Fibroblast Growth Factor Receptor 2 IIIc as a Therapeutic Target for Colorectal Cancer Cells

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Abstract
A high percentage of colorectal carcinomas overexpress a lot of growth factors and their receptors, including fibroblast growth factor (FGF) and FGF receptor (FGFR). We previously reported that FGFR2 overexpression was associated with distant metastasis and that FGFR2 inhibition suppressed cell growth, migration, and invasion. The FGFR2 splicing isoform FGFR2IIIb is associated with well-differentiated histologic type, tumor angiogenesis, and adhesion to extracellular matrices. Another isoform, FGFR2IIIc, correlates with the aggressiveness of various types of cancer. In the present study, we examined the expression and roles of FGFR2IIIc in colorectal carcinoma to determine the effectiveness of FGFR2IIIc-targeting therapy. In normal colorectal tissues, FGFR2IIIc expression was weakly detected in superficial colorectal epithelial cells and was not detected in proliferative zone cells. FGFR2IIIc-positive cells were detected by immunohistochemistry in the following lesions, listed in the order of increasing percentage: hyperplastic polyps < low-grade adenomas < high-grade adenomas < carcinomas. FGFR2IIIc immunoreactivity was expressed in 27% of colorectal carcinoma cases, and this expression correlated with distant metastasis and poor prognosis. FGFR2IIIc-transfected colorectal carcinoma cells showed increased cell growth, soft agar colony formation, migration, and invasion, as well as decreased adhesion to extracellular matrices. Furthermore, FGFR2IIIc-transfected colorectal carcinoma cells formed larger tumors in subcutaneous tissues and the cecum of nude mice. Fully human anti-FGFR2IIIc monoclonal antibody inhibited the growth and migration of colorectal carcinoma cells through alterations in cell migration, cell death, and development-related genes. In conclusion, FGFR2IIIc plays an important role in colorectal carcinogenesis and tumor progression. Monoclonal antibody against FGFR2IIIc has promising potential in colorectal carcinoma therapy. Mol Cancer Ther; 11(9); 2010–20. © 2012 AACR.

Introduction
The prognosis of colorectal carcinoma remains unfavorable when the disease has progressed to the unresectable stage; thus, new therapeutic strategies for advanced colorectal carcinomas, such as molecular-targeted agents, are a high priority (1). Colorectal tumorigenesis is thought to be a multistep process involving the accumulation of genetic alterations and the well-characterized molecular events of the adenoma-to-carcinoma sequence (2). A high percentage of colorectal carcinomas overexpress a number of growth factors and their receptors, including fibroblast growth factor (FGF) and FGF receptor (FGFR; refs. 3–7).

The FGF family consists of FGF-1 to FGF-23 (8–10), and FGF7—a specific ligand of FGFR2IIIb—induces tumor angiogenesis through VEGF-A expression (5) and adhesion to type IV collagen in colorectal carcinomas (15). FGFR2IIIc overexpression is associated with distant metastasis; furthermore, decreasing FGFR2 expression inhibited colorectal carcinoma cell growth, FGF-7–induced cell migration and invasion, and tumor growth in nude mice (14). FGFR2IIIc overexpression is correlated with well-differentiated histologic type (7), and FGF7—a specific ligand of FGFR2IIIb—induces tumor angiogenesis through VEGF-A expression (5) and adhesion to type IV collagen in colorectal carcinomas (15).

There have been no reports about FGFR2IIIc in colorectal carcinoma, but FGFR2IIIc expression has been reported in prostate cancer, ovarian cancer, oral squamous cell carcinoma, breast cancer, bladder cancer, non-small cell lung cancer cells, cervical cancer, and pancreatic cancer (16–21). FGFR2IIIc expression correlated with epithelial-to-mesenchymal transition (EMT) in rat bladder cancer cells, a process associated with tumor progression and invasion (22). Recently, we found abundant FGFR2IIIc in 71% of patients with pancreatic cancer; in addition, FGFR2IIIc-transfected
cells exhibited increased proliferation in vitro and formed larger subcutaneous and orthotopic tumors, the latter producing more liver metastases (23). These findings suggest that FGFR2IIIc may contribute to the aggressive growth of certain cancers and is a novel candidate for a molecular target of cancer therapy.

In the present study, we examined the expression and roles of FGFR2IIlc in colorectal carcinoma to determine the effectiveness of FGFR2IIlc-targeting therapy. Our results indicate that FGFR2IIlc is expressed in colorectal carcinomas and that fully human anti-FGFR2IIlc monoclonal antibody inhibited colorectal carcinoma cell growth. These findings suggest that FGFR2IIlc is a promising novel therapeutic target for colorectal carcinomas.

Materials and Methods

Materials

The following were purchased: EZes labeling kit from Invitrogen Corp.; Matrigel invasion chamber from BD Bioscience; bovine type I collagen from KOKEN Co., Ltd.; bovine fibrinrone, recombinant human FGf-1,-2, and -7 protein, and FGFR2xIIIc(IIb)/Fc and FGFR2xIIIC(IIc)/Fc chimera proteins from R&D Systems, Inc.; anti-GFP antibody (AbyD04652) from AbD Serotec; horseradish peroxidase-conjugated anti-human IgG, F(ab')2 fragment antibody from Jackson ImmunoResearch Lab.; silencer select custom-designed siRNA from Applied Biosystems; Trans IT-siQUEST from Mirus Bio LLC.; Low Input Quick Amp Labeling kit from Agilent Technologies; and Qiagen RNeasy Mini kit from Qiagen. Other reagents were purchased from Sigma Chemical Corp.

Patients and tissues

Sixty-one polypectomy samples (hyperplastic polyps, adenomas, or colorectal carcinoma) and 95 surgically resected colorectal carcinoma samples were obtained at Nippon Medical School Hospital (Tokyo, Japan) from 2007 to 2008 and Chiba-Hokusoh Hospital (Chiba, Japan) from 2001 to 2003 (14). None of the patients received chemotherapy or radiation therapy before surgery or had inflammatory colorectal disease. The pathologic diagnosis was determined according to the criteria of the World Health Organization (24). This study was carried out in accordance with the principles embodied in the Declaration of Helsinki, 2008, and informed consent for the usage of colorectal tissues was obtained from each patient.

Immunohistochemistry

Anti-FGFR2IIlc polyclonal antibody was prepared as described previously (23, 25). Paraffin-embedded sections were subjected to immunohistochemistry (IHC) for FGFR2IIlc (diluted 1:200). To evaluate FGFR2IIlc staining, we analyzed the percentages of positive staining in hyperplastic, adenoma, or adenocarcinoma cells. The colorectal carcinoma cases were divided into 2 groups: low (<50%) and high (>50%) FGFR2IIlc expression. To select a cutoff value, we conducted statistical analyses using 10%, 30%, 50%, and 70% as positive. Data were statistically significant using each of these cutoff values; therefore, we used the most statistically significant level (50%) in this study. Immunohistochemical staining results were evaluated independently by 2 pathologists (Y. Matsuda and T. Ishiwata) who were blind to the clinical and outcome data.

In situ hybridization

Preparation of FGFR2IIlc probes for in situ hybridization (ISH) was conducted as previously reported (23). Tissue sections were deparaffinized and incubated at room temperature for 20 minutes with 0.2 N HCl and then at 37°C for 15 minutes with 100 μg/mL proteinase K. The sections were then post-fixed for 5 minutes in PBS containing 4% paraformaldehyde and incubated twice for 15 minutes each with PBS containing 2 mg/mL glycine at room temperature and then in 50% formamide/2× SSC for 1 hour at 42°C. Hybridization was conducted with 500 ng/mL of the indicated digoxigenin-labeled FGFR2IIlc riboprobe in a moist chamber for 16 hours at 42°C. The sections were washed sequentially with 2× SSC for 20 minutes at 42°C and 0.2× SSC for 20 minutes at 42°C. Then, immunologic detection was conducted using the DIG Nucleic Acid Detection Kit.

Colorectal carcinoma cell lines

DLD-1, SW480, HCT-15, and LoVo cell lines were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Japan). The cells were grown in RPMI-1640 medium containing 10% FBS, at 37°C for 20 minutes with 0.2 N HCl and then at 37°C for 15 minutes with 100 μg/mL proteinase K. The sections were then post-fixed for 5 minutes in PBS containing 4% paraformaldehyde and incubated twice for 15 minutes each with PBS containing 2 mg/mL glycine at room temperature and then in 50% formamide/2× SSC for 1 hour at 42°C. Hybridization was conducted with 500 ng/mL of the indicated digoxigenin-labeled FGFR2IIlc riboprobe in a moist chamber for 16 hours at 42°C. The sections were washed sequentially with 2× SSC for 20 minutes at 42°C and 0.2× SSC for 20 minutes at 42°C. Then, immunologic detection was conducted using the DIG Nucleic Acid Detection Kit.

Quantitative real-time PCR of FGFR2IIlc in colorectal carcinoma cells

The PCR primers used for FGFR2IIlc were nucleotides 1,693–1,716 (5’-GGAGGATCTTCCCTGACGCTGGT-3’) and 1,770–1,794 (5’-TGATAGTTTCTGATATCCCTCCAGGTA-3’) of the human FGFR2IIlc cDNA (22 bp, accession no. NM_000141.4). The TaqMan probe 5’-CACGCTTGGCTTGAGGA-3’ was used for FGFR2IIlc. The 50-μL PCR reaction mixture contained 2 μL template cDNA, 0.9 μmol/L primers, 0.25 μmol/L probe, and 25 μL TaqMan Universal PCR Master Mix. The optimized program for FGFR2IIlc and 18S ribosomal RNA (18S rRNA) involved incubation with uracil N-glycosylase at 50°C for 2 minutes, and AmpliTaq Gold activation at 95°C for 10 minutes, followed by 50 cycles of amplification (95°C for 15 seconds and 60°C for 60 seconds). Results were expressed as an internal standard concentration ratio of target/18S rRNA. Gene expression measurements were conducted in triplicate.

Western blot analysis of FGFR2IIlc in colorectal carcinoma cells

The anti-FGFR2IIlc polyclonal antibody used for IHC was also used for Western blot analysis (23). Protein lysates were subjected to SDS-PAGE under reducing conditions.
The membranes were incubated overnight at 4°C with the rabbit anti-FGFR2IIIc polyclonal antibody (diluted 1:200) and then incubated with horse radish peroxidase-conjugated anti-rabbit IgG antibody (diluted 1:200). To confirm equal protein loading, the membrane was blotted with mouse monoclonal anti-β-actin antibody.

**Construction of FGFR2IIIc expression vector and generation of stably transfected clones**

The full-length FGFR2IIIc cDNA fragment was ligated to the 3' end of the human cytomegalovirus early promoter/enhancer in the eukaryotic expression vector pRES2-EGFP (23, 25). DLD-1 cells (1 × 10⁶/mL) were transfected with the plasmid DNA using FuGene HD and cultured with 1,000 µg/mL of Geneticin. Independent colonies were isolated by ring cloning.

**Flow cytometry of FGFR2IIIc**

Anti-FGFR2IIIc polyclonal antibody was labeled with allophycocyanin using the Zenon Labeling Kit. Cells were incubated for 20 minutes at 4°C in 10% human serum and then incubated (5 × 10⁵ cells/25 µL) with 1 µg of anti-FGFR2IIIc antibody for 60 minutes at 4°C. Dead cells were labeled with the addition of 1 µg propidium iodide. We prepared rabbit IgG isotype control–treated cells as negative controls. FGFR2IIIc expression was analyzed using a BD FACS Aria II flow cytometer (BD Bioscience).

**Immunocytochemistry**

Cells were fixed in 4% paraformaldehyde solution and incubated overnight at 4°C with a polyclonal anti-FGFR2IIIc antibody (1:100 dilution) and Alexa 488-labeled anti-rabbit IgG antibody (1:1,000 dilution). FGFR2IIIc were visualized using a Digital Eclipse C1 TE2000-E microscope (Nikon Inotech Co., Ltd.).

**Anchorage-dependent cell proliferation assay of FGFR2IIIc-transfected DLD-1 cells**

For the nonradioactive cell proliferation assay, cells were plated at a density of 5 × 10⁵ cells per well in a 96-well plate (14). After 24, 48, and 72 hours, the cells were incubated with WST-8 cell counting reagent, and the optical density of the culture solution was measured using a ELISA plate reader (Bio-Rad Laboratory).

**Anchorage-independent proliferation assay of FGFR2IIIc-transfected DLD-1 cells**

In vitro tumorigenicity was determined on the basis of cell growth in a soft agar colony assay (26). The flasks were covered with 2.5 mL RPMI-1640 with 0.5% agar and 10% FBS. The upper layer consisted of 2 mL RPMI-1640 with 0.03% agar and 10% FBS. Two hundred cells per well were seeded and incubated for 25 days and then the number of colonies was counted.

**Cell adhesion to extracellular matrices of FGFR2IIIc-transfected DLD-1 cells**

Bovine type I collagen, human type IV collagen, bovine fibronectin, or murine laminin solutions, at a concentration of 20 µg/mL, were added into the wells of 96-well microplates (26). The cell suspension (2 × 10⁵ cells per well) was incubated for 2 hours at 37°C. Nonadherent cells were removed by washing with serum-free medium. The number of attached cells was determined using a WST-8 cell counting kit. All assays were conducted in triplicate.

**Cell migration and invasion assays of FGFR2IIIc-transfected DLD-1 cells**

Migration assays were carried out using a modified Boyden chamber technique (14). Cells were placed onto the upper component, and the lower compartment was filled with 750 µL medium containing 10% FBS or 100 ng/mL FGF-1, -2, or -7. After 20 hours, the cells that had migrated through the membrane to the lower surface of the filter were stained and were counted in five high-power fields (×200). Cell invasion assays were conducted using Matrigel-coated inserts. All assays were conducted in triplicate.

**Heterotopic and orthotopic implantation of FGFR2IIIc-transfected DLD-1 cells**

To assess the effect of FGFR2IIIc expression on in vivo tumorigenicity, 2 × 10⁶ cells per animal were injected subcutaneously into 6-week-old, male, nude mice (BALB/cA Jcl-nu/nu; CLEA Japan Inc.; n = 6 per cell line). Tumor volume was calculated using the formula: volume = a × b² × 0.5, where a is the longest diameter and b is the shortest. The tumors were removed and cut into 2-mm squares and used for orthotopic implantation into other mice. The mice to undergo implantation were subjected to brief general inhalation anesthesia with isoflurane; then, the 2-mm square tumor fragments were sutured on the surface on cecum wall using 7.0 Protene suture (ref. 27; n = 3 per cell line). The animals were monitored for 9 weeks. The experimental protocol was approved by the Animal Ethics Committee of Nippon Medical School.

**Human monoclonal anti-human FGFR2IIIc antibody**

Human monoclonal anti-human FGFR2IIIc antibody was generated from the HuCAL GOLD collection of human antibody genes (28). Three rounds of selection were conducted using immobilized bovine serum albumin (BSA) or human transferrin coupled with a specific peptide corresponding to amino acids AGVNTTDKEIEVLALFN of the human FGFR2IIIc protein (from the C-terminus half of the Ig loop closest to the transmembrane region; accession no. NM_000141). To deplete antibodies for the other FGFR2 isoforms before each selection, the phage library was blocked with BSA coupled with a peptide corresponding to amino acids SGINSSNAEV-LYIRN of the human FGFR2IIIc protein (from the C-terminus half of the Ig loop closest to the transmembrane region; accession no. NM_000141). After 3 rounds of selection, the enriched pool of Fab genes was isolated and inserted into *Escherichia coli* vectors that contained a short sequence adding a His6-tag at the C-terminus of the Fab genes. After the transformation of *E. coli* TG1 with the ligated expression vectors, individual colonies were
randomly picked and grown in microtiter plates. Antibody expression was induced with overnight incubation with 0.5 mmol/L isopropyl 1-thiogalactopyranoside (IPTG) at 30°C. Then the cells were lysed, and the crude extracts were tested by ELISA with immobilized antigens to determine the presence of binding antibody fragments. The sequences of the antibody VH CDR regions were determined from up to 20 colonies that gave a strong signal in the ELISA; 5 colonies containing antibodies with unique CDR sequences were chosen for subsequent larger scale growth.

To estimate the reactivity of the anti-FGFR2IIIc antibody, the chimera proteins FGFR2α(IIIb)/Fc and FGFR2α(IIIc)/Fc were subjected to Western blotting.

**Effect of monoclonal human anti-human FGFR2IIIc antibody on colorectal carcinoma cell growth**

Cells were plated at a density of 5 × 10^3 cells per well in a 96-well plate and grown overnight. Then, 100 µg/mL of monoclonal human anti-human FGFR2IIIc antibody was added in each well. An equal amount of monoclonal anti-GFP antibody was added in another well as a negative control. After 24 and 48 hours, the cells were incubated with WST-8 cell counting reagent.

Alternatively, cells were plated at a density of 5 × 10^4 cells per well in a 12-well plate and grown overnight and then 100 µg/mL of monoclonal human anti-human FGFR2IIIc antibody was added in each well. After 48 hours, the cell number of each well was counted using C-reader (Digital Bio Technology Co., Ltd.). All assays were conducted in triplicate.

**Effect of anti-human FGFR2IIIc monoclonal antibody on colorectal carcinoma cell migration**

We conducted time lapse analysis with or without administration of monoclonal anti-FGFR2IIIc antibody. Cells were plated in 4-well chamber dishes (5,000 cells per chamber) and grown overnight; then 100 µg/mL of monoclonal anti-human FGFR2IIIc antibody was added in each well. Anti-GFP antibody was added for a negative control. Cell movement was monitored by taking pictures every 5 minutes using a motorized inverted microscope BioStation (Nikon Instech Co., Ltd.). The total distance covered by individual cells within 24 hours was determined using MetaMorph software 7.6 (Universal Imaging Corp., Ltd.; ref. 25).

**Transfection of FGFR2IIIc siRNA**

siRNA was used to induce downregulation of FGFR2IIIc expression in LoVo and HCT-15 cells. We purchased 2 different types of custom-designed siRNAs against a specific IIIc region of FGFR2IIIc; the sense
sequences were 5'-GGA-AUG-UAA-CUU-UUG-AGG-Att-3' (s275290) and 5'-CUC-UAU-AUU-CGG-AAU-GUA-Att-3' (s275292). The cells were plated at a density of 1 × 10^6 cells in a 35-mm dish and transfected with 5 nM siRNA for FGFR2 IIIc and siencer negative control siRNA as a control using Trans IT-siQUEST according to the manufacturer's protocol. To confirm the effective transfection of siRNA in cells, total RNA was prepared at 72 hours after transfection and suppressed FGFR2IIIc mRNA levels were confirmed by quantitative real-time PCR (qRT-PCR).

**Gene expression analysis using DNA microarray**

Cells were plated at a density of 2.5 × 10^5 cells in a 60-mm dish and grown overnight. Then, 100 μg/mL of monoclonal anti-human FGFR2IIIc antibody was added in each dish. For control groups, an equal amount of anti-GFP antibody was added in another dish. After 48 hours, total RNA was isolated from cells. For use in DNA microarray analysis, 50 ng RNA from each group of cells was labeled using the Low Input Quick Amp Labeling Kit. Labeled RNA was further purified using the Qiagen RNeasy Mini kit. Labeled cRNA was hybridized to the Agilent human 44k oligonucleotide microarray and washed using Agilent Gene Expression washing buffer. Microarrays were scanned in an Agilent DNA Microarray Scanner, and expression data were obtained using the Agilent Feature Extraction software. Data were analyzed using Gene Spring GX version 11 (Agilent Technologies) and the Ingenuity Pathways Analysis (IPA) database (Ingenuity Systems, Inc.; ref. 29). Microarray results were submitted to the Gene Expression Omnibus (30) and given the accession number GSE38544.

**Statistical analysis**

Results are shown as mean ± SE. The data between different 2 groups were compared using Student t test or Mann–Whitney U test. Data were compared between multiple groups using a post hoc test. The χ² and Fisher exact tests were used to analyze the clinicopathologic features. Survival rate was calculated by the Kaplan–Meier method. P < 0.05 was considered significant in all analyses. Computations were conducted using the StatView J version 5.0 software package (SAS Institute, Inc.).

**Results**

**FGFR2IIIc in human colorectal tissues**

In normal colorectal tissues, weak FGFR2IIIc expression was detected in superficial colorectal epithelial cells (Supplementary Fig. S1A and S1B), but no FGFR2IIIc expression was detected in the proliferative zone of colorectal epithelium. FGFR2IIIc was very weakly localized in hyperplastic epithelial cells of hyperplastic polyps (Supplementary Fig. S1C and S1D). In contrast, adenoma and adenocarcinoma showed strong immunoreactivity for FGFR2IIIc in the tumor cell cytoplasm (Fig. 1A and D, respectively). Compared with adenomas, adenocarcinomas showed stronger FGFR2IIIc immunoreactivity. FGFR2IIIc mRNA was also expressed in adenomas and adenocarcinomas (Fig. 1B and E, respectively), whereas sense probe did not yield any positive signals (Fig. 1C and F). Immunohistochemical analysis showed FGFR2IIIc-positive cells in the following lesions, listed in the order of increasing percentages: hyperplastic polyps < low-grade adenomas < high-grade adenomas < carcinomas (Fig. 1G).

In colorectal carcinoma cases, FGFR2IIIc immunoreactivity was highly expressed in 26 of 95 patients with colorectal carcinomas (27%), and its expression was correlated with distance metastasis of the cancer (Table 1). Other clinicopathologic factors—including age, gender, serum carcinoembryonic antigen (CEA) level, serum carbohydrate antigen 19-9 (CA19-9) level, Borrmann classification, histologic type, stage, and Duke’s classification—showed no significant differences between low and high FGFR2IIIc groups. The overall survival rate of the high FGFR2IIIc group was significantly shorter than that of low FGFR2IIIc group (Fig. 1H).

**Table 1. IHC for FGFR2IIIc of colon carcinoma cases (N = 95)**

<table>
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<th>High (&gt;50%, n = 26)</th>
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<tr>
<td>+</td>
<td>35</td>
<td>13</td>
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<td>Distant metastasis (liver, lung, or bone)</td>
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FGFR2IIIc in colorectal carcinoma cell lines
We examined whether colorectal carcinoma cells expressed FGFR2IIIc. The level of FGFR2IIIc mRNA expression was highest in LoVo cells and lowest in DLD-1 cells (Fig. 2A) and was 4.2-fold higher in LoVo cells than in DLD-1 cells. Western blot analysis of anti-FGFR2IIIc polyclonal antibody showed FGFR2IIIc expression in all tested colorectal carcinoma cell lines (Fig. 2B, top). β-Actin showed almost equal loading of the proteins (Fig. 2B, bottom).

Stable transfection of DLD-1 cells with FGFR2IIIc
To clarify the exact roles of FGFR2IIIc in colorectal carcinoma cells, we created FGFR2IIIc-overexpressing colorectal carcinoma cells. Among our panel of colorectal carcinoma cell lines, DLD-1 cells expressed the lowest level of FGFR2IIIc; therefore, we transfected the FGFR2IIIc gene expression vector into DLD-1 cells. qRT-PCR showed high FGFR2IIIc levels in 2 FGFR2IIIc-vector–transfected clones (Fig. 2C, FGFR2IIIc-6 and 9), whereas expression levels were low in parental cells and Mock cells that were transfected with empty vector (Fig. 2C, parental, mock-1, and mock-5). Western blotting showed higher FGFR2IIIc protein expression in stably transfected DLD-1 cells than in parental and mock cells (Fig. 2D, top). Flow cytometry analysis revealed increased FGFR2IIIc at the cell surface of FGFR2IIIc-transfected DLD-1 cells, as compared with parental and mock cells (Fig. 2E). Immunocytochemical analysis showed strong FGFR2IIIc expression in FGFR2IIIc-6 cells, especially at the cell membrane (Fig. 2F, top, arrows). FGFR2IIIc-6 cells did not show characteristic histologic alterations, as compared with mock-1 cells (Fig. 2F, bottom).

Effects of FGFR2IIIc expression on anchorage-dependent and -independent cell proliferation
FGFR2IIIc-transfected DLD-1 cells showed a higher cell growth rate than mock and parental cells (P < 0.05; Fig. 3A). Next, we analyzed anchorage-independent cell growth. FGFR2IIIc-6 and 9 cells showed statistically significant increases of soft agar colony-forming activity, as compared with Mock-1 and Mock-5 cells (P < 0.05; Fig. 3B).

Effects of FGFR2IIIc expression on cell adhesion, migration, and invasion
Cell adhesion was examined on 4 major types of extracellular matrix components: collagen types I and IV, fibronectin, and laminin. FGFR2IIIc-6 and 9 cells showed decreased adhesion ability to type I and IV collagen (P < 0.05; Fig. 3C and E, respectively), and only FGFR2IIIc-9 cells showed decreased adhesion to...
fibronectin (Fig. 3D). Both FGFR2IIIc-transfected clones showed similar adhesion to laminin, as compared with parental and mock cells (Fig. 3F).

Cell migration was examined next, using modified Boyden chamber assays. FGFR2IIIc-transfected DLD-1 cells cultured with FBS in the lower chamber migrated similarly to mock cells (Fig. 3A). On the other hand, FGFR2IIIc-transfected DLD-1 cells cultured with FGF-1, -2, or -7 in serum-free medium in the lower chamber exhibited increased cell migration ability compared with mock cells ($P < 0.05$).

The invasion assay using the modified Boyden chamber with a Matrigel-coated insert showed that the invasion ability of FGFR2IIIc-transfected DLD-1 cells was increased by FGF2 in serum-free medium ($P < 0.05$; Fig. 4B) but not affected by FGF-1 or -7.

**Heterotopic implantation of FGFR2IIIc-overexpressing colorectal carcinoma cells in nude mice**

We examined whether FGFR2IIIc expression levels in colorectal carcinoma cells were associated with increased tumor growth in nude mice. FGFR2IIIc-transfected DLD-1 cells (FGFR2IIIc-9) formed larger subcutaneous tumors than mock or parental cells ($P < 0.05$; Fig. 4C). None of the animals showed metastatic lesions, and we did not observe any histologic differences between subcutaneous tumors with FGFR2IIIc-transfected cells and mock cells (data not shown).

**Orthotopic implantation of FGFR2IIIc-overexpressing colorectal carcinoma cells in nude mice**

Next, we analyzed orthotopic tumor formation of FGFR2IIIc-transfected DLD-1 cells and mock cells (FGFR2IIIc-9 and mock-1, respectively). Subcutaneous tumors from mice were cut into small-sized fragments and sutured on the cecum wall surface of other mice (27). FGFR2IIIc-9 cells formed larger tumors in the cecum, with tumor volume that was significantly higher than that of tumors formed by mock-1 cells (Fig. 4D, arrowheads). One of 3 animals in the FGFR2IIIc-9-implanted group exhibited a metastatic nodule on the surface of small intestine (Fig. 4D, arrow), whereas the other animals did not have metastases. We did not observe any histologic differences between tumors of FGFR2IIIc-transfected cells and mock cells (data not shown).

![Figure 3](image-url)
Growth inhibition of colorectal carcinoma cells due to monoclonal human anti-human FGFR2IIIc antibody

To examine the inhibitory effects of FGFR2IIIc on colorectal carcinoma cell behaviors, including growth and migration, we prepared monoclonal human anti-human FGFR2IIIc antibody. Anti-FGFR2IIIc monoclonal antibody reacted with recombinant human FGFR2IIIc protein (rhIIIc; Fig. 5A, top) but not with recombinant human FGFR2IIIb protein (rhIIIb). Anti-human IgG antibody reacted with each recombinant protein on the reblotted membrane (Fig. 5A, bottom). These findings indicate that the anti-FGFR2IIIc antibody was highly specific to FGFR2IIIc.

Next, we examined whether the human monoclonal anti-human FGFR2IIIc antibody inhibited the growth and migration of colorectal carcinoma cells. For this experiment, we used LoVo and HCT-15 cells, which expressed the highest and second highest levels of FGFR2IIIc mRNA of the tested colorectal carcinoma cell lines. Following addition of 100 μg/mL anti-human FGFR2IIIc antibody, the growth rates of LoVo and HCT-15 cells were significantly decreased, as compared with following the addition of the same amount of control anti-GFP antibody for 24 and 48 hours (P < 0.05; Fig. 5B). Using the C-reader cell counting method after 48 hours, we found a significantly decreased cell number of LoVo and HCT-15 cells in the group treated with human monoclonal anti-human FGFR2IIIc antibody, as compared with control cells treated with anti-GFP antibody (P < 0.05; Fig. 5C). Cell migration was also decreased in the LoVo and HCT-15 cells treated with human monoclonal anti-human FGFR2IIIc antibody (P < 0.05; Fig. 5D).

We also analyzed the effect of FGFR2IIIc monoclonal antibody on FGFR2IIIc-overexpressing DLD-1 cells. Cells were treated with FGFR2IIIc monoclonal antibody for 48 hours and then the WST-8 cell growth assay was conducted. Monoclonal anti-FGFR2IIIc antibody significantly inhibited the growth of FGFR2IIIc-transfected DLD-1 cells (Supplementary Fig. S2A; P < 0.05 vs. parental and mock cells), whereas control GFP antibody showed no significant effects on any cells.

To determine the effect of decreased expression levels of FGFR2IIIc, siRNA-targeting FGFR2IIIc was transfected into LoVo and HCT-15 cells. qRT-PCR showed approximately 80% knockdown of FGFR2IIIc mRNA in LoVo cells, whereas HCT-15 cells did not show decreased expression levels of FGFR2IIIc mRNA with 2 different types of siRNAs targeting FGFR2IIIc (data not shown).

Figure 4. Cell migration, invasion assays, and in vivo study of FGFR2IIIc gene-transfected DLD-1 cells. A, cell migration assay following administration of recombinant FGF-1, -2, or -7 (100 ng/mL; * P < 0.05). B, cell invasion assay (* P < 0.05). C, tumor volume of subcutaneously implanted tumors in nude mice (* P < 0.05). D, orthotopic tumor formation in nude mice (* P < 0.05). Arrowheads, tumors of the cecum; arrows, metastatic nodule.
Therefore, we used LoVo cells in the experiment using siRNA-targeting FGFR2IIIc. After 48 hours of transfection, with siRNA-targeting FGFR2IIIc, LoVo cells exhibited suppressed cell growth compared with cells transfected with negative control siRNA (Supplementary Fig. S2B; *, P < 0.05).

**Gene expression analysis using DNA microarray**

To investigate the underlying mechanisms of the inhibitory effects of human anti-human FGFR2IIIc antibody on growth and migration of colorectal carcinoma cells, we used DNA microarray analysis to examine the cell signaling pathway alterations following the administration of anti-FGFR2IIIc antibody. Supplementary Table S1 shows the list of genes whose expressions were increased or decreased more than 2-fold in anti-FGFR2IIIc monoclonal antibody–treated LoVo and HCT-15 cells, as compared with control cells. Administration of anti-FGFR2IIIc monoclonal antibody increased expressions of 34 genes and decreased expressions of 22 genes. Each gene was matched with a representative gene network using the IPA database. Anti-FGFR2IIIc antibody treatment of colorectal carcinoma cells caused altered expression levels of genes involved in cell migration, cell death, and cell development (Supplementary Table S2).

**Discussion**

Here, we found that in colorectal carcinoma cases, expression levels of FGFR2IIIc in tumor cells were correlated with the advances of carcinogenesis stages, similar to previous findings of FGFR2IIIc expression in precancerous lesions in the uterine cervix (25). Increased FGFR2IIIc expression in precancerous lesions may be influenced by the accumulation of genetic and epigenetic alterations of carcinogenesis. Furthermore, FGFR2IIIc expression correlated with metastasis and poor prognosis of colorectal carcinomas, consistent with previous findings in pancreatic cancers (23). On the other hand, FGFR2IIIb expression in colorectal carcinomas did not correlate with survival or metastasis (7). We previously reported that FGFR2 expression, both of FGFR2IIIc and FGFR2IIIb, in colorectal carcinomas tended to correlate with distant metastasis (14); the present data indicate that expression levels of FGFR2IIIc, rather than FGFR2IIIb, may contribute to colorectal carcinoma progression.

FGFR2IIIc gene–transfected DLD-1 cells exhibited increased cell growth and tumor volume, as was previously found for similarly treated pancreatic carcinomas (23). In the attachment assay, FGFR2IIIc–transfected cells showed decreased attachment to type I and IV collagen and increased cell migration and invasion ability under FGF treatments. In our previous reports, FGFR2IIIb–transfected colorectal carcinoma cells showed increased adhesion to type IV collagen and fibronectin, through integrins, extracellular-regulated kinase-1 and -2 phosphorylation, and focal adhesion kinase signaling pathways (15). Knockdown of FGFR2 in colorectal carcinoma cell lines suppressed cell migration and invasion under FGF treatment (14). These results suggest that FGFR2IIIb and FGFR2IIIc have different roles in migration and invasion; specifically, FGFR2IIIc has more malignant effects than FGFR2IIIb. Thus, compared with FGFR2IIIb, FGFR2IIIc has superior potential as a therapeutic target for colorectal carcinoma therapy.

In our previous study, an anti-FGFR2IIIc polyclonal antibody inhibited both proliferation and migration (23). Therefore, we prepared fully human monoclonal anti-FGFR2IIIc antibody using HuCAL phage display technologies that have reported the usefulness and low toxicity in in vivo studies (31) and clinical trials (32, 33). Administration of anti-FGFR2IIIc monoclonal antibody inhibited colorectal carcinoma cell growth and migration through the alteration of cell migration, cell death, and cell development–related genes. Furthermore, anti-FGFR2IIIc antibody effectively inhibited cell growth of FGFR2IIIc–transfected DLD-1 cells, which expressed markedly high FGFR2 levels (20- to 30-fold higher than control cells in mRNA levels). Two different types of siRNA-targeting FGFR2IIIc did not effectively reduce...
expression of FGFR2IIIc mRNA in 1 of 2 high FGFR2IIIc expressing colorectal carcinoma cell lines in this study. However, human monoclonal anti-FGFR2IIIc antibody significantly inhibited the growth and migration of the both colorectal carcinoma cell lines.

In conclusion, FGFR2IIIc plays important roles in colorectal carcinogenesis and progression and that monoclonal antibody against FGFR2IIIc has a potential use in colorectal carcinoma therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Y. Matsuda, T. Ishiwata
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Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): Y. Matsuda, T. Seya, T. Ishiwata
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Matsuda et al.

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