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KIT Signaling Promotes Growth of Colon Xenograft Tumors in Mice and Is Up-Regulated in a Subset of Human Colon Cancers

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BACKGROUND & AIMS: Receptor tyrosine kinase (RTK) inhibitors have advanced colon cancer treatment. We investigated the role of the RTK KIT in development of human colon cancer. **METHODS:** An array of 137 patient-derived colon tumors and their associated xenografts were analyzed by immunohistochemistry to measure levels of KIT and its ligand KITLG. KIT and/or KITLG was stably knocked down by expression of small hairpin RNAs from lentiviral vectors in DLD1, HT29, LS174T, and COLO320 DM colon cancer cell lines, and in UM-COLON#8 and POP77 xenografts; cells transduced with only vector were used as controls. Cells were analyzed by real-time quantitative reverse transcription polymerase chain reaction, single-cell gene expression analysis, flow cytometry, and immunohistochemical, immunoblot, and functional assays. Xenograft tumors were grown from control and KIT-knockdown DLD1 and UM-COLON#8 cells in immunocompromised mice and compared. Some mice were given the RTK inhibitor imatinib after injection of cancer cells; tumor growth was measured based on bioluminescence. We assessed tumorigenicity using limiting dilution analysis. **RESULTS:** KIT and KITLG were expressed heterogeneously by a subset of human colon tumors. Knockdown of KIT decreased proliferation of colon cancer cell lines and growth of xenograft tumors in mice compared with control cells. KIT knockdown cells had increased expression of enterocyte markers, decreased expression of cycling genes, and, unexpectedly, increased expression of LGR5 associated genes. No activating mutations in KIT were detected in DLD1, POP77, or UM-COLON#8 cells. However, KITLG-knockdown DLD1 cells formed smaller xenograft tumors than control cells. Gene expression analysis of single $CD44^+$ cells indicated that KIT can promote growth via KITLG autocrine and/or paracrine signaling. Imatinib inhibited growth of KIT^+ colon cancer organoids in culture and growth of xenograft tumors in mice. Cancer cells with endogenous KIT expression were more tumorigenic in mice. **CONCLUSIONS:** KIT and KITLG are expressed by a subset of human colon tumors. KIT signaling promotes growth of colon cancer cells and organoids in culture and xenograft tumors in mice via its ligand, KITLG, in an autocrine or paracrine manner. Patients with KIT-expressing colon tumors can benefit from KIT RTK inhibitors.

Keywords: CD117; Stem Cell Factor; Colorectal Cancer; Enteroids.

More than 1 million cases of colon cancer are diagnosed annually worldwide.[1] Survival rates for advanced disease remain poor, despite the use of recently developed receptor tyrosine kinase (RTK) inhibitors, such as regorafenib.[2,3] Regorafenib's high-affinity targets include the vascular endothelial growth factor receptor, epidermal growth factor receptor, and the oncogenic kinases KIT, RET, and BRAF. Identifying which of these targets drives colon cancer progression would help identify patients for targeted therapy.[4] Because RTK inhibitors are nonspecific, genetic approaches are required for determining which RTKs are important for colon cancer growth.

KIT (CD117) is an RTK expressed in specialized goblet cells that contribute to the stem cell niche in the murine colon crypt base.[5] In human colon, KIT expression has been reported in several colon cancer cell lines, although KIT expression in primary colon tumors has been controversial.[6–10] KIT signaling occurs when it binds its only known ligand, KITLG (stem cell factor), causing receptor dimerization, autophosphorylation, and intracellular signaling. KIT can promote cell growth, survival, migration, differentiation, and secretion in different biological contexts.[11–13] Activating/oncogenic mutations in KIT are well

Abbreviations used in this paper: **Cy, cyanine; FACS, fluorescence-activated cell sorting; LGR5, leucine-rich repeat-containing G-protein coupled receptor 5; NSG, NOD-scid IL2Rg^{null}; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RTK, receptor tyrosine kinase; shRNA, small hairpin RNA.**

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documented in gastrointestinal stromal tumor, melanoma, and other diseases.[11,13] However, the roles of KIT and KITLG remain incompletely understood in colon cancer. In addition, studies on patient-derived xenografts are lacking.

Here we show that a subset of human colon cancers expresses KIT in the tumorigenic $CD44^+$ fraction.[14] Stable KIT knockdown inhibits colon cancer growth, increases expression of differentiation markers, and, unexpectedly, increases expression of stem cell–associated genes. KIT may drive tumor growth via autocrine and/or paracrine signaling by KITLG, since no activating KIT mutations were found. $CD44^+KIT^+$ cells are also highly tumorigenic. Our findings support the hypothesis that patients with KIT-expressing colon tumors can benefit from KIT inhibition.

Methods

Additional details for methods can be found in the Supplementary Materials.

Mice

Mice were maintained at the Stanford University Research Animal Facility in accordance with Stanford University guidelines. Four-to eight-week-old NOD-scid $IL2Rg^{null}$ (NSG) mice were used (The Jackson Laboratory, Bar Harbor, ME).

Tumor Microarray

A collection of 316 colon tumors were sectioned, processed, and stained with anti-human cKIT (cat# A4502; Dako, Carpinteria, CA) and KITLG (cat #2093, Cell Signaling Technology, Danvers, MA) antibodies. The collection consisted of 137 primary tumors from University of Toronto patients and associated xenografts. Staining was scored as uninterpretable, none, weak, or strong.

Tissue Culture and Xenograft Formation

Cell lines and xenograft-derived organoids were grown in advanced Dulbecco's modified Eagle medium/F12 (Invitrogen, Carlsbad, CA) with 10 mM HEPES, 1× Glutamax (Life Technologies, Carlsbad, CA), 10% heat-inactivated fetal bovine serum, 120 μg/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin-B. Organoids were seeded in growth factor reduced Matrigel (BD Biosciences, San Jose, CA). Xenografts were derived from cells or tumor chunks implanted subcutaneously into flanks of NSG mice.

Small Hairpin RNA Lentiviral Transduction

Small hairpin RNA (shRNA) oligonucleotides containing KIT- or KITLG-targeting sequences (Supplementary Table 1) were designed using pSicoOligomaker 1.5 (The Jackson Laboratory) and Basic Local Alignment Search Tool (National Center for Biotechnology Information, Bethesda, MD) against the human genome to minimize off-target effects. Five oligonucleotides each were screened for KIT and KITLG knockdown, and the best 2 were selected. A modified pSico-Pgk-GFP vector (plasmid# 12093; Addgene, Cambridge, MA) containing the shRNA sequences preceded by the EF1α promoter, along with lentiviral packaging vectors Δ8.9 and VSVG, were transfected into 293T fibroblasts using FuGENE (Promega, Madison, WI).[15]

Flow Cytometry

Methods for flow cytometry have been described previously.[5] Briefly, cells in single-cell suspension were stained in the dark on ice with fluorescently conjugated antibodies: Kit-phycoerythrin (PE) (cat# 340529; BD Biosciences), Kit-phycoerythrin-cyanine 7 (PE-Cy7) (cat# 339195; BD Biosciences), CD44-PE-Cy7 (cat# 560533; BD Biosciences), CD44-allophycocyanin (APC) (cat# 559942; BD Biosciences), epithelial cell adhesion molecule (EPCAM)-Alexa 488 (clone 9C4; BioLegend, San Diego, CA), H2KD-Pacific Blue (clone SF1-1.1; BioLegend), and MKI67-peridinin chlorophyll-cyanine5.5 (PerCP-Cy5.5) (cat# 561284; BD Biosciences). 5-Ethynyl-2′-deoxyuridine (EdU) staining was performed with Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Life Technologies) and BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Bioscences).

Single-Cell Gene Expression Analysis

Double-sorted fluorescence-activated sell sorted (FACS) isolated single cells with purity >95% were sorted into individual wells of 96-well plates containing 5 μL lysis buffer (Cells Direct qRT-PCR mix; Invitrogen) and 2 U (0.1 μL) SuperaseIn (Invitrogen), and processed as described.[5,16]

Bioluminescence Imaging

Colon cancer cells/xenografts engineered to constitutively express luciferase (LUC2) by lentiviral transduction were injected into flanks of NSG mice.[17] Mice received intraperitoneal injections of luciferin (Biosynth, Itasca, IL), and total flux (photons/second) was recorded.

Immunofluorescence Imaging

Paraffin-embedded tissue sections were stained with mouse anti-human Ki67 (Clone MIB-1; Dako) and rabbit anti-human CD117 (cat# A4502; Dako) primary antibodies, with donkey anti-mouse Cy5 (cat# 715-175-150; Jackson ImmunoResearch Laboratories Inc, West Grove, PA) and goat anti-rabbit A568 (cat# SAB4600084; Sigma, St Louis, MO) secondary antibodies. Stained sections were mounted with ProLong Gold+Dapi (Molecular Probes, Eugene, OR).

Imatinib Experiments

Imatinib mesylate (LC Laboratories, Woburn, MA) was dissolved in phosphate-buffered saline at the specified concentrations for each experiment. For in vivo studies, imatinib 50 mg/kg/d or phosphate-buffered saline control was administered to mice via intraperitoneal injections.

Statistical Analysis

Values represent mean, SD, or SEM as indicated. Differences between groups were determined using the 2-tailed Student t test. One-way analysis of variance was performed with Tukey's post-hoc test. L-Calc (Stemcell Technologies, Vancouver, BC, Canada) was used for limiting dilution analysis. All tests used a significance cutoff of $P < .05$. Analysis was performed with GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA).