Original Paper

Evaluation of Angiogenesis in Colorectal Cancer

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ABSTRACT: Purpose: Angiogenesis is an important step in the process of cancer growth. The purpose of this study was to determine the neoangiogenesis with CD31, CD34 and CD105, and tried to observe the differences between these three antibodies. Material/Methods: The blood vessels stained with CD31, CD 34 and CD105 were counted, and we reported their number per square millimeter to obtain microvascular density (MVD). For angiogenesis quantification we determined the neoformation blood vessels with CD105. The CD31 and CD34 were used as control markers, in order to observe the difference between neoformation blood vessels and mature vessels. Results: Comparing the average effective vessels marked with the 3 markers, Student t test showed that the mean number of blood vessels market with CD 34 is higher than blood vessels market with CD31 and CD 105. The value of the Student t test was highly significant in all three cases ($p<0.001$). By calculating the Pearson 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 significant direct correlation between the two factors. Conclusions: An important number of vessels (around 40%) that can be found in tumor area are neoformation vessels, this concept being an important assessment for the choice of the correct and effective treatment in colorectal adenocarcinoma.

KEYWORDS*: angiogenesis, colorectal cancer, CD31, CD34, CD105*

Introduction

Colorectal cancer is one of the most frequent malignant diseases with a raising incidence in Romania, being the second commonest cause of death in Western Europe and in North America [1].

More than 200 papers published in the last 10 years reveal the prognostic significance of angiogenesis in colorectal carcinomas (CRC). The majority of authors revealed that the microvascular density (MVD) had an important role in tumor progression but also in the survival rate [2,3]. There are several studies which do not confirm this idea [4]. The clinical trials proved that the antiangiogenic treatment could prolonge the survival rate with 2–5 months but the results are not observed in all patients with CRC [5,6].

Angiogenesis is an important step in the process of cancer growth. It promotes metastatic spread by providing the means for cells to detach from the primary tumor and to travel in the bloodstream to distant metastatic sites.The angiogenesis could be identify with the panendothelial markers CD31 and CD34, and also with CD105 (Endoglin). CD31 and CD34 show the vascular status of CRC but do not indicate the angiogenic intensity because of the assessment of both neoformation and normal vessels, preexistent vessels in neoplastic and non-neoplastic tissues [7]. Increased microvascular density was found to be negative prognostic factor independent of tumor stage, correlating with a lower overall survival, especially with a shorter disease-free interval [1,8].

CD105 seems to be more specifically for the endothelial cells of neoformation vessels. Its expression increased in the same time with the neoangiogenic progression [2].

Some authors described that the progression of tumor is accompanied by the increasing of vessels' diameter and decreasing of number of these vessels [8]. The diameter of vessels seems to be the primordial parameter in the metastatic spread [9].

In our paper, we determined the neoformation vessels with CD31, CD34 and CD105, and tried to observe the differences between these three antibodies.

Material and Methods

Tumoral tissue samples were obtain from 50 patiens with colorectal adenocarcinoma and embedded in paraffin. 3µm tissue sections were cut, deparaffinized in xylene and rehydrated in graded alcohol solutions. Endogenous peroxidase was blocked using 6% H_2O_2 at 25^0C

for 5 min. For antigens retrieval citrate buffer in 1:10 dilution, pH 7 was used. The solution and the slides were heated using a microwave oven set at 650W. After, slides were washed for 10 min in tap wather, developed using diaminobenzidine for 9 min at 25^0C, counterstained with haematoxylin, dehydrated and mounted.

The slides were examined with optical microscope and were classified after pTNM staging, according with the criteria of World Health Organization (WHO) for colon and rectum [10]. To establish the histological grade we used the criteria of American Joint Committee on Cancer Prognostic [11].

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

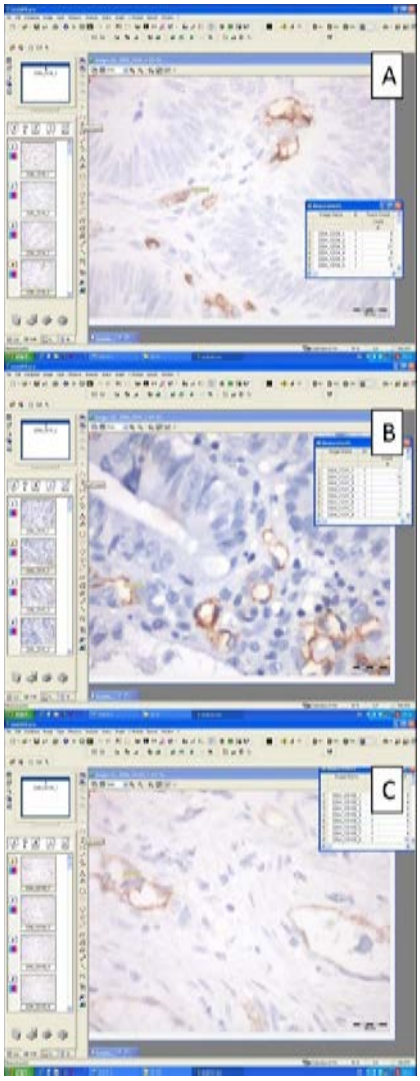

Fig.1. Blood vessels from tumoral area marked with CD34 (A), CD31 (B) and CD105 (C). A, B and C depict the same case. The pictures were realized with the optical microscope Olympus CX31, coupled to a color video camera and were made in the intratumoral area (five pictures for each area).

For angiogenesis quantification we determined the neoformation vessels with CD105. In order to observe the difference between neoformation and mature vessels we used CD31 and CD34 as control markers (Fig.1, Fig.2).

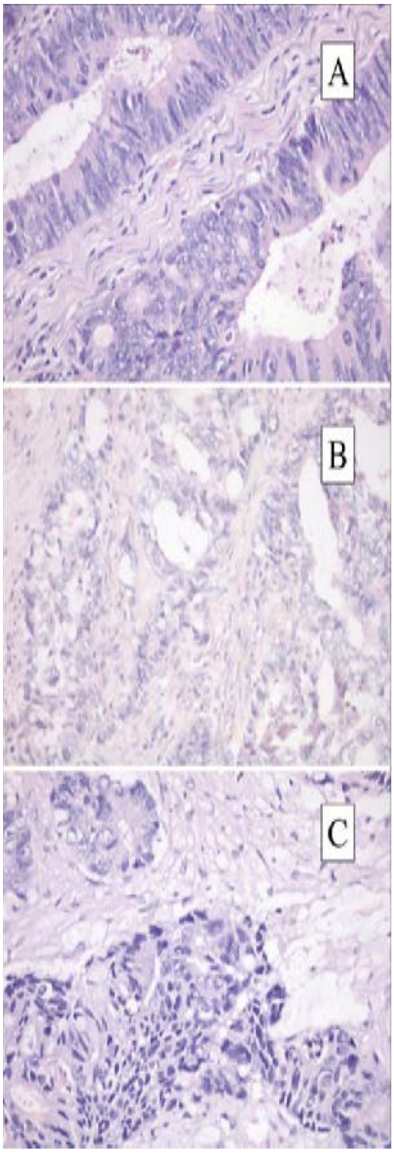

Fig.2.Hematoxilin-Eosin, 20x objective. A - well diferentiated adenocarcinoma. B - moderately diferentiated adenocarcinoma. C - poorly diferentiated adenocarcinoma

Results

The study group consisted of 32 men (64%) and 18 women (36%), the difference between genders being highly significant, when compared to the gender distribution in general population for our region (51.36% females, z test for proportions $p<0.001$).

Age distribution showed a high prevalence of colorectal cancer for 60-69 and 70-79 age groups (Table 1).

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Table 1: Distribution of patients in age classes

Age	40-49	50-59	60-69	70-79	>80	Total
No. case	3	8	22	15	2	50
Percentage	6%	16%	44%	30%	4%	100%

In our study 56% of all patients descent from urban area and the distribution by area of origin is urban/rural = 1.27.

For an effective and comparative analysis of clinical features, treatment and prognosis in colorectal cancer, the large bowel was divided based on embryological, anatomical, clinical, pathogenesis and therapy in four segments: colon, sigmoid, recto-sigmoid junction and rectum. According to this, localization of the primary tumor had the distribution presented in Table 2.

Table 2:Distributionof primary tumor according with localization

Region	Colon	Sigmoid	Rectosigmoid junction	Rectum	Total
No.case	20	6	5	19	50
Percentage	40%	12%	10%	38%	100%

Table 3: Histopathological grading of tumors

Grading	G1	G2	G3	Total
No. case	7	28	15	50
Percentage	14%	56%	30%	100%

All the tumors analyzed were adenocarcinomas, more than half of them having the histopathological grading G2 - moderately differentiated (Table 3).

We counted blood vessels stained with CD31, CD 34 and CD105, and we reported their number per square millimeter to obtain microvascular density (MVD). Analyzing the overall results, we found CD4 values to be almost double, compared with CD31 or CD105. As for CD31 and CD105, they have similar values, but CD31 is mean values are significantly higher than CD105 values (p Student=0.00515<0.05).(Fig.3 and Table 4).

Table 4:Differention between CD31 MVD and CD105 MVD

CD	Mean	Standard dev.
CD34	351.85	85.45
CD31	173.71	63.18
CD105	140.23	53.45

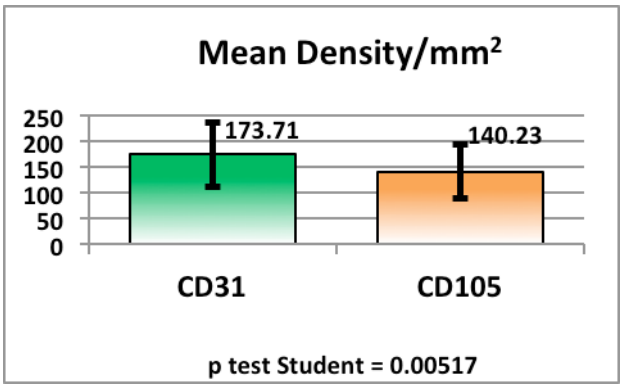

Fig.3. Differention between the mean CD31 MVD and the mean CD105 MVD. Bars depict standard deviation

Table 5: Pearson's correlation for CD 34, CD 31 and CD105.

Factor 1	Factor 2	r Pearson	p
CD 34	CD 31	0.001	1.000
CD 34	CD 105	0.130	0.367
CD 31	CD 105	**0.440**	**0.001**

In order to underline the differentiation between new-formed blood vessels, that are

marked with CD105, and mature blood vessels, that are marked with CD31, we choose to represent the number of vessels marked with CD31 and CD105 as percentage from the number of vessels marker with CD34. The mean percentage of blood vessels marked with CD105 (39.85%) is lower than the mean percentage of blood vessels marked with CD 31 (49.36%).

We couldn't find a statistically significant correlation between CD 34 and CD 31; Pearson's correlation coefficient was r = 0.001, which corresponds to a p≈1.(Fig.4 and Table 5).

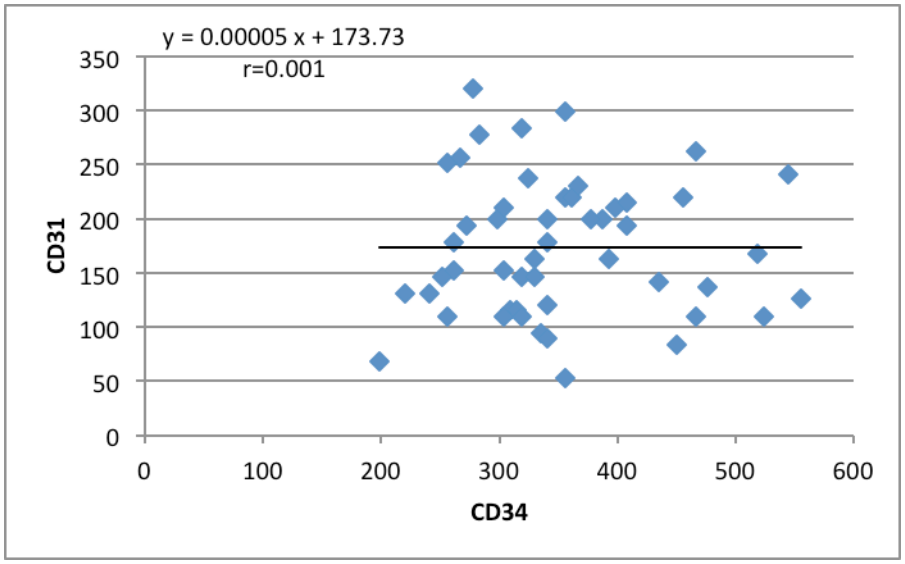

Fig.4.Pearson's correlation for CD 34 and CD 31. Bar shows lack of correlation between the two data sets

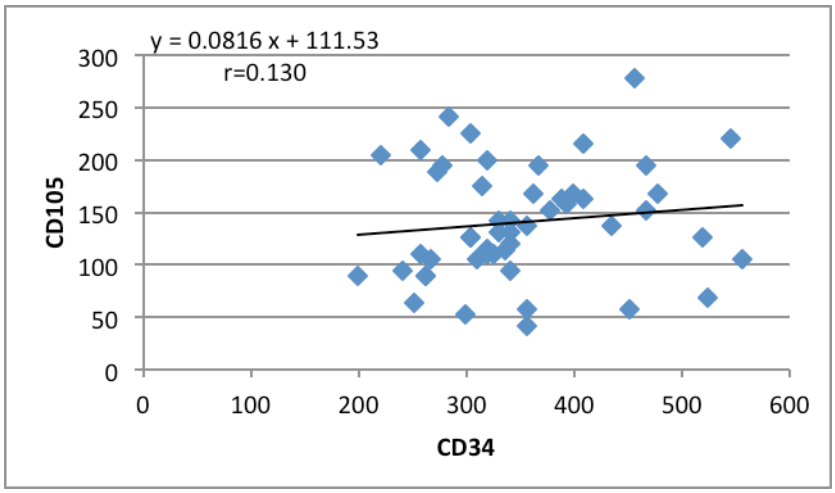

Fig.5.Pearson's correlation for CD 34 and CD 105. Bar shows lack of correlation between the two data sets

Neither between CD34 and CD 105 there is a statistically significant correlation, although Pearson's coefficient value is higher (r = 0.130), but not sufficient for p value to get below the maximum permissible threshold that indicates statistical significance (p = 367> 0.05). (Fig.5 and Table 5)

Calculating the Pearson 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 significant direct correlation between the two factors,

In conclusion, we can say that the CD 31 increases in parallel with the CD 105 for cases analyzed in this study (Fig.6.and Table 5).

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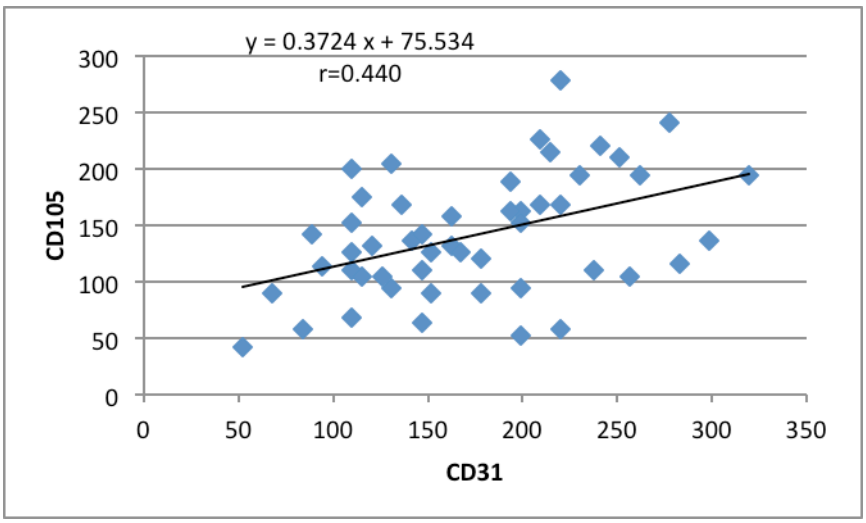

Fig.6.Pearson's correlation for CD 31 and CD 105. Bar shows correlation between the two data sets

The differences of CD34 values for the 3 grading levels proved statistically not significant; the ANOVA test yielded a result of 0.436 > 0.05. As an observation, we found CD34 values for G2 cases to be much lower than for G1, which is somehow unexpected, and lower than for G3, none of the differences being statistically significant.(Fig 7.and Table 6)

Table 6: Short statistics of CD34 MVD reported to tumor grading

Grading	No.cases	Mean	Standard dev.
G1	7	374.33	60.80
G2	28	338.02	79.68
G3	15	367.19	104.11

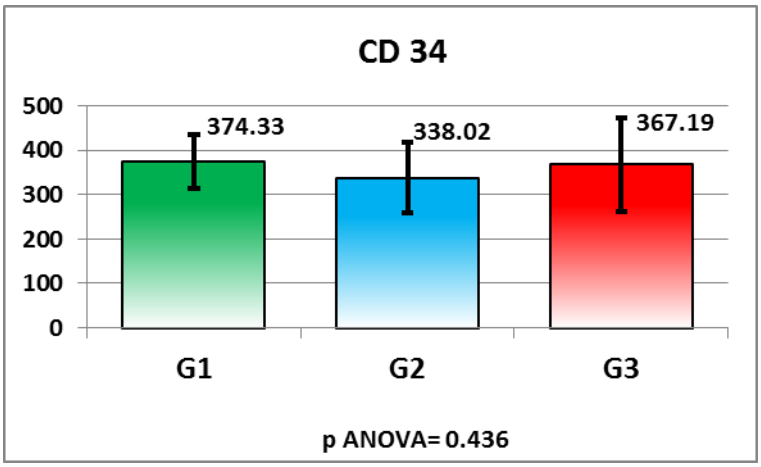

Fig.7: Differences between mean CD34 MVD reported to tumor grading

The differences of CD31 among the 3 grading categories are not very big; even if CD31 levels are slightly lower for G1 than for G2, and lower for G2 than for G3, the observed variability of the measurements makes this comparison irrelevant (p ANOVA=0.964 >0.05).(Fig 8 and Table 7)

Table 7: Short statistics ofCD31 MVD reported to tumor grading

Grading	No.cases	Mean	Standard dev.
G1	7	167.70	93.89
G2	28	174.31	63.51
G3	15	175.39	48.92

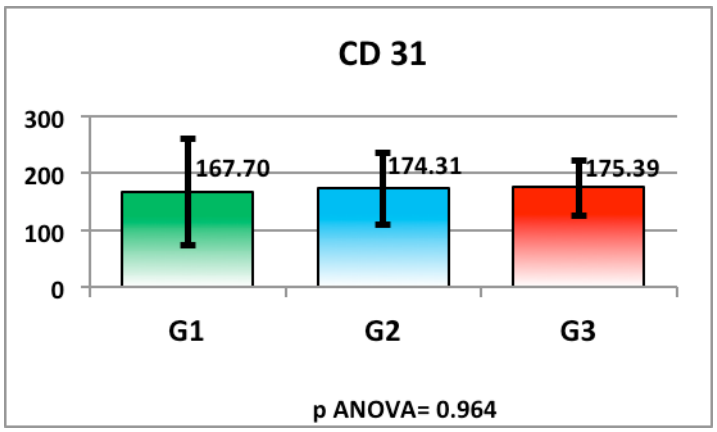

Fig.8.Differences between mean CD31 MVD reported to tumor grading

We found that differences among the 3 grading levels for CD105 are statistically significant (p=0.046<0.05), which makes CD105 a valuable tool for assessing grading differences for colorectal cancer.(Fig 9. And Table 8).

Table 8: Short statistics ofCD105 MVD reported to tumor grading

Grading	No.cases	Mean	Standard dev.
G1	7	107.81	56.35
G2	28	135.11	47.87
G3	15	164.90	54.67

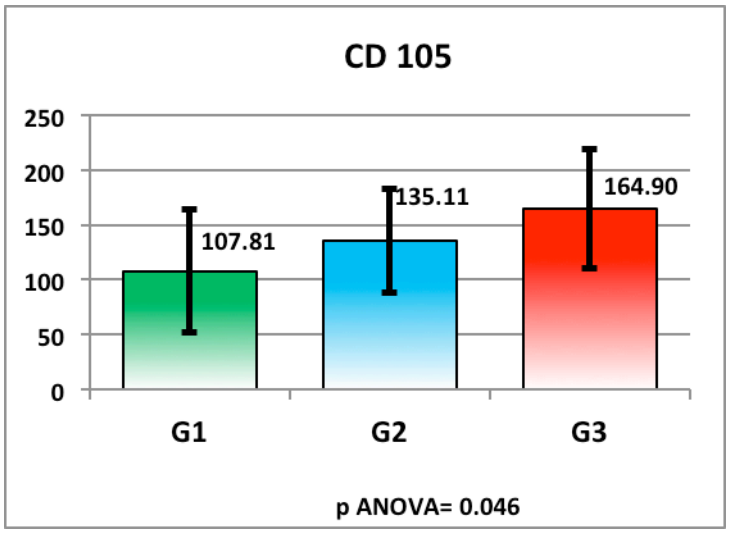

Fig.9.Differences between mean CD105 MVD reported to tumor grading

The CD 31 increases in parallel with the CD 105 in the cases analyzed in this study. In the tumor area an important number of neoformation vessels (around 40%) can be found, with high value in prognosis and treatment of this disease.

Discussion

Controversies regarding 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.

Different studies showed the predominant of male (64%) with a proportion male/female of 1.7:1. This change in the M/F ratio is not statistically significant, although there are European studies that confirm the increased incidence of malignancies particularly in women. In some studies, the MVD determined with CD105 was higher than that for CD31, which demonstrates that the CD105 is the best marker to identify proliferating endothelium involved in tumor angiogenesis [3].

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In our study, we observed that the mean microvascular density for CD31 was higher than CD105. This feature seems to be normally because CD 31 also assess the preexistent mature vessels and neoformation vessel.

Giving the fact 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 neoformation vessels expression of CD105 can be found simultaneously with the expression of CD31, we can conclude that an important number of vessels (around 40%) that can be found in tumor area are neoformation vessels, being an important assessement for the choice of the correct and effective treatment in colorectal adenocarcinoma.

A meta-analysis of the literature on the prognostic role of angiogenesis in colorectal carcinoma, clearly established inverse relationship between this and survival, confirming that like breast cancer colorectal cancer is a dependent cancer [12]. There are obvious pathophysiological reasons for such a relationship, as angiogenesis is a phenomenon which occurs very early in carcinogenesis colorectal cancer, and is also essential in the process of metastasis.

Conclusions

The CD 31 increase in parallel with the CD 105 cases analyzed in this study. An important number of vessels (around 40%) that can be found in tumor area are neoformation vessels, fact that is an important observation for the choice of the correct and effective treatment in colorectal adenocarcinoma.

Acknowledgements

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