Epigenetically Controlled Fibroblast Growth Factor Receptor 2 Signaling Imposes on the RAS/BRAF/Mitogen-Activated Protein Kinase Pathway to Modulate Thyroid Cancer Progression

Tetsuo Kondo,1,2,4 Lei Zheng,2,4 Wei Liu,1,2,4 Junichi Kurebayashi,5 Sylvia L. Asa,1,3,4 and Shereen Ezzat2,4

Departments of1Pathology and Medicine, and the2Ontario Cancer Institute, Princess Margaret Hospital, University Health Network and3University of Toronto, Toronto, Ontario, Canada and4Departments of Breast and Thyroid Surgery, Kawasaki Medical School, Okayama, Japan

Abstract

Fibroblast growth factor (FGF) signals play fundamental roles in development and tumorigenesis. Thyroid cancer is an example of a tumor with nonoverlapping genetic mutations that up-regulate mitogen-activated protein kinase (MAPK). Here, we show that FGF receptor 1 (FGFR1), which is expressed mainly in neoplastic thyroid cells, propagates MAPK activation and promotes tumor progression. In contrast, FGFR2 is down-regulated in neoplastic thyroid cells through DNA promoter methylation. Reexpression of FGFR2 competes with FGFR1 for the immediate substrate FGFR substrate 2 to impede signaling upstream of the BRAF/MAPK pathway. These data unmask an epigenetically controlled FGFR2 signal that imposes precisely on the intragenically modified BRAF/MAPK pathway to modulate thyroid cancer behavior. [Cancer Res 2007;67(11):5461–70]

Introduction

Thyroid cancer is the most common endocrine malignancy and is increasing in incidence (1). The majority of thyroid carcinomas are derived from follicular epithelial cells and show a spectrum of differentiation from indolent well-differentiated papillary and follicular carcinomas to the more aggressive poorly differentiated carcinoma and the rare but rapidly undifferentiated carcinoma (2). This spectrum of progression has been linked with a pattern of cumulative genetic defects that correlates with tumor differentiation, aggressiveness, and metastatic potential (3). Gene rearrangements involving the RET proto-oncogene or activating point mutations along the RAS/BRAF pathway account for the majority of these carcinomas (3). Cancer behavior and progression are modified by dysregulation of growth factor signaling. Alterations of several growth factors and their receptors identified in thyroid tumors include up-regulation of MET, epidermal growth factor receptor, platelet-derived growth factor, and vascular endothelial growth factor (VEGF; ref. 3). Fibroblast growth factors (FGF) and FGF receptors (FGFR) are also implicated in regulating endocrine neoplasia, including thyroid carcinoma (4, 5).

FGFs comprise a family of heparin-binding proteins that currently includes 23 members. They signal through four high-affinity tyrosine kinase receptors (FGFR1–FGFR4; ref. 5). Each receptor has two or three immunoglobulin-like extracellular domains, a transmembrane domain, an intracellular split tyrosine kinase, and a carboxyl-terminal (6). After FGF binding and receptor dimerization, several signal transduction pathways are activated, mainly involving FRS2 and PLCγ. Activated FRS2 recruits the GRB2/SOS complex and ultimately mitogen-activated protein kinase [MAPK or extracellular signal-regulated kinase 1 (ERK1)/ERK2; ref. 6]. Combinations of FGFRs, FGFR isoforms, and adaptor proteins comprise complex signaling networks that play fundamental roles in development, organogenesis, cell differentiation, angiogenesis, and tumor progression (5, 7). Up-regulation of FGFR1 has been identified in astrocytomas, breast carcinomas, prostate carcinomas, melanomas, and malignant salivary gland tumors (6, 7). In thyroid, increased expression of FGFR1 has been observed in benign and malignant tumors (4, 8). FGFR1 is up-regulated in rat thyroid follicular cells upon goitrogen administration, whereas a dominant-negative FGFR1 reduces goitrogenesis in mice (9, 10).

We have previously shown that FGFR expression is dysregulated in human thyroid tumors and cell lines (4). FGFR2 was the only FGFR consistently detected in normal thyroid tissues, and its expression was diminished in a large tissue microarray of thyroid tumors and in six carcinoma cell lines (4). In contrast, FGFR1 was expressed in hyperplastic goiters, benign adenomas, and carcinomas (4). Thus far, no mutations or rearrangements involving FGFRs have been identified in thyroid cancers, suggesting that epigenetic factors are implicated in their dysregulated expression in thyroid tumors.

In this study, we focused on the expression of two principal members of the FGFR family (1 and 2) in thyroid. We hypothesized that FGFR1 promotes thyroid cell growth and that FGFR2 plays a protective role against cancer progression in genetically transformed thyroid cells. We used loss-of-function and gain-of-function approaches to investigate the signaling effect of FGFR1 and FGFR2 on thyroid cancer growth in vitro and in vivo. We also examined the potential role of epigenetic modification as a mechanism implicated in FGFR2 down-regulation in thyroid carcinoma cells.

Materials and Methods

Cell lines and cell culture. Human thyroid carcinoma–derived cell lines, WRO, NPA, and DBO (provided by Dr. J. Fagin, University of Cincinnati, originally established by Dr. G. Juillard, University of California at Los Angeles, Los Angeles, CA), TPC-1 (Dr. S.M. Jhiang, Ohio State University, Columbus, OH), KTC-1 (11), and 8505C (Cell Resource Center for
Biomedical Research, Toho University, Sendai, Japan), were maintained in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum (FBS), streptomycin sulfate (100 units/mL), and penicillin (100 μg/mL). H, N, or K-Ras mutations and BRAF V600E were examined in all cell lines by PCR direct sequencing using primers listed in Supplementary Table S1. The human embryonic kidney (HEK) 293 cells were propagated in DME (Life Technologies) supplemented with 10% FBS and antibiotics.

Vector constructs and stable cell transfection. FGFR1 small interfering RNA (siRNA) target sequence, without homology to other FGFRs and other known human genes, was 5′-AAGAAATTTGCAATG-CAGTGGCCG-3′ situated in the second immunoglobulin-like domain (nucleotide positions 487–507) to cover most of the splice variants of FGFR1. The double-stranded oligonucleotide templates containing the sequence of the hairpin siRNA (top strand 5′-GATCCGAAATTGCAATG-CAGTGGCCGTCGAGGGCACTGCAATTCTTTTGGAAA-3′; bottom strand 5′-AGCTTTTTAAAAAAAGAGTGCACTGGCGGCTCT-GCAAGGACGCTGCACTGGAACCTCG-3′) were synthesized and inserted into the expression vector (pSilencer 2.1-U6neo, Ambion). A circular vector that expresses a control hairpin siRNA without homology to any known human sequence was used as a control.

The cDNA encoding human FGFR2-IIIb, also known as Ksam-IIC1 (established by Drs. M. Terada and T. Yoshida, National Cancer Institute, Tokyo, Japan) in pcDNA1/Neo expression vector (Invitrogen) and a control empty vector (pCDNA1/Neo) were kindly provided by Dr. F. Radvanyi (Center National de la Recherche Scientifique, Paris, France). The full-length FGFR1 cDNA, kindly provided by Dr. J. Rossant (Hospital for Sick Children, Toronto, Canada), was inserted into pcDNA1/Neo (Invitrogen).

The expression vectors were transfected into cells using LipofectAMINE (Invitrogen). Stable clones were selected and maintained in a growth medium containing 1 mg/mL of Genetin (Life Technologies). Alterations in FGFR expression were confirmed by Western blotting. Two independent clones of each manipulation were used for all studies.

Growth factor stimulation. After 24 h starvation in serum-free medium, cells were treated with the nonselective FGFR1 ligand (25 ng/mL, Sigma) or the FGFR2-selective ligand FGF7 (25 ng/mL, Sigma), each with 10 units/mL of heparin (Sigma) in serum-free medium for 15 min at 37°C. Identical volume of vehicle served as control. In other experiments, 10% FBS was used as a source of multiple FGF ligands.

RNA extraction and reverse transcription–PCR analysis. Total RNA was isolated from cultured cells and frozen human thyroid tissues using TRIzol (Invitrogen). cDNA was generated using the TaqMan reverse transcription (RT) reagent kit (Applied Biosystems). Specific PCR primers for FGFR1, FGFR2-IIIb, FGFR2-IIIc, FGFR2, and phosphoglycerate kinase 1 (as an internal control) were used as listed in Supplementary Table S1. Amplicons were designed to cross exon/intron boundaries to exclude genomic DNA contamination. Amplification was done using HotStarTaq DNA polymerase kit (Qiagen). PCR conditions were as follows: (i) 95°C for 10 min; (ii) 30 cycles of 94°C for 1 min, 56°C for 30 s, and 72°C for 1 min; (iii) 72°C for 10 min; and (iv) 4°C hold. Negative controls omitting RT and positive controls were included in each PCR reaction.

Protein isolation and Western blotting analysis. Thyroid tissues were homogenized using a polyvinyl homogenizer in radioimmunoprecipitation assay buffer (RIPA) lysis buffer with proteasine inhibitors [1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 12 μg/mL aprotinin, and 1 mmol/L sodium orthovanadate]. Cultured cells were lysed in a RIPA lysis buffer with proteasine inhibitors and 5 μL/mL of phosphatase inhibitor cocktail 1 and 2 (Sigma).

Equal amounts of protein (40 μg) solubilized in sample buffer were separated on 10% SDS polyacrylamide gels and transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in TBS containing 0.5% Tween 20 plus 5% nonfat dried milk for 1 h at room temperature, probed with primary antibodies at 4°C overnight.

Primary antisera or monoclonal antibodies were used at the specified dilutions: anti-FGFR1 (Santa Cruz Biotechnology; 1:500), anti-FGFR2 (Santa Cruz Biotechnology; 1:500), anti–phospho-MAP2 (Cell Signaling Technology; 1:1000), anti–total FBS2 (Santa Cruz Biotechnology; 1:500), anti–phospho-
fixed in −20 °C 80% ethanol overnight. Fixed cells were washed with ice-cold staining buffer (1× PBS, 0.2% Triton X-100, and 1 mmol/L EDTA (pH 8.0)) and resuspended in staining buffer containing 50 μg/mL RNase A (Sigma) and 50 μg/mL propidium iodide for 1 h. A FACScan (Becton Dickinson) coupled with CellQuest software was used to obtain fluorescence-activated cell sorting (FACS) data.

**Invasion and migration assays.** Cell motility was examined in a transwell assay using 24-well plates with uncoated inserts to examine migration or Matrigel-coated inserts to assess invasiveness (Becton Dickinson). The upper and lower culture compartments were separated by polycarbonate filters with a pore size of 8 μm. After trypsinization, 2.5 × 10⁴ cells were plated in each insert with 500 μL of serum-free medium. The growth medium containing 10% FBS was used as a chemoattractant in the bottom well. After 20 h of incubation, cells on the upper surface were removed by scrubbing with a cotton swab. Cells on the lower surface of the membrane were stained with Diff-Quik stain (Dade Behring) and quantified by light microscopy. Assays were done in triplicate.

Human thyroid cancer cell xenografts in severe combined immune deficiency mice. Subconfluent cells were trypsinized, washed twice with PBS, and harvested by centrifugation. Cell pellets were resuspended in PBS, and 5 × 10⁶ cells in 0.1 mL volume were injected s.c. into the flank of 6-week-old female severe combined immune deficiency (SCID) mice to generate s.c. tumors. Tumor volume was monitored every 2 days. Mice were sacrificed after 14 days after cell implantation; tumors were excised and weighed, and volume was measured. Excised tissue was fixed in 10% formalin and embedded in paraffin for light microscopy and immunohistochemical staining. The mouse protocol was approved by the Ontario Cancer Institute Animal Care and Utilization Committee.

5′-Aza-deoxycytidine and trichostatin A treatment. Cells were plated at a density of 5 × 10⁵ per 10 cm² dish and incubated in growth medium without or with the demethylating agent 5′-aza-deoxycytidine (10 μmol/L, Sigma) for 72 h. For histone deacetylase inhibition, trichostatin A (0.3 μmol/L, Sigma) was applied for 24 h. The equivalent volume of vehicle (50% acetic acid for 5′-aza-deoxycytidine or 100% ethanol for trichostatin A) was applied as control.

Bisulfite treatment and methylation-specific PCR (MSP) assay. Genomic DNA was extracted from 5′-aza-deoxycytidine-treated or untreated cells by proteinase K digestion and phenol/chloroform extraction. Denatured DNA was modified by bisulfite under conditions that convert all unmethylated cytosines to uracils using CpGenome DNA modification kit (Chemicon International). Specific primers are listed in Supplementary Table S1. Amplification was done in a reaction volume of 50 μL containing 40 ng of bisulfite-treated DNA, 1× PCR buffer, 3.0 mmol/L MgCl₂, 0.25 mmol/L of each deoxynucleotide triphosphate, 0.5 μmol/L of each primer, and 1.25 units of HotStarTaq DNA polymerase (Qiagen). PCR conditions were as follows: (a) 95 °C for 15 min; (b) 30 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min, (c) 72 °C for 10 min, and (d) 4 °C hold. Both negative and positive controls were included for all PCR reactions.

**Statistical analysis.** Data are presented as mean ± SE. Statistical analysis was conducted by the Student’s t test. P values of 0.05 or less were considered statistically significant.

**Results**

Expression of FGFR1 and FGFR2 in normal thyroid and thyroid carcinoma cell lines. We previously reported expression of FGFR1 in TPC-1, NPA, and WRO thyroid cancer cell lines and lack of FGFR2 expression in transformed compared with non-transformed thyroid follicular cells (4). In this study, FGFR1 mRNA was detected by RT-PCR in TPC-1, WRO, and 8505C carcinoma cell lines but not in KTC-1 cells; in contrast only a faint signal was found in normal thyroid tissue, a feature consistent with stromal contribution (Fig. 1A). Western blotting confirmed that endogenous FGFR1 protein was up-regulated in WRO and 8505C cells, although it was undetectable in normal thyroid tissues (Fig. 1B).

We previously reported that FGFR2 protein was undetectable in six human thyroid carcinoma cell lines (TPC-1, NPA, WRO, MRO, DRO, and ARO; ref. 4). To complete a search for FGFR2 expression, we examined two additional thyroid carcinoma cell lines (papillary thyroid carcinoma–derived KTC-1 cells and undifferentiated thyroid carcinoma–derived 8505C cells). Alternative splicing of the third immunoglobulin-like extracellular domain of FGFR2 results in two major transcripts, FGFR2-IIIb and FGFR2-IIIc, that exhibit distinct ligand binding specificities (12). Thus, we also examined FGFR2 splicing using primers to specifically amplify either FGFR2-IIIb or FGFR2-IIIc. In contrast to FGFR1 expression, FGFR2-IIIb mRNA was identified in all normal thyroids but only in one thyroid carcinoma cell line, the KTC-1 cell line (Fig. 1A). FGFR2-IIIc mRNA was not detectable in normal thyroids or carcinoma cell lines. FGFR2 protein was detectable in all normal thyroid tissues but was not detectable in thyroid carcinoma cell lines with the exception of KTC-1 cells (Fig. 1B). The doublet migration of FGFR1 and FGFR2 is consistent with variable N-terminal glycosylation (4, 13).

To examine the signaling potential of FGFRs in thyroid cells, we examined expression of the FGFR substrate adaptor protein FRS2. Although FRS2 mRNA was detected in normal and thyroid carcinoma cell lines (Fig. 1A), FRS2 was up-regulated at the protein level in all four thyroid cancer cell lines examined (Fig. 1B).

FGFR1 silencing and FGFR2-IIIb reexpression in WRO cells. To examine the contribution of FGFR1 to cell growth, we established stable cell clones of WRO cells with down-regulated FGFR1 using siRNA-mediated gene silencing. We selected WRO cells based on their endogenous expression of FGFR1 but not FGFR2; these cells do not have confounding growth effects known to be characteristic of FGFR4 that is expressed in more aggressive tumors (4). We also confirmed that they do not harbor a BRAF mutation or an intragenic RAS mutation (data not shown). Western blotting documented silencing of FGFR1 expression, and this was corroborated by diminished immunoreactivity for FGFR1 using immunohistochemistry of cell blocks (Fig. 1C).

Having determined that the FGFR2-IIIb isoform is expressed in normal thyroids but not in the majority of thyroid carcinoma cell lines, we examined the functional properties of FGFR2-IIIb using a gain-of-function approach. WRO cells, which do not endogenously express FGFR2, were forced to express FGFR2-IIIb. Western blotting showed strong expression of FGFR2 in FGFR2-IIIb/WRO cells, whereas pcDNA/WRO cells were negative (Fig. 1D). Similarly, immunohistochemistry confirmed the stable reexpression of FGFR2 (Fig. 1D).

**FGFR1 silencing impedes cell proliferation and invasion.** To test the signaling consequences of FGFR1 silencing, we examined the response of FGFR1–down-regulated WRO cells to FGFR1, a ligand that activates multiple FGFRs (6). As noted in control siRNA/WRO cells, FGFR1 effectively induced phosphorylation of MAPK (ERK1/2), Akt, and Rb (Fig. 2A). In contrast, silencing of FGFR1 prevented FGFR1-induced responses, highlighting the importance of FGFR1 in FGF-mediated signaling.

To assess the effect of FGFR1 down-regulation on thyroid cancer cell behavior, we used transwell assays. FGFR1 underexpression unequivocally suppressed thyroid cancer cell invasion (P = 0.04) with no significant effect on cell migration (Fig. 2B). We also compared cell cycle progression in FGFR1 siRNA/WRO and control cells by FACS analysis. Cell cycle variables showed a consistent and significant decrease of the DNA synthetic S phase (P = 0.003) with corresponding induction of G2 phase arrest (P = 0.002) in FGFR1
siRNA/WRO compared with control cells. Detailed cell cycle profiles are as follows: FGFR1 siRNA/WRO (G0-G1 phase, 41.9 ± 1.4%; S phase, 20.0 ± 0.7%; G2-M phase, 37.7 ± 1.8%) and control (G0-G1 phase, 41.2 ± 1.6%; S phase, 41.3 ± 3.1%; G2-M phase, 14.2 ± 2.9%). For assessment of tumor growth in vivo, we used a SCID mouse model of human thyroid cancer xenografts (14). Tumor volume was significantly reduced in FGFR1 siRNA/WRO tumors compared with control tumors (152.0 ± 29.1 mm³ versus 390.6 ± 107.9 mm³, P = 0.05; Fig. 2C). TUNEL assay identified no significant difference in apoptosis in the two types of xenografts [control 10.6/high power field (HPF) versus FGFR1 siRNA/WRO, 14.1/HPF, P = 0.6; Fig. 2D].

Restoration of FGFR2-IIIb attenuates RAS/BRAF/MAPK phosphorylation. Given the recognized importance of the BRAF/MAPK signaling pathway in human thyroid tumorigenesis (3), we asked whether FGFR2-IIIb can affect this pathway. We chose serum as a stimulus source of multiple ligands. In control WRO cells, serum effectively induced BRAF serine phosphorylation. In contrast, WRO cells expressing FGFR2-IIIb failed to show BRAF phosphorylation (Fig. 3A, left). Immunoblotting analysis using a phospho-BRAF–specific antibody (Thr597/Ser601) also showed BRAF activation under serum stimulation in control cells but restoration of FGFR2-IIIb attenuated serum-induced BRAF phosphorylation. Consistent with its attenuating effect on BRAF activation, expression of FGFR2-IIIb diminished serum-induced MAPK activation (Fig. 3A, right). To determine whether FGFR2-IIIb can override the effect of the BRAF V600E mutation that is characteristic of nearly half of human thyroid carcinomas (3), we did these studies in 8505C cells that we confirmed to harbor this
mutation by PCR direct sequencing. In these cells, expression of FGFR2-IIIb also attenuated serum-induced BRAF and MAPK phosphorylation (Fig. 3B). To determine the site of interruption of signaling mediated by FGFR2-IIIb, we did a Ras-GTP loading assay (Fig. 3C). This showed the ability of FGFR2-IIIb to significantly attenuate Ras activation, suggesting a level of control upstream of BRAF. Consistent with this finding, a corresponding BRAF kinase assay showed diminished intrinsic kinase activity in response to FGFR2-IIIb signaling (Fig. 3D).

**Restoration of FGFR2-IIIb impedes thyroid carcinoma growth and enhances apoptosis.** To determine if FGFR2-IIIb can restrain tumor progression, we examined cell cycle progression by flow cytometry. Forced expression of FGFR2-IIIb significantly decreased the percentage of cell population in S phase \( (P = 0.006) \) with corresponding increases in G2-M phase \( (P = 0.009) \). Detailed cell cycle profiles are as follows: FGFR2-IIIb/WRO (G0-G1 phase, 34.8 ± 0.9%; S phase, 28.4 ± 3.3%; G2-M phase, 36.4 ± 3.9%) and pcDNA/WRO (G0-G1 phase, 33.2 ± 1.5%; S phase, 46.2 ± 0.7%; G2-M phase, 16.8 ± 1.1%). Cell proliferation as determined by an MTT assay showed similar reduction in cell growth in the presence of FGFR2-IIIb (Fig. 4A). Similarly, cell migration and invasion were significantly retarded in response to FGFR2-IIIb expression (Fig. 4B).

In *vivo* studies using xenografts in SCID mice confirmed a significant reduction of tumor volume of cells with forced FGFR2-IIIb expression compared with control FGFR2-IIIb-negative tumors \( (721.7 ± 145.6 \text{ mm}^3 \text{ versus } 1249.0 ± 145.6 \text{ mm}^3, P = 0.03; \) Fig. 4C). TUNEL assay identified a significant increase in apoptosis in tumors expressing FGFR2-IIIb (pcDNA/WRO, 23.1/HPF versus FGFR2-IIIb/WRO, 93.6/HPF, \( P = 0.04; \) Fig. 4D).

**FGFR2-IIIb and FGFR1 compete with each other for FRS2 activation.** To examine the functional signaling relationship between FGFR1 and FGFR2-IIIb, we tested their ability to engage FRS2. WRO cells expressing FGFR2-IIIb or their controls were treated after serum starvation with FGF1 or FGF7 (Fig. 5). As expected, the FGFR2-selective ligand, FGF7, activates FRS2 only in cells expressing FGFR2-IIIb (Fig. 5A). In contrast, the FRS2 response to FGF1 in WRO cells (which endogenously express FGFR1; Fig. 1A and B) transfected with FGFR2-IIIb was significantly diminished compared with control WRO cells. Forced expression of FGFR2-IIIb without or with FGF1 or FGF7 stimulation did not change the total amount of FRS2 in WRO cells. These studies were further extended in HEK293 cells in which FGFR1 and FGFR2-IIIb were independently introduced. In this system, FGF7 resulted in activation of FRS2 in the presence of FGFR2-IIIb alone (Fig. 5B). In contrast, the response to FGF7 was markedly blunted in the concomitant presence of FGFR1 and FGFR2-IIIb. Based on previous observations and these current data, we propose the following models (Fig. 5C).

In the first model (i), FGFR2-IIIb directly mediates inhibitory signals in thyroid carcinoma cells. In the second model (ii), FGFR2-IIIb and FGFR1 compete with each other for FRS2 activation.
forms an inactive heterodimer complex with FGFR1, abrogating the tumor-promoting functions of FGFR1. In the third model (iii), FGFR2-IIIb recruits FRS2 and diverts signaling away from other tyrosine kinase receptors including FGFR1. FGFR2-IIIb is silenced through DNA methylation in thyroid carcinoma.

Given the role for FGFR2-IIIb signaling in modulating thyroid cancer growth, we examined the mechanism underlying its down-regulation in thyroid carcinoma cells. Based on the observation that large CpG islands are present in the human promoter and noncoding region of FGFR2 (15), CpG methylation status was studied by MSP analysis. As shown in Fig. 6A, MSP showed strong amplification of CpG-unmethylated FGFR2 genomic DNA in normal thyroid tissues (which endogenously express FGFR2; Fig. 1A and B), whereas CpG-methylation of FGFR2 was observed in four of six cell lines with corresponding reduction in amplification of the unmethylated product. Next, we treated cells (WRO, TPC-1, NPA, and DRO) with the DNA demethylating agent 5-aza-deoxycytidine. Compared with vehicle treatment, 5-aza-deoxycytidine treatment restored unmethylated DNA and/or reduced the methylation-specific PCR product (Fig. 6B). Furthermore, DNA-demethylation restored FGFR2 protein expression without significantly affecting FGFR1 expression (Fig. 6C). Moreover, histone deacetylase inhibition by trichostatin A failed to alter FGFR1 or FGFR2 expression in these cells. These findings provide evidence of epigenetic silencing through DNA methylation as a putative mechanism for FGFR2-IIIb down-regulation.

Discussion

In this study, we show divergent expression and actions of FGFR1 and FGFR2 in thyroid carcinoma cells, disclosing cancer-promoting roles of FGFR1 and cancer-suppressive properties of FGFR2. Down-regulated FGFR1 through siRNA knockdown decelerated carcinoma cell invasion and suppressed xenograft tumor growth in SCID mice. In marked contrast, reexpression of FGFR2-IIIb interrupted signaling upstream of BRAF and consequently MAPK (ERK1/2) activation in thyroid carcinoma cells and significantly reduced tumor growth.

Overexpression and/or gain-of-function mutations of FGFR1, FGFR3, and FGFR4 have been identified and implicated as oncogenes in a number of human neoplasms, including thyroid tumors (4, 7, 16). We have previously shown that transduction of a dominant-negative FGFR and pharmacologic FGFR tyrosine kinase inhibition using the PD173074 compound attenuates thyroid cancer cell proliferation (4). In the current study, we applied siRNA methodology for FGFR1-selective inhibition to clarify the oncogenic role of FGFR1 and to ask whether FGFR1 is a potential therapeutic target in thyroid carcinoma. Consistent with our hypothesis, FGFR1 silencing inhibited cell signal activation including AKT, cancer cell
growth, and invasiveness of thyroid carcinoma cells. Invasion-promoting properties of FGFR1 are supported by several other studies, involving matrix metalloproteinase (MMP) regulation and cadherin modification. FGF1 increases MMP7 expression through FGFR1 signaling in normal prostate cells, and this is inhibited by an FGFR1-specific inhibitor or through dominant-negative FGFR1 transduction (17). In mouse cells, activation of FGFR1 results in invasive growth accompanied by induction of MMP3 and MMP9, and cleavage of adhesion factors including E-cadherin (18). FGFR1 signaling enhances N-cadherin signaling and activates MMP9 gene transcription to promote cellular invasion of human breast carcinoma cells (19). Our studies also support AKT involvement in FGFR1 signaling in thyroid cancer. Indeed, AKT is frequently overexpressed in thyroid carcinomas, in which it has been implicated in disease progression and invasion (20–22). These findings assign a role for FGFR1 in mediating invasive growth, thus providing a rationale for FGFR1-selective manipulation as a potential therapeutic target for human thyroid carcinoma.

We previously identified expression of FGFR2 in normal thyroid tissue (4). In the current study, we clarified that the FGFR2-IIIb isoform is expressed. FGFR2-IIIb expression is typically restricted to epithelial cells, whereas FGFR2-IIIc is characteristic of mesenchymal lineages (12, 23). Targeted disruption of FGFR2-IIIb causes agenesis of the lungs, anterior pituitary, thyroid, teeth, and limbs (24); in contrast, FGFR2-IIIc knockout mice show severe impairment of skull and bone development (25). Our data are consistent with these patterns of expression.

The role of FGFR2 in tumorigenesis has recently gained interest. Down-regulation of FGFR2 has been noted with tumor progression in astrocytomas, bladder and prostatic carcinomas, pituitary tumors, and thyroid carcinomas (4, 5, 7, 15). Based on these observations, it is reasonable to propose a tumor-suppressive role for FGFR2. Our study clearly shows that forced FGFR2-IIIb expression significantly retards thyroid tumor progression while enhancing apoptosis (15, 26, 27). It should be noted, however, that FGFR2-IIIb down-regulation is not a universal feature in solid

Figure 4. FGFR2-IIIb restoration impedes thyroid cancer cell proliferation and retards tumor progression in mouse xenografts. A, WRO control and FGFR2-IIIb–transfected clones were subjected to MTT assays during 6 days of culture. Each curve represents an independently transfected clone (mean ± SE, 8 wells per clone). Cell growth is significantly delayed by expression of FGFR2-IIIb; *, *P < 0.05 versus pcDNA/WRO clone 1; ††, P < 0.05 versus pcDNA/WRO clone 2. B, WRO control and FGFR2-IIIb–transfected clones as in (A) were subjected to invasion and migration assays as detailed under Materials and Methods. Cell migration and invasion were significantly retarded in response to FGFR2-IIIb expression; †, *P < 0.05. C, xenograft experiments involved the injection of FGFR2-IIIb/WRO cells or control cells into the flank of SCID mice. Tumor volumes are consistently lower in xenografted FGFR2-IIIb–expressing WRO cells (n = 8) than in controls (n = 8, P = 0.04). D, tissue from mouse xenografts was examined by TUNEL staining for apoptosis assessment. Columns, mean of eight samples in each group; bars, SE.
tumors (28, 29). FGFR2 amplification was identified in gastric cancer cell lines with conspicuous absence of gain-of-function mutations (15, 30, 31). In fact, gastric carcinomas exhibit increased expression of the FGFR2-IIIb-C3 splice variant (also called Ksami-IIIC3), in which the COOH terminus is shorter than that of wild-type FGFR2-IIIb and lacks the putative PLCγ binding site (32). This C-terminally truncated FGFR2-IIIb-C3 isoform accelerates cancer cell growth and invasion (33, 34). Thus, alternate splicing of the C-terminal region of FGFR2-IIIb may clarify the controversy regarding FGFR2 expression and function in tumorigenesis.

**BRAF**, located on chromosome 7q24, encodes a serine/threonine protein kinase that transduces regulatory signals through the MAPK signaling cascade. We show that restoration of FGFR2-IIIb inhibits BRAF phosphorylation, resulting in diminished MAPK activation even in the presence of activated BRAF signaling due to point mutation. Gain-of-function **BRAF** mutations, resulting in constitutive activation of MAPK signaling, are found in approximately one third of papillary thyroid carcinomas and one third of undifferentiated thyroid carcinomas (3, 35). **BRAF** is a putative therapeutic target, and transient siRNA–mediated down-regulation of mutant **BRAF** suppresses MAPK activation and cell growth in thyroid carcinoma cell lines (36). However, nearly half of human thyroid carcinomas and several thyroid cancer cell lines (including WRO cells) are negative for **BRAF** mutations (37, 38). Suppressive signals upstream of the BRAF kinase, as shown in the present study, could represent an alternative or complementary therapeutic approach in thyroid carcinomas.

The signaling mechanisms underlying FGFR2-IIIb antitumor action are summarized in three models proposed in Fig. 5C. In one model, FGFR2-IIIb directly mediates an inhibitory signal. Radvanyi’s group suggested that FGFR2-IIIb inhibits cancer cell growth by reducing insulin-like growth factor II via its C-terminal domain, independent of its tyrosine kinase domain (33). Although one group reported that restoration of FGFR2-IIIb inhibits FRS2 activation (27), we and others found that FGFR2-IIIb can engage FRS2 activation (39). These discrepancies may be due to cell specific responses or differences of FGF profiles. In the second model, FGFR2-IIIb forms inactive heterodimers with FGFR1, abrogating the tumor-promoting functions of FGFR1. In the third model, FGFR2-IIIb and FGFR1 compete with each other for FRS2. FRS2 is up-regulated in thyroid carcinoma cells, and transduces signals for a number of kinases including FGFRs (6), RET and RET/PTC (40, 41), and NTRK1 and TRK-T1/T3 (42, 43). We note that RET and NTRK are not constitutively activated in WRO cells. We also observed stable amounts of FRS2 protein after FGFR2-IIIb reexpression. Therefore, the most plausible model is one in which FGFR2-IIIb sequesters limited amounts of FRS2 to divert signaling away from other receptor tyrosine kinases, including FGFR1 as well as the RET/RAS/BRAF/MAPK pathway, to retard tumor progression.

---

**Figure 5.** FGFR1 and FGFR2-IIIb compete with each other for FRS2 activation. A, WRO cells expressing FGFR2-IIIb or their controls (pcDNA) were treated after serum starvation with FGF1 or FGF7. As expected the FGFR2-selective FGF7 activates FRS2 only in the presence of FGFR2-IIIb. Note the diminished FRS2 response to FGF1 in cells expressing FGFR2-IIIb compared with those that do not. B, HEK293 cells were transfected with FGFR2-IIIb alone or with FGFR1. Note the robust effect of FGF7 on FRS2 activation in the presence of FGFR2-IIIb alone (left) which is markedly diminished in the concomitant presence of FGFR1 (right). C, the proposed models are based on previous observations and the current data. In model (i), FGFR2-IIIb directly mediates inhibitory signals in thyroid carcinoma cells. In model (ii), FGFR2-IIIb forms an inactive heterodimer complex with FGFR1, abrogating the tumor-promoting functions of FGFR1. In model (iii), FGFR2-IIIb recruits FRS2 and diverts signaling away from other tyrosine kinase receptors including FGFR1.
Figure 6. DNA methylation regulates FGFR2 expression in thyroid carcinoma. A, the CpG methylation status of the FGFR2 gene was analyzed in normal human thyroids and KTC-1, TPC-1, WRO, 8505C, NPA, and DRO thyroid cancer cells as indicated. After bisulfite modification of genomic DNA, methylation-specific and unmethylation-specific primers were used for MSP analysis. Complete unmethylation of FGFR2 genomic DNA was observed in seven cases of normal thyroids, whereas CpG-methylation of FGFR2 genomic DNA was detected in TPC-1, WRO, NPA, and DRO cell lines with corresponding reduction in amplification of the unmethylated product. B, MSP analysis. The DNA demethylating agent 5’-aza-deoxycytidine (Aza-dc) treatment restored the unmethylated amplicon in WRO, TPC-1, and NPA cells and reduced the methylated product in DRO cells. C, Western blotting confirms that 5’-aza-deoxycytidine treatment restored FGFR2 protein expression in WRO cells without significantly effecting FGFR1 expression. Histone deacetylase inhibition by trichostatin A treatment failed to significantly alter FGFR1 or FGFR2 expression.

Our data also point to a potential mechanism underlying the loss of expression of the FGFR2-IIIb tumor suppressor in thyroid cancer. One previous report implicated CpG methylation in the 5’ region of the human FGFR2 gene in the process of FGFR2-IIIb down-regulation in human bladder carcinoma cell lines (15). We have directly shown this mechanism of FGFR2 gene silencing in thyroid cancer cells and shown restoration of FGFR2 protein expression after treatment with the DNA demethylating agent 5’-aza-deoxycytidine. Epigenetic gene silencing of other tumor-suppressor genes, including E-cadherin, PTEN, and RASSF1A, and of differentiation-related genes, such as thyroid-stimulating hormone receptor and the sodium-iodide symporter, has been reported in thyroid cancers (3, 44). These data suggest that further studies should be pursued to determine the potential application of demethylating agents in the therapy of thyroid cancer.

In conclusion, our data show a reciprocal expression profile of FGFR1 and FGFR2 in thyroid carcinomas. FGFR1 has tumor-promoting actions, whereas FGFR2-IIIb plays a pivotal tumor-suppressive role. The growth suppressive functions are mediated upstream of the well-recognized RAS/BRAF/MAPK pathway. These findings underscore the complex network of the FGFR family of tyrosine kinases in modulating cancer cell growth and predict the need for highly selective inhibitors in the control of disease progression even in the context of distinct intragenic mutations.

Acknowledgments

Received 12/7/2006; revised 3/5/2007; accepted 3/20/2007.
Grant support: Canadian Institutes of Health Research grant MT-14404, Toronto Medical Laboratories, and Ministry of Education, Culture, Sciences, and Technology, Japan grant 16-KJ-194 (T. Kondo).
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Kelvin So for his technical assistance.

References

5. Ezzat S, Asa SL. FGFR receptor signaling at the crossroads of endocrine homeostasis and tumorigene-

42. Ranzi V, Meakin SO, Miranda C, Mondellini P, Pierrotti MA, Greco A. The signaling adapters fibroblast growth factor receptor substrate 2 and 3 are activated by the thyroid TRK oncoproteins. Endocrinology 2003;144:922–8.
Epigenetically Controlled Fibroblast Growth Factor Receptor 2 Signaling Imposes on the RAS/BRAF/Mitogen-Activated Protein Kinase Pathway to Modulate Thyroid Cancer Progression

Tetsuo Kondo, Lei Zheng, Wei Liu, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/67/11/5461

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2007/05/30/67.11.5461.DC1

Cited articles
This article cites 42 articles, 17 of which you can access for free at:
http://cancerres.aacrjournals.org/content/67/11/5461.full#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/67/11/5461.full#related-urls

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://cancerres.aacrjournals.org/content/67/11/5461.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.