poor structural stability, lack of parietal contractile elements, lack of receptors, incomplete formation of basal membrane, lack of lymphatic formation.

Unlike malignant non-transformed cells that secrete angiogenic factors as a result of a hypoxic stimulus or cellular damage, it has been experimentally demonstrated (*in vitro*), in 1995, that the tumor cells secretes, besides any stimulus, a strong complex of angiogenic factors called TAFC [10]. Numerous groups of researchers have since studied this complex, in order to isolate and identificate it. Some molecules have been identified (angiogenin, FGF, angiotropin, etc.), but others have remained unknown until now. Relatively recent studies have highlighted the presence of high platelet activation factor (PAF) in some tumors, a presence that has been linked to the important angiogenesis present in this type of tumor.

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Clinical studies, performed in order to verify the existence of a correlation between angiogenesis and the progression of tumor disease and to establish a possible prognostic role of the presence and quantification of the new vascular network, have produced the following results:

- metastasis in local and distal lymph nodes, as well as prognosis, were strongly correlated with the microvessel density presented in the tumor,

- because neovascularization can be visualized and quantified without using invasive methods, and since, as demonstrated, is correlated with tumor development, it can be an important tool in early detection of tumors as well as in monitoring the effectiveness of antitumor therapies [11].

CD-34 is an endothelial antigen that is used to highlight microvascular density (MVD) as a direct marker of neoangiogenesis degree [12].

As we noted in a previous study, this marker can react not only with *newly formed* vessels but also with normal vessels trapped under the tumor tissue, and overexpression of CD34 has been correlated with unfavorable progression, the transition from localized form to metastatic disease and postoperative recurrence in patients with colorectal neoplasm [13]. It has been shown to be expressed both on the surface of endothelial cells and on the surface of neoplastic cells, their density being correlated with prognosis [14].

The emergence and growth of new capillary vessels from those that already exist is strictly addressed to the tumor, a phenomenon that is required for metastasis processes and which, if not, does not occur. Studies conducted in important groups of patients [15-20] show a deterministic relationship between microvascular density and metastatic risk. The data shown indicates that the expression of the CD-34 marker is inversely proportional to the degree of histological differentiation of the tumors and to the staging of the tumor according to the degree of local extension. This immunohistochemical marker has a high sensitivity and precision [21], but its expression can not play the role of organ diagnosis because it can not localize the tumor.

Experimental part
Materials and methods

We used a study group of 11 patients diagnosed with colorectal cancer in the G1-G3 stages. We evaluated their grading according to known criteria: pTNM stages according to WHO criteria for distal colon and proximal rectum [22]. We used the *American Joint Committee on Prognostic Cancer* to chart the grading [23].

Most of the studied cases, were moderate and poorly differentiated colorectal adenocarcinomas (G2 and G3). Approximately 60% were in advanced stages (T3), with perirectal adipose tissue invasion.

The results obtained were interpreted according to the intensity of fixation of the immunohistochemical reagent. We used the semi-quantitative scale of the color intensity scale as follows:

- Fixing absent in the absence of coloration (*Grade 0*) or Negative Result (-);

- Low intensity fixation when a low intensity stain (*grade 1*) or poorly positive result (+) result is obtained;

- moderate intensity fixation when a medium intensity stain (*grade 2*) or moderately positive result (++) is obtained;

- high intensity fixation when intense staining of the tissue specimen (*Grade 3*) or intense positive result (+++) has been obtained.

We correlated the results obtained for each patient with the imaging aspects of the perilesional neoangiogenesis processes. We have used computer tomography (CT), nuclear magnetic resonance imaging (MRI) and 3D reconstruction techniques in the pelvis, methods we revealed changes in number, appearance and caliber of intra- and extra-colorectal vessels at the level of the area where the malignant formation occurred. The 11 cases studied were evaluated by colonoscopy, CT and MRI. We focused on the anatomical localization of the tumor, the change in local vasculature, the intensity of the angiogenesis process, the depth of the tumor in the colorectal wall, its relations with the mesorectum as well as with the neighboring organs.

On the MRI and CT images we have applied the EMVI score criteria.

Results and discussions

Neovascularization quantifications were performed on sections stained with CD-34 antigen, the expression of which is intense in the neoformation microvascular endothelium, being considered a direct marker of the degree of neoangiogenesis. As mentioned above, the CD34 marker shows the condition of vascular endothelium in colorectal carcinoma, but it marks both neoformed and normal vessels. For this reason only high density areas and intensely colored endotheliums are considered (figs. 1 and 2).
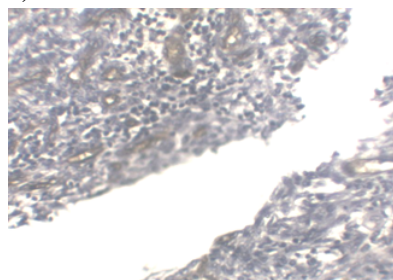

Fig. 1 Tumor stroma in a poorly differentiated colorectal carcinoma with the presence of CD34 positive vessels
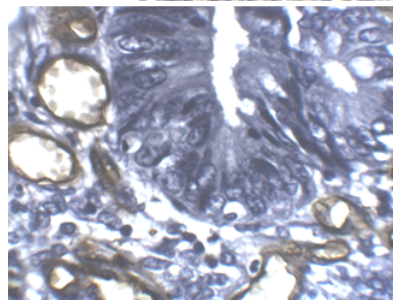

Fig. 2. Overexpression of CD34 in vascular endothelium

The graphical representation of the reported changes highlights the significant increase in tumor microvascular density with increasing grading, inversely proportional to the degree of differentiation. Generally, in metastatic tumors, microvascular density is greater than in non-metastatic tumors. In addition, there are large variations in vascular density between different areas of the same tumor in poorly differentiated forms, whereas in well-differentiated carcinomas the topographic location is more constant. In the central, perinecrotic and extratumoral areas, microvascular densities are similar.

Depending on the degree of adenocarcinoma differentiation, ranging from the well-differentiated to the poorly differentiated form, the density of the microvessels varied as follows: the microvascular density / mm^2 tumor stroma progressively increased from 480.30 / mm^2 to 875.92 / mm^2 and 1495, respectively, 37 / mm^2, and the microvessel density / mm^2 tumor exhibited the same change of magnitude, from 670.54 / mm^2 to 1053.84 / mm^2 and 2250.69 / mm^2 tumor respectively (table 1).

	TUMOR TYPE		
Vascular density	Well-differentiated adenocarcinoma	Moderately differentiated adenocarcinoma	Undifferentiated adenocarcinoma
Density of microvessels / mm^2 of tumor stroma (DM / ST)	480.30	875.92	1495.37
The density of microvessels / mm^2 of tumor (DM / T)	670.54	1053.84	2250.69

Table 1

This microvessel density (MVD) criterion was commonly used as a prognostic marker in immunohistochemical determinations performed with other substances, such as VEGF A, CD105, Ki67.

The degree of extramural vascular invasion on MRI images, ranges from minimal to extended, following four criteria: tumor edge, tumor location, vessel size and its appearance. The lowest score, 0, correlates with the absence of any suggestive characteristic for extramural vascular invasion. The maximum score, 4, is given when the most obvious features of vascular tortuosity, nodular and obstructive appearance are seen (figs. 3, 4 and 5). Larger scores are associated with short survival and the smaller ones are not associated with vascular extramural invasion. Patient stratification in prognostic groups based
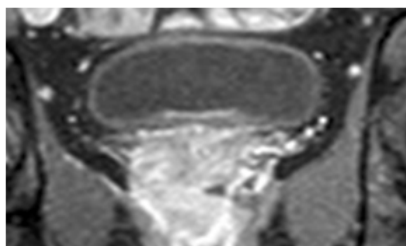

Fig. 3 Neoformation vessels with perivascular adenopathy characteristic of EMM 2 score

Fig. 4 Neoformation vessels with multifocal condensation, characteristic of the EMM score 3

Fig. 5 Rectal vein characteristic of the EMM score 4

on extramural vascular invasion score is clinically relevant for both preoperative treatment of high-risk patients and postoperative follow-up [24, 25].

The correlation between measuring MVD in immunohistochemical determinations with CD34 antibody in colorectal cancers and evaluating the same patients according to EMM staging criteria is the main novelty of this study. Both methods provide information about neoangiogenesis processes in colorectal paraneoplastic syndrome.

The association of this information increases the diagnostic and prognostic value of the two determinations - the weaknesses of the immunohistochemical evaluation with CD34 are counterbalanced by the information provided by EMM staging and vice versa.

We consider that the simultaneous use of immunohistochemical markers linked to vascular endothelial proliferation with quantitative and, above all, qualitative assessment of tumor and peritumoral neovasculogenesis can be used as a protocol for the individualization of pluridisciplinary colorectal cancer treatment.

The use of medical imaging techniques in assessing neovascularization processes is also a criterion for verifying the specificity of an immunohistochemical marker or even the connection between MVD and VEGF receptors [26, 27].

Blood and lymphatic vessels have a rich density to their area of origin (posterior, mesorectal), and this decreases as we move forward, this vascular distribution being consistent with the predisposition for the formation of a rectal neoplasia, especially on the posterior wall or, possibly, the lateral ones [28, 29]. The anterior evolution of a rectal formation is a second step in the evolution of these tumors and is dictated by the pelvis's conformation.

Conclusions

Although the CD34 panendotelial marker does not indicate the angiogenesis intensity because it marks both neoformed and normal vessels, highlighting the state of vascular endothelium in colorectal carcinoma, it is considered that quantization of microvessels density (MVD) could be useful in assessing tumors's aggression. CD34 overexpression was correlated with unfavorable prognosis, transition from localized form to metastatic disease and postoperative recurrence in patients with colorectal neoplasm. The anatomic-imaging staging of colorectal cancers using the EMM score is very close to the accuracy of colonoscopic diagnosis. The current tendencies to minimally invasive techniques, but with very high accuracy, are achieved by the correct, multidisciplinary interpretation

of aspects related to paraneoplastic vascular invasion. EMM scores are correlated with those in the classic TNMG2 and G3 stages. Concomitant assessment of patients using the CD34 marker and the EMM score corrects the shortcomings of both methods and provides a set of important information for both the surgeon and the oncologist.

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