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ORIGINAL ARTICLE

Clinical significance of Leptin receptor (LEPR) and Endoglin (CD105) expressions in colorectal adenocarcinoma

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Summary

Purpose: *Carcinoma of the colon occurs quite more often in obese than in healthy people. The key molecule in the development of obesity is leptin, a product of Ob gene that expresses its effects through a specific receptor (LEPR), so our goal was to investigate the expression of LEPR in colorectal carcinoma and the association of their expression with neoangiogenesis, with local/regional and distant metastases and with tumor stage according to the Astler-Coller classification.*

Methods: *In the paraffin blocks taken from 75 patients treated for colorectal cancer, 3-4 μm thick cuts were made using routine hematoxylin-eosin (HE) and immunohistochemical ABC methods with anti-LEPR and anti-CD105 antibodies. After quantitative analysis of LEPR expression, the microvascular density per mm^2 was calculated stereometrically. For the statistical processing, the SPSS software (version 13.0) was used.*

Results: *Pronounced expression of LEPR in stages B_1 and B_2 was present in 9.1% and in 16% of the cases. In the C_2 and D stages, pronounced LEPR expression was found in 51.6%, i.e. 57.1% of the cases, which was significantly higher than in the stages B_1 and B_2. In the C_2 and D stages, a high neoangiogenesis index was found in a significantly higher number of cases (67.7% and 100%) than in stages B_1 and B_2. LEPR expression had a highly significant correlation coefficient associated with tumor stage, neoangiogenesis index, metastases in the lymph nodes and with distant metastases.*

Conclusion: *The increase of LEPR expression was accompanied by increased neoangiogenesis and an increase in the metastatic potential of colorectal cancer.*

Key words: *colorectal carcinoma, leptin receptors, metastasis, neoangiogenesis*

Introduction

Colorectal carcinoma is the third most common malignant tumor in humans with incidence and mortality steadily rising over the last three decades. Its average annual growth rate was around 3% or more with 400,000 newly diseased people in one year. Only during 2012, 693,900 people died of colorectal carcinoma [1,2].

Colon carcinoma is a multifactorial disease resulting from the interaction of hereditary and environmental factors. Numerous studies and meta-analyses show a marked correlation of colorectal carcinoma with obesity [3-5]. It has been confirmed in the literature that obese people, in comparison to normally nourished people, have a 1.5-3.5 times higher risk of colorectal cancer development, while it is estimated that 15-45% of deaths in Europe are attributed to obesity [6,7].

A crucial molecule in the development of obesity is leptin, the product of Ob gene, which is localized on the long arm of chromosome 7 (7q31).

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In its structure, leptin is a typical neuropeptide with anorexic function and is also called "hormone of satiety" because it plays a key role in food intake and control of energy consumption. Its name comes from the Greek word "leptos" which means slim, thin [8,9]. In addition, leptin participates in the regulation of energy consumption, in reproductive functions, in regulation of immunity, hematopoiesis etc. [10-12], and in the last decade its role in cancer development and metastasis is increasingly emphasized [13-16].

Leptin exhibits its effects through a specific receptor (LEPR), which is encoded by the LEPR/Ob-gene. LEPR initiates descending signalling cascades including the JAK2/STAT3 signaling pathway. Numerous studies confirm that LEPR signalling can cause adhesion, angiogenesis, migration and survival of cancer cells [15,17,18].

The aim in this research was to investigate the expression of Leptin receptors (LEPR) in colorectal carcinoma and the association of their expression with neoangiogenesis, with regional and distant metastases, and with the tumor stage according to the Astler-Coller classification.

Methods

Patients and samples

The biopsy and the operative material of 75 patients were used, obtained by resection of colorectal tumor in the Center for Abdominal Surgery of the Clinical Center of Montenegro (KCCG) between January 2010 to December 2012. In the Institute of Pathology KCCG, according to the established protocol, from each operative preparation, depending on the size of the tumor, 5 to 15 biopsies were taken, including 2 to 3 biopsies of the adjacent, non tumorous colorectal tissue. After fixation in a 4% neutral buffered formaldehyde solution, the bioptic material was routinely processed in autotechnikone, embedded in paraffin and archived. Based on standard pathological reports from that period, an experimental group was formed that consisted of operative biopsies of colorectal adenocarcinoma (n=75). The control group (n=75) consisted of operative biopsies of the adjacent non-tumoral colorectal tissuet. The research was approved by the Ethics Committee of the Clinical Center of Montenegro (Decision number: 03/01-15221/2).

Histopathology and immunohistochemistry

From paraffin blocks, 3-4 μm thick cuts were made, on which routine HE and immunohistochemical ABC method were applied. Representative tissue blocks for immunohistochemistry mounted on highly adherent StarFrost, Waldemar-Knittel glasses were first deparaffined through a series of xylols (4 times per 5 min), and then rehydrated in a series of alcohol (3 times per 5 min). Then, the de-masking of the antigen in citrate buffer (pH 6.00) was initiated in a microwave oven. After rinsing in phosphate buffered saline (PBS), the blocking of endogenous peroxidase for 20 min in 3% methanol solution of hydrogen peroxide (H_2O_2) followed. After washing in PBS, incubation with primary rabbit polyclonal anti-leptin receptor antibody (ABCAM, Bulringame, CA, USA, 1:60) and monoclonal mouse anti-human CD105 (DAKO, Denmark, Clone SNGH, 1:10) was performed overnight at +4°C. The labeled antigens were incubated after rinsing with a biotinized secondary antibody (Vectastain ABC-*Elite* kit, Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. After rinsing in PBS, visualization of LEPR and CD105 expression) was carried out with diamino-benzidine-tetrahydrochloride (DAB), followed by contrasting coloring with Mayer's hematoxylin. After rinsing in distilled water, dehydration was carried out through rising alcohol concentrations (70,95,100%) and rinsing three times in xylol. The procedure ended with the application of Canada-Balsam and the covering of tissue samples with a cover glass.

As positive control, samples of invasive breast carcinoma were used, which had previously been tested multiple times and certainly contained the tested antigen. As negative tissue control, tissue samples treated with non-immune serum were used.

Quantification of immunohistochemical staining

Leptin receptor expression was determined on 10 visual fields (the mean value obtained by counting in 10 visible fields is the final result for the case) and classified as follows: 0, <10% positive cells (negative finding); 1+, 10-50% positive cells; 2+, >50% positive cells [19].

The microvascular density (MVD) was calculated by counting the microvascular CD105 positive structures by selecting sites of the highest microvascular density ("hot spots") at a small microscopic magnification. Each single cell or field colored with an immunohistochemical marker was calculated as microvascular structure. In order to determine MVD per unit area in mm^2, a multifunctional test system M42, according to Weibel, was used, calibrated by an objective micrometer (Reichert Wien 2mm/200), with a determined measuring field of 0.016 mm^2. For testing MVD per mm^2, 10 "hot spots" were counted successively, and the absolute value of the density of positive vascular structures in the "hotspot" was determined stereometrically [20]. The arithmetic mean of the obtained "hot spots" values represented the final number of CD105 positive microvascular structures in mm^2 for the case. Thereafter, a median was defined in relation to which the patients were divided into two groups: those with a low degree of angiogenesis (MVD in the tumor lower than the median value) and those with a high degree of angiogenesis (MVD in the tumor higher than the median value). The MVD index (mvdIDX) was obtained from absolutely determined values of the MVD in relation to the deviation from the median.

Statistics

For the statistical processing of the obtained results, the commercial software package SPSS (version 13.0) was used. The distribution regularity was evaluated by

Kolmogorov-Smirnov test. In addition to the univariate analysis, the analysis of the significance of the differences between parametric and non-parametric features, between and within groups, was carried out using the x^2-test, Mann-Whitney U test, Kruskal-Wallis test, and Student's *t*-test. The dependence between two numerical variables was evaluated using Spearman's rank correlation coefficient and Pearson's correlation coefficient for parametric features. Using the receiver operating characteristic (ROC) analysis, the possibility of CD105 being a marker was investigated, and its limit value was determined. Significance testing was carried out at the probability level $p<0.05$.

Results

Forty-five males (60%) and 30 females (40%) were analyzed. The difference in the proportion of respondents by sex was not statistically significant at the adopted level of reliability ($x^2=3.000$, $p=0.083$).

The distribution of patients by age intervals between men and women was also not significantly different (Mann-Whitney *U*-test=569,500 (p=0.232). Male patients had an average age of 65.9±10.8 years and women 60.8±14.5 years, which was not statistically significant (Student's *t*-test=1.622, p=0.111).

Expression of LEPR in colorectal adenocarcinoma and in the adjacent non-tumor tissue

A statistically significant difference in the expression of LEPR was observed between colorectal adenocarcinoma and adjacent non-tumor tissue (Mann-Whitney U-test=1337.5, $p<0.0001$), as shown in Table 1.

In non-tumor tissue, LEPR expression was not found in significant number of cases (62.7%, $x^2=4.813$, $p=0.028$). Moderate expression of LEPR (10-50% of positive cells) was found in 28 cases (37.3%, $x^2=4.813$, $p=0.028$).

In contrast, in the colorectal adenocarcinoma tissue, a significantly lower number of cases without LEPR expression (22.7%) was found. In this group, moderate expression of LEPR was most common (44%). In one third of the cases (33.3%), pronounced LEPR expression was found, so that the occurrence of LEPR expression was verified in a significant number of cases (77.3%) of colorectal adenocarcinoma.

Expression of CD105 (Endoglin) in colorectal adenocarcinoma and in adjacent non-tumor tissue

By immunohistochemical examination of endoglin expression (CD105) and the determination of MVD, absolute metrics of the distribution of positive cells in mm^2 were obtained. The basic characteristics of these metrics (median, minimum and maximum values, deviations) are shown in the box-plot diagram (Figure 1).

The association between LEPR expression and neoangiogenesis index (mvdIDX)

The MVD, expressed through the neoangiogenesis index was very significantly correlated to the expression of LEPR (Table 2). Low index of

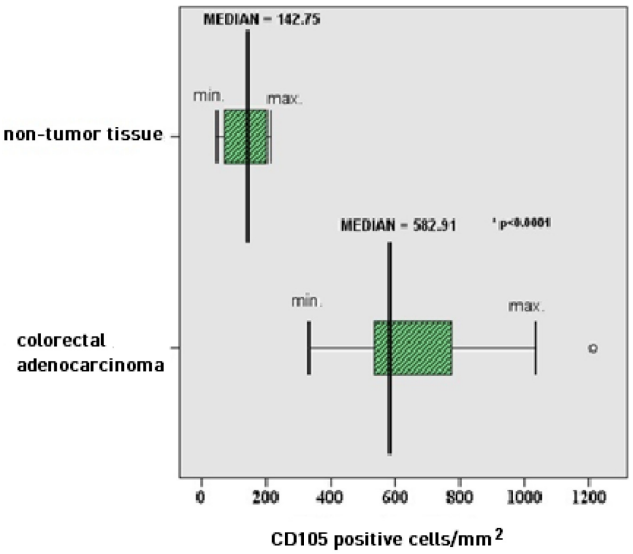

Figure 1. Distribution of CD105-positive cells in mm2 in colorectal carcinoma and in adjacent non-tumor tissue.

Table 1. Leptin receptor expression in colorectal adenocarcinoma and adjacent non-tumor surrounding tissue

LEPR expression	*Groups*	
	Colorectal adenocarcinoma n (%)	*Non-tumor surrounding tissue n (%)*
<10% of positive cells	17 (22.7)	47 (62.7)*
10-50% of positive cells	33 (44.0)	28 (37.3)
>50% of positive cells	25 (33.3)	-
Total	75 (100.0)	75 (100.0)

*x2=4.813, *p=0.028.

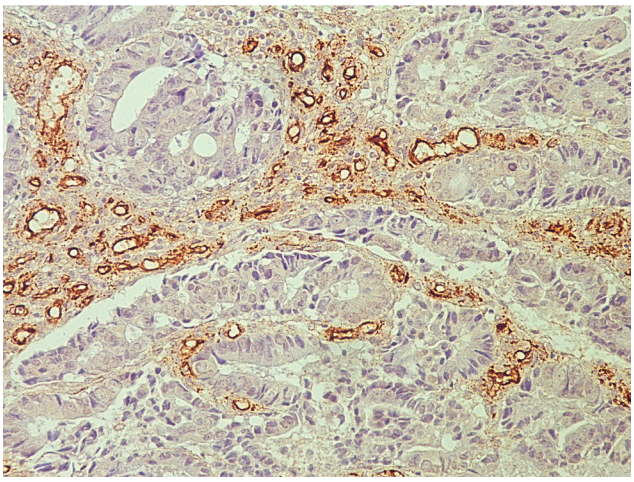

Figure 2. High neoangiogenesis index in colorectal carcinoma (CD105, ABC×200).

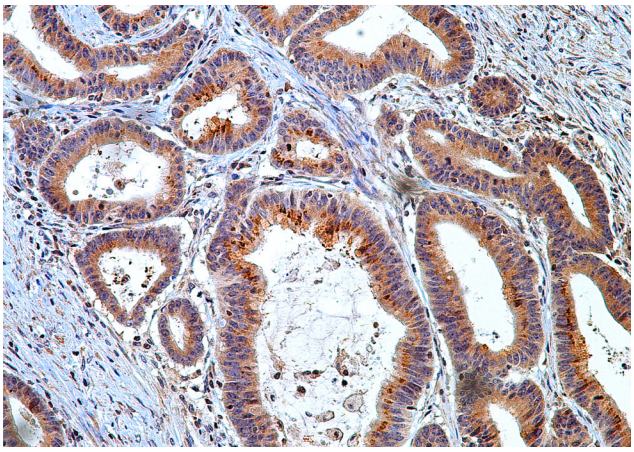

Figure 3. Pronounced intracytoplasmic and intramembranous, microgranular expression of LEPR in colorectal carcinoma (ABCx200).

Table 2. Distribution of the neoangiogenesis index in relation to the expression of LEPR

Neoangiogenesis index	*Expression of LEPR in colorectal adenocarcinoma*		
	<10% of positive cells n (%)	*10-50% of positive cells n (%)*	*>50% of positive cells n (%)*
Low	15 (88.2)	22 (66.7)	2 (8.0)
High	2 (11.8)	11 (33.3)	23 (92.0)*
In total	17 (100.0)	33 (100.0)	25 (100.0)

x2=3.667, *p=0.056.

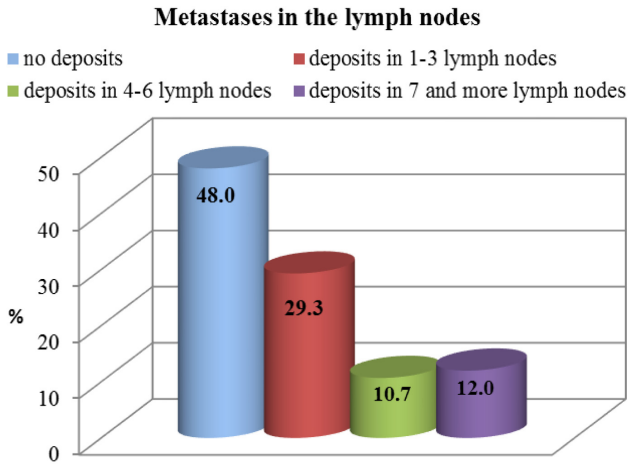

Figure 4. Metastases in the lymph nodes.

neoangiogenesis correlated with the absence of expression of LEPR in a significant number of cases (88.2%), and a high neoangiogenesis index (Figure 2) corresponded to significant 92% of cases with pronounced LEPR expression (Figure 3). In moderate expression of LEPR, there was a more frequent presence of a low neoangiogenesis index (66.7%), than of high (33.3%), but there was no statistically significant difference between these frequencies (x^2=3.667, p=0.056).

Astler-Coller stage of tumor and metastases

The distribution of the stages of the examined colorectal carcinomas according to the Astler-Coller staging showed the lowest incidence of patients in the stage C1 (1 case, 1.3%) and stage D (7 patients, 9.3%). Evaluated by frequency followed stage B1 (11 cases, 14.7%). The highest incidence of patients was in stage B2 (25 patients, 33.3%) and stage C2 (31 cases, 41.3%).

Metastases in lymph nodes were found in 39 patients (52%), while 36 patients (48%) had not nodal metastases (Figure 4). Deposits in 1-3 lymph nodes had 22 patients (29.3%), while deposits in 4-6 lymph nodes were found in 8 patients (10.7%), and in 7 and more nodes were found in 9 patients (12%).

Distant metastases were found in 8 (10.7%) patients, while in 67 patients (89.3%) no distant metastases were found.

Expression of LEPR and neoangiogenesis index (nv-dIDX) in relation to the Astler-Coller tumor stage

Distributions of expression of LEPR and mv-dIDX picturesquely divided the stages according to the Astler-Coller staging classification in two parts.

By comparing the tested parameters (Table 3), using a test to determine the significance of differences in the distributions between groups, it was determined that the distributions within groups B1 and B2, but also within the C1 to D groups, were not statistically different (Mann-Whitney U-test for B1-B2, p=0.074 to p=0.839 and C1 to D, p=0.053 to p=0.658).

However, when the whole group B1-B2 was compared with the C1-D group, a highly significant difference was obtained for all the comparable parameters (p<0.0001).

The distribution of cases without expression and those with moderate LEPR expression in stages B1 and B2 was uniform (90.9% and 84% respectively). Pronounced expression of LEPR in stages B1 and B2 was rare (9.1% and 16% respectively).

In the C2 and D stages, pronounced expression of LEPR was found in 51.6% i.e. 57.1% of the cases. Moderate LEPR expression was found in 35.5% of the cases in C2 stage and 42.9% in D stage, which was significantly higher than in the B1-B2 stages (Fisher exact test=7.2, p=0.007). The absence of LEPR expression in C2 and D stages was a rare occurrence (12.9% i.e. 0%), as opposed to stages B1 and B2, where the frequency of absence of LEPR expression was 36.4% (x^2=4.765, p=0.029).

The neoangiogenesis index, analogously to the LEPR expression assays, correlated with the stages according to the Astler-Coller classification (Table 3). In a statistically significant number of cases, the low neoangiogenesis index (72.7% i.e. 80%) was present in stages B1 and B2, respectively. In stages C2 and D, the neoangiogenesis index was high in a significant number of cases (67.7% i.e. 100% respectively).

LEPR expression and neoangiogenesis index (nvdIDX) in relation to metastases in the lymph nodes

The expression of LEPR and the neoangiogenesis index in relation to the precence of metastatic lymph nodes, as well as the number of affected lymph nodes are shown in Table 4.

By applying the aforesaid method for comparing the distribution between groups, it was observed that groups classified by metastases in the lymph nodes were significantly different only in relation to cases without metastases. A group of cases without metastatic deposits was significantly different in the distribution of LEPR and mvdIDX, and in relation to each individual group with a deposit in the lymph nodes (Kruskal-Wallis Test, p<0.001 to p<0.007).

Table 3. Distribution of LEPR expression and neoangiogenesis index according to Astler-Coller tumor classification

Parameters	*Astler-Coller tumor stage*				
	B1 *n (%)*	*B2* *n (%)*	*C1* *n (%)*	*C2* *n (%)*	*D* *n (%)*
LEPR expression					
<10% of positive cells	4 (36.4)	9 (36.0)	0 (0.0)	4 (12.9)	0 (0.0)
10-50% of positive cells	6 (54.5)	12 (48.0)	1 (100.0)	11 (35.5)	3 (42.9)
>50% of positive cells	1 (9.1)	4 (16.0)	0 (0.0)	16 (51.6)	4 (57.1)
Neoangiogenesis index					
Low	8 (72.7)	20 (80.0)	1 (100.0)	10 (32.3)	0 (0.0)
High	3 (27.3)	5 (20.0)	0 (0.0)	21 (67.7)	7 (100.0)

Table 4. Distribution of LEPR and neoangiogenesis index in relation to metastases in lymph nodes

Parameters	*Metastases in lymph nodes*			
	No deposits *n (%)*	*Deposits in 1-3 LN* *n (%)*	*Deposits in 4-6 LN* *n (%)*	*Deposits in more than 7 LN* *n (%)*
LEPR expression				
<10% of positive cells	13 (36.1)	3 (13.6)	0 (0.0)	1 (11.1)
10-50% of positive cells	18 (50.0)	9 (40.9)	4 (50.0)	2 (22.2)
>50% of positive cells	5 (13.9)	10 (45.5)	4 (50.0)	6 (66.7)
Neoangiogenesis index				
Low	28 (77.8)	5 (22.7)	3 (37.5)	3 (33.3)
High	8 (22.2)	17 (77.3)	5 (62.5)	6 (66.7)

LN: lymph nodes

However, groups with deposits in a different number of lymph nodes did not differ significantly among each other, according to the distribution of certain categories of parameters (Kruskal-Wallis Test, p=0.119 to p=0.755). In lymph nodes without metastatic deposits, absent or moderate expression of LEPR was identified in most cases (86.1%). In this group, the presence of a low neoangiogenesis index (77.8% of the cases) was also highly significant. The occurrence of metastases in the lymph nodes significantly changed the distribution of these parameters, so that with the increase in the number of affected lymph nodes, increased the presence of pronounced LEPR expression (45.5%, 50% and 66.7%). This trend was also followed by an increase in the neoangiogenesis index.

Expression of LEPR and neoangiogenesis index (nvdIDX) in relation to distant metastases

Distant metastases were verified in 8 cases (10.7%). In cases with distant metastases, the expression of LEPR increased significantly, and the neoangiogenesis index was high in all cases (Table 5).

Correlation analysis of LEPR expression, neoangiogenesis index and other parameters

In previous analyses, the cross-section of the parameters pointed to highly significant connections in certain interrelated relationships. Correlation analysis showed the right degree of connection between the tested parameters, where the significance of that connection was proved by the significance of the correlation coefficient and the strength of the connection by its size.

In Table 6, in the correlation matrix, the parameters that were the subject of this analysis were shown via the coefficient of correlation (cc) together with its statistical significance (p).

LEPR expression had significant and high positive correlation coefficients (cc=0.63) associated with the neoangiogenesis index (mvdIDX). Beside the neoangiogenesis index, the absolute value of endoglin expression (CD105) had also significant correlation coefficient associated with the expression of LEPR (Pearson's cc=0.548, p<0.001).

Moderately high and statistically very significant positive correlation coefficients existed among LEPR expression, tumor stages according to Astler-

Table 5. Distribution of LEPR and neoangiogenesis index in relation to distant metastases

Parameters	*Distant metastases*	
	No *n (%)*	*Yes* *n (%)*
LEPR expression		
<10% of positive cells	17 (25.4)	0 (0.0)
10-50% of positive cells	30 (44.8)	3 (37.5)
>50% of positive cells	20 (29.9)	5 (62.5)
Neoangiogenesis index		
Low	39 (58.2)	0 (0.0)
High	28 (41.8)	8 (100.0)

Table 6. Correlation matrix - interdependence of parameters - significance and degree of dependence

		LEPR	*mvdIDX*	*AC stage*	*LN metastases*	*Distant metastases*
LEPR	cc	1.00	0.63*	0.43*	0.44*	0.24*
	p	.	0.00	0.00	0.00	0.04
mvdIDX	cc	0.63*	1.00	0.51*	0.43*	0.36*
	p	0.00	.	0.00	0.00	0.00
Astler-Coller stage	cc	0.43*	0.51*	1.00	0.84*	0.54*
	p	0.00	0.00	.	0.00	0.00
LN metastases	cc	0.44*	0.43*	0.84*	1.00	0.23
	p	0.00	0.00	0.00	.	0.05
Distant metastases	cc	0.24*	0.36*	0.54*	0.23	1.00
	p	0.04	0.00	0.00	0.05	.

*significant p<0.05, cc: Spearman's correlation coefficient, LN: lymph nodes

Table 7. Results of the ROC analysis of the parameter CD105

Parameter	*AUC*	*p*	*CI (95%)*	*Cut off*	*Sensitivity %*	*Specificity %*
CD105	0.854	<0.0001	0.772-0.936	606.72	73.7	81.1

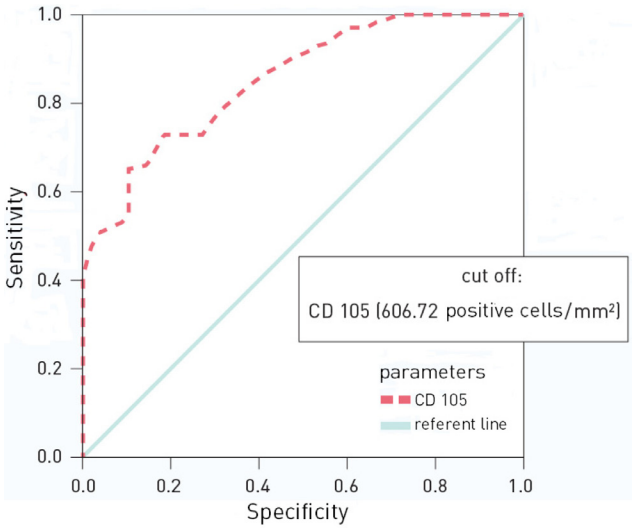

Figure 5. ROC curve determines the cut off value of the neoangiogenesis marker CD105.

Coller classification and the metastatic lymph node involvement.

It has been previously shown that tumor stages according to the Astler-Coller classification show significant changes in all the comparable parameters in relation to the presence of metastatic potential of colorectal carcinoma. This correlation was also confirmed by a very high and significant coefficient of correlation (cc=0.84), between Astler-Coller stage and the presence of metastases in the lymph nodes, as well as the degree of the affected lymph nodes. Related to this was a highly significant correlation of Astler-Coller stages and distant metastases (cc=0.54).

The Astler-Coller stage was in a good and significant correlation with LEPR expression (0.43, p<0.001), but even stronger was the positive correlation between the neoangiogenesis index and the Astler-Coller stage (cc=0.51).

Distant metastases correlated best, beside the proven link to the Astler-Coller stage, with the neoangiogenesis indexes (cc=0.36), and somewhat weaker, but statistically significant was also the correlation of distant metastases with LEPR expression. (cc=0.24, p=0.04).

The cut-off value of angiogenesis marker (CD105) in the prediction of colorectal carcinoma progression

Using ROC analysis, the cut-off values of neoangiogenesis marker (CD105) were determined, above which values could be claimed with high reliability that colorectal adenocarcinoma will progress with the occurrence and increase of metastatic potential. (Figure 5).

Highly significant value of the area under the curve (AUC) showed that the obtained cut-off values with high sensitivity and specificity were reliable diagnostic markers of colorectal carcinoma progression (Table 7).

Discussion

Leptin is a peptide hormone of 16kD molecular weight which, in adults, is mostly produced in white fat tissue. At the same time, leptin, in significantly smaller quantities, is also secreted in numerous non-adipose tissues (lungs, epithelial breast cells, gastric mucosa, brain, placenta, prostate, testicles, ovaries, endometrium, etc.) [9,10,21-24], but it is considered that it has no significance in the endocrine regulation of energy consumption [8].

Leptin is released cyclically, usually 2-3 h after a meal, and serum leptin is in direct correlation with the amount of fatty deposits, that is, increases in obesity and decreases in weight reduction. It has been observed that, when there is an increase in the number and size of the adipocytes, LEP gene begins with the production of leptin that is then secreted into the circulation. Numerous reports from literature indicate that leptin plays an important role in the progression and pathogenesis of colorectal cancer [13,18,25,26], while Tutino et al showed that a high level of serum leptin is an independent risk factor for the development of colorectal cancer [27].

Leptin receptors belong to the first class of the cytokine superfamily receptors, identified as proteins with multiple isoforms from LEP-Ra (leptin receptor isoform a) to LEP-Rf (leptin receptor isoform f). There are three classes of isoforms: short, long and secretory isoforms. The long isoform of LEP-Rb (leptin receptor isoform b) appears as a functional signal-transduction isoform that is responsible for leptin actions and the transmission of information on weight regulation [9]. High levels of expression of LEP-Rb isoform are normally seen in neurons of the hypothalamus, in β-cell pancreatic cells in the vascular endothelium, in epithelial cells of the bowel, etc. LEP-Ra isoform, which has a short cytoplasmic domain, is considered to be responsi-

ble for transporting leptin through the blood-brain barrier [28]. LEP-Re (leptin receptor isoform e) is a soluble isoform that regulates the half-life of leptin and is responsible for transporting leptin through the blood stream. It is the major plasma leptin-binding protein [9].

Leptin receptor expression appears in the cytoplasm and in the cell membrane of many tumor cells, including colorectal carcinoma cells [18,29-31].

By summarizing the literature reports, it could be said that about 77-95.5% of colorectal carcinomas express leptin receptors [19,26,32]. In this study, we have verified the intracytoplasmic and intramembranous expression of LEPR in a significant number of cases (77.3%), with a pronounced expression of LEPR in about one third of the subjects (33.3%).

While examining the neoangiogenesis in colorectal carcinoma, we observed a highly positive correlation of the MVD, expressed through the neoangiogenesis index (mvdIDX), with the expression of LEPR. We noticed that the high neoangiogenesis index correlated with highly pronounced expression of LEPR in 92% of the cases of colorectal carcinoma. At the same time, the low neoangiogenesis index correlated with the absence of LEPR expression in 88.2% of cases.

It is well known that the depth of tumor invasion and the metastatic tumor potential are crucial for prognosis and survival. The metastatic potential of the tumor depends on the presence or absence of metastases in the regional lymph nodes. Metastases usually spread from one to the other lymph node, following lymphatic drainage [33,34]. Metastases in lymph nodes were found in 52% of the subjects. Metastatic deposits in 1-3 lymph nodes had 29.3% of the patients, deposits in 4-6 lymph nodes were found in 10.7% of the subjects, and deposits in 7 and more lymph nodes had 12% of the patients. Distant metastases were found in 10.7% of the patients. The results obtained are in agreement with the results of other authors [34,35].

This study showed that LEPR expression was significantly associated with nodal metastases and with distant metastases. Absent or moderate expression of LEPR existed in the majority of subjects with lymph nodes without metastatic deposits (86.1%). With the increase in the number of affected lymph nodes, the presence of moderate and pronounced LEPR expression was increased, so that the pronounced expression of LEPR was present in 45.5% of the cases of tumors in which metastatic deposits were present in 1-3 lymph nodes; in cases where deposits were present in 4-6 lymph nodes, pronounced LEPR expression was present in 50% of the cases, while in tumors in which the deposits affected more than 7 lymph nodes the pronounced expression of LEPR was present in 66.7% of the cases. In cases with distant metastases, LEPR expression was significantly increased (100%) and was present in all cases with distant metastases.

These results are consistent with the findings of other studies in which a highly significant correlation of LEPR expression with metastases in regional lymph nodes and distant metastases was also demonstrated [19,26].

The highest number of examined tumors (41.3%) was in Astler-Coller C2 stage. As C2 tumor stage were classified all tumors in which the primary tumor affected the peritoneum, was ingrown into the surrounding organs and in which more than 4 lymph nodes contained tumor cells. The B2 stage tumors (33.3%) followed by frequency, which broke through the muscle layer, subserosa, and infiltrated nonperitoneally pericolic tissue. In B1 stage 14.7% of the cases were recorded, in D stage 9.3%, while the lowest incidence of colorectal carcinoma observed was in C1 stage (1.3%).

The LEPR expression in our study was significantly correlated with Astler-Coller tumor stages, with a significant difference between the stage B and the C2 and D stages. Namely, moderate and pronounced expression of LEPR was found in 87.1% of the cases in stage C2 and 100% of the cases in stage D, while moderate and pronounced expression of LEPR in stage B1 was 63.6%, and in B2 stage it was found in 64% of the cases. A significant increase (100%) of LEPR expression in D stage indicated that LEPR expression is a good indicator of metastases in colorectal carcinoma. We have also noted that there was an even stronger positive association of these stages with the neoangiogenesis index. At the same time, a significant increase in neoangiogenesis from stages B (1 and 2) to stages C and D has created the possibility to determine the limit threshold of angiogenesis (via CD105 marker) using ROC analysis, beyond which the threshold a progressive metastatic disease can be expected with high reliability. For our respondents, the limit value for neoangiogenesis was 606.72 CD105 positive cells/mm^2.

In the present study, there was no significant association of LEPR expression with the patient demographic characteristics (gender and age), which was in line with the results of Wang et al and Uddin et al, who examined the expression of LEPR in colorectal carcinoma in relation to demographic parameters [26,32]. On the other hand, Koda et al reported statistically significant positive correlation of LEPR expression with female gender and subjects older than 60 years [36].

In the end, our results show that the LEPR expression is highly dependent on the Astler-Coller tumor stage and is significantly associated with tumor neoangiogenesis. A significant increase in the expression of LEPR in patients with metastatic forms of colorectal cancer indicates that LEPR activity is a valuable diagnostic indicator of the metastatic potential of colorectal carcinoma.

Conflict of interests

The authors declare no conflict of interests.

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