Disrupted Expression of CXCL5 in Colorectal Cancer Is Associated with Rapid Tumor Formation in Rats and Poor Prognosis in Patients

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Abstract Purpose: We isolated a subline (CC531M) from the CC531S rat colon carcinoma cell line, which grows and metastasizes much more rapidly than CC531S. We found, using RNA expression profiling, that one of the major changes in the CC531M cell line was a 5.8-fold reduction of the chemokine CXCL5. The purpose of this study was to determine the effect of CXCL5 expression on colorectal tumor growth and metastasis.

Experimental Design: CC531 clones were generated with either knockdown or restored expression of CXCL5. These clones were inoculated in the liver of rats. In addition, in two independent cohorts of colorectal cancer patients, the level of CXCL5 expression was determined and associated to clinical variables.

Results: Knockdown of CXCL5 expression in CC531S resulted in rapid tumor growth and increased number of metastasis, whereas restored expression of CXCL5 in CC531M resulted in a return of the "mild" tumor growth pattern of the parental cell line CC531S. In vitro, no difference was found in proliferation rate between clones with either high or low expression of CXCL5, suggesting that environmental interactions directed by CXCL5 determine tumor outgrowth. Finally, the importance of our findings was established for patients with colorectal cancer. We found that low expression of CXCL5 was significantly associated with poor prognosis for colorectal cancer patients. CXCL5 showed a trend (P = 0.05) for a positive correlation with intratumoral CD8+ T-cell infiltration, suggesting a possible explanation for the observed poorer prognosis.

Conclusions: Our results show that CXCL5 is important in growth and development of colorectal cancer, implicating a future role in both cancer therapy and diagnosis.

Colorectal cancer is one of the three leading causes of cancer-related death among men and women in the western world (1, 2). Despite curative surgical resection of the primary tumor, 40% to 50% of the patients ultimately die of metastases (3). Tumor growth and metastasis result from a complex cascade of biological processes. Therefore, knowing key factors in these processes is crucial to design new treatment modalities.

In a previous paper, we reported the in vivo selection of an aggressive rat colorectal cell line (CC531M) from the well-described CC531S cell line (4, 5). The present study was initiated to identify factors that contribute to rapid growth and metastatic capacity of CC531M. In this study, we focus on the chemokine CXCL5.

CXCL5 is a member of the subfamily of lipopolysaccharide-inducible ELR+ CXC chemokines (6). It functions, mainly through interaction with the CXCR2 receptor, both as a chemoattractant and as an angiogenic factor (7–10). CXCL5 is expressed in the epithelial cells of the colon and overexpressed in colorectal cancer (11, 12). It has been reported that CXCL5 plays a role in development and metastasis of several cancer types (13–15). CXCL5 contributes to the in vivo growth and angiogenic potential of non–small cell lung cancer. Homogenates of human non–small cell lung cancer specimens were angiogenic in the rat corneal micropocket assay, and the development of vasculature can be blocked by antibodies that neutralize CXCL5 (14). The role of CXCL5, produced by colorectal tumors, in relation to cancer progression and prognosis is poorly understood.

In this study, we investigated the expression of CXCL5 on tumor growth and metastasis in a colorectal tumor rat model. CC531 cells, expressing different levels of CXCL5, were inoculated in the livers of syngenic rats, and both tumor formation and metastasis were determined. CXCL5 expression was determined in two different independent large panels of human colorectal tumors and correlated with clinical follow-up and T-cell infiltration data.
Materials and Methods

CC531S and CC531M cell lines and culture conditions. The rat colon carcinoma cell line CC531S was originally developed using dimethylhydrazin in Wag/Rij rats (5). The aggressive CC531M was isolated from CC531S using an in vivo selection protocol (4, 16). Cells were cultured at 37°C and 5% CO2, in cell culture flasks (Corning) containing culture medium, composed of RPMI1640 (Life Technologies), supplemented with 10% heat-inactivated FCS, 100 μg/mL streptomycin, 100 IU/mL penicillin, and 2 mmol/L L-glutamine (all Life Technologies).

Development of CXCL5 knockdown and CXCL5-expressing CC531 clones. RNAi techniques were used to generate CC531S CXCL5 knockdown clones. A 19-nucleotide sequence (AACGGAGCTACGCTGTGTT), separated by a 9-nucleotide noncomplementary spacer (TTCAAGAGA) from the reverse complement of the 19-nucleotide sequence, was cloned and sequenced after digestion with BglII and HindIII and inserted into the pSUPER backbone (OligoEngine), using standard procedures. To obtain stably transfected CC531S CXCL5 knockdown and control clones, the pSUPER-CXCL5 siRNA or empty vectors were cotransfected with the pcDNA3 vector, using Lipofectamine 2000 (Invitrogen). Three CXCL5 knockdown CC531S clones (S1CXCL5-, S2CXCL5-, and S3CXCL5-) and three control clones (S4Control, S5Control, and S6Control) were selected.

To restore CXCL5 expression in CC531M clones, CXCL5 was amplified by routine PCR using cDNA derived from CC531S. Forward and reversed primers were designed, using the first or last complementary 20 bp in addition of a HindIII or EcoRI sequence, respectively. In front of the initial ATG code, a KOZAK sequence was placed. PCR products were directly cloned with the pGEM-T-Easy cloning kit (Promega) and sequenced. Expression plasmids for CXCL5 were obtained through unidirectional cloning of the sequence into the mammalian expression vector pcDNA3 (Invitrogen). To obtain stably transfected CC531M CXCL5-expressing and control clones, CC531M cells were transfected with pcDNA3-CXCL5 or control empty vector. Two CC531M clones expressing CXCL5 (M1CXCL5+ and M2CXCL5+) and two control clones (M3Control and M4Control) were selected. Selection was based on expression of CXCL5 as indicated by immunostaining. Stably transfected clones were grown under selective pressure, in culture medium supplemented with 200 μg/mL G418 (Sigma).

Immunocytochemistry. Cells were cultured on 12-mm glass coverslips and stained as previously described (17) using the primary antibody rabbit antimurine LIX (Peprotech EC Ltd; ref. 18) and Cy3-conjugated goat anti-rabbit secondary antibody (Jackson) in TATA box-binding protein (1 h in room temperature). Finally, cells were stained with 2 μg/mL Hoechst 33258 (Invitrogen). Cells were analyzed using a Nikon Eclipse E600 fluorescence microscope with a 40 plan fluor Nikon objective (Nikon). The percentage of CXCL5-positive tumor cells and the CXCL5 pixel intensity per cell were determined. For every clone or cell line, at least five randomly chosen fields were analyzed to determine the percentage of CXCL5-positive cells.

Cell proliferation. DNA content was used to determine the proliferation rate of CC531 cells and clones by a method previously described (19). In short, 25,000 cells were seeded into a 24-well plate. Medium was replaced daily. From 24 h up to 144 h after the start of seeding, each day plates were removed, rinsed with PBS, and stored at -80°C until assayed. On the day of assay, cells were

Fig. 1. CXCL5 expression is reduced in CC531M cells. A, immunofluorescent staining of CXCL5 expression of CC531S and CC531M (red, CXCL5; green, nuclei). B, percentage of CXCL5-positive CC531S and CC531M cells; columns, mean; bars, SE. C, in vitro proliferation rate of CC531S and CC531M cells on different time points; points, mean; bars, SE. *, statistically significant differences.
thawed and 200 μL of distilled water was added (1 h at 37°C). The plates were frozen at -80°C and thawed. From each well, 50 μL were taken and placed into a 96-well plate. DNA content was determined after addition of 50 μL of 20 μg/mL Hoechst 33258 fluorochrome (Invitrogen) and measured on a fluorostar optima plate reader (BMG Labtech GmbH).

**Rat experiments.** All animal experiments were approved by the animal experiment committee of Leiden University. Animals were kept in our own animal facilities. Male Wag/Rij rats (Charles River) were anesthetized with halothane, underwent laparotomy, and were double-blind randomized for induction of a liver tumor. A suspension of 5 × 10^6 viable CC531 tumor cells in 50 μL was injected subcapsulary at four sites into the liver. Per clone, four rats were inoculated. Rats were sacrificed by abdominal bleeding under halothane anesthesia, liver and tumor were removed, and weight was determined. To determine the number of lung metastasis, lungs were removed and filled with an ink solution, as previously described (20, 21).

**Reverse transcriptase PCR of CXCL5 in a patient cohort.** Tumors from a cohort of 70 patients that were curatively treated by surgery for colorectal cancer, between 1990 and 2001, were used to associate level of CXCL5 RNA expression with prognosis. Fifty percent of the patients were female. The mean age at the time of surgery was 67.2 y. Tumors were staged according to the American Joint Committee on Cancer criteria [47 (67%), stage I/II; 23 (33%), stage III; ref. 22]. At the time of censoring, 41 (59%) had died of whom 22 (54%) died from their disease, and 29 patients were still alive; 4 of them were alive with recurrence of the tumor. Mean follow up was 99 mo (range, 50-172 mo). Patient material was obtained with approval of local medical ethics committee. RNA from snap-frozen tumors, containing at least 60% tumor cells as determined by a pathologist, was isolated using RNeasy columns (Qiagen Sciences). Quantitative reverse transcriptase PCR (RT-PCR) primers for the detection of housekeeping genes (cleavage and polyadenylation specificity factor subunit 6, heterogeneous nuclear ribonucleoprotein M, and TATA-binding protein and CXCL5) were designed in PRIMER Express (Applied Biosystems) and span at least one exon-exon boundary. The primers used were as follows: cleavage and polyadenylation specificity factor subunit 6: 5′-AAGATTTGGCCTTCATGGAATTGAG-3′, 5′-TCGGTGATCTACTATGGTGCCTCCTCTC-3′; heterogeneous nuclear ribonucleoprotein M: 5′-GAGCCGATCATCCTCGG-3′, 5′-TTTGATGCCATCCTGGAATCTCG-3′; TATA box-binding protein: 5′-CAGGAACCAGGCCACTGAT-3′, 5′-TTTCTGTGCTGCCAGTCTGGAC-3′; CXCL5: 5′-CTGTGTTGAGACGCTGCGT-3′, 5′-GTTTTCCTTGTTTCCACCGTC-3′. RT-PCR reactions were performed on an ABI Prism 7900ht (Applied Biosystems) using the SybrGreen RT-PCR core-kit (Eurogentec). Cycle conditions were 10 min at 94°C followed by 40 cycles of 10 s at 94°C and 1 min at 60°C. Cycle threshold extraction was performed using the SDS software (version 2.2.2; Applied Biosystems). For all PCRs, a standard curve was generated using a five-step, 5-fold dilution of pooled cDNA from the HCT81 colorectal cancer cell line. Relative concentrations of mRNA for CXCL5 were expressed relative to the housekeeping genes.

**Fig. 2.** Knockdown of CXCL5 expression results in massive tumor outgrowth and formation of metastasis in vivo. A, the percentage CXCL5-positive cells of all knockdown and control clones was determined. Top bars, the average (av) number of CXCL5-positive cells; columns, mean; bars, SE. B, in vitro growth rate of all knockdown and control CC531S clones on several time points connected by a line. C, the average liver and tumor weight of both control clones versus the knockdown clones after inoculation in the liver of rats at sacrifice. Top bars, average of the knockdown versus the control clones. D, the number of lung metastases found after inoculation of the knockdown and control clones in the liver. *p < 0.001.
each gene were calculated from the standard curve. After RT-PCR, dissociation curves were made to check the quality of the reaction. Reactions with more than one peak in the dissociation curve were discarded. For normalization, the expression values for each gene were divided by the normalization factor of the gene (the average of the three house-keeping genes).

**Immunohistochemistry of CXCL5 in a patient cohort.** In a second independent cohort of 58 patients, curatively operated for colorectal cancer was used to associate protein level of CXCL5 to prognosis. The cohort comprised 43% females; mean age at the time of surgery was 66.2 y [29 stage I/II (50%) and 29 (50%) stage III colorectal tumors]. At the time of censoring, 46 (79%) had died; mean follow-up was 49 mo (range, 1.2-162 mo). Standard two-step, indirect immunohistochemistry was performed on 4-μm paraffin tissue sections, including blockage of endogenous peroxidase, EDTA antigen retrieval (not for CXCL5 detection), and diaminobenzidine development. To be able to distinguish intraepithelial from stromal infiltration, an additional staining for laminin was performed on CD4- and CD8-stained sections, including trypsin antigen retrieval and development using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution, as previously described (23). The primary antibodies used were as follows: the monoclonal antibody anti-CXCL5 (clone MAB254; R&D Systems), the monoclonal antibody anti-CD4 (clone 1F6; Novocastra), the monoclonal antibody anti-CD8 (clone 4B11; Novocastra), and rabbit anti-human laminin polyclonal Ab (Sigma-Aldrich). Secondary reagents used were anti-mouse horseradish peroxidase EnVision+ (K4001; Dako), biotinylated swine anti-rabbit IgG antibodies (DAKO Cytomation), and biotinylated-peroxidase streptavidin complex (SABC; DAKO Cytomation). CXCL5 expression was scored by microscopically assessing the percentage of CXCL5-positive tumor of the whole section. Infiltration in the tumor tissue was scored in three compartments of the tumor tissue, i.e., intraepithelially, intrastromally, and in the advancing tumor margin. Method of scoring has previously been described (23).

**Statistical analysis.** All analyses were performed with SPSS statistical software (version 12.0 for Windows; SPSS, Inc.). Mann-Whitney U test was used to compare variables. Kaplan-Meier analyses were performed to analyze patient survival. The entry date for the survival analyses was the time of surgery of the primary tumor. Events for time to disease-free survival were defined as follows: from time of surgery to time of disease relapse or death. Events for time to cancer-specific survival were defined as follows: from time of surgery to time of disease relapse or death by disease. Cox regression analyses were used to calculate hazard ratios (HR) with 95% confidence intervals (CI). Variables with a P value of ≤0.10 in the univariate analyses were subjected to a multivariate analysis. Pearson’s product-moment correlation was used to analyze correlations between level of CXCL5 expression and T-cell infiltration.

**Results**

**Expression of CXCL5 is lost in CC531M compared with CC531S cells.** We showed previously that subcapsular inoculation of the colorectal cell line, CC531M, in the liver of rats resulted in rapid tumor growth and induction of larger number of metastases compared with inoculation of the parental CC531S (4). Affymetrix microarray analysis was performed in

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**Fig. 3.** Examples of tumor outgrowth in the rat of a CXCL5 knockdown and a control CC531S clone. Top, tumor outgrowth of CXCL5 knockdown clone S1cxcl5- after inoculation of the clone in the liver of a rat at sacrifice (A-C). A, displays the peritoneal cavity with tumor throughout the liver and peritoneal metastasis. B shows the same liver after resection, overgrown with tumor mass; individual sites of inoculation cannot be distinguished. C, lungs of this rat after ink injection, with four metastases (one is indicated by an arrow). Bottom, tumor growth after inoculation with CXCL5-positive CC531S control clone S5control (D-F). D, tumor is only found in the liver at the four sites of inoculation and not in other places in the peritoneal cavity. E, the liver in detail, with four individual tumors (1-4). F, no lung metastases were found in this rat.
triplicate to determine differences in gene expression between the parental cell line CC531S and CC531M. The major change was a 5.8-fold (SD, 0.7) reduction of CXCL5 RNA content in CC531M cells compared with expression in CC531S. To confirm RNA expression data, cells were stained for the presence of CXCL5 protein using immunofluorescence techniques. In CC531S cells, a strong cytoplasmic staining was found, whereas CC531M cells hardly showed any staining (Fig. 1A). The percentage of CXCL5-positive cells was significantly ($P < 0.0001$) higher in CC531S cells compared with CC531M cells (Fig. 1B). In vitro, CC531S and CC531M showed the same proliferation rate (Fig. 1C).

Knockdown of CXCL5 expression results in aggressive tumor growth in vivo. To study the contribution of CXCL5 to tumor outgrowth and metastatic potency of CC531S cells, RNAi technology was used to knock down CXCL5 in this cell line. Three CXCL5-siRNA–transfected CC531S clones (S1CXCL5-, S2CXCL5-, and S3CXCL5-) and three control clones (S4Control, S5Control, and S6Control) transfected with the empty vector were selected. Immunofluorescence staining for CXCL5 expression showed a significant ($P < 0.0001$) CXCL5 down-regulation in S1CXCL5-, S2CXCL5-, and S3CXCL5- compared with S4Control, S5Control, and S6Control (Fig. 2A). In vitro, no significant difference in mean proliferation rate was found between CC531S CXCL5 knockdown clones and control clones (Fig. 2B). To study the effect of loss of CXCL5 in CC531S on tumor formation, each individual clone was subcapsularly injected in the liver of four rats. At sacrifice, rats injected with clones S1CXCL5- or S2CXCL5- showed large tumors overgrowing the whole liver and, in addition, also large tumor masses in the peritoneal cavity and lungs were found. Due to the massive tumor outgrowth, it was impossible to determine weight and surface of the individual tumors of rats inoculated with clones S1CXCL5- or S2CXCL5-. Therefore, the weight of both tumor and liver of all rats was determined. The third clone, CC531S clone S3CXCL5-, showed somewhat less aggressive outgrowth: four solitary liver tumors at the site of inoculation were found at sacrifice. Only one of the three control clones, S5Control, showed four small tumors at the place of inoculation, whereas the others (S4Control and S6Control) did not show any tumor formation in the liver. The average weight of tumor and liver of the three CXCL5 knockdown clones was significantly ($P = 0.001$) higher than the control clones (Fig. 2C). Furthermore, injection of CXCL5 knockdown clones resulted in significant more lung metastases compared with the control CC531S clones ($P = 0.007$; Fig. 2D). Images of in vivo tumor growth are displayed in Fig. 3. Together, these results show that although knockdown of CXCL5 in vitro did not result in difference in proliferation rate, in vivo CXCL5 knockdown in CC531S resulted in aggressive tumor growth accompanied with increased formation of metastases.

Restoration of CXCL5 expression results in less aggressive tumor growth in vivo. Stable transfection of CXCL5 into CC531M cells was used to study whether restored expression of CXCL5 would inhibit tumor growth and metastasizing capacity of CC531M in vivo. Two CXCL5-transfected clones (M1CXCL5+ and M2CXCL5+) and two control clones (M3Control and M4Control) transfected with the empty vector were used. Analysis showed significant ($P < 0.0001$) up-regulation of CXCL5 in CXCL5-transfected clones (Fig. 4A). Restoration of CXCL5 expression had no significant effect on in vitro proliferation rate (Fig. 4B). To determine the in vivo growth capacity of the different clones, each individual clone was inoculated in the liver of four rats. All clones showed solitary tumors at the site of inoculation, as determined at sacrifice. Tumors were enucleated from the liver, and tumor weight was determined. The mean tumor weight of the CXCL5-transfected clones was significantly less compared with the tumor weight of the control clones ($P = 0.005$; Fig. 4C). Only very few lung metastases were found, not differing among clones. These results showed that CXCL5 reconstitution in CC531M resulted in inhibition of tumor growth in vivo.
Low expression of CXCL5 in human colorectal cancer is associated with decreased survival. The relation between expression of CXCL5 in human colorectal tumors and prognosis was studied in two cohorts of colorectal cancer patients, using different techniques to determine the level of CXCL5 expression. The RNA level of expression of CXCL5 in the first cohort was determined using quantitative RT-PCR and linked to clinical follow-up data. The effect of high versus low expression of CXCL5 was assessed using the 25th percentile as cut off point, leaving 53 patients with high expression of CXCL5 (11.2 ± 2.1; mean ± SD) and 17 patients with low expression (7.1 ± 1.3) of CXCL5. CXCL5 expression levels were distributed equally with regard to clinical and pathologic variables (Table 1). Univariate Cox regression analyses were performed to identify prognostic factors for disease-free survival. Advanced patient age, advanced pathologic stage, and low CXCL5 expression proved to be significant predictors of poor prognosis in the univariate analyses (Table 1). The Kaplan-Meier curve for disease-free survival is shown for low versus high CXCL5 expression (Fig. 5) and revealed that low expression was associated with a significantly worse prognosis (P = 0.016). Variables, significant in univariate analysis, were subjected to Cox multivariate analysis. Patient age above the median (HR, 2.3; CI, 1.2-4.2; P = 0.01), advanced pathologic stage (HR, 3.1; CI, 1.6-5.7; P < 0.001), and low CXCL5 expression (HR, 2.3; CI, 1.2-4.4; P = 0.016) all retained their strength as independent prognostic factors for disease-free survival (Table 1).

In a second independent cohort of colorectal cancer patients, the protein level of CXCL5 expression was determined using immunohistochemical staining of tissue sections. Staining confirmed previous data showing that tumor cells displayed increased expression of CXCL5 compared with normal colon epithelium (Fig. 6; ref. 11, 12). Fifty tumors showed CXCL5 expression in >50% of the tumor cells, whereas 8 tumors showed expression of CXCL5 in <50% of the tumor cells. CXCL5 levels were distributed equally to clinicopathologic variables (Table 2). Univariate analysis showed that CXCL5

<table>
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<tr>
<th>CXCL5 expression</th>
<th>Relation CXCL5 to:</th>
<th>Disease-free survival</th>
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<tr>
<td></td>
<td>M-W-Univariate</td>
<td>Multivariate analysis</td>
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<td></td>
<td>P</td>
<td>P</td>
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Table 1. RNA level of CXCL5 in relation to clinicopathologic and prognostic variables

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<tr>
<th>Gender</th>
<th>Male (%)</th>
<th>Low n (%)</th>
<th>0.78</th>
<th>0.78</th>
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<th>—</th>
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<tr>
<td>Location tumor</td>
<td>Proximal (%)</td>
<td>29 (55%)</td>
<td>7 (41%)</td>
<td>0.34</td>
<td>0.51</td>
<td>—</td>
</tr>
<tr>
<td>Median age at diagnosis (y)</td>
<td>&lt;68.5</td>
<td>27</td>
<td>8</td>
<td>0.78</td>
<td>0.006</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>&gt;68.5</td>
<td>26</td>
<td>9</td>
<td>2.3 (1.2-4.2)</td>
<td>0.010</td>
<td>—</td>
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Stage

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<th>Stage</th>
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<th>36 (68%)</th>
<th>11 (65%)</th>
<th>0.81</th>
<th>0.0001</th>
<th>1</th>
<th>0.001</th>
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<tr>
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<td>III</td>
<td>17 (32%)</td>
<td>6 (35%)</td>
<td>3.1 (1.6-5.7)</td>
<td>&lt;0.001</td>
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Pathway

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<tr>
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<th>MSI</th>
<th>1 (6%)</th>
<th>11 (21%)</th>
<th>0.16</th>
<th>0.60</th>
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<td></td>
<td>MSS</td>
<td>16 (94%)</td>
<td>42 (79%)</td>
<td>2.3 (1.2-4.4)</td>
<td>0.016</td>
<td>1</td>
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CXCL5

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<tr>
<th>CXCL5</th>
<th>High</th>
<th>53 (76%)</th>
<th>—</th>
<th>—</th>
<th>0.016</th>
<th>1</th>
<th>0.016</th>
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</thead>
<tbody>
<tr>
<td>Low</td>
<td>—</td>
<td>17 (24%)</td>
<td>—</td>
<td>—</td>
<td>—</td>
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</table>

NOTE: Table 1 displays level of CXCL5 in a panel of colorectal cancer patients determined by quantitative RT-PCR. The 25th percentile was used to define high versus low expression of CXCL5. On the left side of both tables, the distribution of high versus low expression of CXCL5 with respect to clinical and pathologic characteristics and the relation of CXCL5 to clinicopathologic factors are displayed. On the right side of the table, prognostic factors are displayed. Univariate Cox regression analyses were done to identify prognostic factors for survival. All factors with a P value of <0.10 were subjected to Multivariate Cox regression analysis. Statistically significant P values are in bold.

Abbreviations: MSS, microsatellite stable; MSI, microsatellite instable.
Cox multivariate analysis confirmed the value of low level of CXCL5 (HR, 3.6; CI, 1.3-9.9; \( P = 0.01 \)) as independent predictor of poor prognosis in addition to advanced pathologic stage (HR, 2.6; CI, 1.1-6.3; \( P = 0.04 \); Table 2). Of the latter cohort, also the CD4 and CD8 infiltration was scored in three compartments of the tumor (intraepithelial, stromal, and advancing margin). Using Pearson's product-moment correlation, a trend was found for significant positive correlation between level of CXCL5 and intraepithelial and stromal infiltration of CD8\(^+\) T cells (\( r = 0.21, P = 0.12; r = 0.26, P = 0.05 \), respectively; Table 3). Neither CD4\(^+\) T-cell infiltration, nor CD8\(^+\) T-cell Infiltration, scored at the border of the tumor (advancing margin) was correlated with expression of CXCL5. Low CXCL5 expression was an independent predictor of poor prognosis in colorectal cancer patients, showing a trend for a positive correlation for level of CXCL5 and intratumoral cytotoxic T-cell infiltration.

### Discussion

Many chemokines play a pivotal role in colorectal cancer (24). We decided to study CXCL5 because our initial rat experiments indicated that the absence of this chemokine was associated with an aggressive tumor phenotype. CXCL5, an important homeostatic factor in the colon, is mainly produced in epithelial cells and is in general more highly expressed in cancer tissue compared with normal tissue (11, 12). This pattern, higher expression in tumor tissue than in normal tissue, was also found in our experiments. However, absence of CXCL5 expression in tumor tissue was correlated with poor prognosis. To our knowledge, the only report

| Table 2. Protein level of CXCL5 in relation to clinicopathologic and prognostic variables |
|---------------------------------------------|--------|----------------------|------------------------|------------------|---------------------|---------------------|
| CXCL5 expression | Relation CXCL5 to: | Cancer-specific survival |
| High n (%) | Low n (%) | M-W | P | Univariate analysis | Multivariate analysis |
| Gender | | | | P | HR (95% CI) | P |
| Male (%) | 27 (54%) | 6 (75%) | 0.27 | 0.15 | — | — |
| Median age at diagnosis (y) | | | | | | |
| <68.5 | 25 | 4 | 1.0 | 0.83 | — | — |
| >68.5 | 25 | 4 | | | | |
| Stage | | | | | | |
| I and II | 24 (48%) | 5 (62%) | 0.45 | 0.03 | 1 | 0.04 |
| III | 26 (52%) | 3 (38%) | | 2.6 (1.1-6.3) | | |
| CXCL5 | | | | | | |
| High | 50 (86%) | — | — | 0.009 | 1 | 0.01 |
| Low | — | 8 (14%) | | 3.6 (1.3-9.9) | | |

**NOTE:** Table 2 displays the results after immunohistochemical staining and scoring the percentage of CXCL5-positive tumor cells. For immunohistochemical staining, high was defined as <50% of tumor cells expressing CXCL5, and low was defined as <50% of tumor cells expressing CXCL5. On the left side of both tables, the distribution of high versus low expression of CXCL5 with respect to clinical and pathologic characteristics and the relation of CXCL5 to clinicopathologic factors are displayed. On the right side of the table, prognostic factors are displayed. Univariate Cox regression analyses were done to identify prognostic factors for survival. All factors with a \( P \) value of <0.10 were subjected to Multivariate Cox regression analysis. Statistically significant \( P \) values are in bold.
This is in contrast with other reports that may indicate that CXCL5 functions as an autocrine growth factor in colorectal cancer. Expression of CXCR2 has also been found in CC531 cells.3 This receptor has been found on colorectal tumor cells (41–43). colorectal tumor growth is based on the fact that the CXCR2 receptor contributes to an antitumor response.

CXCL5 is involved in growth and development of colorectal cancers. The importance of CXCL5 for tumor formation in vivo was confirmed by comparing the growth of transfected CC531 clones that expressed either high or low levels of CXCL5. Importantly, our findings in the rat proved to be relevant for colorectal cancer patients, as in two different tumor tissue cohorts of these cancer patients, low expression of CXCL5 was associated with shorter survival.

Well-established is the chemotraction of neutrophils into inflamed regions after CXCL5-CXCR2 interaction (9). Antagonists to the CXCR2 receptor prevent neutrophil attraction and reduce the inflammatory response (26, 27). CXCR2 is also involved in chemokine-induced migration of natural killer and T cells (27, 28). CXCL5 produced by tumor cells may attract CXCR2-expressing leukocytes as T cells, natural killer cells, and neutrophils, triggering an antitumor immune response. A trend for positive correlation between level of vascularitzation in human non–small cell lung cancer. In addition, they showed, using a severe combined immunodeficient mouse model that expression of CXCL5 in developing human non–small cell lung cancer associated with a better prognosis compared with immortal tumors having a poorer prognosis (44). In other cancers, a tumor-promoting role for CXCL5 has been reported (12–14, 45). Arenberg et al. (13) found a strong correlation between levels of CXCL5 and expression had no influence on proliferation rate of any of our cell lines. Moreover, we found in vivo that low expression of CXCL5 promotes tumor growth. These data indicate that the effect of CXCL5 is not very likely to depend on an autocrine signaling pathway.

CXCL5 may play opposing roles in tumor formation in general. On one hand, CXCL5 may induce an antitumor response by chemotraction of immune cells; on the other hand, it may promote angiogenesis that supports tumor growth. Our results indicate that in colorectal cancer formation, the antitumor response is dominant. In support of our results, for head and neck squamous cell carcinoma higher expression of CXCL5 was also found in mortal tumors associated with a better prognosis compared with immortal tumors having a poorer prognosis (44). In other cancers, a tumor-promoting role for CXCL5 has been reported (12–14, 45). Arenberg et al. (13) found a strong correlation between levels of CXCL5 and expression had no influence on proliferation rate of any of our cell lines. Moreover, we found in vivo that low expression of CXCL5 promotes tumor growth. These data indicate that the effect of CXCL5 is not very likely to depend on an autocrine signaling pathway.

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In conclusion, our results show that CXCL5 is an important factor in growth and development of colorectal cancer. Our data suggest that expression of CXCL5 by tumor cells enhances the recruitment of tumor-infiltrating lymphocytes, thereby bringing about better prognosis in colorectal cancer patients. Therefore, CXCL5 should be further studied for its potential role as a therapeutic target and prognostic biomarker in colorectal cancer.

**Table 3.** Correlation between expression of CXCL5 and infiltrative T-cell markers

<table>
<thead>
<tr>
<th>Location infiltrate</th>
<th>CD8⁺</th>
<th>CD4⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pearson correlation (r)</td>
<td>P</td>
</tr>
<tr>
<td>Intraepithelial</td>
<td>0.21</td>
<td>0.12</td>
</tr>
<tr>
<td>Stromal</td>
<td>0.26</td>
<td>0.05</td>
</tr>
<tr>
<td>Advancing margin</td>
<td>-0.02</td>
<td>0.87</td>
</tr>
</tbody>
</table>

NOTE: A trend for positive correlation between expression of CXCL5 and intratumoral T-cell infiltration was found. T-cell infiltration was scored in different compartments of the tumor: intraepithelial, stromal, and at the advancing margin. Infiltration in each of these different compartments was associated to protein expression of CXCL5 using Pearson’s product-moment correlation.

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3 Unpublished data.
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