# The growth differentiation factor-15 (GDF-15) can be useful in the detection of distant metastases in sera of colorectal cancer patients

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## ABSTRACT

**Purpose:** Growth differentiation factor-15 (GDF-15) protein belongs to a transforming growth factor-β family which determines the growth and differentiation of cells. In cancers, GDF-15 influences on the proliferation, differentiation, viability, migration and invasiveness of cancer cells. The aim of our study was to evaluate the expression of GDF-15 in the tissue and its levels in sera of patients with colorectal cancer.

**Materials and methods:** The level of GDF-15 in the sera of 55 patients diagnosed with colorectal cancer was determined using the ELISA method whereas expression of this protein was performed by immunohistochemical method.

**Results:** The mean value of GDF-15 levels in the sera of patients with colorectal cancer was significantly higher than in healthy control group ($p<0.001$). The expression of GDF-15 in the tissue was weak, moderate and strong in 23.6%, 15.7% and 60.7% cases, respectively. Statistical analysis showed that the expression of GDF-15 correlated with patients' age ($p<0.005$) and non-mucinous type of cancer ($p<0.001$). The high GDF-15 levels in the serum was associated with tumor size ($p<0.01$) and distant metastases ($p<0.05$).

**Conclusions:** According to our results, we postulate that the level of GDF-15 in serum can be use to assess the metastatic behavior of colorectal cancer.

**Key words:** Colorectal cancer, Growth differentiation factor-15, macrophage inhibitory cytokine-1, serum.

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## INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies among the Polish population and its morbidity rate is still growing. Due to high mortality rate it is the second cause of death due to malignancies in men and third in women [1]. Risk factors include genetic predispositions, the presence of various intestinal diseases and environmental factors [2]. The CRC incidence in Poland is estimated to increase twice in men and by 1/3 in women within the next few years [3]. Detection of early neoplastic lesions is very rare. Despite prophylactic colorectal cancer screening, these tumors often infiltrate the muscle layer into the subserous membrane of the intestinal wall, and are accompanied by lymph node involvement or distant metastases. Tumors with mucus-secreting cells have much worse prognosis [4]. CRC patients undergo surgical treatment with adjuvant therapy, which reduces complaints and decreases the risk of relapse [5]. However, the 5-year survival rate in patients with advanced lesions is low and despite widely available prophylactic examinations colorectal cancer still remains a diagnostic challenge, its early detection being too rare [6]. Therefore, detailed studies are undertaken to investigate the pathomechanism of CRC growth, including processes of proliferation, differentiation and metastasizing.

Growth/differentiation factor-15 (GDF-15), also known as macrophage inhibitory cytokine-1 (MIC-1), prostate-derived factor (PDF), placental TGF-β (PTGF-β), placental bone morphogenetic protein (PLAB) or non-steroidal autoinflammatory drug-activated gene-1 (NAG-1), belongs to the TGF-β family of proteins. Members of this family are responsible for growth and differentiation of cells during embryogenesis and in adult tissues [7,8]. This protein occurs mainly in the placenta, but is also found in the mammary, prostate glands, lung, liver, gastrointestinal tract, pancreas and kidney [9,10]. GDF-15 is involved in the maintenance of homeostasis in normal cells, but also plays a role in pathological conditions, such as cell stress, sudden injury, inflammatory reaction and neoplastic process [11,12].

GDF-15 plays pleiotropic functions in cancer cells. It originates both from cancer cells and from the extracellular matrix enclosing them [13]. The complex of GDF-15 with latent TGF-β-binding protein-1 helps maintain stability of MMP and conditions their expression in breast cancer and kidney cancer cell lines. Therefore, GDF-15 takes part in extracellular remodeling and tissue permeability, conditions metastasizing and regulates tumor growth [14]. Due to its chemopreventive activity, the protein is thought to be mainly responsible for growth inhibition and apoptosis of cancer cells [15,16]. GDF-15 overexpression in epithelial cancer cells has been shown to be associated with a drop in their viability and tumor growth inhibition [17,18]. On the other hand, this protein is responsible for proliferation of cancer cells and tumor vascularization [19,20]. It has been also found that the increase in GDF-15 expression is related to the greater metastatic capacity in cell lines of gastric and breast cancer [21]. Moreover, GDF-15 has been found to cause local and distant metastases to the liver, kidneys and bones in prostate cancer cells [22,23]. Perhaps, GDF-15, due to its significant involvement in the growth and progression of various types of cancers, will become a prognostic marker also in CRC patients. Therefore, the objective of the current study was to assess GDF-15 expression in the tissue and serum of CRC patients.

## MATERIALS AND METHODS

### Patients

The study group consisted of 55 patients (20 women and 35 men) treated surgically in the 2nd Department of General and Gastroenterological Surgery in the Medical University of Bialystok. The pathological diagnosis confirmed colorectal cancer and its stage (TNM) according to WHO classification. The adenocarcinoma type was diagnosed in 48 individuals, whereas adenocarcinoma with mucous component was identified in 7 individuals. The investigated tumors were classified as moderately differentiated (G2) in 48 patients, and poorly differentiated (G3) in 7 patients. The pT1 tumor was observed in 1 case, pT2 in 3 patients, pT3 in 49 patients and pT4 in 2 patients. At the time of the diagnosis, metastases to local lymph nodes were observed in 29 out of 55 cases, whereas the presence of metastases to distant organs was noted in 25 out of 55 of the studied cases. The control group consisted of 22 healthy volunteers (12 males and 10 females aged 45-75).

Study material consisted of serum samples obtained from both the blood of the patients with colorectal carcinoma collected prior to the surgery and the healthy controls. Blood serum was stored at $-80^0$C immediately after centrifugation until the assay was performed.

The study received the approval of the local Bioethics Committee. All the participants (study group and controls) signed informed consent forms prior to the examination.

### Enzyme-linked immunosorbent assay (ELISA)

GDF-15 level was determined by means of the enzyme-linked immunosorbent assay (ELISA) method. Serum samples were prepared according to the manufacturer's instructions. Prior to the assay, the samples were 100-fold diluted with Calibrator Diluents. A monoclonal antibody specific for

GDF-15 had been pre-coated onto a microplate and incubated with serum samples. After the first washing, an enzyme-linked polyclonal antibody specific for GDF-15 was added to the wells. Following the second wash, a substrate solution was added. Next, the color development was stopped. The reaction measurement was based on the intensity of the sample color. All the specimens were assayed twice. No statistically significant differences between the measurements were found. The minimum detectable dose (MDD) of GDF-15 ranged from 0.0 to 4.4 pg/mL. Mean MDD was 2.0 pg/mL. The serum GDF-15 level was expressed in picogram per mililiter (pg/mL).

**Immunohistochemistry**

The immunohistochemistry method was carried out 38 of 55 patients with colorectal cancer. Formalin-fixed and paraffin-embedded tissue specimens were cut on a microtome into 4 µm sections. The sections were deparaffinized in xylenes and hydrated in alcohols. To visualize the antigens of GDF-15 protein, the sections were heated in a microwave oven for 20 min in a citrate buffer (pH=6.0). Then, they were incubated with 3% hydrogen peroxide solution for 5 min in order to block endogenous peroxidase. Next, incubation was performed with polyclonal rabbit antibody against human GDF-15 (Acris Antibodies, No AP07912PU-N, dilution 1:50) and incubated at $4^0$C temperature over night. The reaction was carried out using Novocastra Novolink Polymer Detection System (NCL- Novocastra, Leica Biosystems). A colour reaction for peroxidase was developed with chromogene DAB. The expression of investigated protein was found in cytoplasm of the tumour cells and determined using the semiquantitative method. Expression was defined in relation to the intensity of staining (0 - absent, 1 - weak, 2 - moderate, 3 - strong) and the percentage of positive tumour cells (0-lack of reaction, <10%-weak, 10-50%-moderate, > 50%- strong).

**Statistical analysis**

Statistical analysis was conducted based on the STATISTICA 10.0 program. In order to compare the two groups, the U Mann-Whitney test was used. Correlations between the parameters were calculated by the Spearman's correlation coefficient tests. The value of $p<0.05$ was considered statistically significant. The missing data was removed in pairs. The analysis of ROC curve was performed using MedCalc statistical software.

## RESULTS

**The levels of GDF-15 in the sera of patients with colorectal cancer and healthy controls**

The mean serum level of GDF-15 was 2307.23 pg/mL (range 613.55-6300.00 pg/mL) and was statistically significantly higher as compared to healthy volunteers (1305.62 pg/mL; range 274.48-5285.24 pg/mL) (Table 1).

**Table 1.** The level of GDF-15 protein in the serum of patients with colorectal cancer and healthy controls

| | | GDF-15 (pg/mL) | | | | |
|---|---|---|---|---|---|---|
| | **N** | **Mean** | **Median** | **SD** | **Range** | **P- value** |
| **Normal** | 22 | 1305.62 | 832.07 | 1175.12 | 274.48-5285.24 | <0.01 |
| **Tumor** | 55 | 2307.23 | 1638.31 | 1623.58 | 613.55-6300.00 | |

Mann-Whitney U-test. Missing data is removed in pairs

The relationship between diagnostic sensitivity and specificity of GDF-15 levels was assessed by ROC curve analysis. The discrimination between colorectal cancer patients (n=55) and healthy controls (n=22) showed an area under ROC curve for GDF-15 was 0.76 (Standard Error[a], 0.066; p<0.001) (Figure 1a). A ROC curve for GDF-15 as a discriminator between colorectal cancer patients with distant metastases (n=25) and cancer subjects without distant metastases (n=30) showed an area under ROC curve to be 0.70 (Standard Error[a], 0.074; p<0.0074). (Figure 1b).

No statistical significance was noted between the serum level of GDF-15 and its expression in the tissue of CRC patients.

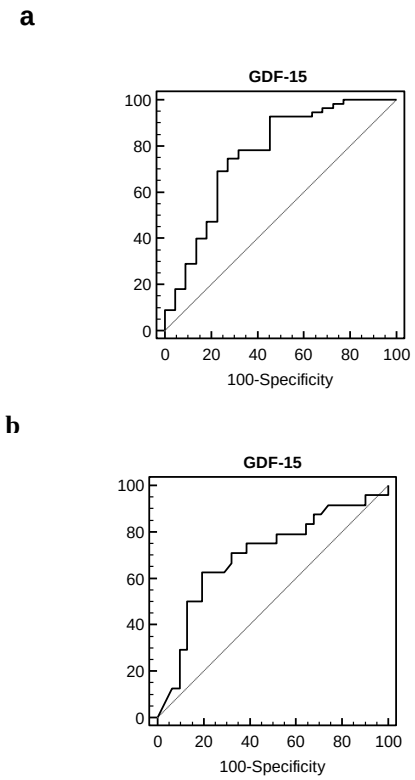



**Figure 1.** ROC curve analysis for GDF-15: (a) as a discrimator between colorectal cancer subjects healthy controls (AUC-0.76; p<0.001), (b) colorectal patients with distant metastasis via carcinoma subjects without distant metastases (AUC-0.70; p=0.0074).

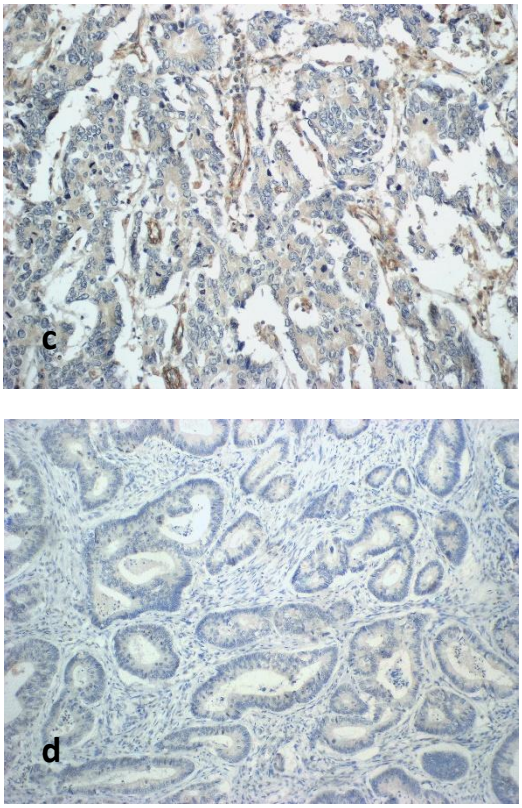
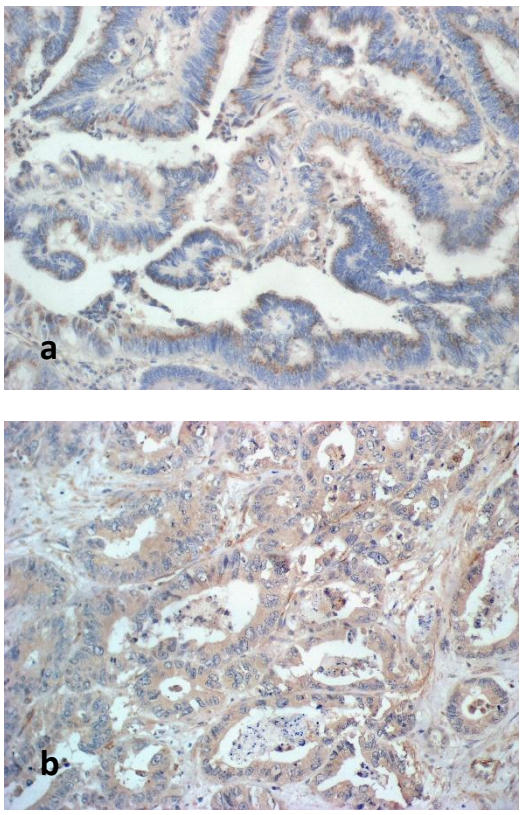



**Figure 2.** Immunohistochemical expression of GDF-15 in colorectal cancer tissue. The positive reactions were strong (a,b), moderate (c) and weak (d) in cytoplasm of cancer cells. Original magnification 200x for a,b,c; 100x for d.




**Correlations between the levels of GDF-15 in the sera of patients with CRC and chosen clinicopathological parameters**

The serum level of GDF-15 was found to correlate with tumor size (p<0.05). In patients with <5cm tumor, the serum level of the protein was significantly higher. Moreover, increased level of GDF-15 was noted to show a positive correlation with the presence of distant metastases in CRC patients (p<0.05). No other correlations were observed of serum GDF-15 with such parameters as age, sex, location, histological type, malignancy grade, tumor stage, Duke's classification and local lymph node involvement. Details are shown in (Table 2).

**The correlation between serum GDF-15 concentrations and morphological blood parameters in patients with CRC**

The serum level of GDF-15 in CRC patients showed a negative correlation with the level of sodium (p<0.01) and positive correlation with potassium (p<0.01). The level of sodium was decreasing and that of potassium increasing along with a rise in serum GDF-15.

**Table 2.** The correlations between the GDF-15 levels in serum and clinical-pathological parameters in patients with colorectal cancer

| **Parameter** | | **GDF-15 (pg/mL)** | | | | |
|---|---|---|---|---|---|---|
| | | **N** | **Median** | **Range** | **Coefficient** | **P-value** |
| **Age** | ≤60 | 19 | 2202.25 | 638.67-6300 | 0.042 | NS |
| | >60 | 36 | 2362.64 | 613.55-6300 | | |
| **Gender** | Male | 35 | 2186.85 | 638.67-6300 | –0.099 | NS |
| | Female | 20 | 2517.89 | 613.55-6300 | | |
| **Localization** | Colon | 33 | 2360.78 | 681.37-6300 | 0.002 | NS |
| | Rectum | 22 | 2370.04 | 638.67-6300 | | |
| **Adenocarcinoma type** | Nonmucinous | 48 | 2281.40 | 638.67-6300 | 0.129 | NS |
| | Mucinous | 7 | 2898.82 | 724.07-6300 | | |
| **Grade of malignancies** | 2 | 48 | 2402.36 | 638.67-6300 | -0.106 | NS |
| | 3 | 7 | 1815.13 | 724.07-2952 | | |
| **Tumor size** | <5cm | 30 | 2203.93 | 638.67-6300 | 0.367 | **<0.01** |
| | >5cm | 25 | 2553.19 | 724.07-6300 | | |
| **pT stage** | 1 | 1 | 1638.31 | - | 0.195 | NS |
| | 2 | 3 | 1615.70 | 638.67-2592 | | |
| | 3 | 49 | 2311.88 | 681.37-6300 | | |
| | 4 | 2 | 4138.69 | 1977.38-6300 | | |
| **Duke stage** | A | 2 | 1138.49 | 681.37-1638 | 0.228 | NS |
| | B | 16 | 2223.37 | 638.30-6300 | | |
| | C | 14 | 1568.82 | 1022.95-3665 | | |
| | D | 23 | 2854.71 | 613.55-6300 | | |
| **Lymph node metastasis** | Absent | 24 | 2485.48 | 638.67-6300 | 0.462 | NS |
| | Present | 29 | 2233.74 | 681.37-6300 | | |
| **Distant metastasis** | Absent | 30 | 1889.23 | 638.67-6300 | 0.294 | **<0.05** |
| | Present | 25 | 2848.61 | 613.55-6300 | | |
| **IHC expression of GDF-15 in cancer cells** | Weak<br>Moderate<br>Strong | 9<br>6<br>23 | 2828.42<br>1972.86<br>2011.60 | 1103.32-6300<br>638.67-4664.84<br>681.37-6300 | -0.201 | NS |

Spearman's correlation coefficient test. Missing data is removed in pairs. NS- non significant

Statistical analysis revealed a positive correlation between the serum level of the protein and aspartate transaminase ($p<0.01$). Moreover, when the level of GDF-15 increased, glucose level in the blood decreased ($p<0.05$) (Table 3).

**The expression of GDF-15 in colorectal cancer tissue and its association with selected clinicopathological parameters**

GDF-15 expression was observed in the cytoplasm of cancer cells. It was weak, moderate or strong in 23.6% (9/38), 15.7% (6/38) and 60.7% (23/38), respectively (Figure 2). In statistical analysis, the expression of GDF-15 correlated with patients' age ($p<0.005$). GDF-15 expression increased in patients over 60. A rise in the protein expression was also noted in the tissue of patients with mucinous type cancer ($p<0.001$) (Table 4).

**Table 3.** The correlations between the level of GDF-15 protein in serum and morphological blood parameters

| **Parameter** | **GDF-15** | | |
|---|---|---|---|
| | **N** | **Coefficient** | **P- value** |
| **Red Blood Cell Count** | 29 | -0.085 | NS |
| **White Blood Cell Count** | 29 | 0.200 | NS |
| **PLT** | 29 | 0.238 | NS |
| **Hematocrit** | 29 | –0.121 | NS |
| **Hemoglobin** | 29 | –0.161 | NS |
| **Sodium** | 29 | –0.497 | **<0.01** |
| **Potassium** | 29 | 0.468 | **<0.01** |
| **Prothrombin time** | 29 | -0.076 | NS |
| **Wsk protr** | 28 | 0.013 | NS |
| **Total proteins** | 20 | 0.370 | NS |
| **Aspartate Transaminase** | 22 | 0.621 | **<0.01** |
| **Alanine Transaminase** | 22 | 0.094 | NS |
| **Glucose** | 13 | –0.552 | **<0.05** |
| **Urea** | 26 | –0.042 | NS |
| **Creatinine** | 23 | –0.192 | NS |

Spearman's correlation coefficient test. Missing data is removed in pairs. NS- non significant.

**Table 4.** The correlations between the GDF-15 expression in tumor tissue and clinical-pathological parameters in patients with colorectal cancer

| **Parameter** | | **GDF-15 expression (Number of cases/ percentage)** | | | |
|---|---|---|---|---|---|
| | | **Absent/weak** | **Moderate** | **Strong** | ***p* value** |
| **Age** | ≤60 | 1 (2.6%) | 5 (13.1%) | 7 (18.4%) | <0.005 |
| | >60 | 8 (21%) | 1 (2.6%) | 16 (41.3%) | |
| **Gender** | Male | 3 (7.8%) | 2 (5.2%) | 10 (27%) | NS |
| | Female | 6 (15.7%) | 3 (7.8%) | 13 (36.5%) | |
| **Localization** | Colon | 4 (10.5%) | 2 (5.2%) | 18 (46.7%) | NS |
| | Rectum | 5 (13.1%) | 4 (10.5%) | 5 (13.1%) | |
| **Adenocarcinoma type** | Nonmucinous | 7 (18.4%) | 6 (15.7%) | 22 (58.1%) | <0.001 |
| | Mucinous | 2 (5.2%) | 0 (0%) | 1 (2.6%) | |
| **Grade of malignancies** | 2 | 8 (21%) | 6 (15.7%) | 21 (55.2%) | NS |
| | 3 | 0 (0%) | 0 (0%) | 2 (5.2%) | |
| **Tumor size** | <5cm | 3 (7.8%) | 4 (10.5%) | 13 (36.5%) | NS |
| | >5cm | 5 (13.3%) | 2 (5.2%) | 9 (26.8%) | |
| **pT stage** | 1 | 0 (0%) | 0 (0%) | 1 (2.6%) | NS |
| | 2 | 0 (0%) | 1 (2.6%) | 2 (5.2%) | |
| | 3 | 8 (21%) | 15 (39.4%) | 20 (52.6%) | |
| **Duke stage** | A | 0 (0%) | 1(2.6%) | 1 (2.6%) | NS |
| | B | 2 (5.2%) | 2 (5.2%) | 7 (18.4%) | |
| | C | 2 (5.2%) | 2 (5.2%) | 6 (15.7%) | |
| | D | 4 (10.5%) | 1 (2.6%) | 9 (26.8%) | |
| **Lymph node metastasis** | Absent | 4 (10.5%) | 3 (7.8%) | 9 (26.8%) | NS |
| | Present | 5 (13.1%) | 3 (7.8%) | 13 (36.5%) | |
| **Distant metastasis** | Absent | 4 (10.5%) | 5 (13.1%) | 14 (33.9%) | NS |
| | Present | 5 (13.1%) | 1 (2.6%) | 9 (26.8%) | |

Spearman's correlation coefficient test. Missing data is removed in pairs. NS- non significant

## DISCUSSION

According to recent literature data, GDF-15 protein is present in various types of cancer cells, and its expression and level in body fluids increase as compared to normal control [12,24]. We found markedly higher serum level of GDF-15 in CRC patients than in healthy volunteers. In turn, Brown et al. [25] noted a rise in serum level of GDF-15 both in patients with adenomic polyposis and CRC as compared to healthy subjects. Later, this author also confirmed the rise in patients with polyps in comparison with polyp-free subjects [26]. Research conducted to assess the serum level of GDF-15 in CRC patients showed a gradual increase in the subsequent stages of tumor advancement [27].

Colorectal cancer growth is a complex process, closely associated with secretion of numerous factors of growth, angiogenesis and stromal enzymes [28,29]. It has been proven that GDF-15 plays a double role in the neoplastic process [30]. This protein can decrease viability of HCT-116 colon cancer cell line in vitro and inhibit tumor growth *in vivo* [17,18]. In turn, observations of gastric cancer cell lines revealed that GDF-15 conditioned substantial invasiveness of these cells *in vitro* [31]. In our study, the serum level of GDF-15 was found to increase along with a growing tumor diameter. This seems to confirm the protein involvement in tumor progression and its increased level may indicate further expansion of cancer. However, our analysis did not show any statistical significance between serum GDF-15 and TNM. The correlation was confirmed in a study conducted by Brown et al. [25], in which the serum level increased along with TNM stage. Similar findings have been also reported in the plasma of CRC patients [27].

The likelihood of metastasizing increases with rapid tumor growth and its further spread in the stroma. Serum level of GDF-15 has been proven to correlate with the presence of distant metastases in various types of cancers [22,23]. We also observed the correlation between serum level of GDF-15 with CRC and the presence of distant metastases. Our observations remain in accordance with the findings reported by Brown et al. [25], showing the protein increase in CRC patients with metastases as compared to non-metastatic carcinoma. The risk of metastases in these patients also increased when they had D Alle of GDF-15. Moreover, these authors have shown that the assessment of serum GDF-15 together with TNM stage, lymph node involvement and A allele of GDF-15 can be an independent predictive factor of metastases in CRC patients [25]. Xue et al. [32] confirmed the correlation between serum GDF-15 in CRC patients and lymph node involvement. Moreover, the assessment of GDF-15 and other available diagnostic markers has shown high specificity [22-23,25]. However, the analysis of ROC curve confirmed that GDF-15 shows pretty high specificity and sensitivity as a prognostic parameter in CRC patients. As revealed by the analysis of the field under the ROC curve, serum GDF-15 in CRC patients may allow detection of metastases using simple ELISA test. Our results concerning serum level of GDF-15 in CRC patients confirm that this protein is involved in CRC, determining tumor mass growth in the primary focus and the ability of cancer cells to metastasize.

Moreover, in our study the serum level of the protein was found to correlate with such blood parameters as the level of potassium, sodium, glucose and asparinian transferase. Perhaps, the stimulation of growth factors in neoformation, including GDF-15, disturbs the whole organism and thus changes the level of body fluid parameters. However, no reports have confirmed our observations.

The presence of GDF-15 has also been confirmed by research concerning its expression in the tissues of patients with CRC and colon cancer cell lines [17,18,33-35]. Proapoptotic properties have been indicated in human colorectal cell lines and antitumorogenic activity in the development of colorectal cancer in the Gdf15 transgenic overexpression mouse model [18,21]. The assessment of GDF-15 gene expression has revealed that this protein is up-regulated in colonic adenomas as compared to normal glands [36]. Our immunohistochemical assessment of the protein showed moderate or strong cytoplasmic expression in most CRC patients. Also, Wallin et al. [27] have noted increased expression of GDF-15 in cancer cells as compared to the normal intestinal epithelium. The above findings seem to be consistent with the observations made by Brown et al. [25], who found a strong protein reaction in the cytoplasm of tumor cells in 90% of cases. In our study, the statistical analysis revealed a correlation between the elevated expression of GDF-15 and age and non-mucinous type of tumor. In turn, Wallin et al. [27] noted a relationship between the increased protein expression and vascular invasion. Moreover, they also observed increased expression of GDF-15 in the primary tumor as compared to metastases in stage III patients. As revealed by immunohistochemical evaluation of the increased expression of GDF-15, the protein takes part in colorectal carcinogenesis and probably conditions its spread.

## CONCLUSIONS

Concluding, GDF-15 plays a key role in the development of colorectal cancer, which seems

to be confirmed by the results of our immune-histochemical study and assessment of its serum level in CRC patients. Moreover, determination of serum GDF-15 is strictly related to distant metastases, which may allow application of this protein as a prognostic metastatic marker in colorectal cancer patients. However, broader studies are required, based on a larger group of patients, to prove a definite diagnostic value of serum GDF-15 in the clinical assessment of CRC patients.

## Conflicts of interest

None declared.

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