Original paper

# Evaluation of CXCL12 and CXCR4 to predict poor survival in lymph node-positive colorectal cancer patients

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It is well known that interactions in the tumour microenvironment are very important in the progression of tumours. We investigated the relationship between chemokine ligand type 12 (CXCL12), chemokine receptor type 4 (CXCR4) and survival in advanced colorectal cancers (CRC).

Primary tumour samples of stage III-IV CRC patients were investigated for CXCL12 and CXCR4.

Chemokine ligand type 12 and CXCR4 expressions were significantly associated with poor prognostic factors (e.g. for CXCL12: lymphatic invasion [p = 0.009], positive surgical margin [p = 0.006], advanced stage [p = 0.028], etc.). Also, these parameters were independent risk factors for low LIR (e.g. for CXCL12: Odds ratio [OR] = 2.27, p = 0.001) and low tumour stroma-ratio (TSR; e.g. for CXCL12: OR = 1.18, p = 0.003). In univariate analysis, 5-year RFS and OS were poor (e.g. for CXCL12: RFS, p < 0.001 and OS, p = 0.001). Multivariate analysis showed that these parameters were independent poor survival parameters for RFS and OS (e.g. for CXCL12: Hazard ratio [HR] = 3.54 [CI: 1.52-4.67], p = 0.001 and HR = 2.74 [1.48-4.71], p = 0.025).

We showed that CXCL12 and CXCR4 expressions are poor prognostic factors in lymph node-positive CRC patients and are associated with low TSR and low LIR.

**Key words:** colorectal cancers, CXCL12, CXCR4, prognostic markers, stage III-IV.

## Introduction

Colorectal cancers (CRC) are one of the most common tumours with cancer-related deaths in both sexes worldwide [1]. Colorectal cancers cases mostly admit to the hospital as an advanced stage. Although results in CRCs have improved significantly with advances in treatment approaches and imaging methods, the 5-year survival rates in lymph node-positive patients are still low [1, 2]. And although current treatment approaches recommend adjuvant chemotherapy as a standard for this patient population, the absolute improvement in survival is less than 5%, and some subpopulations in these patients may benefit from targeted therapies [2, 3]. While the TNM system suggests traditional pathological staging parameters when deciding on prognosis in these patients, their value alone in demonstrating direct outcome and response to treatment is still limited [3, 4]. Therefore, new prognostic markers are needed for

better clinical management. Chemokines, tumour microenvironment and local lymphocytic response are among the most promising pathological parameters in the literature.

Chemokine ligand type 12 (CXCL12), also known as stromal-derived factor 1, is one of the most important chemokines belonging to the human chemokine superfamily. It is genetically located at the 10q11.1 *locus* [5]. Chemokine ligand type 12 is widely expressed by a large number of normal and tumoral tissues such as B cells, endothelial cells, stromal fibroblasts, stem cells and many cancers [5, 6]. Chemokine receptor type 4 (CXCR4) is the most common chemokine receptor of CXCL12 in many cells. It is genetically located at the 2q21 *locus*. The interactions between CXCR4 and CXCL12 affect many biological effects such as cell growth, differentiation and angiogenesis in normal and tumoral tissues [5, 6]. Chemokine ligand type 12 and CXCR4 expressions have been identified in many types of cancer such as CRC, breast cancer, gastric cancer, pancreatic cancer, ovarian cancer, lung cancer, and squamous cell cancer [7, 8, 9, 10, 11, 12, 13]. Also, the expressions of these two parameters were found to be significantly associated with metastases and poor overall and relapse-free survival rates in many tumours [7, 8, 9, 10, 11, 12, 13]. In addition, recent studies have reported that the CXCL12- CXCR4 pathway is closely related to the initiation and progression of some tumours, and inhibiting this pathway makes tumours more susceptible to anticancer treatments [14]. However, the exact role of these two parameters in the tumour microenvironment and associated molecular mechanisms remain unclear.

One of the most important factors in the tumour microenvironment is the intratumoral stroma [15]. It has been shown that many tumours with a large intratumoral stroma, including CRC, have a worse prognosis. It has also been reported that tumour stroma may affect the response to adjuvant chemotherapy [15, 16, 17]. Therefore, evaluation of the tumour-stroma ratio (TSR), defined as the microscopic measurement of the intra-tumour stroma ratio in primary resection materials, can be a simple, successful and reliable prognostic parameter. Another important factor in the tumour microenvironment is the local inflammatory response (LIR). It is known that LIR plays an important role in the production of chemokines secreted during the growth, angiogenesis, invasion and metastasis stages of cancer cells [18]. With these expressed chemokines, inflammatory cells in the tumour microenvironment can attack tumour cells, produce different cytokines and consequently lead to tumour regression [19]. Many studies in the literature have confirmed the prognostic value of inflammatory cells for many tumours, including CRC [18, 19, 20]. However, the complex interactions between these parameters in the tumour microenvironment are still not elucidated.

The aim of this study is to examine the relationships between CXCL12, CXCR4, and survival in advanced-stage CRC and to clarify the role of these parameters in prognosis.

## Materials and methods

This study was designed in accordance with REMARK [21] recommendations.

### Patients

Ethics committee approval was obtained from Kırıkkale University health research ethics committee for our study (2020.09.01), and all procedures were carried out in accordance with the 1964 Helsinki declaration and national and institutional ethics committee standards.

This study was retrospective and was carried out in Kırıkkale University Faculty of Medicine, Department of Pathology. All cases operated with the diagnosis of CRC between 2005 and 2015 were collected from the hospital electronic database. A total of 17 patients who died or recurred within 1 month after surgery, had more than one tumour and received neoadjuvant therapy were excluded from the study. Paraffin blocks and hematoxylin-eosin (HE) stained slides of 355 cases were collected and all of the slides were re-examined. Of these cases, 5 patients with limited tumour tissue in paraffin blocks, 8 patients with paraffin blocks could not be reached, and 72 patients with different tumour stages were excluded from the study, and a total of 260 patients were included. In our study, 103 (39.6%) of the population were female and 157 (60.4%) were male. The median value of age was 69 (range: 37-92 years). Clinical, pathological and survival data of the cases were obtained from the hospital database. The prognostic parameters used in the study were age, size, gender, localization, stage, perineural invasion, lymphatic invasion, surgical margin, TSR, LIR, tumour budding, and MSI status.

### Methods

Paraffin blocks and HE stained preparations of 260 patients stored at room temperature were removed from the archive of the Department of Pathology. Tumour blocks of the cases varied between 2 and 18. Hematoxylin and eosin stained preparations of the cases were evaluated and a paraffin block with the deepest tumour area was selected. Three sections of 4-micron thickness were taken from the chosen paraffin block. These sections were stained with CXC12, CXCR4 and HE. The stained preparations were evaluated by two experienced pathologists (M.Z, O.O) in

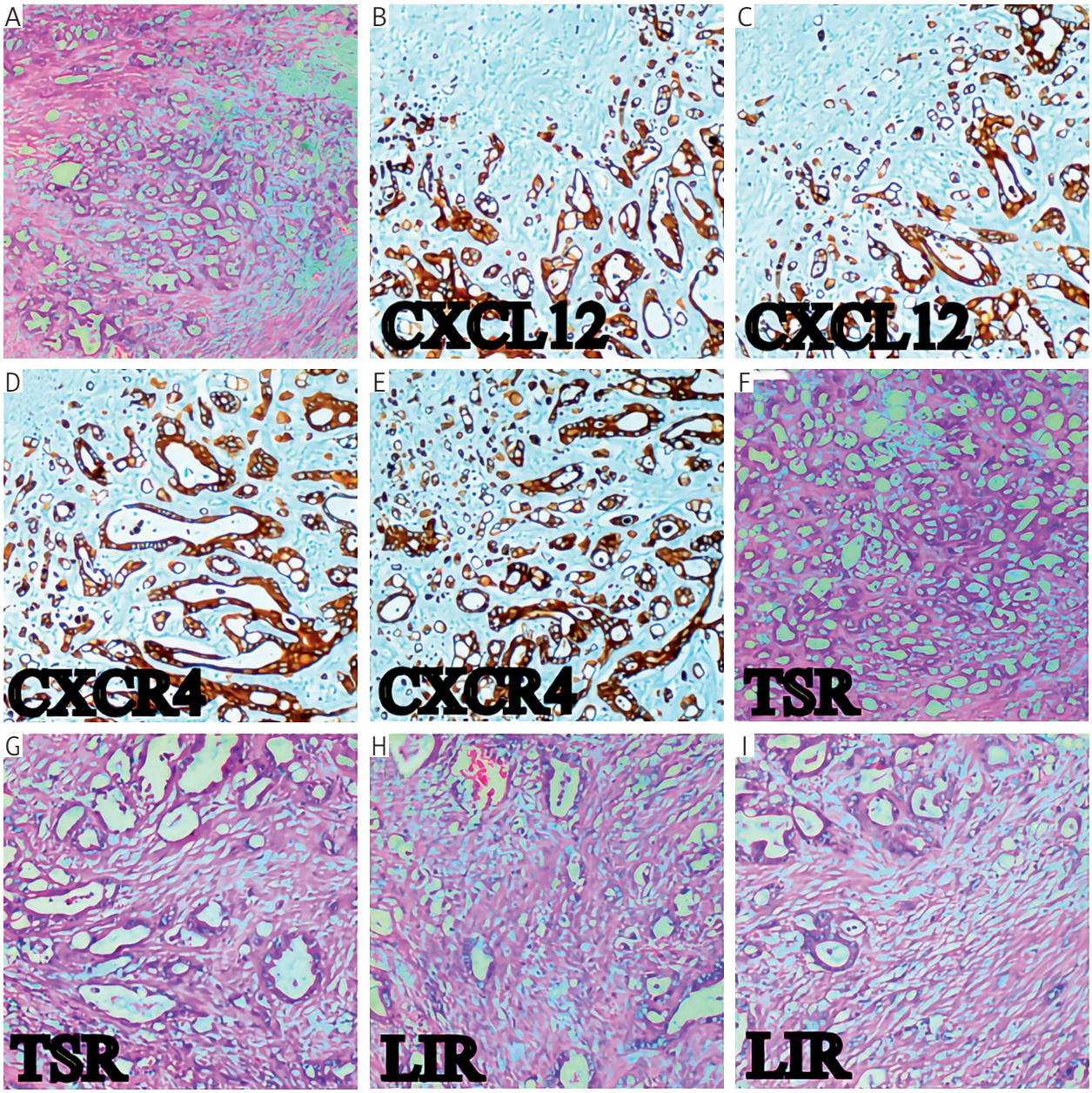


**Fig. 1.** Microscopic image samples of chemokine ligand type 12 (CXCL12), chemokine receptor type 4 (CXCR4), tumour stroma-ratio (TSR) and local inflammatory response (LIR)

accordance with the guidelines of the American Joint Cancer Classification Committee [22], blindly from the clinical and pathological information.

Evaluation of CXCL12 and CXCR4 was noted semi-quantitatively on IHC stained slides using a conventional microscope and an × 10 lens (Nikon Eclipse E600, Switzerland). Initially, all sections were scanned with an × 4 lens to determine the staining distribution. Tumour glands showing cytoplasmic or membranous staining, albeit focal and weak, were accepted as positive. If staining was common, an area containing stromal and tumour tissue together was selected to assess both TSR and LIR in the same area. Care was taken to ensure that the tumour cells are in many borders of this field of view. If staining was focal, the evaluation was made on this focal stained area. Adenocarcinoma cells without a clearly stained blue nucleus were omitted to avoid false immunohistochemical (IHC) staining. Finally, all cases were divided into two groups as positive and negative.

Tumour stroma-ratio and LIR were evaluated semi-quantitatively using an ×20 lens on the HE stained sections. While evaluating LIR, recommendations of International Tumor-Infiltrating Lymphocytes Working Group were taken into consideration [23]. Multiples of five were used when recording the TSR, e.g. 5%, 10%, 15%. Cases with more than 50 lymphocytes for LIR and cases with more than 50%

stroma for TSR were considered positive [24]. Tumour budding was defined as the presence of small tumour cells in the invasive area of the tumour, and cases with more than 10 tumour buds were classified as positive [24]. Representative examples of research are shown in Figure 1.

### Follow-up of patients

The follow-up period of the cases was determined as ten years. Survival and recurrence times were calculated considering the day of surgery. All events over 60 months were considered 60 months. Overall survival (OS) time was accepted as the time between the day of surgery and the day of death, and the relapse-free survival (RFS) time was accepted as the time between the day of surgery and the day of local and regional recurrence.

### Evaluation of reproducibility

Heterogeneity of tumours and interobserver agreement were evaluated for reproducibility of histopathological parameters. Heterogeneity of tumours was evaluated by Intra-Class Correlation (ICC), a ratio of variation calculated by differences between tumours [25]. ICC ratio is expected to be high if it arises from inter-tumour differences such as biological variation, and low if it is derived from intra-tumour variability such as heterogeneity. The agreement between the observers was evaluated by the kappa test (κ). Kappa value is a variable ratio calculated by differences between observers and classified according to the Landis [26].

### Immunohistochemical study

Two 4-micron sections from the paraffin blocks of the tumours were taken on lysine slides. After deparaffinization and rehydration, sections were boiled in a citrated buffer (pH = 8) for 10 minutes in the microwave to recover the antigen. To prevent endogenous peroxidase activity, sections were kept in 0.3% hydrogen peroxide-methanol solution for 10 minutes at room temperature. Mouse monoclonal CXC12 (R&D systems, 1 : 60, clone: 79018) and mouse monoclonal CXCR4 (R&D systems, 1 : 120, clone: 44716) were used for primary antibodies. These primary antibodies were incubated overnight at room temperature. The secondary antibody (Dako) was applied for one hour. Sections were then stained with 3,3'-diaminobenzidine (Dako) for 5 minutes. Finally, sections were stained with hematoxylin (Merck, Germany, Darmstadt) and covered with Pertex (Histolab, Gothenburg Sweden).

### Statistical analysis

Statistical variables were recorded as frequency, percentage, standard deviation, range, and median. Univariate and multivariate analysis of prognostic parameters were performed by Chi-square test and Logistic regression test [95% CI and 1.0 odds ratio (OR)]. Spearman correlation test was used when analyzing the correlation between estimates, and Wilcoxon Signed-Rank test was used when analyzing the differences. As we mentioned above, intra-tumour heterogeneity was investigated by ICC test and the inter-observer agreement was investigated by the κ test. Univariate and multivariate analysis of survival groups were analyzed by Log-Rank test and Cox regression test [95% CI and 1.0 hazard ratio (HR)]. Kaplan-Meier analysis was used when presenting survival curves. Significance limit for p values was accepted as 0.05. SPSS 21.0 (IBM Institute, North Castle, USA) was used for analyzes.

## Results

### General features

The median value of tumour size was 6.50 (range: 3-12 cm). Ninety-nine (38.0%) of the cases were stage IV, 161 (62.0%) were stage III. Sixty-eight stage IV patients had liver metastases. Tumours were located in the right colon in 104 (40.0%) patients, and in the left colon in 156 (60.0%) patients.

### Histopathological examination

Expressions were heterogeneous and increased in invasive areas. When categorical data were examined, there was a significant relationship between these two parameters and poor prognostic parameters (for CXCL12: lymphatic invasion [$p = 0.009$], positive surgical margin [$p = 0.006$], advanced stage [$p = 0.028$], low LIR [$p < 0.001$], low TSR [$p < 0.001$], high tumour budding [$p < 0.002$]; for CXCR4: lymphatic invasion [$p = 0.007$], positive surgical margin [$p = 0.003$], advanced stage [$p = 0.026$], low LIR [$p < 0.001$], low TSR [$p < 0.001$], high tumour budding [$p < 0.002$]). In logistic regression analysis, CXCL12 and CXCR4 were found to be independent risk factors for LIR (for CXCL12: OR = 1.18 [1.03-2.66], $p = 0.003$; CXCR4: OR = 1.22 [1.26-2.86], $p = 0.005$) and TSR (for CXCL12: OR = 2.27 [1.13-3.47], $p = 0.001$; CXCR4: OR = 2.35 [1.18-3.54], $p = 0.001$) (Tables I, II).

### Analysis of reproducibility

When continuous data were examined, the analysis of correlation (for CXCL12: $r = 0.709$, $p < 0.001$; for CXCR4: $r = 0.697$, $p < 0.001$) and difference (for CXCL12: $d = 0.322$, $p < 0.001$; for CXCR4: $d = 0.338$, $p < 0.001$) were well. When heterogeneity

**Table I.** The relationship between CXCL12, CXCR4 and clinicopathological characteristics

| Parameter | CXCL12 | | | CXCR4 | | |
|---|---|---|---|---|---|---|
| | Positive | Negative | P-value | Positive | Negative | P-value |
| Age | | | 0.760 | | | 0.721 |
| < 69 | 50 (40%) | 54 (39%) | | 47 (41%) | 57 (39%) | |
| ≥ 69 | 72 (60%) | 84 (61%) | | 67 (59%) | 89 (61%) | |
| Gender | | | 0.351 | | | 0.326 |
| Female | 52 (42%) | 51 (36%) | | 49 (42%) | 54 (36%) | |
| Male | 70 (58%) | 87 (64%) | | 65 (58%) | 92 (64%) | |
| Size | | | 0.530 | | | 0.380 |
| < 6.5 cm | 44 (36%) | 55 (40%) | | 40 (35%) | 59 (40%) | |
| ≥ 6.5 cm | 78 (64%) | 83 (60%) | | 74 (65%) | 87 (60%) | |
| Localization | | | 0.286 | | | 0.540 |
| Right | 53 (43%) | 51 (36%) | | 48 (42%) | 56 (38%) | |
| Left | 69 (57%) | 87 (64%) | | 66 (58%) | 90 (62%) | |
| Stage | | | 0.028* | | | 0.026* |
| Stage IV | 55 (45%) | 44 (31%) | | 52 (45%) | 47 (32%) | |
| Stage III | 67 (55%) | 94 (69%) | | 62 (55%) | 99 (68%) | |
| Perineural invasion | | | 0.853 | | | 0.806 |
| No | 50 (40%) | 55 (40%) | | 47 (41%) | 58 (40%) | |
| Yes | 72 (60%) | 83 (60%) | | 67 (59%) | 88 (60%) | |
| Lymphatic invasion | | | 0.009* | | | 0.007* |
| No | 60 (49%) | 46 (33%) | | 57 (50%) | 49 (43%) | |
| Yes | 62 (51%) | 92 (67%) | | 57 (50%) | 97 (57%) | |
| Surgical margin | | | 0.006* | | | 0.003* |
| Negative | 61 (50%) | 46 (33%) | | 58 (51%) | 48 (32%) | |
| Positive | 61 (50%) | 92 (67%) | | 56 (49%) | 98 (68%) | |
| LIR | | | < 0.001* | | | < 0.001* |
| Negative | 62 (51%) | 42 (30%) | | 60 (52%) | 44 (30%) | |
| Positive | 60 (49%) | 96 (70%) | | 54 (48%) | 102 (70%) | |
| TSR | | | < 0.001* | | | < 0.001* |
| Negative | 58 (47%) | 38 (27%) | | 55 (49%) | 40 (27%) | |
| Positive | 64 (53%) | 100 (73%) | | 59 (51%) | 106 (73%) | |
| Tumour budding | | | 0.002* | | | 0.002* |
| Negative | 62 (51%) | 45 (32%) | | 59 (51%) | 48 (32%) | |
| Positive | 60 (49%) | 93 (68%) | | 55 (49%) | 98 (68%) | |
| MSI Status | | | 0.992 | | | 0.582 |
| MMR-P | 54 (44%) | 61 (44%) | | 45 (39%) | 50 (34%) | |
| MMR-D | 68 (56%) | 77 (56%) | | 69 (61%) | 76 (66%) | |

** P-values below 0.05 were considered statistically significant.*

*CXCL12 – chemokine ligand type 12; CXCR4 – chemokine receptor type 4; TSR – tumour stroma-ratio; LIR – local inflammatory response; MSI – microsatellite instability; MMR-D – mismatch repair proteins deficiency; MMR-P – mismatch repair proteins proficiency*

was examined, it was seen that most of the variation was due to biodiversity between different tumours. For example, the ICC value of 0.684 in Table III means that the variation between different tumours accounts for 68.4% of the total heterogeneity. Also, the results of the interobserver agreement were clinically useful (for CXCL12: $\kappa = 0.71$; for CXCR4: $\kappa = 0.69$ (Table III).

**Table II.** Regression analysis between CXCL12, CXCR4 and clinicopathological features

| | CXCL12 | | CXCR4 | |
|---|---|---|---|---|
| | OR (95% CI) | P-value | OR (95% CI) | P-value |
| Lymphatic invasion | 1.77 (0.78-5.12) | 0.164 | 2.61 (0.79-4.53) | 0.229 |
| Surgical margin | 1.56 (0.55-3.98) | 0.657 | 1.78 (0.73-4.35) | 0.458 |
| Stage | 2.77 (0.96-5.78) | 0.092 | 2.67 (0.98-5.43) | 0.076 |
| LIR | 2.27 (1.13-3.47) | 0.001* | 2.35 (1.18-3.54) | 0.001* |
| TSR | 1.18 (1.03-2.66) | 0.003* | 1.22 (1.26-2.86) | 0.005* |
| Tumour budding | 2.46 (0.89-9.53) | 0.112 | 2.97 (0.91-8.36) | 0.080 |

** P-values below 0.05 were considered statistically significant.*

*CXCL12 – chemokine ligand type 12; CXCR4 – chemokine receptor type 4; TSR – tumour stroma-ratio; LIR – local inflammatory response; OR – odds ratio; CI – confidence interval*

**Table III**. Reproducibility of the study

| | Correlation | Difference | ICC (95% CI) | Kappa values |
|---|---|---|---|---|
| CXCL12 | 0.709, $p < 0.001$ | 0.322, $p < 0.001$ | 0.692 (0.556-0.784) | 0.71 |
| CXCR4 | 0.697, $p < 0.001$ | 0.338, $p < 0.001$ | 0.676 (0.569-0.756) | 0.69 |

** P-values below 0.05 were considered statistically significant.*

*CXCL12 – chemokine ligand type 12; CXCR4 – chemokine receptor type 4; ICC – intra-class correlation coefficient; CI – confidence interval*

### Follow-up

During our study, two hundred and nine patients died (CXCL12 positive, n = 143; CXCR4 positive, n = 141), and two hundred eighteen patients relapsed (CXCL12 positive, n = 151; CXCR4 positive, n = 148). The 5-year RFS and OS rates were 12% and 15% in CXCL12 cases, 10% and 15% in CXCR4 cases (Table IV).

### Survival analyses

In univariate survival analysis, there was a significant relationship between CXCL12 (RFS, $p < 0.001$; OS, $p = 0.001$) and CXCR4 (RFS, $p < 0.001$; OS, $p = 0.001$). In multivariate survival analysis, these two parameters have an independent poor survival parameter for RFS (for CXCL12: HR = 3.54 [CI: 1.52-4.67], $p = 0.001$; for CXCR4: HR = 3.45 [CI: 1.48-4.77], $p = 0.003$) and OS (for CXCL12: HR = 2.74 [1.48-4.71], $p = 0.025$; for CXCR4: HR = 2.84 [1.42-4.68], $p = 0.023$). Tumour stroma-ratio, LIR, and surgical margin were the other independent poor prognostic parameters (Table IV, Fig. 2).

## Discussion

In this study, we investigated the role of CXCL12 and CXCR4 on survival in advanced CRC cases. We found that these two parameters could be a reliable marker in determining patients with poor prognosis in CRC. We also found that these parameters are related to TSR and LIR.

Tumour tissue communicates with each other through a mixed signalling system called chemokines. Chemokines control lymphoid organ development and immune cell activity in normal tissues [27]. In the tumour microenvironment, they are expressed by immune and stromal cells and are responsible for cell migration and cell-cell interaction. In other words, they regulate the proliferation, invasion, angiogenesis and metastasis of tumour cells [27, 28]. On the other hand, tumour cells also secrete chemokines that increase their division and therefore growth. Namely, cancer and host cells in the tumour microenvironment induce the release of many different chemokines, leading to the migration and activation of different cell types, leading to antitumor and pro-tumour responses [28, 29]. In this study, we studied one of the most promising of these chemokines in terms of prognostic parameters, CXCL12 and its receptor CXCR4.

Since tumour cells proliferate rapidly, they must accelerate vascular invasion and neoangiogenesis to provide the necessary oxygen and nutrients. Therefore, vascular invasion and angiogenesis are the most important steps in tumour progression [30]. Chemokines and their receptors play an important role in these processes. The strongest angiogenic chemokine is CXCL4 and its ligand is CXCR12. Chemokine receptor type 4 stimulates angiogenesis by activating VEGF, thereby increasing the vascular invasion of tumour cells [30, 31]. Also, CXCL12 affects these processes by stimulating the migration of angiogenic factor-producing leukocytes into the tumour microenvironment [32, 33]. In this study, we found that

**Table IV.** Univariate and multivariate survival analysis

| | Univariable Analysis | | Multivariable Analysis | |
|---|---|---|---|---|
| | **OS** | **RFS** | **OS** | **RFS** |
| | **P-value (5-year survival,%)** | **P-value (5-year survival,%)** | **P-value (HR 95% CI)** | **P-value (HR 95% CI)** |
| Age | 0.312 | 0.227 | NC | NC |
| < 69 | 45 | 47 | | |
| ≥ 69 | 24 | 25 | | |
| Gender | 0.472 | 0.379 | NC | NC |
| Female | 44 | 46 | | |
| Male | 25 | 26 | | |
| Size | 0.571 | 0.453 | NC | NC |
| < 6.5 | 43 | 45 | | |
| ≥ 6.5 | 27 | 27 | | |
| Localization | 0.496 | 0.363 | NC | NC |
| Right | 44 | 46 | | |
| Left | 26 | 26 | | |
| Stage | 0.427 | 0.312 | NC | NC |
| Stage III | 43 | 45 | | |
| Stage III | 25 | 25 | | |
| Lymphatic invasion | 0.196 | 0.106 | NC | NC |
| No | 47 | 48 | | |
| Yes | 23 | 24 | | |
| Perineural invasion | 0.529 | 0.464 | NC | NC |
| No | %42 | %44 | | |
| Yes | %24 | %26 | | |
| Surgical Margin | 0.003* | 0.001* | 0.011* | 0.002* |
| Negative | 51 | 53 | 2.64 | 2.17 |
| Positive | 15 | 13 | (1.15-4.47) | (1.18-3.96) |
| TSR | 0.009* | 0.004* | 0.037* | 0.026* |
| Negative | 50 | 50 | 2.11 | 2.28 |
| Positive | 16 | 14 | (1.21-4.46) | (1.28-4.63) |
| LIR | 0.005* | 0.001* | 0.028* | 0.016* |
| Negative | 51 | 52 | 2.76 | 3.19 |
| Positive | 15 | 12 | (1.32-4.55) | (1.49-4.64) |
| Tumour budding | 0.102 | 0.096 | NC | NC |
| Negative | 49 | 48 | | |
| Positive | 20 | 21 | | |
| MSI | 0.852 | 0.798 | NC | NC |
| MMR-P | 54 | 43 | | |
| MMR-D | 22 | 10 | | |
| CXCL12 | 0.001* | < 0.001* | 0.025* | 0.001* |
| Negative | 54 | 53 | 2.74 | 3.54 |
| Positive | 15 | 12 | (1.48-4.71) | (1.52-4.67) |

**Table IV.** Cont.

| | Univariable Analysis | | Multivariable Analysis | |
|---|---|---|---|---|
| | OS | RFS | OS | RFS |
| | P-value (5-year survival,%) | P-value (5-year survival,%) | P-value (HR 95% CI) | P-value (HR 95% CI) |
| CXCR4 | 0.001* | < 0.001* | 0.023* | 0.003* |
| Negative | 53 | 54 | 2.84 | 3.45 |
| Positive | 15 | 10 | (1.42-4.68) | (1.48-4.77) |

** P-values below 0.05 were considered statistically significant.*

*CXCL12 – chemokine ligand type 12; CXCR4 – chemokine receptor type 4; TSR – tumour stroma-ratio; LIR – local inflammatory response; MSI – microsatellite instability; MMR-D – mismatch repair proteins deficiency; MMR-P – mismatch repair proteins proficiency; HR – hazard ratio; CI – confidence interval; NC – not calculable*

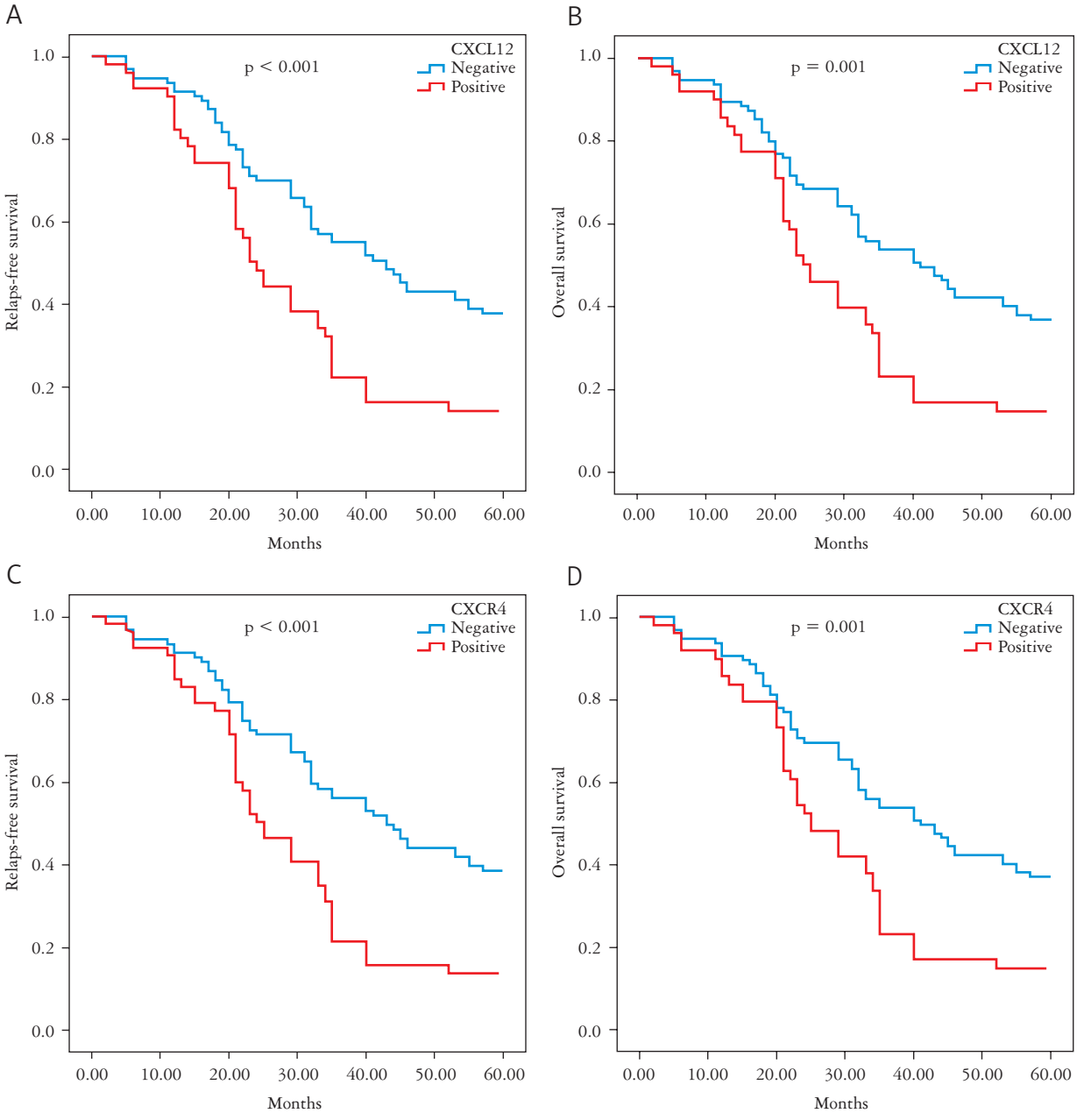


**Fig. 2.** Survival curves of CXCL12 and CXCR4. Kaplan-Meier survival curves of CXCL12 (A, B) and CXCR4 (C, D)

*P-values below 0.05 were considered statistically significant*

*CXCL12 – **chemokine ligand type 12; CXCR4 – chemokine receptor type 4***

these two parameters were significantly associated with vascular invasion. This finding is invaluable and can guide targeted therapies. For example, the proliferation of tumours can be limited by the CXCL12 or CXCR4 antagonists. Success can be achieved with further studies on this subject.

The CXCL12-CXCR4 axis plays an important role in organ targeted metastasis. CXCL12 is normally expressed in lung, bone, liver and lymph nodes and these organs are prominent in metastases. In tumour cells, CXCL12 and its receptor CXCR4 control chemotaxis, migration and metastasis. For example, studies have shown that anti-CXCR4 antibodies reduce tumour extravasation and metastasis [34]. Also, the CXCR4/CXCL12 axis has been reported to play a role in prostate cancer, melanoma and colorectal cancer liver metastases [35, 36]. In addition, blocking this axis has been shown to prevent metastasis to the lungs in breast cancer patients [37]. Moreover, increased CXCR4 expression has been shown to increase the risk of liver metastasis in primary breast cancer patients with axillary lymph node metastasis [34, 37]. In our study, we found that these two parameters were higher in patients with distant organ metastasis. This finding may guide target therapies. For example, specific metastases can be reduced or limited with anti-CXCR4 and anti-CXCL12 antibodies. Further studies are needed on this subject.

Leukocytes facilitate the recruitment of cell types specific to the tumour microenvironment with chemokine receptors, thereby providing an appropriate and effective immune response [33]. In general, the presence of leukocytes is assumed to be a manifestation of an effective immune response, although it is unclear whether this reflects different tumour biology or specific host properties. It is known that the infiltration of inflammatory cells is associated with survival in CRC tumours [33, 29]. For example, in CRC patients, whether in tumour stroma or cancer cell nests, a strong T cell infiltration is consistently associated with cancer-specific prognosis, regardless of nodal state and stage. That is, a massive T-cell infiltration, indicative of a coordinated adaptive immune response, appears to be one of the most important factors in predicting the outcome for patients resected for CRC [38]. In this study, we found that LIR was significantly lower in tumours expressing CXCL12 and CXCR4. This finding suggests that tumours use chemokines for progression. It also points out that these pathways can be used in treatment. We hope that larger studies will contribute to this issue.

Stromal cells around the tumour play a central role in the invasion-metastasis cascade, where cancer cells detach from the primary tumour, invade the surrounding stroma, penetrate the blood vessel wall, and move with the bloodstream to form a distant metastasis [16]. This stromal host tissue changes in a complex way during the progression of cancer cells. Also, these stromal cells play a key role in the growth, progression, and metastasis of cancer cells by producing various chemokines [16, 29]. Tumour stroma-ratio is an estimate of the ratio of tumoral and stromal cells. It has been reported in the literature that increasing tumour stroma increases the epithelial-mesenchymal transition (migration of the tumour to normal tissue), facilitates invasion into surrounding tissues, and leads to aggressive behaviour [39, 40]. Many studies of CRC have identified TSR as an independent prognostic marker and associated high amounts of stroma with adverse outcome. Also, it has been reported that the stroma of the tumour may affect the response to chemotherapy [39, 40]. On the other hand, the increase in immature stroma has been associated with tumour budding, which is known as an indicator of epithelial-mesenchymal transition [24]. In this study, we found that the rate of stroma was significantly higher in tumours expressing CXCL12 and CXCR4. This finding indicates that the metastasis stages of the tumours are related to chemokines. It also shows that these pathways can be useful in targeted therapies.

This research has many strengths. We tried to shed light on the tumour microenvironment studied in many studies. For this, we examined the relationship between reliable parameters associated with the tumour microenvironment. We conducted our study in a very homogeneous population. And we carried out our work in accordance with REMARK recommendations.

The limitations of our study are as follows. All retrospective studies have an internal limitation. In other words, it is not possible to overcome sampling bias. We know that the area we are evaluating is a small part of the tumour. Since our cases are treated according to the approaches before 2015, there may be differences according to the current treatment protocols.

In conclusion, in this study, we showed that CXCL12 and CXCR4 are poor survival factors in patients with lymph node-positive CRC and are associated with TSR and LIR. According to our findings, these parameters can be powerful biomarkers that can be easily used to improve risk stratification in daily practice. Also, these biomarkers can play a key role in understanding the tumour microenvironment and can guide target therapies.

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*The authors declare no conflict of interest.*

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