ORIGINAL PAPER

The differences between the endothelial area marked with CD31 and CD105 in colorectal carcinomas by computer-assisted morphometrical analysis

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Abstract

The aim of our study was to compare the CD31 and CD105 endothelial area (EA) by computer-assisted morphometrical analysis in colorectal carcinomas (CRC). Two hundred and eleven surgical specimens with CRC were immunohistochemical analyzed with markers for angiogenesis CD31 and CD105 (Endoglin). We determined the area of endothelial cells occupied in the microscope field (EA). *Results:* The median area was 6.93 ± 4.25% for CD31, respectively 5.65 ± 2.23% for CD105. In the majority of cases, the CD31 EA was higher than CD105 EA. In the cases with the predominance of mature vessels, and also in the cases after radiotherapy, the CD105 EA was higher than CD31 (5.69 ± 2.49%, respectively 10.23 ± 5.93%). In our study, we tried to describe the clinico-morphological features of these cases. *Conclusions:* The CD105 seems to be the best marker for study of neoangiogenesis in CRC. Sometime, CD105 marks the activated endothelium of preexistent mature vessels. The radiotherapy destroys the neoformed but also the preexistent vessels. For the antiangiogenic treatment, it is important to determine the intensity of angiogenesis but also the type of neoformed vessels.

***Keywords*:** angiogenesis, colorectal cancer, endothelial area, CD31, CD105.

Introduction

More than 200 papers published in the last 10 years reveal the prognostic significance of angiogenesis in colorectal carcinomas (CRC). The majority of authors observed that the microvascular density (MVD) had an important role in tumor progression but also in the survival rate [1, 2]. This idea is infirmed by other studies [3]. The clinical trials proved that the anti-angiogenic treatment could prolong the survival rate with 2–5 months but the results are not observed in all patients with CRC [4, 5]. One of the reasons for resistance at this treatment seems to be the heterogeneity of vessels [6]. The mature neoformed vessels are resistant at antiangiogenic drugs [7], but the pericytes limit tumor cell metastasis [8].

The angiogenesis could be identifying with the panendothelial markers CD31 and CD34, and also with CD105 (Endoglin). CD31 and CD34 show the vascular status of CRC but they do not indicate the angiogenic intensity because they mark both neoformed vessels and normal, preexistent vessels in neoplastic and non-neoplastic tissues [6].

CD105 seems to be more specifically for the endothelial cells of neoformed vessels. Its expression increased in the same time with the neoangiogenic progression [1].

Some authors described that the progression of tumor is accompanied by the increasing of vessels' diameter and decreasing of number of these vessels [9]. The diameter of vessels seems to be the primordial parameter in the metastatic spread [9].

The quantification of angiogenesis was made in the majority of studies with the classical "hot-spot" Weidner's method, which supposes the counting of positive microvessels with and without vascular lumen [10]. With this method it is determines the MVD. Other possibility is to determine the endothelial area (EA) which is defined as the percentage area occupied by the positive endothelial cells in the microscope field [11].

In our paper, we tried to determine the positive EA by computer-assisted method and to compare the CD31 and CD105 endothelial area. In this way, we made a morphometrical analysis.

Material and Methods

Surgical specimens from 211 patients with CRC diagnosed in the Department of Pathology of Emergency County Hospital of Targu Mures, Romania, were used for IHC staining.

All cases were adenocarcinomas. The histopathological interpretation supposed the fixation of pieces in

10% formalin solution, inclusion on paraffin, sectioning at microtome at 3–5 μm thickness and a staining with Hematoxylin–Eosin.

The lesions were examined with optical microscope and were classified after pTNM staging, according with the criteria of *World Health Organization* (WHO) for colon and rectum [12], and also for anal canal [13]. To establish the histological grade we used the criteria of *American Joint Committee on Cancer Prognostic* [14].

We considered mucinous carcinomas the cases with more than 30% mucinous component.

The number of cases in different colon segments is observed in Table 1.

Table 1 – ***The distribution of cases included in the study***

Clinicopathologic factors		Right colon [n]	Rectum and sigma [n]	Descendent colon [n]	Anal canal [n]	Total [n]
Age [years]	≤40	10	40	7	0	57
	≥40	51	73	19	11	154
Sex	Men	37	72	20	3	132
	Women	24	41	6	8	79
pT	pT1	1	2	1	0	4
	pT2	6	20	5	1	32
	pT3	48	79	15	9	151
	pT4	6	12	5	1	24
pN	pN0	36	53	16	11	116
	pN1, 2, 3	25	60	10	0	95
Histologic grade and type	G1	12	48	12	4	76
	G2	15	24	6	3	48
	G3	13	8	1	1	23
	Mucinous carcinomas	21	33	7	3	64

The angiogenesis was analyzed through immuno-histochemical staining in CRC with and without lymph node metastases.

We used the following antibodies, provided by LabVision: CD31 clone JC/70A, dilution 1:40; CD105 clone SN6h, dilution 1:50; VEGF-A, clone VG1, dilution 1:50; p53 clone DO-7, dilution 1:50; Ki67 clone Ki-S5, dilution 1:200.

The antibodies Ki67 and p53 were used to determine the proliferative activity of tumoral cells. With VEGF, CD31 and CD105 we quantified the angiogenesis.

We used the Ultra Vision system by LabVision, in formalin-fixed, paraffin-embedded tissues. Sections were deparaffinized, were incubated at 100^0C in citrate solution, pH 6 (CD31, Ki67) or in EDTA, pH 9 (CD105, VEGF, p53), and were washed with distilled water before the hydrogen peroxide incubation.

After this, all sections were washed with Tris Buffered Saline (TBS) and were incubated with primary antibodies for 60 minutes, then were washed with TBS and were covered by Streptavidin Peroxidase Solution for 5 minutes.

After this, they were washed with TBS and were covered with Biotinylated Goat Anti-Polyvalent Solution for other 5 minutes.

The development was performed with substrate-chromogen solution (DAB) for 3–5 minutes. The nuclei were stained with Mayer's Hematoxylin.

For angiogenesis quantification we determined the endothelial area and compared the values of EA determined with CD31 with that for CD105, trying to observe the differences between these two antibodies.

We identified the "hot-spot" regions at 100 high-power fields and realized the digital photo capture at 400× high-power fields.

The pictures were realized with the optical microscope Nikon 800E, coupled to a color video camera and were made in the intratumoral area (five pictures for each area).

The count was made using NIH's ImageJ software for image processing. We batch-measured the EA *versus* total tissue area ratio. We eliminated the ulcerated regions, and also the regions rich in lymphocytes.

The intensity of VEGF-A was scored according to the following criteria:

- score 0, no staining;
- score 1+, weak diffuse cytoplasmic staining <10% of tumoral cells;
- score 2+, moderate cytoplasmic staining in 10–70% of cells;
- score 3+, strong cytoplasmic staining >70% of tumoral cells.

For statistical analysis, we used the Statistical Program Graph Pad In Stat 3-Trial Version. We used the two-tails unpaired *t*-test, chi square test and the contingency tables, determining the values of *p* and *chi*. We considered the significant association when $p<0.05$, with 95% confidence interval.

Results

The mean endothelial area (EA) for both CD31 and CD105 was higher in the cases located in the anal canal, and the smaller EA was observed in the right colon (Table 2).

Table 2 – ***The mean endothelial area marked with CD31 and CD105 in different segments of colon***

Marker	Right colon	Rectum and sigma	Descendent colon	Anal canal	Whole colon
CD31	5.62 ± 2.51%	6.91 ± 4.81%	7.51 ± 3.58%	7.73 ± 3.54%	6.93 ± 2.35%
CD105	3.95 ± 3.24%	5.08 ± 3.92%	6.30 ± 3.27%	7.27 ± 4.12%	5.65 ± 4.25%

In all colon segments, except the rectum and sigma, the expression of CD31 was not correlated with that of CD105.

In rectum and sigma the correlation was significant ($p = 0.002$).

The VEGF-A intensity was not correlated with EA for CD31 ($p = 0.19$) neither with EA for CD105 ($p = 0.15$).

The CD31 and CD105 EA were not correlated with p53 ($p = 0.15$, respectively 0.19).

EA for CD31 decreased in parallel with the increasing of Ki67-positivity ($p<0.001$).

EA for CD105 presented oscillating values related with Ki67-positivity (Table 3).

The most interesting aspect was observed when we compared the EA for CD31 and CD105. In the majority of cases, the EA for CD31 was higher than CD105. In these cases, the mean EA was 6.93 ± 2.35% for CD31 respectively 5.65 ± 4.25% for CD105.

Table 3 – ***The correlation between endothelial area, VEGF-A, p53 and Ki67***

Immunohistochemical marker		Endothelial area determined with CD31	Endothelial area determined with CD105
VEGF-A intensity	negative	7.86 ± 3.32%	5.72 ± 2.70%
	1+	6.67 ± 4.36%	4.44 ± 3.45%
	2+	6.62 ± 3.47%	5.17 ± 3.12%
	3+	6.76 ± 4.63%	6.09 ± 3.82%
Ki67-positivity	<10%	7.07 ± 4.13%	3.65 ± 2.47%
	<50%	7.69 ± 5.39%	6.51 ± 4.07%
	>50%	4.66 ± 1.82%	3.27 ± 2.79%
p53-positivity	<10%	6.67 ± 4.07%	4.22 ± 2.14%
	<50%	6.55 ± 4.51%	5.12 ± 2.17%
	>50%	4.32 ± 2.16%	4.23 ± 1.87%

In 25 from the 211 cases (11.85% of cases), the CD105 EA was higher than CD31.

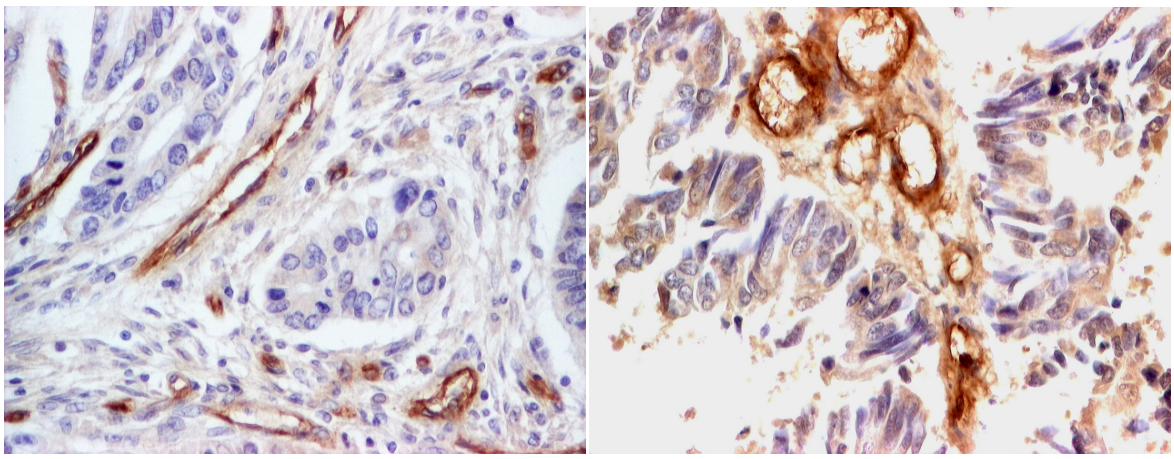
The median EA was 5.69 ± 2.49% for CD31 and 10.23 ± 5.93% for CD105.

The number of cases with this particularity, related to colon segments was:

- six cases in the anal canal (54.54% of cases);
- four cases in the descendent colon (16%);
- 12 cases in the rectum and sigma (10.61%);
- three cases in the right colon (4.92%).

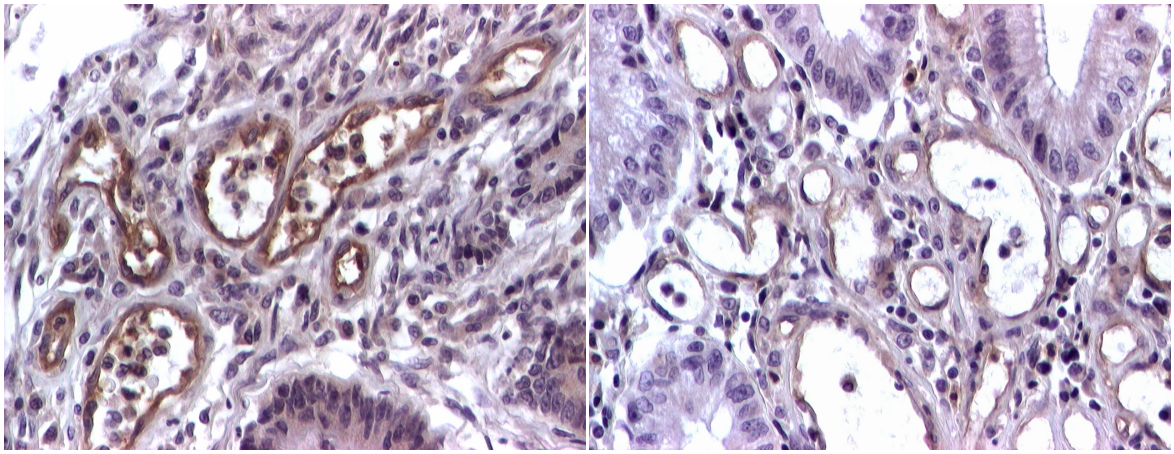
We analyzed the clinical, histological and immunohistochemical features of these 25 cases and we observed that:

- in all cases the large mature vessels, with big lumen and thin wall were predominated (Figures 1 and 2);
- all cases located in the right and descendent colon presented mucinous component;
- the rectum and sigma tumors were non-mucinous adenocarcinomas;
- the surgical intervention of the anal canal carcinomas was performed after radiotherapy;
- the lymphangiogenic invasion was observed in all cases;
- the majority of cases (20) penetrated the muscularis layer but had not lymph node metastases;
- all cases were diagnosed after 46-year-old, no differences between male and women;
- in all cases VEGF had a strongly intensity and p53 and Ki67-positivity was higher than 50% of nuclear cells.



(A) **(B)**

Figure 1 – ***The large mature vessels, marked with CD105 (ob. ×10 – A, and ×20 – B).***



(A) **(B)**

Figure 2 – ***The mature neoformed vessels, marked with CD105 (A) and mature preexistent vessels, CD105-negative (B), in one well-differentiated adenocarcinoma (ob. ×20).***

Discussion

Conflicting results are found in the published literature regarding the angiogenesis in CRC. One of the reasons is the large panel of antibodies, and also the different methods utilized for quantification.

Few papers reveal the prognostic role of positive endothelial area (EA) occupied in the microscopic field [9, 11], and the differences between CD31/CD34 and CD105 EA [11, 15].

In some studies, the MVD determined with CD105 was higher than that for CD31, which demonstrated that the CD105 is the best marker to identify proliferating endothelium involved in tumor angiogenesis [2].

In according with other studies we believe that the computerized method is more accurately than conventional MVD determination because in the latter it is determined only the number of vessels and the diameter, perimeter or size of vessels are not evaluated [16].

In our study, we observed that the median EA for CD31 was higher than that from CD105. This feature seems to be normally because CD31 also marks the preexistent mature vessels and neoformed vessels, the thrombocytes, plasmocytes and megakaryocytes. On the other hand, CD105 do not mark the endothelial cells from the preexistent vessels. Because the EA for both antibodies was highest in the anal canal carcinomas, we tried to explain this feature according to the radiotherapy performed before surgical intervention.

It is known that the radiotherapy decreases the number of proliferating tumor cells and proliferating endothelial cells [17], but also increase the pressure of oxygen, especially in the tumor rim [18].

Because in our study, in the carcinoma of the anal canal the majority of vessels were mature and 54.54% of cases presented a higher CD105 EA than CD31, we tend to believe that the radiotherapy destroys the neoformed but also the preexistent vessels.

We must mention that in the biopsies performed before radiotherapy and surgical intervention the CD31 EA was higher than CD105 and all types of vessels where present.

Conclusions

Because in the 25 cases the mature vessels were predominated we have two possibility for interpretation:

- CD105 marked the endothelium of preexistent mature vessels, which is activated like first step in angiogenesis. It is difficult to believe this idea because, in this situation, the vessels would be marked also by CD31.
- CD31 non-marks all the mature neoformed vessels and CD105 remains the best marker for angiogenesis.

The small number of cases with the CD105 EA higher than CD31 EA compared with that in which CD31 was highest do not allow yet to state definite conclusions.

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