Interplay between expression of leptin receptors and mucin histochemical aberrations in colorectal adenocarcinoma

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Abstract

Background. There is no information on the effects of leptin receptors expression on mucin-histochemical alterations in human colorectal adenocarcinoma. Aim: Testing the correlation of leptin receptors expression with histochemical dysregulation of mucins in colorectal adenocarcinoma. Patients and Methods: The study included 75 patients with colorectal adenocarcinoma who underwent surgical resection. Following a routine histopathological tissue analysis, 3–4 μm thick cuts were made onto resected tumors, which underwent a routine Hematoxylin–Eosin, histochemical Alcian Blue–Periodic Acid Schiff, pH 2.5, and High Iron Diamine–Alcian Blue, pH 2.5, methods for mucin differentiation and immunochemical Avidin–Biotin peroxidase complex method with anti-Ki67 and anti-leptin receptor antibodies. Following the quantification of results for the statistical analysis, the statistical software package SPSS for Windows (13.0) was used, and the tests for analyzing the significance of differences and correlation analysis – Spearman’s rank correlation coefficient, were conducted. Results: Increased expression of leptin receptors is with highly significant correlation coefficient associated with hypersecretion of sialomucins. Significant positive correlation coefficient exists between the leptin receptors expression against neutral-fucomucins secretion. With weak and negative, but a significant correlation coefficient, leptin receptors expression is associated to the sulfomucins generation. Conclusions: Increased expression of leptin receptors in colorectal adenocarcinoma is associated with mucin-histochemical abnormalities that are manifested by sialomucins hypersecretion and reduction, ultimately resulting in the absence of sulfomucins secretion.

Keywords: leptin receptors, mucins, histochemistry, colorectal adenocarcinoma.

Introduction

Colorectal cancer is the most common malignant tumor of the gastrointestinal tract, and pursuant to the estimates from 2011 and 2012, it is the third most frequently diagnosed malignant cancer after lung and prostate cancer in men and after breast and lung cancer in women [1, 2].

Many epidemic studies and meta-analysis are mostly indicating the most consistent linkage of colorectal cancer with obesity [3–8]. The literature confirms that obese people are exposed to 1.5–3.5 times higher risk of developing colorectal cancer compared with the ones having normal weight [3], and it is estimated that 15–45% of deaths in Europe is attributed to the obesity consequences [4–8].

A key molecule in the development of obesity is leptin, a product of Ob gene, which is localized on the long Q arm of chromosome 7 (7q31) [9]. It is generated in white adipose tissue; nevertheless, numerous non-adipose tissues are synthesizing and secreting leptin in small quantities [10–14]. The main leptin function is to regulate the body weight through a negative feedback between adipose tissue and the satiety center in the hypothalamus [3, 14]. It participates in the regulation of energy consumption, in proliferation of many normal and neoplastic tissues, and it plays a role in hematopoiesis and reproduction [3, 10, 11, 15–17]. Leptin is released cyclically, usually 2–3 hours after a meal, and its serum half-life is 30 minutes. Leptin level is increased in the serum of obese people, and recent reports have shown that leptin high levels in serum represents an independent risk factor for developing colorectal cancer [18]. Leptin exerts its action through a specific receptor (LEPR) which is encoded by the Ob gene. Leptin receptors have been identified as proteins with multiple isoforms ranging from LEPRα to LEPRf. Expression of leptin receptor was detected in many tissues, whereas it was observed that leptin has a stimulatory effect on the proliferation, migration and angiogenesis of malignant tumor cells of different localization [3–6, 11–14, 16–18, 19–22].

It has been observed long ago that, in addition to uncontrolled proliferation and angiogenesis, during colorectal carcinogenesis, alterations in the structure and/or the amount of epithelial mucins are emerging, causing alterations to the barrier function of mucus and abnormal cell signal transduction [23]. These changes in mucins expression or glycosylation are affecting cellular growth,
Materials and Methods

Patients and samples

The survey covered 75 patients (45 men, mean age 65.9, range 34–89 years and 30 women, mean age 60.8, range 27–83 years) with colorectal adenocarcinoma who were submitted to surgical resection at the Surgical Clinic of the Clinical Center of Montenegro (CCMNE) in the period from January 2010 to December 2012.

In the Pathology Institute of CCMNE out of each operating resection, depending on the size of the tumor, 5–15 biopsies were taken, including also 2–3 biopsies of the adjacent non-tumoral colorectal tissue. After fixation in 10% neutral buffered formaldehyde, biopsy material was routinely processed, embedded in paraffin and archived.

Tissue samples of colorectal cancer were analyzed after conducting surgical biopsy, making a testing group, whereas the cases of operating biopsy of adjacent non-tumoral tissues represented a control group. The study protocol was approved by the local Ethics Committee.

Histopathology and mucins-histochemistry

Serial 3–4 μm thick cuts were made on all paraffin blocks of all resected tumors and regional lymph nodes, subjected to a routine Hematoxylin–Eosin method for histopathological lesions verification, histochemical AB–PAS (Alcian Blue–Periodic Acid Schiff), pH 2.5 method for the differentiation of neutral (red) from acid (blue) mucins and histochemical HID–AB (High Iron Diamine–Alcian Blue), pH 2.5 technique for differentiation of poorly acid, sulfate-free (blue) si alomucins from highly acid, sulfated (dark brown), colonic sulfomucins [26].

Immunohistochemical examination

Representative tissue 3 μm thick cuts were made on all paraffin blocks of all resected tumors and regional lymph nodes, subjected to a routine Hematoxylin–Eosin method for histopathological lesions verification, histochemical AB–PAS (Alcian Blue–Periodic Acid Schiff), pH 2.5 method for the differentiation of neutral (red) from acid (blue) mucins and histochemical HID–AB (High Iron Diamine–Alcian Blue), pH 2.5 technique for differentiation of poorly acid, sulfate-free (blue) sialomucins from highly acid, sulfated (dark brown), colonic sulfomucins [26].

Statistical analysis

The statistical software package SPSS for Windows (13.0) has been used for conducting statistical analysis. For conducting the analysis of the significance of differences of non-parametric features, between and within the groups, χ²-test (chi-square test), was used. Afterwards, correlation analysis (Spearman’s rank correlation coefficient) was used. Significance testing was carried out at the probability level of p<0.05.

Results

Histological expression of epithelial mucins in colorectal adenocarcinoma and adjacent non-tumoral tissue

Histochemical epithelial mucins expression in colorectal carcinoma and adjacent non-tumoral tissue is shown in the Table 1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Colorectal cancer (n=75)</th>
<th>Adjacent non-tumoral tissue (n=75)</th>
<th>Neutral-fuco mucins (FM)s</th>
</tr>
</thead>
<tbody>
<tr>
<td>No secretion</td>
<td>(48.0)</td>
<td>73 (97.3)*</td>
<td></td>
</tr>
<tr>
<td>(+/-)</td>
<td>(45.3)</td>
<td>2 (2.7)</td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>(6.7)</td>
<td>-</td>
<td></td>
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<td>(++)</td>
<td>-</td>
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</tbody>
</table>

Quantification of mucinohistochemical and immunohistochemical staining

We have quantified the histochemical expression of epithelial mucins according to the following scale: - (minus), mucins no secretion; +/- (plus/minus), mucins secretion in trace; +, moderate mucins secretion; ++, highly pronounced secretion (hypersecretion).

Expression of leptin receptor was measured in 10 visible fields of microscopic magnification 40× (mean value represents the final result for the case) and classified in the following manner: 0, no cell stained or <10% positive cells (negative finding); +, 10–50% positive cells – moderate expression; ++, >50% positive cells – highly pronounced expression [27].

For the reason of evaluating the expression of Ki67, only staining nuclei were taken into account, while for the designation of density of Ki67-positive cells per mm² by area was used the multipurpose test system M42 by Weibel. Objective micrometer (Reichert Wien 2 mm/200) was used to determine the measuring area of 0.016 mm². For the purpose of density, testing of Ki67-positive cells/mm² were counted successively by 10 “hot spots”. The absolute value of the density of positive cells in the “hot spot” was determined stereometrically [28]. The arithmetic mean of the obtained values of the “hot spots” represents the final number of Ki67-positive cells in mm² per case. The median was subsequently determined and the respondents were divided into two groups: those with low expression level (value below or equal to the median value) and those with high levels of expression (values above the median value). The expression of the aforementioned markers was evaluated by the two independent researchers (pathologists).

Table 1 – Mucins secretion distribution in colorectal carcinoma and adjacent non-tumoral tissue

<table>
<thead>
<tr>
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<td>(6.7)</td>
<td>-</td>
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<td>(++)</td>
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</tbody>
</table>
Interplay between expression of leptin receptors and mucin histochemical aberrations in colorectal adenocarcinoma

### Table

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<tr>
<th>Parameter</th>
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<th>Adjacent non-tumoral tissue (n=75)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weakly acid-sialomucins (SiMs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No secretion</td>
<td>-</td>
<td>14 (18.7)</td>
</tr>
<tr>
<td>(+/-)</td>
<td>(12.0)</td>
<td>51 (68.0)*</td>
</tr>
<tr>
<td>(+)</td>
<td>(45.3)</td>
<td>9 (12.0)</td>
</tr>
<tr>
<td>(+++)</td>
<td>(42.7)</td>
<td>1 (1.3)</td>
</tr>
</tbody>
</table>

| Highly acid-sulfomucins (SuMs)   |                          |                                    |
| No secretion                     | 25 (33.3)                |                                    |
| (+/-)                            | 40 (53.3)                |                                    |
| (+)                              | 10 (13.3)                | 19 (25.3)                          |
| (+++)                            |                          | 56 (74.7)*                         |

*Significant, p<0.05, n(%).

In colorectal adenocarcinoma, a moderate secretion (+) of neutral fucomucins (FMs) was found, while no secretion was identified in 48% of cases. In the adjacent non-tumoral tissue, in 97.3% of cases the FMs secretion was not present.

Hypersecretion (+++) of sialomucins (SiMs) (Figure 1A) was found in 42.7% of cases, moderate secretion (+) (Figure 1B) was found in 45.3%, secretion in trace (+/-) was found in 12% of cases, and SiMs no secretion (-) was not identified in either case. Contrary to this distribution, the existence of SiMs hypersecretion in non-tumoral tissue is extremely rare occurrence (1.3%), but in a significant number of cases in relation to other categories, the most common finding of SiMs secretion is in the trace (68%, $\chi^2$-test = 9.720, $p=0.002$).

**Figure 1** – Histochemical sialomucins expression in colorectal adenocarcinoma: (A) Hypersecretion sialomucins (AB–PAS staining, pH 2.5, ×200); (B) Moderate focal sialomucins secretion (AB–PAS staining, pH 2.5, ×200).

In non-tumoral colorectal tissue, significant hypersecretion (74.7%) of excessively acid, sulfomucins (SuMs) is present. Only around quarters of respondents (25.3%) had moderate secretion (+), while the secretion in trace and SuMs no secretion has not been determined. Contrary to this finding, in a group of colorectal adenocarcinoma, hypersecretion of SuMs has not been identified in any of the case. In most of the cases (53.3%), SuMs secretion has not been found, or it has been present the secretion in trace (33.3% of cases) (Figure 2).

**Figure 2** – Excessive sulfomucins reduction in colorectal adenocarcinoma (HID–AB staining, pH 2.5, ×200).

Leptin receptors expression in colorectal cancer and adjacent non-tumoral tissues

Microgranular leptin receptor expression was detected in the cytoplasm and cell membrane. A statistically significant difference in the prevalence of leptin receptor positive cells was found in non-tumoral tissue and colorectal adenocarcinoma ($\chi^2$-test = 39.47, $p<0.001$).

The non-tumoral tissue does not include the expression of LEPR in a significant number of 47 cases. Moderate expression of the LEPR (+) was found in 28 cases (62.7% vs. 37.3%, $\chi^2$-test = 4.813, $p=0.028$), and pronounced expression of LEPR (++) was not found in non-tumoral colorectal tissue (Figure 3).

**Figure 3** – Expression of the LEPR in colorectal adenocarcinoma and in non-tumoral tissue.
In colorectal carcinoma tissue, LEPR expression was verified in a significant number (77.3%) of cases, while a significantly lower number (22.7%) of cases were found without LEPR expression. This group is most commonly featured (44%) by a moderate expression of the leptin receptor (+). Pronounced LEPR expression (+++) was found in one-third (33.3%) of the cases (Figures 3 and 4).

Figure 4 – Pronounced immunohistochemical expression of the leptin receptors in colorectal adenocarcinoma (ABC method, ×200).

Association between leptin receptor expression and mucin-histochemical expression of colorectal cancer and adjacent non-tumoral tissue

The absence of leptin receptor expression in colorectal adenocarcinoma has been associated with no secretion of FMs in a significant number (94.1%) of cases. Pronounced LEPR expression is associated with FMs no secretion in 32%, with the secretion in trace in 60% and a moderate FMs secretion in 8% of the cases.

In the adjacent non-tumoral tissue, FMs secretion is an extremely rare occurrence. With the high significance, this tissue is not secreting FMs, both in the absence of expression and at a moderate expression of the leptin receptor (in 97.9% and 96.4% of cases).

In colorectal carcinoma tissue, the absence of expression of the LEPR, in a significantly high number (82.4%) of cases was associated with moderate secretion (+) SiMs. The higher the LEPR expression is the greater is the SiMs secretion, thus a moderate expression (+) resulted in SiMs hypersecretion in 33% of cases. In excessive expression (+++) of LEPR, there is a significantly high incidence (84%) of SiMs hypersecretion in colorectal adenocarcinoma.

In the absence of expression of LEPR in non-tumoral colorectal tissue, SiMs are usually present in trace (68.1% of cases), while the hypersecretion of SiMs is extremely rare (2.1%). At moderate expression of the LEPR in non-tumoral tissue, in a significantly lower number (10.7%) of cases, there is no SiMs secretion, yet there is still a significant number (67.9%) of secretion in trace, whereas the incidence of moderate secretion of SiMs is increasing (21.4%).

In excessive secretion of LEPR in colorectal cancer, in a significant number of cases SuMs no secretion was found (60%, χ²-test = 10.160, p=0.006). In cases with moderate expression of LEPR, SuMs exists in trace in 60.6%. In the absence of expression of leptin receptor, SuMs exists in trace in 70.6% of cases (Figure 5A).

In cases without and with moderate expression of the LEPR in non-tumoral colorectal tissue, hypersecretion of SuMs is significant occurrence with the probability that is clearly significant (72.3% and 78.6%, respectively). The share of SuMs moderate secretion is significantly lower in this group of analyzed tissues, thus its amount in the lack of expression of LEPR is 27.7%, whereas in the cases of moderate expression of leptin receptor it amounts to 21.4% of cases (Figure 5B).

Linkage between the leptin receptor and immunohistochemical expression of Ki67

A significant correlation between the proliferation degrees was determined, expressed as the proliferation index and the expression of the LEPR in colorectal carcinoma tissue. The low level of proliferation correlates with the absence of expression of the LEPR in a highly significant number of cases (94.1%). High proliferation index corresponds to the significant 92% of cases with excessively high expression of the LEPR (Figures 6 and 7).

Figure 5 – Association of LEPR expression and sulfomucins secretion: (A) In colorectal adenocarcinoma; (B) In adjacent non-tumoral tissue.
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Correlation analysis of leptin receptor, mucin secretion and proliferation index

Table 2, in its correlative matrix and through the correlation coefficients ($r$) with its statistical significance ($p$), is illustrating all parameters, which were the subject of this analysis. Significant and high positive correlation coefficient ($r=0.63–0.66$) is associating LEPR expression with the index expression of nuclear proliferation antigen (proIDX). Next in importance is the relationship of LEPR expression and sialomucins (SiMs). Highly significant is the correlation coefficient, which is showing that the increase in LEPR expression is in positive correlation with the hypersecretion of SiMs in colorectal adenocarcinoma ($r=0.59; p<0.001$).

As it can be seen from Table 2, moderately high and statistically highly significant positive coefficient of correlation exists between the expression of the LEPR and secretion of neutral fucomucins (FMs) ($r=0.40; p<0.001$).

Somewhat weaker and negative correlation coefficient ($r=-0.27$), but statistically significant, at the adopted level of confidence $p<0.05$, LEPR expression is related to the production of sulfomucins (SuMs) in colorectal cancer.

Mucins secretion is in good and substantial interconnectivity (0.37 to 0.44), only the SuMs secretion is negatively correlated both to other mucins and the LEPR expression and proliferative index ($r=-0.34$). SIms production is in a stronger relation with the proliferative activity ($r=0.44$) comparing to the FMs ($r=0.27$), expressed with significant correlation coefficients.

Table 2 – Correlation of expression of the LEPR, proliferation index and secretion of mucins in colorectal carcinoma

<table>
<thead>
<tr>
<th></th>
<th>Leptin receptors</th>
<th>Proliferation index</th>
<th>Fucomucins (FMs)</th>
<th>Sialomucins (SiMs)</th>
<th>Sulfomucins (SuMs)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leptin receptors</strong></td>
<td>$r$ 1.00</td>
<td>0.66*</td>
<td>0.40*</td>
<td>0.59*</td>
<td>-0.27*</td>
</tr>
<tr>
<td><strong>Proliferation index</strong></td>
<td>$p$ 0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Fucomucins (FMs)</strong></td>
<td>$r$ 0.40*</td>
<td>0.27*</td>
<td>1.00</td>
<td>0.37*</td>
<td>-0.34*</td>
</tr>
<tr>
<td><strong>Sialomucins (SiMs)</strong></td>
<td>$p$ 0.00</td>
<td>0.02</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Sulfomucins (SuMs)</strong></td>
<td>$r$ -0.27*</td>
<td>-0.32*</td>
<td>-0.34*</td>
<td>-0.44*</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>$p$ 0.02</td>
<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

*Significant, $p<0.05$, $r$ – Spearman’s correlation coefficient.

**Discussion**

Mucins represent a selective molecular barrier on the epithelial surface, ensuring the protection of the cell surface and participating in the morphogenetic cell transduction [29]. They represent a complex macromolecular compounds composed of proteins and polysaccharides linked with each other with strong covalent bonds. Protein component is made by a polypeptide chain, and over 70% of the molecular weight is composed of polysaccharides in the form of carbohydrate side chains, which are attached to the protein component [23, 29, 30]. Constituents of the carbohydrate side chains are the N-acetylgalactosamine, N-acetylglucosamine, galactose, fucose and sialic acid [23].

Based on its histochemical features, mucins may be divided into neutral-fucomucins (containing hexosamine, galactose and fucose), weakly acid sialomucins (containing hexosamine and sialic acid) and highly acid sulfomucins (containing hexosamine, immersion acid and sulfate group). Mucins with acid carbohydrate groups are realizing negative charge, and therefore are associated with AB (Alcian Blue) and HID (High Iron Diamine) reagents, which is the reason behind using these combined histochemical methods for the purpose of conducting qualitative and semi quantitative analysis of acid mucins [26].

It is well known that the secretion of sulphated, highly acid mucins, is predominant in the colonic mucosa, while sialo and fucomucins are presented in trace [31, 32]. In the non-tumoral tissue of our respondents, in all cases, the hyper and moderate sulfomucins secretion was verified, while sialo and fucomucins usually exist in traces or are completely missing. This natural redistribution is
understandable if one bears in mind the significant protective effect of hyperviscose sulfated mucin [25, 31].

Based on our results, it is evident that a qualitative and quantitative disruption of epithelial mucins secretion is generated in colorectal adenocarcinoma. Alteration of mucins are manifested primarily by hyper and moderate sialomucins secretion in a high percentage (88%) of cases and a significant reduction in excessively acid sulfomucins which are usually present in traces, if they exist at all. These results are in agreement with the observations of many authors [26, 31–34]. Sialomucins hyperecretion with the sulfomucins reduction has been observed long ago in the adenoma-carcinoma sequence in humans [35] and in the “aberrant cryptal focus” (ACF) in rats [36]. The emergence of sialomucins hyperecretion with sulfomucins reduction in ACF, the earliest known pre-cancer lesion, goes in favor of the understanding that the aberrant secretion of mucins is an early event in colorectal carcinogenesis. There is an opinion that mucins expressed in colorectal adenocarcinoma are indicating changes in peripheral carbohydrate chains that include the reduction of O-acetyl-sialic acid and sulphating reduction, but on the other hand there is an increase in sialomucins quantity and associated structures on mucins that can act as ligands for selectins, which increases metastatic potential of carcinoma cells of colorectal carcinoma [37, 38]. Alterations in the structure and/or the amount of mucins cause changes to the barrier function of the mucus in the intestine, as well as the disturbance of signal transduction in which the mucin molecules are included. This triggers inflammatory processes in the intestinal mucosa, causing the development of inflammatory bowel disease and predisposing the development of cancer [39]. Studies have shown that chronic inflammation in the gut leads to changes in expression and glycosylation of mucins, and suggest that the aberrant and deregulated expression of mucins represent a link between cancer and inflammation. Excessive expression of cell surface mucins, is directly causing the damage of the close ties between the epithelial cells through activation of HER2 [40, 41].

Expression of leptin receptor is, in our study, verified in 77.3% of cases of colorectal adenocarcinoma. Excessive LEPR expression, in a high percentage of cases, was also found by other authors [27, 42]. Our results indicate that the expression of the LEPR is, by significant and high positive correlation coefficient, associated with the proliferative index in colorectal adenocarcinoma, which is consistent with observations of other authors [16, 18–20].

This study is for the first time shedding a light on a significant correlation between the histochemical alterations of mucins and LEPR expression in human colorectal adenocarcinoma. Our results are clearly indicating that the secretion of sialomucins is following the upward trend in the expression of leptin receptors in both non-tumoral tissue and in colorectal adenocarcinoma, with greater intensity in cases of colorectal adenocarcinoma. Therefore, excessive LEPR expression entails also the expression of weakly acid SiMs with significantly higher frequency (84%).

The increase in the expression of the LEPR is simultaneously reducing the sulfomucins secretion. It was also observed that the increase in the leptin receptor expression is increasing the secretion of neutral fucomucins. The literature has for long been consistent with the opinion that there is a positive correlation between aggressive phenotype of colorectal cancer and secretion of low-acid sialomucins [25, 26], what is confirmed also in this study. There is an opinion that, in the case of sialomucins hypersecretion and excessive expression, the adhesion between cancer cells is reducing, thus it is facilitating the infiltration of the colonic wall and early metastasis [26].

Plaisancié et al. [43] experiment had shown that the locally administered leptin is potent secretagogue of colonic mucosa and that is capable of stimulating the secretion of mucins glycoprotein in the colon, through the expression of a functional LEPR. Another study has also shown that systemic administration of high doses of leptin increases gastric mucus secretion [44]. A study that dealt with the influence of leptin on mucus secretion of sublingual salivary glands had shown that leptin prevents decrease in mucin synthesis induced by Porphyromonas gingivalis lipopolysaccharide [45].

Several studies have shown that leptin, through his receptors can activate different signal pathways for growth and proliferation of cancer cells [46–49]. It is emphasized that long isof orm of LEPRb is responsible for leptin actions and that this isof orm activates intracytoplasmic transduction pathways [47, 48]. However, in literature there is still lack of concordance regarding the way that leptin stimulates mucin secretion. However, Plaisancié et al. [43] and El Homssi et al. [50], suggest that functional LEPR acts through the activation of PKC, PI3K and MAPK signal pathways.

Conclusions

In its concluding remarks, the study had shown that an excessive expression of the leptin receptors in colorectal adenocarcinoma is associated with the mucin-histochemical abnormalities that are manifesting with hypersecretion of weakly acid sialomucins and reduction of excessively acid sulfomucins. It is necessary to conduct further researches that will shed a light on the precise signaling molecules that mediate in this leptin effect.

Conflict of interests

The authors declare that they have no conflict of interests.

References

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