ORIGINAL PAPER

The angiogenesis in colorectal carcinomas with and without lymph node metastases

SIMONA GURZU[1)], J. JUNG[1)], L. AZAMFIREI[2)], T. MEZEI[1)],
ANCA MARIA CÎMPEAN[3)], Z. SZENTIRMAY[4)]

[1)]Department of Pathology
[2)]Department of Intensive Care
University of Medicine and Pharmacy of Targu Mures, Romania
[3)]Department of Histology,
"Victor Babeş" University of Medicine and Pharmacy Timisoara, Romania
[4)]Department of Pathology and Molecular Biology,
National Institute of Oncology, Budapest, Hungary

Abstract

Many clinical trials revealed that the anti-angiogenic treatment could improve prognosis in patients with metastatic colorectal carcinomas (CRC), when added to standard chemotherapy. In this paper, we tried to find out if the microvascular density (MVD) determined with CD31, CD105 was correlated with lymph node status, and if the intensity of angiogenesis was different in right versus left colon segments. We studied 187 CRC, with and without lymph node metastases, 128 from left and 59 from right colon. *Results*. In the right colon, the MVD was higher in the cases where the lymph nodes did not present metastases (pN0) but also when four or more lymph nodes were involved (pN2). In the rectum and sigma, the angiogenesis presented the highest intensity in pN0 and pN1 stage (1–3 lymph nodes with metastases), decreasing in pN2 stage. In the descendent colon segment, the MVD did not present differences between the cases with and without lymph node metastases. *Conclusions*. Our study reveals that the most indicated cases for antiangiogenic treatment seem to be the pN0 and pN1 cases in the rectum and sigma, respectively pN0 and pN2 cases in the right colon. We tend to believe that the angiogenesis intensity in CRC is higher in early-stages of the tumoral proliferation but it is not an increasing process, having rather an oscillating character. Therefore, the angiogenesis remains an independent prognostic and predictive factor and the antiangiogenic treatment is necessary to be individualized for each patient.

Keywords: colorectal carcinoma, lymph node metastases, angiogenesis, immunohistochemistry, antiangiogenic treatment.

⊡ Introduction

Many clinical trials revealed that the anti-VEGF (vascular endothelial growth factor) agents could improve prognosis in patients with metastatic colorectal carcinomas (CRC), when added to standard chemotherapy [1, 2]. The antiangiogenic treatment could prolong the survival time with 2–5 months [3], but the results are not the same in all cases. This is the reason why many studies regard the angiogenesis in CRC.

High angiogenesis intensity seems to be correlated with CRC aggressive histopathological features and poor patients' survival [1]. Some studies reveal that a high microvessel density (MVD) predicts the occurrence of metastatic disease. All authors do not sustain these ideas, the reason being that in various studies different antibodies for immunohistochemical (IHC) analysis for angiogenesis were used [4].

CD105 (endoglin), a co-receptor of the TGF-beta family, is a marker for neovascularization in CRC. It is correlated with a high risk for lymph node metastases [5]. Some studies reveal that the MVD determined with CD105 is not correlated with recurrence rate or survival but it is correlated with the patients' sex, having a higher intensity in women [4].

In this paper, we tried to find if the MVD was correlated with lymph node status and if the intensity of angiogenesis was different in the right versus left colon segments.

⊡ Material and methods

Tissue preparation

Surgical specimens from 187 patients with CRC diagnosed in the Department of Pathology of Emergency Hospital Targu Mures, were used for immunohistochemical (IHC) staining.

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

The angiogenesis was analyzed in CRC with and without lymph node metastases.

After pTNM staging, the cases were classified in:
▪ pN0: no lymph node metastases;
▪ pN1: 1–4 lymph nodes with metastases;
▪ pN2: more than four lymph nodes with metastases.

Primary antibodies

We used the following antibodies, provided by LabVision: CD31 clone JC/70A and CD105 clone SN6h.

Immunohistochemical staining

We used the immunoperoxidase staining, in formalin-fixed, paraffin-embedded sections. Sections were deparaffinized, were incubated at 100^{0}C in citrate solution, pH 6 (CD31) or in EDTA, pH 9 (CD105) and were washed with distillated 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-chromogene solution (DAB) for 3–5 minutes.

The nuclei were stained with Mayer's Hematoxylin.

Morphometrical analysis

To determine the microvascular density (MVD) we used the pictures by "hot-spot" regions, at 200× and 400× high power fields.

The pictures were realized with Nikon 800E microscope and were made in the intratumoral area (five pictures for each area).

The count was made using NIH's ImageJ program-Trial Version.

We batch-measured the positive area *versus* total tissue area ratio.

We eliminated the ulcerated regions and the regions rich in lymphocytes.

Statistical analysis

For statistical analysis, we used the Statistical Program Graph Pad In Stat 3-Trial Version.

First, we collected the data with the program Microsoft Excel.

We used the t-test, chi square test and the contingency tables, Fischer's test, One way ANOVA test, determining the values of p and chi.

We considered the significant association when $p < 0.05$, with 95% confidence interval.

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

Segment of colon	pN0 (n)	pN1 (n)	pN2(n)	Total
Right colon	36	17	6	**59**
Recto and sigma	54	35	15	**104**
Descendent	16	8	0	**24**
Total	**106**	**60**	**21**	**187**

⊟ Results

In all colon segments CD31 immunostain was correlated with CD105 when the counting was made at both 200× and 400× magnification ($p < 0.05$).

The angiogenesis in the right colon

If we make a first sight analysis of Table 2, we would observe that the average area for CD31 is higher in pN1 but for CD105 is higher in pN2 cases.

The ratio CD31/CD105 was smaller in pN2, which means that the neoangiogenesis intensity increased with the number of lymph node, which presents metastases. When we statistically analyzed the results, we observed completely different aspects.

Therefore, there were no differences between the cases with and without metastases regarding neither CD31 nor CD105 average area ($p = 0.94$ respectively 0.52).

A statistical difference regarded the ratio **CD31/CD105** which **was higher in pN1 than pN0 and pN2** cases ($p = 0.04$ respectively 0.03). This difference was not observed between pN0 and pN2 cases ($p = 0.66$).

The angiogenesis in the rectum and sigma

With both CD31 and CD105 we observed that **the angiogenesis was higher in pN0 and pN1 than in pN2 cases** ($p = 0.003$ respectively 0.02).

These differences were also observed between the ratio CD31/CD105 at 400× magnification ($p = 0.03$ respectively 0.01). We did not observe differences between pN0 and pN1 cases regarding MVD (Table 3).

The particularity of angiogenesis in the descendent segment of the colon

Out of 24 cases localized in this segment, 16 were in pN0 and eight in pN1 stage. There was no difference between the cases with and without lymph node metastases regarding MVD.

The most interesting aspect was observed when we made a comparison among the right, the recto-sigmoidian and the descendent segment of colon (Table 4).

Therefore, the **CD31 average area in the descendent colon segment was higher than in the right colon or in the rectum and sigma** ($p < 0.0001$ respectively 0.007). This difference **was also observed for CD105** average area ($p = 0.004$ respectively 0.01). Regarding the ratio CD31/CD105 differences between descendent and right or left colon were not observed ($p = 0.07$) (Figures 1–4).

Table 2 – *The values of microvessel density in the right colon*

Tumor stage	Average area for CD31		Average area for CD105		CD31/CD105	
	200×	**400×**	**200×**	**400×**	**200×**	**400×**
pN0	3.83 ± 2.25%	5.30 ± 2.66%	2.74 ± 1.97%	3.22 ± 1.48%	1.67 ± 0.91	**2 ± 0.86**
pN1	**4.06 ± 1.73%**	**7.25 ± 2.71%**	2.25 ± 1.80%	4 ± 2.24%	2.18 ± 1.05	**2.87 ± 1.64**
pN2	4 ± 2%	5.5 ± 3.5%	**3.33 ± 2.65%**	**5.5 ± 3.53%**	**1.50 ± 0.83**	**1.33 ± 0.57**
Total	**3.86 ± 1.99%**	**6 ± 2.76%**	**2.58 ± 1.94%**	**3.6 ± 2.98%**	**1.85 ± 1.03**	**2.35 ± 1.34**

Table 3 – *The values of microvessel density*
in the rectum and sigma

Tumor stage	Average area for CD31		Average area for CD105		CD31/CD105	
	200×	*400×*	*200×*	*400×*	*200×*	*400×*
pN0	5.69 ± 3.39%	8.55 ± 3.21%	3 ± 2.31%	4.86 ± 3.66%	2.46 ± 1.83	**2.56 ± 2.53**
pN1	5.15 ± 3.09%	8.55 ± 3.78%	2.82 ± 2.61%	5 ± 3.13%	2.33 ± 2.01	**2.53 ± 2.36**
pN2	**2.93** ± 1.79%	**5.87** ± 3.09%	**1.47** ± 1.73%	**2.47** ± 1.20%	2.13 ± 1.24	**3.40 ± 2.09**
Total	**4.5 ± 3.12%**	**7.27 ± 5.04%**	**2.38 ± 2.24%**	**4.03 ± 2.97%**	**2.22 ± 1.66**	**2.55 ± 2.28**

Table 4 – *The values of microvessel density*
in different colon segments

Colon segment	Average area for CD31		Average area for CD105		CD31/CD105	
	200×	*400×*	*200×*	*400×*	*200×*	*400×*
Right colon	3.86 ± 1.99%	6 ± 2.76%	2.58 ± 1.94%	3.6 ± 2.98%	1.85 ± 1.03	**2.35 ± 1.34**
Recto and sigma	4.5 ± 3.12%	7.27 ± 5.04%	2.38 ± 2.24%	4.03 ± 2.97%	2.22 ± 1.66	2.55 ± 2.28
Descendent	**6.33 ± 2.77%**	**8.20 ± 3.58%**	**3.96 ± 1.29%**	**5.48 ± 2.74%**	**2.43 ± 1.01**	**2.56 ± 1.02**
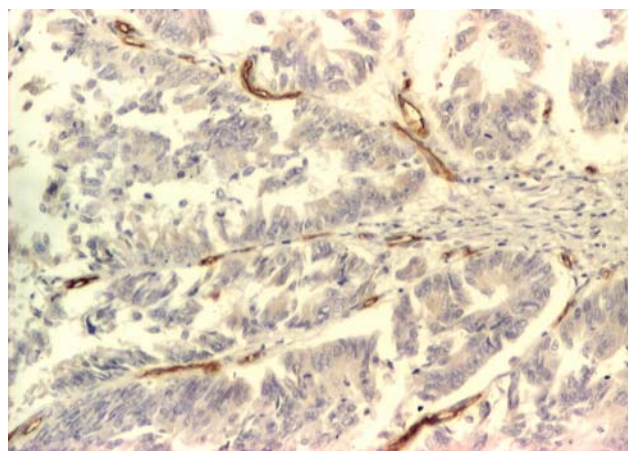
Figure 1 – *Immature and intermediary vessels*
marked with CD31 (ob. 10×)
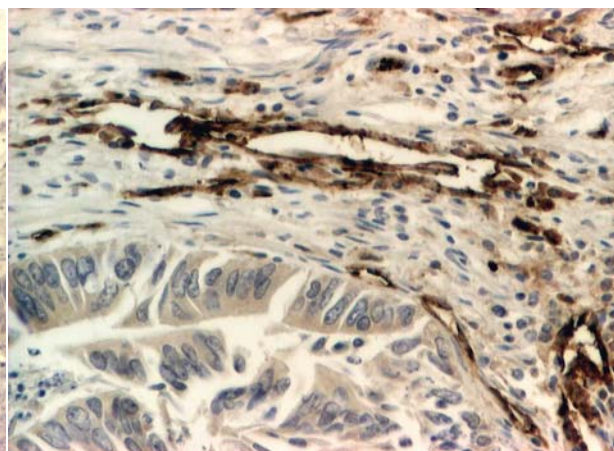
Figure 2 – *Vessels marked*
with CD31 (ob. 20×)
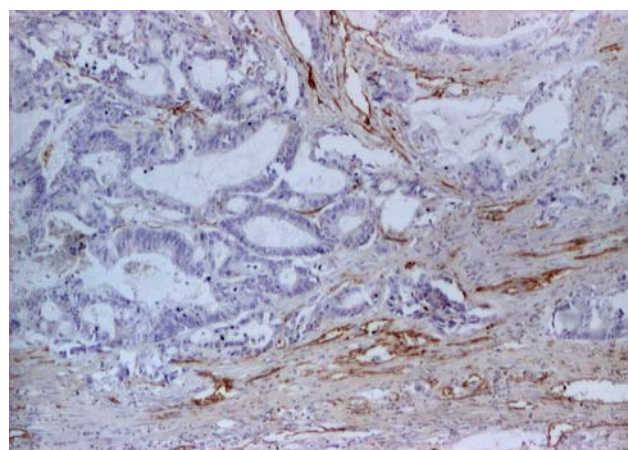
Figure 3 – *Neoformed vessels marked*
with CD105 (ob. 10×)
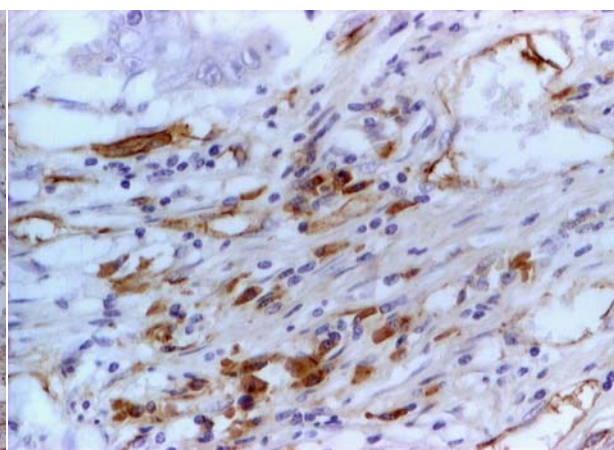
Figure 4 – *Neoformed vessels marked with CD105*
and mature vessels CD105 negative (ob. 20×)

⮒ Discussions

In CRC conventional prognostic parameters such as tumor grade, depth of invasion, angiolymphatic invasion and lymph node involvement are important prognostic factors [6]. The role of angiogenesis like prognostic factor is very controversial. Some authors reported that the MVD was reversely correlated with the survival and directly correlated with the patients' age or neoplastic relapse, lymph node status, parietal invasion and tumor stage. Other papers revealed that angiogenesis did not provide any significant information [4, 7]. Saad RS *et al.* observed that both CD31 and CD105 microvessels counts were correlated with lymph node metastases independent of tumor stage. They also state that CD31 could predict the recurrence but only endoglin counts is correlated significantly with liver metastasis [8].

Few papers presented the same conclusions with our study revealing that the angiogenesis is higher in the cases with no lymph nodes involvement [9]. We did not

find a study with a correlation between angiogenesis, lymph node metastases and localization of tumor on colon segments.

The CD105 microvessels counts are very important in the angiogenesis quantification but it is necessary to be compared with CD31 immunoexpression. In the cases where the ratio CD31/CD105 is smaller, the angiogenesis is higher because CD105 marks only the neoformed vessels [10] but CD31 is also expressed in normal vessels [8]. So, the angiogenesis can be quantified only by making a comparison between the MVD determined with both CD31 and CD105.

In our study, after the statistical analysis in 59 cases, we observed that in the right colon the angiogenesis was higher in the cases where the lymph nodes did not present metastases (pN0) but also when four or more lymph nodes were involved (pN2). Therefore, in this colon segment, the antiangiogenic treatment could have the best results in pN0 and pN2 cases. In the rectum and sigma, the angiogenesis presented the highest intensity in pN0 and pN1 stage, decreasing in pN2 stage. Therefore, in these colon segments the antiangiogenic treatment could have the best results in pN0 and pN1 cases. Compared with the right colon, we observed that the angiogenesis was higher in the left colon segments, especially in the descendent colon. In the latter segment, the MVD did not present differences between the cases with and without metastases. Our results prove that the angiogenesis intensity in CRC is higher in early-stages of the tumoral proliferation but it is not an increasing process having rather an oscillating character.

⯗ Conclusions

Although many studies reveal that the antiangiogenic treatment prolongs life in CRC with metastases we believe that this treatment could have very good results in non-metastatic CRC and could reduce the spread in the lymph nodes. Because the angiogenesis is not the only parameter, which determines the spread, we conclude that the angiogenesis remains an independent prognostic and predictive factor and the antiangiogenic treatment is necessary to be individualized for each patient.

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Corresponding author
Simona Gurzu, Assistant Professor, MD, PhD candidate, Department of Pathology, University of Medicine and Pharmacy, 38 Gheorghe Marinescu Street, 540 139 Târgu Mureş, Romania; Phone +40745–673 550, Fax +40265–210 933, E-mail: simonagurzu@yahoo.com