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ORIGINAL ARTICLE

Involvement of leptin receptors expression in proliferation and neoangiogenesis in colorectal carcinoma

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Summary

Purpose: *This study tested whether there exists a correlation between leptin receptors (LEPR) expression with proliferation and neoangiogenesis in colorectal carcinoma.*

Methods: *Enrolled were 75 patients with colorectal carcinoma, who underwent surgical tumor resection. After routine histopathological preparation, sections 3-4 μm thick were prepared. Routine H&E and immunohistochemical ABC method with anti-LEPR, anti-Ki67 and anti-CD 105 antibodies were performed.*

Results*: Pronounced or moderate LEPR expression in colorectal carcinoma was found in 77.3% of the cases. Absence of expression of LEPR correlated with low rate of proliferation in 94.1% of the cases, while high proliferation rate showed 92% of the cases with pronounced LEPR expression. Low grade neoangiogenesis correlated with absence of LEPR expression in 88.2% of the cases. In 92% of the cases with pronounced LEPR expression, high rate of angiogenesis was observed. The LEPR expression correlated significantly (p<0.001) with proliferation index (proIDX) and neoangiogenesis index (mvdIDX). The corresponded correlation coefficients indicated considerable strength of association between variables (r=0.63 and r=0.66).*

Conclusion: *Our results demonstrated that LEPR expression in colorectal carcinoma significantly corresponded to proliferation index of tumor cells and neoangiogenesis, which could have significant therapeutic and prognostic implications.*

Key words: *angiogenesis, colorectal carcinoma, leptin/leptin receptors, proliferation*

Introduction

Colorectal carcinoma is a multifactorial disease being a consequence of intereaction between genetic and environmental factors. Numerous epidemiologic studies have demonstrated close correlation of this tumor with eating habits, because high incidence of colorectal carcinoma is observed in obese persons with "western type diet" (highly caloric food, rich in fats of animal origin), reduced physical activity and reduced intake of fibers [1,2]. It is confirmed that obese persons have 1.5 -3.5-fold increased risk for development of colorectal carcinoma compared to normal-weight persons [3], and it is estimated that 15-45% of deaths in Europe are a consequence of obesity [4].

Key molecule in obesity development is leptin, product of obese gene (ob-gene), a peptide of 16kDa, primarily secreted by adipocytes. The main function of leptin is regulation of energy consumption and appetite [2,4]. Leptin acts via its receptors (LEPR), which belong to the first class of cytokine family receptors, identified as transmembrane proteins with multiple isoforms, from LEPRa to LEPRf. The second isoform LEPRb appears as functional signal-transduction isoform, activating intracytoplasmatic transductional pathways, and it is responsible for leptin actions. The role of shorter isoforms is still unclear [5,6].

Expression of leptin and LEPR is observed in

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different tissues, and it has been reported that leptin stimulates the proliferation of different malignant cells [3,7-9]. Besides, there is more evidence that leptin has an important role in tumor invasion, metastasis, angiogenesis and refractoriness to chemotherapy [9]. It has been shown that LEPR are expressed on endothelial cells and adipocytes, and that leptin increases the proliferation of malignant cells of breast , oesophagus, stomach,colon and prostate cancer [3,7,8].

In prostate and endometrial carcinoma, it has been determined that leptin not only promotes the proliferation, but it also stimulates invasiveness and migration of malignant cells [7,9]. Upon these reports, it is suggested that leptin serves as multi-functional growth factor in carcinogenesis and promotes more aggressive phenotypes of carcinomas [3,8].

The aim of this research was to investigate the influence of LEPR expression on the proliferation and angiogenesis in colorectal carcinoma.

Methods

Patients and tissue samples

The survey covered 75 patients (45 men, mean age 65.9 years, range 34-89 and 30 women, mean age 60.8 years, range 27-83) with colorectal adenocarcinoma who underwent surgical treatment at the Surgical Clinic of the Clinical Center of Montenegro (CCMNE) between January 2010 to December 2012. Depending on the size of the tumor, 5-15 biopsies were taken, including also 2-3 biopsies of the adjacent , non tumorous colorectal tissue. After fixation in 10% neutral buffered formaldehyde, the bioptic material was routinely processed, embedded in paraffin and archived.

Tissue samples of colorectal cancer composed the study group whereas the cases of adjacent non tumorous tissues composed the control group. The study protocol was approved by the local Ethics Committee which gave permission for using paraffin embedded tissues.

Histopathology

Serial sections 3-4μm thich were made on all paraffin blocks of all resected tumors and regional lymph nodes, and subjected to routine H&E staining for histopathological lesions' verification.

Immunohistochemical examination

The nuclear proliferation antigen Ki67 was used for testing the proliferative activity in colorectal carcinoma tissue, whereas for angiogenesis testing, we used Endoglin (CD105, Dako, Glostrup, Denmark), and according to its expression, we determined microvascular density (MVD) in tumor and peritumoral stroma.

Representative tissue sections 3μm thick were heated at 55 ^{O}C to melt the paraffin, deparaffinised in xylene (3x5min) and then rehydrated through graded ethanols. Antigen retrieval was enhanced by autoclaving slides in sodium citrate buffer (pH 6.0) for 30 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide-methanol buffer for 25 min. The examined tissue sections were incubated with rabbit polyclonal anti-Leptin receptor antibody (Abcam, Burlingame, CA, USA; 1:60), rabbit monoclonal Ki67 antibody (Abcam; 1:100) and monoclonal mouse anti-human CD 105 (Dako, Clone SNGh, 1:10) at +4 ^{0}C overnight. Immunostaining was performed by the avidin-biotin peroxidase complex (ABC) method (Vectastain ABC-*Elite* kit, Vector Laboratories, Burlingame, CA, USA). Staining was visualized with 3,3diaminobenzidine tetrachloride (DAB). The slides were counterstained with Mayer hematoxylin and mounted in Canada balsam. In negative controls the primary antibody was replaced with phosphate buffered solution (PBS).

Quantification of immunohistochemical staining

Expression of LEPR was measured in 10 fields of microscopic magnification 400x and classified as follows: 0, no cell stained or <10% positive cells (negative finding); +, 10-50% positive cells (moderate expression); + +, > 50% positive cells (high expression) [10].

In evaluating the expression of Ki67, only stained nuclei were taken into account, while for the evaluation of Ki67-positive cells per mm^2 by area the multipurpose test system M42 by Weibel was used. The objective micrometer (Reichert Wien 2mm/200) was used to determine the measuring area of 0.016 mm^2 . For testing Ki67, positive cells/mm^2 were counted successively by 10 "hot spots". The absolute value of the density of positive cells in the "hot spot" was determined stereometrically [11]. The arithmetic mean of the obtained values of the "hot spots" represented the final number of Ki67 positive cells per mm2 per case. The median was subsequently determined and the absolute values of the density of positive cells were divided into two groups: those with low expression level (value ≤ the median value) and those with high level of expression (values > the median value). These values represented the proliferative activity (proIDX).

MVD was calculated by counting microvascular CD105 positive structures, applying microscopic magnification of 400x, whereas first were selected areas with highest MVD ("hot spots"). Every single cell or field marker was counted as microvascular structure. For the determination of MVD it was also used the multitask test system M42 according to Weibel and measure field of 0.016 mm^2 with Olympus BH-2 microscope. For the investigation of MVD per mm^2, 10 "hotspots" were counted successively, and the absolute value of positive vascular structures density in "hotspot"

was determined stereometrically [12]. The final result was from the study of 10 consecutive fields on average. After having obtained data regarding the number of microvascular structures for each patient, the median was determined, according to which, the patients were divided into two groups: those with low grade of angiogenesis (MVD in tumor ≤ than median value), and those with high grade of angiogenesis (MVD>than median value). From absolute determined values of MVD regarding deviation from median, MVD index was obtained.

The expression of the aforementioned markers was evaluated by two independent pathologist.

Statistics

The statistical software package SPSS for Windows (13.0) (SPSS Inc, Chicago, Ill) was used for conducting statistical analyses. Chi-square test and Mann-Whitney U-test were used to estimate significant differences of parameters between the groups. Then, the Kolmogorov-Smirnov normality test and correlation analysis (Spearman's rank correlation coefficient and Pearson's correlation coefficient) were used. A $p<0.05$ was considered as statistically significant.

Results

LEPR expression in colorectal cancer and adjacent non tumorous tissues

Microgranular LEPR expression was detected in the cytoplasm and cell membrane. A statistically significant difference in the prevalence of LEPR- positive cells was found between non tumorous tissue and colorectal adenocarcinoma (Mann-Whitney U-test = 1337.5, $p<0.0001$).

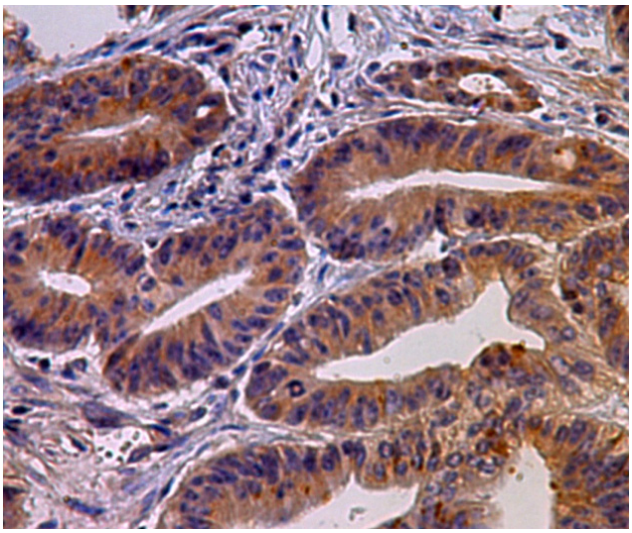

Figure 1. Pronounced intracytoplasmic and intramembranous LEPR expression in colorectal carcinoma areas (ABC x200).

In non tumorous tissues LEPR expression was negative in a significant number of the cases (62.7%, $x^2=4.813$, $p=0.028$), moderate expression [LEPR (+)] in 28 cases (37.3%, $x^2=4.813$, $p=0.028$), and no high expression [LEPR (++)] was found in nontumor colorectal tissue (Table 1).

In colorectal carcinoma tissue, LEPR expression was verified in a significant number of cases (77.3%), while a significantly lower number of cases (22.7%) had negative LEPR expression. This group was most commonly characterised (44%)

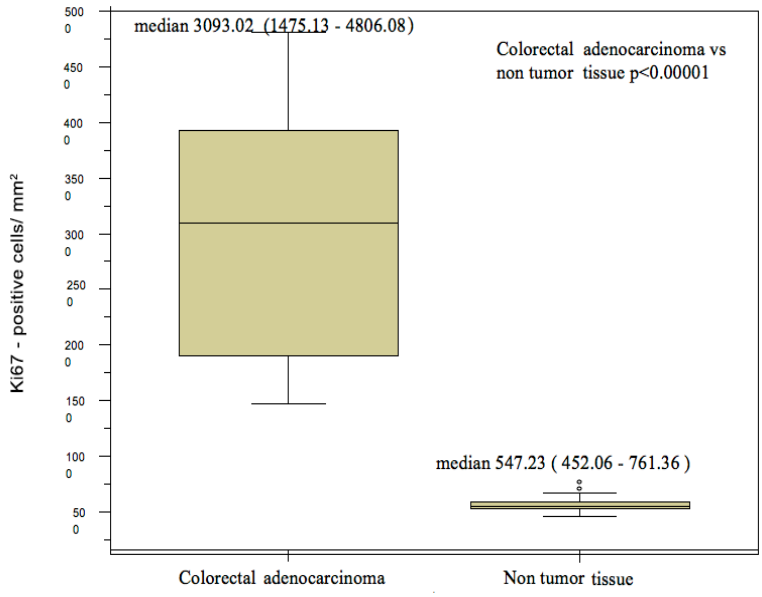

Figure 2. Ki67 positive cells per mm^2 of the investigated tissues.

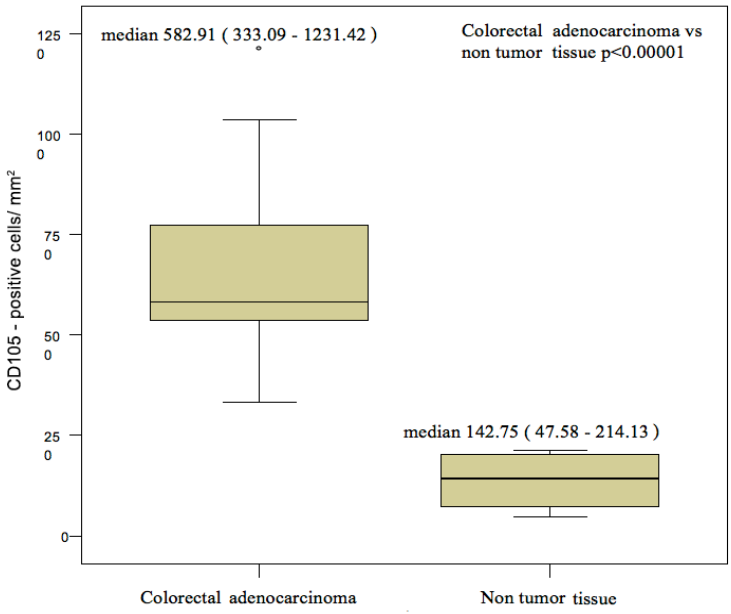

Figure 3. Microvascular density (MVD) per mm² of the investigated tissues.

by moderate expression of LEPR (+). Pronounced expression of LEPR (++) was found in one third of the cases (33.3%; Table 1, Figure 1).

Immunohistochemical expression of Ki67 and CD105 in colorectal cancer and adjacent non tumorous tissues

Box plots show the comparison of immunohistochemical expression of Ki67 positive cells per mm² in colorectal cancer and adjacent non tumorous tissues (Figure 2), and of CD105 positive cells per mm2 in colorectal cancer and adjacent non tumorous tissues (Figure 3). The comparison revealed significant differences for both immunohistochemical parameters between cancerous and non cancerous tissues. The median number of Ki67 positive cells per mm2 in colorectal carcinoma was 3093.03 (range 1475.13-4806.08) compared to 547.23 (range 452.06-761.36 for non tumorous tissues (p<0.0001). Similarly, the median number of CD105 positive cells per mm2 was 582.91 (range 333.09-1231.42) compared to 142.75 (range 45.58-214.13) for non tumorous tissues (p<0.00001).

Table 1. LEPR expression in colorectal adenocarcinoma and adjacent non tumor tissue

LEPR expression	*Colorectal adenocarcinoma*		*Non tumor tissue*	
	N	*%*	*N*	*%*
<10% positive cells	17	22.7	47	62.7*
10-50% positive cells	33	44.0	28	37.3
>50% positive cells	25	33.3	-	-

*p=0.028

Association between LEPR and Ki67 expressions

A significant correlation between the proliferation levels was determined, expressed as the proliferation index and the expression of LEPR in colorectal carcinoma tissue. The low proliferative level correlated with absence of expression of LEPR in a very high number of cases (94.1%). High proliferation index corresponded to a significant 92% of the cases with pronounced expression of the LEPR (Figure 4). Moderate LEPR expression had a greater share of low proliferation index (63.6%), whereas high proliferation index was determined in 36.4% of the cases. (Figure 5)

Association between LEPR expression and MVD

The link between MVD, as expressed through neoangiogenesis index (mvdIDX) and LEPR expression was very obvious. Low grade of neoangiogenesis correlated with absence of LEPR expression in most (88.2%) of the cases, and high index of neoangiogenesis was connected with pronounced LEPR expression in 92% of the cases. In moderate LEPR expression, more common was

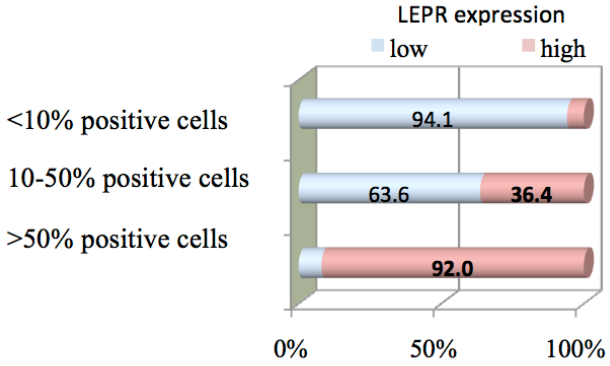

Figure 4. Proliferation index in relation to LEPR expression in colorectal carcinoma.

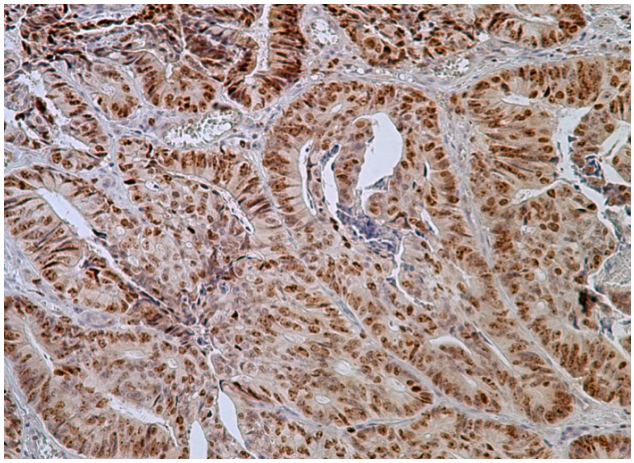

Figure 5. Pronounced intracytoplasmic and intramembranous LEPR expression in colorectal carcinoma areas (ABC x200).

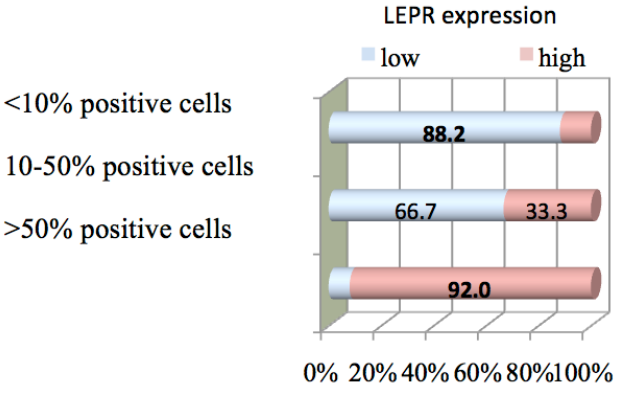

Figure 6. Neoangiogenesis in relation to LEPR expression in colorectal carcinoma.

the proportion of low index of neoangiogenesis (66.7%), compared to high neoangiogenesis index (33.3%) (Figures 6,7).

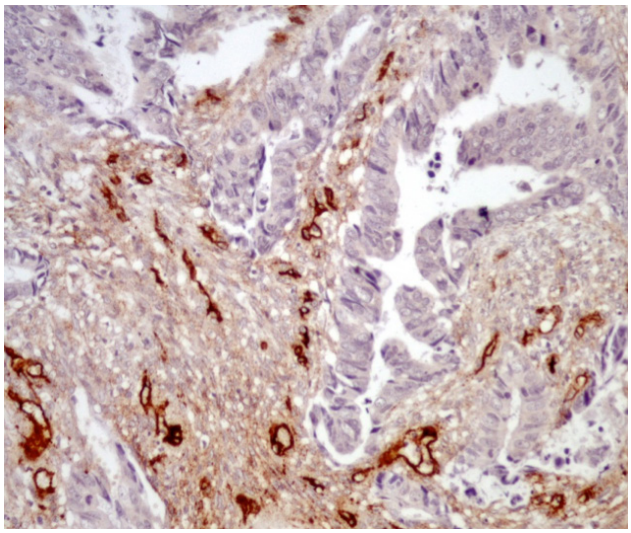

Figure 7. Intensive CD105 expression in microcirculation endothelium (ABC x200).

Table 2. Correlation matrix – correlation parameters – significance and degree of dependence

		LEPR	*proIDX*	*mvdIDX*
LEPR	r	1.00	0.66*	0.63*
			0.00	0.00
proIDX	r	0.66*	1.00	0.47*
mvdIDX	r	0.63*	0.47*	1.00
		0.00	0.00	

*p<0.001, r: Spearman's correlation coefficient.
For other abbreviations see text

Correlation analyses of LEPR expression, proliferation index and neoangiogenesis

Table 2 illustrates all the parameters which were the subject of this analysis. LEPR expression with significant and highly positive correlation coefficients (Spearman's correlation coefficient, r=0.63 and r=0.66, p<0.001) was related to expression index of nuclear proliferative antigen (proIDX) and neoangiogenesis index (mvdIDX). Besides the indexes of proliferation and neoangiogenesis, the absolute values of Ki67 and Endoglin (CD105) showed highly significant correlation coefficients related to LEPR expression (Pearson's correlation coefficient r=0.514 and r=0.548, p<0.001). This implies that increased LEPR expression corresponds to increased proliferative activity and creation of new vascular structures in colorectal adenocarcinoma.

Discussion

In recent years, the obesity and physical inactivity are the most emphasized and pronounced risk factors in the development of colorectal carcinoma [4,12-15]. It is already said that obese persons have higher risk of developing this type of tumor, compared to those with normal weight [3]. It is considered that high level of physical activity reduces the risk of colorectal carcinoma up to 50%, whereas an explanation is that physical activity improves bowel movements [16]. Numerous studies have shown that leptin has great influence in development of obesity, through activation of its receptors [5,6].

In this research, microgranular LEPR expression was detected in the cytoplasm and cell membrane of cancer cells in a significant number of cases (77.3%), while in non tumorous tissue moderate expression was observed rated in 37.3% of cases. Pronounced LEPR expression in high percents of colorectal carcinoma has been reported by other authors also. These data are, reasonably, heterogeneous. Koda et al. detected LEPR expression in 65.2% [10], Wang et al. in 76.5% [17], and Uddin et al. detected LEPR expression in 87.3 % of colorectal carcinoma [18].

Numerous reports in the literature imply that leptin has important role in the genesis and progression of colorectal carcinoma [17,19]. Leptin serum level is increased in obese people, and it is directly correlated with the amount of fat deposits. This could be an important link between obesity and colorectal carcinoma, and a recent report, that high leptin serum level represents an independent risk factor for development of colorectal carcinoma, supports this hypothesis [20].

Although some authors deny relations between plasma soluble LEPR and risk for colorectal carcinoma [21], yet many authors have well documented a link between serum leptin and tumors of epithelial origin, including colorectal carcinoma [22,23]. Numerous reports confirm the opinion that leptin has stimulatory effects on malignant cells proliferation in different localizations [3,7-9,19,24,25], and the results of correlation analyses in our study demonstrated that LEPR expression had highly positive correlation related to proliferative index, in concordance to these reports. In our research, in a very high number of cases (94.1%), absence of LEPR expression correlated with low proliferation index, and concurrently, high proliferation index corresponded to 92% of cases with pronounced LEPR expression. In tissue culture it has also been demonstrated that leptin stimulates proliferation and promotes cell migration and angiogenesis of human colorectal carcinoma cells [26].

There are numerous reports emphasizing the predictive value of MVD in different malignant tumors [27-30], but regarding the role of angiogenesis in colorectal carcinoma, results from different studies are controversial. While Bossa et al. [31] and Pietra et al. [32] denied a predictive importance of angiogenesis, Saad et al. using Endoglin, claimed that significant correlation existed between increased MVD with recurrence, metastasis and survival [30]. These conflicting results can be explained by the fact that different markers were used, and different immunohistochemical techniques as well. Namely, in the research of Bossi et al. [31] and Pietra et al. [32] used were the panendothelial markers CD34, CD31 and von Willebrand factor, which show pronounced expression in normal tissue also and are not specific for blood vessels, because they stain lymphatics as well [28,33]. In this context, Endoglin as marker of angiogenesis has some advantages; first, Endoglin antibody binds mainly to activated endothelial cells that take part in tumor angiogenesis, and only to 20% of non neoplastic blood vessels, and doesn't bind to inflammatory or stromal cells [30,34]. In our study, for investigation of MVD in tumor tissue, Endoglin (CD105) was used. Pronounced Endoglin expression was present in tumor and peritumoral vasculature in all cases of colorectal carcinoma, while its expression was weak or negative in adjacent non tumorous tissue , which is in concordance with the results of Saad et al. [30] and Minhajat et al. [34].

The results of our research indicate that high index of neoangiogenesis (92% of the cases) was related to high LEPR expression, while low index of angiogenesis (88.2%) was related to absence of LEPR expression. In cases of moderate LEPR expression, low angiogenesis index was more frequent (66.7%), compared to high index of angiogenesis (33.3%). Correlation analysis demonstrated that LEPR expression correlated significantly with the proliferation and neoangiogenesis indices. The corresponding correlation coefficients indicated considerable strength of association between LEPR expression and proliferative activity and neoangiogenesis in colorectal carcinoma. Our results are convergent to the studies emphasizing that leptin takes part in tumor spreading and stimulation of angiogenesis, like VEGF [3,9,19,22,26,35].

It is observed that, when it comes to the in-

crease of number and size of adipocytes , it starts with production of leptin that is secreted in the circulation [20]. Circulating leptin levels increase at night, and reach peak in the middle of night hours. Leptin secretion in adipocytes and its circulatory values are mainly regulated by insulin, glucocorticoids and catecholamines [5]. Leptin is released cyclically, usually 2-3 hours after meals, and its half-life is 30 min. During aging, leptin levels decrease slowly, and this reduction is higher in women compared to men, not depending on body mass index and other endocrine changes connected to aging [36].

The main function of leptin is the regulation of body weight by negative feedback between adipose tissue and satiety center in hypothalamus [3,37]. Leptin acts on metabolism directly, by increasing the metabolism in adipocytes and non adipose tissue with increased oxidation of fatty acids, and indirectly, by decreasing the plasma insulin level and decreasing the sensitivity of peripheral tissue (first of all adipocytes) to insulin [22,38]. Leptin gives information to brain about fat deposits in the body, and acts like part of reversal mechanism that can work as lipostat [39,40]. Besides, leptin acts in regulation of energy consumption, in the proliferation of many normal and neoplastic tissues, in angiogenesis, but also has role in hematopoiesis and reproduction [3,22,39,41]. Experimentally, it has been demonstrated that leptin administration to female rats results in ovulation, pregnancy and lactation [35]. It is also observed that during the reproductive years, leptin level is higher in the middle of lutein phase, and that estrogens increase leptin level, while in menopauseal women leptin level decreases [35,42].

Leptin action in energetic metabolism is well known, but its role becomes more complex after information about LEPR expression in many tumor tissues. Koda et al. demonstrated positive correlation between leptin, LEPR and hypoxia-inducible-factor-1alpha (HIF-1α) in endometrial carcinoma, which clearly implies contribution of tissue hypoxia on the expression of leptin and LEPR [10]. It is known that HIF-1α positive tumors can be resistant to chemotherapy and radiotherapy because of accelerated transcription processes that confront apoptosis and favor tumor cells survival [43,44].

Except colorectal carcinoma, LEPR expression is found in tumor cells of the stomach, breast, lung, prostate, endometrial and thyroid carcinomas [3,7,8,9,25,37,45]. Also, it is observed that endometrial , breast and prostate carcinoma are associated with obesity, so it is suggested that this phenomenon can be associated with production of biologically active substances secreted in adipose tissue [46,47].

Several authors, using different cell lines of colorectal carcinoma have shown that leptin through its receptors can activate different signal pathways for growth and proliferation of cancer cells, including JAK/STAT,PI3K/AKT and MAP-kinase pathway [48]. Other researchers have shown that leptin stimulates phosphorylation and activation of STAT3 protein on JAK2 dependent pathway, whereby activated STAT3 is involved in proliferation, inhibition of apoptosis and cell transformation [49,50]. Similar are the results of Abubaker et al. [51], who determined that inhibition of JAK2 activity breaks the signal pathway of STAT3 activation. It is also demonstrated that activation of PI3K/AKT with leptin happens through activation of JAK2 [51]. According the reports of Wang et al. [17,52], it can be presumed that leptin modulates the survival of colorectal cancer cells through activation of PI3K/AKT/mTOR signal pathways. For PI3K/AKT signal pathway, it is known that it regulates multiple cell processes, including cell proliferation, growth and viability [53].

In conclusion, this study has demonstrated that LEPR expression in colorectal carcinoma is significantly related with the proliferation index of tumor cells and neoangiogenesis , which could have significant therapeutic and prognostic implications.

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