

Analysis of the expression of SDF-1 splicing variants in human colorectal cancer and normal mucosa tissues

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Abstract. C-X-C motif chemokine ligand 12 (CXCL12), also termed stromal cell-derived factor-1 (SDF-1) is a small protein 8-14 kDa in length that is expressed as six isoforms, consisting of SDF-1 α , SDF-1 β , SDF-1 γ , SDF-1 δ , SDF-1 ϵ and SDF-1 θ . All six isoforms are encoded by the single CXCL12 gene on chromosome 10. This gene regulates leukocyte trafficking and is variably expressed in a number of normal and cancer tissues. The potential role of the novel CXCL12 splice variants as components of the CXCR4 axis in cancer development is not fully understood. The present study aimed to analyze the expression profile of the various SDF-1 isoforms and SDF-1 polymorphisms, and the association with the clinicopathological features and overall survival of patients with colorectal cancer (CRC). SDF-1 polymorphism analysis was performed using restriction fragment length polymorphism (RFLP) analysis in 73 histologically confirmed human CRC tissue samples at various stages of disease. The expression pattern of the SDF-1 isoforms was analyzed by reverse transcription-polymerase chain reaction in 40 histologically confirmed human CRC tissue samples obtained at various stages of disease, as well as in matched adjacent normal mucosa samples. The presence of the CXCL12 gene polymorphism rs1801157 demonstrated an association with local progression of the primary tumor, as indicated by the T stage. The frequency of the GG genotype was slightly increased in patients with stage 3 and 4 tumors (78.0%) compared with

the incidence of the GA/AA genotype (69.5%; P=0.067). The expression of SDF-1 β was associated with the presence of metastases (P=0.0656) and the expression of SDF-1 γ was significantly associated with tumor size (P=0.0423). The present study is the first to analyze the association between the expression profile of the chemokine CXCL12 splice variants in human CRC tissues and their clinical relevance. The present results reveal that the CXCL12 G801A polymorphism is a low-penetrance risk factor for the development of CRC, and was associated with the T stage. All six isoforms of SDF-1 were expressed in CRC tissues. The expression of SDF-1 β was found to be associated with metastases and SDF-1 γ appears to be a possible tumor marker for local tumor progression.

Introduction

Colorectal cancer (CRC) is one of the most frequent cancers in Western countries, and the disease is delineated by local recurrence and lymphatic and hematogenous dissemination (1,2). The results of *in vivo* and *in vitro* studies performed in various tumor entities indicate that tumor progression and organ-specific metastasis is partially affected by interactions between chemokine receptors on cancer cells and the corresponding chemokines expressed in target organs. Therefore, chemokine receptors direct the spread of tumor cells and affect the sites of metastatic growth (3). Chemokines and the corresponding G-protein-coupled receptors have previously been reported to mediate various immunoresponses (4). The high expression of stromal cell derived factor 1 (SDF-1), also termed C-X-C motif chemokine ligand 12 (CXCL12), in endothelial cells, biliary epithelial cells, bone marrow stromal cells and lymph nodes results in a chemotactic gradient that attracts lymphocytes expressing C-X-C chemokine receptor type 4 (CXCR4) to the respective organs (4-6).

CXCR4 has become a focus of studies, as it is the most common chemokine receptor expressed on cancer cells (3). CXCR4 has been indicated to play an important role in tumor dissemination in colorectal, breast and oral squamous cell carcinoma, as these tumors all commonly metastasize

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to SDF-1-expressing organs (7,8). Previous studies that analyzed the metastatic ability of CXCR4-expressing cancer cells using murine tumor models underlined the key role of CXCR4 in tumor cell malignancy (9-13). The activation of CXCR4 by SDF-1 α has been reported to induce the migration, invasion and angiogenesis of cancer cells (14-17). SDF-1 proteins are found as monomers in living organisms. Six isoforms of SDF-1 exist, consisting of SDF-1 α , SDF-1 β , SDF-1 γ , SDF-1 δ , SDF-1 ϵ and SDF-1 θ , due to alternative splicing. All isoforms possess the same initial three exons and then differ from exon 4 onwards (18,19). The best-known isoforms are SDF-1 α and SDF-1 β . The functional diversity and differential proteolytic processing properties of these two isoforms has been extensively investigated and characterized (20,21). However, little is known about the other isoforms.

In the present study, the expression of the various SDF-1 isoforms and SDF-1 polymorphisms in tissue specimens obtained from CRC patients was analyzed. These results were assessed for associations between the clinicopathological parameters and overall survival of the patients.

Materials and methods

Tissue samples. CRC tissue samples were intraoperatively obtained from 73 patients, according to the ethical committee of the State Chamber of Medicine in Rhineland-Palatinate, and were derived from 30 (41%) females and 43 (59%) males, with a median age at diagnosis of 66.5 years. Written informed consent was obtained from all patients. The morphological classification of the carcinoma was performed according to World Health Organization (WHO) specifications for the tumor-node-metastasis (TNM) classification. The patients were followed up on a regular basis, depending on the procedure performed.

Restriction fragment length polymorphism (RFLP) assay. The presence of a polymorphism at codon 801 of the SDF-1 gene was analyzed by RFLP analysis using cDNA obtained from 73 human CRC tissue specimens. Polymerase chain reaction (PCR) was performed as described by Dimberg *et al* (22), using the 5'-CAGTCAACC TGGGCAAAGCC-3' and 5'-AGC TTTGGTCCTGAGAGTCC-3' primers (Eurofins Genomics, Ebersberg, Germany). The resulting double-stranded DNA was then digested using the restriction enzyme *MspI*. To visualize the resulting DNA fragments, agarose gel electrophoresis was performed (Fig. 1).

Analysis of CXCL12 splice variant expression in human CRC and normal mucosa tissues by reverse transcription (RT)-PCR. The expression of the CXCL12 splice variants SDF-1 α , SDF-1 β , SDF-1 γ , SDF-1 δ , SDF-1 ϵ and SDF-1 θ was analyzed in 40 CRC and 20 human mucosa cDNA samples obtained from a subgroup of the aforementioned tissue samples. In order to determine the expression patterns of the human SDF-1 isoforms in CRC and normal mucosal tissues, PCR primers (Eurofins Genomics) that specifically detected each SDF-1 isoform were designed as follows: SDF-1 α forward, 5'-ATGAACGCCAAGGTCGTGGTC-3' and reverse, 5'-AAGTGCTTACTTGTTTAAAGCTTTCTC-3';

SDF-1 β forward, 5'-ATGAACGCCAAGGTCGTGGTC-3' and reverse, 5'-ACCCTCTCACATCTTGAACCTCTT-3'; SDF-1 γ forward, 5'-ATGAACGCCAAGGTCGTGGTC-3' and reverse, 5'-AGATAACTAGTTTTTTCCTTTT CTGGGC-3'; SDF-1 δ forward, 5'-ATGAACGCCAAGGTCGTGGTC-3' and reverse, 5'-ACACCATTACAC ATCCCCAGGAGA-3'; SDF-1 ϵ forward, 5'-CGCCATGAACGCCAAGGTCGTGGTCG-3' and reverse, 5'-CTAATTAC TTCAGTGGCAGATCATAC-3'; and SDF-1 θ forward, 5'-CGCCATGAACGCCAAGGTCGTGGTCG-3' and reverse, 5'-CACACTAATTACTTTCAGTGGCAGATC-3'. β -actin was used as an internal control for the tissue samples (23). The PCR reactions were performed using an automated thermal cycler (PTC-200; Bio Rad Laboratories GmbH, Munich, Germany).

Statistical analysis. The association between the SDF-1 G801A polymorphism or isoform expression and the clinicopathological features of patients was assessed using box plots and the Wilcoxon test if the explanatory variable had 2 levels, or the Kruskal-Wallis test if there were >2 levels of the explanatory variable. The association between the patient age, β -actin expression and isoform expression was determined using Pearson product-moment correlation coefficient and reported as scatterplot matrices. $P \leq 0.05$ was considered to indicate a statistical significant difference. Statistical analysis was performed using SAS 9.2 software from SAS Institute, Inc. (Cary, NC, USA).

Results

Association between SDF-1 isoforms and clinicopathological parameters. The expression of the CXCL12 splice variants SDF-1 α , SDF-1 β , SDF-1 γ , SDF-1 δ , SDF-1 ϵ , and SDF-1 θ was evaluated in 40 established CRC and 20 normal mucosa tissue specimens. RT-PCR analysis revealed that the mRNA of these SDF-1 splice variants was expressed in CRC and normal mucosa tissues (Table I; Fig. 1). The expression rates of the SDF-1 α , SDF-1 β , SDF-1 γ , SDF-1 δ , SDF-1 ϵ and SDF-1 θ splice variants were 80, 70, 50, 37.5, 30 or 32.5%, respectively. By contrast, the expression rates of the SDF-1 α , SDF-1 β , SDF-1 γ , SDF-1 δ , SDF-1 ϵ or SDF-1 θ splice variants in the normal mucosa were 65, 50, 25, 40, 45 and 35%, respectively (23).

In total, 40 tumors obtained from 39 patients were included in the present study. For 1 patient, clinical data was not available. Out of the total 39 patients, 22 patients (56%) were male. The analyzed tumors were located in the caecum in 3 patients (8%), colon ascendens in 5 patients (13%), colon transversum in 1 patient (3%), colon descendens in 2 patients (5%), sigma in 13 patients (33%) and rectum in 15 patients (38%). The tumors were stage T3 in 29 patients (74%), whereas 2 patients possessed T1 tumors (5%), 5 patients possessed T2 tumors (13%) and 3 patients possessed T4 tumors (8%).

The median patient age was 65.7 years (standard deviation, 10.7), with a range of 41-83 years. There was little association between the categorical clinical parameters, such as gender, tumor site or TNM stage and the expression of the SDF-1 isoforms, with the exception of two associations, consisting of SDF-1 β being associated with the presence of

Table I. Expression of SDF-1 isoforms in CRC and normal mucosa tissues.

Isoform	Expression of SDF-1 isoforms in CRC tissue samples, n (%)	Expression of SDF-1 isoforms in normal mucosa tissue samples, n (%)
Total	40 (100.0)	20 (100.0)
SDF-1 α	32 (80.0)	13 (65.0)
SDF-1 β	28 (70.0)	10 (50.0)
SDF-1 γ	20 (50.0)	5 (25.0)
SDF-1 δ	15 (37.5)	8 (40.0)
SDF-1 ϵ	12 (30.0)	9 (45.0)
SDF-1 θ	13 (32.5)	7 (35.0)

SDF-1, stromal cell derived factor 1; CRC, colorectal cancer.

Table II. Tumour characteristics in patients expressing the CXCL12 genotypes.

Characteristics	CXCL12 genotype		P-value
	GG, n (%)	GA or AA, n (%)	
Total	50 (100.0)	23 (100.0)	
Median age, years	66	67	0.035
Gender			
Male	27 (54.0)	16 (69.6)	0.579
Female	23 (46.0)	7 (30.5)	NS
T stage			
1+2	11 (22.0)	7 (30.5)	0.067
3+4	39 (78.0)	16 (69.5)	
N stage			
0	12 (24.0)	6 (26.0)	0.364
1-3	37 (76.0)	17 (74.0)	NS
M stage			
0	33 (66.0)	13 (56.5)	0.509
+	17 (34.0)	10 (43.5)	NS
R status			
0	32 (64.0)	11 (47.8)	0.395
+	18 (36.0)	12 (52.2)	NS
T stage			
1-3	39 (78.0)	13 (56.5)	0.214
4	11 (22.0)	10 (43.5)	NS

N-status could not be obtain from one patient. CXCL12, C-X-C motif chemokine ligand 12; NS, not significant; T stage, tumor stage; N stage, stage of lymph node involvement; M stage, metastasis stage; R status, residual tumor status.

distant metastases ($P=0.0656$) and SDF-1 γ being significantly positively associated with tumor size ($P=0.0423$) (23).

Genotype frequency. The CXCL12 splice variants were successfully detected in all patient samples. The frequency of the GG CXCL12 allele was compared with the frequency of the GA and AA CXCL12 alleles for the patient gender, patient age and clinical presentation (Table II). Out of the 73 patients with CRC, GG homozygosity was observed in 50 patients with

CRC (68.5%), whereas 23 CRC patients possessed the GA and AA genotypes (31.5%; Fig. 2). With the exception of the local progression of the primary tumor, no significant associations were identified between the rs1801157 CXCL12 gene polymorphism and the clinicopathological parameters. The rs1801157 CXCL12 gene polymorphism was associated with the local progression of the primary tumor, as indicated by the T stage. The frequency of the GG genotype was notably increased in patients with stage 3 and 4 tumors (78.0%)

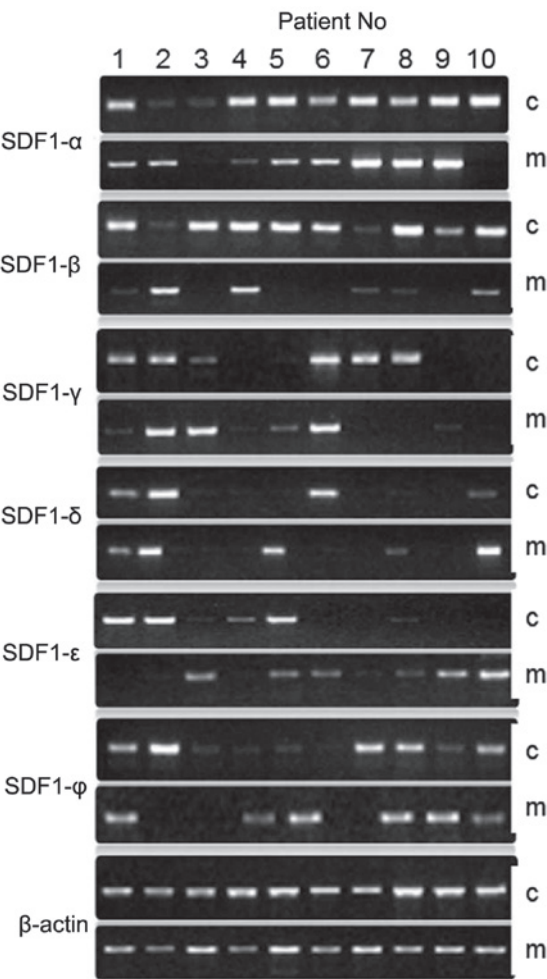


Figure 1. Expression of SDF-1 isoforms in colorectal carcinoma and human normal mucosa tissues. Polymerase chain reaction analysis of the SDF-1 isoforms in cDNA samples obtained from colorectal carcinoma tissues and matched normal mucosa tissues adjacent to the tumor. The experiments were repeated twice and identical results were obtained. SDF-1, stromal cell derived factor 1; c, colorectal carcinoma tissue; m, normal mucosa tissue.

compared with the incidence of the GA or AA genotypes (69.5%; $P=0.067$) (23).

Discussion

SDF-1 is considered to be important for the pathogenesis of certain diseases (24,25). The known CXCL12 isoforms, SDF-1 α , SDF-1 β , SDF-1 γ , SDF-1 δ , SDF-1 ϵ and SDF-1 ϕ result from alternative splicing events of the same gene (26). In addition, a single-nucleotide polymorphism (SNP), termed CXCL12-G801A or rs1801157, was identified in the 3'-untranslated region of the CXCL12 gene transcript. Several studies have examined the association between disease susceptibility and the CXCL12-G801A polymorphism(27-33). These studies indicated that the CXCL12-G801A polymorphism was associated with an increased risk of HIV infection, lung cancer, breast cancer, acute leukemia, prostate cancer, non-Hodgkin's lymphoma and oral squamous cell carcinoma (27-34). However, only a small number of studies focused on the presence of the CXCL12-G801A polymorphism in CRC patients to clarify whether the CXCL12-G801A

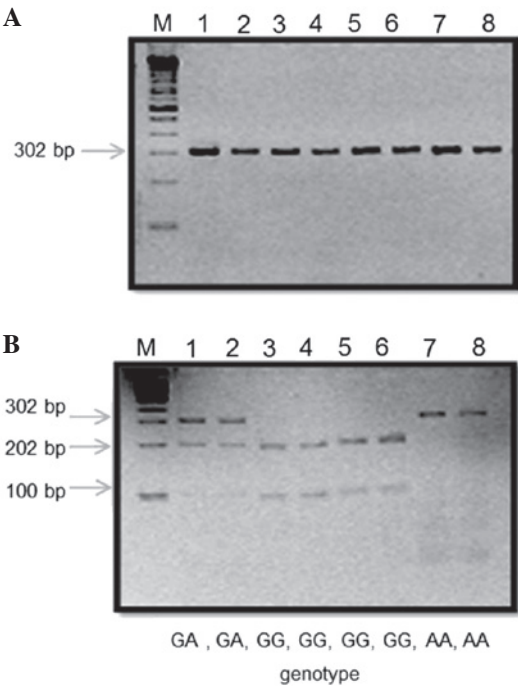


Figure 2. (A) Polymerase chain reaction products for CXCL12 from representative CRC patients. Lane M, molecular marker (100 bp). (B) Restriction fragment length polymorphism analysis of CXCL12 gene polymorphism in representative CRC patients. Lane M, molecular marker (100bp); lanes 1 and 2, mutated heterozygote GA genotype (100, 202 and 302 bp); lanes 3-6, wild type homozygous GG genotype (100 and 202 bp); lanes 7 and 8, mutated homozygous AA genotype (302 bp). CXCL12, C-X-C motif chemokine ligand 12; CRC, colorectal cancer.

polymorphism is associated with disease susceptibility and clinical characteristics in CRC (31,35). A total of 73 patients were included in the present study, and the G/G polymorphism was identified in 50 patients with CRC (68.5%), and the G/A and A/A genotypes were identified in 23 patients with CRC (31.5%). Comparison of the results that were obtained in the present analysis of the CXCL12 gene polymorphism in human CRC with results obtained previously is of importance. The results of the present study were consistent with the results of Dimberg *et al* (22), who studied the CXCL12 gene polymorphism in 151 patients with CRC and 141 control individuals. This study reported that the frequency of the G/G, G/A and A/A genotypes were 55.6% (84/151), 41.1% (62/151) and 3.3% (5/151), respectively, in the patients with CRC. The genotype distribution was not significantly different between the CRC patients and control individuals (22). The present study attempted to determine the association between the presence of the CXCL12 gene polymorphism and the development of CRC. The data in the present study revealed that the GG genotype distribution demonstrates a strong association with the local progression of CRC. Previous studies have reported that the effect of the CXCL12 gene polymorphism may contribute differentially to CRC by mediating tumor progression, angiogenesis, metastasis and leukocyte migration (22). Dimberg *et al* (22) and Hidalgo-Pascual *et al* (36) did not identify a significant correlation between the CXCL12-AA genotype and various prognostic markers, such as the development of metastasis and disease-free survival time. The present

data were consistent with these studies, as the current results only identified an association between this genotype and the presence of larger primary tumors at primary diagnosis.

However, the study by Chang *et al* (37) compared the frequency of six SDF-1 α SNPs in 424 patients with primary T3 stage CRC, and reported that the SDF-1 α -G801A polymorphism was associated with lymph node metastasis. The frequency of GA/AA genotypes was significantly increased (54.8%) in the group of patients with lymph node metastasis compared with the group of patients without lymph node metastasis (40.7%). In the patients without lymph node metastasis, the disease-free survival rate was decreased in patients with the GA/AA genotype (74%) compared with patients with the GG genotype (87.6%). This previous study concluded that the SDF-1 α -G801A polymorphism may increase the expression of SDF-1 α mRNA and be a predictive marker of lymph node metastasis in CRC (37). The differences observed between the studies, including the present study, are likely to be due to a difference in patient numbers and tumor stages.

To assess the strength of the association between the presence of the CXCL12-G801A polymorphism and the risk of cancer, Gong *et al* (38) performed a meta-analysis of 17 studies comprising a total of 3,048 cancer patients and 4522 control individuals. The odds ratio (OR) and 95% confidence interval (CI) were used to determine the association. Compared with the GG genotype, the results revealed that the variant genotypes, consisting of GA (OR, 1.38; 95% CI, 1.18-1.61) and GA/AA (OR, 1.36; 95% CI, 1.17-1.59), were associated with a significantly increased risk of all cancer types. This meta-analysis indicated that the CXCL12 G801A polymorphism is a low-penetrance risk factor for cancer development (23,38).

Furthermore, the present study analyzed the correlation between SDF-1 isoforms and clinicopathological parameters. It was demonstrated that SDF-1 β was associated with the presence of metastases and that SDF-1 γ was significantly associated with tumor size. To the best of our knowledge, the present study is the first to analyze the expression profile of the chemokine CXCL12 splice variants in a larger series of human CRC tissues and normal human mucosa.

Investigation of the expression of novel CXCL12 splice variants and understanding of the potential pathophysiological relevance of the variants under various conditions is required. Different splice variants in certain cases may exhibit similar functions but are active in different tissues or physiological conditions. An improved understanding of the functional diversity of SDF-1 splicing variants may lead to novel therapies that target these variants, which may delay or inhibit the metastatic process in cancer cells (23).

In the present study, SDF-1 α was the most predominant splicing variant in CRC and normal mucosa tissues, followed by SDF-1 β , whereas the other splice variants γ , δ , ϵ and θ were expressed less abundantly. The present findings are consistent with previous data from the literature (26,39,40), which demonstrated that SDF-1 α is a ubiquitously expressed splicing variant with multiple functions in various organs. The SDF-1 β isoform appears to be involved in inter-organ communication through blood-mediated gradients and also in the support of angiogenesis (26,39,40). SDF-1 β was mainly found in highly vascularized organs, such as the liver, spleen, bone marrow and kidneys, but was absent in the brain (26,41,42).

The data obtained in the present study therefore suggested that SDF-1 α , SDF-1 β and SDF-1 γ may act as tumor markers, but are not necessarily associated with the patient prognosis.

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