Involvement of the FGF18 Gene in Colorectal Carcinogenesis, as a Novel Downstream Target of the β-Catenin/T-Cell Factor Complex

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Abstract

To search for potential molecular targets for development of novel anticancer drugs, we have been analyzing expression profiles of clinical samples from cancer patients, using a genome-wide cDNA microarray. In experiments with colon cancer cells, the gene encoding fibroblast growth factor factor 18 (FGF18) was among those that showed elevated expression. The promoter region of this gene was found to contain putative Tcf4-binding motifs; moreover a reporter-gene assay using luciferase activity as a marker and an electromobility shift assay indicated that FGF18 is a downstream transcription target in the β-catenin/Tcf4 pathway. We showed that exogenous FGF18 promoted growth of NIH3T3 cells in an autocrine manner and that transfection of FGF18 short interfering RNAs suppressed growth of colon cancer cells in culture. Our results indicate that FGF18 is activated in colon cancers as a direct downstream target of the Wnt signaling pathway and that it might represent a marker for early diagnosis and a molecular target for treatment of this life-threatening tumor.

Introduction

CRC is one of the most common solid tumors worldwide. In 2000, nearly 94,000 individuals were diagnosed with colon cancer, and ~579,000 died from it (1, 2). Although great progress has been made in recent years with regard to diagnosis and treatment, the prognosis for patients with advanced colon cancers remains poor. Hence, discovery of a sensitive and specific diagnostic biomarker for detection of early-stage carcinomas and development of more effective but less harmful therapeutic drugs are matters of pressing concern. Furthermore, effective preventive strategies would release many people from fear of this life-threatening disease. To achieve those goals, the detailed molecular mechanisms underlying colorectal carcinogenesis must first be well understood.

Recent molecular studies have revealed that colorectal carcinogenesis involves an accumulation of genetic alterations within a cell lineage, which include not only mutations that inactivate tumor suppressor genes and activate proto-oncogenes, but also amplifications of DNA and/or losses of DNA in certain chromosomal regions. In addition to these types of changes, epigenetic events such as methylation, loss of imprinting, and/or dysregulated expression resulting from genetic changes or unknown mechanisms underlie the genesis of colorectal tumors.

Genes in the Wnt/wingless signaling pathway play critical roles in differentiation and morphogenesis during embryogenesis. Impaired regulation of this pathway often is a feature of tumors arising in the colon, liver, prostate, stomach, brain, endometrium, or elsewhere (3). One of the key mediators of the pathway is β-catenin, which plays a pivotal role in cell-to-cell adhesion and signal transduction. In the absence of Wnt signaling, β-catenin is phosphorylated by a multimolecular complex composed of β-catenin, adenosomatous polyposis coli protein, Axin1, Axil/conductin (AXIN2), and glycogen synthase kinase 3β. β-Catenin is normally down-regulated through ubiquitination and subsequent degradation in the proteosome, but Wnt signaling allows β-catenin to accumulate in the cytoplasm and/or nucleus as a result of inhibition of glycogen synthase kinase 3β. Abnormal intracellular accumulation of β-catenin as a consequence of genetic alterations in the APC, AXIN1, AXIN2, or β-catenin (CTNNB1) genes has been observed in various human cancers, including CRC and hepatocellular carcinoma (4). Accumulated β-catenin forms a complex with the Tcf/LEF transcription factor and up-regulates downstream target genes such as c-myc (5) and cyclinD1 (6, 7). Activation of one or more of these genes can contribute to processes that confer malignant properties on colon-carcinoma cells.

The family of FGFs comprises a group of 23 secreted polypeptides that mediate their signals on binding with one or two of five types of cognate receptors. FGFs play important roles in embryonic development, cell growth, morphogenesis, tissue repair, inflammation, and angiogenesis (8). For example, FGF4, FGF8, FGF10, FGF18, and FGF20 are involved in limb development (9, 10); FGF8 participates in the signaling cascade in the organogenesis of midbrain-hindbrain (11); and FGF10 appears to be essential for development of the lung. Wnt signals control some of the FGFs involved in limb initiation and tooth organogenesis (12, 13). Apart from a crucial role in organogenesis, FGF2 stimulates tissue repair in the adult (14, 15). However, inappropriate expression of FGFs and/or their receptors occurs in many human tumors including bladder, cervical, and gastric cancers (16, 17). Among members of the FGF family, FGF18 is the one that most closely resembles FGF8, and like FGF2, FGF18 stimulates proliferation of NIH3T3 cells (18), osteoblasts (19), chondrocytes (19), and glial cells (20) and induces neuropeptide outgrowth of PC12 rat pheochromocytoma cells (21).

In an ongoing effort to clarify the molecular mechanisms underlying colorectal carcinogenesis, we previously analyzed expression profiles of 9 adenomas and 11 adenocarcinomas of the colon. Here we report that FGF18, one of the genes up-regulated in the earlier microarray experiments, is a direct downstream target of the β-catenin/Tcf4 complex. We also reveal that its elevated expression promotes the growth of colon cancer cells in culture. These data may add to our understanding of colorectal carcinogenesis and may be useful for development of novel diagnostic and therapeutic strategies.

Materials and Methods

Cell Lines and Clinical Materials. Human colon cancer cell lines SW480 HCT116, and DLD1 and the murine fibroblast line NIH3T3 were obtained.

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3 The abbreviations used are: CRC, colorectal cancer; Tcf, T-cell factor; FGF, fibroblast growth factor; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; siRNA, short interfering RNA; EMSA, electrophoretic mobility-shift assay; FGFR, fibroblast growth factor receptor.
from the American Type Culture Collection (Rockville, MD). Human colon cancer cell lines SNUC4 and SNUC5 were obtained from the Korean cell line bank (KCLB, Seoul, Korea). All of the cells were cultured as monolayers in appropriate media, as follows: Leibovitz’s L-15 (Invitrogen, Carlsbad, CA) for SW480; MaCoy’s 5A (Invitrogen) for HCT116; RPMI 1640 (Sigma-Aldrich Corporation, St. Louis, MO) for DLD1, SNUC4, and SNUC5; and DMEM (Sigma-Aldrich) for NIH3T3. Each was supplemented with 0.5% or 10% fetal bovine serum (Cansera International Inc., Ontario, Canada) and 1% antibiotic/antimycotic solution (Sigma-Aldrich). Cells were maintained at 37°C in an atmosphere of humidified air with 5% CO2 (HCT116, DLD1, SNUC4, and SNUC5) or without CO2 (SW480). Cancersous tissues and corresponding noncancersous mucosa were excised from 12 patients during surgery, after informed consent had been obtained.

Semiquantitative RT-PCR. Total RNA was extracted from cultured cells and clinical tissues by use of TRIZOL reagent (Invitrogen), according to the manufacturer’s protocol. RNA extracted was treated with DNase I (Roche Diagnostics, Mannheim, Germany) and reverse transcribed to single-stranded cDNAs by use of oligo(dT)12,18 primer with Superscript II reverse transcriptase (Invitroscript). We prepared appropriate dilutions of each single-stranded cDNA for subsequent PCR amplification by monitoring GAPDH gene as a quantitative control. Primer sequences were as follows: 5′-ACAA-CAGCCTCAAGATCTACG-3′ and 5′-GGTCCACCACTGACACGTG-3′ for GAPDH, and 5′-GGACATGTGCGGCTGGGCTA-3′ and 5′-GTA-GAATTCCTCGTCTCCTGCTT-3′ for FGFR. All of the reactions involved initial denaturation at 94°C for 2 min, followed by 18 (for GAPDH) or 33 cycles (for FGFR) at 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s on a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster, CA).

Northern Blotting. Human multiple-tissue blots (BD Bioscience, Palo Alto, CA) were hybridized with a 32P-labeled PCR product of FGFR, which had been isolated by random-oligonucleotide priming with a Mega Label kit (Amer sham Biosciences, Buckinghamshire, United Kingdom). The product was prepared by RT-PCR using primers 5′-GGACATGTGCGGCTGGGCTA-3′ and 5′-GTA-GAATTCCTCGTCTCCTGCTT-3′. Prehybridization, hybridization, and washing were performed according to the supplier’s recommendations. The blots were autoradiographed with intensifying screens at −80°C for 240 h.

Preparation of Polyclonal Antibody against FGFR. Plasmids expressing His-tagged COOH-terminal FGFR protein (codons 167–207) were prepared by using pET28 vector (Novagen, Madison, WI). The recombinant protein was expressed in Escherichia coli, BL21 codon-plus strain (Strategene, La Jolla, CA), and purified using TALON resin (BD Bioscience) according to the supplier’s protocol. The protein was inoculated into rabbits, and the immune sera were purified on affinity columns according to standard methodology.

Immunohistochemistry. Immunohistochemical staining was carried out using affinity-purified antibody against human FGFR. Frozen tissue sections were subjected to the SAB-PO peroxidase immunostaining system (Nichirei, Tokyo, Japan) according to the manufacturer’s recommended method.

Effect of FGFR18 on Cell Survival in Vitro. The entire coding region of human FGFR18 was amplified by RT-PCR, using primers 5′-CTCACTGCTTACCCCTGTCG-3′ and 5′-GCAGCTCCGTCTTTCCTGCAGTTC-3′, and cloned into appropriate cloning sites of expression vectors pcDNA3.1 (+) (Invitrogen) or pFlagCMV5 (Sigma-Aldrich). Plasmids expressing FGFR18 (pcDNA-FGFR18 or pFlag-FGFR18) or empty vector (pcDNA or pFlagCMV5) were transfected into murine fibroblast NIH3T3 cells for a focus formation assay. One week after transfection, the cells were fixed with 100% methanol and stained with Giemsa solution. Viable cells were measured with a cell-counting kit (DOJINDO, Kumamoto, Japan). Expression of FGFR18 in the treated cells was examined by semiquantitative RT-PCR 24 h after transfection.

MTT Assay. Cells (1 × 103) on 6-well plates were transfected with expression vector or control vector using FuGene6 (Roche diagnostics) according to the supplier’s protocol. Cell viability was evaluated by MTT assay 7 days after transfection. Cell-counting kit (DOJINDO) was added to each dish at a concentration of 1/10 volume, and the plates were incubated at 37°C for an additional 4 h. Absorbance was then measured at 490 nm, and at 630 nm as a reference, with a Microplate Reader 550 (Bio-Rad Laboratories, Hercules, CA).

Reportor Assay. An initiation site for transcription of FGFR18 was determined by comparing the human genomic sequence (GenBank accession no. AC093246) with the cDNA sequence of FGFR18 (GenBank accession no. NM_003862). To examine the activity of the FGFR18 promoter we amplified by PCR four fragments, each corresponding to part of the region flanking FGFR18 on the 5′ side, and cloned each product into an appropriate enzyme site of pGL3-Basic vector (Promega, Madison, WI). Plasmids expressing an activated form of β-catenin (mut β-catenin) and wild-type and dominant-negative forms of Tcf4 (wtTcf4 and TnTc4) were prepared as described previously (22). One microgram of each reporter plasmid and 1 μg of each expression construct were cotransfected with 0.2 μg of pRL-TK plasmid (Promega) into SW480 cells by use of FuGENE6 to normalize the efficiency of transfection. Reporter assays were carried out using a dual-luciferase reporter assay system according to the supplier’s recommendations (Promega).

EMSA. The EMSA was performed as described previously (23), using nuclear extracts from SW480 cells. A double-stranded 16-nucleotide DNA probe was prepared by annealing FGFR18 (5′-CGCCCTTTAGGCTGGG-3′) to FGFR3 (5′-GGCCACTCAGAAGCCCGG-3′) and labeled with [32P]ATP and T4 polynucleotide kinase.

Statistical Analysis. Statistical significance was analyzed by ANOVA with Scheffe’s F test, using commercially available software (Statview; SAS Institute, Cary, NC).

Results

Up-Regulation of FGFR18 in CRC. We had previously analyzed expression profiles of 9 adenomas and 11 adenocarcinomas of the colon by means of a cDNA microarray representing 23,040 genes (24). Among the genes whose expression levels were commonly up-regulated in cancer cells, a spot corresponding to FGFR18 showed high tumor/normal intensity ratios in the majority of the cases examined. Subsequent semiquantitative RT-PCR corroborated enhanced expression of this gene in 10 of 12 additional colon cancer tissues examined (Fig. 1A). To examine expression of FGFR18 in human adult normal tissues, we carried out Northern blot analysis and identified a transcript of ~1.8 kb that was abundantly expressed in the heart but not in any of 22 other tissues examined (data not shown).

Accumulation of FGFR18 in Tumor Cells. To analyze the function of FGFR18, we prepared an anti-FGFR18 antibody that would recognize endogenous FGFR18 protein in cells and investigated expression of this
protein in four CRC tissues by immunohistochemical staining. In all four cases, FGF18 was stained in the cytoplasm of cancerous cells (Fig. 1B); staining in the cytoplasm of noncancerous epithelial cells from corresponding mucosae was significantly weaker and localized mainly at the bottom of crypts.

Assay of the FGF18 Promoter in Colon Cancer Cells. Because transactivation of the β-catenin/Tcf4 complex is a relatively common feature of colon cancer cells, we tested whether this complex regulates expression of FGF18 by infecting SW480 cells with adenovirus expressing a dominant-negative form of Tcf4 (dnTcf4) or with a control gene (LacZ). Expression of FGF18 was significantly decreased in response to dnTcf4 compared with the control, suggesting that Tcf4-mediated transcriptional activity was correlated with expression of FGF18 (data not shown). We then searched for consensus Tcf4-binding motifs, 5'-CTTTGWW-3' or 5'-WWCAAAG-3', within a 2-kb genomic fragment of the 5'-region flanking FGF18 and identified three possible candidate sites: between −1631 and −1625 (TBMI), between −1348 and −1342 (TBM2), and between −190 and −184 (TBM3; Fig. 2A).

To examine which of these binding sites might be responsible for the promoter activity of FGF18, we cloned fragments of various lengths from its 5'-flanking region upstream of the luciferase gene and performed a reporter assay using SW480 cells (Fig. 2B). pGL3-P1 (containing nucleotides between −1644 and +26), pGL3-P2 (containing nucleotides between −1354 and +26), and pGL3-P3 (containing nucleotides between −195 and +26) revealed −5-fold increases in luciferase activity compared with pGL3-P4 (containing nucleotides between −181 and +26), suggesting that the region between −195 and −182 was responsible for the transcriptional activity. To further clarify the role of TBM3, we assayed the luciferase activity after introducing a 2-base mutation (CTTTGAT to CTT-TGGC) at the TBM3 site (pGL3-P3mt). As expected, mutation at the TBM3 site (P3mt) reduced luciferase activity by more than 75%.

These results suggested that TBM3 indeed contained the promoter sequence for FGF18. Notably, the TBM3 sequence was conserved in the 5'-flanking region of the mouse Fgf18.

Association between the Putative Tcf4 Binding Site and the β-Catenin/Tcf Complex. To examine whether the β-catenin/Tcf4 complex associates with the TBM3 site in the promoter region of FGF18, we carried out an EMSA, using oligonucleotides correspond-
ing to the TBM3 sequence. A single band was shifted by addition of anti-β-catenin antibody but not by an unrelated (control) antibody. This binding was abrogated by addition of wild-type unlabeled oligonucleotide but not by mutant unlabeled oligonucleotides, indicating direct interaction between the binding sequence and the β-catenin/Tcf4 complex (Fig. 3).

**Growth Advantage Conferred by Overexpression of FGF18 in NIH3T3 Cells.** Because overexpression of FGF18 is known to promote growth of fibroblasts and osteoblasts, we hypothesized that FGF18 could render oncogenic effects in an autocrine manner. In line with that hypothesis, our immunoblotting experiments detected Flag-tagged FGF18 protein in the culture medium of murine fibroblast cells transfected with pFlagCMV-FGF18 (Fig. 4A). As expected, the NIH3T3 cells proliferated at a significantly higher rate in conditioned medium with FGF18 than cells in conditioned medium without FGF18 (Fig. 4B).

**Effect of FGF18 siRNA on Growth of Cancer Cells.** To evaluate the potentially oncogenic role of FGF18, we prepared plasmids expressing siRNA of FGF18 and transfected them into five lines of CRC cells expressing abundant amounts of FGF18. Among the constructed plasmids, psiH1BX-FGF18 significantly reduced expression of FGF18 compared with a control plasmid (psiH1BX-EGFP) and markedly decreased the number of viable cells compared with psiH1BX-EGFP (Fig. 4, C–E). This growth suppression was partially rescued by addition of the conditioned medium containing FGF18 (Fig. 4E). These data support the notion that enhanced FGF18 expression is involved in the growth of cancer cells and implied that inhibition of its activity could be an effective therapeutic strategy for CRC.

**Discussion**

We have demonstrated here that FGF18 is frequently up-regulated in CRCs, as a direct target of the β-catenin/Tcf4 complex. Because FGF18 is a secreted protein, it might serve as a novel marker for early detection of colorectal tumors. Moreover, because in our experiments FGF18 protein promoted growth of NIH3T3 cells in an autocrine manner and its down-regulation suppressed growth or survival of colon cancer cells, FGF18 may also represent a promising molecular target for novel anticancer drugs.

Fig. 3. EMSA of the β-catenin/Tcf4 complex using TBM3-oligonucleotide as a probe. A supershifted band was observed after addition of anti-β-catenin antibody (Lanes 2 and 7) but was not elicited by anti-P53-antibody (Lane 3). Bands corresponding to the DNA-protein complex were reduced by addition of nonlabeled wild-type probe (Lanes 4 and 5), but not by nonlabeled mutant probe (Lanes 6 and 7).

Fig. 4. A, immunoblotting of Flag-tagged FGF18 protein secreted into culture medium. Proteins in the medium and cell lysate were immunoblotted with anti-Flag antibody. NIH3T3 cells were transfected with either pFlagCMV or pFlagCMV-FGF18. Filled and open triangles indicate the cellular or secreted forms, respectively, of tagged FGF18 protein. B, microscopic appearance of NIH3T3 cells after incubation in DMEM containing 0.5% fetal bovine serum (left), in the conditioned medium after transfection with pFlagCMV-FGF18 (center), and in the conditioned medium after transfection with pFlagCMV (right). C, effect of FGF18 siRNAs on expression of FGF18. Semiquantitative RT-PCR was carried out with RNAs from cells transfected with siRNA-expressing or control plasmids. D, Giemsa staining of viable HCT116 cells in response to EGFP-siRNA or FGF18-siRNA in the conditioned medium transfected with mock or pFlagCMV-FGF18 plasmids. E, MTT assay of viable HCT116 cells in response to EGFP-siRNA or FGF18-siRNA in the conditioned medium transfected with mock or pFlagCMV-FGF18 plasmids. The conditioned medium was changed every other day. MTT assays were carried out in triplicate. Error bars, SD.
FGF18 expression has been observed in the developing lung, surrounding developing bones, and in the cerebral cortex of the developing brain during embryonic stage E15.5 (18). Intraperitoneal injection of recombinant FGF18 protein induced significant gains in the weights of liver and intestine in mice (18). Additionally, FGF18 stimulated growth of NIH3T3 cells in a heparan sulfate-dependent manner (18). These observations agree well with our conclusion that elevated expression of FGF18 stimulates growth and/or prevents death of epithelial and mesenchymal cells in an autocrine manner.

FGF function by binding with FGFRs; five FGFR genes and their splicing variants have been identified to date. A BLACORE assay demonstrated that FGFR-3c and FGFR-2c, but not FGFR-1c, have affinity for FGF18 (20). These data suggest that FGF18 exerts its growth-promoting effect by interacting with some receptors. However, the phenotypes of FGF18-deficient mice, which show delayed ossification and decreased expression of osteogenic markers, do not conform completely with the phenotype of mice lacking FGFR-3 (27). Therefore, antagonizing FGFR-3c and/or other FGFRs, such as FGFR-2c, might be an effective strategy for suppressing FGF18-mediated cell growth.

Our experiments also revealed that FGF18 is the second member of the FGF family to be confirmed as a direct target of Tcfe4/LEF1. Previously FGF4 was reported to be a direct target of LEF1; recombinant FGF4 fully rescued the Lef1 mutant phenotype for tooth development in mice (13). Wnt signals also control FGF-dependent limb initiation via FGF8 and FGF10 (12, 25). Because FGF18 is expressed in the right side of Hensen’s node before expression of FGF8 occurs and expression of FGF18 also precedes FGF8 in the isthmus in the developing brain, Wnt signals may recruit FGF18 as an initial mediator of organogenesis of the limb and brain. Like FGF4, which has transforming activity (17), FGF18 probably can become oncogenic when inappropriately overexpressed.

Immunohistochemical staining of FGF18 in human colon cancer tissues and corresponding noncancerous mucosas showed a pattern similar to that of β-catenin and other β-catenin/Tcf downstream proteins, such as ENS1, CD44, and EPHB2 (28). This evidence supports a view that activated β-catenin/Tcf4 complexes in colonic tumors have switched on the proliferative signals that are usually restricted to progenitor cells located in the lower third of colonic crypts. Therefore, FGF18 expressed in nontumorous crypts may play restricted to progenitor cells located in the lower third of colonic crypts. However, because in published experiments FGF18-knockout mice did not reveal any abnormalities in intestinal structure, other factors may redundantly affect the development of mucosa (27). Furthermore, FGF18 could exert different functions in other tissues, specifically bone and brain. Further studies on the function of genes downstream of the β-catenin/Tcf4 transcription complex could help to clarify which factors are required for maintenance of progenitor cells in colonic epithelium.

In conclusion, our data underscore the importance of elevated expression of FGF18 in colorectal tumorigenesis. Because FGF18 functions as growth factor by binding its receptor(s) at least, but not solely, in an autocrine manner, our data clearly indicate that FGF18 could be a good candidate as a tumor marker as well as a molecular target for the development of reagents, such as specific neutralizing antibodies or antagonists against the receptor(s), for treatment of patients with colorectal tumors.

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