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# Evaluation of the associations between GDF-15, OPG, and IL-15 levels in CRC: preliminary results

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## ABSTRACT

**Introduction:** Osteoprotegerin (OPG), Interleukin-15 (IL-15), and Growth/Differentiation Factor-15 (GDF-15) are all proven to take part in the processes associated with colorectal carcinoma (CRC) including tissue remodeling, inflammation modulation, and metastasis.

**Aim of the study:** To investigate the concentrations of GDF-15, OPG and IL-15 in tumor and margin tissues of CRC in relation to clinicopathological features of patients.

**Material and methods:** The study used 50 specimens of tumor and tumor-free margin tissues obtained from CRC patients. To determine the GDF-15, OPG and IL-15 concentrations commercially available enzyme-linked immunosorbent assay (ELISA) kits were used.

**Results:** Concentrations of GDF-15, OPG and IL-15 were significantly higher in the tumor in comparison with the margin. The tumor levels of GDF-15 were positively associated with those of OPG and IL-15, while tumor levels of OPG correlated positively with those of IL-15. There was no association between levels of investigated molecules and clinical features of patients.

**Conclusions:** The levels of GDF-15, OPG and IL-15 are elevated in patients suffering from CRC. More studies are needed to establish a specific role of these cytokines in the development, tumor growth, progression, and prognosis of CRC and to examine their role as possible CRC treatment candidates.

**Key words:** colorectal cancer (CRC), growth/differentiation factor-15 (GDF-15), osteoprotegerin (OPG), interleukin-15 (IL-15)

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## Introduction

Colorectal cancer (CRC) is the third most common cancer diagnosed worldwide accompanied by high morbidity and mortality. Amount of newly diagnosed cases is still rising and reached 2 million in 2020 [1]. According to epidemiological prognosis by 2030 the incidence will increase by even 60% [2].The number of new cases of CRC varies significantly between the regions — the highest one is observed in Europe, North America, Australia, and New Zealand, while much lower incidence is reported in Africa and South-Central Asia countries. This observation seems to be associated with different dietary patterns, environmental factors, and lower socioeconomic status [3]. Among all the CRC cases, 70% are sporadic, 20% are associated with genetic susceptibility to CRC, and only 5% are classified as inherited (including Lynch Syndrome and FAP- familiar adenomatous polyposis) [4]. Although the awareness of the prevalence, risk factors, effective screening methods, and therapeutic options improved remarkably, CRC is still responsible for about 900,000 deaths per year [5].

Numerous factors have been proven to increase the risk of CRC development including inflammatory bowel disease, CRC in a first-degree relative, high BMI, low physical activity, smoking, high red meat consumption, and low intake of fruits and vegetables [2].

Due to the rapid development of immunooncology and targeted therapy accomplished in recent years, the

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associations between cytokines involved in immune response and other molecules participating in epithelial to mesenchymal transition (EMT), tissue remodeling, and angiogenesis are extensively investigated.

Growth/differentiation factor-15 (GDF-15), also known as macrophage inhibitory cytokine-1, is a member of TGF- βsuperfamily. It is a cytokine expressed in low concentrations in most organs, it is also abundant in placenta. GDF-15 is also upregulated under stress conditions, such as liver, kidney, heart, and lung injuries [6]. The cytokine might maintain cell and tissue homeostasis. GDF-15 might work as a serum marker in patients with metastatic CRC [7]. Its potential as a biomarker in the prognosis of cancer is still under investigation as it might work as a potential target in cancer immunotherapy [6].

Osteoprotegerin (OPG), also known as osteoclastogenesis inhibitory factor (OCIF), is a cytokine receptor of the tumor necrosis factor (TNF) receptor superfamily encoded by the TNFRSF11B gene. It is expressed in many tissues, such as osteoblasts, heart, spleen, liver, kidney, and bone marrow [8]. It is proven to have a role in different processes, one of them being tumor growth and metastasis. OPG is a part of the RANKL/RANK/OPG pathway which is associated with bone formation and resorption. OPG works as an intrinsic inhibitor of the receptor activator of RANKL allowing it to bind with its membrane-bound receptor RANK [9]. The same pathway also plays an important role in pathological angiogenesis and inflammation. Additionally, it takes part in cell survival. A connection has been proven between angiogenesis and osteogenesis by showing that there is a crosstalk between endothelial cells and osteoblasts during osteogenesis [10]. OPG also inhibits apoptosis by neutralising the function of TNF-related apoptosis-inducing ligand, which is why the cytokine might be associated with aggressiveness of different types of cancer, including CRC [11].

Interleukin-15 is a cytokine structurally similar to IL-2, that is secreted by mononuclear phagocytes and induces the proliferation of NK cells in viral infections. IL-15 is expressed by monocytes, macrophages, dendritic cells, fibroblasts, myocyte cells, keratinocytes, and nerve cells. One of its functions is to regulate the activation and proliferation of T cells. A balance between IL-15 and IL-2 is shown to control the number of CD8+ memory cells. It is suggested that IL-15 might prevent apoptosis by increasing the expression of apoptosis inhibitor BCL2L1/BCL-x(L). IL-15 also regulates tissue repair and modulates inflammation. The role of IL-15 in CRC is still under investigation [12].

In this study, we aimed to investigate the correlations between levels of GDF-15, OPG and IL-15 taking into consideration interplay between tumor and tumor-free margin tissue of CRC.

## Material and methods

The study involved 50 samples of colorectal tumor tissue and surgical margin tissue obtained during surgeries in the 1st Specialistic Hospital in Bytom (Research Ethics Committee PCN/0022/KB1/42/VI/14/16/18/19/20). Inclusion criteria included the confirmation of colorectal adenocarcinoma and surgical "tumor-free" margin tissue confirmed in histological examination, patients' age > 18 years, and signed written consent. Patients with cancer other than adenocarcinoma, tumor infiltration of margin, age < 18 years, lack of signed consent, and with history of chemo- or radio-therapy were excluded. The tumor stage was classified using the TNM scale and grading. Research sample characteristics are provided in Table 1.

### Preparation of samples for the evaluation of GDF-15, OPG and IL-15

Fragments of the tumor tissue and surgical tissue margin were weighted and homogenized using a PRO 200 homogenizer (PRO Scientific Inc., Oxford, CT, USA) at 10,000 rpm in nine volumes of phosphate-buffered saline (BIOMED, Lublin, 06, Poland). The suspensions were sonicated with an ultrasonic cell disrupter (UP 100H, Hielscher Ultrasonics GmbH, Teltow, BB, Germany). Subsequently, the homogenates were centrifuged at 12,000 rpm for 5 min at 4°C. The total protein level was determined using a Universal Microplate Spectrophotometer (μQUANT, Biotek Inc., Winooski, VT, USA).

To measure the levels of the studied proteins, an enzyme-linked immunosorbent assay (ELISA) was used, following the manufacturer's instructions. GDF-15 levels were evaluated by a human GDF-15/MIC-1 ELISA kit (BioVendor, Czech Republic) with a sensitivity of 16 pg/mL. Levels of OPG were assayed using human Osteoprotegerin ELISA Kit (Biovendor, Czech Republic) with a sensitivity 0.03 pg/mL. Levels of IL-15 were determined using human IL-15 Quantikine ELISA kit (R&D, USA) with sensitivity 2 pg/mL. The absorbance of the samples was assessed using a Universal Microplate Spectrophotometer (μQUANT, Biotek Inc., Winooski, VT, USA). The measurement was performed at a wavelength of 450 nm. The obtained results were calculated to the corresponding total protein level and presented as ng/mg of protein.

### Statistical analyses

Data distribution was assessed using Shapiro-Wilk test. The log transformation of the levels of the examined molecules was performed to provide a better fit to the Gaussian distribution. Data are presented as mean ± SD for variables with normal distribution and as

**Table 1.** Characteristic of the patients

| | Female | Male | Total |
|---|---|---|---|
| | 22 (44%) | 28 (56%) | 50 (100%) |
| Age | 63.05 ± 11.27 | 63.81 ± 8.61 | 63.47 ± 9.75 |
| **T parameter** | | | |
| T1 | 0 (0%) | 0 (0%) | 0 (0%) |
| T2 | 7 (31.82%) | 5 (17.86%) | 12 (24.00%) |
| T3 | 12 (54.55%) | 14 (50.00%) | 26 (52.00%) |
| T4 | 3 (13.64%) | 9 (32.14%) | 12 (24.00%) |
| **N parameter** | | | |
| N0 | 9 (40.91%) | 12 (42.86%) | 21 (42.00%) |
| N1 | 10 (45.45%) | 9 (32.14%) | 19 (38.00%) |
| N2 | 3 (13.64%) | 7 (25.00%) | 10 (20.00%) |
| **M parameter** | | | |
| M0 | 18 (81.82%) | 19 (67.86%) | 37 (74.00%) |
| M1 | 4 (18.18%) | 9 (32.14%) | 13 (26.00%) |
| **TNM stage** | | | |
| I | 6 (27.27%) | 4 (14.29%) | 10 (20.00%) |
| II | 3 (13.64%) | 7 (25.00%) | 10 (20.00%) |
| III | 9 (40.91%) | 8 (28.57%) | 17 (34.00%) |
| IV | 4 (18.18%) | 9 (32.14%) | 13 (26.00%) |
| **Grading** | | | |
| G1 | 1 (4.55%) | 0 (0%) | 1 (2.00%) |
| G2 | 20 (90.91%) | 28 (100%) | 48 (96.00%) |
| G3 | 1 (4.55%) | 0 (0%) | 1 (2.00%) |

**Table 2.** Levels of GDF-15, OPG and IL-15 proteins in tumor and margin presented as log-transformed ng/mg of protein. Paired T-student's test

| | Tumor | | Margin | | P-value |
|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | |
| log GDF-15 | 0.29 | 0.43 | –0.63 | 0.44 | < 0.0001 |
| log OPG | –0.05 | 0.30 | –0.66 | 0.36 | < 0.0001 |
| log IL-15 | –2.49 | 0.32 | –2.76 | 0.37 | < 0.0001 |

median with interquartile range for variables with nonnormal distribution. To compare the tumor and margin levels, paired Student's t-test was used. Independent variables were also compared using Student's t-test. To assess the relationships between the variables with normal distributions Pearson's coefficient was used. For those with nonnormal distributions, Tau-Kendall's tau rank correlation coefficient was used. P values < 0.05 were considered significant. The statistical analysis was performed using STATISTICA 13software (Statsoft) and the ggplot2-R package dedicated to data visualization in RStudio software (Integrated Development for R. RStudio, PBC, Boston, MA, USA).

## Results

We observed significantly higher levels of all the studied proteins: GDF-15, OPG, and IL-15 in the tumor in comparison with the margin (Tab. 2, Fig. 1).

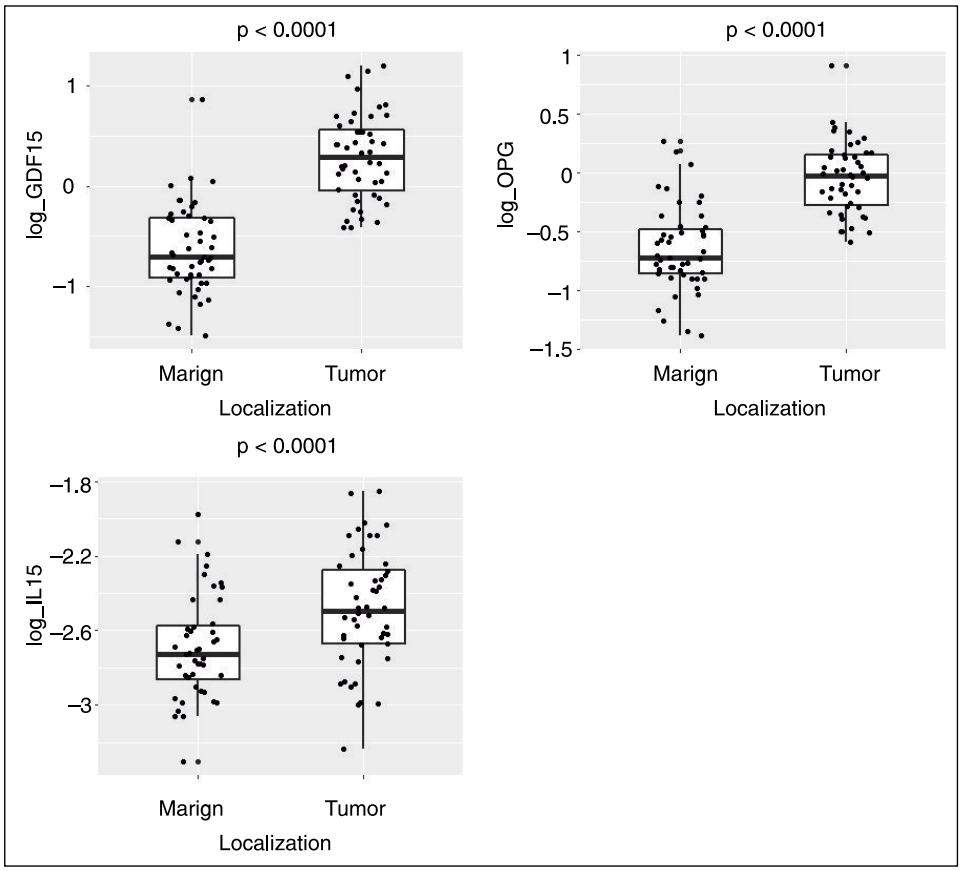


**Figure 1.** Box-plot- levels of GDF-15, OPG and IL-15 molecules in tumor and tissue margins; protein levels are presented as log-transformed values as ng/mg. Paired T-student's test

**Table 3.** Correlations between the GDF-15, OPG and IL-15

| Pair of variables | R | P-value |
|---|---|---|
| Tumor log GDF-15 and tumor log OPG | 0.64 | < 0.0001 |
| Tumor log GDF-15 and tumor log IL-15 | 0.48 | 0.001 |
| Tumor log OPG and tumor log IL-15 | 0.55 | < 0.0001 |
| Tumor log GDF-15 and margin log GDF-15 | 0.4 | 0.006 |

R — Pearson's correlation coefficient

The tumor levels of GDF-15 were positively associated with tumor levels of OPG and IL-15 (Tab. 3, Fig. 2). The positive relationship was also found between tumor levels of OPG and IL-15 (Tab. 3, Fig. 2). Additionally, tumor concentrations of GDF-15 correlated positively with the corresponding margin concentrations (Tab. 3, Fig. 2). We did not found any association between the levels of studied molecules and clinicopathological features of patients.

## Discussion

### Comparison of concentration of studied proteins between margin and tumor tissue

GDF-15 levels are increased in various pathological conditions, such as inflammation and injuries. They are also up-regulated in digestive system tumours including CRC, gastrointestinal cancer, pancreatic cancer, esophageal carcinoma, and liver cancer [13].

In our study, the concentrations of GDF-15 were higher in tumour tissue than in margin tissue. The cytokine is said to be a possible diagnostic and prognostic biomarker in CRC and its role has been described as promising in the diagnosis of CRC in a meta-analysis created in 2016 [7]. It however still needs to be investigated.

Osteoprotegerin was found to be able to bind to and inhibit the activity of TNF-related apoptosis-inducing ligand (TRAIL) [14]. TRAIL is produced by monocytes in tumours in response to interferon-β and -β and me-

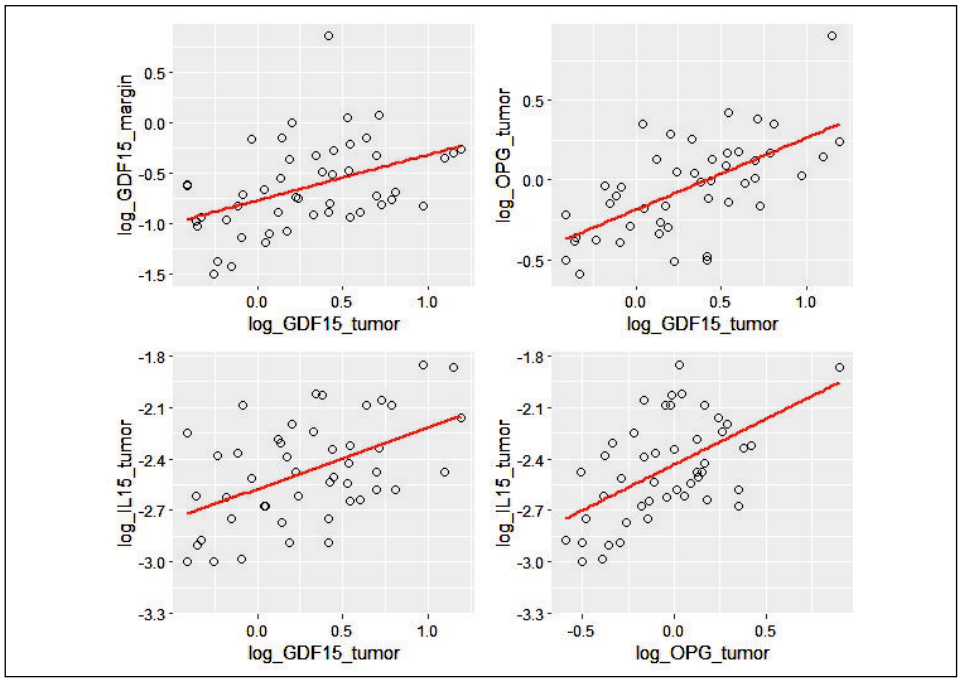


**Figure 2.** Correlations between tumor and margin levels of GDF-15 ($r = 0.4$, $p = 0.006$), tumor levels of GDF-15 and OPG ($r = 0.64$, $p < 0.0001$), tumor levels of GDF-15 and IL-15 ($r = 0.45$, $p = 0.001$), tumor levels of OPG and IL-15 ($r = 0.55$, $p < 0.0001$)

diates acquired tumour cell killing activity. Secretion of OPG by tumour cells could be a way to inhibit the apoptosis signaling cascade and would give tumour cells a higher chance to survive. That suggests that OPG might have a role in survival of CRC cells, but it is yet to be established [14]. OPG serum levels may work as a prognostic marker in several cancer types. OPG has also been proven to positively regulate tumor microvessel formation [15, 16].

In our study, the concentrations of osteoprotegerin in tumour were higher than the concentrations of the OPG in margin tissue. Different studies have also reported an OPG expression in CRC, two of them finding OPG mRNA and proteins in CRC cell lines, stating that OPG might be involved in tumorigenesis and the progression of colon cancer [17, 18]. In one of the previously stated studies, RANKL (which selectively binds OPG) was added to cell cultures along with recombinant TRAIL leading to a significant increase in apoptosis [18]. The same study also demonstrated that OPG is strongly up-regulated by inflammatory cytokines such as TNF-β and IL-1, which are known to be present in CRC tissues. That leads to a conclusion that OPG secretion can be up-regulated by the production of pro-inflammatory cytokines in tumour cells as a way to inhibit apoptosis and thus increase their chance of survival. This process is however yet to be further examined.

IL-15 plays a dual role as it induces both tumour cell growth and antitumor immunity. The role of this cytokine in inflammation-induced cancers, such as CRC, remains unclear.

In our study, the concentrations of IL-15 were higher in tumour than in margin tissue. It might be associated with the fact that IL-15 has antitumour effects on cancer tissues, as well as with the fact that it might promote tumor growth, invasion, and metastasis, preventing apoptosis of tumour cells. Its role in CRC pathogenesis might depend on the balance between pro- and anti-inflammatory activities. One study has found a connection between single nucleotide polimorphism (SNPs) from several cytokines, including IL-15, and colon cancer risk [8]. Interestingly, a different study has shown promise for treating CRC with an experimental new drug the IL-15 plasmid and DMA complex. That leads to a conclusion that the role of IL-15 in CRC needs to be thoroughly investigated [19].

## Correlations between investigated proteins

According to the results of our study, tumour levels of GDF-15 positively correlated with tumour levels of OPG. It might be a result of the fact that OPG may be an inhibitor of apoptosis in tumour cells [20], including CRC cells, and levels of GDF-15 are elevated under stress conditions, one of them being a tumour.

In the present study, there was a positive correlation between tumour levels of GDF-15 and tumour levels of IL-15. The result is probably as they are both elevated in different kinds of tumours. They can be treated as prognostic biomarkers of CRC after further investigation.

There was also a positive correlation between tumour levels of OPG and tumour levels of IL-15. The results may have an association with the fact that OPG might play a protumerogenic role in CRC and IL-15 might either have an antitumour effect on cancer tissues or promote tumor growth, invasion, and metastasis. As it was stated before, the role of told cytokines is still under investigation.

### Lack of correlation between our markers and clinicopathological features of patients

In our study, no correlation between the levels of IL-15, OPG, GDF-15 and clinicopathological features of patients was observed. The reason for this may be the small number of patients participating in the study.

However, another study has shown that the mRNA expression of OPG in CRC was significantly higher in patients with distant metastases than those without them, showing an association between tumour levels of the cytokine and clinicopathological features of patients [11]. A different study has also stated that OPG levels above the median concentration of 51ng/mL correlated with a poorer life expectancy of CRC patients [21]. The life expectancy was even poorer in patients with high levels of both OPG and CEA (Carcinoembryonic antigen) which indicated that they may have addictive prognostic significance [21].

Some studies have also stated that IL-15 may be a promising treatment candidate in CRC, used in combination with Cetuximab [22]. IL-15 induces antitumour immunity and suppresses colitis-associated colon carcinogenesis [23].

GDF-15 has been found to be a negative prognostic marker in CRC in different studies. One of them has proven that moderate to high intensity of GDF-15 immunostaining in CRC patients increased reaccurance rate compared to patients with no or low intensity in all stages [24]. The time to reaccurance as well as overall survival were reduced in patients with high plasma levels of GDF-15 [24]. A different study has shown that serum levels of GDF-15 correlated with the extent of liver involvement and patients with higher GDF-15 levels had a significantly worse outcome than patients with lower levels [25]. It has been demonstrated in one of the studies that GDF-15 may promote CRC metastasis through activating epithelial-mesenchymal transition [26].

Whereas our study showed no significant correlation between levels of OPG, IL-15, GDF-15, and clinicopathological features of CRC patients, different studies show an increased expression of GDF-15 and OPG in advanced stages of CRC. This may be associated with the possible promotion of metastasis of colorectal carcinoma cells. Higher levels of these cytokines are also associated with poorer prognosis for CRC patients. That leads to a conclusion that all of researched cytokines could possibly work as prognostic factors as well as biomarkers in CRC occurrence and progression. However, they still need to be thoroughly investigated.

## Conclusions

The expression of GDF-15, OPG and IL-15 is upregulated in CRC. GDF-15 may be involved in processes associated with immune response, tumor growth and metastasis. IL-15 can work dually and its role in CRC needs further investigation. Potential of targeting GDF-15, OPG, IL-15 in CRC therapies will need to be determined in further studies.

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