Intratumoral Heterogeneity for Expression of Tyrosine Kinase Growth Factor Receptors in Human Colon Cancer Surgical Specimens and Orthotopic Tumors

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The design of targeted therapy, particularly patient-specific targeted therapy, requires knowledge of the presence and intratumoral distribution of tyrosine kinase receptors. To determine whether the expression of such receptors is constant or varies between and within individual colon cancer neoplasms, we examined the pattern of expression of the ligands, epidermal growth factor, vascular endothelial growth factor, and platelet-derived growth factor-B as well as their respective receptors in human colon cancer surgical specimens and orthotopic human colon cancers growing in the cecal wall of nude mice. The expression of the epidermal growth factor receptor and the vascular endothelial growth factor receptor on tumor cells and stromal cells, including tumor-associated endothelial cells, was heterogeneous in surgical specimens and orthotopic tumors. In some tumors, the receptor was expressed on both tumor cells and stromal cells, and in other tumors the receptor was expressed only on tumor cells or only on stromal cells. In contrast, the platelet-derived growth factor receptor was expressed only on stromal cells in both surgical specimens and orthotopic tumors. Examination of receptor expression in both individual surgical specimens and orthotopic tumors revealed that the platelet-derived growth factor receptor was expressed only on stromal cells and that the patterns of epidermal growth factor receptor and vascular endothelial growth factor receptor 2 expression differed between tumor cells. This heterogeneity in receptor expression among different tumor cells suggests that target-

ing a single tyrosine kinase may not yield eradication of the disease. (Am J Pathol 2008; 172:358–366; DOI: 10.2353/ajpath.2008.070625)

Growth factors and their receptors play a major role in the regulation of progressive cancer growth and metastasis.^{1,2} For this reason, the expression of growth factors and their receptors by cancer cells and host stromal cells, including tumor-associated endothelial cells, has been an attractive target for therapy.^{3,4} Recently, specific inhibitors of tyrosine kinase receptors have been targeted in patients with chemotherapy-refractory neoplasms, and this approach has led to prolongation of median survival in some patients.^{5–7} The failure of this approach to produce improved clinical outcome in a larger proportion of the treated patients indicates the necessity of establishing better criteria for patient selection and evaluation of therapy.

Tumor progression, angiogenesis, and metastasis are regulated by the interaction of tumor cells with the host organ microenvironment.⁸ The expression of growth factors and their receptors varies in different zones of any given organ and may differ among different cells of the same tumor.^{9,10} Overexpression of transforming growth factor- α (TGF- α), epidermal growth factor (EGF), and the EGF receptor (EGFR) has been reported to be associated with poor prognosis in multiple neoplasms.^{11–15} Similarly, expression of vascular endothelial growth factor (VEGF) is associated with increased vascular permeability, cell proliferation, and survival^{16–19} of endothelial cells. The expression of VEGF has been correlated with mi-

Supported in part by Cancer Center Support grant CA16672 and SPORE in Prostate Cancer grant CA902701 from the National Cancer Institute, National Institutes of Health.

Accepted for publication November 13, 2007.

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crovessel density of neoplasms,^{20,21} metastasis, and, hence, poor prognosis.^{22–26} The expression of platelet-derived growth factor (PDGF) and its receptor (PDGFR) by tumor cells, tumor-associated endothelial cells,^{3,27,28} and pericytes and myofibroblasts^{4,29–32} is common to many neoplasms. Our recent data clearly established that in clinical specimens of human colon carcinomas, although PDGF is produced by tumor cells, the expression of PDGFR is restricted to stromal cells, including tumor-associated endothelial cells.³⁰ The significance of these findings is that tumor-associated stromal cells generate a microenvironment favorable to the survival and progressive growth of tumor cells.³³ For example, PDGFR signaling is related to recruitment of pericytes and control of interstitial fluid pressure,^{33–36} favorable to the survival and progressive growth of neoplasms.³⁷

The design of targeted therapy directed against tyrosine kinase receptors, particularly the design of patient-specific therapy (“customization”), requires identification of the presence and intratumoral distribution of tyrosine kinase receptors. To determine whether the expression of these receptors is constant or varies between and within different colon neoplasms, we examined the expression of the ligands TGF- α , EGF, VEGF, and PDGF-B and their respective receptors in surgical specimens from several human colon cancers of different pathological stages and in orthotopic human colon cancers growing in nude mice. Our finding of intratumoral heterogeneity in receptor expression dictates the use of multiple tyrosine kinase inhibitors for effective therapy.

Materials and Methods

Surgical Specimens

Twelve surgical specimens of colon carcinoma (seven cases Duke's stage B; two cases Duke's stage C; and three cases Duke's stage D) were obtained from The University of Texas M. D. Anderson Cancer Center Tissue Bank. The specimens were frozen in liquid nitrogen or fixed in neutralized-buffered formalin within 30 minutes after surgical resection, making the analysis of phosphorylated receptors possible. The approval of the Institutional Review Board was obtained before we conducted the study.

Human Colon Cancer Cell Lines

The highly metastatic human colon cancer cell lines KM12SM³⁸ and HT29^{4,30} were maintained in minimal essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, a twofold vitamin solution (Life Technologies, Grand Island, NY), and penicillin/streptomycin (Flow Laboratories, Rockville, MD). Adherent monolayer cultures were maintained at 37°C in a mixture of 5% CO₂ and 95% air. The cultures were free of *Mycoplasma* species and pathogenic murine viruses (assayed by Science Applications International Co., Fred-

erick, MD). The cultures were maintained for no longer than 12 weeks after recovery from frozen stock.

Reagents

The primary antibodies used were rabbit anti-phosphorylated VEGFR1 (Flk-1, Oncogene, Boston, MA), rabbit anti-VEGFR2 (C1158, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-VEGFR2 (A3, Santa Cruz Biotechnology), rabbit anti-VEGF (A20, Santa Cruz Biotechnology), rabbit anti-phosphorylated EGFR (pEGFR, Tyr¹¹⁷³, Biosource, Camarillo, CA), mouse anti-EGFR (Zymed, San Francisco, CA), rabbit anti-EGFR (Santa Cruz Biotechnology), rabbit anti-TGF- α (Santa Cruz Biotechnology), rabbit anti-EGF (Santa Cruz Biotechnology), rabbit anti-PDGFR- β (Santa Cruz Biotechnology), rabbit anti-phosphorylated PDGFR- β (Santa Cruz Biotechnology), rabbit anti-PDGF-B (Santa Cruz Biotechnology), rat anti-mouse CD31 (BD PharMingen, San Diego, CA), and mouse anti-human CD31 (Dako A/S, Copenhagen, Denmark). Peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was a secondary antibody used for colorimetric immunohistochemical analysis. Fluorescent secondary antibodies were Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories), Cy5-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories), and Cy5-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories).

Animals and Orthotopic Implantation of Tumor Cells

Male athymic nude mice (NCI-nu) were purchased from the Animal Production Area of the National Cancer Institute Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and the National Institutes of Health. The mice were used in accordance with institutional guidelines when they were 8 to 12 weeks old.

To produce cecal tumors, KM12SM or HT29 cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% fetal bovine serum, and the cells were washed once in serum-free medium and resuspended in Ca²⁺/Mg²⁺-free Hanks' balanced salt solution. Only suspensions consisting of single cells with >90% viability were used. KM12SM cells (5×10^5) and HT29 cells (1×10^6) in 50 μ l of Hanks' balanced salt solution were injected into the cecal wall of nude mice under a dissecting microscope as described previously.³⁸

Necropsy and Histopathological Processing

Six weeks after intracecal injection of tumor cells, mice were euthanized with methoxyflurane and necropsied. Body weight, tumor incidence, tumor weight, and incidence of lymph node metastasis were recorded. Tumor tissues were fixed in 10% neutralized buffered formalin to prepare paraffin sections or embedded in ornithine carbamyl transferase compound (Miles, Elkhart, IN) to prepare frozen sections for hematoxylin and eosin staining and immunohistochemical analysis.

Immunohistochemical Staining for Ligands

Paraffin-embedded tissues were used for immunohistochemical analyses of TGF- α , EGF, VEGF, and PDGF-B. The sections were deparaffinized in xylene and rehydrated with alcohol and phosphate-buffered saline (PBS). Endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS. The slides were placed in a humidified chamber and incubated with protein blocking solution (5% normal horse serum and 1% normal goat serum in PBS) for 20 minutes at room temperature and incubated overnight at 4°C with primary antibody for TGF- α (1:00), EGF (1:00), VEGF (1:50), or PDGF-B (1:100). Slides were washed with PBS for 3 minutes three times and incubated for 1 hour with peroxidase-conjugated secondary antibody (1:500). Positive reaction was detected by exposure to stable 3,3'-diaminobenzidine (Phoenix Biotechnologies, Huntsville, AL). The slides were counterstained with Gill's no. 3 hematoxylin. Slides stained by diaminobenzidine or hematoxylin and eosin were examined in a Nikon Microphoto-FX microscope equipped with a three-chip charge-coupled device color video camera (model DXC990, Sony Corp., Tokyo, Japan). Digital images were captured using Optimas image analysis software (Media Cybernetics, Silver Spring, MD).

Double Immunofluorescence Staining for Receptors and Phosphorylated Receptors

Frozen sections of human colon cancers from the cecum of nude mice were assayed. Specimens were cut into 4- μ m sections, mounted on positively charged slides, and stored at -80°C. Slides were fixed for 10 minutes in cold acetone, placed in a light-shielded humidified chamber, incubated for 20 minutes with protein blocking solution (5% normal horse serum and 1% normal goat serum in PBS) at room temperature, and incubated overnight at 4°C with primary antibody against CD31 (1:800). The slides were washed three times with PBS and then incubated for 1 hour at room temperature with goat anti-rat Cy-5 secondary antibody (1:500). Nuclear counterstain with Sytox green was applied for 10 minutes, and mounting medium (90% glycerol, 10% PBS, and 0.1 mol/L propyl gallate) was placed on each sample with a glass coverslip (Fisher Scientific, Pittsburgh, PA).

Endothelial cells (CD31-positive cells) were identified by green fluorescence, whereas cells positive for EGFR, pEGFR, VEGFR2, pVEGFR2, PDGFR- β , or pPDGFR- β

were identified by red fluorescence. The expression of EGFR, pEGFR, VEGFR2, pVEGFR2, PDGFR- β , or pPDGFR- β on endothelial cells was detected by co-localization of red and green fluorescence, which produced yellow staining.

Double Immunofluorescence Staining for EGFR and PDGFR- β , VEGFR2 and PDGFR- β , and EGFR and VEGFR2

Frozen sections of cecal tumors were used for double immunofluorescence staining. Specimens were cut into 4- μ m sections, mounted on positively charged slides, and stored at -80°C. Slides were fixed for 10 minutes in cold acetone, placed in a light-shielded humidified chamber, incubated for 20 minutes with protein blocking solution (5% normal horse serum and 1% normal goat serum in PBS) at room temperature, and incubated overnight at 4°C with goat anti-mouse IgG, Fab fragment (Jackson ImmunoResearch), to block endogenous immunoglobulins. The slides were again blocked briefly in protein blocking solution and incubated overnight at 4°C with primary mouse anti-EGFR (1:100) or mouse anti-VEGFR2 (1:400). The slides were washed three times with PBS and then incubated for 1 hour at room temperature with goat anti-mouse Cy-5 secondary antibody (1:500). Then, the slides were incubated overnight at 4°C with rabbit anti-VEGFR2 (1:400) or rabbit anti-PDGFR- β (1:400). The slides were washed with PBS for 3 minutes three times and then incubated for 1 hour at room temperature with goat anti-rabbit Cy-3 secondary antibody (1:500). Nuclear counterstain with Sytox green was applied for 10 minutes, and mounting medium was placed on each sample with a glass coverslip (Fisher Scientific). EGFR- or VEGFR2-positive cells were identified by green fluorescence, whereas VEGFR2- or PDGFR- β -positive cells were identified by red fluorescence. Co-localization of EGFR and PDGFR- β , VEGFR2 and PDGFR- β , or EGFR and VEGFR2 was detected by yellow staining.

Confocal Microscopy

Confocal fluorescence images were collected using 20 $\times$ or 40 $\times$ objectives on a Zeiss LSM 510 laser scanning microscopy system (Carl Zeiss Inc., Thornwood, NY) equipped with a motorized Axioplan microscope, argon laser (458/477/488/514 nm, 30 mW), HeNe laser (413 nm, 1 mW, and 633 nm, 5 mW), LSM 510 control and image acquisition software, and appropriate filters (Chroma Technology Corp., Brattleboro, VT). Confocal images were exported to Adobe Photoshop software for preparation of montages.

Assessment of Intensity of Cytoplasmic Staining

The intensity of cytoplasmic staining for TGF- α , EGF, VEGF, PDGF-B, EGFR, pEGFR, VEGFR2, pVEGFR2,

Figure 1. Expression of TGF- α , EGF, VEGF, PDGF-B, EGFR, pEGFR, VEGFR2, pVEGFR2, PDGFR- β , and pPDGFR- β in surgical specimens of human colon carcinoma. **A:** The ligands were mainly expressed on tumor cells, and their expression was higher in more advanced cases (eg, case 10) than in less advanced cases (eg, case 6). **B:** Case 10 (stage D). **C:** Case 6 (stage B). Fluorescence double-labeled immunohistochemical analysis for receptors and phosphorylated receptors (red) and CD31 (green). Co-localization of the receptors and CD31 (endothelial cells) produced yellow staining. Expression of EGFR and VEGFR2 was detected on endothelial cells (yellow) as well as tumor cells (red). PDGFR- β and activated PDGFR- β were not expressed on tumor cells but rather only on stromal cells, including endothelial cells (yellow).

Table 1. Expression of Ligands and Their Receptors in Surgical Specimens of Human Colon Cancer

	Case no.	TGF- α	EGF	VEGF	PDGF-B	EGFR		pEGFR		VEGFR2		pVEGFR		PDGFR- β			pPDGFR- β		
						TC	EC	TC	EC	TC	EC	TC	EC	TC	EC	SC	TC	EC	SC
Duke's B	1	2	1	2	2	2	2	2	3	1	2	1	2	0	1	3	0	1	2
	2	2	0	1	1	0	2	0	2	3	3	2	1	0	0	3	0	0	2
	3	1	0	2	1	1	2	1	2	3	3	3	2	1	0	1	0	0	1
	4	1	2	0	2	2	1	2	1	0	2	0	0	1	1	3	1	1	2
	5	2	0	3	2	2	2	2	2	0	3	0	2	0	2	2	0	1	1
	6	1	1	1	0	1	0	1	0	0	2	0	2	0	0	2	0	0	0
	7	0	0	2	1	2	2	0	0	2	3	1	3	0	2	1	0	1	1
Duke's C	8	3	1	3	3	2	2	2	2	1	2	2	1	0	2	2	0	2	2
	9	2	2	1	1	2	2	2	2	2	2	1	2	0	1	3	0	1	2
Duke's D	10	3	3	3	3	3	3	3	3	3	3	3	3	0	3	3	0	3	3
	11	1	1	2	2	1	2	1	2	1	3	1	2	0	2	3	0	2	2
	12	3	3	3	3	3	2	2	2	1	2	1	2	0	3	3	0	2	2
High-expression cases (%)		58	33	67	58	67	83	58	75	42	100	33	75	0	50	83	0	33	67

PDGFR- β , and pPDGFR- β was evaluated in 10 random fields at 200 $\times$ magnification and confirmed by an image analyzer using the Optimas image analysis software program (Bioscan, Edmonds, WA; Media Cybernetics, Silver Spring, MD). For negative controls, nonspecific IgG was used as the primary antibody. The staining intensity of the negative control was considered the background staining intensity and was measured and subtracted from the value of each sample. Intensity of staining for TGF- α , EGF, VEGF, PDGF-B, EGFR, pEGFR, VEGFR2, pVEGFR2, PDGFR- β , and pPDGFR- β was graded on a scale of 0 to 3+, with 0 representing no detectable staining and 3+ representing the strongest staining. The expression was defined as high if the score was 2+ or 3+ and low if the score was 0 or 1+.

Results

Expression of TGF- α , EGF, VEGF, and PDGF-B in Surgical Specimens of Human Colon Carcinomas

In the 12 surgical specimens of colon carcinomas that we examined, TGF- α , EGF, VEGF, and PDGF-B were mainly expressed on tumor cells, and expression of these ligands correlated with advanced stages of the disease (Figure 1A; Table 1).

Expression of EGFR, pEGFR, VEGFR2, pVEGFR2, PDGFR- β , and pPDGFR- β on Tumor Cells and Stromal Cells, Including Tumor-Associated Endothelial Cells

Surgical specimens were analyzed for expression of EGFR, pEGFR, VEGFR2, pVEGFR2, PDGFR- β , and pPDGFR- β . Receptors and phosphorylated receptors were labeled red, and endothelial cells (CD31-positive cells) were labeled green. The expression of tyrosine kinase receptors by tumor-associated endothelial cells was detected by co-localization, ie, yellow color. In all cases, EGFR and pEGFR were expressed by both tumor cells and endothelial cells; rates of expression varied by cell type. The percentage of cells expressing

EGFR and pEGFR on tumor cells in stage D and stage B surgical specimens was 67% and 58%, respectively. On tumor-associated endothelial cells in stage D and stage B surgical specimens, it was 83% and 75%, respectively. VEGFR2 and pVEGFR2 were expressed on tumor cells in only a few neoplasms but were expressed on tumor-associated endothelial cells in all neoplasms. PDGFR- β and pPDGFR- β were not expressed on tumor cells in any neoplasm; these molecules were expressed only on stromal cells (red) and tumor-associated endothelial cells (yellow) (Figure 1, B and C; Table 1).

In the next series of experiments, we determined whether tumor-associated endothelial cells expressed multiple protein tyrosine kinase receptors. Images captured in identical regions of specimens clearly demonstrated heterogeneity with respect to expression of EGFR, VEGFR2, and PDGFR- β on tumor-associated endothelial cells (Figure 2).

Figure 2. Heterogeneity for expression of EGFR, VEGFR2, and PDGFR- β on tumor-associated endothelial cells in a surgical specimen of Duke's stage D colon carcinoma. Sections were stained with antibodies against EGFR, VEGFR2, or PDGFR- β (red fluorescence) and CD31 (green fluorescence). Double immunofluorescence staining revealed that endothelial cells were heterogeneous in expression of EGFR, VEGFR2, and PDGFR- β .

Figure 3. Expression of TGF- α , EGF, VEGF, PDGF-B, and their receptors in orthotopic human colon cancers (KM12SM and HT29) growing in the cecal wall of nude mice. **A:** Immunohistochemical analysis showed that the KM12SM and HT29 cells expressed TGF- α , EGF, VEGF, and PDGF-B. **B and C:** Tumor sections were stained with antibodies against EGFR, pEGFR, VEGFR2, pVEGFR2, PDGFR- β , or pPDGFR- β (red fluorescence) or CD31 (green fluorescence). Co-localization of the receptors and CD31 (endothelial cells) produced yellow staining. EGFR and VEGFR2 were highly expressed on tumor cells (red) as well as tumor-associated endothelial cells (yellow), and these receptors were phosphorylated in both KM12SM and HT29 tumor cells. High expression of PDGFR- β and pPDGFR- β was detected only on stromal cells (red), including endothelial cells (yellow), but not on tumor cells. These data closely agree with data from surgical specimens of human colon carcinoma.

Immunohistochemical Analysis for Expression of Ligands and Their Receptors in Orthotopic Human Colon Carcinomas in the Cecum of Nude Mice

Human KM12SM and HT29 colon cancer cells were implanted into the cecum of nude mice, and 6 weeks later tumors were resected and processed for immunohistochemical analysis. Both KM12SM and HT29 cells from orthotopic human colon carcinomas growing in nude mice expressed high levels of TGF- α , EGF, VEGF, and PDGF-B (Figure 3A). EGFR and VEGFR2 were highly expressed on both tumor cells (red) and tumor-associated endothelial cells (yellow), and these receptors were phosphorylated in both KM12SM cells (Figure 3B) and HT29 cells (Figure 3C). As in clinical specimen of human colon carcinomas, PDGFR- β and pPDGFR- β were not expressed on tumor cells but rather were expressed on stromal cells (red) and tumor-associated endothelial cells (yellow) (Figure 3, B and C). These data confirmed that the orthotopic colon cancer model reflected colon tumors in patients.

Intratumoral Heterogeneity for EGFR, VEGFR2, and PDGFR- β Expression in Surgical Specimens of Colon Carcinoma and Orthotopic Human Colon Carcinomas in the Cecum of Nude Mice

To determine the extent of intratumoral heterogeneity for expression of EGFR, VEGFR2, and PDGFR- β in colon carcinomas, we performed double immunofluorescence staining for EGFR and PDGFR- β , EGFR and VEGFR2, and VEGFR2 and PDGFR- β in a surgical specimen of colon carcinoma (case 10, Duke's D) and in orthotopic KM12SM and HT29 carcinomas growing in the cecal wall of nude mice. EGFR and VEGFR2 were expressed on both tumor cells and tumor-associated endothelial cells, whereas PDGFR- β was expressed only on stromal cells, including tumor-associated endothelial cells (Figure 4). On tumor cells, expression of EGFR and VEGFR2 was not uniform; many tumor cells expressed both EGFR and VEGFR2, some cells expressed only EGFR, and other cells expressed only VEGFR2 (Figure 4).

Discussion

In this study, we examined the expression of TGF- α , EGF, VEGF, and PDGF- β and their respective receptors in 12 human colon cancer surgical specimens and in orthotopic tumors produced by two distinct human colon carcinoma cell lines. The present data clearly demonstrate inter- and intratumoral heterogeneity in expression of these growth factors and their receptors and confirmed that orthotopic colon cancer animal models reflect the clinical reality.

Different proteins produced by tumor cells regulate the interaction between tumor cells and the organ microen-

Figure 4. Double immunofluorescence staining for EGFR and PDGFR- β , VEGFR2 and PDGFR- β , and EGFR and VEGFR2 in a human colon cancer surgical specimen (Duke's stage D) and orthotopic human colon cancers (KM12SM and HT29) growing in the cecal wall of nude mice. Tumor tissues were stained with antibody against EGFR or VEGFR2 with green fluorescence or antibody against VEGFR2 or PDGFR- β with red fluorescence. Co-localization of receptors appears as yellow staining. Many tumor cells expressed both EGFR and VEGFR2; others expressed only EGFR, and still others expressed only VEGFR2. The expression of EGFR and VEGFR2 by stromal cells was also heterogeneous. PDGFR was expressed only on stromal cells, including tumor-associated endothelial cells. This expression appeared uniform.

vironment.³⁹ Many protein tyrosine kinase receptors on tumor cells and tumor-associated endothelial cells can be induced or up-regulated by ligands produced by tumor cells via autocrine and paracrine pathways.⁴⁰ Treatment of cancer by inhibition of protein tyrosine kinase receptors has produced promising results.⁵⁻⁷ However, in many clinical trials, the presence of targets in patients was not confirmed, and the response rate was unpredictable.⁴¹ For inhibition of phosphorylated EGFR, the presence of mutated receptors was reported to be a predictable variable,⁴² but significant therapeutic responses of cancer cells with wild-type receptors has also been reported.^{4,43} These confusing criteria for selection of patients strongly indicate the need for better methodologies.

The data reported here demonstrating inter- and intratumoral heterogeneity for expression of EGFR, VEGFR2, and PDGFR- β in different human colon cancer specimens of different stages suggest that targeting a single tyrosine kinase receptor is not likely to provide significant therapeutic results. In other words, targeted therapy is effective only against its target, and eliminating tumor cells that are dependent on one pathway, eg, EGFR, is not likely to prevent the proliferation of tumor cells that are independent of this pathway.

Our results agree with a recent report demonstrating biological heterogeneity of tyrosine kinase receptors in other cancers.^{44,45} These data suggest that more effective therapy for human colon cancers will require inhibition of multiple receptor pathways. This issue is not unique to colon cancer. We previously reported that simultaneous blocking of two protein tyrosine kinase pathways produced more efficient therapeutic effects in prostate³ and pancreatic⁴⁶ carcinoma than did blocking of a single such pathway. Therapeutic efficacy was further increased when three protein tyrosine kinase pathways were inhibited.⁴

Cancers are biologically heterogeneous for multiple properties, including antigenicity, sensitivity to chemotherapeutic agents, invasion, and metastasis.^{47,48} The present data demonstrate intratumoral heterogeneity in expression of growth factors and their receptors in colon cancers. The progressive growth of metastasis and survival of tumor cells depend on their interaction with the organ microenvironment. The expression of multiple tyrosine kinase receptors by different tumor cells within a single neoplasm indicates that targeting a single tyrosine kinase may not produce eradication of the disease.

Acknowledgments

We thank Stephanie Deming for critical editorial review and Arminda Martinez for expert assistance with the preparation of the manuscript.

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