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

Yoko Matsuda¹, Masahito Hagio¹, Tomoko Seya², Toshiyuki Ishiwata¹

¹Departments of Pathology and Integrative Oncological Pathology, Nippon Medical School, Tokyo, Japan, ²Department of Surgery, Chiba-Hokusoh Hospital, Nippon Medical School, Chiba, Japan

Short running title: FGFR2IIIc in colorectal cancer

Keywords: adenocarcinoma; adenoma; cell growth; cell migration; colorectal cancer; FGFR2IIIc; monoclonal antibody

Financial support: This work was supported by a Grant-in-Aid for Young Scientists (A, No. 22689038 to Y. Matsuda), a Grant-in-Aid for Challenging Exploratory Research (No. 23650604 to Y. Matsuda), and a Grant-in-Aid for Scientific Research (C, No. 22591531 to T. Ishiwata) from the Japan Society for the Promotion of Science.

Address correspondence to:
Toshiyuki Ishiwata, M.D., Ph.D.,
Departments of Pathology and Integrative Oncological Pathology, Nippon Medical School 1-1-5, Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan, Phone: +81-3-3822-2131 ext. 5232, Fax: +81-3-5685-3067, e-mail: ishiwata@nms.ac.jp

Disclosure/conflict of interest
The authors declare no conflict of interest.

Word count (excluding references): 4619 words.

Number of Figures: 5 figures

Table: 1 table
Abstract

A high percentage of colorectal carcinomas (CRCs) 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 histological 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 CRC 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 CRC cases, and this expression correlated with distant metastasis and poor
prognosis. FGFR2IIIc-transfected CRC cells showed increased cell growth, soft agar colony formation, migration, and invasion, as well as decreased adhesion to extracellular matrices. Furthermore, FGFR2IIIc-transfected CRC 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 CRC cells, through alterations in cell migration, cell death, and development-related genes. In conclusion, FGFR2IIIc plays important roles in colorectal carcinogenesis and tumor progression. Monoclonal antibody against FGFR2IIIc has promising potential in CRC therapy.
Introduction

The prognosis of colorectal carcinoma (CRC) remains unfavorable when the disease has progressed to the unresectable stage; thus, new therapeutic strategies for advanced CRC, 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 CRCs overexpress a number of growth factors and their receptors, including fibroblast growth factor (FGF) and FGF receptor (FGFR) (3-7).

The FGF family consists of FGF-1 to FGF-23 (8-10), which bind to four high-affinity FGF receptors (FGFR1 to FGFR4) (9). The extracellular FGFR domain is composed of three immunoglobulin-like domains (I–III). In FGFR1–3, alternative splicing of the C-terminal half of the third Ig-like domain generates IIIb and IIIc isoforms. FGF-1, 3, 7, 10, and 22 reportedly bind to FGFR2IIIb, while FGF-1, 2, 4, 6, 9, 17, and 18 bind to FGFR2IIIc with high affinity (11-13). We recently reported that FGFR2 overexpression in CRC is associated with distant metastasis; furthermore,
decreasing FGFR2 expression inhibited CRC cell growth, FGF-7-induced cell migration and invasion, and tumor growth in nude mice (14). FGFR2IIIb overexpression is correlated with well-differentiated histological type (7), and FGF7— a specific ligand of FGFR2IIIb—induces tumor angiogenesis through VEGF-A expression (5), and adhesion to type-IV collagen in CRCs (15).

There have been no reports about FGFR2IIIc in CRC, 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 pancreatic cancer patients; additionally, 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 FGFR2IIIc in CRC to determine the effectiveness of FGFR2IIIc-targeting therapy. Our results indicate that FGFR2IIIc is expressed in CRCs, and that fully human monoclonal anti-FGFR2IIIc antibody inhibited CRC cell growth. These findings suggest that FGFR2IIIc is a promising novel therapeutic target for CRC.
Materials and Methods

Materials

The following were purchased: Zenon labeling kit from Invitrogen Corp. (Carlsbad, CA); matrigel invasion chamber from BD Bioscience (Franklin Lakes, NJ); bovine type I collagen from KOKEN Co., Ltd. (Tokyo, Japan); bovine fibronectin, recombinant human FGF1, FGF2, and FGF7 protein, and FGFR 2α(IIIb)/Fc and FGFR 2α(IIIc)/Fc chimera proteins from R&D Systems, Inc. (Westerville, OH); anti-GFP antibody (AbyD04652) from AbD Serotec (Martinsried, Germany); HRP-conjugated anti-human IgG, F(ab’)2 Fragment antibody from Jackson ImmunoResearch Lab. (West Grove, PA); Silencer Select Custom Designed siRNA (s275290 and s275292) and Silencer Negative Control siRNA from Applied Biosystems (Foster City, CA); Trans IT-siQUEST from Mirus Bio LLC. (Madison, WI); Low Input Quick Amp Labeling kit from Agilent Technologies (Palo Alto, CA), and Qiagen RNeasy Mini kit from Qiagen (Valencia, CA). Other reagents were purchased from Sigma Chemical Corp. (St. Louis, MO).
Patients and tissues

Sixty-one polypectomy samples (hyperplastic polyps, adenomas, or CRC) and ninety-five surgically resected CRC samples were obtained at Nippon Medical School Hospital from 2007 to 2008 and Chiba-Hokusoh Hospital from 2001 to 2003 (14). None of the patients received chemotherapy or radiation therapy prior to surgery, or had inflammatory colorectal disease. The pathological 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-FGFR2IIIc polyclonal antibody was prepared as described previously (23, 25). Paraffin-embedded sections were subjected to immunohistochemistry (IHC) for FGFR2IIIc (diluted 1:200). To evaluate FGFR2IIIc staining, we analyzed the percentages of positive staining in hyperplastic, adenoma, or adenocarcinoma cells.
The CRC cases were divided into two groups: Low (≤50%) and High (>50%) FGFR2IIIc expression. To select a cut-off value, we performed statistical analyses using 10%, 30%, 50%, and 70% as positive. Data was statistically significant using each of these cut-off values; therefore, we used the most statistically significant level (50%) in this study. IHC staining results were evaluated independently by two pathologists (Y.M. and T.I.) who were blind to the clinical and outcome data.

**In situ hybridization**

Preparation of FGFR2IIIc probes for *in situ* hybridization (ISH) was performed as previously reported (23). Tissue sections were deparaffinized and incubated at room temperature (RT) for 20 min with 0.2 N HCl and then at 37°C for 15 min with 100 μg/mL proteinase K. The sections were then postfixed for 5 min in PBS containing 4% paraformaldehyde (PFA), and incubated twice for 15 min each with PBS containing 2 mg/mL glycine at RT, and once in 50% formamide/2× SSC for 1 hr at 42°C. Hybridization was performed with 500 ng/mL of the indicated digoxigenin-labeled FGFR2IIIc riboprobe in a moist chamber for 16 hr at 42°C. The sections were
washed sequentially with 2× SSC for 20 min at 42°C, and 0.2× SSC for 20 min at 42°C. Then, immunological detection was performed using the DIG nucleic acid detection kit.

**CRC 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% fetal bovine serum (FBS), at 37°C under a humidified 5% CO₂ atmosphere. These cell lines were authenticated by short tandem repeat profiling analysis in May 2012.

**Quantitative real-time PCR of FGFR2IIIc in CRC cells**

The PCR primers used for FGFR2IIIc were nts 1693-1716 (5’-GGA-TAT-CCT-TTC-ACT-CTG-CAT-GGT-3’) and nts 1770-1794 (5’-TGC-AGT-AAA-TGG-CTA-TCT-CCA-GGT-A-3’) of the human FGFR2IIIc cDNA (102 bp, Accession No.
NM_000141.4). The TaqMan probe 5’-CAG-TTC-TGC-CAG-CGC-CTG-GAA-GA-3’ was used for FGFR2IIIc. The 50-μL PCR reaction mixture contained 2 μL template cDNA, 0.9 μM primers, 0.25 μM probe, and 25 μL TaqMan Universal PCR Master Mix. The optimized program for FGFR2IIIc and 18S ribosomal RNA (18S rRNA) involved incubation with uracil N-glycosylase at 50°C for 2 min, and AmpliTaq Gold activation at 95°C for 10 min, followed by 50 cycles of amplification (95°C for 15 sec and 60°C for 60 sec). Results were expressed as an internal standard concentration ratio of target/18S rRNA. Gene expression measurements were performed in triplicate.

**Western blot analysis of FGFR2IIIc in CRC cells**

The polyclonal anti-FGFR2IIIc antibody used for IHC was also utilized 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 polyclonal anti-FGFR2IIIc antibody (diluted 1:200), and then incubated with HRP-conjugated anti-rabbit IgG antibody (diluted 1:200). To confirm equal protein loading, the membrane was rebotted 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 pIRES2-EGFP (23, 25). DLD-1 cells (1 × 10^5/mL) were transfected with the plasmid DNA using FuGene HD, and cultured with 1000 µg/mL of Geneticin. Independent colonies were isolated by ring cloning.

Flow cytometry of FGFR2IIIc

Polyclonal anti-FGFR2IIIc antibody was labeled with allophycocyanin using the Zenon labeling kit. Cells were incubated for 20 min at 4°C in 10% human serum, and then incubated (5 × 10^5 cells/25 µL) with 1 µg of anti-FGFR2IIIc antibody for 60 min 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 FACSARia II flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA).

**Immunocytochemistry**

Cells were fixed in 4% PFA solution, and incubated overnight at 4°C with a polyclonal anti-FGFR2IIIc antibody (1:100 dilution) and Alexa 488-labeled anti-rabbit IgG antibody (1:1000 dilution). FGFR2IIIc were visualized using a Digital Eclipse C1 TE2000-E microscope (Nikon Instech Co., Ltd., Tokyo, Japan).

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

For the non-radioactive cell proliferation assay, cells were plated at a density of $5 \times 10^3$ cells/well in a 96-well plate (14). After 24, 48, and 72 hr, the cells were incubated with WST-8 cell counting reagent, and the optical density of the culture solution was measured using an ELISA plate reader (Bio-Rad Laboratory Hercules, CA, USA).
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/well were seeded and incubated for 25 days; then the number of colonies was counted.

Cell adhesion to extracellular matrices (ECMs) 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 × 10^4 cells/well) was incubated for 2 hr at 37°C. Non-adherent 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 performed 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 FGF1, FGF2, or FGF7. After 20 hr, 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 performed using Matrigel-coated inserts. All assays were performed in triplicate.

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

To assess the effect of FGFR2IIIc expression on *in vivo* tumorigenicity, 2 × 10^6 cells/animal were injected subcutaneously into 6-week-old, male, nude mice (BALB/cA Jcl-nu/nu; CLEA Japan Inc, Tokyo, Japan) (*n* = 6 per cell line). Tumor
volume was calculated using the formula: volume = \(a \times b^2 \times 0.5\), where \(a\) is the longest diameter and \(b\) 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 Prolene suture (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 performed using immobilized BSA or human transferrin coupled with a specific peptide corresponding to amino acids AGVNTTDKEIEVLYIRN 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 prior to each selection, the phage library was blocked with BSA coupled with a peptide corresponding to amino acids SGINSSNAEVLANFLN of the human FGFR2IIIb protein (from the carboxyl-terminal half of the Ig loop closest to the transmembrane region; Accession No. NM_022970). After three rounds of selection, the enriched pool of Fab genes was isolated and inserted into *E. 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* TG1F 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 mM isopropyl β-D-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; five 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 blot.

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

Cells were plated at a density of $5 \times 10^3$ cells/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-green fluorescent protein (GFP) antibody was added in another well as a negative control. After 24 and 48 hr, the cells were incubated with WST-8 cell counting reagent.

Alternatively, cells were plated at a density of $5 \times 10^4$ 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 hr, the cell number of each well was counted using C-reader (Digital Bio Technology Co., Ltd., Kyungki-do, Korea). All assays were performed in triplicate.
Effect of anti-human FGFR2IIIc monoclonal antibody on CRC cell migration

We performed time-lapse analysis with or without administration of monoclonal anti-FGFR2IIIc antibody. Cells were plated in 4-well chamber dishes (5000 cells/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 min using a motorized inverted microscope BioStation (Nikon Instech Co., Ltd.). The total distance covered by individual cells within 24 hr was determined using Metamorph software 7.6 (Universal Imaging Corp., Ltd., Buckinghamshire, UK) (25).

Transfection of FGFR2IIIc siRNA

siRNA was used to induce down-regulation of FGFR2IIIc expression in LoVo and HCT-15 cells. We purchased two different types of custom-designed siRNA 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 × 10^5 cells in a 35-mm dish and
transfected with 5 nM siRNA for FGFR2 IIIc, and Silencer 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 hr after transfection, and suppressed FGFR2IIIc mRNA levels were confirmed by qRT-PCR.

**Gene expression analysis using DNA microarray**

Cells were plated at a density of $2.5 \times 10^5$ 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 hr, 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, Santa Clara, CA, USA) and the Ingenuity Pathways Analysis (IPA) database (Ingenuity Systems, Inc., Redwood, CA, USA) (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 two groups were compared using Student’s t-test or Mann-Whitney U test. Data were compared between multiple groups using a post-hoc test. The chi-square test and Fisher’s exact test were used to analyze the clinicopathological features. Survival rate was calculated by the Kaplan-Meier method. \( P < 0.05 \) was considered significant in all analyses. Computations were performed using the StatView J version 5.0 software package (SAS Institute, Inc., Cary, NC, USA).
Results

FGFR2IIIc in human colorectal tissues

In normal colorectal tissues, weak FGFR2IIIc expression was detected in superficial colorectal epithelial cells (Supplemental Figs. 1A and B), 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 (Supplemental Figs. 1C and D). In contrast, adenoma and adenocarcinoma showed strong immunoreactivity for FGFR2IIIc in the tumor cell cytoplasm (Figs. 1A and D, respectively). Compared to adenomas, adenocarcinomas showed stronger FGFR2IIIc immunoreactivity. FGFR2IIIc mRNA was also expressed in adenomas and adenocarcinomas (Figs. 1B and E, respectively), while sense probe did not yield any positive signals (Figs. 1C and F). IHC 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 CRC cases, FGFR2IIIc immunoreactivity was highly expressed in 26 of 95 CRC patients (27%), and its expression was correlated with distance metastasis of the
cancer (Table 1). Other clinicopathological factors—including age, gender, serum
carcinoembryonic antigen (CEA) level, serum carbohydrate antigen 19-9 (CA19-9)
level, Borrmann classification, histological 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).

**FGFR2IIIc in CRC cell lines**

We examined whether CRC 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 polyclonal anti-FGFR2IIIc antibody showed FGFR2IIIc expression in all
tested CRC cell lines (Fig. 2B, upper panel). β-actin showed almost equal loading of
the proteins (Fig. 2B, lower panel).

**Stable transfection of DLD-1 cells with FGFR2IIIc**
To clarify the exact roles of FGFR2IIIc in CRC cells, we created FGFR2IIIc-overexpressing CRC cells. Among our panel of CRC 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 two 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, upper panel). 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, upper panels, arrows). FGFR2IIIc-6 cells did not show characteristic histological alterations, as compared to Mock-1 cells (Fig. 2F, lower panels).
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 four major types of extracellular matrix (ECM) components: collagen types I and IV, fibronectin, and laminin. FGFR2IIIc-6 and 9 cells showed decreased adhesion ability to type I and IV collagen ($P < 0.05$; Figs. 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 to 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. 4A). On the other hand, FGFR2IIIc-transfected DLD-1 cells cultured with FGF1, FGF2, or FGF7 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 FGF1 or FGF7.

**Heterotopic implantation of FGFR2IIIc-overexpressing CRC cells in nude mice**

We examined whether FGFR2IIIc expression levels in CRC 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 histological differences between subcutaneous tumors with FGFR2IIIc-transfected cells and Mock cells (data not shown).

Orthotopic implantation of FGFR2IIIc-overexpressing CRC 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 out of three animals in the FGFR2IIIc-9-implanted group exhibited a metastatic nodule on the surface of small intestine (Fig. 4D, arrow), while the other animals did not have metastases. We did not observe any histological differences between tumors of FGFR2IIIc-transfected cells and Mock cells (data not shown).
Growth inhibition of CRC cells due to monoclonal human anti-human FGFR2IIlc antibody

To examine the inhibitory effects of FGFR2IIlc on CRC cell behaviors, including growth and migration, we prepared monoclonal human anti-human FGFR2IIlc antibody. Anti-FGFR2IIlc monoclonal antibody reacted with recombinant human FGFR2IIlc protein (rhIIIc; Fig. 5A, upper panel), but not with recombinant human FGFR2IIlb protein (rhIIib). Anti-human IgG antibody reacted with each recombinant protein on the reblotted membrane (Fig. 5A, lower panel). These findings indicate that the anti-FGFR2IIlc antibody was highly specific to FGFR2IIlc.

Next, we examined whether the monoclonal human anti-human FGFR2IIlc antibody inhibited the growth and migration of CRC cells. For this experiment, we used LoVo and HCT-15 cells, which expressed the highest and second-highest levels of FGFR2IIlc mRNA of the tested CRC cell lines. Following addition of 100 μg/mL anti-human FGFR2IIlc antibody, the growth rates of LoVo and HCT-15 cells were significantly decreased, as compared to following the addition of the same amount of control anti-GFP antibody for 24 and 48 hr ($P < 0.05$; 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 monoclonal human 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 hr, and then the WST-8 cell growth assay was performed. Monoclonal anti-FGFR2IIIc antibody significantly inhibited the growth of FGFR2IIIc-transfected DLD-1 cells (Supplemental Fig. 2A, *$P < 0.05$ vs. parental and mock cells), while 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, while HCT-15 cells did not show decreased expression levels of FGFR2IIIc mRNA with two different types of siRNA targeting FGFR2IIIc (data not shown). Therefore, we used LoVo cells
in the experiment using siRNA targeting FGFR2IIIc. After 48 hr of transfection, with siRNA targeting FGFR2IIIc, LoVo cells exhibited suppressed cell growth compared to cells transfected with negative control siRNA (Supplemental Fig. 2B, *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 CRC cells, we used DNA microarray analysis to examine the cell signaling pathway alterations following the administration of anti-FGFR2IIIc antibody. Supplemental Table 1 shows the list of genes whose expressions were increased or decreased more than two-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 CRC cells caused altered expression levels of genes involved in cell migration, cell death, and cell development (Supplemental Table 2).
Discussion

Here, we found that in CRC cases, expression levels of FGFR2IIIc in tumor cells were correlated to 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 to metastasis and poor prognosis of CRC, consistent with previous findings in pancreatic cancers (23). On the other hand, FGFR2IIIb expression in CRC did not correlate with survival or metastasis (7). We previously reported that FGFR2 expression, both of FGFR2IIIc and FGFR2IIIb, in CRCs tended to correlate with distant metastasis (14); the present data indicates that expression levels of FGFR2IIIc, rather than FGFR2IIIb, may contribute to CRC 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 CRC cells showed increased adhesion to type-IV collagen and fibronectin, through integrins, extracellular-regulated kinase-1 and -2 (ERK1/2) phosphorylation, and focal adhesion kinase (FAK) signaling pathways (15). Knock-down of FGFR2 in CRC 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 to FGFR2IIIb, FGFR2IIIc has superior potential as a therapeutic target for CRC 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 which has been reported the usefulness and low-toxicity in in vivo studies (31) and clinical trials (32, 33). Administration of anti-FGFR2IIIc monoclonal antibody inhibited CRC 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 one of two high FGFR2IIIc expressing CRC cell lines in this study. However, human monoclonal anti-FGFR2IIIc antibody significantly inhibited the growth and migration of the both CRC cell lines.

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

We express our appreciation to Dr. Yoshiharu Ohaki (Department of Pathology, Chiba-Hokusoh Hospital, Nippon Medical School) and Dr. Shin-ichi Tsuchiya (Division of Surgical Pathology, Nippon Medical School Hospital) for preparing tissue blocks and for helpful discussions. We thank for Dr. Zenya Naito (Departments of Pathology and Integrative Oncological Pathology, Nippon Medical School) for helpful discussion, Dr. Atsushi Watanabe (Department of Biochemistry and Molecular Biology, Nippon Medical School) for providing Gene Spring software, and Dr. Toshihiro Takizawa (Department of Molecular Anatomy and Medicine, Nippon Medical School) for providing IPA software.
References


23. Ishiwata T, Matsuda Y, Yamamoto T, Uchida E, Korc M, Naito Z. 
Enhanced expression of fibroblast growth factor receptor 2 IIIc promotes human

Tumours of the colon and rectum. 4 ed. Lyon: IARCPress; 2010.

Expression of fibroblast growth factor receptor 2 IIIc in human uterine cervical

Nestin is a novel target for suppressing pancreatic cancer cell migration, invasion

not Grb2 mediates tumor progression in Met overexpressing colorectal cancer

human combinatorial antibody library HuCAL GOLD combines diversification
of all six CDRs according to the natural immune system with a novel display method for efficient selection of high-affinity antibodies. J Mol Biol 2008; 376: 1182-200.


Table

Table 1. Immunohistochemistry for FGFR2IIIc of colon carcinoma cases (n = 95)

<table>
<thead>
<tr>
<th>FGFR2IIIc expression</th>
<th>Low (≤50%, n = 69)</th>
<th>High (&gt;50%, n = 26)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤65</td>
<td>39</td>
<td>11</td>
<td>0.2161</td>
</tr>
<tr>
<td>&gt;65</td>
<td>30</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>34</td>
<td>13</td>
<td>0.9498</td>
</tr>
<tr>
<td>M</td>
<td>35</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td><strong>Histological type</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>well differentiated</td>
<td>37</td>
<td>10</td>
<td>0.0996</td>
</tr>
<tr>
<td>moderately differentiated</td>
<td>28</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>poorly differentiated</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>mucinous adenocarcinoma</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Lymph node metastasis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−</td>
<td>34</td>
<td>13</td>
<td>0.9837</td>
</tr>
<tr>
<td>+</td>
<td>35</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td><strong>Distant metastasis (liver, lung, or bone)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−</td>
<td>61</td>
<td>18</td>
<td>0.0260</td>
</tr>
<tr>
<td>+</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>14</td>
<td>4</td>
<td>0.3407</td>
</tr>
<tr>
<td>II</td>
<td>26</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>IIIa</td>
<td>15</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>IIIb</td>
<td>7</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>
Titles and legends to figures

Figure 1.

FGFR2IIIc in colorectal adenomas and adenocarcinomas. A, B, and C, adenoma. D, E, and F, adenocarcinoma; A and C, IHC for FGFR2IIIc; B and E, ISH using anti-sense probe; C and F, ISH using sense probe; original magnification, ×600. G, percentages of FGFR2IIIc-positive cells. *P < 0.05 vs. hyperplastic polyp, #P < 0.05 vs. low grade adenoma. Data represent mean ± SE. H, the overall survival rate of the FGFR2IIIc-low group (dotted line, ≤50%) and FGFR2IIIc-high group (continuous line, >50%). P = 0.0265.

Figure 2.

Expression level of FGFR2IIIc in four CRC cell lines. A, qRT-PCR analysis; B, western blot analysis; C, qRT-PCR analysis; D, western blot analysis; E, flow cytometry analysis (blue line, FGFR2IIIc; red line, isotype control); F, (upper panel) immunofluorescence images of FGFR2IIIc, (lower panels) phase contrast images of
FGFR2IIIc-transfected clones. Original magnification: upper panels, $\times 1000$; lower panels, $\times 200$.

**Figure 3.** Cell proliferation and adhesion assays of FGFR2IIIc-gene-transfected DLD-1 cells. A, WST-8 cell growth assay ($*P < 0.05$); B, soft-agar colony formation assay ($*P < 0.05$); C, D, E, and F, cell adhesion activity to type I and IV collagen, fibronectin, and laminin ($*P < 0.05$).

**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 FGF1, FGF2, or FGF7 (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.
**Figure 5.**

Effects of monoclonal FGFR2IIIc antibody on CRC cell growth and migration. A, western blot analysis of anti-FGFR2IIIc monoclonal antibody; recombinant FGFR2IIIc protein (rhIIIc); recombinant FGFR2IIIb protein (rhIIIb); anti-human IgG antibody, loading control; B, WST-8 cell growth assay of cells treated with monoclonal anti-FGFR2IIIc antibody or control anti-GFP antibody (*P < 0.05); C, cell numbers of the CRC cells treated with monoclonal anti-FGFR2IIIc antibody for 48 hr (*P < 0.05); D, cell migration assay (*P < 0.05).
Fig. 1

A
B
C
D
E
F

G
H

Immunoreactivity of FGFR2IIIc (%)  

Hyper  | Low adenoma  | High adenoma  | Carcinoma  

0  | 50  | 100  

Dashed line: low (≤50%)  
Solid line: high (>50%)  
P = 0.0265
Fig. 3

A

Ratio of cell growth

B

Mock-1

FGFR2IIlc-9

Colonies/well

C

Absorbance at 450 nm

D

Absorbance at 450 nm

Type I collagen (mg/mL)

Fibronectin (mg/mL)

E

Absorbance at 450 nm

Type IV collagen (mg/mL)

F

Absorbance at 450 nm

Laminin (mg/mL)
Fig. 4

A

FBS

FGF1

FGF2

FGF7

Migrated cells/field

Mock-1
Mock-5
FGFR2IIlc-6
FGFR2IIlc-9

Migrated cells/field

Mock-1
Mock-5
FGFR2IIlc-6
FGFR2IIlc-9

Migrated cells/field

Mock-1
Mock-5
FGFR2IIlc-6
FGFR2IIlc-9

Migrated cells/field

Mock-1
Mock-5
FGFR2IIlc-6
FGFR2IIlc-9

B

FBS

FGF1

FGF2

FGF7

Invaded cells/field

Mock-1
Mock-5
FGFR2IIlc-6
FGFR2IIlc-9

Invaded cells/field

Mock-1
Mock-5
FGFR2IIlc-6
FGFR2IIlc-9

Invaded cells/field

Mock-1
Mock-5
FGFR2IIlc-6
FGFR2IIlc-9

Invaded cells/field

Mock-1
Mock-5
FGFR2IIlc-6
FGFR2IIlc-9

C

Parental
Mock-1
FGFR2IIlc-9

Tumor volume (mm³)

7 9 12 14 16 19 (days)

0 100 200 300 400 500 600

D

Mock

FGFR2IIlc-9

Tumor volume (mm³)

0 1000 2000

Mock-1

FGFR2IIlc-9

Downloaded from mct.aacrjournals.org on January 7, 2018. © 2012 American Association for Cancer Research.
Fig. 5

A

rhlIib  rhlIic

FGFR2IIlc

human IgG

B

LoVo

HCT-15

Absorbance at 450nm

24  48 (hrs)

non-treat  control  FGFR2IIlcAb

C

LoVo  HCT-15

Cells/well

x10^5

non-treat  control  FGFR2IIlcAb

D

LoVo  HCT-15

Mean distance (µm)

control  FGFR2IIlcAb
Molecular Cancer Therapeutics

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

Yoko Matsuda, Masahito Hagio, Tomoko Seya, et al.

Mol Cancer Ther  Published OnlineFirst July 9, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-12-0243

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2012/07/09/1535-7163.MCT-12-0243.DC2

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://mct.aacrjournals.org/content/early/2012/07/07/1535-7163.MCT-12-0243. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.