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Correlations between endothelial cell markers CD31, CD34 and CD105 in colorectal carcinoma

IONELA CRISTINA DELIU[1)], CARMEN-DANIELA NEAGOE[1)], MARINELA BEZNĂ[1)], AMELIA-VALENTINA GENUNCHE-DUMITRESCU[1)], SEBASTIAN CONSTANTIN TOMA[2)], BOGDAN SILVIU UNGUREANU[1)], CONSTANTIN DANIEL USCATU[3)], MARIA CRISTINA BEZNĂ[4)], CRISTIAN VIRGIL LUNGULESCU[5)], VLAD PĂDUREANU[1)], DAN IONUŢ GHEONEA[1)], TUDOREL CIUREA[1)], MARIA FORŢOFOIU[6)]

[1)]*Department of Gastroenterology, University of Medicine and Pharmacy of Craiova, Romania*
[2)]*Department of Surgery, University of Medicine and Pharmacy of Craiova, Romania*
[3)]*Department of Cellular and Molecular Biology, University of Medicine and Pharmacy of Craiova, Romania*
[4)]*Department of Cardiology, University of Medicine and Pharmacy of Craiova, Romania*
[5)]*Department of Oncology, University of Medicine and Pharmacy of Craiova, Romania*
[6)]*Department of Emergency Medicine, University of Medicine and Pharmacy of Craiova, Romania*

Abstract

Purpose: Colorectal carcinoma is an important cause of mortality worldwide. The fact that tumor growth is dependent on angiogenesis has supported researches for new prognostic parameters and the development of novel therapeutic strategies. Accordingly, we sought to evaluate angiogenesis quantitatively by assessing microvessel density in colorectal cancer. *Materials and Methods*: The blood vessels stained with CD31, CD34 and CD105 were counted, and we reported their number per square millimeter in order to obtain microvascular density (MVD). Then, we aimed at comparing the performance of three endothelial cell markers (CD31, CD34, and CD105) on formalin-fixed tissues from 58 patients diagnosed with colorectal cancer. *Results*: Following the comparison of the average effective vessels marked with the three markers, Student's *t*-test showed that the mean number of blood vessels marked with CD34 is higher than the blood vessels marked with CD31 and CD105. A significant difference that has been registered between the three levels of the T stage was found in the patients in our study, in terms of value marker CD105, ANOVA p=0.049, which returns to a value <0.05. Quick time decreases the pT stage, the observed differences being close to statistical significance. However, the result of ANOVA test does not allow us to say that differences can be generalized and not just a particular result, valid only for the study group, p=0.061 >0.05. There is a significant difference between patients with stage T, in terms of value: hemoglobin (ANOVA p<0.001), hematocrit (ANOVA p<0.001), mean corpuscular volume (MCV) (ANOVA p<0.001), mean corpuscular hemoglobin (MCH) (ANOVA p=0.002 <0.01 – significant difference with 99% confidence). By calculating the Pearson's correlation coefficient for the relationship CD31–CD105, we obtained a value r=0.440, which corresponds to p=0.0013 <0.05, indicating a statistically noteworthy direct correlation between the two factors. *Conclusions*: CD31 marker increases simultaneously with the CD105, in the cases analyzed throughout the present study. The ability of tumors to maintain a high vascular blood density in their inner portions may represent a reliable parameter to evaluate tumor angiogenesis and a finding relevant for future development of therapeutic angiogenesis strategies.

Keywords: colorectal cancer, CD34, CD31, CD105.

Introduction

Colorectal carcinoma (CRC) is an important cause of mortality worldwide [1]. The fact that tumor growth is dependent on angiogenesis has supported researches for new prognostic parameters and the development of novel therapeutic strategies. This is a welcome development, as 20–30% of patients with CRC treated with potentially curative surgery, will succumb from recurrent disease. This suggests that the conventional prognostic factors may not be sufficient and that additional parameters, either morphological or molecular, are needed for clinical management [2].

Vascular quantification has been a matter of diverging results with regard to prognostication in CRC. Some manuscripts have shown correlation of microvessel counting with poorer outcome or lymph nodes metastases [3, 4]. Others have reported that vessel ramification and total vascular area were related to the outcome. In contrast, it has even been shown that higher microvascular counting was correlated with favorable outcome [5, 6].

In order to detect neovascular microvessels, various markers have been applied for immunohistochemical staining of endothelial cells. CD31, CD34, and CD105 have often been used on human cancer tissues, including urothelial cancer (UC) [7–13]. Taking into consideration that the biology of these markers differs, no single endothelial marker can be considered the most appropriate one to fulfill all the conditions requested. Admittedly, in recent years a series of studies carried out to compare if these markers have been performed on different cancer types [7–11]. Thus, in investigating a variety of cancers, microvascular density (MVD) assessment using CD105 as a marker (CD105–MVD) has been recommended as a better progression and prognosis predictor than other

assessments where CD31 or CD34 were used [7, 8, 11–13]. Several studies have investigated the clinical significance and the pathological role of CD105–MVD in bladder cancer patients [14, 15].

The key objective of the present research study is to highlight the role of CD105–MVD in pathological assessment and its clinical significance in CRC. Accordingly, we set out to assess the role of CD105–MVD in CRC patients and then to compare the results obtained with those provided by CD31–MVD and CD34–MVD.

Materials and Methods

The tumoral tissue samples were obtained from 65 patients with colorectal adenocarcinoma and embedded in paraffin. Three μm tissue sections were cut, deparaffinized in xylene and rehydrated in graded alcohol solutions. Endogenous peroxidase was blocked using 6% hydrogen peroxide at 25^0C for 5 minutes. The solution and the slides were heated using a microwave oven set at 650 W. Then, the slides were washed for 10 minutes in tap water, prepared using 3,3'-Diaminobenzidine (DAB) for 9 minutes at 25^0C, counterstained with Hematoxylin, dehydrated and mounted (Figure 1, A and B).

The slides were examined by means of an optical microscope and classified in line with the pTNM staging, in accordance with the criteria put forward by the *World Health Organization* (WHO) for colon and rectum [9]. Thus, the cancer grade was divided into three stages (G1, G2, and G3), based on the classifications provided by WHO. Moreover, in order to establish the histological grade, we have applied the criteria endorsed by the *American Joint Committee on Cancer Prognostic* [10].

Aiming at establishing the angiogenesis quantification, we sought to determine the neoformation vessels by means of CD105. In order to register the variation between the neoformation vessels and the mature vessels, we used CD31 and CD34 as control markers (Figure 2).

Normality was evaluated in relation to the normal distribution and the histograms of each variable, and the results were indicated as mean ± standard deviation (SD) unless otherwise stated. Student's *t*-test was performed for continuous variables. Pearson's correlation was used to assess the relationships between the continuous variables and the correlation coefficient (*r*).

Results

The study group encompassed 31 men and 34 women, thus the difference between genders was highly significant, if compared to the gender distribution of the overall population in our region (51.36% females, *Z*-test for proportions $p<0.001$).

Age distribution indicated a high prevalence of CRC in 60–69 and 70–79 age groups. In our study, 67% of all patients descent from urban area and the distribution by area of origin is urban/rural = 1.27. All the tumors analyzed were adenocarcinomas and more than half were labeled with the G2 histopathological grading – moderately differentiated.

Aiming at an effective and moreover at a comparison-based analysis of clinical features in the treatment and the prognosis of CRC, we classified the large bowel according to embryological, anatomical, clinical, pathogenesis and therapy features, thus establishing four segments, *i.e.*, colon, sigmoid, recto-sigmoid junction and rectum. Having analyzed the survival length according to tumor location, we reached the conclusion that survival in colic tumors is higher and statistically more significant compared to the rectum segment. Conversely, the Kaplan–Meier curves indicated considerable higher survival rates for the colon segment in comparison to the rectum segment.

Then, following our counting of blood vessels stained with CD31, CD34 and CD105, we could establish their frequency per square millimeter, obtaining MVD. Having analyzed the overall results, we found CD34 values to be almost double, compared with CD31 or CD105. As for CD31 and CD105, they indicated similar values, though CD31 mean values are significantly higher than CD105 values (Student $p=0.00515 <0.05$) (Figure 3).

In order to highlight the dissimilarities registered between newly formed blood vessels, marked with CD105, and the mature blood vessels, marked with CD31, we choose to represent the number of vessels marked with CD31 and CD105 as the percentage of the number of vessels marked with CD34. The results indicated that the mean percentage of blood vessels marked with CD105 (39.85%) is lower than the mean percentage of blood vessels marked with CD31 (49.36%).

Hence, no statistically significant correlation could be established between CD34 and CD31; Pearson's correlation coefficient was $r=0.001$, which corresponds to a $p\approx1$ (Figure 4).

No statistically significant correlations could be established either between CD34 or between CD105. Even though Pearson's coefficient value was higher ($r=0.130$), it was still not sufficient for the p value to go below the maximum permissible threshold that indicates statistical significance ($p=0.367 >0.05$) (Figure 5).

If calculating the Pearson's correlation coefficient for the relationship CD31–CD105 we obtain a value $r=0.440$ that corresponds to $p=0.0013 <0.05$, which indicates a statistically significant direct correlation between the two factors. In conclusion, we could envisage that CD31 increases simultaneously with CD105 for the analyzed cases throughout this study (Figure 6).

Moreover, the most effective classification of the patients in the study group that also provided highly conclusive results in relation to the different characteristics of the patients in each category proved to be the pT stage. Thus, a significant difference that has been registered between the three levels of the T stage was found in the patients in our study, in terms of value marker CD105, ANOVA $p=0.049$, which returns to a value <0.05.

Quick time decreases the pT stage, the observed differences being close to statistical significance. However, the result of ANOVA test does not allow us to say that differences can be generalized and not just a particular result, valid only for the study group, $p=0.061 >0.05$.

There is a significant difference between patients with stage T, in terms of value: hemoglobin (ANOVA $p<0.001$), hematocrit (ANOVA $p<0.001$), mean corpuscular volume (MCV) (ANOVA $p<0.001$), mean corpuscular hemoglobin (MCH) (ANOVA $p=0.002 <0.01$ – significant difference with 99% confidence) (Table 1).

Table 1 – ***Correlations between endothelial cell markers and pT colorectal cancer***

Parameter	Total	pT2	pT3	pT4	ANOVA *p*-value
CD34	351.41±83.08	340.41±96.64	353.74±72.29	363.26±73.56	0.704
CD31	175.11±61.15	170.05±57.91	174.59±56.44	185.6±76.97	0.767
CD105	140.9±54.2	121.98±51.77	153.96±55.08	158.9±50.04	0.049
Direct bilirubin [mg/dL]	0.22±0.13	0.24±0.17	0.2±0.08	0.21±0.11	0.455
Indirect bilirubin [mg/dL]	0.34±0.18	0.37±0.18	0.33±0.14	0.32±0.26	0.582
Total bilirubin [mg/dL]	0.57±0.26	0.64±0.26	0.51±0.2	0.52±0.34	0.197
Circumference [cm]	0.79±0.28	0.72±0.33	0.79±0.27	0.87±0.18	0.373
Blood glucose [mg/dL]	116.95±65.69	108.96±35.88	125.76±86.91	115.92±67.73	0.665
GOT [U/L]	23.46±24.83	22.92±13.19	27.88±36.64	15.09±7.26	0.370
GPT [U/L]	18.06±14.59	18.97±15.47	18.97±16.4	13.95±6.12	0.595
Total proteins [g/dL]	6.75±0.79	6.76±0.91	6.85±0.79	6.49±0.57	0.768
Urea [mg/dL]	37.09±17.63	33.27±15.5	41.27±18.33	36.6±20.17	0.272
Creatinine [mg/dL]	0.89±0.3	0.9±0.32	0.86±0.28	0.91±0.32	0.892
Amylasemia [IU/mL]	60.97±28.41	66.8±33.99	59±20.38	31±16.97	0.238
Hemoglobin [g%]	11.48±2.31	12.05±2.12	12.18±1.71	8.75±1.88	<0.0001
Hematocrit [%]	34.5±6.89	35.94±5.74	36.82±4.94	26.36±7.19	<0.0001
MCV [fL]	81.57±10.11	84.43±9.9	83.35±7.06	71.52±10.59	0.000
MCH [pg/cell]	32.85±1.84	33.48±1.59	32.54±1.84	32.16±2.04	0.062
Hemoglobin [g/dL]	26.98±4.27	28.32±4.25	27.48±3.18	23.03±4.29	0.001
Erythrocytes	4.24±0.63	4.27±0.6	4.46±0.56	3.71±0.59	0.002
Thrombocytes/mm^3	300 637.5± 112714.14	280 265.38± 106655.48	291 703.85± 80083.75	364 133.33± 163600.34	0.086
Leukocytes/mm^3	8080.25±3004.58	7292.35±2246.3	8298.35±3256.43	9314.83±3590.5	0.139
Lymphocytes	25.66±26.24	24.79±6.24	29.8±40.78	18.93±8.62	0.494
Monocytes [%]	7.07±2.81	7.86±2.9	6.74±3.12	6.01±1.21	0.129
Segmented neutrophils [%]	70.38±8.68	68.12±8.66	70.64±8.65	74.74±7.62	0.088
INR	1.12±0.46	1.04±0.09	1.1±0.11	1.34±1.03	0.175
Howell's time [s]	22.67±5.23	22.91±5.26	22.31±4.33	22.88±6.99	0.913
Quick's time [%]	94.86±16.52	100.27±11.08	92.72±12.36	87.11±30.06	0.061
K^+ [mmol/L]	4.42±0.59	4.58±0.7	4.35±0.33	4.34±0.78	0.459
Na^+ [mmol/L]	137.35±3.87	138.63±3.11	137.72±2.86	135.13±5.39	0.052
Cl^- [mmol/L]	103.28±3.89	103.63±4.72	102.7±3.67	103.87±3.24	0.660

GOT: Glutamate oxaloacetate transaminase; GPT: Glutamate pyruvate transaminase; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; INR: International normalized ratio.

The variations of CD31 among the three grading categories are not quite considerable; however, the CD31 levels are slightly lower for G1 in comparison to G2, and lower for G2 in comparison to G3. Subsequently, the recorded measurements' variation invalidates any comparison among them, making it irrelevant (ANOVA p=0.964 >0.05 (Figure 7).

Thus, we have found that the differences among the three grading levels for CD105 are statistically significant (p=0.046 <0.05), which makes CD105 a valuable tool for the assessment of the grading differences in CRC (Figure 8).

CD31 increases simultaneously with CD105, according to the cases analyzed in our study. Thus, we could encounter a considerable number of neoformation vessels (around 40%) in the tumor area, which may weight considerably in the prognosis and the treatment of this disease.

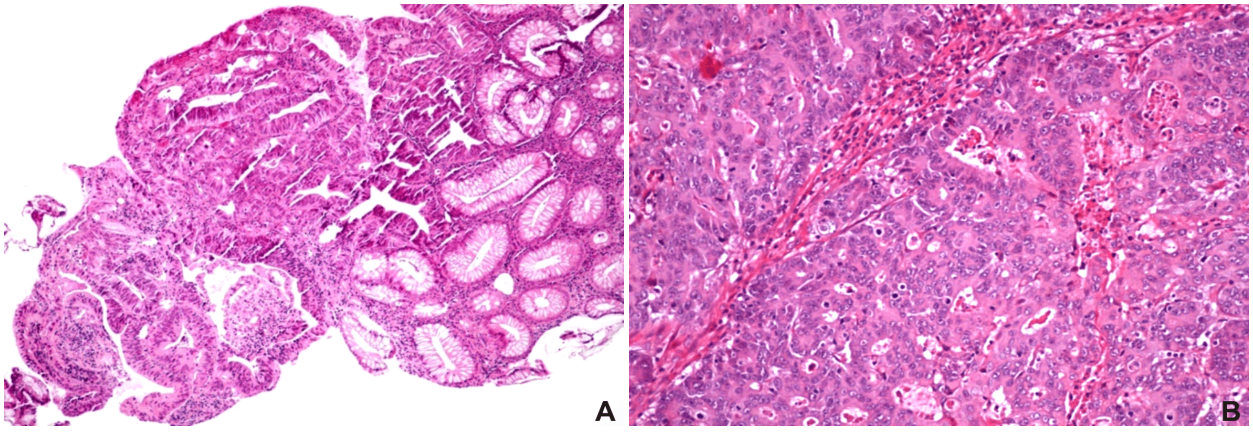

Figure 1 – ***(A) Moderately differentiated adenocarcinoma; (B) Poorly differentiated adenocarcinoma. Hematoxylin–Eosin (HE) staining, ×200.***

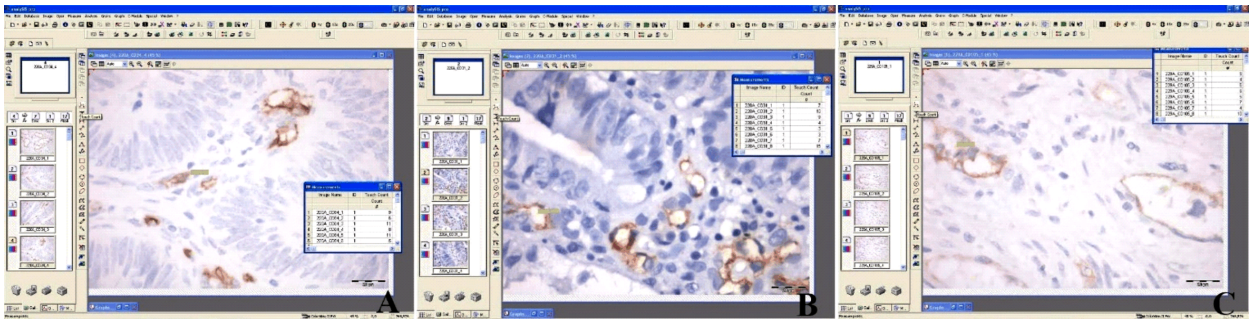

Figure 2 – ***Blood vessels from tumoral area marked with CD34 (A), CD31 (B) and CD105 (C). (A), (B) and (C) depict the same case. The images were obtained by means of the optical microscope Olympus CX31 attached to a color video camera and were made in the intratumoral area (five images per each area).***

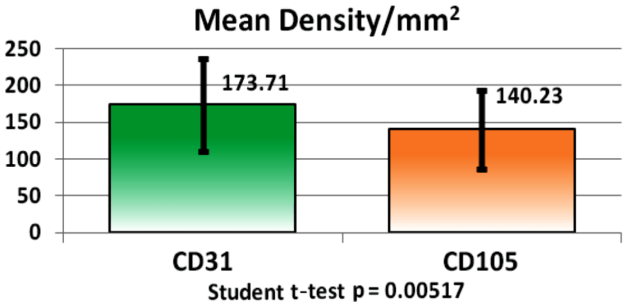

Figure 3 – ***Dissimilarities between the mean CD31–MVD and the mean CD105–MVD.***

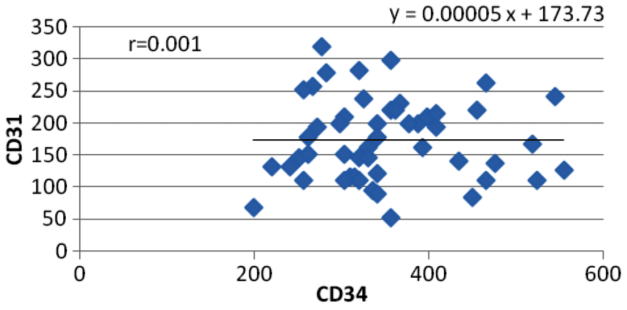

Figure 4 – ***Pearson's correlation for CD34 and CD31.***

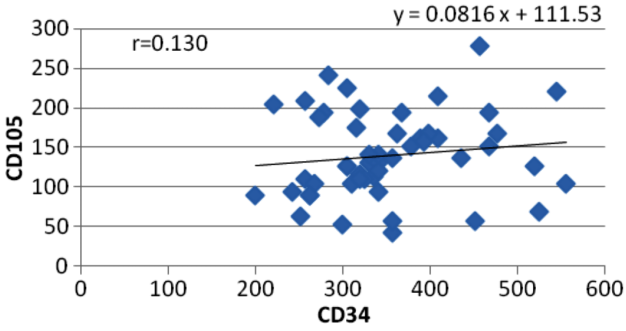

Figure 5 – ***Pearson's correlation for CD34 and CD105.***

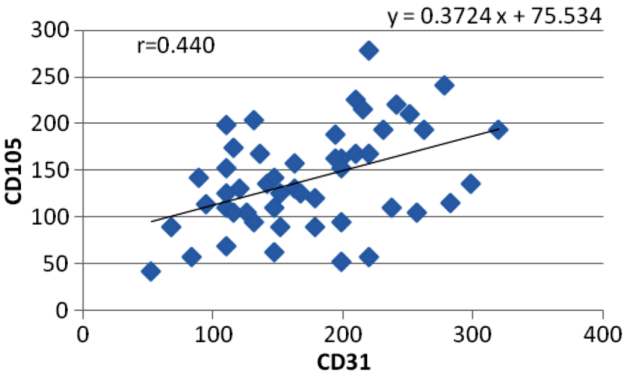

Figure 6 – ***Pearson's correlation for CD31 and CD105.***

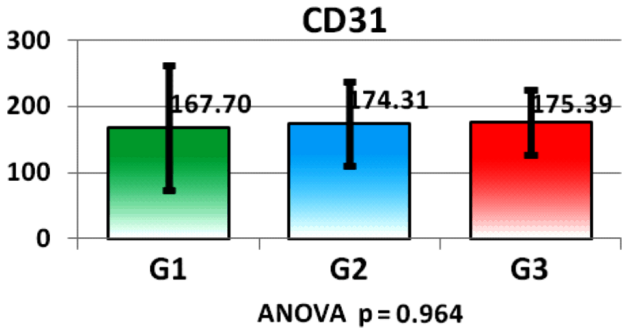

Figure 7 – ***Variation between mean CD31–MVD reported to tumor grading.***

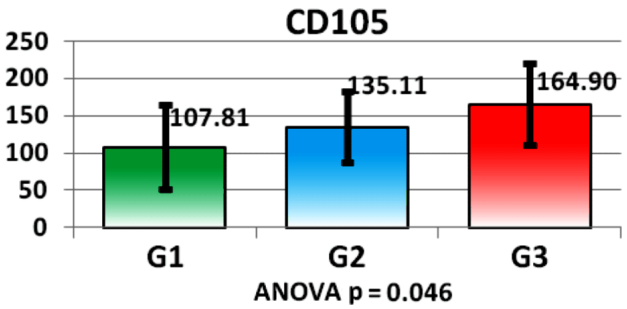

Figure 8 – ***Variation between mean CD105–MVD reported to tumor grading.***

Discussion

CRC incidence and mortality are much more frequent in urban areas, indicating no tendencies towards any change from one year to another. The distribution of death rates in a series of regions in Romania indicates that here there are also places with similar incidence and mortality levels as in Central and Northern Europe, *i.e.*, Arad, Bucharest and Timiş regions and areas such as Vaslui, Ialomiţa, etc. displaying a significantly reduced mortality rate, similar to the Mediterranean countries (Greece, Albania).

Vertical tumor growth, as highlighted by means of the T classification, stands as the most noteworthy prognostic variable in CRC. The tumor size proved to be an independent prognostic parameter for patients with CRC.

Optimal cut-off values vary among different parts of the large bowel. While the prognostic values proved to be significant within the colon, only a minor value could be registered within the rectum [16]. A retrospective study established that the number of lymph nodes removed in patients with rectal carcinoma (RC) was significantly lower than in the colon carcinoma (CC) ($p<0.001$), while the invaded lymph nodes in the RC group was significantly higher than the CC group ($p<0.001$).

Two-thirds of the colorectal malignancies are localized on the left colon and the rectum. Recent studies advocate an increasing trend in right colic tumors, thus leading to considerable implications for screening and surveillance, with total colonoscopy indication in such cases. Cancer incidence displayed an overall increase. In Modena (Italy) Cancer Registry, an increase of 33.7% in all colonic segments was shown, whereas rectal tumors were likely to decrease. TNM staging showed a gradual proliferation of the localized lesions (41.2% in 1984 to 53.3% in 1998), with a proportionate reduction of advanced tumors. Tumor studies indicated an increased incidence of colon tumors in all segments, and especially a "migration" to the right side. TNM classification highlights a downward trend in stages, with an appreciable increase of localized lesions. These findings could be due to a wider use of total colonoscopy [17].

In CRC, expression of CD105, but not of CD34, presented significantly higher values in the adenoma–carcinoma sequence [18]. The microvessel counting assessed by anti-CD105 was revealed as an independent prognostic parameter for survival in CRC, in contrast to blood vessel pan-endothelial marker CD34 [19]. CD105+ vessel counts have been equally strongly correlated with the occurrence of metastatic disease [20]. Our findings corroborate the clinical value of the assessment of intratumoral MVD using anti-CD34 as an additional prognostic parameter in patients with CRC.

Even more, we have established that the MVD for CD31 was higher than CD105. This feature seems to be normal because CD31 also assesses the preexistent mature vessels and the neoformation vessel.

Provided that the mean percentage of the MVD marked by CD105 and CD31 are relatively close to each other, and the fact that in the maturation process of the neoformation vessels expression of CD105 can be found simultaneously with the expression of CD31, we could reach the conclusion that a considerable number of vessels (around 40%) which are within tumor area are neoformation vessels.

A meta-analysis on the prognostic role of angiogenesis in CRC has clearly highlighted an inversed relationship between this and the survival rate. Accordingly, like breast cancer, CRC has been validated as a dependent cancer [21]. There are obvious pathophysiological reasons for such an association, as angiogenesis is a phenomenon that occurs very early in CRC carcinogenesis, being also essential in the process of metastasis.

Conclusions

CRC tends to increase in terms of incidence and prevalence. The CD31 increases in parallel with the CD105 according to the cases analyzed within this research study. An important number of vessels (around 40%) that can be found in tumor area are neoformation vessels, which leads to important observations when deciding upon the most appropriate and effective treatment in colorectal adenocarcinoma. The ability of tumors to maintain a high vascular blood density in their inner portions may represent a reliable parameter to evaluate tumor angiogenesis and a finding relevant for future development of therapeutic angiogenesis strategies.

Conflict of interests

The authors declare that they have no conflict of interests.

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Corresponding author
Dan Ionuţ Gheonea, Associate Professor, MD, PhD, MSc, Department of Gastroenterology, University of Medicine and Pharmacy of Craiova, 2 Petru Rareş Street, 200349 Craiova, Romania; Phone +40751–268 731, e-mail: digheonea@gmail.com

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