ORIGINAL ARTICLE

Preoperative serum chemokine (C-C motif) ligand 2 levels and prognosis in colorectal cancer

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KEY WORDS

chemokine (C-C motif) ligand 2, chemokine (C-C motif) ligand 5, chemokines, colorectal cancer

ABSTRACT

INTRODUCTION Chemokines are cytokines with chemotactic functions in the initiation and maintenance of immune reactions. They have also been shown to regulate other processes such as cancer progression and cancer cell migration.

OBJECTIVES The aim of this study was to determine the prognostic role of serum levels of chemokine (C-C motif) ligand 2 (CCL2) and chemokine (C-C motif) ligand 5 (CCL5) in patients with colorectal cancer.

PATIENTS AND METHODS The study included a group of 45 patients with colorectal cancer. The serum concentrations of CCL2 and CCL5 were measured preoperatively. Peripheral blood mononuclear cells (PBMC) from patients' blood were isolated and cultured alone or with cancer cells. The concentrations of chemokines in serum and culture supernatants were measured using the cytometric bead array method. The cut-off points for serum chemokine levels were set based on the receiver-operating characteristic curve analysis at a level of 103.6 pg/ml for CCL2 and of 11933.2 pg/ml for CCL5. The survival analysis and multivariate analysis of prognostic factors were performed.

RESULTS The 5-year survival was 57.5% for the group with low CCL2 levels and 23.87% for the group with high CCL2 levels. For the groups with low and high CCL5 levels, the survival was 18.3% and 49.3%, respectively. For CCL2, the survival of the low-level group was significantly better than that of the high-level group ($P = 0.0028$). In the Cox proportional hazard model, radicality of resection ($P = 0.001$) and CCL2 levels ($P = 0.029$) were independent prognostic factors.

CONCLUSIONS The serum level of CCL2 in patients with colorectal cancer may have prognostic value. One of the possible mechanisms of CCL2 production is the interaction of PBMC with cancer cells.

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INTRODUCTION Chemokines are the subclass of cytokines with chemotactic functions. These proteins with a molecular weight of 8 to 13 kDa and length of 70 to 130 amino acids are traditionally divided into 4 subfamilies on the basis of cysteine residues in proteins (C-C, C, C-X-C, and C-X-3C). Chemokines have an important function in the initiation and maintenance of immune reactions, as well as in regulating the mobility and homing of immune competent cells in the lymphatic system and sites of inflammation. They have also been shown to regulate other processes such as embryonic implantation and development, angiogenesis, and stem cell migration.[1-3] Chemokine receptors are expressed on various types of cells, and more than 50 chemokines have been identified so far.

Chemokines were initially investigated as the factors influencing the development of inflammation in carcinogenesis in patients with, for example, ulcerative colitis, gastritis related to *Helicobacter pylori* infection, or chronic pancreatitis.[4-8] There is also evidence suggesting that chemokines take part in the recruitment of M2 or Tie2+ macrophages to the tumor bed.[9,10] Some authors have also demonstrated that when chemokines interact with these cells, they can maintain their migration towards the tumor as well as promote

new vessel formation (neoangiogenesis).[11-14] Neoplastic cells are also able to produce chemokines that regulate the cellular content of the tumor microenvironment.[13-15] However, these observations are not universal for all cancer types. The impact of chemokines on cancer progression may vary in different types of cancer.[16,17] In addition, evidence indicates that the so called proinflammatory chemokines are involved in the mechanisms of tumor progression and neoangiogenesis, mostly through the involvement of chemokine (C-C motif) ligand 2 (CCL2) and chemokine (C-C motif) ligand 5 (CCL5).[15,16]

CCL2 is produced by monocytes, endothelial cells, osteoblasts, and some cancer cells. It is chemotactic for monocytes and mast cells; however, it does not recruit neutrophils. CCL2 promotes the release of some proinflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin 1. In experimental studies, CCL2 may increase the cytotoxic activity of monocytes against cancer cells, but in clinical studies, overexpression of CCL2 is related to ovarian and breast cancer progression.[3] Overexpression of CCL2 in luminal B breast cancer cells inhibits necrosis and autophagy of tumor cells, which correlates with poor prognosis of patients with luminal B breast cancer.[18] CCL2 is also a target in immunotherapeutic studies on 6-shogaol. In vitro 6-shogaol inhibits tumor-associated dendritic cell secretion of CCL2, which decreases tumorigenesis and metastatic properties of lung and breast cancer cells. This finding was also observed in vivo in mice.[19]

CCL5 is produced by T-lymphocytes, fibroblasts, and other cell types. Its production is activated by TNF-α. CCL5 increases the number of $CD4^+$ cells in the peritumoral infiltrate, but $CD8^+$ cells are affected to a lesser degree.[20] Moreover, the CCR5–CCL5 axis promotes vascular endothelial growth factor (VEGF) expression in human osteosarcoma cells[19] and human chondrosarcoma cells,[21] which results in an increase in angiogenesis in experimental models in vitro and in vivo. The observations of the 2 above cytokines may explain their activity in the progression of gastrointestinal malignancies. Increased expression of CCL2 was observed in colonic adenomas, and its level of expression correlated with the grade of dysplasia. This, however, was not observed in the normal mucosa of the same patients.[22] The increase of CCL2 expression was also correlated with VEGF production by macrophages in the tumor microenvironment.[22] The overexpression of CCL2 in liver metastases of colorectal cancer was a negative prognostic factor.[23] CCL2 blocks migration of cytotoxic T lymphocytes towards the tumor, thus inhibiting colon cancer cell apoptosis in vitro.[24] In gastric cancer, an increased expression of CCL2 correlated with the depth of cancer infiltration, vessel density, and with the number of macrophages in the peritumoral area.[25] CCL5 was also overexpressed in colorectal cancers, though its prognostic value was not determined.[26] However, CCL5 in vitro increases growth and migration of human colon cancer cells. In addition, knockdown of CCL5 from CT26 mouse colon tumor cells leads to apoptosis of tumor cells and reduces tumor growth in mice.[27] In gastric cancer patients, serum CCL5 levels correlated with the tumor's clinical stage,[20] and in experimental studies, downregulation of the CCR5–CCL5 axis was shown to block migration of gastric cancer cells.[28] The vast majority of the studies have focused on tissue expression of CCL2 and CCL5. In a study on serum CCL5 levels in gastric cancer patients, higher CCL5 levels correlated with lower histological differentiation and advanced tumor stage. What is more, the overall survival of patients with high serum CCL5 levels was significantly lower.[29]

The serum level of chemokines may be easily measured in cancer patients. However, there are no data concerning serum CCL2 and CCL5 levels in patients with colorectal cancer and its relation to the tumor stage and prognostic significance. Therefore, because the increased incidence of colorectal cancer and the outcomes of treatment are not satisfying,[30] the aim of this study was to assess the prognostic role of serum CCL2 and CCL5 levels in patients with colorectal cancer, which may allow to identify a group of patients who require specific treatment. The production of CCL2 and CCL5 by peripheral blood mononuclear cells (PBMCs) of patients was assessed to elucidate the mechanism of chemokine production.

PATIENTS AND METHODS

A group of 45 patients with a diagnosis of colorectal cancer were operated between 2006 and 2008 in a single institution. Only the patients who signed informed consent were included into the study.

In all cases, the diagnosis was established preoperatively. There were 18 women and 27 men, and all TNM stages were present (TABLE 1).

In this group of patients, no preoperative infections, chronic inflammatory diseases, or autoimmune diseases were diagnosed. No chemotherapy, radiotherapy, or immunotherapy was administered before the surgery. Among this group, 32 patients underwent partial colectomy with colonic anastomosis; 9, Hartmann's procedure or abdominoperineal resection; and 4, palliative bypass or colostomy. Tumor staging was performed according to the TNM classification, 7th edition. The radicality of surgery was assessed by TNM residual tumor (R) classification.

The study was approved by the Bioethics Committee of the Jagiellonian University, Kraków, Poland, and was conducted according to the ICH GCP – Good Clinical Practice and the Declaration of Helsinki guidelines.

Chemokine concentration measurement

CCL2 and CCL5 levels were measured in serum or in culture supernatants of PBMCs isolated from patients' blood and cultured with or without stimulation

TABLE 1 Clinical and pathological characteristics of the patients

age, y	65.4 ±9.2
sex, male/female	27/18
TNM stage I/II/III/IV	5/14/12/14
T 1/2/3/4	5/8/20/12
N–/N+	21/24
M–/M+	31/14
tumor grade G1/G2	21/24
radicality R0/R1/R2	27/4/14

Data are presented as mean ± standard deviation or number of patients.

Abbreviations: M, metastases; N, lymph nodes; T, primary tumor

with HPC-4 cancer cell line cells. The HPC-4 cell line was derived from human pancreatic adenocarcinoma, and its characteristics were described previously.[31] Blood samples were collected 24 hours before the surgery. Blood was centrifuged and serum samples were stored at –80°C until the time of measurement.

Concentrations of chemokines (CCL2 and CCL5) in the serum of patients or culture supernatants were analyzed using the cytometric bead array method and flow cytometry (FACSCanto, BD Biosciences, San Jose, California, United States). The following Flex Sets (BD) were used: CCL5 (#558324) and CCL2 (#558287). The specified beads were discriminated in the FL-4 and FL-5 channels, while the concentration of individual chemokine was determined by the intensity of FL-2 fluorescence. The amount of chemokine was computed using the respective standard reference curve and the FCAP Array software (#641488, BD Biosciences).

Isolation and stimulation of peripheral blood mononuclear cells Blood samples were collected in EDTA-containing tubes (BD Biosciences), and PBMCs were isolated using standard Ficoll/Isopaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation.

PBMCs were suspended in the sterile culture medium (RPMI1640 + glutamine + gentamycin + fetal calf serum [10%]) at a concentration of 1×10^6/ml.

PBMCs were cultured for 18 hours in round bottom 96-well plates (Nunc, Denmark) at 37°C, CO_2 atmosphere (5%). The cells (1×10^5) were cultured without stimulation or stimulated with HPC-4 cancer cells: 3×10^4 per well in the 200 µl of the final culture medium volume.

Statistical analysis Differences in median chemokine levels between the groups were tested using the Mann–Whitney test. The survival was presented using the Kaplan–Meier curves, and the difference between the curves was tested using the log-rank test. For the assessment of the prognostic factors, a Cox proportional hazard model was used. For a multivariate analysis, the following covariates (which were prognostic factors in the univariate analysis) were used: TNM stages I–II vs III–IV, sex, age >65 years or <65 years, radicality R0+R1 vs R2, and CCL2 levels. A P level of less than 0.05 was considered statistically significant. The statistical analysis was performed using Statistica v. 10 software package (CBA Flex Sets, BD Biosciences).

RESULTS

Serum chemokine (C-C motif) ligand 2 and chemokine (C-C motif) ligand 5 levels The median level of CCL2 in the entire group was 82.53 pg/ml, with a range of 26.06 to 69118.0 pg/ml. For CCL5, the median level was 5411.14 pg/ml with a range of 72.5 to 17399.8 pg/ml. There was 1 patient with an exceptionally high level of CCL2, without a known cause; the second highest CCL2 level was 287.73 pg/ml. The analysis of the subgroups according to demographic and clinical parameters (TABLE 2) showed no significant differences in chemokine concentrations between the subgroups. There was a tendency towards a

TABLE 2 Median, minimum, and maximum serum levels of chemokines CCL2 and CCL5 in the study subgroups

	CCL2, pg/ml median (min–max)	CCL5, pg/ml median (min–max)
age <65 years	76.48 (28.89–287.73)	5060.35 (274.46–17399.83)
age >65 years	93.02 (26.06–69118.01)	5548.33 (72.56–13895.47)
sex, female/male	49.92 (26.54–287.73) / 94.30 (26.06–69118.01)	5339.34 (464.11–13865.95) / 5773.30 (274.46–17399.83)
T1–2	76.91 (26.06–69118.01)	5411.14 (464.11–13865.95)
T3–4	87.78 (26.54–287.73)	5668.81 (72.56–17399.83)
N–	82.53 (26.06–69118.01)	5411.14 (72.56–13895.47)
N+	70.44 (26.54–281.49)	5998.27(464.11–17399.83)
M–	76.35 (26.06–69118.01)	5411.14 (72.56–13895.47)
M+	114.44 (26.85–161.79)	6138.50 (274.46–17399.83)
R0+R1	70.44 (26.06–69118.01)	5411.14 (72.56–15895.41)
R2	113.13 (28.79–161.79)	5546.74 (274.46–17399.83)
G1	73.39 (26.06–69118.01)	4174.41 (72.56–13895.47)
G2	98.00 (26.54–287.83)	6068.38 (464.11–17399.83)

Abbreviations: see TABLE 1

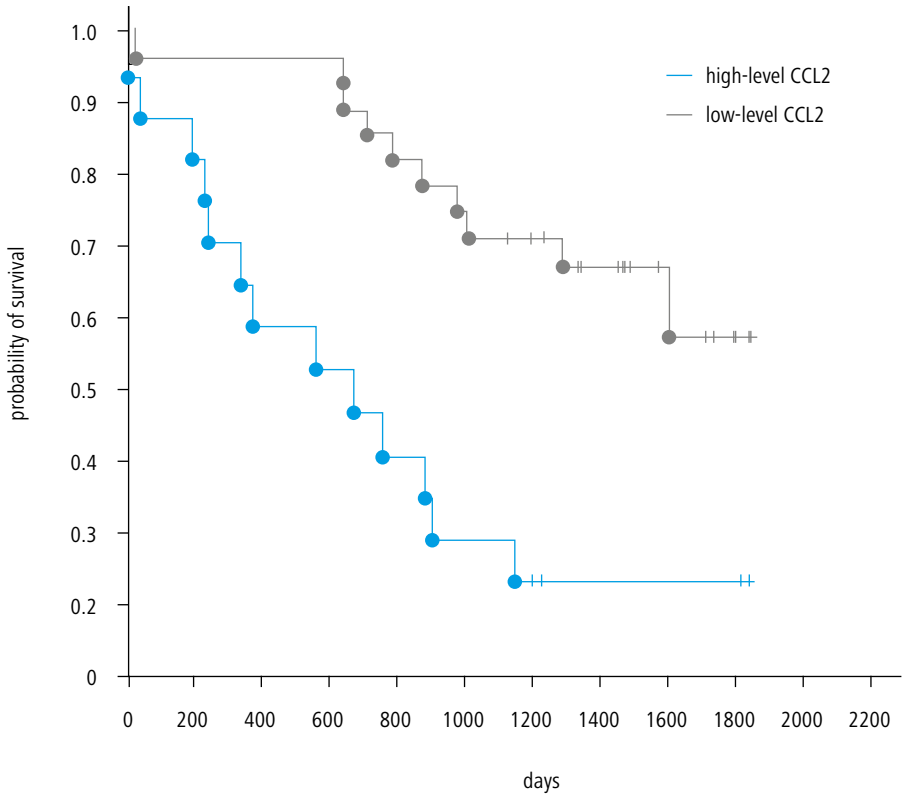

FIGURE 1 Survival of chemokine (C-C motif) ligand 2 (CCL2) in high--level and low-level groups

higher serum CCL2 level in patients with distant metastases, less differentiated tumor histology, and in patients with nonradical (R2) resections. However, probably due to a small number of the patients in the subgroups, the difference did not achieve significance.

Categorization of serum chemokine levels For further analysis, cut-off levels for each chemokine were computed using a receiver-operating characteristic curve analysis. For CCL2, a level of 103.21 pg/ml or less was categorized as low and of more than 103.21 pg/ml—as high. CCL5 levels of 11 933.22 pg/ml or less were classified as low and of more than 11 933.22 pg/ml—as high. The survival curves for low and high levels were plotted using the Kaplan–Meier method. The group with low CCL2 levels included 28 patients, and that with high CCL2 levels—17 patients. For CCL5, the number of patients in the low- and high-level groups were 37 and 8, respectively.

The median follow-up time was 46 months.

The 5-year survival for the group with low CCL2 levels was 57.5% and for the group with high CCL2 levels—23.8%. For CCL5, the 5-year survival was 18.3% for the low-level group and 49.3% for the high-level group.

For CCL2, the survival of the low-level group was significantly better than that for the high -level group (P = 0.0028; log-rank test; FIGURE 1). For CCL5, the survival did not differ significantly between the low- and high-level groups (FIGURE 2).

A univariate analysis of prognostic factors revealed that CCL2 levels were a prognostic factor for survival in the analyzed group of patients (P = 0.002), but not CCL5 levels. The multivariate analysis was performed with the following covariates: TNM stages I–II vs III–IV, sex, age >65 years or <65 years, radicality R0+R1 vs R2, and CCL2 levels.

In the Cox proportional hazard model, the radicality of resection (hazard ratio [HR], 6.55; 95% confidence interval [CI], 2.01–21.35; P = 0.001) and CCL2 levels (HR, 2.8; 95% CI, 1.10–7.08; P = 0.029) were independent prognostic factors.

Chemokine production as the result of interaction between peripheral blood mononuclear cells and HPC-4 cancer cells PBMCs isolated from the patients' blood were cocultured with HPC-4 cancer cells, and the concentrations of CCL2 and CCL5 in culture supernatants were compared with the concentration in the supernatants of PBMC culture alone (control) (FIGURES 3 and 4). The mean concentration of CCL2 in the coculture was 24 550 ±22 862 pg/ml vs 14 353 ±17 745 pg/ml in controls. The mean CCL5 concentration in the coculture was 4189 ±1827 pg/ml vs 3138 ±2096 pg/ml in controls. The concentration of CCL2 was significantly higher after stimulation with cancer cells than in controls (P = 0.003) but there was no significance for CCL5 (P = 0.06).

DISCUSSION The problem of prognostic or predictive factors in patients with colorectal cancer is clinically important because these factors may influence postoperative decisions. The guidelines for colorectal cancer are mainly based on the TNM stage and molecular characteristics of the tumor. In many individual cases, the decision as to whether to use adjuvant chemotherapy

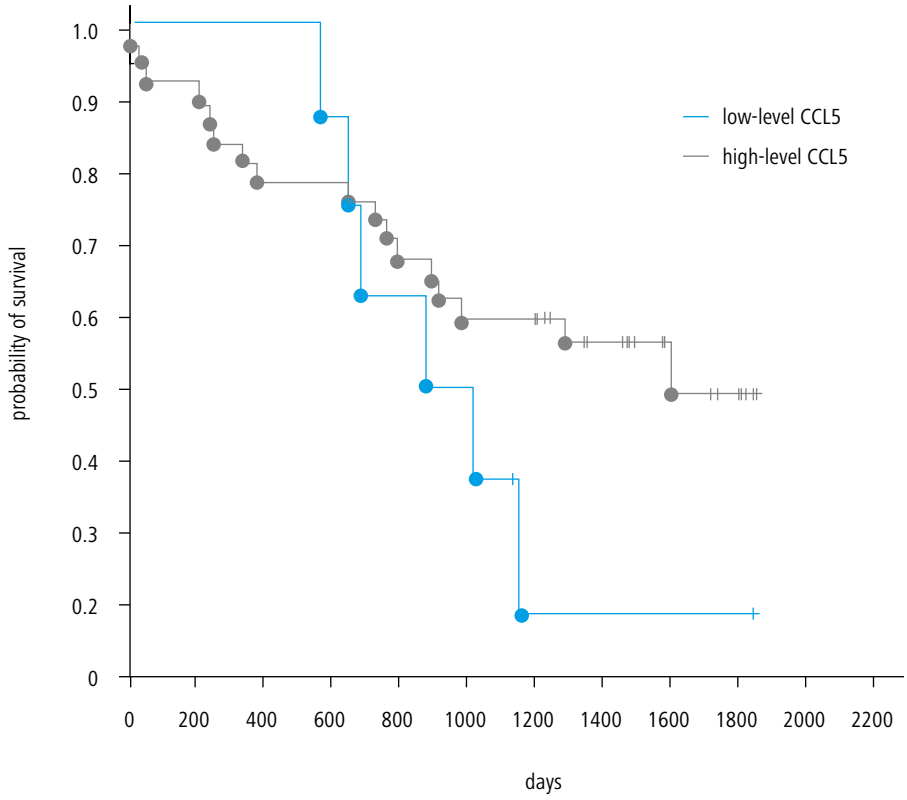

FIGURE 2 Survival of chemokine (C-C motif) ligand 5 (CCL5) in high--level and low-level groups

or not, or a regimen of adjuvant treatment, requires additional information. Therefore, a number of additional measures are used, such as the serum carcinoembryonic antigen level, p53, or Ki67 expression to predict the biology and behavior of the tumor.[32,33] CCL2 and CCL5 are proinflammatory chemokines; therefore, their overexpression or increased serum levels likely drive a shift towards a proinflammatory reaction in a subgroup of cancer patients. The phenomenon of the effect of coexisting inflammation on the prognosis of colorectal cancer patients has been described, and the grade of inflammation was implemented as the one of prognostic factors.[34,35] Quite a simple Glasgow Prognostic Score based on C-reactive protein levels and hypoalbuminemia as the markers of inflammation has been assessed as a valuable prognostic tool in colorectal cancer and other gastrointestinal cancers.[36-38] In our study, we did not asses chemokine levels in healthy subjects. However, one of the studies reported a median serum CCL2 level of 62.74 pg/ml in healthy subjects.[39] In another analysis, the median level of CCL2 in healthy subjects fluctuated between 39.74 pg/ml and 53.76 pg/ml. For CCL5, the respective levels were from 10 264.21 pg/ml to 11 740.25 pg/ml.[40] These levels are coherent with our results.

In the present study, there were no significant correlations between chemokine levels and tumor stage or other clinical and pathological factors. This is the pilot study in a relatively small cohort of patients with different stages of colorectal cancer. This is a limitation of this study because of many possible covariates in such inhomogeneous groups. Importantly, within the group of patients, there is a substantial number of nonradical resections, mainly due to disseminated disease. Nevertheless, we decided to publish our results to enable further investigation by the scientific community.

The observed trend of higher CCL2 levels in patients with R2 resections could possibly be caused by the inflammation related to tumor invasion to the surrounding tissues.

Our findings may suggest that high serum levels of CCL2 may be considered a prognostic factor for colorectal cancer. This finding is in line with other observations, which indicated that the CCL2 expression in the tumor tissue is associated with a negative prognosis of patients with colorectal cancer.[38] Moreover, there are some data indicating that colorectal cancer cells with increased metastatic potential overexpress CCL2.[41] In contrast, in a group of 76 gastric cancer patients, low CCL2 serum levels were associated with a more advanced stage and worse prognosis.[42] Nevertheless, numerous studies on other cancer types corroborate our findings. Serum CCL2 levels were significantly higher in patients with more advanced ovarian cancer and in patients with ovarian cancer versus controls.[43] Additionally, in patients with nasopharyngeal carcinoma, high serum levels of CCL2 were associated with shorter survival and shorter metastasis-free survival.[44] In breast cancer, the expression of CCL2 within the tumor tissue has been shown to have prognostic value in the determination of relapse-free survival time and was suggested to be involved in the mechanism of bone metastases.[15,45] The proposed mechanism of CCL2 impact on cancer

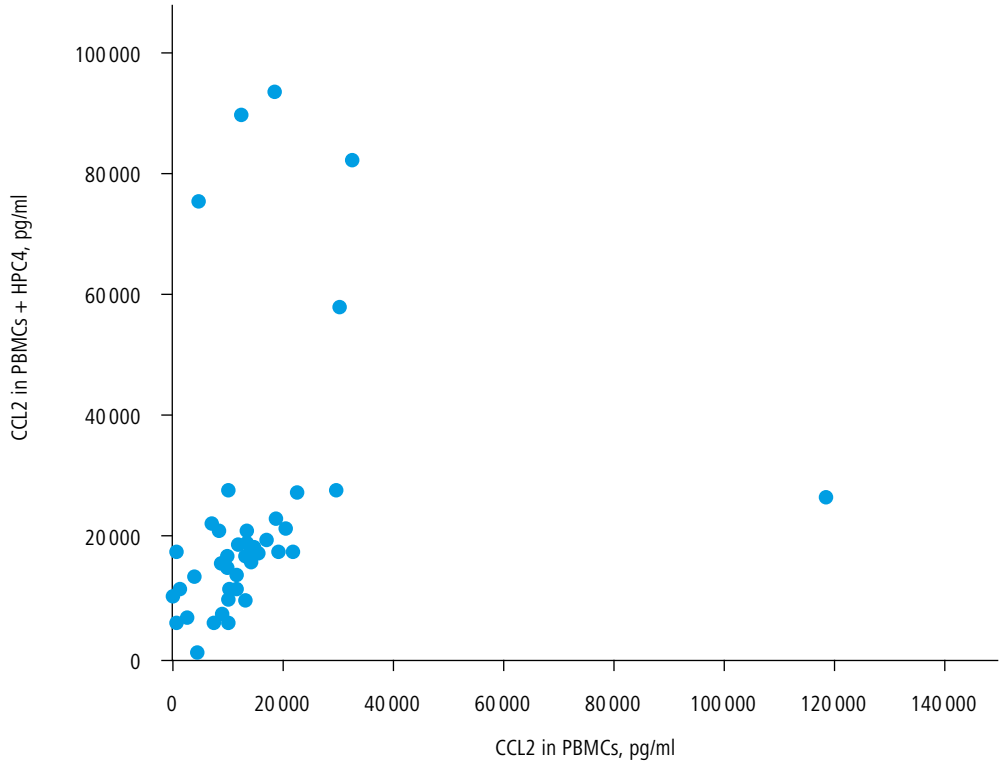

FIGURE 3 Distribution of chemokine (C-C motif) ligand 2 (CCL2) levels in culture supernatants
Abbreviations: PBMCs, peripheral blood mononuclear cells

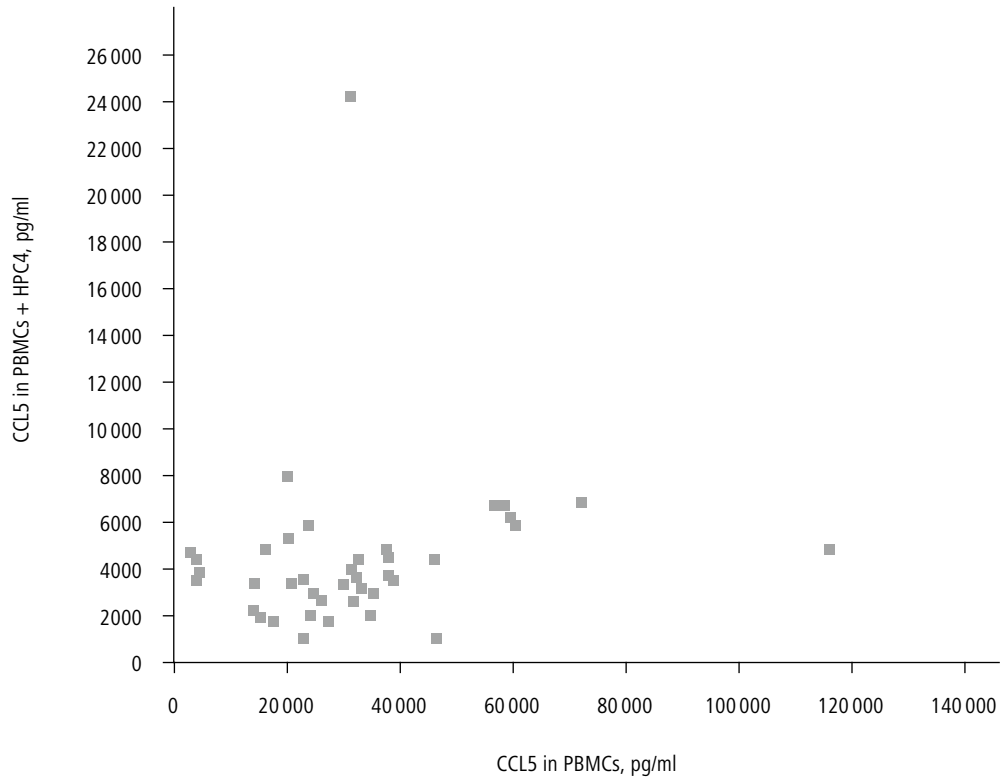

FIGURE 4 Distribution of chemokine (C-C motif) ligand 5 (CCL5) levels in culture supernatants
Abbreviations: see **FIGURE 3**

progression is the modulation of the balance of different leukocyte subpopulations in peritumoral infiltrates, promotion of the rate of tumor-associated macrophages, angiogenesis, and the increase in tumor cell migration.[15,46,47]

There are a number of studies on the prognostic role of chemokines other than CCL2 in colorectal cancer, such as the C-X-C subfamily, but the studies on the role of CCL5 role are sparse. Our findings about the lack of the prognostic role of CCL5 cannot be directly compared to others.

We have investigated one of the possible mechanisms of the CCL2 and CCL5 production in colorectal cancer, namely, straight stimulation of patients' PBMCs for chemokine production following contact with cancer cells. The results may indicate that one of the sources of CCL2 may be the interaction of PBMCs with cancer cells or their derivatives. This indicates that not only the

cells of tumor microenvironment but also peripheral cells may be responsible for CCL2 overproduction in cancer patients. The results of the studies on the role of CCL2 in various cancers did not lead to the implementation of CCL2-targeted therapies at the time of writing this manuscript. Nevertheless, some experimental studies are ongoing. In one of these studies, in which a mouse model of hepatocellular carcinoma is being employed, inhibition of CCL2 activity by recombinant VP1, viral capsid protein, suppressed cancer growth.[48] More data about the clinical significance of CCL2 in cancer may contribute to the development of such strategies. Because most of the currently published studies analyze the role of tissue expression of CCL2 and CCL5, the significance of their serum levels requires further studies. Serum levels are easier to obtain and if their prognostic value is confirmed, they may have wider clinical application than tissue expression.

To conclude, serum CCL2 levels in the cohort of patients with colorectal cancer has been the prognostic factor in this pilot study. This observation is supported by observations in different cancer patient series. It also may confirm the prognostic significance of inflammatory reactions in patients with colorectal cancer. One of the possible mechanisms of CCL2 production is the interaction of PBMCs with cancer cells.

Contribution statement AMS, MS, and MS made substantial contributions to the conception, design, drafting, and critical revision of the article; WK, PB, and JK analyzed the data and contributed to drafting of the paper; KB contributed to data acquisition and interpretation and contributed to drafting; all authors approved the final version of the paper.

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Przedoperacyjny poziom chemokiny (motyw CC) ligand 2 a prognoza w raku jelita grubego

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SŁOWA KLUCZOWE

chemokina (motyw CC) ligand 2, chemokina (motyw CC) ligand 5, chemokiny, rak jelita grubego

STRESZCZENIE

WPROWADZENIE Chemokiny to cytokiny pełniące funkcje chemotaktyczne w inicjowaniu i utrzymaniu reakcji immunologicznej. Wykazano również, że biorą one udział w regulacji innych procesów, takich jak progresja nowotworu i migracja komórek nowotworowych.

CELE Celem badania było określenie prognostycznej roli poziomu chemokiny (motyw CC) ligand 2 (CCL2) i chemokiny (motyw CC) ligand 5 (CCL5) w surowicy u chorych na raka jelita grubego.

PACJENCI I METODY W badaniu wzięło udział 45 pacjentów z rakiem jelita grubego. Stężenie CCL2 i CCL5 w surowicy mierzono przed operacją. Jednojądrzaste komórki krwi obwodowej (*peripheral blood mononuclear cells* – PBMC) z krwi pacjentów izolowano i hodowano osobno lub z komórkami nowotworowymi. Stężenie chemokin w surowicy i w hodowli mierzono za pomocą metody *cytometric bead array*. Punkty graniczne dla stężenia chemokin w surowicy zostały ustalone za pomocą krzywej ROC na poziomie 103,6 pg/ml dla CCL2 i 11 933,2 pg/ml dla CCL5. Przeprowadzono analizę przeżycia i wielowymiarową analizę czynników prognostycznych.

WYNIKI Pięcioletnie przeżycie dla grupy z niskim poziomem CCL2 wyniosło 57,5%, a dla grupy z wysokim poziomem – 23,8%. Dla grup z niskim i wysokim poziomem CCL5 pięcioletnie przeżycie wynosiło odpowiednio 18,3% i 49,3%. W przypadku CCL2 przeżycie grupy z niskim poziomem cytokiny było znamiennie lepsze niż w przypadku grupy z wysokim poziomem cytokiny ($p = 0,028$). W modelu proporcjonalnego hazardu Coxa radykalność resekcji ($p = 0,001$) i poziom CCL2 ($p = 0,029$) były niezależnymi czynnikami prognostycznymi.

WNIOSKI Poziom CCL2 w surowicy pacjentów z rakiem jelita grubego może mieć wartość prognostyczną. Jednym z możliwych mechanizmów wytwarzania CCL2 jest interakcja PBMC z komórkami nowotworowymi.

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