CANCER GENOMICS & PROTEOMICS *21*: 54-64 (2024)
doi:10.21873/cgp.20429

CXCL10 Expression in Human Colorectal Cancer Tissue and its Correlation With Serum Levels of CXCL10

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Abstract. *Background/Aim: CXCL10, a member of the CXC chemokine family, plays a crucial role in immune response by facilitating the chemotaxis of CXCR3-positive immune cells. We examined the expression of CXCL10 to unravel its functional significance in colorectal cancer. Materials and Methods: Bioinformatics analysis was performed to investigate CXCL10 expression and its clinicopathological relevance. Subsequently, we examined the correlation between the serum levels of CXCL10 and its expression within cancer tissues. Results: Analysis of the TCGA database revealed that elevated CXCL10 expression in CRC tissues correlates with improved long-term survival and is inversely associated with lymph node infiltration and metastasis. Insights from Gene Ontology and Kyoto Encyclopedia of Genes and Genomes further established a connection between increased CXCL10 and co-regulated gene expression with enhanced immune activation and regulation, mediated by the inhibition of the NOD-like receptor signaling pathway. Single-cell analysis pinpointed myeloid cells and macrophages as the primary sources of CXCL10. Immunohistochemical assessments revealed that a subset of cancer cells and macrophages are positive for CXCL10 expression. CXCL10-positive cells are predominantly located at the invasive front of the tumor. Intriguingly, our findings reveal an inverse correlation between serum CXCL10 levels and its expression in cancer tissues. Conclusion: The expression of CXCL10 may play a role in mediating the inflammatory responses at the invasive front in colorectal cancer and is observed to be inversely correlated with serum CXCL10 levels. It is pivotal to elucidate the distinct roles of CXCL10 in colorectal cancer, particularly different functions of cancer-tissue CXCL10 from serum CXCL10.*

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Key Words: CXCL10, colorectal cancer, tumor microenvironment; macrophages, invasive front.

Colorectal cancer (CRC) ranks as the third most common cancer diagnosis and the second leading cause of cancer-related death (1). A rising trend in early-onset CRC has been highlighted in recent studies (2). While the overall five-year survival rate for early-stage colon cancer is commendable at approximately 80%, the disease-free survival rate lingers at a subdued 55% (3). Alarmingly, progression to stage IV correlates with a precipitous drop in the five-year overall survival rate to a mere 16.9% (4, 5). These statistics underscore the critical necessity to elucidate the factors influencing tumor progression.

CXCL10, part of the CXC chemokine family, is a small protein integral to immune responses (6). Its significance in immunology is underscored by recent findings highlighting its role in directing the accumulation of immune cells, notably CXCR3-positive effector cytotoxic T lymphocytes (CTLs),

within the cancer microenvironment (7, 8). In kidney cancer cases, elevated serum levels of CXCL10 have been associated with an increased incidence of immune-related adverse events triggered by immune checkpoint inhibitors (9-11). CXCL10's pivotal role extends to mediating inflammation, guiding immune cells to sites of infection or injury. Emerging evidence implicates CXCL10 in tumor development and metastasis, signifying its influence on cancer progression (12, 13). It is posited that CXCL10 fosters tumorigenesis in inflammatory bowel diseases via several signaling pathways, including the JAK-STAT signal (14).

While the role of CXCL10 in colorectal cancer has been acknowledged, as previously described, its specific expression patterns within this cancer type remain unexplored. The advent of commercially available anti-human CXCL10 antibodies for immunohistochemistry, coupled with our recent findings of CXCL10 expression in kidney cancer cells, presents an opportunity for detailed examination (9). Consequently, we undertook an immunohistochemical analysis of CXCL10 in human colorectal cancer specimens and augmented this with bioinformatics analysis. We also assessed serum CXCL10 concentrations to explore the correlation with its expression in primary cancer tissues.

Materials and Methods

The study design is illustrated in Figure 1. Bioinformatics analysis of colorectal cancer (CRC) was conducted using data obtained from an open database, and samples of CRC patients, including serum and paraffin-embedded blocks, were sourced from Kumamoto University for this investigation.

TCGA database analysis. Differential expression of *CXCL10* between tumor and adjacent normal tissues across all TCGA tumors was assessed using the TIMER 2.0 database (http://timer.cistrome.org/). Patients were categorized into low or high CXCL10 expression levels based on a cutoff percentage of 25%. Kaplan-Meier survival analyses were conducted to evaluate short- and long-term survival in CRC patients. Biological replicates of 616 CRC and 45 paracancer tissues RNAseq datasets were downloaded, accompanied by clinical characteristics, from TCGA for analysis. Utilizing the original count for downstream analysis, we employed DESeq2 (Version 1.40.2) and clusterProfiler (Version 4.8.3) packages. Transcripts per million (TPM) metrics facilitated the analysis of clinically related data. In the linear regression analysis between *CXCL10* and all other genes, the top 3000 genes with the smallest p-values were earmarked for further Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis.

Human tissue specimens. Immunohistochemistry was performed on 43 paraffin-embedded CRC tissue blocks, while clinical data and biological replicates of 49 blood samples were analyzed to determine serum CXCL10 concentrations using the KE00128 Human CXCL10/IP-10 ELISA Kit (Proteintech, Rosemont, IL, USA), following the manufacturer's protocol. These specimens were previously collected from patients who underwent colorectal resections for CRC at Kumamoto University Hospital in Kumamoto, Japan, with CRC diagnoses confirmed *via* histopathological examination. Informed consent for inclusion in the study was secured from the patients' caregivers. The study's design and proposal received approval from the Institutional Review Board (IRB) at Kumamoto University (IRB No. #1016).

Single-cell clustering analysis. We employed pre-quality controlled single-cell RNA-seq data (GSE132465) derived from 23 colorectal cancer (CRC) patients, encompassing 23 primary colorectal cancer samples and 10 corresponding normal mucosa samples. Additionally, we utilized Visium gene expression data (GSE226997), which includes samples from 4 CRC patients with primary colorectal cancer. These datasets were obtained from the Gene Expression Omnibus (GEO) for our analysis. The criteria for quality control included cells with >1,000 unique molecular identifier (UMI) counts, >200 and <6,000 genes, and <20% mitochondrial gene expression in UMI counts. The Seurat package (Version 4.9.9.9039) in R (Version 4.3.1) facilitated the clustering and analysis of cells captured in the single-cell RNA sequencing. We categorized samples into "Tumor" and "Normal" groups. A global-scaling normalization method (LogNormalize) was applied to normalize each cell's gene expression. We identified the top 2000 variably expressed genes among cells and conducted a PCA, retaining the first 100 PCs for further analysis. To select significant PCs for the K-nearest neighbors cluster analysis, we implemented the JackStraw resampling method, involving 100 replicates of PCA on a 1% data subset. For graph-based clustering, we included all 100 PCs, and the "clustree" package (Version 0.5.0) aided in resolution-level selection. We opted for a 1.2 resolution, resulting in 23 cell clusters. Using the FindAllMarker function in the Seurat package, we identified the DEGs in each cluster. Wilcoxon's test, followed by Bonferroni correction, was employed to estimate the corresponding *p*-values.

Immunohistochemistry (IHC). Biological replicates of 43 colorectal cancer samples from the Kumamoto University have already been preserved as paraffin-embedded blocks. These were sliced into 3-μm thick sections using a microtome and then deparaffinized with xylene and ethanol. For immunohistochemistry, we used primary antibodies mouse monoclonal NCNP24 (WAKO Pure Chemical Industries, Ltd.) to detect Ionized calcium-binding adapter molecule 1 (Iba1) and goat polyclonal AF-266-NA (biotechne) to identify CXCL10. The secondary antibody was horseradish peroxidase-labeled anti-mouse or anti-rabbit immunoglobulin antibody (Nichirei, Tokyo, Japan), and positive signals were visualized using 3,3'-diaminobenzidine (Nichirei).

Statistical analysis. We utilized the ImageJ 1.53k software (National Institutes of Health, USA) for the relative quantification of stained IHC samples by measuring pixel density. For statistical analyses, GraphPad Prism 9.4.0 (GraphPad, San Diego, CA, USA) and R (version 4.3.1) were employed. Continuous data are expressed as mean ± SD. We applied one-way ANOVA coupled with a post-hoc Tukey's HSD test for a comprehensive comparison of means across different groups, considering a *p*-value of less than 0.05 as indicative of statistical significance.

Ethics approval statement. The study design was approved by the Institutional Review Boards (IRBs) of Kumamoto University (#1016) in accordance with the principles laid out in the Declaration of Helsinki of 1964 and later versions.

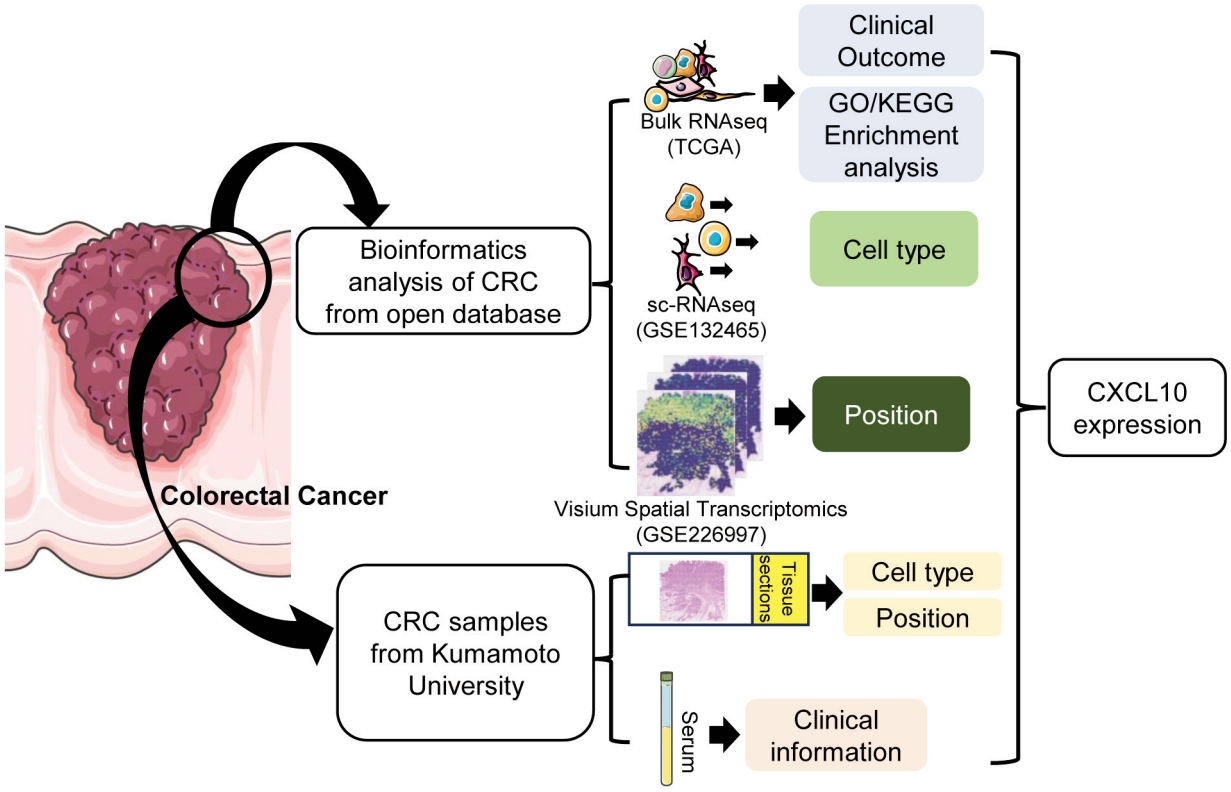

Figure 1. *Flow chart of the study design.*

Patient consent for publication statement. Informed consent for all procedures in this study was obtained from all patients. All samples (plasma and peripheral blood mononuclear cells) were obtained from patients in accordance with protocols approved by the IRBs of Kumamoto University (#1016).

Results

Increased expression of CXCL10 was associated with a better clinical outcome in colorectal cancer. We initially investigated the significance of *CXCL10* in colorectal cancer using TCGA database. Our findings revealed a notable increase in *CXCL10* expression in CRC tissues compared to normal colorectal tissues (Figure 2A). This upregulation was statistically significant ($p<0.001$) (Figure 2B). We stratified CRC patients into two groups based on *CXCL10* expression levels for further analysis. Kaplan-Meier survival analysis showed no significant impact of *CXCL10* expression on short-term survival outcomes at 3 months and 1 year. However, a significant disparity emerged in long-term survival at 3 and 5 years, with the *CXCL10* high group exhibiting better outcomes (Figure 2C). Analysis by TNM stage supported these findings; early-stage CRC (Stage II) patients had significantly higher *CXCL10* expression compared to those with advanced-stage disease (Figure 2D). Additionally, cases without lymph node infiltration or metastasis displayed elevated *CXCL10* expression levels compared to those with lymph node involvement or metastatic disease.

RNAseq revealed that CXCL10 expression correlates with the tumor microenvironment. Through the utilization of *CXCL10* and its co-regulated genes in GO analysis, we identified their involvement in the activation of immune response and regulation of innate immune response as part of biological processes. Additionally, these genes appeared to influence immune receptor activity within the domain of molecular function (Figure 3A). We further examined the average gene expression levels of various immune cell types to understand their association with *CXCL10* regulation in the tumor microenvironment. These cell types included macrophages (*AIF1*, *CD163*, *SIGLEC1*, *MRC2*, *ARG1*), T cells (*CD8A*, *CD8B*, *CD4*, *FOXP3*), NK cells (*ITGAM*), DC cells (*CD40*, *ITGAX*), neutrophils (*S100A8*, *S100A9*), monocytes (*FCN1*), CAFs (*ACAT2*), and B cells (*MS4A1*). We noted that macrophages, T cells, and neutrophils exhibited higher expression in the *CXCL10* high CRC group (Figure 3B). Linear regression modeling supported a positive association between *CXCL10* and the related genes (Figure 3C). KEGG pathway analysis suggested that *CXCL10* and its co-regulated genes

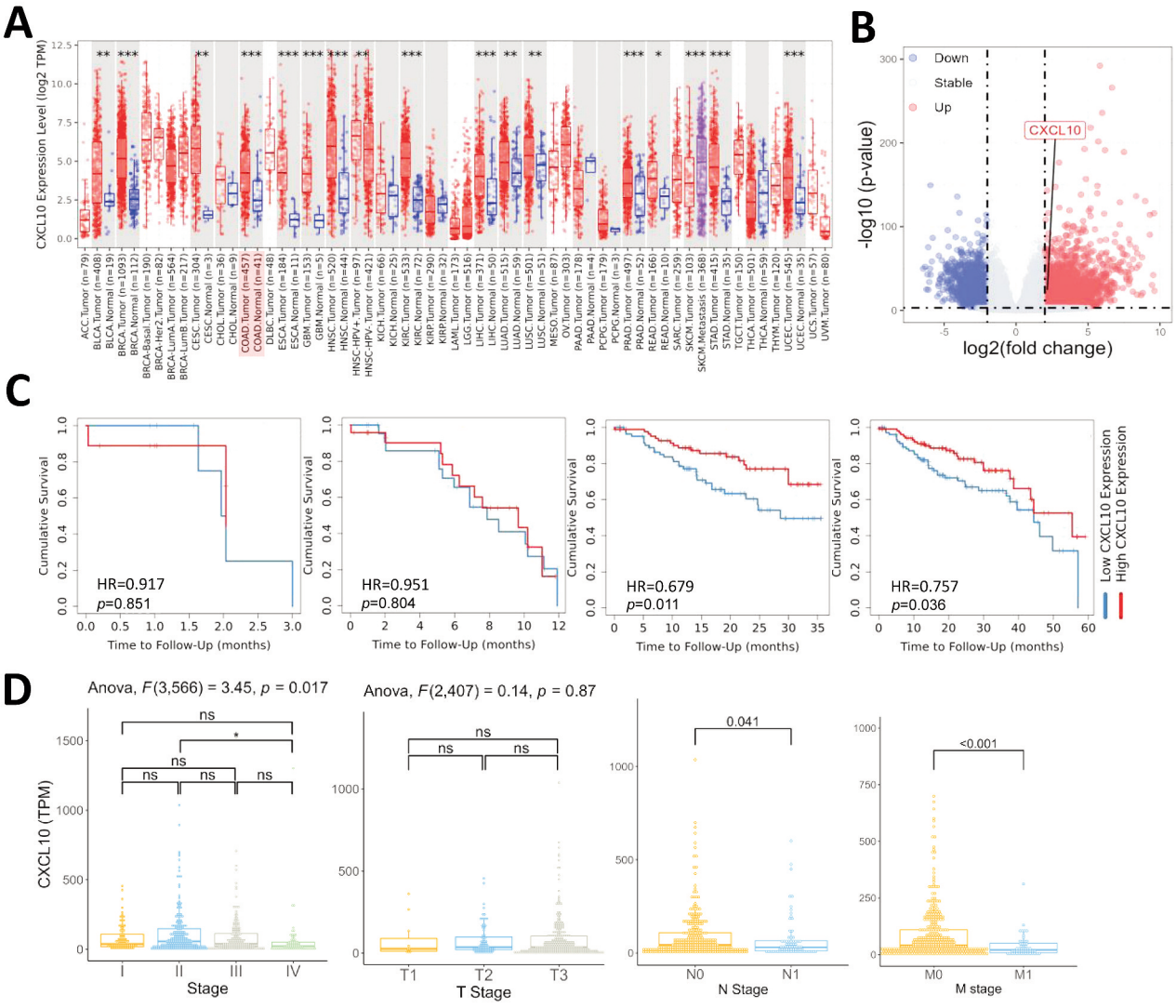

Figure 2. *Expression profile and prognostic significance of CXCL10. (A) The TCGA database showed that CXCL10 is highly expressed in colorectal cancer (marked with red box). (B) Differential genes in 45 normal paracancer tissues and 616 CRC tissues from TCGA database were analyzed by R language to target CXCL10 based on log|FC| >2, p<0.001. A total of 5,026 up-regulated and 1,251 down-regulated genes were identified, mapped by a volcano plot. (C) CXCL10 level is not related with short-term survival rate (3 month and 1-year), but high CXCL10 expression have a higher long-term survival rate (3 and 5-year). (D) Relationship between CXCL10 expression level and TNM stage. *p<0.05, **p<0.01, ***p<0.001.*

might function through the NOD-like receptor signaling pathway (Figure 3D). We delved deeper into investigating *NOD1*, *NOD2*, and their co-regulated gene *RIP2*, uncovering that *CXCL10* might exert inhibitory effects on the entire NOD-like receptor signaling pathway (Figure 3E, F).

Single-cell analysis indicated that myeloid cells expressed high levels of CXCL10, while cancer cells, B cells, T cells, and stromal cells exhibited lower levels of this cytokine. Subsequently, an *in silico* analysis was conducted using published single-cell sequencing data of colorectal cancer to investigate the expression of *CXCL10* mRNA in specific cell types. Pre-annotated cells were categorized into six distinct groups (Figure 4A). Notably, both myeloid and stromal cells exhibited an increased presence in tumor tissues, while B cells demonstrated decreased abundance in tumor tissues compared to normal tissue (Figure 4B). The expression of the *CXCL10* gene was detected in various cell types, with prominent presence observed in myeloid cells (Figure 4C, D). Both the positive cell rate and the level of *CXCL10* expression in tumors surpassed those in the corresponding normal control cells among four cell types, including epithelial cells, myeloid

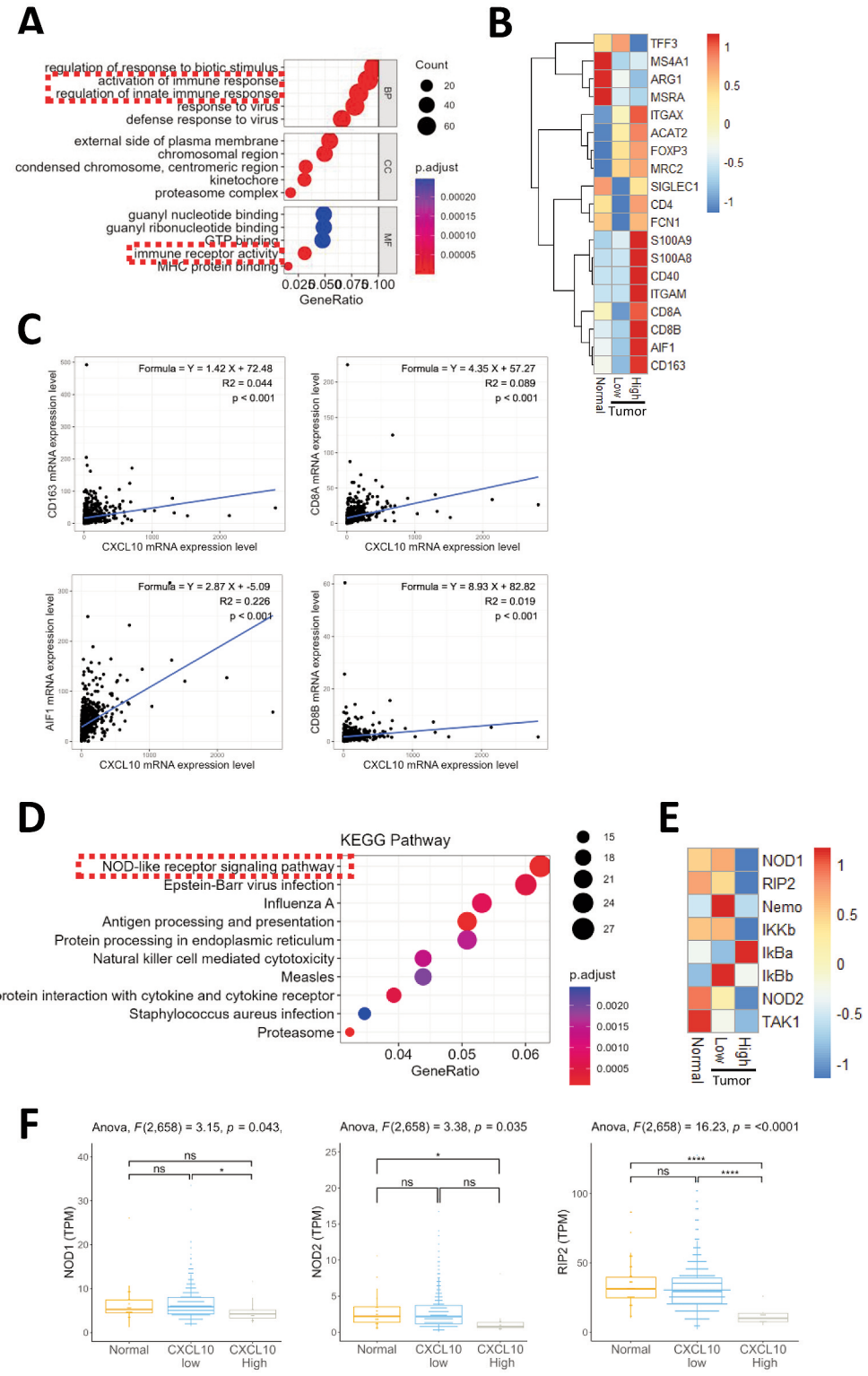

Figure 3. *GO and KEGG analysis of CXCL10 and its co-regulation genes. (A) GO analysis is presented by point charts. (B) Gene expression level of tumor and tumor microenvironment cells in normal and tumor tissues. (C) Linear regression of CXCL10 with macrophages (CD163, AIF1) and T-cells (CD8A, CD8B). (D) KEGG analysis is presented by point charts. (E) Gene expression level of NOD-like receptor signaling pathway related genes in normal and tumor tissues. (F) Expression levels of key NOD-like receptor signaling pathway genes. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

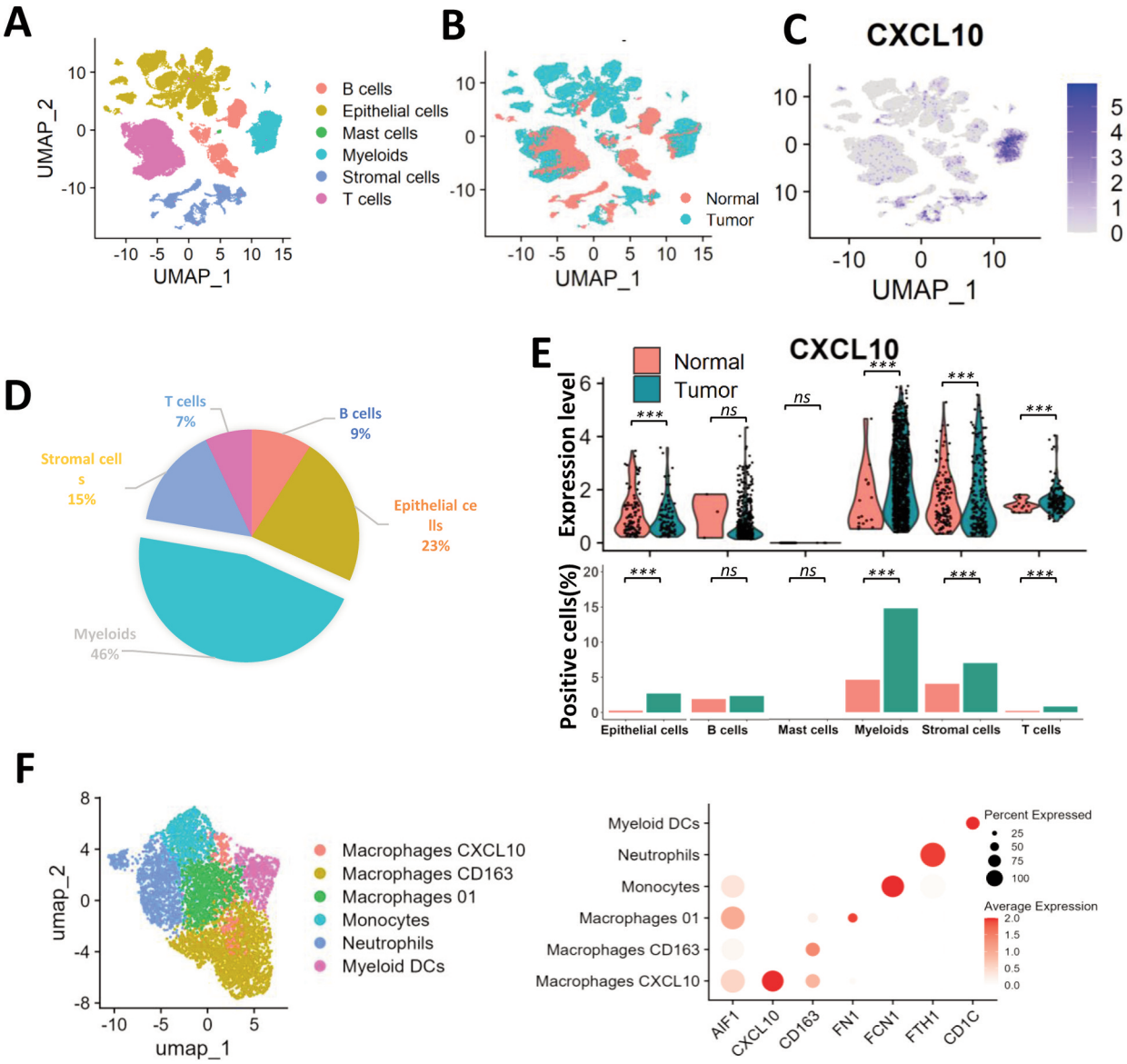

Figure 4. *Single cell analysis of colorectal cancer. Uniform manifold approximation and projection (UMAP) of colorectal cancer cells, annotated by cell type (A), and cell source (B). (C) Feature plot of CXCL10 expression. (D) Composition of positive CXCL10 cells. (E) Expression levels and positive rate of CXCL10 in each cell type. (F) UMAP of re-clustered myeloid cells and its marker.*

cells, stromal cells, and T cells. However, a majority of the positive cells were concentrated within the myeloid cluster (Figure 4E). Further subdivision of myeloid cells into six groups revealed that *CD163*-negative macrophages exhibited robust *CXCL10* expression (Figure 4F).

CXCL10 was detected at the invasive front, consistent with the results of the single-cell analysis. IHC revealed that CXCL10-positive cells were detected in tumor cells and macrophages at the invasive front but were not observed in the tumor central nest (Figure 5A, B). This observation was corroborated by results from the Visium single-cell analysis (Figure 5C). Additionally, double IHC showed that the majority of CXCL10-positive non-tumor cells were identified as macrophages (Figure 5D). In all examined normal paired colorectal cancer (CRC) tissues, CXCL10 expression was absent. However, a significant proportion of cases displayed positive CXCL10 expression within tumor cells. Specifically, 18.6% of the cases expressed CXCL10 in the tumor nest, while a markedly higher percentage, 46.5%, exhibited CXCL10-positive tumor cells at the invasive

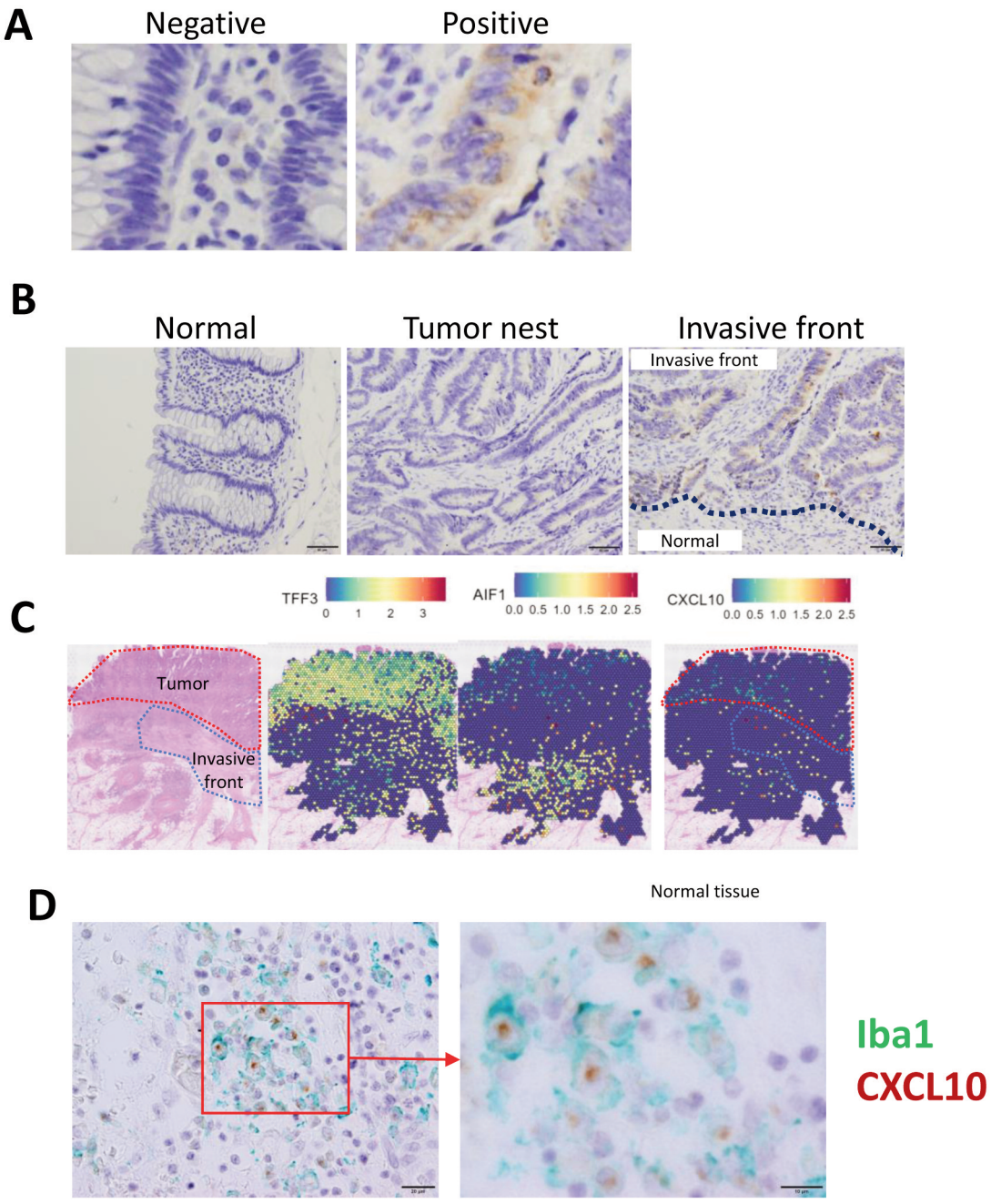

Figure 5. *Immunohistochemical staining of CXCL10 in colorectal cancer samples. (A) Negative and positive CXCL10 part in colorectal cancer samples. (B) Increased expression of CXCL10 in invasive front. (C) RNA expression level of CXCL10 in Visium single-cell analysis. (D) Double IHC of Iba1 and CXCL10.*

fronts (p<0.01). A detailed examination of the tumor nest also revealed the presence of CXCL10-positive macrophages in 46.5% of the cases, and this expression increased dramatically to 83.7% at the invasion front (p=0.010). These findings suggest a potential spatial pattern of CXCL10 expression, with a noticeable increase at the invasive fronts of the tumors (Table I).

Serum CXCL10 levels were inversely correlated with CXCL10 expression at the primary site. In the final step, we tested the CXCL10 level in blood and analyzed its correlation with CXCL10 expression in cancer tissues and other clinicopathological profiles. An increased CXCL10 level in blood was observed in cases with right-side colon cancer

Table I. *CXCL10 expression in normal epithelium, tumor cells, and TAMs.*

	CXCL10 expression	
	Negative	Positive
Normal epithelium	43 (100%)	0 (0%)
Tumor cells in tumor nest	35 (81.4%)	8 (18.6%)
Macrophages in tumor nest	23 (53.5%)	20 (46.5%)
Tumor cells in invasive front	23 (53.5%)	20 (46.5%)
Macrophages in invasive front	7 (16.3%)	36 (83.7%)

TAMs: Tumor associated macrophages.

Table II. *Clinicopathological profiles of CRC patients in different CXCL10 expression groups.*

Variables	*CXCL10* expression level, n		*p*-Value
	High	Low	
Sex			
Male	11	14	
Female	13	10	0.386
Position			
Left	13	21	
Right	11	3	0.011
Size			
≥50 mm	6	11	
<50 mm	12	6	0.063
Tumor stage			
T3,4	14	15	
T1,2	10	9	0.768
Lymph node metastasis			
Positive	8	7	
Negative	16	17	0.755

CRC: Colorectal cancer. Statistical analyses were performed with the Chi-square test or fisher exact test.

(Figure 6A). However, no association was found between CXCL10 levels and sex, tumor size, stage, or lymph node metastasis (Table II). Serum CXCL10 concentrations did not correlate with the presence of CXCL10 in tumor nests or the invasive tumor front (Figure 6B). Notably, an elevated level of serum CXCL10 was distinctly observed in instances where macrophages lacked CXCL10 expression, both within tumor nests and at the invasive fronts (Figure 6C).

Discussion

In the present study, we initially assessed the significance of *CXCL10* mRNA expression using TCGA data and found that increased *CXCL10* expression was associated with a better clinical course in colorectal cancer. A similar result has been reported by Li *et al*. (15). They demonstrated that a high immune cell score and low stromal cell score were linked to favorable prognosis in colorectal cancer. They identified 15 hub genes, including *CXCL10*, associated with immune and stromal scores. In kidney cancer, increased *CXCL10* expression correlated with a high density of infiltrating immune cells, but was not associated with prognosis (16). *CXCL10* expression has been implicated in the formation of tertiary lymphoid structures (TLS) by interacting with *CXCL13* and *TNFSF13* in melanoma (17). TLS has been recognized as a prognostic factor for cancer patients treated with immunotherapy across various cancer types (18). Consequently, CXCL10 plays a crucial role in the immune microenvironment of colorectal cancer.

An *in silico* analysis using published single-cell RNA sequencing data revealed *CXCL10* gene expression in cancer cells and various types of immune cells, with the highest expression detected in macrophages. Macrophages infiltrating cancer tissues are known as tumor-associated macrophages (TAMs), which are typically associated with pro-tumor functions (19, 20). We observed an increased density of TAMs expressing osteopontin/SPP1 at the invasive front, linked to cancer cell expression of HLA-G, which is associated with immune system evasion in colorectal cancer (21). SPP1 and CXCL10 have been suggested as markers of monocyte-derived TAMs (22), indicating that these TAMs may express both markers. In our study, IHC analysis revealed CXCL10 expression in cancer cells and TAMs, with particularly strong expression at the invasive front. This suggests that TAMs in the invasive front of colorectal cancer exhibit both pro-tumor and anti-tumor activities. Identifying the key factors that regulate the balance between these opposing functions in TAMs is a subject of interest for future research.

Hematological examinations have been reported as validated for the screening of CRC patients (23). Thus, we hypothesized that the serum level of CXCL10 reflects its expression in colorectal cancer tissues and tested this correlation. However, we found a significant negative correlation between serum CXCL10 levels and CXCL10 expression in macrophages within cancer tissues. We have previously shown that circulating monocytes and lymph node macrophages express the *CXCL10* gene (13, 24). Interestingly, *CXCL10* expression was negatively correlated with the frequency of CD14+HLA-DRlow myeloid-derived suppressor cells. Although the source of serum CXCL10 has not yet been identified, circulating and lymph node monocytes are suggested to be the primary cells producing CXCL10.

In this study, positive CXCL10 expression was also found in tumor cells, predominantly at the invasive front. Colorectal cancer with neuroendocrine differentiation is known to secrete CXCL10, which recruits TAMs and leads to an unfavorable prognosis (25). Although no evaluation of neuroendocrine differentiation was conducted in our study, it is noteworthy that this differentiation is implicated in cancer cell invasion and

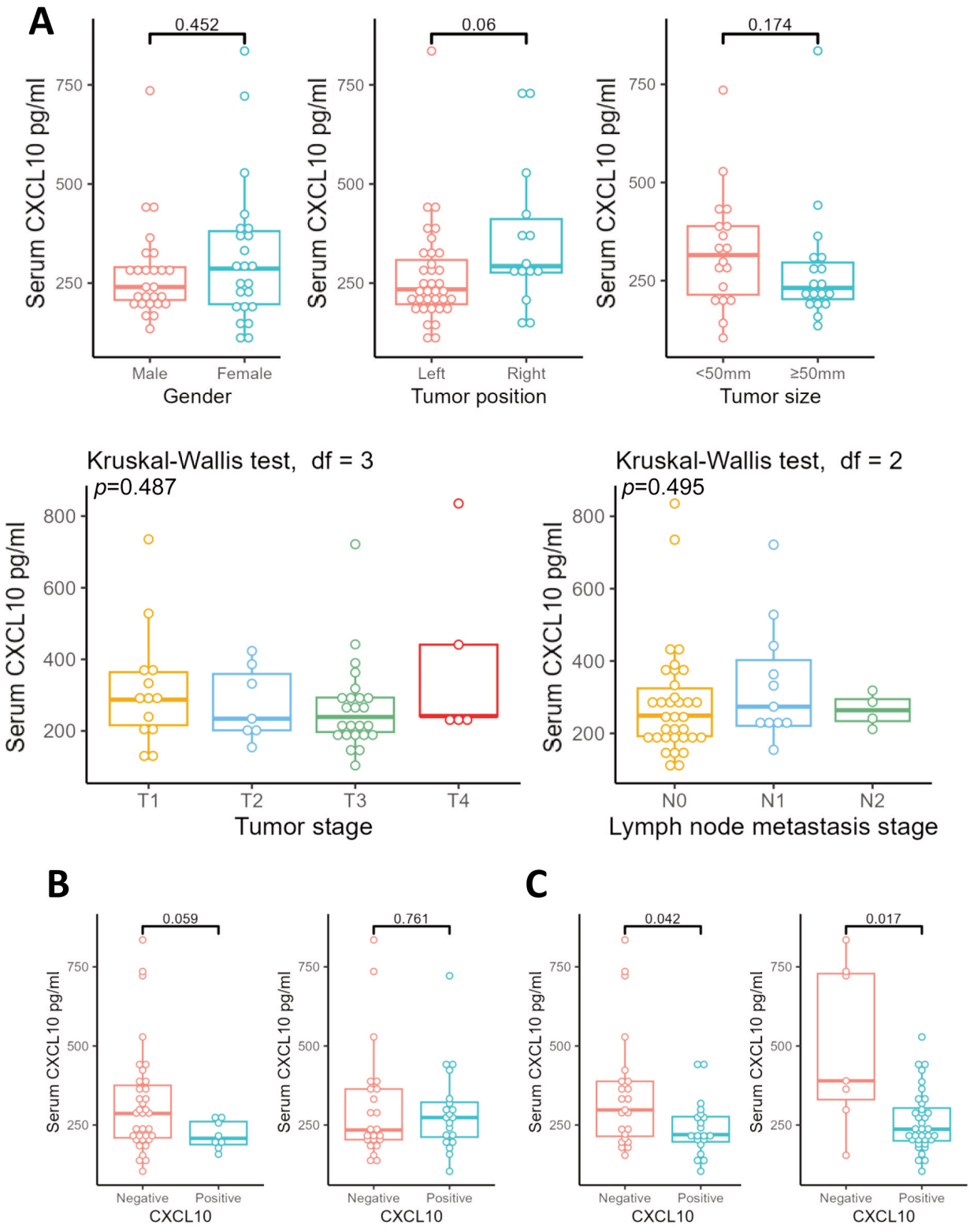

Figure 6. *Relationship between serum CXCL10 and patient clinicopathological profiles. (A) Serum CXCL10 level and its relationship with sex, tumor positivity, tumor size, tumor stage, and invasion of lymph nodes. (B) There is no correlation between serum CXCL10 levels and its expression within the tumor nest or at the invasive tumor front (C). A higher concentration of serum CXCL10 is observed in cases where macrophages are CXCL10 negative, both within the tumor nest and at the invasive front of the tumor.*

metastasis through autocrine and paracrine mechanisms (26). Consequently, the predominant presence of CXCL10-positive cancer cells at the invasive front might be associated with neuroendocrine differentiation.

In conclusion, we discovered that both cancer cells and TAMs, especially at the invasive front, express CXCL10, which is potentially involved in immune cell infiltration. CXCL10 appears to have two opposing functions: inducing antitumoral CTLs and pro-tumoral TAMs. The association of increased CXCL10 expression with a better clinical course suggests that the anti-cancer function of CXCL10 is predominant in colorectal cancer. Moreover, serum levels of CXCL10 were found to be negatively correlated with its expression in cancer tissues, indicating it may reflect the inflammatory activity of monocytes. Further research to uncover the detailed functions of CXCL10 at the invasive front will be of significant interest.

Conflicts of Interest

All Authors have no financial competing interests to declare.

Authors' Contributions

Lianbo Li conducted the TCGA and RNAseq data analysis and contributed to writing the manuscript. Kanemitsu Kosuke and Rin Yamada were responsible for the formal analysis. Koji Ohnishi and Yoshiki Mikami prepared the human tissue samples. Hiromu Yano and Yukio Fujiwara ensured the quality of the experiments. Taizo Hibi and Hideo Baba also contributed to writing the manuscript. Yoshihiro Komohara managed and coordinated the research activity planning and execution, in addition to writing the manuscript.

Acknowledgements

Lianbo Li was supported by the Otsuka Toshimi Scholarship Foundation, enabling the successful completion of this project. Figure 1 was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

Funding

This work was supported by Grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Nos. 20H03459).

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Received October 9, 2023
Revised November 20, 2023
Accepted November 30, 2023