

## Autophagy and Neovascularization in Colorectal Cancer

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**ABSTRACT:** Purpose: This study aims to determinate the microvessel density at the base of the tumor, as well in tumor's mass, in order to determinate the number of neovascularization vessels (marked with CD105) in comparison with presence or absence of autophagy puncta. Material/Methods: Standard immunohistochemistry was performed on 38 samples of colorectal adenocarcinoma, in order to determinate the presence of autophagy and neovascularization blood vessels with the help of LC3, CD34, CD31 and CD105 antibodies. Results: The autophagy process was observed in the cancerous cells and was noted as present in both regions of interest from the tumor. The mean number of blood vessels marked with CD105 is higher in tumor mass than at its base, p value of the Student t test being highly significant ( $p < 0.0001$ ). Conclusions: The presence of autophagy puncta was noticed in every case, both in the mass of the tumor and at its base. Microvascular density of new-grown blood vessels is higher in the mass of the tumor compared with the base of the tumor.

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

### Introduction

Autophagy is the basic catabolic mechanism which provides recycling of aged and degraded cellular components [1]. There are three forms of autophagy described: macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy is the most studied one, and in this form of autophagy the cytoplasmic constituents that need to be digested, are wrapped in double-membrane vesicles named autophagosomes [2]. Autophagosomes fuse with lysosomes to ensure the degradation of engulfed cytoplasmic components with the help of lysosomal enzymes [3]. This fusion is mediated by a key component of autophagy machinery: microtubule-associated protein 1 light chain 3 (LC3), which is considered a marker for autophagy detection [4].

Autophagy is an important survival pathway for cancer cells under conditions of nutrient or oxygen limitation [5]. Numerous studies tried to find a link between the number of neofunctional blood vessels and the autophagy activity in the cancerous cells. Most of these papers study the microvessel density (MVD) with the help of three endothelial markers: CD34, CD31 (PECAM-1) and CD105 (endoglin). From these three the most reliable immunohistochemical marker for neofunctional blood vessels detection

is CD105 which is specific for activated endothelium [6,7,8]. During blood vessel maturation CD105 expression is graduated diminishing and CD31 expression is increasing. Therefore CD 31 and CD34 (which is expressed in blood vessel-mature and new-grown-and lymphatic vessels, too) are panendothelial cell markers for blood vessel endothelium [7,9].

In this study we aim to determinate the microvessel density at the base of the tumors, as well in tumor's mass, in order to determinate the number of neovascularization vessels (marked with CD105) in comparison with presence or absence of autophagy puncta.

### Material and Methods

Colorectal adenocarcinoma tissue samples were collected from 38 patients that went through surgery at Emergency County Hospital, Surgery Clinic II, Craiova, Romania from November 2013 to May 2014. All patients were informed about their participation in this study and a written consent was provided by every patient.

All the samples were processed using classical histopathological technique (fixation in 10% buffered formalin and embedding in paraffin). Serial sections of 3µm in thickness were cut from each paraffin block.

First section was stained for LC3 expression using Anti-LC3A/B antibody (rabbit anti-human

polyclonal, ab58610, Abcam, dilution 1:50). The next three sections were stained with antibodies in order to detect the presence of blood vessels, especially the neovascularization vessels, in the following order: the first of the three sections was stained for CD34 expression using Novocastra Lyophilized Mouse Monoclonal Antibody Endothelial Cell Marker (CD34), dilution 1:70; the second was stained for PECAM-1 expression using Novocastra Lyophilized Mouse Monoclonal Antibody CD31 (PECAM-1), dilution 1:70; the third section was stained for endoglin expression using Novocastra Lyophilized Mouse Monoclonal Antibody Endoglin (CD105), dilution 1:70.

A standard immunohistochemistry technique was used: dewaxing (xylene); rehydration in graded ethanol solutions; blocking of endogenous peroxidase (6% H<sub>2</sub>O<sub>2</sub>); antigen retrieving (microwaving slides in citrate buffer - pH 6.0, 20 minutes, 650W); washing PBS (pH 7.0); blocking nonspecific binding sites: 3% Nonfat-Dried Milk Bovine (Sigma-Aldrich)-30 minutes at 25°C; incubating with primary antibody (30 minutes at 25°C); washing PBS; incubating with EnVision (Dako), 30 minutes at 25°C; antibody detecting: diaminobenzidine (DAB) 9 minutes at 25°C; counterstaining: Hematoxylin-Eosin.

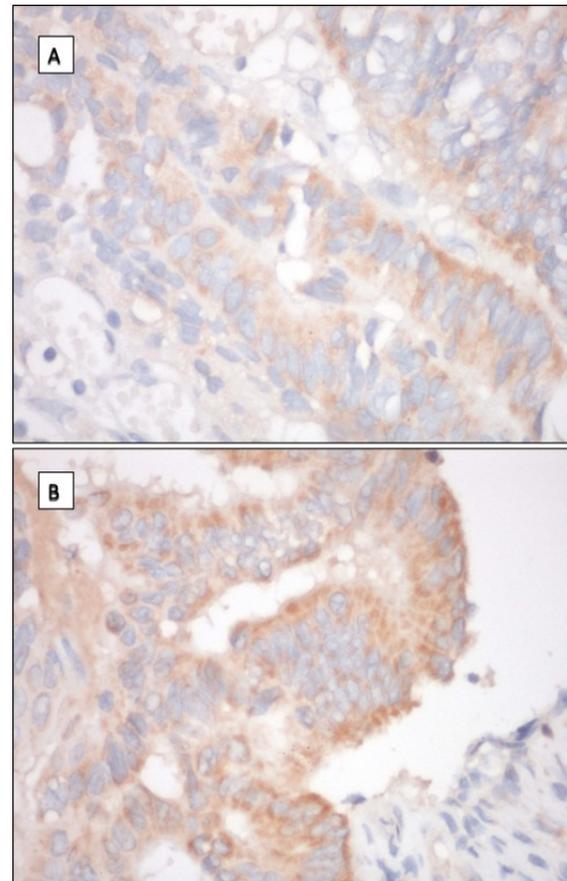
After staining all tissue slides were analyzed with the help of an Olympus CX 31 microscope equipped with a ColorView II camera and AnalySis Pro 5.0 software that is calibrated for this microscope. Using the 40x objective of the microscope, ten photographs were selected, approximately from the same area, for each slide stained for blood vessels presence (five photographs capture the vascularization at the base of the tumor and the other five capture the vascularization from the mass of the tumor). For the evaluation of autophagy process presence, another ten photographs were capture from the LC3 stained slides (five of them from the base of the tumor and five from the mass of the tumor) using the same 40x objective. All measurements done with Analysis Pro were exported in Excel (Microsoft Office, Microsoft Corporation). Statistical analysis of the data were done with GraphPad Prism version 6.

## Results

The group in this study is composed of 38 patients (24 males), age ranging from 45 to 85 years old.

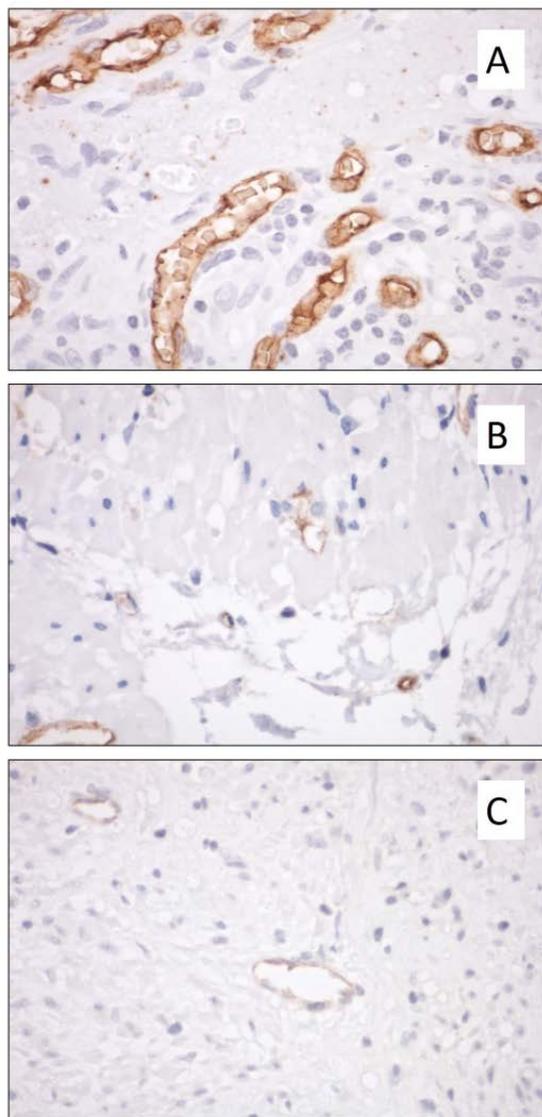
First, we analysed each case in order to determinate the presence or absence of

autophagy puncta (LC3 agglomerations fawned in a diffuse LC3 area) [10] in the cytoplasm of the cancerous cells from the base of the tumor and in its mass. Autophagy puncta were present in both regions of the tumor (example Fig.1), this aspect applying in each and every one of the study cases, autophagy puncta being detected in a much higher number in the cancerous cells from tumor mass then the ones from tumor base.

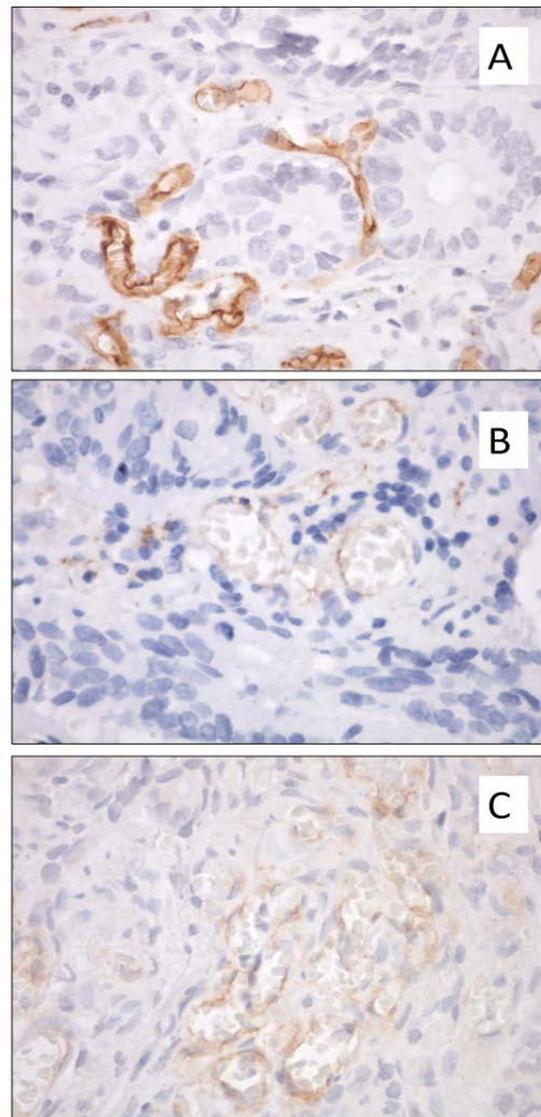


**Fig.1. LC3 stain for autophagy puncta detection (A and B represent the same case). A-base of tumor; B-tumor mass**

Second, we counted blood vessels stained with CD34, CD31 and CD105, and we reported their number per square millimeter both at the base of the tumor (Fig.2) and its mass (Fig.3) in order to obtain MVD.



**Fig.2. Blood vessels in the base of the tumor (A, B, C represent the same case). A-CD34; B-CD31; C-CD105**



**Fig.3. Blood vessels in the mass of the tumor (A, B, C represent the same case). A-CD34; B-CD31; C-CD105**

General statistics of the blood vessel count per square millimeter are illustrated in Table 1 and Fig.4. As it can be observed the mean number of vessels marked with CD34 is higher at the base of the tumors then in its mass. Nonetheless the mean number of blood vessels

market with CD105 is higher in tumor mass then at its base, and a slightly decies in the mean number of blood vessels market with CD31 can be observed in the mass of the tumor compared with its base. P value of the Student t test was highly significant in all three cases ( $p < 0.0001$ ).

**Table 1. General statistics of blood vessels count**

Blood vessels per square millimeter	Number of values	Mean	Std. Deviation
CD34 tumor base	38	405.1	109.2
CD34 tumor mass	38	312.2	81.03
CD31 tumor base	38	206.0	71.93
CD31 tumor mass	38	137.3	73.25
CD105 tumor base	38	110.7	45.99
CD105 tumor mass	38	169.9	63.97

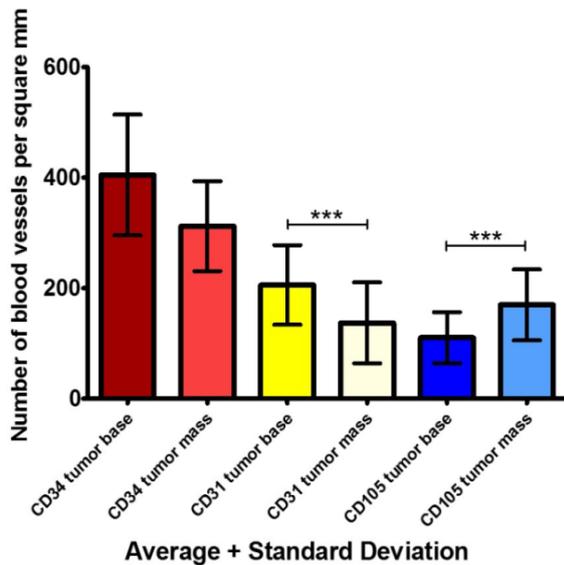


Fig.4. Blood vessel count per mm<sup>2</sup>

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 chose to represent the number of vessels marked with CD31 and CD105 as percentage from the number of vessels marker with CD34, both for the base and the mass of the tumor. The results can be observed in Fig.5. With this approach the difference between the mean percentage (56.60) of blood vessels marked with CD105 from the mass of the tumor and the mean percentage from the base of the tumor (29.24) is more clear. Also the mean percentage (45.61) of CD31 marked blood vessels from the mass of tumor is clearly lower then the mean percentage (54.54) from the base of tumor.

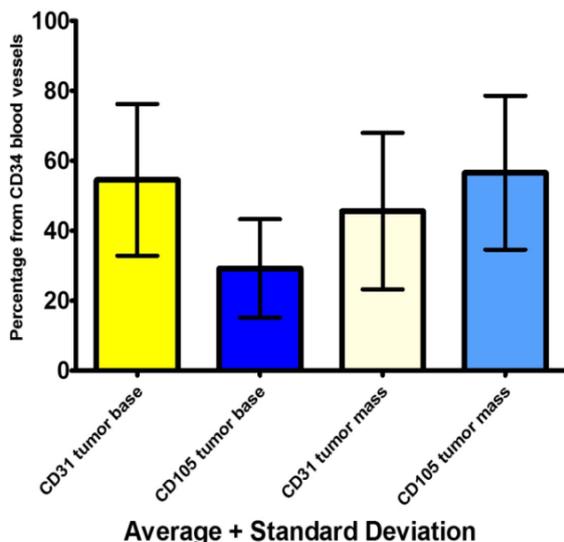


Fig.5. Percentages of CD34 and CD105 positive blood vessels from CD34 positive vessels

## Discussion

The presence of autophagy puncta was notice in every case, both in the mass of the tumor and at its base. The number of autophagy puncta appeared to be increased in the cancerous cells from the mass of the tumor compared with the ones from the base, but giving the lack for a quantitative evaluation of autophagy puncta on immunohistochemical slides, we cannot speculate that a more intense autophagic process is present in the mass of the tumor compared with the base of the tumor.

Regarding vascularization, we demonstrated the presence of a higher number of neovascularization vessels in the mass of the tumor compared with the base, fact confirmed by other authors [7,8,9]. This observation was ever clear when we compared the percentages of CD105 positive blood vessel calculated from the total amount of CD34 positive vessels. The impediment that CD 34 marks both blood and lymphatic vessels was taken in account and a second control stain with CD31 was performed. This control helped us to overcome the above mentioned problem, and, also helped us to better correlate the number of new-grown blood vessels and mature ones.

Taking into account the observation about the increased number of new-grown blood vessels from tumor's mass and the presence of autophagy puncta we can speculate that the autophagic process is not influenced by MVD, and it may be used by the cancerous cells to assist the new metabolic changes that occur in the malignant tissue, including increased division cycle.

According with these conclusions, for the treatment of colorectal carcinoma a complex scheme may be used (conclusion reached by other authors, too [4,5]), scheme that include classic chemotherapy, antiangiogenic therapy and autophagy inhibitors.

## Conclusion

The presence of autophagy puncta was notice in every case, both in the mass of the tumor and at its base.

Microvascular density of new-grown blood vessels is higher in the mass of the tumor compared with the base of the tumor.

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