Overexpression of cathepsin B correlates with angiogenesis in colon adenocarcinoma

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Some studies have shown the influence of proteases and vascular density in colorectal primary tumors on spreading and on the course of colorectal cancer. In the present study we have analyzed the relationships between overexpression of cathepsin B protein and angiogenesis intensity in resected colon tumors and their impact on prognosis. It has been investigated in a series of 90 colon cancer patients. Immunohistochemistry was used to evaluate cathepsin B overexpression in cancer cells and to visualize microvessels with antibodies against von Willebrand factor. Overexpression of cathepsin B was observed if more than 50% of cancer cells in searched field showed immunoreactivity with antibody against cathepsin B. Intensity of angiogenesis was evaluated as a mean number of microvessels from three fields with highest vessel number. In 36 cases (40%) overexpression of cathepsin B was detected. Increased angiogenesis (above median 31 vessels per 0.785 mm^2) correlated positively with cathepsin B overexpression (p=0.0006). Higher vascular density associated with the presence of metastases in regional lymph nodes (p=0.01). Overexpression of cathepsin B was observed more often in group of older people (age above median 65 years; p=0.005). According to univariate analysis metastases in regional lymph nodes (p=0.0007), increased angiogenesis (p=0.0085), and distant metastases (p=0.02) were the features potentially influencing prognosis. Multivariate analysis revealed independent prognostic value only in case of metastases in regional lymph nodes (p=0.013) and when distant metastases were present (p=0.021), but not when increased angiogenesis in primary colon adenocarcinoma was observed (p=0.078). In conclusion we can say that there is a close relationship between intensity of angiogenesis and overexpression of cathepsin B protein in cancer cells in resected colon adenocarcinoma.

Key words: colon, adenocarcinoma, cathepsin B, angiogenesis, prognosis

Colorectal cancer is a neoplasm with one of the highest incidence rate [15]. In Poland it is the third most common cause of cancer mortality in women and the fourth in men [8]. Treatment of this neoplasm can be more effective, if the prognostic and predictive factors were evaluated more precisely, and in that way the indications for particular diagnostic tools and treatment methods are identified, especially in patients after radical surgery. It is well known that angiogenesis plays an important role in growth and spreading of colorectal cancer [12, 37]. Since many years angiogenesis remains the subject of intensive studies looking for factors affecting that process in benign adenomas as well as in carcinomas and their metastases [17, 23, 32, 35, 49]. For determination of angiogenesis in evaluated tumor usually vascular density method is prefered [37, 45]. In the widely used method, introduced by Weidner, endothelial cell antigens are detected immunohistochemically to visualize microvessels [45, 47]. Most often tissues are stained for CD31, CD34 and von Willebrand factor (vWf) [37, 45, 49]. Published results of assessment of angiogenesis in primary tumor of colorectal cancer do not lead to one conclusion. Many articles describe the negative influence of increased angiogenesis on prognosis [5, 6, 11, 17, 37, 42–44]. On the other hand there are many reports, that do not reveal this correlation [1, 3, 9, 30, 33].

There is a large group of proteolytic enzymes which help neoangiogenesis in growing tumor [23]. They also enable spreading of neoplastic cells from epithelium into surroundings, by basement membrane degradation and participation in the process of stroma invasion. They contribute to me-

tastases origination [17]. Cathepsin B (CatB), a member of the cathepsins family, belongs to this group [14, 21, 23, 38]. CatB is one of lysosomal cysteine proteases taking part in the intracellular degradation of proteins. Neoplastic transformation may lead to overproduction and increased secretion of several cathepsines [14, 41]. In many neoplasms overexpression of CatB seems to influence worse prognosis [13, 27, 31, 46].

In our study we tried to find out any correlation between primary tumor angiogenesis, and overexpression of CatB in cancer cells in relation to long-term results in patients who underwent surgical resection for colon cancer. The relationships between these parameters and clinical and pathological features of prognostic value were evaluated.

Material and methods

A total of 90 patients with operable colon adenocarcionoma were enrolled in this study. They underwent colon resection between 02.1996 and 02.1998. The specimens were obtained from: Department of Surgical Oncology of Medical University of Gdańsk, Navy Hospital, City Hospital, County Hospital and Railway Hospital in Gdańsk and from City Hospital in Gdynia. The patients with rectal and recto-sigmoid cancer were not included into the study. No neoadjuvant chemotherapy was used. Patients have undergone postoperative chemotherapy, according to standard indications. The study population consisted of 42 women (47%), and 48 men (53%). Patients' age ranged from 33 to 87 years (average 64 years, median 66). Follow-up was finished on 15th November 2002. The cause of death in all patients during the follow-up was cancer recurrence. Average time of the follow-up was 46.9 months (ranged from 4 to 81.8 months, median 53.1). The calculated survival rate according to Kaplan-Meier method was 46.4%. All tumors were evaluated according to pTNM staging [40]. In Table 1 all pathological data are described. The histological classification of the tumors was based on the WHO criteria [15].

The study was prospective. Specimens were fixed in 4% buffered formalin and embedded in paraffin wax. For analyses specimen tissue sections were routinely stained, then all stained sections were revieved in the Pathology Department, Medical University of Gdańsk. Each specimen was cut in 5 μm thin slices. For morphologic analyses, tissue sections were routinley stained with hematoxylin and eosin. After staining from each case sections were chosen without necrosis to perform immunohistochemical staining. Staining was performed in Pathology Department, Medical University of Gdansk. The microvessels were visualized using monoclonal antibody against vWf. Immunohistochemical staining was performed as required by antibodies manufacturers (anti-vWf: Dako M 616, dilution 1:25, skin tissue sections as positive control slices; anti-CatB: The Binding Site PC 049, liver tissue sections as negative control slices). To localize antigen-antibody complexes sections were incubated with diaminobenzidine substrate and hydrogen peroxide to develop color reaction. Additionally sections were counterstained with Meyer hematoxylin. The sections were dehydrated in alcohol and xylene and then embedded. The expresion of CatB was evaluated in the whole section. Expresion was described as CatB(+) when more than 50% of the cancer cells were stained for antigen-antibody complexes (Fig. 1). Microvessel density was evaluated according to WEIDNER method [47]. Area of the highest microvessel density was localised at magnification of x40 and x100. At magnification of x200 all vessels were counted, three areas were assessed, and the mean was calculated as microvessel density of each tumor. (1 area = 0.785 mm^2, Olympus BX50 microscope). Each endothelial cell or group of cells with brown chromogen deposits (staining for vWf) clearly separated from neighbouring vessels, from cancer cells and connective tissue was counted as separate vessel. The lack of vessel lumen was not an obstacle to count it as a vessel (Fig. 2).

Data analysis was performed with the use of Statistica 5.0. The relation between data was analyzed using chi-square statistics of Pearson with the Yates exact test correction if expected values were smaller than 5. Differences were considered significant at $p \leq 0.01$. The survival curves were generated using the Kaplan-Meier method and statistical evaluation was performed using the log-rang test ($p < 0.05$). Univariate and multivariate relationships were analyzed according to the Cox's proportional hazard regression model.

Table 1. Pathomorphological features of primary tumor, n=90

Investigated feature and number of cases
Localization:
Right colon n=42 (46.7%)
Left colon n=48 (53.3%)
T (pTNM):
T_1 – 2 (2%); T_2 – 11 (12%); T_3 – 26 (29%); T_4 – 51 (57%)
N (pTNM):
N_0 – 52 (58%); N_1 – 25 (28%); N_2 – 13 (14%)
M (pTNM):
M_0 – 84 (93%); M_1 – 6 (7%)
Staging:
I – 9 (10%); II – 41 (46%); III – 34 (37.8%); IV – 6 (7%)
Histologic type (WHO):
adenocarcinoma – 81 (90%);
adenocarcinoma mucinosum – 9 (10%)
Grading (adenocarcinoma, n=81):
G_1 – 29 (36%); G_2 – 43 (53%); G_3 – 9 (11%)

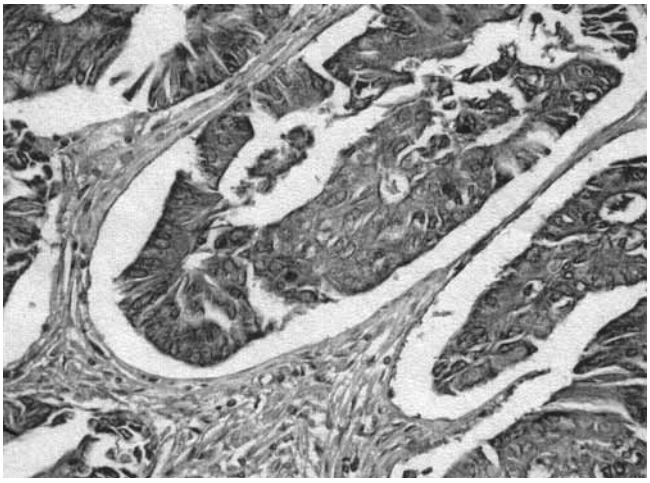

Figure 1. Cathepsin B overexpression in neoplastic cells (x200).

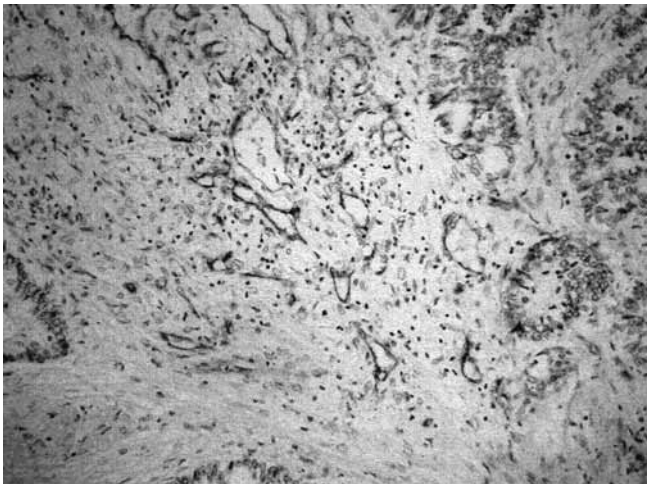

Figure 2. Angiogenesis in the stroma of colon adenocarcinoma (vWf; x200).

Table 2. Statistically significant correlations according to chi-square test

	CatB(-)	CatB(+)	N_0	N_{1+2}
angio(-)	35	10	32	13
angio(+)	19	26	20	25
n=90	p=0.0006		p=0.01	
≤65 yrs.	33	11		
>65 yrs.	21	25		
n=90	p=0.005			

Table 3. Cox regression analyses of investigated features

Variable	p value
Univariate analysis	
Age: ≤65 years vs. >65 years	0.095
Gender: male/female	0.72
Right colon/left colon	0.62
WHO type	0.74
Grading G_1/G_{2+3}	0.51
T_{1+2+3}/T_4	0.14
N_0/N_{1+2}	0.0007
M_0/M_1	0.022
angio(-)/angio(+)	0.0085
CatB(-)/CatB(+)	0.93
Multivariate analysis	
Age: ≤65 years vs. >65 years	0.61
T_{1+2+3}/T_4	0.35
N_0/N_{1+2}	0.013
M_0/M_1	0.021
angio(-)/angio(+)	0.078

Results

CatB overexpression was observed in cytoplasm of the cancer cells and in some from stromal cells, mainly in cases when inflammation was present. In the most tumor samples moderate activity of CatB in a few cells was observed (CatB(-)). Only in 36 cases (40%) we noted distinctly positive reaction in more than 50% of cancer cells (CatB(+); Fig. 1). In the specimens immunohistochemical staining with monoclonal antibody against vWf showed very irregular distribution of microvessel density. Fields with distinctly increased vessel number with different vessel lumen were observed (Fig. 2). Mean vessel number in a single microscopic field (0.785 mm^2) was 33 (42 vessels per mm^2), with median 31 vessels in a single field (n=90, SD 15.09, range from 5.5 to 80). Angiogenesis below median was estimated as low (angio(-)), in the remaining cases as high (angio(+)). In Table 2 we collected features with occurrence relationship statistically significant (p≤0.01). We observed relationship between high vascular density and CatB overexpression in cancer cells (p=0.0006). Relationship between high vascular density and metastases in lymph nodes was observed (p=0.01). More often we detected overexpression of CatB in cancer cells in older patients (median 65 years, p=0.005). Table 3 presents comparison of survival time in selected groups according to Kaplan-Meier method, with "p" value for a log-rank test in univariate analysis and "p" value in multivariate analysis. The results from Table 3 suggest that following factors may influence the survival: the presence of metastases in regional lymph nodes, distant metastases, and intensity of angiogenesis. Figure 3 illustrates survival curves for patients with tumors of different angiogenesis intensity. Results of univariate analysis revealed data necessary for multivariate analysis. Data with p<0.2 were used. In this study only metastases in regional lymph nodes and the presence of distant metastases were

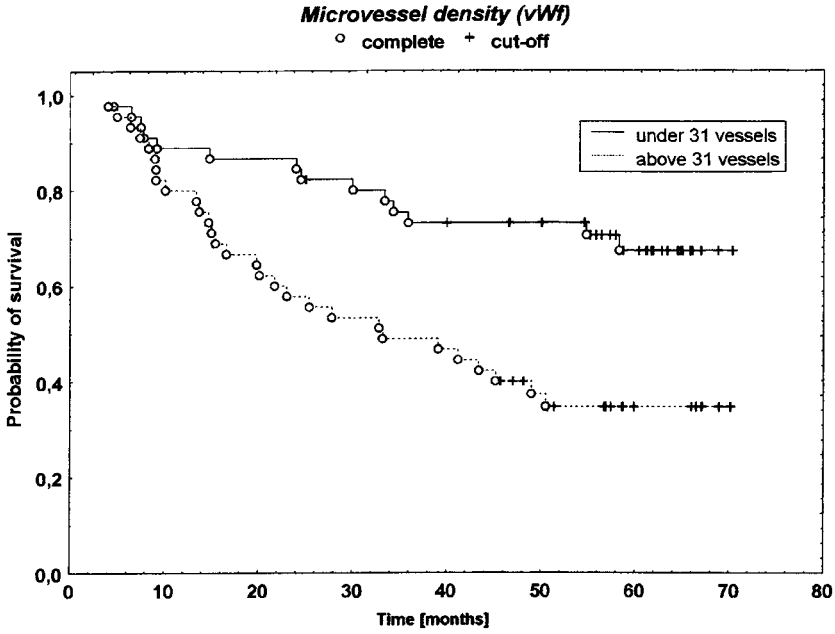

Figure 3. Survival curves of patients according to angiogenesis intensity.

independent prognostic factors. The multivariate analysis did not confirm the influence of microvessel density in primary colon cancer on prognosis (p=0.078).

Discussion

There is a great divergence in evaluation of prognostic and predictive value of angiogenesis in primary tumor of colorectal cancer. We studied the material of more uniform colon cancer, without rectal cancers. In our study we used vWf as a marker of endothelium of microvessels. In our previous study we described the value of vWf as a marker of angiogenesis in tumor, similarly to other authors [26, 49]. Mean number of vessels in evaluated material was 42 vessels/mm^2. This value resembles the results presented by other authors [1, 5, 6, 11, 28, 33, 42]. Relationship between intensive angiogenesis and shorter survival time in univariate analysis (Fig. 3), and with presence of metastases in regional lymph nodes (Tab. 2) may suggest the influence of angiogenesis on the growth and course of colon cancer. However, we did not reveal independent prognostic value of this feature in multivariate analysis. We observed no significant differences in intensity of angiogenesis in different T status. It can confirm to data from literature suggesting the constant intensity of angiogenesis in colorectal tumor in every stage of cancer course [1, 3]. Except constant pattern of vascular architecture in primary tumor and similarity to the pattern of vascular architecture of metastases, the differences in vascularization of normal colon mucosa and cancer are clearly described [25, 36]. It may confirm the usefullness of research on angiogenesis inhibitors in prophylaxis and treatment of colon cancer [39]. The constant vascular architecture confirms studies which analyze its three-dimensional picture [25]. These observations may explain the lack of distinct correlation between intensity of angiogenesis in resected primary tumor of colorectal cancer and the survival time, what has been observed by different authors [3, 30, 33]. There are many studies, that show that microvessel density is an independent prognostic factor in colorectal cancer [5, 11, 42, 43]. Analysis of our material does not confirm the relationship between increased vessel density and poorer prognosis (Tab. 3). The number of cases with very variable amount of vessels in the tumor provokes the researches on correlation between number of the vessels, factors stimulating angiogenesis and course of this disease. We observed the relationship between high vascular density and overexpression of CatB in cancer cells. The influence of CatB on neoangiogenesis is well described [23]. This protein takes part in primary tumor growth in colon cancer and its dissemination [14, 17]. CatB activity and the activity of other cathepsines is an integral part of cascade in the process of cancer spreading using the ability to degradate basement membrane and extracellular stroma [14, 19, 21, 38]. Higher exspression of CatB and other proteases is observed in cancer tissue and in adenoma than in normal colon mucosa [16, 18, 29]. The level of CatB in the serum of patients with colon cancer is also elevated [18]. In the literature higher protein concentration of CatB is observed in the regions with higher microvessel density of many cancer types, but authors emphasize that the CatB enzyme activity is the most important factor to understand its role [2, 10]. It is increased in colon especially in invasive fronts of the tumor and in the areas of intensive neoangiogenesis [16]. According to some authors the elevated activity of CatB is observed mainly in Dukes' A and Dukes' B tumors in comparision to Dukes' C tumors [34]. Other authors describe the highest activity of CatB in metastases of colon cancer in comparison to smaller activity in primary tumor and the smallest in normal colon mucosa [29]. Immunohistochemical staining of CatB protein does not detect activity of this protease [2]. This may be the reason of lack of correlation in our study between overexpression of CatB and long-term results of the treatment or the factors with prognostic value (Tab. 3). There are, however, articles describing the relationship between overexpression of CatB and the cancer stage and poor prognosis [4, 24]. Interesting results were described in the material of 80 cases of colon cancer [20].

Higher expression of CatB was observed in these cases where metastases in liver were present. In patients without metastases higher expression was observed in lower stages of the dissease. According to the authors of the quoted study it may confirm the role of CatB in cancer spreading and progression of the dissease. As concerns CatB protein overexpression according to our estimation method we did not observe this relationship in our material.

Still the most important prognostic factor in colon cancer is staging according to pathologic TNM scale [7]. Parameters describing pTNM, as nodal status or presence of distant metastases studied separately also possess independent prognostic significance [5, 48]. Analysis of our material revealed similar results. TNM staging is helpful in deciding about the adjuvant treatment in colon adenocarcinoma, however, it does not provide detailed information about real cancer aggressiveness and the course of the disease [22]. Neoangiogenesis and proteolytic activity are essential for cancer progression. We observed in our study interdependence between increased angiogenesis and overexpression of CatB protein. We also detected in univariate analysis the potential influence of decreased angiogenesis on better prognosis. Results of our study confirm the need for further research on influence of proteases on carcinogenesis and angiogenesis in colon cancer.

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