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Chapter 4

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 sub-line (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 impact of CXCL5 expression on colorectal tumor growth and metastasis.

Experimental design: CC531 clones were generated with either knock-down 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 parameters.

Results: Knock-down of CXCL5 expression in CC531S resulted in rapid tumor growth and increased number of metastasis, while 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 intra-tumoral $CD8^+$ 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.

INTRODUCTION

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 percent 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 lipopolysacharide (LPS)-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 over-expressed 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 (NSCLC). Homogenates of human NSCLC 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 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.

EXPERIMENTAL DESIGN

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% CO_2, in cell culture flasks (Corning, NY, USA) containing culture medium, composed of RPMI1640

(Gibco, Paisley, Scotland), supplemented with 10% heat-inactivated FCS, 100 µg/ml streptomycin, 100 IU/ml penicillin and 2 mM L-glutamine (all Gibco, Paisley, Scotland).

Development of CXCL5 knock-down and CXCL5 expressing CC531 clones

RNAi techniques were used to generate CC531S CXCL5 knock-down clones. A 19-nucleotide sequence (AACGGAGCTACGCTGTGTT), separated by a 9-nucleotide non-complementary 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, Seattle, USA), using standard procedures. To obtain stably transfected CC531S CXCL5-knock-down and control clones, the pSUPER-CXCL5 siRNA or empty vectors were co-transfected with the pcDNA3 vector, using Lipofectamine2000 (Invitrogen, California, USA). Three CXCL5-knock-down CC531S clones ($S1^{CXCL5-}$, $S2^{CXCL5-}$ and $S3^{CXCL5-}$) and three control clones ($S4^{Control}$, $S5^{Control}$ and $S6^{Control}$) 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 base pairs 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, Wisconsin, USA) and sequenced. Expression plasmids for CXCL5 were obtained, through unidirectional cloning of the sequence into the mammalian expression vector pcDNA3 (Invitrogen, California, USA). 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 ($M1^{CXCL5+}$ and $M2^{CXCL5+}$) and two control clones ($M3^{Control}$ and $M4^{Control}$) were selected. Selection was based upon expression of CXCL5 as indicated by immunostaining. Stably transfected clones were grown under selective pressure, in culture medium supplemented with 200µg/ml G418 (Sigma, St. Louis, MO, USA).

Immunocytochemistry

Cells were cultured on 12-mm glass coverslips and stained as previously described [17], using the primary antibody rabbit anti-murine LIX [18] (Peprotech EC Ltd, London, UK) and Cy3-conjugated goat anti rabbit secondary antibody (Jackson, Suffolk, UK) in TBP (1h RT). Finally, cells were stained with 2 µg/ml Hoechst 33258 (Invitrogen, California, USA). Cells were analyzed using a Nikon Eclipse E600 fluorescence microscope with a 40x plan fluor Nikon objective (Nikon, Tokyo, Japan). The percentage CXCL5 positive tumor cells and the CXCL5 pixel intensity per cell were determined. For every clone or cell line at least 5 randomly chosen fields were analysed 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: 25000 cells were seeded into a 24 well plate. Medium was replaced daily. From 24h up to 144h after the start of seeding cells, each day plates were removed, rinsed with PBS, and stored at -80°C until assayed. On the day of assay, cells were thawed, 200 µl of distilled water was added (1h at 37°C). The plates were frozen at -80°C and thawed. From each well 50µl was taken and placed into a 96 well plate. DNA content was determined after addition of 50µl of 20µg/ml Hoechst 33258 fluorochrome (Invitrogen, California, USA) and measured on a fluostar optima platereader (BMG Labtech GmbH, Offenburg, Germany).

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, Zeist, The Netherlands) were anesthetized with halothane, underwent laparotomy, and were double blind randomized for induction of a liver tumor. A suspension of $5*10^4$ viable CC531 tumor cells in 50µl was injected sub-capsulary 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].

RT-PCR of CXCL5 in a patient cohort

Tumours 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 years. Tumors were staged according to the American Joint Committee on Cancer (AJCC) criteria [22]: 47 (67%) stage I/II; 23 (33%) stage III. At the time of censoring 41 (59%) had died of whom 22 (54%) died from their disease, and 29 patients were still alive; four of them were alive with recurrence of the tumor. Mean follow up was 99 months (range 50-172 months). 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, Germantown, MD, USA). Quantative reverse transcriptase PCR (RT-PCR) primers for the detection of housekeeping genes (Cleavage and polyadenylation specificity factor subunit 6 (CPSF6), Heterogeneous nuclear ribonucleoprotein M (HNRPM) and TATA-binding protein (TBP) and CXCL5 were designed in PRIMER Express (Applied Biosystems, Foster City, CA, USA) and span at least one exon-exon boundary). The primers used were:

CPSF6, 5'-AAGATTGCCTTCATGGAATTGAG-3', 5'-TCGTGATCTACTATGGTCCCTCTCT-3'; HNRPM, 5'-GAGGCCATGCTCCTGGG-3', 5'-TTTAGCATCTTCCATGTGAAATCG-3', TBP, 5'-CACGAACCACGGCACTGAT-3', 5'-TTTTCTTGCTGCCAGTCTGGAC-3' CXCL5, 5'- ctgt-gttgagagagctgcgt-3', 5'-gttttccttgtttccaccgtc-3'. RT-PCR reactions were performed on an ABI Prism 7900ht (Applied Biosystems) using the SybrGreen RT-PCR core-kit (Eurogentec, Seraing, Belgium). Cycle conditions were 10 minutes at 94°C followed by 40 cycles of 10 s at 94°C and 1 minute 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, five-fold dilution of pooled cDNA from the HCT81 colorectal cancer cell line. Relative concentrations of mRNA for 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 years; 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 months (range 1.2-162 months). Standard two-step, indirect immunohistochemistry was performed on 4-µm paraffin tissue sections, including blockage of endogenous peroxidise, EDTA antigen retrieval (not for CXCL5 detection) and di-aminobenzidine development. To be able to distinguish intra-epithelial from stromal infiltration, an additional staining for laminin was performed on CD4 and CD8 stained sections, including trypsin antigen retrieval and development using NBT/BCIP solution, as previously described [23]. The following primary antibodies were used: the mAb anti- CXCL5 (clone MAB254, R&Dsystems, Minneapolis, USA), the mAb anti-CD4 (clone 1F6, Novocastra, UK), the mAb anti-CD8 (clone 4B11, Novocastra, UK) and rabbit anti-human laminin polyclonal Ab (Sigma-Aldrich, USA). Secondary reagents used were anti Mouse HRP EnVision+ (K400111, Dako, USA), biotinylated swine anti-rabbit IgG antibodies (DAKO Cytomation, Denmark), and biotinylated-peroxidase streptavidin complex (SABC; DAKO Cytomation, Denmark). CXCL5 expression was scored by microscopically assessing the percentage CXCL5 positive tumor of the whole section. Infiltration in the tumor tissue was scored in three compartments of the tumor tissue, i.e. intra-epithelially, intra-stromally 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, Chicago, IL). 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 $\leq$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 to 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 as compared to inoculation of the parental CC531S [4]. Affymetrix micro array analysis was performed in 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 as compared to expression in CC531S. To confirm RNA expression data, cells were stained for the presence of CXCL5 protein using immuno-fluorescence techniques. In CC531S cells a strong cytoplasmic staining was found, while CC531M cells hardly showed any staining (figure 1A). The percentage of CXCL5 positive cells was significantly ($p<0.0001$) higher in CC531S cells compared to CC531M cells (figure 1B). *In vitro*, CC531S and CC531M showed the same proliferation rate (figure 1C).

Knock-down 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 ($S1^{CXCL5-}$, $S2^{CXCL5-}$ and $S3^{CXCL5-}$) and three control clones ($S4^{Control}$, $S5^{Control}$ and $S6^{Control}$) transfected with the empty vector, were selected. Immuno-fluorescence staining for CXCL5 expression showed a significant ($p<0.0001$) CXCL5 down-regulation in $S1^{CXCL5-}$, $S2^{CXCL5-}$ and $S3^{CXCL5-}$ compared to $S4^{Control}$, $S5^{Control}$ and $S6^{Control}$ (figure 2A). *In vitro*, no significant difference in mean proliferation rate was found between CC531S CXCL5 knock-down clones and control

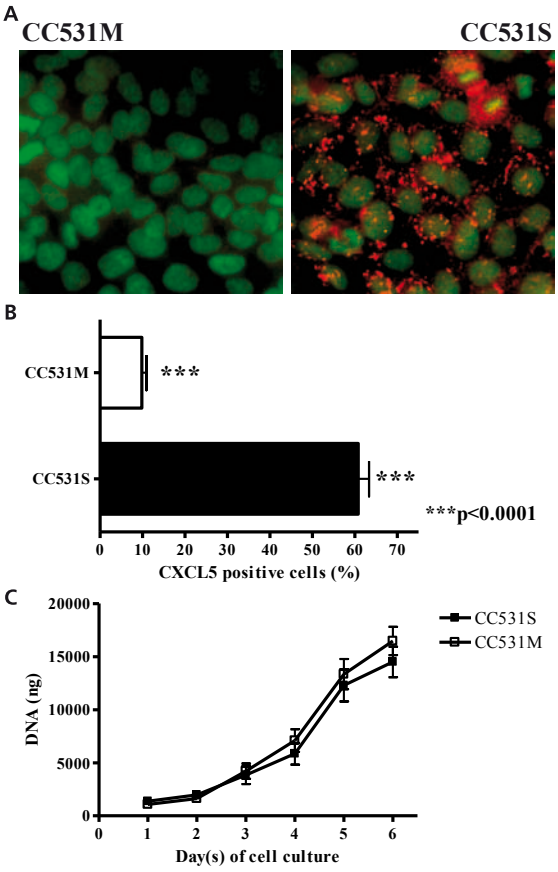

Figure 1. CXCL5 expression is reduced in CC531M cells.
(A) Immuno-fluorescent staining of CXCL5 expression of CC531S and CC531M (CXCL5 – red; nuclei – green). *(B)* Displays the percentage CXCL5 positive CC531S and CC531M cells; columns - mean; error bars - standard error mean (SEM). *(C)* Represents *in vitro* proliferation rate of CC531S and CC531M cells on different time points; error bars - SEM. Statistically significant differences are marked (*).

clones (figure 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 $S1^{CXCL5-}$ or $S2^{CXCL5-}$ showed large tumors over-growing 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 $S1^{CXCL5-}$ or $S2^{CXCL5-}$. Therefore, the weight of both tumor and liver of all rats was determined. The third clone, CC531S clone $S3^{CXCL5-}$, showed somewhat less aggressive outgrowth: 4 solitary liver tumors at the site of inoculation were found at sacrifice. Only one of

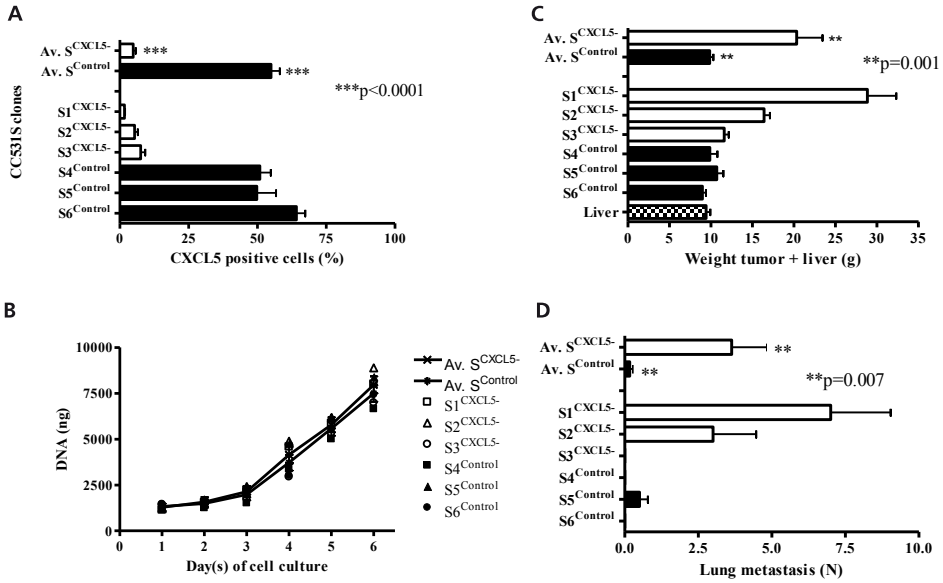

Figure 2. Knock-down of CXCL5 expression results in massive tumor outgrowth and formation of metastasis *in vivo.*

(A) The percentage CXCL5 positive cells of all knock-down and control clones was determined. Top bars represent the average (Av.) number of CXCL5 positive cells, error bars - SEM. *(B) In vitro* growth rate of all knock-down and control CC531S clones on several time points connected by a line. *(C)* Represents the average liver and tumor weight of both control clones versus the knock down clones after inoculation in the liver of rats at sacrifice. Top bars show the average (Av.) of the knock-down versus the control clones. *(D)* The number of lung metastases found after inoculation of the knock-down and control clones in the liver. Statistically significant differences are marked (*).

the 3 control clones, $S5^{Control}$, showed 4 small tumors at the place of inoculation, the others ($S4^{Control}$, $S6^{Control}$) did not show any tumor formation in the liver. The average weight of tumor and liver of the three CXCL5 knock-down clones was significant (p=0.001) higher than the control clones (figure 2C). Furthermore, injection of CXCL5 knock-down clones resulted in significant more lung metastases compared to the control CC531S clones (p=0.007) (figure 2D). Images of *in vivo* tumor growth are displayed in figure 3. Together, these results show that while knock-down of CXCL5 *in vitro* did not result in difference in proliferation rate; *in vivo* CXCL5 knock-down 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 ($M1^{CXCL5+}$ and $M2^{CXCL5+}$) and two

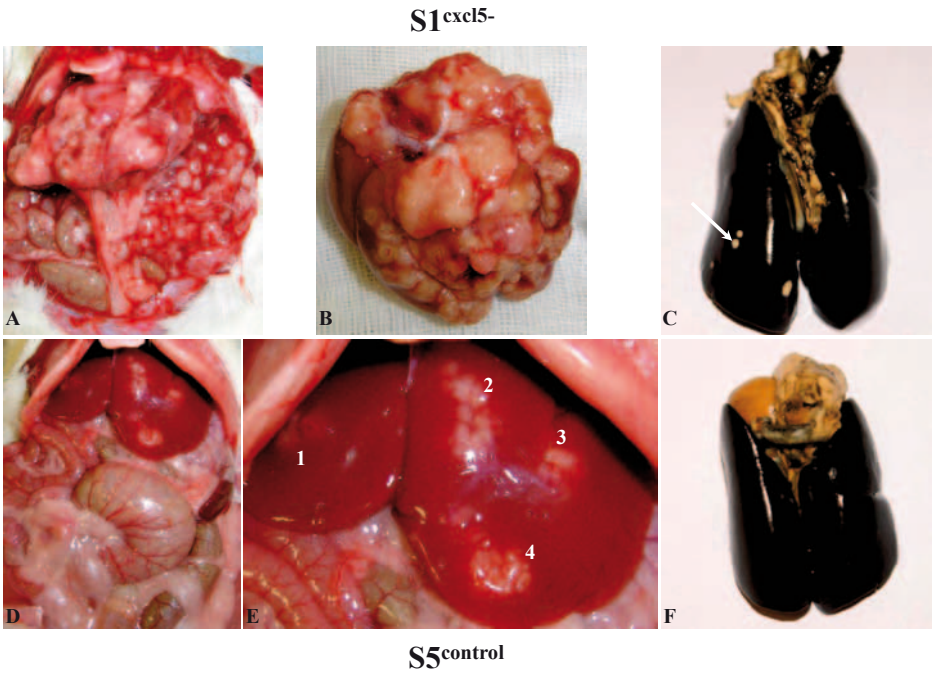

Figure 3. Examples of tumor outgrowth in the rat of a CXCL5 knock-down and a control CC531S clone.
Top panels *(A-C)* display tumor outgrowth of CXCL5 knock-down clone $S1^{cxcl5-}$ after inoculation of the clone in the liver of a rat at sacrifice. *(A)* Displays the peritoneal cavity with tumor throughout the liver and peritoneal metastasis. *(B)* Shows the same liver after resection, overgrown with tumor mass, individual sites of inoculation cannot be distinguished. *(C)* Displays the lungs of this rat after ink injection, with 4 metastases (one is indicated by an arrow). Bottom panels *(D-F)* display tumor growth after inoculation with CXCL5 positive CC531S control clone $S5^{control}$. *(D)* Tumor is only found in the liver at the four sites of inoculation and not in other places in the peritoneal cavity. *(E)* Shows the liver in detail, with 4 individual tumors (numbered 1-4). *(F)* No lung metastases were found in this rat.

control clones ($M3^{Control}$ and $M4^{Control}$) transfected with the empty vector were used. Analysis showed significant ($p<0.0001$) up-regulation of CXCL5, in CXCL5-transfected clones (figure 4A). Restoration of CXCL5 expression had no significant impact on *in vitro* proliferation rate (figure 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 to the tumor weight of the control clones ($p=0.005$) (figure 4C). Only very few lung metastases were found, not differing among clones. These results demonstrated that CXCL5 reconstitution in CC531M resulted in inhibition of tumor growth *in vivo*.

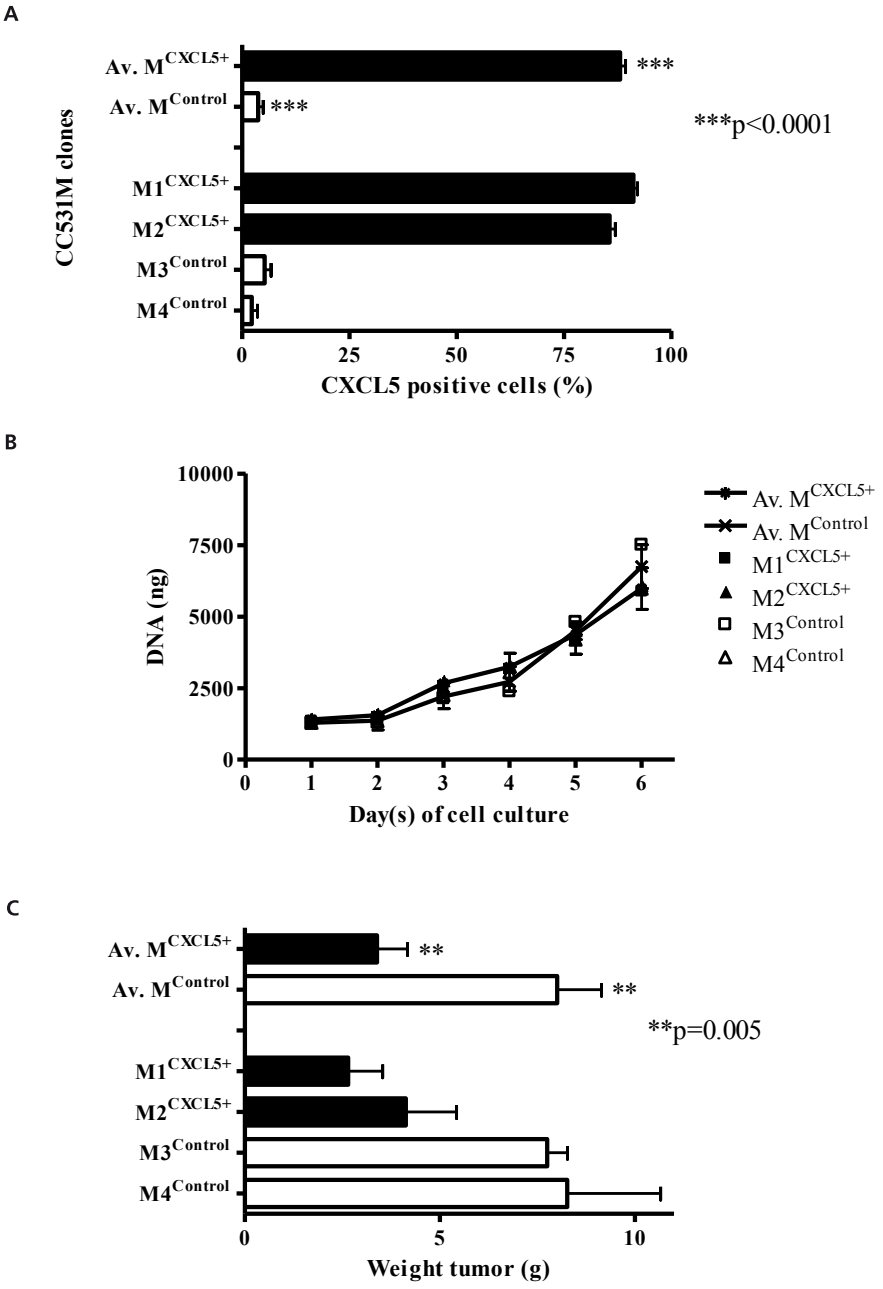

Figure 4. Restoration of CXCL5 expression inhibits tumor growth*, in vivo.*
(A) CXCL5 expression of the CXCL5 and control CC531M clones; columns - mean; bars - SEM. *(B) In vitro* growth rate of all CXCL5 transfected versus control clones at several time points. *(C)* The mean weight of the total tumor mass per clone in four rats at sacrifice is shown. The two columns on top represent the average weight of the CXCL5 transfected versus the control clones; columns - mean; bars - SEM. Statistically significant differences are marked (*).

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 impact 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 pathological parameters (table 1). Univariate cox regression analyses were performed to identify prognostic factors for disease free survival. Advanced patient age, advanced pathological stage, and low CXCL5 expression proved to be significant predictors of poor prognosis in the univariate analyses (table 1). The Kaplan Meyer curve for disease free survival is shown for low versus high CXCL5 expression (figure 5) and revealed that low expression was associated with a significantly worse prognosis (p=0.016). Parameters, significant in univariate analysis, were subjected to Cox multivariate analysis. Patient age above the median (HR: 2.3, C.I.: 1.2-4.2, p=0.01), advanced pathological stage (HR: 3.1, C.I.: 1.6-5.7, p<0.001), and low CXCL5 expression (HR: 2.3, C.I.: 1.2-4.4, p=0.016) all retained their strength as independent prognostic factors for disease free survival (table 1).

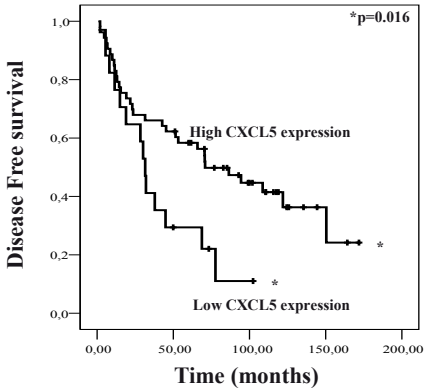

Figure 5. Correlation between disease free survival and expression of CXCL5 assessed by RT-PCR in a cohort of colorectal cancer patients.
Kaplan Meier survival curve is displayed, patients with low expression of CXCL5 have a significant (p=0.016) decreased disease free survival compared to patients with high expression of CXCL5.

Table 1. RNA level of CXCL5 in relation to clinicopathological and prognostic parameters

	CXCL5 expression		Relation CXCL5 to:	Disease Free Survival		
			M-W	Univariate analysis	Multivariate analysis	
	High N (%)	Low N (%)	p-value	p-value	HR (95% CI)	p-value
Gender						
Male (%)	27 (51%)	8 (47%)	0.78	0.78	---	---
Location tumor						
Proximal (%)	29 (55%)	7 (41%)	0.34	0.51	---	---
Median age at diagnosis (years)						
<68.5	27	8	0.78	**0.006**	1	**0.010**
>68.5	26	9			2.3 (1.2 – 4.2)	
Stage						
I and II	36 (68%)	11 (65%)	0.81	**0.0001**	1	**<0.001**
III	17 (32%)	6 (35%)			3.1 (1.6 – 5.7)	
Pathway						
MSI	1 (6%)	11 (21%)	0.16	0.60	---	---
MSS	16 (94%)	42 (79%)				
CXCL5						
High	53 (76%)	---	---	**0.016**	1	**0.016**
Low	---	17 (24%)			2.3 (1.2 – 4.4)	

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 $\leq$0.10 were subjected to Multivariate Cox regression analysis. Statistically significant *P*-values are in bold.

Abbreviations: MSS, microsatellite stable; MSI, microsatellite instable.

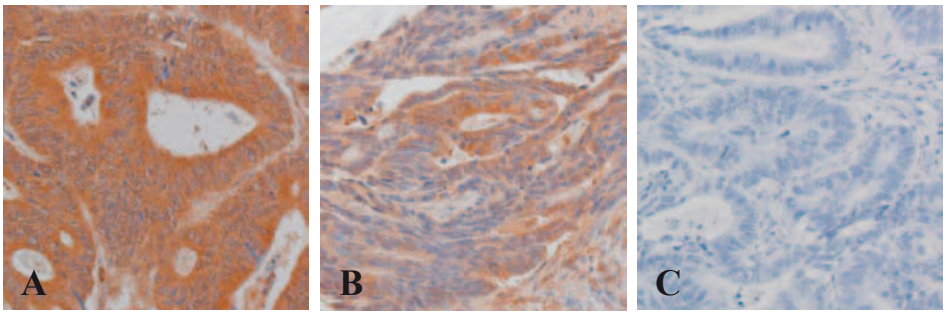

Figure 6. Examples of CXCL5 immunohistochemical staining of human colorectal tumors.
(A) displays CXCL5 expression in almost colon cancer cells; *(B)* shows heterogeneous expression of CXCL5 in a colorectal tumor; *(C)* displays a negative PBS control; original magnification x200

In a second independent cohort of colorectal cancer patients, the protein level of CXCL5 expression was determined using immunohistochemical staining of tissue sections. After staining, the percentage positive tumor cells was scored. Staining confirmed previous data showing that tumor cells displayed increased expression of CXCL5 compared to normal colon epithelium (figure 6) [11;12]. Fifty tumors showed CXCL5 expression in more than 50% of the tumor cells, while 8 tumors showed expression of CXCL5 in less than 50% of the tumor cells. CXCL5 levels were distributed equally to clinicopathological parameters (table 2). Univariate analysis showed that CXCL5 (p=0.009) and stage (p=0.03) both predicted prognosis. Cox multivariate analysis confirmed the value of low level of CXCL5 (HR: 3.6, C.I.: 1.3-9.9, p=0.01) as independent predictor of poor prognosis in addition to advanced pathological stage (HR: 2.6, C.I.: 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 (intra-epithelial, stromal and advancing margin). Using Pearson's product-moment correlation a trend was found for significant positive correlation between level of CXCL5 and intra-epithelial 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 decreased disease free survival in colorectal cancer patients, showing a trend for a positive correlation for level of CXCL5 and intra-tumoral cytotoxic T-cell infiltration.

Table 2. Protein level of CXCL5 in relation to clinicopathological and prognostic parameters

	CXCL5 expression		Relation CXCL5 to:	Cancer Specific Survival		
			M-W	Univariate analysis	Multivariate analysis	
	High N (%)	Low N (%)	p-value	p-value	HR (95% CI)	p-value
Gender						
Male (%)	27 (54%)	6 (75%)	0.27	0.15	---	---
Median age at diagnosis (years)						
<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 showing CXCL5 expression and low was defined as <50% of tumor cells showing CXCL5 expression. On the left side of both tables, the distribution of high versus low expression of CXCL5 with respect to clinical and pathologic characteristic 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 $\leq$0.10 were subjected to Multivariate Cox regression analysis. Statistically significant *P* values are in bold.

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

Location infiltrate	$CD8^+$		$CD4^+$	
	Pearson correlation (r)	p-value	Pearson correlation (r)	p-value
Intra-epitelial	0.21	0.12	0.12	0.38
Stromal	0.26	0.05	0.15	0.27
Advancing margin	-0.02	0.87	-0.93	0.50

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.

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 to 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 describing mechanisms by which CXCL5 expression is abrogated is provided by Dimberg *et al.* showing that CXCL5 gene variants are related to expression of CXCL5 protein in colorectal cancer [12]. Besides (epi-)genetic explanations, other mechanisms influencing CXCL5 expression might be involved in the nuclear factor-kappaB (NF-κB) that controls expression of CXCL5 [25]. Functions of CXCL5 include chemo-attraction and promotion of angiogenesis, mainly by interaction with the CXCR2 receptor [7;8]. Our data indicate that 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 chemo-attraction of neutrophils into inflamed regions after CXCL5-CXCR2 interaction [9;26]. Antagonists to the CXCR2 receptor prevent neutrophil attraction and reduce the inflammatory response [27;28]. CXCR2 is also involved in chemokine-induced migration of NK and T-cells [28;29]. CXCL5 produced by tumor cells may attract CXCR2 expressing leukocytes as T-cells, NK cells and neutrophils, triggering an anti-tumor immune response. A trend for positive correlation between level of CXCL5 and intratumoral cytotoxic T-cell infiltration was found. This trend was not found for infiltration in the advancing border of the tumor, suggesting that CXCL5 indeed especially contributes to intratumoral infiltration of cytotoxic T-cells. High tumor infiltration of these inflammatory immune cells is positively associated with good prognosis in colorectal cancer [30-32]. This concept that over-expression of specific chemokines causes tumor infiltration by distinct leukocyte subsets, resulting in tumor regression and tumor specific immunity, has also been described for other chemokines [33-41]. Thus, CXCL5 may contribute to an anti-tumor response.

Another mechanism by which CXCL5 may be involved in colorectal tumor growth is based on the fact that the CXCR2 receptor has been found on colorectal tumor cells [42-44]. Expression of CXCR2 has also been found in CC531 cells (unpublished data).

This may indicate that CXCL5 functions as an autocrine growth inhibitory factor. This is in contrast with other reports that described a positive effect of CXCR2 ligands on tumor growth [43;44]. In our results the presence or absence of CXCL5 expression had no influence *in vitro* 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 the one hand CXCL5 may induce an anti-tumor response by chemo-attraction of immune cells; on the other hand it may promote angiogenesis that supports tumor growth. Our results indicate that in colorectal cancer formation, the anti-tumor response is dominant. In support of our results for head and neck squamous cell carcinoma HNSCC higher expression of CXCL5 was also found in mortal tumors associated with a better prognosis compared to immortal tumors having a poorer prognosis [45]. In other cancers a tumor promoting role for CXCL5 has been reported [13-15;46]. Arenberg *et al.* found a strong correlation between levels of CXCL5 and the level of vascularitzation in human NSCLC. In addition they showed, using a SCID mouse model that expression of CXCL5 in developing human NSCLC correlated with tumor growth [14]. The data presented by Arenberg and others may seem in contrast with our findings. However, in addition to the different tumor type studied, our findings were derived from a syngeneic rat model for colorectal cancer, with a competent immune system, while their results were obtained in immune deficient mouse models. Therefore, in the study by Arenberg *et al.* the potential effect of a CXCL5-dependent immune response on tumor development would not have been manifested.

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.

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