

# Comparative analysis of microvessel density quantified through the immunohistochemistry expression of CD34 and CD105 in rectal cancer

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## Abstract

Endothelial cells are highlighted using a variety of endothelial markers. One of the best known markers is CD34, a surface antigen. The most used immunohistochemical marker for identification of activated endothelial cells is CD105. We chose to compare these two markers in order to evaluate angiogenesis of the rectal cancers by determining the microvessel density (MVD). Our study included 31 patients with rectal cancer between 2010–2014, who underwent rectal resection at Arad and Timisoara Counties Hospitals, Romania. We used MVD quantification by highlighting the tumor blood vessels with two different endothelial markers using the immunohistochemical protocols. The CD34 evaluation of MVD was 37 vessels/field/×200 peritumoral (PT), compared with normal rectal mucosa with 17 vessels/field/×200. Intratumoral (IT) MVD for CD34 positive vessels was between 7 and 120 vessels/field/×200. Average IT MVD CD105+ was 13.7 vessels/field/×200, the PT MVD CD105+ was 10 vessels/field/×200. Usually, IT MVD CD105 is smaller than PT MVD CD105, a pattern that was not respected in our study. There was a statistical significant correlation between IT MVD CD34 and PT MVD CD34 with  $p=0.008$ , also IT MVD CD34 and IT MVD CD105 with  $p=0.009$ , PT MVD CD34 with PT MVD CD105,  $p=0.001$ . PT MVD CD34 had a statistical significant correlation with T,  $p=0.004$ . IT MVD CD105 associated with T,  $p=0.004$ , and with N,  $p=0.004$ . The evaluation of both CD34–CD105 showed the role of angiogenesis in the cancer proliferation and local spread, the angiogenesis level being maintained high even in the advanced stages of the disease. There was observed a difference between the intratumoral and peritumoral MVD, the study of this difference possibly leading to a better assessment of prognosis and adjusted therapies in the future.

**Keywords:** CD34, CD105, rectal cancer, endoglin, angiogenesis.

## Introduction

Endothelial cells are highlighted using a variety of endothelial markers with variable specificity and sensitivity, depending on their development stage, pathological or physiological condition and on the assessed organ. One of the best known markers is CD34, a surface antigen that is expressed in hematopoietic progenitor cells during the embryonic phase and then in the vascular endothelial cells, subsequently having a lifelong expression within these cells. Although CD34 is characterized by a high sensitivity and specificity, it does not distinguish between the endothelial cells of normal vessels and those of the vessels of a tumor [1]. Other endothelial markers under research in order to isolate these activated endothelial cells, did not present general acceptance, so many possible advances in the cancer therapy are held back by this lack of knowledge [2]. The most used immunohistochemical marker for the identification of activated endothelial cells is CD105 or endoglin, which only has an expression in the endothelial cells of the tumor blood vessel, without being present in the normal endothelial cells [3, 4]. We chose to compare these two markers in order to evaluate the

angiogenesis of rectal cancers. Direct evidence that tumor growth is angiogenesis dependent was presented in several studies where different methods of specifically inhibiting angiogenesis (which are not cytostatic to tumor cells *in vitro*) clearly inhibited *in vivo* tumor growth [5–10]. In CRC (colorectal cancer), a high microvessel density (MVD) has been shown to predict disease recurrence and survival in some studies [11, 12], whereas other studies have shown an association between high MVD and improved survival [13] or have concluded that MVD failed to provide any additional prognostic information [14].

## Materials and Methods

Our study included 31 patients with rectal cancer that underwent different types of rectal resection for malignant lesions between 2004 and 2010 within Arad and Timișoara Counties Hospitals, Romania.

All the patients included in our study refused pre-operative neo-adjuvant therapy.

Our study intended to evaluate angiogenesis in rectal cancer in its natural stages of evolution. For this purpose, we used the microvessel density quantification by high-

lighting the tumor blood vessels with two different endothelial markers using the immunohistochemical (IHC) protocols.

The antibodies with affinity for pan-endothelial marker CD34 and endoglin (CD105) for activated endothelial cells were used in an immunohistochemical protocol, including incubation with primary antibody for 30 minutes, followed by the use of NOVOLINK Visualisation system (Novocastra, UK).

Three areas of the highest microvessel density were chosen both for normal mucosa and tumor areas, and an arithmetic mean was obtained. We assessed both intra-tumoral and peritumoral microvessel density and we correlated our results with the TNM parameters.

All immunohistochemical procedures were performed in an automatic manner by using Bond MAX Immunostainer (Leica, Microsystem, UK). The microvessel density quantification was performed manually, by three independent experts in the field, by using Nikon Eclipse E600 microscope, equipped with a camera for image acquisition. The image processing was performed with Lucia G System and the statistical analysis was performed with SPSS Software ver. 20.

## Results

The unaffected rectal mucosa near the tumors presented blood vessels mostly distributed in the lamina propria and submucosa. These blood vessels had a reduced caliber and a regular lumen, with evidence of blood perfusion; they did not present any collateral branches or signs of endothelial growth in their walls. The microvessel density (MVD) at the normal mucosa level was 23 vessels/field/ $\times 200$ , and the distribution of these vessels was mainly in the submucosa (Figure 1A). The Hematoxylin–Eosin (HE) staining only managed to highlight the blood vessels in the inflammation areas at the tumor site and we had difficulties to detect these vessels in the normal rectal tissue. One thing that we noticed in the normal rectal wall was at the lamina propria layer was that the vessels were dilated and stasis was present. The border between normal mucosa and tumor tissue was marked by the appearance of dilated blood vessels inside the lower part of the mucosa and increase in number of submucosal blood vessels with a high-grade of hyperemia (Figure 1B). The lymphoid structures in the rectal wall presented more vessels that were CD34-positive with 36 vessels/field/ $\times 200$ . The fibroblast-like cells inside submucosa were also positive for CD34 (Figure 1, C and D).

The CD105 expression at the level of the normal rectal mucosa was observed only in a limited number of vessels in the lymphoid structures. There were no other CD105 expression at the level of normal rectal mucosa.

The difficulties in the MVD evaluation with HE staining led us to use immunohistochemistry in order to assess these rectal tissues for CD34 and CD105. The CD34 expression was measured inside the tumor and in the peritumoral tissues. Inside the tumor (IT), the CD34 positive vessels were present and had a heterogeneous morphology. The small blood vessels and structures between the tumoral cells were observed as being positive

for CD34 (Figure 2A) compared with those from normal rectal tissue (Figure 2B). The positive blood vessels for CD34 presented an irregular lumen; they had signs of new vascular growth in their walls, also CD34 positive; this was observed for most of the IT vessels (the sprouting phenomenon).

The IT CD34 positive vessels presented a split lumen because of some endothelial cells pillars, which were also positive for CD34. Near the tumor, the superficial layers of the mucosa had a higher density of CD34 positive blood vessels than the normal mucosa.

The MDV value was 37 vessels/field/ $\times 200$  in the peritumoral (PT) tissues, compared with the normal rectal mucosa, which had a MVD of 17 vessels/field/ $\times 200$ . IT MVD for CD34 positive vessels was between 7 and 120 vessels/field/ $\times 200$ . In a limited number of cases, positive CD34 endothelial structures looking like a cordon, without a visible lumen, were mixed with CD34 positive blood vessels IT.

Thirty-six percent of the studied cases did not have IT CD34 positive blood vessels and they were assigned with a MVD value of 0.

CD34+ PT vessels were quantified without the vessels from the muscularis propria. PT vessels presented an obvious lumen, had a bigger caliber than the IT vessels, and presented the sprouting phenomenon. Because of an increased presence of myofibroblast-like CD34+ cells in the stroma, not only the blood vessels were numbered, but also the structures with cordon type disposition and the structures that presented a tendency to form a lumen. PT MVD varied between 10–40 vessels/field/ $\times 200$ , significantly reduced compared to IT MVD. Local inflammation increased MVD.

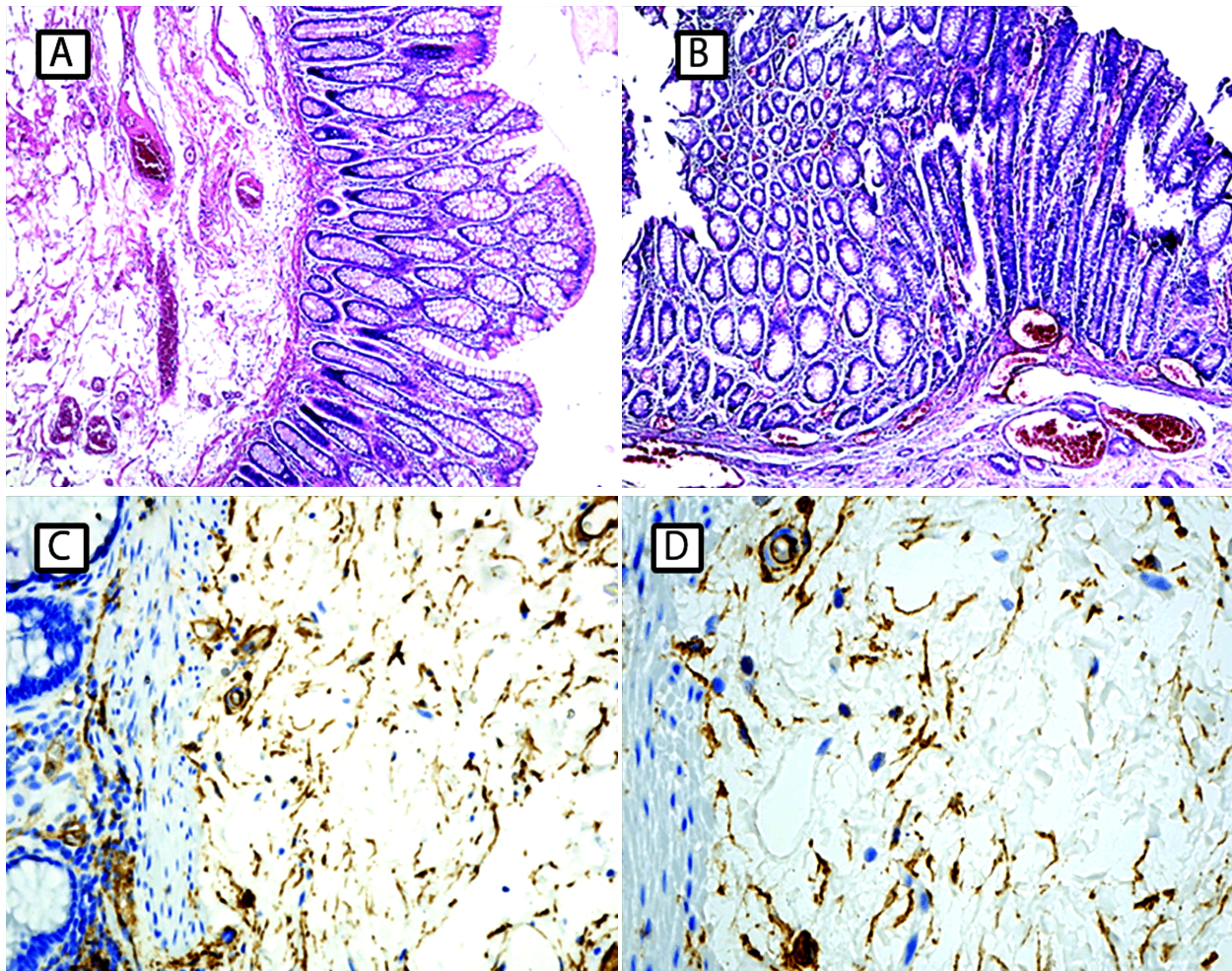
IT CD105 expression was significantly increased compared to the normal mucosa. An intense reaction was observed in the endothelial cells of tumoral blood vessels, but also a moderate reaction in the tumoral cells. IT MVD CD105+ was between 6–50 vessels/field/ $\times 200$ . The morphology of the blood vessels did not differ from the one described for the CD34+ vessels, both for IT and PT localization. In the PT bigger blood vessels, CD105 had a focal expression in the endothelium. There was a mix of CD105+ cells with negative CD105 cells, these negative cells usually appearing in their endothelium. There was a difference between IT and PT MVD CD105+. Average IT MVD CD105+ was 13.7 vessels/field/ $\times 200$ , the value for PT MVD CD105+ was 10 vessels/field/ $\times 200$ . Although these differences were not important, we found it interesting because in most tumors, IT MVD CD105 is smaller than PT MVD CD105, a pattern which was not respected in our study. Comparative assessment of CD34 and CD105 in tumor tissue is highlighted in Figure 3 (A–D).

We observed more frequent intra-vascular tumoral embolus when we used CD105 markers, than when using CD34. IT CD105+ vessels were disposed mainly in the epithelial area and usually presented an obvious lumen. Sixteen percent of the cases presented CD105+ marker inside the tumor cells. CD105 expression inside the tumor cells was of focal disposition, of medium or high intensity and more pronounced on the cell apex and margins.

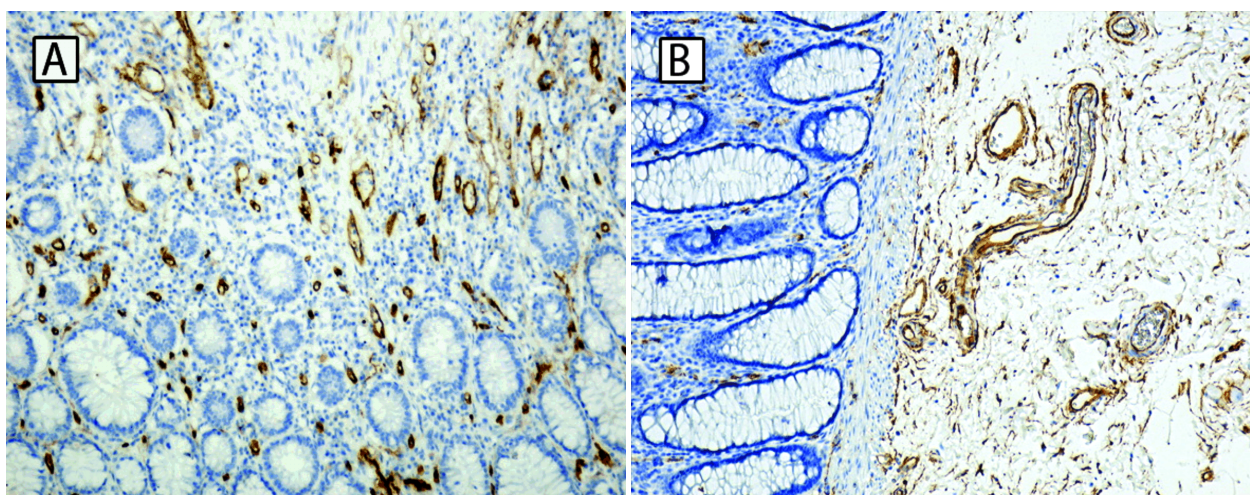


There was a statistical significant correlation between IT MVD CD34 and PT MVD CD34 with a  $p$ -value equal to 0.008. There was a strong statistical significance between IT MVD CD34 and IT MVD CD105 with  $p=0.009$ . PT MVD CD34 correlated with PT MVD CD105 with a  $p=0.001$ .

There was no correlation between age and MVD. IT MVD CD34 did not correlate with any TNM feature. PT MVD CD34 had a statistical significant correlation with T,  $p=0.004$ . IT MVD CD105 associated with T,  $p=0.004$ , and with N,  $p=0.004$ . No association was found for PT MVD CD105.

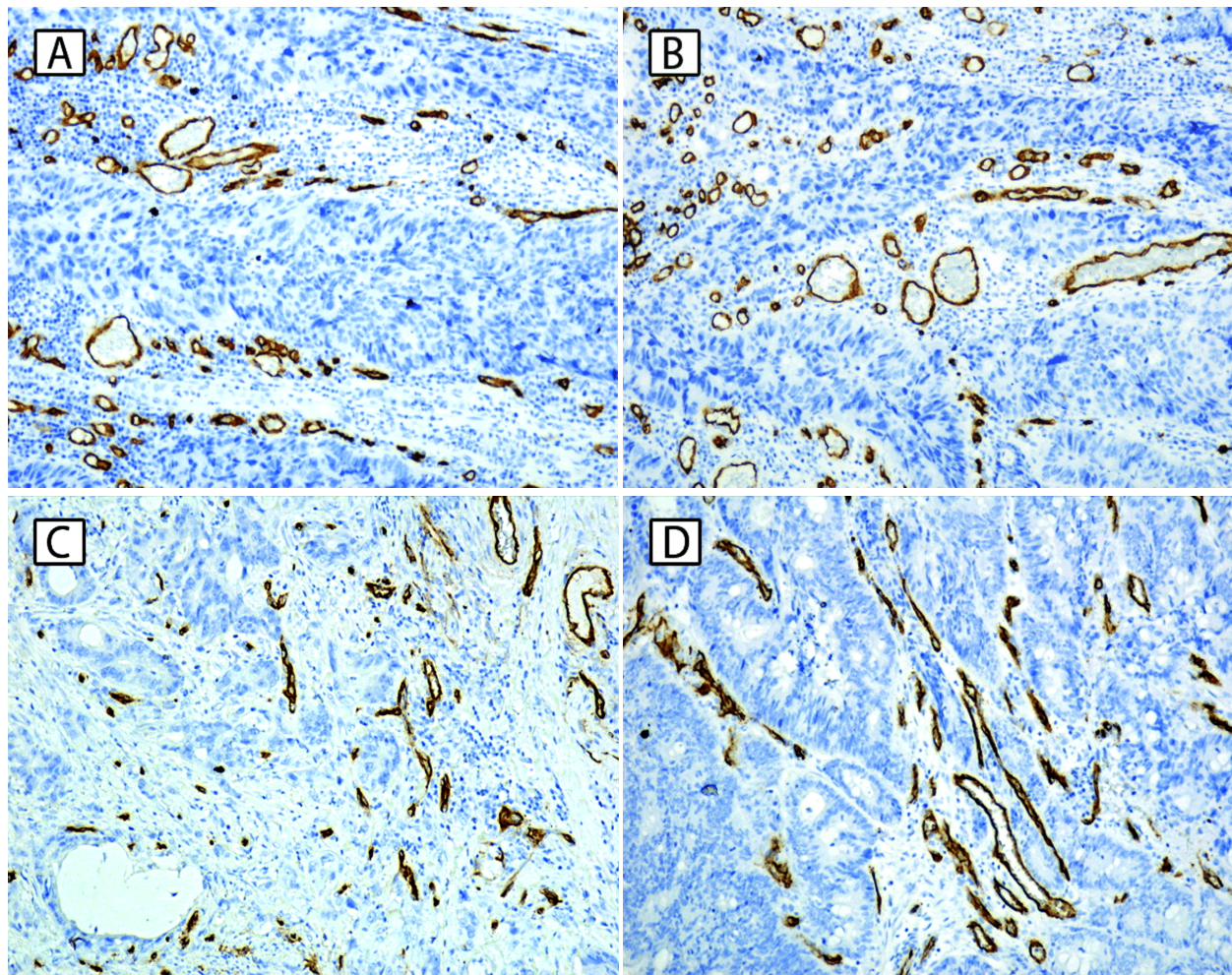


**Figure 1** – Normal rectal mucosa with a high vascularity inside submucosa (A – HE staining,  $\times 100$ ). Border between normal and tumor mucosa with a particular distribution of morphology of blood vessels from mucosa and submucosa of these two different zones (B – HE staining,  $\times 200$ ). CD34 positive fibroblast like cells and blood vessels in submucosa from normal rectal tissue (C and D – IHC, CD34,  $\times 400$ ).



**Figure 2** – Immunohistochemical staining of blood vessels highlighting by CD34. We observed a high microvessel density in tumor area (A – IHC,  $\times 400$ ) compared with normal rectal tissue (B – IHC,  $\times 400$ ).





**Figure 3** – Comparative evaluation of CD34 positive (IHC, CD34 – A and B,  $\times 400$ ; D,  $\times 200$ ) and CD105 positive blood vessels inside the tumor and in the peritumoral area (C – IHC, CD105,  $\times 200$ ).

## Discussion

Our study revealed the expression of CD34 in the normal mucosa endothelial cells and the absence of CD105 in the same cells. This data matches the known negative expression of CD105 markers in the normal non-tumoral tissues, excepting the endothelial cells from the small capillaries veins from the lymph nodes [15, 16]. The measurement of MVD using CD34 showed a low value compared to all other types of evaluation, only in the PT mucosa an increase of level was present. IT MVD CD34 had an increased value as reported by others previously [17, 18]. Despite these similarities, there are controversies linked to the prognostic importance of CD34 marker in the patients with rectal cancers. These days, the use of MVD CD34 as a single marker of prognosis for rectal cancer, for liver metastasis of rectal cancer is not accepted anymore [19]. Other investigated p53 protein researching factors of prognostic in rectal cancer concluding that more than two-thirds of adenocarcinomas were strongly positive to the p53 antibody, colorectal carcinoma with overexpression of p53 protein have a halved five-year survival rate, although the immunohistochemical expression of p53 does not correlate with the degree of differentiation, tumor stage or recurrence, gender and age [20–22]. Other authors found that positive markers of tumor (CEA, CA 19-9, CA 72-4) might have a high of false rate – 45%; their study confirmed the value for diagnosis of oncological

serum markers regarding metastasis and/or recurrent loco-regional colorectal carcinomas – 71% [23].

We quantified IT and PT MVD CD34+. The analysis of these two different areas of the affected rectum showed a statistical significance between PT MVD CD34 and the T parameter of TNM classification and no correlation between IT MVD CD34 and any TNM parameter, demonstrating once more the role of peritumoral angiogenesis in the local spread of the tumor. The differences between IT and PT MVD associated with TNM classification for rectal cancers using ultrasound methods for evaluation of the IT and PT blood vessels network concluded that global MVD CD34 evaluation does not correlate with any TNM parameters [24]. Lollert *et al.* reported a significant statistical association between global MVD and N parameter [25, 26]. Their results confirmed our study results, but in our study, this was valid only for PT MVD and the N parameter. Hinganu *et al.* performed studies regarding tumoral microvessels, by immunohistochemistry and quantitative microanatomical methods, their findings supported the significant increase of tumoral microvascular density related to histological degree, with an inverse proportion with differentiation degree. In addition, they noted a variation of intratumoral vascular density depending on the degree of differentiation [27, 28]. We found an association between PT MVD CD34 and T. There were no other reports in concordance with ours so far in the literature,

although this association was studied before [29, 30]. There is a correlation between tumor perfusion and T parameter, but other factors might influence this association. Our study regarding the association between PT MVD CD34 and T parameter concluded with a significant statistical meaning, our results turning out to be different from the results in other reports. Despite the lack of studies proving the role of CD34 as a prognostic marker for rectal cancer, we observed that an increased MVD led to a more favorable outcome after radiation therapy, being a predictor of global survival rate after long radiation course therapy.

In our study, we found a statistical significance between IT and PT MVD CD34+ and CD105+ in rectal tumors. These facts suggest that CD34+ vessels also have a CD105 expression and are characterized by a high level of endothelial activation. We consider this is one of the main reasons why the angiogenesis is active even in advanced rectal cancers. These aspects were mentioned in the literature by Zhou *et al.*; they showed that vascular invasion with CD34 and CD105+ vessels represent an independent factor for lowering the survival rates for stage I and II rectal cancers [31]. Li *et al.* determined the plasmatic level of CD105 and correlated these levels with MVD CD105 finding a statistical significance between tissue expression, plasmatic levels and survival rates after the Duke classification, showing once more that CD105 is an independent prognosis factor for survival in rectal cancer [32]. There is no published data to correlate MVD CD105 with the TNM classification. Our data suggested that IT MVD CD105 is associated with T and N.

## ✉ Conclusions

The assessment of both CD34–CD105 showed the role played by angiogenesis in the cancer proliferation and local spread, the angiogenesis level being maintained high even in the advanced stages of the disease. There is a difference between the intratumoral and peritumoral MVD and the study of this difference might lead in the future to a better assessment of prognosis and adjusted therapies.

## Conflict of interests

The authors declare that they have no conflict of interests.

## Author contribution

All authors have contributed equally in preparing this manuscript and thus share first authorship.

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