

High-Level Expression of Chemokine CXCL16 by Tumor Cells Correlates with a Good Prognosis and Increased Tumor-Infiltrating Lymphocytes in Colorectal Cancer

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Abstract

CXCL16 is a new member of the chemokine superfamily, which exists in a transmembrane as well as a soluble form. Its receptor CXCR6 is detected on CD4⁺ T cells, CD8⁺ T cells, and natural killer T cells. Here, we report a significant correlation of CXCL16 expression by tumor cells with the infiltration of T cells and prognosis in colorectal cancer (CRC). We first found that CXCL16 expression was consistently up-regulated more in tumor tissues than in normal mucosa derived from the same CRC patients. Four human CRC cell lines also expressed CXCL16 mRNA and secreted soluble CXCL16. We next examined the expression of CXCL16 and infiltration of lymphocytes in CRC specimens ($n = 58$) by immunohistochemistry. CRC patients with high levels of CXCL16 expression ($n = 43$) had higher levels of CD4⁺ and CD8⁺ tumor-infiltrating lymphocytes (TIL; $P < 0.01$) than those with low levels of CXCL16 expression ($n = 15$). Furthermore, the high CXCL16 expression group showed significantly better prognosis than the low CXCL16 expression group ($P < 0.05$). Collectively, our data suggest that the expression of CXCL16 by tumor cells enhances the recruitment of TILs, thereby bringing about a better prognosis in CRC. Thus, CXCL16 is a new prognostic biomarker and may be useful for the development of a more effective therapeutic strategy for CRC. [Cancer Res 2007;67(10):4725–31]

Introduction

Colorectal cancer (CRC) is one of the most common malignancies and a frequent cause of cancer-related death in developed countries. Almost half of all patients with CRC are cured by radical surgical resection of the primary tumor together with the corresponding lymphatic drainage area. At the time of initial diagnosis, ~20% of patients present with synchronous liver metastases and ~10% to 15% develop metachronous liver metastases. The 5-year survival rates are ~90% for Dukes' A, 50% to 65% for Dukes' B, and 15% to 25% for Dukes' C (1–3). Availability of an effective prognostic biomarker will help design a better therapeutic strategy for CRC patients.

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Chemokines are a family of a large number of small cytokines and two cleavable transmembrane proteins that induce the direct migration of cells through interactions with a group of seven transmembrane G protein-coupled receptors (4). It is now known that chemokines play important roles in the rapid recruitment of leukocytes in inflammatory responses as well as in homeostatic migration and tissue homing of lymphocytes (4). Recently, it has been shown that various types of cancer cells express chemokine receptors and that chemokines may play a role in cancer progression and/or organ-selective metastasis (5–7). In CRC cases, chemokine receptors such as CXCR4, CCR6, and CCR7 have recently been shown to be involved in the development of metastasis (8–10). High CXCR4 expression in the primary tumor also correlated with an increased risk of recurrence and a shorter survival time (8); however, relatively little is known about the role of chemokines produced by CRC tumor cells in cancer progression and prognosis.

CXCL16 (also called SR-PSOX) is a unique CXC chemokine that exists both in a transmembrane form and a soluble form (11–13). CXCL16 has been shown to possess multiple biological activities. Soluble CXCL16 is chemotactic for cells expressing its receptor CXCR6 (14) such as CD8⁺ T cells, CD4⁺ T cells, and natural killer (NK) T cells (13, 15, 16), whereas cell surface-anchored CXCL16 can function as a cell adhesion molecule for CXCR6-expressing cells and also as a scavenger receptor for phosphatidylserine and oxidized lipoprotein (SR-PSOX; refs. 12, 17). The expression of CXCL16 is observed on macrophages (12, 18), dendritic cells (19), fibroblast cells in rheumatoid arthritis synovium (20), and liver sinusoidal endothelial cells (21). In this study, we have shown that CRC tissues generally express CXCL16 at levels much higher than normal mucosa. We have further shown that high CXCL16 expression by tumor cells significantly correlates with a high level of tumor-infiltrating lymphocytes (TIL) and a better prognosis of CRC.

Materials and Methods

Cells. Four human CRC cell lines (Colo205, LS174T, SW480, and T84) were maintained in RPMI 1640 (Colo205 and SW480), EMEM (LS174T), and D/F-12 (T84) medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. The mouse L1.2 pre-B-cell line was kindly provided by Dr. E. Butcher (Stanford University School of Medicine, Stanford, CA). L1.2 cells stably expressing CXCR6 (L-CXCR6) or CXCL16 (L-CXCL16) were maintained in RPMI 1640 supplemented with 10% FBS and antibiotics. Peripheral blood mononuclear cells obtained from three healthy donors were stimulated with phytohemagglutinin for 7 days.

Tissue preparations. CRC tissue samples were obtained from 58 patients during surgery at the Toyama University Hospital and an affiliated

Table 1. Primer sequences used in RT-PCR analysis of human chemokines

Targets	Sequences of primers
CXCL16	5'-GCCATCGGTTTCAGTTCA-3' (forward) 5'-CAATCCCCGAGTAAGCAT-3' (reverse)
CX3CL1	5'-GGCAGAGCAGAGACCATCCCT-3' (forward) 5'-TGGGGACAAAGGTGAAGGAGA-3' (reverse)
XCL1	5'-ACACCATCACGGAAGGCTCCTTGAGA-3' (forward) 5'-AGTGAAATGAGCTGGCTGGCTGGAGA-3' (reverse)
CCL5	5'-CCCCGTGCCACATCAAGGAGTATT-3' (forward) 5'-CGTCCAGCCTGGGGAAGGTTTTTGTGA-3' (reverse)
CCL28	5'-AGAAGCCATACCTCCCATTCG-3' (forward) 5'-AGCTTGCACTTTCATCCACTG-3' (reverse)
CXCL12	5'-CCCTCTGTGAGATCCGCTCTTTGGCCT-3' (forward) 5'-TCTGATGGAACCTGAAACCCTGCTG-3' (reverse)
CXCL13	5'-TGATCGAATTCAAATCTGCCCGTG-3' (forward) 5'-AAGCTTGAGTTTGCCCCATACGCTCC-3' (reverse)
CCL11	5'-ACCACCTCTCACGCCAAAGCTCACAC-3' (forward) 5'-CGCCACAGATATCCTTGCCAGTTTG-3' (reverse)
CCL19	5'-CCAGCCTCACATCACTCACACCTTGC-3' (forward) 5'-TGTGGTGAACACTACAGCAGGCACCC-3' (reverse)
CXCR6	5'-ATGGCAATGTCTTTAATCTCGACAA-3' (forward) 5'-TGAAGCTGGTTCATGGCAGTATT-3' (reverse)
GAPDH	5'-TGAAGGTCGGAGTCAACGGATTGGT-3' (forward) 5'-CATGTGGCCATGAGGTCCACCAC-3' (reverse)

hospital between 1998 and 2001. The surgical materials were fixed in 10% neutral-buffered formalin, divided into tumor and nontumor areas, and then embedded in paraffin.

Immunohistochemical analysis for the validation of CXCL16 staining was conducted on optimum cutting temperature compound-embedded sections of frozen CRC tissues or formalin-fixed CRC tissues.

This study was approved by the institutional ethics committee and written informed consent was obtained from each patient.

Reverse transcription-PCR. This was done as previously described (7). In brief, total RNA was extracted using an RNeasy Mini Kit (QIAGEN GmbH)

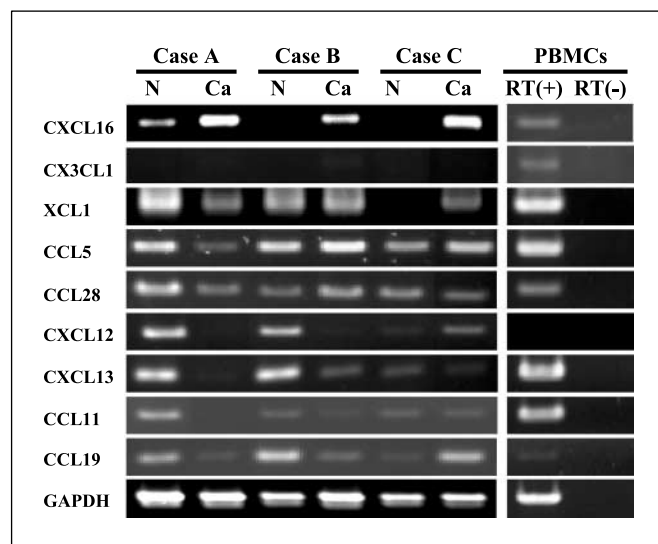


Figure 1. RT-PCR analysis of chemokine expression in CRC and normal mucosa. *N*, normal mucosa; *Ca*, CRC tissue. In all three cases, CXCL16 expression was stronger in cancer tissues than in normal mucosa. Other chemokines did not show such an expression pattern. GAPDH served as a loading control.

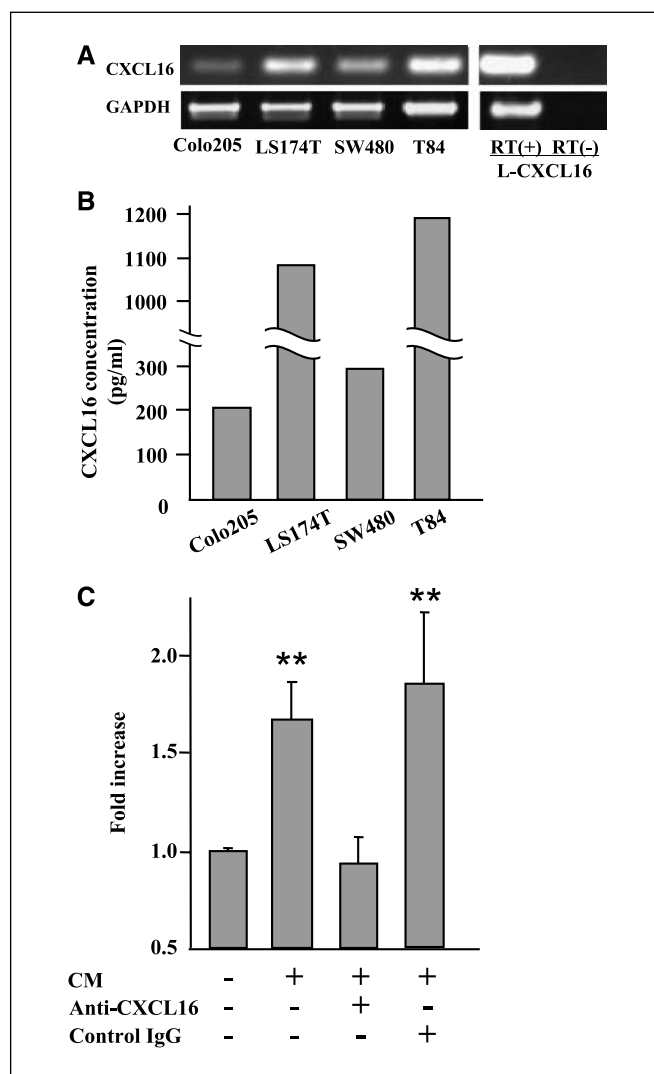


Figure 2. CXCL16 expression in human CRC cell lines. **A**, RT-PCR analysis of CXCL16 expression in four human CRC cell lines. L-CXCL16 was used as a positive control. **B**, measurement of soluble CXCL16 in conditioned media of four human CRC cell lines by ELISA. **C**, chemotactic activity of CXCL16 secreted by LS174T. LS174T supernatants were added to the lower wells with or without neutralizing anti-CXCL16 antibody. DiA-labeled L-CXCR6 cells were placed in the upper compartments of transwell chambers. After 6-h incubation, cells that migrated into lower wells were lysed and quantitated by fluorescence intensity measurement. Conditioned media (CM) of LS174T cells induced chemotaxis in L-CXCR6 cells and anti-CXCL16 antibody completely abrogated chemotactic activity.

according to the manufacturer's directions. First-strand cDNA was prepared from an RNA template (2 μ g) using oligo(dT)₁₈ primer and SuperScript II reverse transcriptase (Invitrogen). Reverse transcription was done at 42°C for 50 min and then at 70°C for 15 min. PCR amplification was done by denaturation at 94°C for 30 s, annealing at 60°C for 60 s, and extension at 72°C for 90 s for 28 cycles [24 cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] using a TaKaRa Ex Taq HS PCR kit (TaKaRa Shuzo Co. Ltd.). The primer sequences are listed in Table 1. All primers were verified to yield the expected products under the indicated conditions. PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide.

ELISA. Conditioned media of human CRC cell lines were collected in 1.5-mL tubes, centrifuged at 2,000 rpm for 5 min to remove cell debris, and kept at -80°C until assay. CXCL16 was measured by ELISA using a Human CXCL16 ELISA Development Kit (PeproTech). Assays were done in

duplicate and absorbance at 450 nm was read on a Wako/Tecan immunoplate reader.

Chemotaxis assay. LS174T cells were cultured in serum-free AIM-V medium (Life Technologies) for 48 h and conditioned media were collected. L-CXCR6 labeled with DiA were applied to the upper wells of transwell chambers of 5- μ m pore size (KURABO) in 100 μ L of assay buffer [RPMI 1640 supplemented with 20 mmol/L HEPES (pH 7.4), 0.5% bovine serum albumin (BSA)]. Aliquots of conditioned media were pretreated with or without antihuman CXCL16 antibody (R&D Systems, Inc.) or control immunoglobulin G (IgG) for 30 min at 37°C and then applied to the lower wells in a volume of 350 μ L. After 6 h at 37°C, cells that migrated into lower wells were lysed with 0.2% TX-100 (WAKO) in 20% ethanol and quantitated by fluorescence intensity measurement (ex, 595 nm; em, 485 nm).

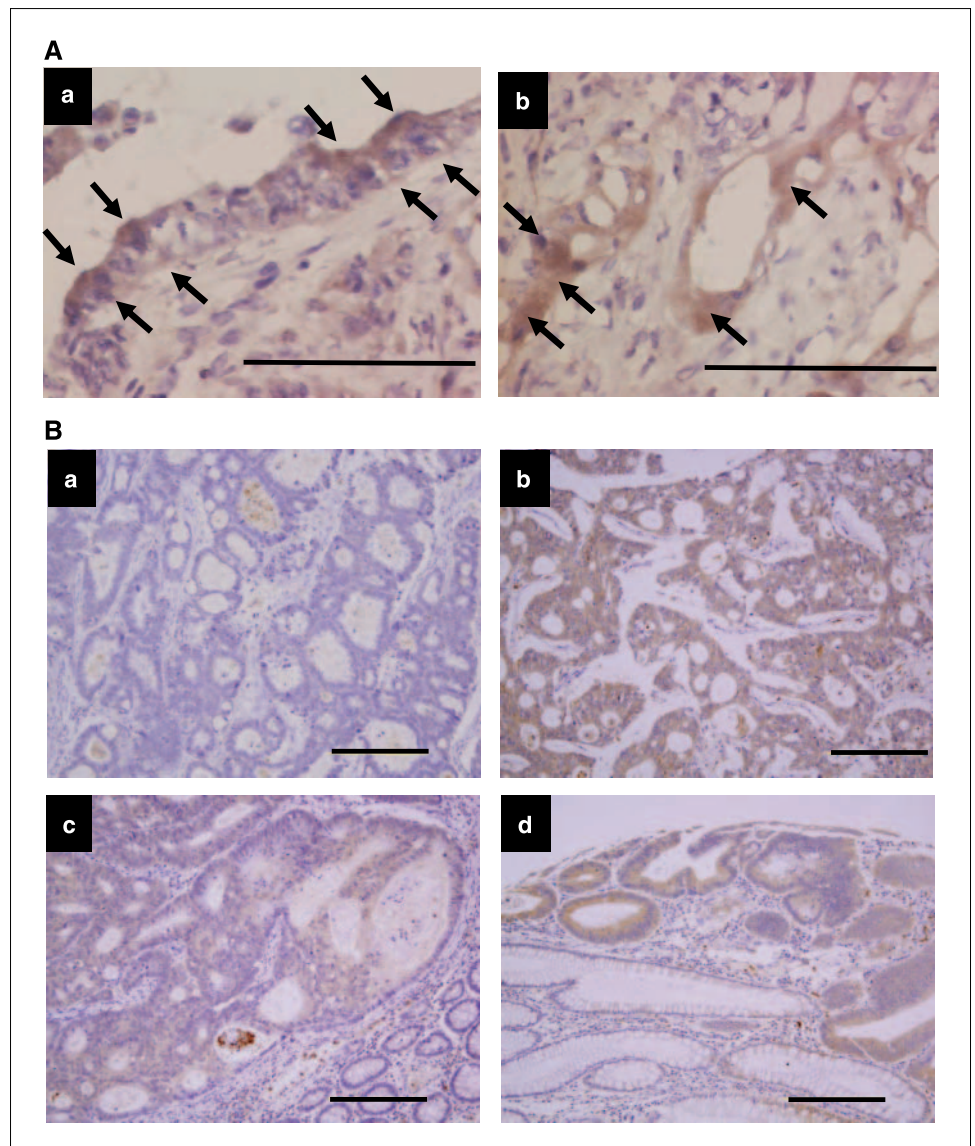
Immunohistochemistry. Cryostat sections of the frozen CRC tissues or formalin-fixed CRC tissues were rehydrated in TBS for 5 min thrice. Endogenous peroxidase was blocked with 3% H₂O₂ in methanol for 5 min and then with 5% bovine serum albumin in TBS for 30 min. Serial sections were then incubated for 1 h at room temperature with goat polyclonal antiserum against human CXCL16 (R&D Systems) or normal goat serum as a control. The samples were then washed twice for 5 min in TBS and expression was detected using the peroxidase-conjugated immune polymer reagent for goat polyclonal antibody (Simple Fine Stain for goat, Nichirei).

Sections were developed with 3,3'-diaminobenzidine (DAB; Sigma) and counterstained with hematoxylin.

Immunostaining of paraffin-embedded tissues from 58 CRC patients was done with our own procedure using microwave irradiation for antibody incubation (22). In brief, deparaffinization and standard antigen retrieval by microwave irradiation were done on paraffin sections. All sections were soaked in 3% H₂O₂ for 5 min. Goat polyclonal anti-CXCL16 antibody and mouse monoclonal anti-CD68 antibody (clone KP1, DakoCytomation) were applied to the specimens in a plastic moist chamber for 15 min under intermittent microwave irradiation (250 W, on for 4 s and off for 3 s; MI-77, Azuyama) followed by incubation at room temperature for an additional 45 min. Peroxidase-conjugated immune polymer reagent for goat polyclonal antibody (Simple Fine Stain for goat, Nichirei) or for mouse monoclonal antibody (EnVision⁺-PO kit, DAKO) was hybridized as the secondary antibody. Sections were developed with DAB (Sigma) and counterstained with hematoxylin.

To assess the distribution and frequency of CD4⁺ T lymphocytes and CD8⁺ T lymphocytes in CRC tissues, double immunostaining of CD4 and CD8 was done. First, tissues were incubated with mouse monoclonal anti-CD4 antibody (Novocastra) and developed with DAB as described above. The specimens were then soaked in boiled water for 10 min to denature the applied antibodies (23). Second, mouse monoclonal anti-CD8 antibody

Figure 3. Immunohistochemical staining of CXCL16 in CRC. *A, a*, nonfixed frozen section. CXCL16 expression was apparently observed on the surface of cancer cells (arrow). *b*, formalin-fixed frozen section. CXCL16 was expressed in the cancer membrane as well as the cytoplasm (arrow). *B, a*, no CXCL16 expression case (absent). *b*, strong expression case. CXCL16 expression was observed in the cell membrane and cytoplasm of CRC cells. *c*, strong expression case. Note that, in the lower right part, normal colon epithelial cells were negative for CXCL16. *d*, the cytoplasm and cell membrane of the colon adenoma showed strong expression of CXCL16 but normal colon epithelial cells were negative (lower part). Magnification, $\times 200$ (A), $\times 100$ (B). Bar, 200 μ m.



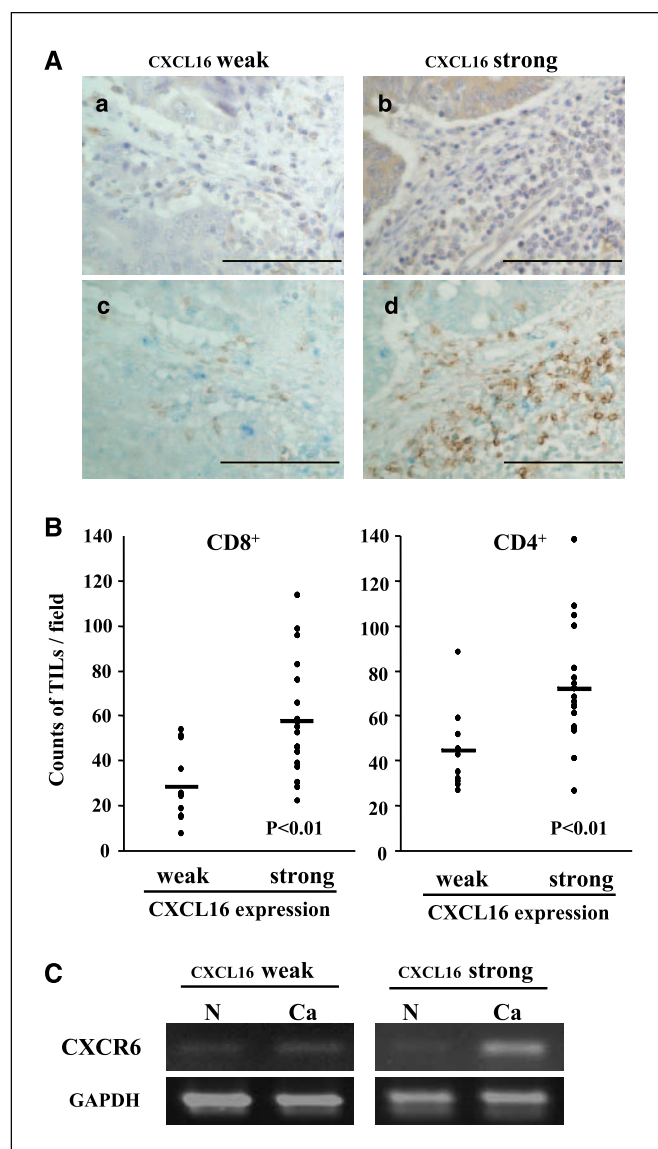


Figure 4. Immunohistochemical staining of T cells in CRC. *A*, *a* and *c*, weak CXCL16 expression case. *b* and *d*, strong expression case. TILs were observed at the tumor border. *c* and *d*, double immunohistochemical staining for CD8 (blue) and CD4 (brown). In this staining, the majority of TILs were identified as CD8⁺ or CD4⁺ cells. A larger number of TILs infiltrated CXCL16 strong cases. *d*, $\times 200$ magnification. Bar, 200 μ m. *B*, TIL counts. CD8⁺ and CD4⁺ TILs were counted in five randomly selected areas at the tumor border for each case. The average counts of TILs were plotted and compared between weak and strong CXCL16 expression groups. The number of CD8⁺ cells was significantly increased in the strong group (mean \pm SD, 58.2 \pm 25.2 per field) as compared to the weak group (29.3 \pm 16.2 per field). Similarly, CD4⁺ cells were significantly increased in the strong group (71.9 \pm 26.1 per field) as compared to the weak group (44.2 \pm 17.8 per field). *C*, CXCR6 mRNA expression was analyzed by RT-PCR in a strong CXCL16 expression case and a weak expression case.

(DAKO), diluted 50-fold in TBS including 5% BSA, was applied, followed by incubation with alkaline phosphatase-conjugated immune polymer reagent for mouse (Envision-AP, DAKO), and then visualized with Fast Blue (Vector). Hematoxylin counterstaining was omitted. CD4⁺ T cells were labeled brown whereas CD8⁺ T cells were labeled blue.

Statistics. The association of staining intensity with clinicopathologic patterns was assessed with χ^2 test and unpaired Student's *t* test, when appropriate. Differences in the migration and count of TIL were evaluated with unpaired Student's *t* test. Survival rates were visualized by applying

Kaplan-Meier curves. *P* values were determined by the log-rank test. *P* < 0.05 was considered significant and *P* < 0.01 highly significant in all statistical analyses.

Results

Higher CXCL16 expression in CRC tissues than in normal mucosa. We first compared the expression of various chemokines in normal mucosa and tumor tissues derived from the same CRC patients by reverse transcription-PCR (RT-PCR; Fig. 1). Tumor tissues consistently contained *CXCL16* mRNA at much higher levels than normal mucosa. On the other hand, chemokines such as *XCL* (also called lymphotactin), *CCL5* (also called regulated on activation, normal T-cell expressed and secreted), and *CCL28* (also called mucosa-associated epithelial chemokine) were detected at mostly comparable levels in normal and tumor tissues, whereas chemokines such as *CXCL12* (also called stromal cell-derived factor 1 α), *CXCL13* (also called B-lymphocyte chemokine), *CCL11* (also called eotaxin), and *CCL19* (also called Epstein-Barr virus-induced molecule 1 ligand chemokine) were mainly detected in normal mucosa. Thus, only *CXCL16* was consistently up-regulated in CRC tumor tissues compared with normal mucosa.

Production of CXCL16 by CRC cell lines. Given that the expression of CXCL16 was selectively up-regulated in CRC cancer tissues compared with normal mucosa, we next examined the expression of CXCL16 in human CRC cell lines. All four CRC cell lines examined expressed *CXCL16* mRNA (Fig. 2*A*) and secreted CXCL16 protein in the culture supernatants (Fig. 2*B*). The mRNA expression levels and soluble protein levels were in good agreement, varying from high (LS174T and T84) to low (Colo205 and SW480). We also examined whether soluble CXCL16 produced by LS174T cells was chemotactic for mouse L1.2 cells stably expressing human CXCR6, the receptor for CXCL16 (19). As shown in Fig. 2*C*, the conditioned media significantly induced the migration of L-CXCR6 cells, and anti-CXCL16 but not control IgG completely neutralized the chemotactic activity of the conditioned media.

Immunohistochemical staining of CXCL16 in CRC tumor tissues. To begin with, immunohistochemical staining in CRC was done to compare CXCL16 expression patterns between frozen and formalin-fixed tissues because of the validation of the CXCL16 staining method.

As shown in Fig. 3, CXCL16 expression was apparently observed on the cell membrane of a frozen tissue section in CRC (Fig. 3*A-a*). Although it was the same tissue, CXCL16 was expressed in the cell membrane as well as the cytoplasm against formalin-fixed tissue section (Fig. 3*A-b*). The same studies done in 10 CRC tissue samples gave similar results. As the result of the validation, we next examined *in situ* localization of CXCL16 in 58 formalin-fixed CRC tissues. Representative results are shown in Fig. 3*B*. CXCL16 was clearly observed in the cell membrane and cytoplasm of cancer cells in 51 cases. Staining intensity was negative (7 cases), weak (8 cases), intermediate (22 cases), and strong (21 cases). Furthermore, we observed that some colon adenoma tissues were clearly positive for CXCL16 in the cell membrane and cytoplasm (Fig. 3*B-d*). In contrast, no clear positive staining of CXCL16 was observed in normal colorectal mucosa except for two weakly positive cases (data not shown).

Correlation between increased TILs and strong CXCL16 expression in CRC. Given that CXCL16 is chemotactic for cells expressing CXCR6 such as Th1 cells and NK T cells (11, 13, 17), the high level expression of CXCL16 by tumor cells may attract these types of immune cells to CRC. We therefore carried out

immunohistochemical staining of CD4 and CD8 in CRC tissues. Representative results are shown in Fig. 4A. As summarized in Fig. 4B, the numbers of CD8⁺ T cells and CD4⁺ T cells were significantly increased in the high CXCL16 expression group (58.2 ± 25.2 and 71.9 ± 26.1 per field, respectively) compared with the weak expression group (29.3 ± 16.2 and 44.2 ± 17.8 per field, respectively). Given that the levels of CXCL16 expression by tumor cells significantly correlated with the levels of TILs, it would be interesting to see the expression of its receptor, CXCR6, by TILs in CRC tissues. Unfortunately, however, anti-CXCR6 suitable for immunohistochemical staining was not available. Therefore, we examined the expression of *CXCR6* mRNA in normal mucosa and tumor tissues from the same CRC patients by RT-PCR (Fig. 4C). Tumor tissues with strong CXCL16 expression indeed contained CXCR6 mRNA at higher levels than those with weak CXCL16 expression and normal mucosa.

As shown in Fig. 5A, CXCL16-positive mononuclear cells were observed in CRC tissue, and we therefore investigated to identify these intermediate cells and whether CXCL16 secreted from the cells might contribute to guiding TILs.

CXCL16-positive intermediate cells were identified as macrophages by immunohistochemical staining of CD68 antigen (Fig. 5B).

We next analyzed the association of CXCL16-positive macrophages and TILs. As shown in Fig. 5C and D, there was no significance between the numbers of macrophages and CD8⁺ and CD4⁺ TILs in CRC.

Accordingly, these results suggest that the main driving force of TILs into CRC is CXCL16 derived not from macrophages but from cancer cells in this study.

Correlation between high CXCL16 expression and a favorable prognosis of CRC. We grouped the 58 CRC cases into two groups according to the level of CXCL16 expression [i.e., the weak (absent/

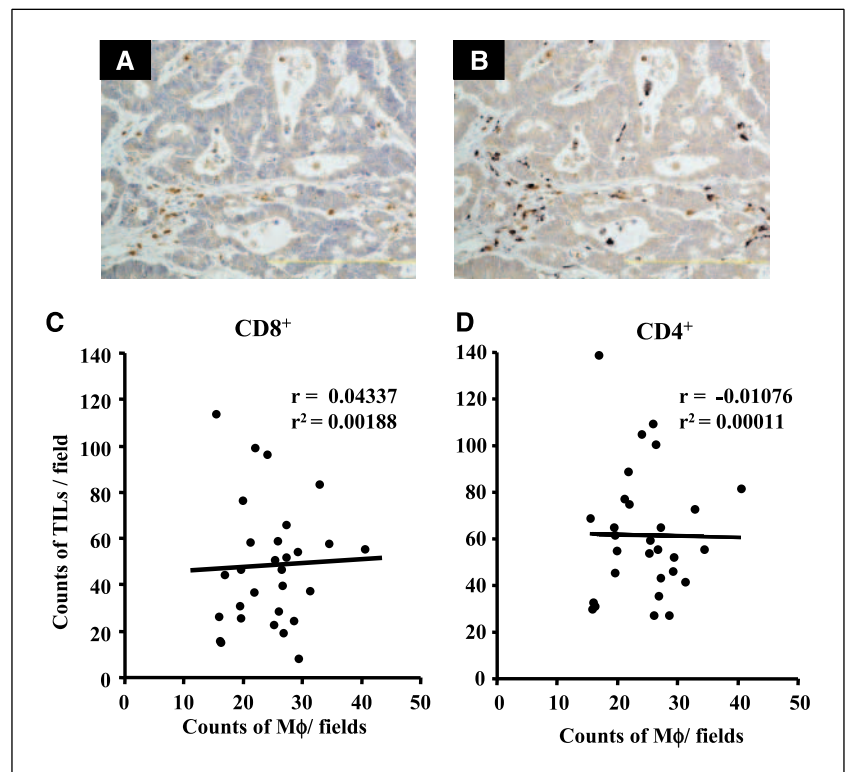
weak) expression group ($n = 15$) and the strong (intermediate/strong) expression group ($n = 43$)]. We compared these two groups for age, sex, location of main tumor, histologic type, depth of tumor invasion, lymph vessel permeation, venous permeation, lymph node metastasis, liver metastasis, and clinical stage. As summarized in Table 2, the two groups showed no significant difference in any of these variables; however, we found a notable difference in prognosis between these two groups. As shown in Fig. 6, the strong expression group had a significantly better long-term prognosis than the weak expression group ($P < 0.05$).

Discussion

Immunohistochemical analyses of CRC specimens have amply shown that higher levels of TILs can be regarded as a favorable prognostic sign (24, 25); however, the molecular mechanisms whereby such TILs are recruited into tumors are still poorly understood.

CXCL16 is a unique transmembrane-type chemokine that can function as a chemoattractant as well as a cell adhesion molecule for cells expressing CXCR6 (13). In the present study, we have shown for the first time that CXCL16 is selectively up-regulated in tumor cells in CRC tissues (Figs. 1 and 3). Furthermore, we showed that CRC tumors with strong CXCL16 expression contained higher levels of CD4⁺ and CD8⁺ TILs than those with weak CXCL16 expression (Fig. 4). Consequently, Fig. 5 showed that the strong CXCL16 expression group had a significantly better prognosis than the weak CXCL16 expression group in CRC. The exact relationship between high CXCL16 expression by tumor cells and high levels of TILs in CRC is not clear yet; however, given that CRC tumors with high CXCL16 expression also contained higher levels of *CXCR6* mRNA (Fig. 4), it is likely that CXCL16 produced by

Figure 5. CXCL16-positive macrophages and TILs. A, immunohistochemical staining of CXCL16. CXCL16-positive mononuclear cells were observed in cancer tissue. B, immunohistochemical staining of CD68. CD68-positive macrophages were identical to CXCL16-positive mononuclear cells (A). C and D, no correlation between the number of CXCL16-positive macrophages and CD4⁺ and CD8⁺ TILs.



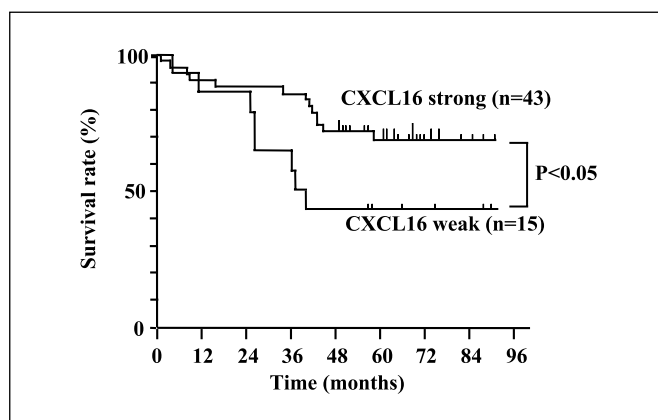


Figure 6. Kaplan-Meier survival curves of 58 CRC patients. The CXCL16 strong expression group had significantly longer survival than the weak expression group (log-rank, $P = 0.041$).

tumor cells attract CXCR6-expressing T cells such as Th1 cells and NK T cells (11, 13, 17, 26). A fraction of TILs attracted by CXCL16 may be directed to cancer cells, leading to a certain degree of antitumor immunity. Thus, CXCL16 is a new prognostic marker for CRC and may be, in part, responsible for the accumulation of TILs in CRC.

We also observed CXCL16-positive macrophages in CRC (Fig. 5). There was no significance between CXCL16-positive infiltrating macrophages and increased TILs. In consequence, no significance was confirmed in the relation between the numbers of macrophages and the prognosis in CRC (data not shown); however, given that the TILs were located around CXCL16-positive macrophages in some cases, this phenomenon implies that T-cell-mediated tumor immunity is triggered by CXCL16 secreted from macrophages in the local cancer region.

Recently, a number of studies have tested the antitumor effects of chemokines. The concept is that the expression of specific chemokines at the tumor site may attract T cells, NK cells, and dendritic cells bearing relevant chemokine receptors, which possibly leads to the induction of antitumor immunity. Significant tumor suppressive activity was reported for chemokines such as CCL3 (also called macrophage inflammatory protein-1 α), CCL21 (also called secondary lymphoid tissue chemokine), CCL27 (also called interleukin-11 receptor α -locus chemokine), and CX₃CL1 (also called Fractalkine) by transducing their genes into a variety of experimental tumors (27–31). It remains to be tested whether CXCL16 has any strong antitumor effect in such experimental tumors.

The major cause of death from CRC is liver metastasis. Thus, it is one of the independent prognostic factors in CRC. NK T cells, present at trace levels (<1%) in many organs, are highly enriched in the liver, where they represent up to 30% of lymphocytes (32). Geissmann et al. (33) reported that NK T cells patrol liver sinusoids to provide intravascular immunosurveillance, and CXCR6 contributes to liver-based immune responses by regulating their abundance. Thus, CXCR6⁺ NK T cells may play an important role in immunosurveillance against CRC metastasis in the liver. A future study focusing on CXCL16 expression by tumor cells and infiltration of NK T cells in CRC liver metastasis in connection with prognosis will be necessary to confirm this possibility.

CXCL16 expression is strongly up-regulated in CRC tumor cells compared with normal colon epithelium in the majority of CRC

cases (Fig. 1) and adenoma also expressed CXCL16 in the cytoplasm (Fig. 3B-d). In a previous study, no difference was found in the expression of CX₃CL1, another membrane-bound chemokine similar to CXCL16, between normal mucosa specimens and CRC specimens (34). Similarly, we also observed CX₃CL1 expression in both normal mucosa and CRC tissues (data not shown). These results have shown that the expression of CXCL16, but not that of CX₃CL1, is associated with colorectal carcinogenesis. Recently, remarkable elevation of soluble CXCL16 in the synovial fluids of rheumatoid arthritis patients was reported (35). It is thus interesting to see whether soluble CXCL16 in serum can also be elevated in CRC patients and is a good biomarker for prognosis.

It also remains to be seen why CRC tumor cells up-regulate CXCL16. Up-regulation of CXCL16 by CRC tumor cells could be potentially disadvantageous for their growth. Several molecules produced by cancer cells are known to have potent immunosuppressive activity. For example, transforming growth factor- β (TGF- β) produced by tumor cells typically induces immunosuppressive effects on T cells, especially on CD4⁺ T helper subsets, and cancer

Table 2. Clinicopathologic characteristics of CRC patients dependent on intensity of CXCL16 expression

CXCL16 expression	Weak	Strong	<i>P</i>
Number	15	43	
Immunohistochemical intensity	Absent/weak, 7/8	Intermediate/strong, 22/21	
Age (y)			
Average	66.3	65.9	0.928
Range	44–83	40–85	
Gender			
Female	3	13	0.522
Male	12	30	
Location			
Colon	11	27	0.671
Rectum	4	16	
Histology			
Well	8	29	0.266
Moderate	5	12	
Poor (Mucinous)	2 0	0 2	
Tumor invasion			
pT ₁ /pT ₂	4	12	0.808
pT ₃ /pT ₄	11	31	
Lymph vessel permeation			
Negative	6	15	0.966
Positive	9	28	
Venous permeation			
Negative	9	16	0.218
Positive	6	27	
Lymph node metastasis			
Negative	4	19	0.375
Positive	11	24	
Liver metastasis			
Negative	11	37	0.468
Positive	4	6	
Dukes			
A/B	4	19	0.375
C/D	11	24	

growth progresses to the immunosuppressed state induced by TGF- β (36). CXCL16 may have an unknown role in CRC tumor progression. Otherwise, up-regulation of CXCL16 by CRC tumor cells may represent a novel self-check mechanism against cancer progression through a chemokine expressed by cancer cells.

In conclusion, we have revealed that high CXCL16 expression by tumor cells significantly correlates with increased TILs and a better prognosis in CRC. Thus, CXCL16 is a new prognostic marker for CRC and may be useful for the future development of a more effective therapeutic strategy for CRC.

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