KIT/stem cell factor expression in premalignant and malignant lesions of the colon mucosa in relationship to disease progression and outcomes

GRAZIELLA BELLONE[1], CARLO SMIRNE[1], ANNA CARBONE[2], ALESSANDRA BUFFOLINO[1],
TIZIANA SCIRELLI[1], ADRIANA PRATI[1], DINO SOLERIO[1], MARIO PIRISI[3],
GUIDO VALENTE[3], MARIO NANO[1] and GIORGIO EMANUELLI[1,2]

[1]Department of Clinical Physiopathology, University of Turin; [2]Department of Gastroenterology and Clinical Nutrition,
S. Giovanni Battista Hospital, Turin; [3]Department of Medical Sciences, University of Eastern Piedmont, Novara, Italy

Abstract. Autocrine/paracrine stimulation of KIT has been observed in colorectal carcinoma (CRC) cell lines. We investigated the expression of KIT and stem cell factor (SCF) in CRC in comparison with premalignant colon lesions and normal colonic mucosa to assess the prognostic and therapeutic relevance of this receptor/ligand system in CRC. Transcript levels of *c-kit* and the two *SCF* splicing variants were determined quantitatively by real-time RT-PCR using cDNA obtained from normal, premalignant and malignant snap frozen colon tissue specimens. Immunohistochemistry with specific anti-KIT and anti-SCF antibodies was performed on paraffin-embedded tissue sections in order to localize the relative protein expression in epithelial compartments. Approximately 10% of patients expressed KIT in their adenoma or primary tumor. The majority of KIT-positive carcinomas co-expressed SCF. Real-time RT-PCR showed expression of *c-kit* and *SCF* transcripts in all cDNA specimens examined. A significant association between the co-expression of KIT/SCF and a worse clinical outcome was found. In conclusion, KIT expression was observed in a proportion of premalignant and malignant colonic lesions, while it was virtually absent in normal colon mucosa. Moreover, the majority of KIT-positive carcinomas co-expressed SCF, suggesting the possibility of aberrant signaling by an autocrine loop, as confirmed by the negative prognostic value of this association. Therefore, in the subset of CRC patients with concomitant KIT/SCF expression, the activity of Imatinib mesylate, a selective

inhibitor of specific tyrosine kinases including KIT, may be exploited in combination with standard therapy.

Introduction

Neoplastic conversion of human colorectal cells occurs in a stepwise fashion, from benign lesions to clinically-aggressive tumors. Tumor progression in this system, as defined by clinical and histopathological criteria, has been correlated with cumulative genetic events and with additional molecular abnormalities including mutation of cell oncogenes, inactivation of tumor suppressor genes (1-3), defects in DNA mismatch repair and aberrant DNA methylation (4-7).

Recently, aberrant expression of the proto-oncogene *c-kit* has been reported in a sub-group of colorectal carcinomas (CRC) (8-10). *c-kit* encodes a type III receptor tyrosine kinase, initially identified as the homologue of the Hardy-Zuckerman 4 feline sarcoma virus oncogene (11,12). Triggering of the KIT receptor by its ligand stem cell factor (SCF) (13) exerts multiple effects, both during development and in adult life, supporting the survival, proliferation, migration, and homing of hemato-poietic cells, mast cells, melanocyte precursors, and primordial germ cells (14).

Abnormal KIT expression has been observed in a variety of human tumors, including myeloid leukaemia (15,16), melanoma (17,18), glioblastoma (19), germ cell cancer (20,21), breast cancer (22,23) and small cell lung cancer (24,25). Furthermore, gain-of-function mutations in *c-kit* have been described primarily in leukemias and gastrointestinal stromal tumors (GIST) (26). Two general mechanisms of *c-kit* activation in malignant cells have been described: acquisition of activating mutations or autocrine or paracrine stimulation of the receptor by its ligand SCF (27).

Previously, we (28) and others (29) have shown that SCF supports anchorage-independent growth, cell survival and migration/invasion of selected KIT-positive colon carcinoma cells in an autocrine/paracrine fashion. To assess the significance of these findings in the development of CRC, we decided to analyze mRNA expression patterns of SCF and c-kit and the relative *in situ* protein distribution at successive stages of CRC progression.

Correspondence to: Dr Graziella Bellone, Department of Clinical Physiopathology, Via Genova 3, I-10126 Torino, Italy
E-mail: graziella.bellone@unito.it

Materials and methods

Tissue specimens and patients. The study group comprised 126 patients who were diagnosed either with colon adenoma (n=32) or adenocarcinoma (n=94) at the General Surgery Section of Department of Clinical Physiopathology (University of Turin, Turin, Italy) between January 1995 and June 2004. None underwent treatment before entering the study, which was conducted under strict observance of the principles of the Declaration of Helsinki. Adenoma tissue specimens were obtained through endoscopy and cancer specimens through surgical resection. All cancer patients had histopathologically-confirmed primary colorectal adenocarcinomas and were staged using the Dukes' system, as revised by Astler and Coller (30). Adenoma specimens were graded for degree of dysplasia (GD) (n=10 low, L; n=12 moderate, M; n=10 high, H).

Histopathologically-confirmed normal colon biopsies (n=73) were used as controls. Informed consent was obtained from patients for the experimental use of blood and surgical specimens, in accordance with the institutional ethics guidelines. The main clinical, demographic and pathological characteristics of the patients are shown in Tables I and II.

Aliquots of fresh normal, premalignant and malignant colon tissue were fixed in formalin and paraffin-embedded for immunohistochemical analysis or placed in liquid nitrogen prior to mRNA extraction. Frozen sections (6-μm) were taken from blocks of tissue and, starting with the first section, every fifth section was routinely stained with hematoxylin and eosin and evaluated histopathologically by an experienced pathologist (GV). Sections were pooled for *c-kit* and *SCF* mRNA analysis from areas estimated to have at least 80% malignant cells. In the same way, colon mucosa specimens from patients free of neoplastic or inflammatory diseases were examined histopathologically and defined as 'normal' tissue.

Complete follow-up data were available for all patients. Among patients with polyps, the median follow-up of surviving patients was 44 months (range, 35-96 months). When the study was concluded, 5 (16%) patients had died of causes non-attributable to the primary disease. Among cancer patients, 69 (73%) were still alive, whereas 25 (27%) had died [19/25 (76%) from causes attributable to the primary disease]. The median follow-up period of surviving patients was 58 months (range, 8-102 months): 43 patients remained disease-free, whereas 26 had a recurrence. The median time to recurrence was 13.6 months (range, 3-60 months).

RNA extraction and reverse transcription. RNA was extracted from frozen tissue specimens, and from appropriate positive controls consisting of DLD-1 and M07 cell lines, using TRIzol reagent (Invitrogen, Life Technologies, Gaithersburg MD) following the manufacturer's instructions. To remove traces of genomic DNA, total RNA (1 μg) was treated with DNase I (Invitrogen) and reverse-transcribed to cDNA using SuperScript II (Invitrogen) as described elsewhere (31).

Real-time reverse transcriptase (RT)-PCR. Real-time quantitative RT-PCR analysis was performed on the iCycler iQ system (Bio-Rad, Hercules, CA) with SYBR-Green I dye detection. Amplification of 18S, *c-kit* and *SCF* was performed

Table I. Main clinical and demographic features of the studied population.

	Controls	Adenoma patients	Carcinoma patients
n	73 (37%)	32 (16%)	94 (47%)
Male	35 (48%)	21 (66%)	48 (51%)
Age, years	52 (25-85)	63 (44-78)	63 (31-87)

Data are presented as medians (range) for continuous variables, and as frequencies (%) for categorical variables.

in duplicate on a PCR optical 96-well reaction plate (Bio-Rad). PCR mixture (25 μl) in each well contained 5 μl of cDNA (corresponding to 100 ng of total RNA), 2.5 μl of each sequence-specific primer (300 nM for rRNA *18S*, *c-kit*, *SCF-1* and *SCF-2*), 12.5 μl of 1X iQ SYBR-Green Supermix (Bio-Rad) and 2.5 μl of nuclease-free water. Primer sequences were designed to be cDNA specific and to work under equivalent reaction conditions using Beacon Designer 2 Software (Bio-Rad); primers were synthesized by Invitrogen and reconstituted in nuclease-free water before use. The sequences of sense and antisense primers used for SCF were designed to detect transcripts corresponding to the transmembrane isoforms SCF-1 (long form) and SCF-2 (short form). Primer sequences and reaction efficiencies are listed in Table III. A negative PCR control without cDNA template and a positive control specimen with a known cycle threshold (C_t) value were included in each assay. Optimized thermal cycling conditions were as follows: 5 min at 95˚C followed by 40 cycles of 15 sec at 95˚C and 1 min at 60˚C (two-step PCR). Specificity of the PCR products was confirmed by the melting curve program at the end of the reaction (55-95˚C with a heating rate of 0.5˚C/10 sec and continuous fluorescence measurements). PCR efficiency (E) was determined using the iCycler iQ software and the method described by Ramakers *et al* (32). For each specimen the C_t was acquired by the fit point method (33). The mRNA expression data for 18S showed no significant differences between control and patient groups. The relative expression ratio of the target genes was computed using the Relative Expression Software Tool (REST) (34). This software calculates an expression ratio relative to the control group (normal colon tissue) normalized by a reference gene (rRNA 18S). The expression ratio (R) is: $R = E_{target}^{\Delta Ct\ target\ (mean\ control-mean\ specimen)} / E_{reference}^{\Delta Ct\ reference\ (mean\ control-mean\ specimen)}$. An expression ratio of 1 means no regulation, >1 is up-regulation and <1 is down-regulation.

Immunohistochemical detection of c-KIT and SCF. For immunohistochemistry, consecutive formalin-fixed, paraffin-embedded tissue sections were immunostained for KIT, using a rabbit anti-KIT polyclonal antibody (Dako, Carpinteria, CA, USA, 1:100 dilution) which recognizes a peptide corresponding to amino acids 963-976 at the cytoplasmatic C-terminal portion, and for SCF, by means of an affinity-purified goat polyclonal antiserum raised against a peptide mapping at the

Table II. Main pathological features of the studied population.

Carcinoma Dukes' stages	A (%)	B1 (%)	B2 (%)	C1-C3 (%)	D (%)	Total (%)
n	15 (16)	13 (14)	25 (27)	24 (25)	17 (18)	94 (100)
Grading						
G1	3	1	1	0	0	5 (6)
G2	9	10	21	17	11	68 (72)
G3	3	2	3	7	6	21 (22)
Tumor site						
Right colon	4	6	10	6	7	33 (35)
Transverse colon	0	1	2	1	0	4 (4)
Descending and Sigmoid colon	8	3	11	12	5	39 (42)
Rectum	3	3	2	5	5	18 (19)

Data are presented as medians (range) for continuous variables, and as frequencies (%) for categorical variables.

Table III. Primer sequences for cytokine quantification by real-time RT-PCR.

Primer set	GenBank Accession no.	Primer sequence (5'→3')	RT-PCR E (%)
18S sense	X03205	CTGCCCTATCAACTTTCGATGGTAG	1.99
18S antisense		CCGTTTCTCAGGCTCCCTCTC	
c-kit sense	X06182	GATTATCCCAAGTCTGAGAATGAA	1.97
c-kit antisense		CGTCAGAATTGGACACTAGGA	
SCF-1 sense	NM_000899	CAGAGTCAGTGTCACAAAACCATT	1.98
SCF-1 antisense		TTGGCCTTCCTATTACTGCTACTG	
SCF-2 sense	NM_003994	CTGAGAAAGGGAAGGCCAAA	2.02
SCF-2 antisense		GCTGTCTCTTCTTCCAGTATAAG	

E = E = Efficiency deducted from the slope (s) of the standard curve based on E = e $^{\ln 10/-s}$ − 1.

N-terminus (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:100 dilution). The latter antibody does not discriminate between SCF-1 and SCF-2 isoforms. To detect the presence of mast cells, mouse anti-tryptase (Chemicom International, Temecula, CA; 1:1000 dilution) was used. Tissue sections were deparaffinized and rehydrated. Heat-induced epitope retrieval was performed with Target Retrieval Solution (Dako) using an electric pressure-cooker for 20 min at 120°C, with cooling before immunostaining. All tissues were then exposed to 3% hydrogen peroxide for 5 min, to quench endogenous peroxidase activity, and to primary antibody for 1 h. Immuno-staining for both proteins was then performed with the peroxidase-based visualization LSAB® kit (Dako), following the manufacturer's recommendations. Diaminobenzidine tetra-hydrochloride was used as chromogen. Incubation was at room temperature; between incubations sections were washed with Tris-buffered saline buffer. The slides were then counterstained with Mayer hematoxylin for 5 sec, dehydrated and mounted in Clarion (Biomeda, Foster City, CA, USA). A KIT-positive GIST tumor of the ileum and chorial villi were used as external positive controls for KIT and SCF expression, respectively (35,36). Substitution of the primary antibody with normal rabbit or goat serum served as a negative control for all immuno-staining tests. Specific staining for KIT and SCF, defined as the presence of stain in the plasma membrane and/or cytoplasm, was evaluated in the epithelial compartment. Internal controls for KIT immunostaining were inflammatory cells and interstitial cells of Cajal. Inflammatory cells were also considered as internal controls for SCF. Immunostaining was scored by the immunoreactive score (IRS) system proposed by Remmele and Stegner (37) in which IRS = SI (staining intensity) x PP (percentage of positive cells). SI was classified

Table IV. Normalized mRNA expression patterns in colon adenoma and carcinoma specimens.

mRNA expression	LGD[a]	MGD[a]	HGD[a]	A[b]	B1/B2[b]	C/D[b]
kit						
Up-regulation	9/10 (90)	10/12 (83)	9/10 (90)		23/38 (61)	28/41 (68)
Down-regulation	1/10 (10)	2/12 (17)	1/10 (10)	15/15 (100)	15/38 (39)	13/41 (32)
SCF-1						
Up-regulation	5/10 (50)	5/12 (42)	4/10 (40)	8/15 (53)	33/38 (87)	27/41 (66)
Down-regulation	5/10 (50)	7/12 (58)	6/10 (60)	7/15 (47)	5/38 (13)	14/41 (34)
SCF-2						
Up-regulation	7/10 (70)	9/12 (75)	10/10 (100)	14/15 (93)	32/38 (84)	27/41 (66)
Down-regulation	3/10 (30)	3/12 (25)		1/15 (7)	6/38 (16)	14/41 (34)

[a]Degree of dysplasia; [b]Dukes' stage. Patient numbers are presented as frequencies (%). Up-regulation, expression ratio >1; down-regulation, expression ratio <1.

as 0, negative; 1, weak; 2, moderate; and 3, strong. PP was defined as 0, negative; 1, 1-20% positive cells; 2, 21-50% positive cells; and 3, 51-100% positive cells. Ten visual fields from different areas of each specimen were chosen at random for IRS evaluation, and the average IRS was calculated.

Determination of SCF concentrations in sera. SCF concentrations were determined using an ELISA kit (R&D Systems, Abingdon, UK) in serum specimens from control subjects, adenoma and cancer patients, following the manufacturer's instructions. All specimens were evaluated in duplicate. The minimum detectable dose was below 9.0 pg/ml.

Statistical analysis. Statistical data analysis was performed with the biomedical statistical software package BMDP Dynamic, Rel. 7.0 (Statistical Solutions, Cork, Ireland) and MedCalc, version 7.6.0.0 (Med-Calc Software, Mariakerke, Belgium). The compatibility of data from continuous variables with a normal distribution was checked by means of Shapiro and Wilk's W-test. Means and SDs are given throughout as measures of central tendency and dispersion for data with normal distribution, medians and range otherwise. Differences between continuous variables were analyzed by the Student's t-test or One-way analysis of variance when data were normally distributed, and by means of the Mann-Whitney or the Kruskal-Wallis test otherwise. Associations between categorical variables were explored by means of Fisher's exact test and, when appropriate, the χ^2 test for linear trend. Correlation between mRNA expression (determined by real-time PCR) and protein expression (by IHC), as well as between the IHC patterns of c-kit and SCF, were quantified in terms of the corresponding Spearman correlation coefficients. Survival data for patients still alive at the end of the study were calculated to the time of the last follow-up examination. The log-rank (Mantel-Cox) test was used to test the null hypothesis of similarity of survival times among patients with different IHC patterns. The statistical significance of differences in mRNA expression of the cytokines examined in patients and control groups was analyzed by the REST software package for group-wise comparison, and this was also used for statistical analysis of relative expression results in real-time PCR (34). A level of 0.05 (two-tailed) was chosen to indicate statistical significance.

Results

Expression of c-kit mRNA and protein in normal, dysplastic and malignant colorectal tissues. Quantitative RT-PCR showed that, in comparison to normal colon mucosa, in almost all adenomas *kit* message was overexpressed (expression ratio >1). In locally extended tumors (Dukes' stage A) *kit* mRNA was down-regulated (expression ratio <1), while in more advanced tumors *kit* message was upregulated (Table IV). Moreover, in general, *c-kit* mRNA was expressed at lower levels in cancer specimens than in adenomas (median ratio 1.236, range 0.119-32.806; ratio 4.180, range 0.120-29.807, respectively; p=0.001 by the Mann-Whitney test). Nevertheless, when cancer patients were divided into the following three categories: Dukes' stages A, B1 or B2, C or D, a progressively increasing *c-kit* mRNA expression was observed (median ratio 0.473, range 0.371-0.791; ratio 1.249, range 0.119-29.807; and ratio 1.964, range 0.196-32.806, respectively; Kruskal-Wallis test =15.14, p=0.0005). By contrast, different degrees of dysplasia were not associated with significant variations in *c-kit* mRNA expression (p=0.66).

Moderate (3<IRS≤6) *in situ* cytoplasmic KIT protein expression was observed in 10/94 (10.6%) tumor specimens. In adenoma tissues, weak KIT immunostaining (1≤IRS≤3) was observed only in 4/32 (12.5%) of specimens, while it was virtually absent in epithelial cells of normal specimens. KIT was detected in the cytoplasm and/or on the cell membrane of inflammatory cells. When specimens were ordered in the following four categories: normal colon tissue, polyp, locally-advanced tumor (Dukes' stages A, B1 and B2), nodal or distally-spread tumor (Dukes' stages C and D), a strong linear trend for progressively higher KIT expression emerged (χ^2 for trend =17.36, p<0.0001). Representative KIT immunostaining in normal, and in positive dysplastic and malignant colorectal tissues is shown in Fig. 1.
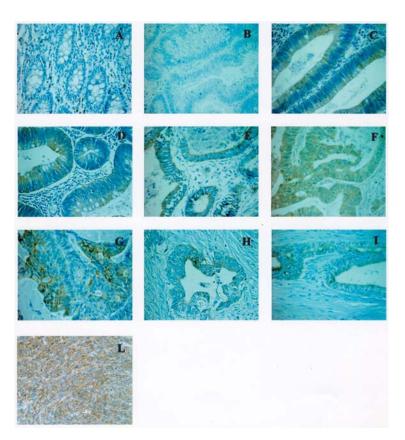
Figure 1. Representative immunohistochemical analysis of KIT protein expression in normal (A, negative staining), premalignant [degrees of dysplasia: low (B, negative staining), moderate (C, positive staining), and high (D, positive staining)] and malignant [Dukes' stages A (E, positive staining), B1 (F, positive staining), B2 (G, positive staining), C (H, positive staining), and D (I, positive staining)] colonic mucosa. L is the positive external control (human ileal GIST). (Original magnification x250).
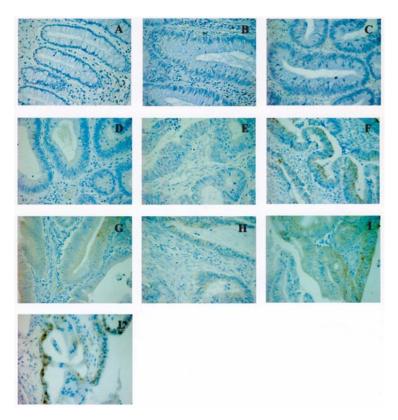

Figure 2. Representative immunohistochemical analysis of SCF protein expression in normal (A, negative staining), dysplastic [degrees of dysplasia: low (B, negative staining), moderate (C, negative staining), and high (D, negative staining)] and malignant [Dukes' stages A (E, negative staining), B1 (F, positive staining), B2 (G, positive staining), C (H, positive staining), and D (I, positive staining)] colorectal tissues. L is the positive external control (human placenta). (Original magnification x250).

Expression of SCF mRNA and protein in normal, dysplastic and malignant colorectal tissues. We assessed *SCF* transcript levels quantitatively by means of real-time RT-PCR. Relative to normal mucosa samples, a slight majority of adenoma cases revealed relatively lower *SCF-1* mRNA levels. In tumors, *SCF-1* mRNA was generally higher, with the highest levels observed in more advanced forms (Table IV). In general, *SCF-1* mRNA was expressed in cancer specimens at significantly higher levels than in adenomas (median relative expression 2.05, range 0.07-34.19; and median relative expression 0.60, range 0.07-8.09, respectively; p=0.007 by the Mann-Whitney test). When cancers specimens were subdivided into the following three categories: Dukes' stages A, B1 or B2, C or D, significantly increased *SCF-1* mRNA expression was observed in more advanced cases compared to the locally-advanced ones (respectively, medians of 1.03, 3.04 and 2.72; p=0.0003 by the Kruskal-Wallis test).

By contrast, compared with normal colon mucosa, *SCF-2* message was overexpressed in both, adenoma and the majority of carcinoma samples (Table IV). In general, cancer and adenoma specimens had similar *SCF-2* mRNA relative expression values (median 2.20, range 0.15-30.72; and 2.15, 0.20-27.82, respectively; p=0.33 by the Mann-Whitney test). Moreover, Dukes' stage and degree of dysplasia did not affect *SCF-2* mRNA expression in cancer and polyp specimens, respectively.

In situ protein expression for SCF was observed in 69/94 (73.4%) cancer specimens and in 8/32 (25%) adenoma specimens; all control specimens were negative for SCF immunostaining; in these cases staining was confined to the cytoplasm of the occasionally-present mononuclear inflammatory cells. Moreover, only cancer specimens possessed an IRS >1 (p<0.0001 by Fisher's exact test). When cancer specimens were ordered in four categories, Dukes' stages A, B1, B2, and C or D, the trend toward progressively increased SCF staining was very strong (χ^2 for trend = 53.32, p<0.0001). Representative SCF immunostaining in normal, dysplastic and malignant colorectal tissues is shown in Fig. 2.

No significant statistical correlation was found between SCF-2 real-time RT-PCR expression ratios and immuno-histochemistry scores. On the contrary, a strong correlation existed between these two methods with regard to SCF-1 (*R*=0.401, p<0.0001).

KIT and SCF protein co-expression. No co-expression of KIT and SCF proteins was observed in adenomas. By contrast, in malignant tumors, 8/10 (80%) of KIT-positive cases also strongly expressed SCF. The 20% of KIT-positive cases that did not co-express both proteins were all locally-advanced carcinomas (Dukes A and B1).

KIT and SCF immunostaining scores and clinical course. No significant association was found between KIT protein expression and survival (Mantel-Cox =2.013, p=0.15) or disease recurrence (Mantel-Cox =1.706, p=0.19), after stratification for possible confounding variables such as gender or age. Similarly, no association was found between SCF immunohistochemical scores and survival (Mantel-Cox =0.261, p=0.60) or disease recurrence (Mantel-Cox =0.384, p=0.53), after allowing for the same confounding variables.

An association was found between KIT and SCF protein co-expression and survival (Mantel-Cox =4.462, p=0.04) or recurrence (Mantel-Cox =9.783, p=0.01), after stratification for the same possible confounding variables.

SCF levels in control and patients sera. Mean serum SCF concentrations were 1008±196 pg/ml, 1035±207 pg/ml and 961±190 pg/ml, in controls, adenoma and cancer patients, respectively (One-way ANOVA, p=0.07). Serum SCF concentrations were not affected by cancer stage or degree of dysplasia.

Discussion

Several reports have indicated that KIT tyrosine kinase is rarely detected *in situ* in CRC. Reed *et al*, using stage-oriented human cancer tissue microarrays, reported that *in situ* KIT expression is sparse in colorectal carcinomas with only 1.6% of positive tumors, being poorly-differentiated carcinomas arising at the anorectal junction (9). In another study, faint cytoplasmic KIT positivity was found in 4.8% of CRC cases (8). More recently, Sammarco *et al* detected KIT-positive staining in the epithelium of the neoplastic colon in 25% of patients examined (10). In a previous preliminary analysis conducted on a small number of patients, we reported KIT immunoreactivity in several malignant mucosa specimens compared to normal counterparts (38). The present evaluation of KIT expression using a polyclonal antibody recommended for accurate GIST diagnosis (39) and using a larger number of CRC cases at different disease stages as well as adenomas, confirms that KIT overexpression is rare in these tumor forms. In premalignant disorders and tumor specimens, respectively, we found weak and moderate KIT immunostaining only in 12.5 and 10.6%. Nevertheless, when colon specimens were ordered by disease progression, a strong linear trend for progressively higher KIT expression emerged.

The discrepancy compared to our preliminary results may arise from the use of different reagents. Unfortunately, the quality of the earliest polyclonal anti-KIT antibodies produced and made available commercially was not very satisfactory, and different studies using a wide variety of different KIT antibodies, protocols, and scoring systems to identify KIT-positive tumors often yielded discrepant results (40).

Interestingly, unlike the immunohistochemistry expression pattern, when *c-kit* mRNA levels were quantitatively examined in total tissue by real-time PCR, expression was lower in CRC than in adenoma or normal mucosa. Alongside the technical issues related to the sensitivities of the two techniques, a possible explanation for this reduced expression could relate to the immunohistochemical localization of the KIT protein in non-epithelial cells. Immunohistochemistry performed on consecutive sections of a limited number of the tissue specimens analyzed showed the mononuclear inflammatory cells expressing KIT to be mostly tryptase-positive mast cells (data not shown). A cell count determined the mean number of mast cells, due to extension of the neoplastic epithelium compartment in patients' tumor tissue specimens, to be significantly fewer in cancer specimens than in premalignant polyps or normal tissue sections. Similar decreased *c-kit* expression in the progression from normal

colon to cancer has also been reported by another group, using standard RT-PCR (10). However, unlike in this study, we did not find a complete absence of *c-kit* mRNA in any of the tissue specimens examined.

The present study examined, for the first time, expression of the KIT ligand in normal, premalignant and malignant colon tissues. SCF immunoreactivity was not detected in normal control mucosa specimens; it was detected at a very low level in a few adenoma tissue specimens and in most cancer specimens, with a positive significant trend for progressive increase. Similar to c-kit expression, different expression patterns were observed for SCF when mRNA and protein abundance were compared in colorectal mucosa specimens by quantitative real-time RT-PCR and immunohistochemistry. SCF is produced by many cell types including mast cells (41), fibroblasts, stromal cells (42), and endothelial cells (43). The relative content of these cells and of epithelial cells may be altered in adenomatous and neoplastic tissues compared to normal tissues.

Moreover, two alternatively-spliced forms of membrane-bound SCF, a larger form of 45 kDa (SCF-1) and a smaller form of 31 kDa (SCF-2) exist, both equally biologically active (44). The antibody used to detect SCF recognizes both membrane-bound long and short forms as well as soluble SCF, while analysis at the mRNA level used two specific primers for SCF-1 and SCF-2 forms.

The discrepancy between SCF and c-kit mRNA and protein expression levels observed might also reflect distinct rates of receptor-mediated regulation and protein turnover in benign and malignant lesions, as discussed in more detail below.

We observed that normal and malignant mucosa specimens contained two *SCF* transcripts most likely generated by differential splicing of the *SCF* message and encoding functionally-distinct isoforms of this cytokine. These two isoforms are distinguished by the presence (SCF-1) or absence (SCF-2) of exon 6 sequences which encode an amino acid sequence required for efficient cleavage of soluble SCF from its transmembrane precursor (44). Because the major proteolytic cleavage site is absent in SCF-2, it remains cell-associated to a greater degree than SCF-1 (44). Importantly, we observed variations in the ratio of steady-state transcript levels corresponding to *SCF-1* and *SCF-2* mRNA in benign and malignant lesions. After normalizing to controls, *SCF-1* mRNA was found to be expressed in cancer specimens at significantly higher levels than in adenomas, and a significantly increased *SCF-1* mRNA expression was observed in more advanced cases compared to locally-advanced ones. By contrast, cancer and adenoma specimens had a similar *SCF-2* mRNA relative expression and Dukes' stage and degree of dysplasia did not affect *SCF-2* mRNA expression in cancer and polyp specimens, respectively.

The distinct patterns of SCF isoform expression may have functional consequences related to the dynamics of ligand-induced receptor activation and turnover. Studies in SCF negative cells established from *Sl/Sl* homozygous murine embryo fetal liver, and engineered to produce either soluble or transmembrane SCF, give support to this idea (45). Both SCF isoforms thus produced equally activated KIT on M07e cells. However, cell-associated SCF-1 induced sustained KIT phosphorylation, contrasted with transient phosphorylation of KIT induced by soluble SCF-2. Importantly, soluble but not

cell-associated KIT rapidly down-regulated KIT expression and accelerated KIT protein degradation. Moreover, soluble recombinant human SCF added to cells expressing cell-associated SCF also markedly reduced cell-surface KIT expression and induced KIT protein degradation. Several lines of evidence suggest that preferential expression of either the long or the short form of SCF has consequences beyond receptor trafficking and dynamics. For example, mice expressing soluble SCF but lacking transmembrane SCF have a severe phenotype affecting SCF-dependent cell lineages (46). Conversely, mice producing SCF-2 only reveal increased sensitivity to sublethal doses of γ-irradiation (47). Furthermore, transmembrane SCF is more potent than soluble SCF in maintaining the survival of multipotent hemopoietic stem cells *in vitro* (48). Experiments in *Sl/Sl^d* mouse embryos (48-50) have shown that, while transmembrane SCF plays a critical role in the proliferative response of primordial germ cells, soluble SCF is able to sustain cell migration but not cell proliferation.

Co-expression of KIT and SCF has been reported in several non-hematological malignancies including gynecological tumors (51), small cell lung cancers (25,52) and testicular tumors (53). Simultaneous expression of KIT and its ligand SCF has been reported in colon carcinoma cell lines (54,55), in which a growth/survival/migration stimulating autocrine loop has been demonstrated (28,29). *In vitro* studies indicated that KIT activation by autocrine or paracrine SCF is not a necessary requirement for colon carcinoma cell survival and growth in anchorage-independent conditions, although it can significantly amplify these malignant traits (28). The present study describes *in vivo* co-expression of SCF in 80% of KIT positive primary CRCs, all at more advanced tumor stages (Dukes' C and D). These results are consistent with the hypothesis that a KIT-dependent autocrine loop is established in a small number of nodal or distally-spread colorectal neoplasias. However, at present, the biological significance of this co-expression in colon carcinogenesis remains unclear.

It is noteworthy that our previous study showed that three primary cell lines derived from Dukes' D carcinomas, expressing *SCF* mRNA, but negative or weakly positive for KIT in immunohistochemistry, in culture expressed KIT at both mRNA and protein levels (56). The discrepancy between observations on paraffin-embedded tissue and primary cell lines might be explained by the assumption that the *kit* gene may be somehow up-regulated in colon carcinoma cells during *in vitro* cell maintenance.

Unlike in other tumors (57,58), the clinical impact of deregulated expression of this ligand/receptor system in CRC remains, at least in part, controversial. As a matter of fact, when KIT or SCF protein expression was analyzed separately, no significant association was found between either patient survival or disease recurrence. This unexpected result may be influenced by the very low incidence of CRCs aberrantly expressing *c-kit*, or it may be explained by the minor role played by KIT/SCF-dependent loop in the pathogenesis of this neoplasm. However, when KIT and SCF co-expression was taken into account, a negative prognostic value for both survival and cancer recurrence was found.

From the standpoint of medical treatment, CRC has been considered for decades a single-drug cancer type, since

5-fluorouracil (FU)-based palliative chemotherapy was the only choice. During the past five years the use of several cytotoxic drugs, sometimes in combination, has provided significant benefit to the patients, particularly in the metastatic setting (59). An additional increase in the effectiveness of systemic therapies can be expected from the new group of drugs targeting specific cellular pathways, such as tyrosine kinase inhibitor imatinib mesylate (STI-571, Gleevec®) (60). STI-571 has been used with interesting results to treat GIST (61). Although the above considerations suggest that KIT is not an attractive target in the majority of CRCs, it is possible that inhibition of KIT kinase activity might make colon carcinoma cells more susceptible to targeting another pathway. Our previous findings *in vitro* demonstrating that, in DLD-1 cells, STI-571 enhances the cytotoxic activity of 5-FU (56), support the clinical attractiveness of STI-571 in combination with conventional anticancer agents for a selected group of CRC patients, not responding to conventional therapies. The lesson learned from clinical trials of imatinib in GIST is that the mutational status of *c-kit* is required to achieve a successful response and improve survival (62). The presence of a truncated *c-kit* form with potentially strong activation capacity has been reported in some colon carcinoma cell lines (54,63) and, at the moment, in a single CRC patient (10). However, since we detected SCF in the majority of KIT-positive CRCs, it is conceivable that, in this tumor, activation of the receptor may be supported by this autocrine circuit. Therefore, based on these findings, it is tempting to speculate that the subgroup of CRCs co-expressing KIT and SCF may have the potential to benefit the most from treatment with tyrosine kinase inhibitors.

Acknowledgements

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