Studies on Fibroblast Growth Factor Receptor 3 IIIc in Esophageal cancer

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論　文　要　旨

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線維芽細胞増殖因子(FGF)とその受容体（FGFR）は，細胞増殖，分化，遊走，生存など多彩な生理作用を制御している。FGF は，ヒトでは 22 種類の異なる遺伝子にコードされている。FGFR は 4 種類の異なる遺伝子にコードされている（FGFR1～FGFR4)。FGFR は細胞外に 3 個の免疫グロブリン様ドメイン(Ig)，細胞膜貫通部位，細胞内にチロシンキナーゼドメインを持つ。FGF が FGFR の 2 番目と 3 番目の Ig に結合すると，FGFR の自己リン酸化が誘導され細胞内にシグナルが伝達される。FGFR1 から FGFR3 の FGFR は，３番目の Ig は異なったエクソン IIIb と IIIc にコードされており，組織特異的な選択的スプライシングにより IIIb アイソフォームまたは IIIc アイソフォーム を生じる。IIIb アイソフォームは主に上皮系細胞に発現し，IIIc アイソフォームは主に間葉系細胞に発現している。

さまざまながんにおいて線維芽細胞増殖因子(FGF)とその受容体（FGFR）の変異，発現異常はがん悪性化に関与していることが報告されている。脳腫瘍において FGFR1 の N546K(546 番目のアスパラギンからリシン)または K656E(656 番目のリシンからグルタミン酸)の変異によりキナーゼ活性が亢進することが知られている。また，膵臓がんにおいて FGFR2IIIc の発現上昇が，がん細胞の細胞増殖を亢進することが報告されている。FGFR3 の変異や発現上昇は主に多発性骨髄腫，大腸がん，膀胱がんの悪性化に関与していることが報告されており，様々ながんにおいて FGFR3 は分子標的薬のターゲット分子として期待されている。日本における食道がん患者の 90%以上は扁平上皮がんである。食道がんの生存率は他の消化器系がん（胃がん : 約 60%，大腸がん : 約 70%）と比較して 30%と低く，効果的な治療薬の開発が必要である。

本論文において，未だ報告されていない食道がん患者のがん組織における FGFR 選択的スプライシングアイソフォームの発現異常について検証した。食道がん患者の非がん性粘膜とがん部位の凍結試料を用いて RT-PCR 法により FGFR の mRNA 発現頻度を比較した。その結果，FGFR3IIIc 以外の FGFR アイソフォーム(FGFR1IIIb，FGFR1IIIc，FGFR2IIIb，FGFR2IIIc および FGFR4)の発現頻度は非がん性粘膜とがん部位とで差は認められなかったが、FGFR3IIIc は，非がん性粘膜においてその mRNA 発現頻度が 38% (6 例/16 例)に対してがん部位で 75%（12 例/16 例）を示し，がん部位は非がん性粘膜と比較して mRNA 発現頻度が 2 倍上昇していた。一方、FGFR3IIIb の mRNA 発現頻度は非がん性粘膜で 69% (11 例/16 例)，がん部位で 75%(12 例/16 例)を示し，がん部位と非がん性粘膜における FGFR3IIIb の mRNA 発現頻度はほぼ同程度を示した。食道がんにおける FGFR3IIIc 発現頻度の上昇が他の消化器系がんにおいても生じるかどうか検証するため，大腸がん（18 例）および胃がん患者（15 例）の凍結試料を用いて FGFR3IIIc 発現について同様に検証した。しかし，大腸がんおよび胃がんのがん部位において，非がん性粘膜と比較して FGFR3IIIc 発現頻度の上昇は認められなかったことから，消化器系がんの中で食道がんのみにおいて FGFR3IIIc の発現頻度が上昇していることが示唆された。大腸がんにおいてがん部位における FGFR3IIIb の発現頻度（11 例/18 例, 61%）が非がん性粘膜（5 例/18 例, 28%）と比較して上昇していたことから，FGFR3IIIb の発現は大腸がんの悪性化に関与している可能性が示唆された。

次に，食道がん患者由来のがん組織パラフィン切片を用いて FGFR3IIIc 特異的抗体による免疫染色法で，FGFR3IIIc の発現を検討した。その結果，stage0 のがん組織において SCC-112（扁平上皮がん細胞マーカー）陽性細胞に FGFR3IIIc の発現が認められたことから，がん細胞に FGFR3IIIc が発現していることが明らかになった。また，stageIA, stageIB, stageIIB および stageIIIA のがん組織においてもがん細胞特異的に FGFR3IIIc の発現が認められた。さらに，FGFR3IIIc 発現細胞は細胞増殖のマーカーである Ki67 陽性であったことから，FGFR3IIIc は細胞増殖能を亢進することにより，がんの悪性化を促進している可能性が示唆された。

FGFR3IIIc 発現が食道がんの細胞増殖能の亢進に貢献している可能性について，食道がん細胞株 EC-GI-10 を用いた強制発現実験により検証した。FGFR3 の非翻訳領域を標的とした siRNA を用いて EC-GI-10 細胞の内在性 FGFR3 をノックダウンすることにより (siFGFR3 細胞), sicontrol 処置群と比較して細胞増殖能が約 25%抑制されたことから，食道がんの細胞増殖能に FGFR3 が関与している可能性が示唆された。また，siFGFR3 細胞に FGFR3IIIb (FGFR3IIIb 細胞)または FGFR3IIIc (FGFR3IIIc 細胞)を強制発現させ，それぞれのアイソフォームの細胞増殖能の亢進作用について検討した。FGFR3IIIc 細胞の細胞増殖能は、siFGFR3 細胞と比較して有意に亢進したが（約 1.4 倍），FGFR3IIIb 細胞の細胞増殖能の亢進は認められなかった。食道がん患者のがん部位および EC-GI-10 細胞で FGFR3IIIc 特異的なリガンドである FGF2 の mRNA 発現が確認されたことから，FGFR3IIIc 細胞の細胞増殖能の亢進は FGF2 などの FGFR3IIIc 特異的に結合する FGF のオートクライン効果である可能性が示唆された。さらに，FGFR キナーゼ選択的阻害剤である AZD4547 により，FGFR3IIIc 細胞の細胞増殖能が抑制されたことから，食道がんにおける FGFR3IIIc 発現による細胞増殖能の亢進は FGFR3IIIc シグナルを介していることが予想された。

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1. General Introduction

The mitogenic polypeptides, or fibroblast growth factors (FGFs), were first discovered in the 1970s and are essential for development and maintenance of life processes in multicellular organisms. Since their discovery, at least 22 FGFs have been identified as heparin-binding factors and shown to constitute one of the largest families of growth and differentiation factors. FGFs play a number of biological roles, including mediating stimulation of cell proliferation and differentiation in diverse. In addition to their abilities to stimulate proliferation of a wide variety of cells, FGFs exhibit potent neurotrophic and angiogenic activities in mammalian cells [1-4].

The biological effects of FGFs are mediated by the cell surface receptor molecules that span the plasma membrane. FGF receptors (FGFRs), which were encoded by four genes (FGFR1, FGFR2, FGFR3, and FGFR4), have receptor tyrosine kinases (RTKs) in their intracellular domains and generate an intracellular signal when FGFs bind to the extracellular domain. FGFR consists of a single polypeptide chain with extracellular, transmembrane and intracellular domains. The extracellular ligand-binding domain consists of three Ig-like domains [4-8]. Between the first and second Ig-like domains, there is a stretch of acid amino acids, referred to as the 'acid box' [4-6]. On the other hand, the catalytic domain, a tyrosine specific kinase, is contained in the cytoplasmic, carboxy-terminal portion of the receptor. Furthermore, the intracellular domain is divided into the two subdomains of tyrosine kinase, the ATP binding and catalytic domains, by a short intervening sequence (Kinase insertion). FGF-binding causes the FGFRs to assemble into dimmers, which enables the two receptor cytoplasmic domains to cross-phosphorylate each other on multiple tyrosine residues. The 'autophosphorylated' tyrosines on the intracellular domain of FGFRs serve as high affinity binding sites for a number of intracellular signaling proteins in the target cells [7]. Thus, tyrosine autophosphorylation serves as a switch to trigger the transient assembly of an intracellular signaling complex, which serves to relay the signal into the cell interior, and eventually to the nucleus.

An important characteristic of FGFRs is that their molecular diversity occurs by alternative splicing of their transcripts. Mainly, two alternatively spliced isoforms of FGFR1, FGFR2, and FGFR3, but not FGFR4, possess an alternative sequence for the C-terminal half of the third Ig domain (IgIII), encoded by a separate 5'-exon IIIa and 3'-exon, either IIIb or IIIc, which determines FGF

specificity [9-11]. The FGFRIIIb isoform is predominantly expressed in epithelial and the FGFRIIIc isoform is predominantly expressed in mesenchymal, with their corresponding ligands only activating either the epithelial or mesenchymal isoforms [8, 12, 13]. The expression of FGFR isoforms is temporal and spatially regulated in embryos as well as in normal adult organs.

In cancer, the overexpression or amplification of FGFR, switching between alternatively spliced isoforms, or mutation of FGFR isoforms has been reported to trigger carcinogenesis of cancer or malignant progression [14]. The overexpression or amplification of FGFR has been found in numerous human cancers such as cancer of the brain, head and neck, lung, breast, stomach, prostate and in sarcomas and multiple myeloma [14]. Amplification of the chromosomal region 8p11-12, where FGFR1 is located, appears in about 10% of breast cancers, and is associated with poor prognosis [14-17]. In prostate cancer, it has been reported that FGFR1 is expressed in approximately 20% of moderately differentiated cancers and 40% of poorly differentiated cancers, but was not expressed in well-differentiated cancers and prostatic luminal epithelial cells, which are normal prostatic cells. These suggest that there is increasing expression of FGFR1 with transformation and progressive loss of differentiation [18]. Moreover, deactivation of FGFR1 in early cancer progression led to regression. On the other hands, inhibition of FGFR1 in late cancer progression reduced cell proliferation and progression of adenocarcinoma, but not lead to regression, indicating that FGFR1 is necessary for both maintenance and progression of prostatic intraepithelial neoplasias [14]. About 15-20% of multiple myeloma patients harbor a chromosomal translocation, t(4;14), which brings FGFR under the influence of a strong IgH enhancer region leading to FGFR3 overexpression [19-21]. The overexpressing FGFR3 correlates with shorter overall survival [20]. FGFR4 was expressed in RMS and it has been reported that the expression correlates with advanced stage and poor survival [14].

The switching between alternatively spliced isoforms leads to autocrine signaling FGFR signaling by altered FGF-binding capacity. The switching from FGFR2IIIb to FGFR2IIIc has been reported in rat models of prostate and bladder cancer [22-24], resulting that FGFR2IIIc was activated by autocrine pathway. Activated FGFR2IIIc disturbed the signaling between the epithelial and mesenchymal cells, leading to epithelial-mesenchymal transition (EMT) [14]. Enhanced expression of FGFR2IIIc in pancreatic cancer induced cell proliferation without exogenous FGFs [25]. The cell proliferation was suppressed

by a neutralizing antibody against FGFR2IIIc or siRNA against FGFR2IIIc mRNA, indicating that the activation of FGFR2IIIc contributes to the malignant progression of pancreatic cancer [25].

Many mutations of FGFRs have been found in human cancers [14]. Activating mutations in FGFR1 has been identified in glioblastoma with N546K (an Asn to Lys replacement at position 546) or K656E (a Lys to Glu replacement at position 656), enhancing kinase activity [13]. Two mutations in FGFR2 have been identified in endometrial cancer cell lines with N549K (an Asn to Lys replacement at position 549) or K659N (a Lys to Asn replacement at position 659). The cell proliferation and survival in these cell lines were blocked with an FGFR inhibitor (PD173074) or knockdown of FGFR2, suggesting that activated FGFR2 plays an important role in endometrial carcinogenesis [26, 27]. Somatic activating mutations in FGFR3 have been identified in bladder cancer with S249C (a Ser to Cys replacement at position 249) and Y373C (a Tyr to Cys replacement at position 373). The mutations in the extracellular domain generating an additional cysteine residue lead to the formation of an intermolecular cysteine disulfide bridge, which results in constitutive receptor dimerization and activation, resulting that the mutated FGFR induces cell proliferation without FGFs [14, 28]. Many mutations in FGFR4 were identified in about 7-8% of rhabdomyosarcoma (RMS) [29]. Two mutation, N535K (an Asn to Lys replacement at position 535) or V550E (a Val to Glu replacement at position 550), promotes auto phosphorylation of FGFR4, increasing invasiveness, metastasis and poor survival [29].

Several pharmaceutical companies have developed FGFR tyrosine kinase inhibitors for cancer therapy. Initially, multi-kinase inhibitors such as brivanib, nindtanib, cediranib, lenvatinib, sulfatinib, dovitinib, ponatinib, pazopanib, regorafenib, and lucitanib were developed to compete for the adenosine triphosphate (ATP)-binding site and reduce the kinase activity in FGFR, VEGFR, PDGFR and so on [30, 31]. Already, pazopanib (for advanced renal cell carcinoma and soft tissue sarcoma), ponatinib (for chronic myelogenous leukemia and acute lymphoblastic leukemia), regorafenib (for advanced gastrointestinal stroma tumor and colorectal cancer), and lenvatib (for thyroid cancer) were FDA-approved for use in human cancer [31]. FGFR-specific inhibitors such as AZD4547, NVP-BGJ398, LY2874455, JNJ-42756493, Debio1347 and TAS120 have high affinity for FGFR compared with multi-kinase inhibitors [30]. AZD4547 (for FGFR1 or FGFR2-amplified cancers containing

squamous non-small-cell lung cancer, gastric cancer, esophageal cancer, breast cancer) and NVP-BGJ398 (for FGFR amplified advanced cancer containing melanoma, cholangiocarcinoma, glioblastoma) are evaluated in phase II clinical trials, and the other compounds are evaluated in phase I clinical trials [32-34]. MGFR1877S and FGF401 are FGFR isoform-specific inhibitors. MGFR1877S, human monoclonal antibody for FGFR3, is evaluated in phase I clinical trials for advanced urothelial carcinoma, and FGF401, a highly selective and potent FGFR4 inhibitor, is evaluated in phase I clinical trials for FGF19-FGFR4-βklotho overexpressed carcinomas. Thus, FGFR is a molecular target for cancer therapy [30].

In Japanese cancer death, 2nd rank is colorectal cancer containing colon and rectum, and 3rd rank is gastric cancer [35]. The 5-year relative survival rates for colorectal cancer patients are approximately 70% and for gastric cancer patients are approximately 60%. On the other hands, the 5-year relative survival rates for esophageal cancer patients are approximately 30%, suggesting that the esophageal cancer is poor prognosis compared with gastric and colorectal cancer [35].

Risk factors of the esophageal carcinoma rely on tobacco, alcohol, achalasia, caustic injury to the esophagus and so on [36]. Esophageal carcinoma is well known as an invasive and progressive cancer and being easy to lead lymph node metastasis, resulting that esophageal carcinoma patients show poor prognosis. Therefore, it is important to find a beneficial molecular target for therapy of esophageal carcinoma.

In the present study, I found the esophageal carcinoma cells in the early stage patients expressed FGFR3IIIc isoform by RT-PCR and immunohistochemical analysis. FGFR3IIIc expression promoted the cell proliferation, probably creating autocrine FGF signaling by expressing FGFR3IIIc-specific FGFs such as FGF2. These results suggest that FGFR3IIIc may have the potential to be an early stage tumor marker and a molecular target for ESCC therapy.

2. Abbreviations

FGF, Fibroblast growth factor
FGFR, Fibroblast growth factor receptor
RTK, Receptor tyrosine kinase
MM, Multiple myeloma
AZD4547, N-[5-[2-(3,5-Dimethoxyphenyl)ethyl]-2H-pyrazol-3-yl]-4-(3,5-diemthylpiperazin-1-yl)benzamide
NVP-BGJ398, 3-(2,6-dichloro-3,5-dimethoxyphenyl)-1-(6-((4-(4-ethylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)-1-methylurea
EC, Esophageal carcinoma
NCM, Non-cancerous mucosa
ESCC, Esophageal squamous cell carcinoma
MAPK, Mitogen activated protein kinase
PI3K, Posphoinositide 3-kinase
PLC-γ, Phospholipase C gamma
PKC, Protein kinase C
RT-PCR, Revese transcription-polymerase chain reaction
RNA, Ribonucleic acid
BSA, Bovine serum albumin
PBS, Phosphate buffered saline
DMEM, Dulbecco's Modified Eagle medium
STR-PCR, Short tandem repeat- polymerase chain reaction
siRNA, Short-interfering RNA
3'-UTR, Three prime untranslated region
DAPI, 4', 6-diamidino-2-phenylindole
EAC, Esophageal adenocarcinoma
SCCA, Squamous cell carcinoma-related antigen
CEA, Carcinoembryonic antigen
PD173074, *N*-[2-[[4-(Diethylamino)butyl]amino]-6-(3,5-dimethoxyphenyl)pyrido[2,3-*d*]pyrimidin-7-yl]-*N*'-(1,1-dimethylethyl)urea
GAPDH, Glyceraldehyde-3-phosphate dehydrogenase
EMT, Epithelial-mesenchymal transition

3. Introduction

As described in General introduction, the human genome contains 22 genes that code for fibroblast growth factors (FGFs). FGFs induce cell proliferation, differentiation and migration by binding to specific receptors [37-39]. Fibroblast growth factor receptors (FGFRs) are encoded by four genes. The consensus structure of FGFRs consists of three Ig-like domains in the extracellular region, a single spanning transmembrane domain, and a split tyrosine kinase domain in the cytoplasmic region (Fig. 1A) [8]. When FGF binds to FGFRs, the tyrosine kinase in the cytoplasmic region of the receptors is activated and generates signals through, for example the Ras-MAPK, PI3K-Akt and PLC-γ-PKC pathway to induce cell proliferation, differentiation, migration and oncogenesis [40]. FGFR3 in particular is known to harbor oncogenic activity in several types of cancers. Malignant progression by the enhanced expression or variations in FGFR3 has been reported for bladder cancer, colon cancer and multiple myeloma [13, 14, 20, 41, 42]. In recent studies, the cell proliferation in these cancers was shown to be suppressed by a neutralizing antibody against FGFR3 or a tyrosine kinase inhibitor of FGFR3, indicating that the activation of FGFR3 contributes to the malignant progression of cancers [20, 43, 44].

In Japan, approximately 90% of esophageal carcinoma (EC) is esophageal squamous cell carcinoma (ESCC) [45], and the overall death rate in the general population of esophageal cancer patients has been reported to be 15.7 per 100,000 for men and a 2.6 per 100,000 for women. The prognosis of patients with EC is known to be poor, with 5-year relative survival rates remaining at approximately 30%. This rate is lower than those of patients with other gastrointestinal cancers containing stomach, colon and rectum [35, 46].

Two alternatively spliced isoforms of FGFR3 possess an alternative sequence for the C-terminal half of the third Ig domain (IgIII), encoded by a separate 5'-exon IIIa and 3'-exon, either IIIb or IIIc (Fig. 1A, Fig. 1B), which determines FGF specificity. FGFR3IIIc has been shown to have a broader ligand spectrum (FGF1, 2, 4, 5, 6, 8, 9, 16, 17, 18, 19, 20, 21, and 23) than that of FGFR3IIIb (FGF1, 9, 16, and 20) [9-11] (Fig. S1).

The expression of FGFR isoforms is temporally and spatially regulated in embryos as well as in normal adult organs. The expression of FGFR3IIIb has been associated with an epithelial lineage, while FGFR3IIIc was shown to be predominantly expressed in nonepithelial cells and tissues [47]. Ligands are produced in either epithelial or mesenchymal tissue and generally activate

receptors of the opposite tissue specificity. Pathological states can result from a breakdown in binding specificity, as is common in cancer in which FGFs are overexpressed [12]. For example, in colorectal cancer, FGFR3IIIc expression was upregulated with increasing stage, whereas FGFR3IIIb expression was downregulated with increasing stage, resulting that a ratio of IIIc/IIIb expression was increased with higher stages [42]. FGF18, which is a specific ligand to FGFR3IIIc, was also upregulated in colorectal cancer [48], resulting that FGFR3IIIc exerts cell proliferation and migration by mediating FGF18 effects in colorectal cancer [42]. In esophageal cancer, a previous study demonstrated that the expression of FGF2, a specific ligand for FGFR3IIIc, was upregulated in the patients who had a poor prognosis [49].

In this study, the gene expression of FGFR1IIIb, FGFR1IIIc, FGFR2IIIb, FGFR2IIIc, FGFR3IIIb, FGFR3IIIc, and FGFR4 was assessed in esophageal carcinoma (EC) and neighbouring non-cancerous mucosa (NCM) by RT-PCR analysis. It was found that the incidence of FGFR3IIIc expression in EC was 2-fold higher than in NCM. On the other hand, no difference was observed in the incidence of other FGFRs containing FGFR3IIIb expression between EC and NCM. Moreover, the incidence of FGFR3IIIc expression in colorectal cancer and gastric cancer was not different between tumor and NCM, either. However, in colorectal cancer, but not gastric cancer, the incidence of FGFR3IIIb expression was about 2-fold higher in tumor than that in NCM.

To further investigate whether the increased expression of FGFR3IIIc is indeed in the esophageal cancer cells but not in mesenchymal cells, an immunohistochemical analysis in consecutive sections from esophageal carcinoma specimens was performed. As a result, the cells expressing SCC-112, a tumor marker, were positively stained by FGFR3IIIc in the early stage of ESCC, as well as Ki67, a cell proliferation marker, colocalized. These results suggest that the enhanced expression of FGFR3IIIc in ESCC may promote cell proliferation.

To examine whether the enhanced expression of FGFR3IIIc gives the advantage for esophageal cancer cell proliferation, EC-GI-10 cells, an esophageal carcinoma cell line was used for an *in vitro* proliferation analysis. The knockdown of endogenous FGFR3 in, EC-GI-10 cells by siRNA significantly reduced cell proliferation. Interestingly, the overexpression of FGFR3IIIc in the cells by lentiviral infection enhanced cell proliferation, whereas that of FGFR3IIIb did not. The enhanced cell proliferation induced by FGFR3IIIc expression was

significantly suppressed by AZD4547, a FGFR kinase inhibitor, suggesting that the upregulation of FGFR3IIIc promoted cell proliferation. RT-PCR analysis showed that EC-GI-10 cells, ESCC and NCM obtained from ESCC patients expressed FGF2. Therefore, this study reports in the first time that the enhanced expression of FGFR3IIIc in esophageal squamous cell carcinoma (ESCC) expressing such as FGF2 as endogenous FGF may create autocrine signaling pathway to stimulate their own proliferation. These findings provide clear evidence for the importance of FGFR3IIIc as an early stage-tumor marker as well as a molecular target for ESCC therapy.

4. Materials and Methods

4-1 Tissue specimens

Fresh samples of EC (16 patients), colorectal cancer (18 patients), gastric cancer (15 patients) and NCM for RT-PCR analysis were obtained from patients undergoing surgery for these cancers at Kitano Hospital in Osaka, Japan, after obtaining informed consent from each patient enrolled in the study. The 16 EC patients contained 14 ESCC patients including 1 patient diagnosed as spindle cell carcinoma, and 2 esophageal adenocarcinoma patients (EAC). ESCC specimens of the all stages were obtained (Table 1, Table 2). Formalin-fixed and paraffin-embedded tissue sections of ESCC for immunohistochemical analysis were retrieved from the pathology files of Shinshu University Hospital in Matsumoto, Japan (Table 3). The study plan including the use of human samples was approved by the Ethics Committees of Kitano Hospital, Shinshu University School of Medicine, and Kyoto Sangyo University. Moreover, it was conducted in full conformity with the current revision of the Declaration of Helsinki.

4-2 RNA extraction

Total RNA was extracted from tissue specimens and human cancer cell lines using ISOGEN (Nippon gene, Tokyo, Japan) according to the manufacturer's protocol.

4-3 RT-PCR analysis

First strand cDNA was synthesized using the Superscript First-strand Synthesis System for RT-PCR (Invitrogen, cat#11904-018, Carlsbad, CA, USA) according to the manufacturer's protocol. PCR for the expression of GAPDH, FGFRs and FGF2 in EC, colorectal cancer, gastric cancer, and neighboring NCM from patients and EC-GI-10 cells was performed by TaqDNA polymerase (Promega, Fitchburg, WI, USA) or Gotaq® Green Master Mix (Promega). PCR was performed in 25 μL of reaction mixture containing cDNA, 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 9.0, 0.1% Triton X-100, 200 μmol/L each dNTP, 0.025 units/μL of TaqDNA polymerase (Promega, Fitchburg, WI, USA) and 0.5 pmol/μL each of the forward and reverse sequence-specific primers. The reaction was carried out for 30 cycles of 1 min at 94 °C, 2.5 min at 60 °C, 3.5 min at 72 °C, with an extra 7-min extension at 72 °C for the last cycle (RoboCycler Gradient96, Agilent Technologies, Santa Clara, CA, USA). The final Mg^{2+}

concentrations for PCR of GAPDH, FGFR2IIIb, and FGFR2IIIc were 1 mmol/L and, FGFR4 was 1.75 mmol/L, and FGFR1IIIb, FGFR1IIIc, FGFR3IIIb, FGFR3IIIc, and FGF2 were 2.5 mmol/L. Aliquots of the PCR products were electrophoresed on 2 or 3.5% agarose gels in Tris acetate EDTA buffer, pH 8.0, and stained with 0.5 μg/mL ethidium bromide. The amount of cDNA for FGFRs expression was normalized by GAPDH expression. The primer sequences and the expected size of PCR products by using these primer pairs are summarized in Table 4.

4-4 Antigen adsorption

A recombinant human FGFR3IIIc Fc chimera (R&D systems, cat#766-FR-050, lot.CWZ0713081, Minneapolis, MN, USA), which contains the extracellular region of FGFR3IIIc and Fc region of human IgG_1, was incubated with a monoclonal anti-FGFR3IIIc antibody (R&D systems, cat#MAB7662, lot.GHK0411041, Minneapolis, MN, USA) in PBS containing 0.1% Tween 20, 3% BSA without γ-globulin (Wako, Kyoto, Japan), and 3% horse serum for 1 h at room temperature, then the solution was used for western blot analysis and immunohistochemistry as an antigen-absorbed primary antibody. The concentration of the recombinant human FGFR3IIIc Fc chimera was 10-fold higher than that of the anti-FGFR3IIIc antibody.

4-5 Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue sections from esophageal squamous cell carcinoma patients were deparaffinized and rehydrated in grade alcohols. Tissue sections were incubated with 10 mM citrate buffer, pH6.0, at 95˚C for 20 min for epitope retrieval. The sections were blocked with PBS containing 0.1% Tween 20, 3% BSA without γ-globulin (Wako, Kyoto, Japan), and 3% horse serum for 30 min at room temperature to eliminate non-specific binding. In immunoperoxidase staining, the sections were treated by 3% hydrogen peroxide for 12 min at room temperature before blocking. The sections were incubated with a monoclonal anti-FGFR3IIIc antibody (R&D systems, cat#MAB7662, lot.GHK0411041, Minneapolis, MN, USA, 1:25 dilution), rabbit anti-SCC-112 antibody (Bethyl Laboratories, Inc., cat#A3000-088A, lot.A300-088A-1, Montgomery, TX, USA, 1:100 dilution) or/and monoclonal anti-Ki67 antibody (Agilent Technologies, cat#M7240, lot.00040373, Santa Clara, CA, USA, 1:100 dilution) at 4 °C overnight. The sections were incubated with

biotinylated anti-mouse IgG (VECTOR LABORATORIES, Burlingame, CA, USA, 1:50 dilution) or biotinylated anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA, 1:50 dilution) for 1 h at room temperature. The sections were incubated with an Alexa Fluor 488-conjugated secondary antibody and Alexa Fluor 594-conjugated streptavidin (Life Technologies, Inc., DriveRockville, MD, USA, 1:100 dilution) for 1 h at room temperature. The nucleus was stained by 4', 6-diamidino-2-phenylindole (DAPI, Invitrogen).

Regarding immunoperoxidase staining, sections were stained by using the VECTASTAIN Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, cat#PK-6100) and Peroxidase substrate kit DAB (Vector Laboratories Inc., Burlingame, CA, cat#PK-4100) according to the manufacturer's protocol. Sections were counterstained by Mayer's hematoxylin solution (Wako, Osaka, Japan). Microphotographs were taken with ECLIPSE E800 (Nikon, Tokyo, Japan) or DM5500 Q (Leica Microsystems, Wetzlar, Germany).

4-6 Cell lines

The EC-GI-10 esophageal squamous carcinoma cell line, which was established from Japanese male (65-year old, ESCC patient), was obtained from the RIKEN cell bank (Tsukuba, Japan) and cultured in Dulbecco's Modified Eagle medium (NISSUI, Tokyo, Japan) containing 10% fetal bovine serum (GE Healthcare, Little Chalfont, UK), 4.5 g/L glucose, 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin [50]. The authenticity of EC-GI-10 cells used in this study was validated by STR-PCR method by RIKEN cell bank, where the cells were tested for mycoplasma by DNA staining method and PCR method and validated as negative. I also sent the cells to the ICLAS Monitoring Center (Kawasaki, Japan) to test mycoplasma infection by PCR method and validated as negative. HEK293T cells were purchased from ATCC (Manassas, Va, USA) and cultured in DMEM supplemented with 10% FBS. BaF3 cells are a pro-B cell line that is IL-3-dependent. BaF3 cells were cultured in RPMI1640 containing 10% FBS and 10% conditioned medium from WEHI-3 cells, which contained IL-3. DJM-1 cells (skin squamous cell carcinoma), Caco2 cells (colorectal adenocarcinoma), and T3M1 (oral squamous cell carcinoma) were cultured in Dulbecco's Modified Eagle medium (NISSUI, Tokyo, Japan) containing 10% fetal bovine serum (GE Healthcare, Little Chalfont, UK), 4.5 g/L glucose, 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin.

4-7 Lentivirus Construction and Induction

Human FGFR3IIIb and FGFR3IIIc cDNAs were amplified by PCR using FGFR3IIIb or FGFR3IIIc specific primers and subcloned into the pHAGE lentiviral backbone vector at the NotI/BamHI sites. FGFR3 primer pairs were 5'-GGGGATCCGGCCCTTCACGTCCGCGAGCCCC-3' and 5'-GGGCGGCCGCGCCGCCATGGGCGCCCCTGCCTGCGCC-3'. Each FGFR3IIIb and FGFR3IIIc in the pHAGE lentiviral back bone vector was co-transfected with the helper plasmids (tat, rev, gag-pol and VSV-G) to HEK293 cells as described previously [51-53]. Viral supernatants were assembled and concentrated at 38,000 x g for 1.5 h at 4 ˚C. The virus was infected with 10 μg/mL of polybrene (Millipore, St Louis) to express FGFR3IIIb or FGFR3IIIc in EC-GI-10 cells or HEK293T cells.

4-8 Design of siRNAs

I designed the original siRNA sequences which targeted the three prime untranslated region (3'-UTR) in human FGFR3 mRNA sequences (FGFR3 siRNA) to knock down the endogenous FGFR3 in EC-GI-10 cells, since the lentivirus vector encoding FGFR3IIIb or FGFR3IIIc do not include 3'-UTR. The siRNA sequenses was designed by the Whitehead siRNA Selection Web Server [54]. I validated that the siRNA knocked down the endogenous FGFR3 in EC-GI-10 cells, but not lentivirus expressed FGFR3IIIb or FGFR3IIIc (Fig. 11A). I ordered the siRNA synthesis to Japan Bio Services Co.,LTD. (Saitama, Japn). The FGFR3 siRNA pairs were 5'-GAUGCUGUGUAUAUGGUAUTT-3' and 5'-AUACCAUAUACACAGCAUCTT-3'. The sicontrol pairs were 5'-UGGUUUACAUGUCGACUAATT-3' and 5'-UUAGUCGACAUGUAAACCATT-3'.

4-9 Cell proliferation assay

EC-GI-10 cells were seeded on a 6 cm dish (3 x 10^5 cells/4 mL) or 10 cm dish (9 x 10^5 cells/10 mL). Three nM of FGFR3 siRNA or control siRNA (sicontrol) were transfected by siLentFect™ Lipid (BIO-RAD, Hercules, CA, USA) according to the manufacturer's protocols. After 24 h, these cells were infected with a lentivirus encoding either FGFR3IIIb or FGFR3IIIc with 10 μg/mL of polybrene (Millipore, St Louis). After 24 h, these cells were plated onto a well (6 x 10^3 cells/500 μL) in a 24 well plate with DMEM containing 1% fetal bovine serum (FBS), 4.5 g/L glucose, 2 mM L-glutamine, 50 U/mL penicillin, and 50

μg/mL streptomycin. After 3 h, AZD4547 (final concentration: 30 and 100 nmol/L) was added. After a 6-day culture, the cells were collected by trypsinization and then the number of cells was counted with a Coulter Counter ZM type (Beckman Coulter Inc., Brea, CA, USA).

4-10 Immunocytochemistry

HEK293T cells (3 x 10^5 cells/4 mL) were seeded on a 6 cm dish. After 24 h, these cells were infected by a lentivirus encoding either FGFR3IIIb or FGFR3IIIc with 10 μg/mL of polybrene (Millipore, St Louis). After 24 h, these cells were plated onto a well (5 x 10^4 cells/100 μL) in a 96 well plate coated by 2.7 μg/mL of poly-D-lysine hydrobromide (Sigma-Aldrich, St. Luis, MO). EC-GI-10 cells were plated onto a well (2 x 10^5 cells/2 mL) in a 6 well plate. After 24 h, these cells were fixed by 4% paraformaldehyde phosphate buffer solution (Wako, Kyoto, Japan) for 20 min, and were blocked with PBS containing 0.1% Tween 20, 3% BSA without γ-globulin (Wako, Kyoto, Japan), and 3% horse serum for 30 min at room temperature. The cells were incubated with a monoclonal anti-FGFR3IIIc antibody (R&D systems, cat#MAB7662, lot.GHK0411041, Minneapolis, MN, USA, 1:50 dilution) or rabbit anti-SCC-112 antibody (Bethyl Laboratories, Inc., cat#A3000-088A, lot.A300-088A-1, Montgomery, TX, USA, 1:100 dilution) at 4°C overnight. EC-GI-10 cells were treated with 0.2% TritonX-100 for 5 min at room temperature before blocking. The sections were incubated with an Alexa Fluor 594-conjugated secondary antibody (Life Technologies, Inc., DriveRockville, MD, USA, 1:100 dilution) for 1 h at room temperature. The nucleus was stained by 4', 6-diamidino-2-phenylindole (DAPI, Invitrogen) in 0.2% TritonX-100. Microphotographs were taken with ECLIPSE Ti-U (Nikon, Tokyo, Japan).

4-11 Western blot analysis

EC-GI-10 cells were lysed with a cold RIPA buffer containing 50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 100 mM NaCl and 1% protease inhibitor (Nacalai Tesque, Kyoto, Japan). The cell lysates were electrophoresed through 7.5% polyacrylamide gels and transfered to PVDF membranes (Immobilon-P, Millipore Co., Billerica, MA, USA), and were analyzed by immunoblotting with the monoclonal anti-β-actin antibody (Sigma-Aldrich, St. Luis, MO, 1:2000 dilution), anti-FGFR3 antibody (C-15) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA, 1:2000

dilution) and monoclonal anti-FGFR3IIIc antibody (R&D systems, cat#MAB7662, lot.GHK411041, Minneapolis, MN, USA, 1:500 dilution). After incubation with primary antibodies overnight at 4˚C, the membrane was incubated with peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, Suffolk, UK, cat#715-035-150, lot. 120343, 1:5000 dilution) or peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, Suffolk, UK, cat#711-035-152, lot. 117895, 1:5000 dilution) for 1 h at room temperature. The blots were treated with chemiluminescent substrate solution (Thermo Fisher Scientific, cat#34080, Waltham, MA, USA) according to the manufacturer's protocol and exposed to LAS-4000 mini (Fujifilm Co., Tokyo, Japan) to reveal immunoreactive bands.

4-12 Statistical analysis

Data were presented as means ± S.D. or means ± S.E. One-way analysis of variance (ANOVA) followed by post-hoc testing was performed. Data were analyzed using Excel 2008 with the t test. Fisher exact test was carried out using the JMP Statistical Discovery Software version 9.0 (SAS Institute Inc., Cary, NC, USA). Statistical significance was set at $p<0.05$.

5. Results

5-1 Enhanced expression of FGFR3IIIc in human esophageal carcinoma (EC), and enhanced expression of FGFR3IIIb in human colorectal cancer

The gene expression of FGFR3IIIb and FGFR3IIIc was determined by RT-PCR. Fig. S2A shows the gene expression of FGFR3IIIb and FGFR3IIIc in DJM-1 cells (skin squamous cell carcinoma), EC-GI-10 cells (esophageal squamous cell carcinoma), Caco2 cells (colorectal adenocarcinoma), and T3M1 (oral squamous cell carcinoma). FGFR3IIIb was expressed in all human cancer cell lines (blue square). FGFR3IIIc was expressed in EC-GI-10 cells and Caco2 cells (red square). Fig. S2B and Fig. 2 show the expression of FGFR3IIIb and FGFR3IIIc in 16 ESCC specimens (E1-E16). The gene expression of FGFR3IIIc was confirmed in 38% of all NCM (6/16) and 75% of all EC (12/16, p = 0.073) including ESCC (11/14, 79%) and EAC (1/2, 50%), respectively (Table 5). The incidence of FGFR3IIIc expression was 2-fold higher in EC than that in NCM. However, no difference was observed in the incidence of FGFR3IIIb expression between EC (12/16, 75%, p = 1.000) and NCM (11/16, 69%) (Table 5). Although the expression of the other FGFRs (FGFR1, FGFR2, and FGFR4) was analyzed in both NCM (N) and ESCC (T), the incidence of FGFR1IIIb, FGFR1IIIc, FGFR2IIIb, FGFR2IIIc, and FGFR4 expression in EC was not higher than that in NCM (Fig. 3, Table 6).

In colorectal cancer and gastric cancer as the other digestive cancers, an RT-PCR analysis was performed to determine whether the incidence of FGFR3IIIc expression was higher in tumor than in NCM. Fig.4 and Fig. 5 shows the gene expression of FGFR3IIIb and FGFR3IIIc in 18 colorectal cancer patients (C1 to C18) and 15 gastric cancer patients (G1 to G15). In both colorectal cancer and gastric cancer, the incidence of FGFR3IIIc in tumor specimens (T) was not higher than that in NCM (N) (Table 7). Interestingly, in colorectal cancer, the incidence of FGFR3IIIb expression was about 2-fold higher in tumor specimens (11/18, 61%, p = 0.092) than that in NCM (5/18, 28%) (Table 7), although, in gastric cancer specimens, there was no difference between tumor and NCM. No difference was observed in the incidence of the other FGFR isoforms containing FGFR1IIIb, FGFR1IIIc, FGFR2IIIb, FGFR2IIIc, and FGFR4 expression between tumor (T) of colorectal cancer and gastric cancer and NCM (N) (Fig. 6, Table 8).

These results suggested that FGFR3IIIc isoform may play a role in EC progression, whereas FGFR3IIIb isoform may play a role in colorectal cancer

progression.

5-2 FGFR3IIIc has the potential to be a new biomarker of ESCC from the early stage

FGFR3IIIc mRNA was expressed in the early stage of ESCC (Table 5). An immunohistochemical analysis with anti-FGFR3IIIc antibody was performed to determine whether FGFR3IIIc was expressed in esophageal carcinoma cells. Anti-FGFR3IIIc antibody used in this study detected FGFR3IIIc isoform from FGFR3IIIc-overexpressed EC-GI-10 cells by western blot analysis (Fig. 7A). On the other hand, anti-FGFR3IIIc antibody after pre-absorbed with recombinant human FGFR3IIIc Fc chimera, it did not detect FGFR3IIIc isoform (Fig. 7A).

Anti-FGFR3IIIc antibody stained FGFR3IIIc-overexpressed HEK293T cells, but not FGFR3IIIb-overexpressed HEK293T cells by immunofluorescence (Fig. 7B). Anti-FGFR3IIIc antibody after pre-absorbed with human FGFR3IIIc chimera did not stain FGFR3IIIc-overexpressed HEK293T cells (Fig. 7B), indicating that anti-FGFR3IIIc antibody recognizes FGFR3IIIc isoform, but not FGFR3IIIb isoform. In this study, anti-FGFR3IIIb antibody did not detect FGFR3IIIb-overexpressed HEK293T cell lysate by western blot analysis (Fig. S3B) and did not stain FGFR3IIIb isoform in FGFR3IIIb-overexpressed EC-GI-10 cells (Fig. S3C).

An immunohistochemical analysis of ESCC from the patients diagnosed with TNM classification stage 0 showed that the cells expressing SCC-112, a tumor marker, were positively stained by FGFR3IIIc [55] (Fig. 8A, e, f, g, h: white arrows). Neither SCC-112- nor FGFR3IIIc-positive cells were detected in normal esophageal epithelium cells (Fig. 8A, a, b, c, d). Most esophageal carcinoma cells (SCC-112 positive) presented Ki-67, a proliferating cell marker (Fig. 8B, d, e, f: white arrows). FGFR3IIIc-expressing cells also expressed Ki-67 in serial sections of the specimens (Fig. 8C, c, f: white arrows). These results indicated that the expression of FGFR3IIIc was enhanced in esophageal carcinoma cells but not in the normal mucosa or in other types of cells, and that FGFR3IIIc may contribute to signal enhanced cell proliferation.

The expression of FGFR3IIIc in the esophageal tissues was analyzed with other patients diagnosed with TNM classification stage IA containing both esophageal carcinoma cells and normal esophageal epithelium cells (Fig. 9A, B, C). The strong staining of FGFR3IIIc was observed in esophageal carcinoma cells (Fig. 9B: white arrows). Cells positively stained for FGFR3IIIc were clearly

distinguished from negatively stained cells with a clear border, indicating that the normal esophageal epithelium cells did not express FGFR3IIIc (Fig. 9B: black arrows). The FGFR3IIIc-positive area was consistent with the same area of strong staining for SCC-112 in nuclei in consecutive sections (Fig. 9C: white arrows), but was not observed in SCC112-negative areas (Fig. 9C: black arrows). The strong staining of FGFR3IIIc in the esophageal carcinoma area (Fig. 9E and G: white arrows) was also observed in tissue samples from different patients who were diagnosed with TNM classification stage IB (Fig. 9D and E) and stage IIIA (Fig. 9F and G), but was absent in the normal esophageal epithelium area (Fig. 9E and G: black arrows). The staining of FGFR3IIIc in esophageal carcinoma area was specific, because FGFR3IIIc was not stained by anti-FGFR3IIIc antibody after pre-absorbed with recombinant human FGFR3IIIc Fc chimera (Fig. 9H).

In all ESCC patients, the strong staining of FGFR3IIIc was observed only in esophageal carcinoma cells (6/6, 100%), but was not observed in the normal esophageal epithelium cells (0/5, 0%) (Table 3).

These results indicate that FGFR3IIIc has the potential to be a new biomarker for ESCC at the early stage, because the expression of FGFR3IIIc was clearly restricted in esophageal carcinoma cells at stage 0 (Fig. 8A, d, h).

5-3 Enhanced expression of FGFR3IIIc accelerated cell proliferation by an endogenous FGFR3IIIc-specific FGFs such as FGF2

FGFR3IIIc was detected in the cells which also expressed Ki-67, a cell proliferation marker in serial sections of the specimens (Fig. 8C, c, f). To determine whether the expression of FGFR3IIIc indeed contribute to cell proliferation in esophageal cancer cells, cell proliferation assays using EC-GI-10 cells as a model of esophageal cancer cells were performed. EC-GI-10 cells are an esophageal squamous carcinoma cell line [56, 57] that endogenously expresses FGFR3IIIc and FGFR3IIIb by RT-PCR analysis (Fig. 10A, Fig. S2B), and endogenously expresses SCC-112, a tumor marker (Fig. 10B). Anti-FGFR3 (c-15) detected FGFR3IIIb and FGFR3IIIc isoforms. However, anti-FGFR3IIIb or FGFR3IIIc did not detect FGFR3IIIb or FGFR3IIIc, respectively, because these monoclonal antibodies had low sensitivity in western blot analysis (Fig. S3A, Fig. S4). First, to evaluate whether the endogenous expressions of FGFR3IIIb and FGFR3IIIc contribute to the proliferation of the cells, the both isoforms were knocked down by FGFR3 siRNA (siFGFR3) which targets the three prime

untranslated region (3'-UTR) of FGFR3 mRNA. Endogenous FGFR3 protein expression levels were lower in EC-GI-10 cells treated with siFGFR3 (siFGFR3-EC-GI-10 cells) than in cells treated with sicontrol (sicontrol-EC-GI-10 cells) (Fig. 11A). Cell proliferation was significantly weaker (by 25%) in siFGFR3-EC-GI-10 cells than in sicontrol-EC-GI-10 cells, indicating that endogenously FGFR3 in EC-GI-10 cells enhanced cell proliferation. Furthermore, FGFR3IIIc or FGFR3IIIb was exogenously overexpressed in siFGFR3-EC-GI-10 cells by lentiviral infection (FGFR3IIIc-overexpressed EC-GI-10 cells or FGFR3IIIb-overexpressed EC-GI-10 cells). A western blotting analysis showed that the expression level of FGFR3IIIc in FGFR3IIIc-overexpressed EC-GI-10 cells was similar to that of FGFR3IIIb in FGFR3IIIb-overexpressed EC-GI-10 cells (Fig.11A). Cell proliferation was significantly stronger (by 1.4-fold) in FGFR3IIIc-overexpressed EC-GI-10 cells than in siFGFR3-EC-GI-10 cells (Fig.11B). In contrast, cell proliferation was not enhanced in FGFR3IIIb-overexpressed EC-GI-10 cells over siFGFR3-EC-GI-10 cells (Fig. 11B). These results indicated that the enhanced expression of FGFR3IIIc in ESCC accelerated cell proliferation, whereas that of FGFR3IIIb did not.

Moreover, AZD4547, a FGFR kinase inhibitor (Fig. S5A), was tested to evaluate whether the enhanced cell proliferation in FGFR3IIIc-overexpressed EC-GI-10 cells was induced via FGFR3IIIc signaling. The cell proliferation was significantly stronger (by 2-fold) in FGFR3IIIc-overexpressed EC-GI-10 cells than in siFGFR3-EC-GI-10 cells, but not in FGFR3IIIb-overexpressed EC-GI-10 cells (Fig. S5B). The enhanced cell proliferation in FGFR3IIIc-overexpressed EC-GI-10 cells was significantly suppressed with AZD4547 (Fig. S5B). 30 nmol/L and 100 nmol/L of AZD4547 treatment suppressed about 60% and 70% of the enhanced cell proliferation in FGFR3IIIc-overexpressed EC-GI-10 cells, respectively. These results demonstrated that FGFR3IIIc signaling in ESCC promotes cell proliferation.

As a ligand, FGF2 specifically binds to FGFR3IIIc, but not to FGFR3IIIb (Chellaiah et al. 1994; Terada 2009). The expression of FGF2 in esophageal cancer has been reported to be up-regulated and involved in the poor prognosis of patients (Barclay et al. 2005). An RT-PCR analysis was performed in order to confirm whether FGF2 was expressed in parental EC-GI-10 cells. The expression of FGF2 was detected in parental EC-GI-10 cells (Fig. 11C), suggesting that enhancements in cell proliferation by the expression of FGFR3IIIc were induced by an endogenous FGFR3IIIc-specific FGFs such as

FGF2 via an autocrine pathway.

5-4 FGF2 was expressed in both EC and NCM

An RT-PCR analysis was performed to determine whether the expression of FGF2 was higher in EC (T) than in NCM (N). No significant difference between was observed in the incidence of FGF2 expression between EC (6/7, 86%) and NCM (6/7, 86%) (Fig. 12), indicating that FGF2 was expressed in NCM, and the incidence of FGF2 expression in EC was not upregulated compared with NCM.

6. Discussion

Although several studies showed that FGFR1 and FGFR2 amplification were observed in ESCC patients [58, 59], the expression of FGFR isofoms containing FGFR1IIIb, FGFR1IIIc, FGFR2IIIb, FGFR2IIIc, FGFR3IIIb, FGFR3IIIc, and FGFR4 in EC has not yet been examined. In this study, it was demonstrated that the incidence of FGFR3IIIc expression was higher in EC than in NCM by RT-PCR analysis. In contrast, the incidence of FGFR3IIIb expression was higher in NCM, but remained unchanged in EC. Moreover, the incidence of FGFR3IIIc expression was not upregulated in both colorectal cancer and gastric cancer compared with that in the NCM, suggesting that FGFR3IIIc expression was upregulated in EC among digestive cancers. In this study, due to the limited number of the patients who received a curative resection, it was difficult to adjust known prognostic factors, including age, sex, T and N stages for multivariable and univariable analyses of the survival outcomes (i.e., overall survival or progression free survival) based on the expression levels of FGFR3IIIc.

In colorectal cancer, the incidence of FGFR3IIIb expression was higher in tumor specimens than in NCM, suggesting that upregulated FGFR3IIIb may promote malignant progression in colorectal cancer. As described in General introduction, it has been reported that FGFR3IIIc expression, but not FGFR3IIIb, exerts oncogenic functions in colorectal cancer [42]. In this study, all patients were Japanese, but all patients in the report were probably European, suggesting that the expression of FGFR3 isoforms in colorectal cancer may be different between Japanese and European.

Tumor markers including the squamous cell carcinoma-related antigen (SCCA), carcinoembryonic antigen (CEA), CA19-9, CYFRA, CA125, and mutated p53 are widely used to diagnose esophageal cancer (Mealy et al. 1996; Song et al. 2009). A clinical study demonstrated that human EC markers had low sensitivity for cancer screening, and tumor marker levels of CEA, CA19-9, CA125, and SCC did not correlate with the stage of disease or short-term survival [60].

An immunohistochemical analysis of ESCC from patients diagnosed with stage 0, which is defined as high-grade dysplasia by the TNM classification, revealed that the expression of FGFR3IIIc was only observed in esophageal carcinoma cells, indicating that, in the early stage of the carcinogenesis, FGFR3IIIc conferred some benefits to enhance proliferative activities for the progression of malignancy. Indeed, in this study, FGFR3IIIc was detected in the

esophageal carcinoma cells which also expressed Ki-67. Thus, FGFR3IIIc may have the potential to be an early stage tumor marker because the expression of FGFR3IIIc was clearly restricted in esophageal carcinoma cells of stage 0. Certain molecular mechanisms associated with carcinogenesis in the esophageal epithelium may upregulate the expression of FGFR3IIIc, but not FGFR3IIIb.

The constitutive activation of FGFR3 has been shown to drive malignant progression in multiple myeloma, colorectal cancer, and bladder cancer [20, 41, 42]. A blockade of activated FGFR3, using highly specific antibody-targeting of FGFR3, reduced tumor growth in t(4;14)-positive MM mouse models [20]. An antibody against FGFR3 or the use of PD173074, a selective tyrosine kinase inhibitor of FGFR3, suppressed cell proliferation in human bladder carcinoma and tumor growth in a mouse xenograft model [20, 41, 43]. The overexpression of FGFR3IIIc induced cell proliferation in colon carcinoma cell lines and tumor growth in a mouse xenograft model [42]. Thus, these findings demonstrated that FGFR3 is tumorigenic.

SCC-112 has been reported to promote cell proliferation and contributes to tumorigenesis by interacting with p63, transcription factor [61], which affect the survival and proliferative capacity of squamous epithelia, and promoting cell cycling [55, 62]. SCC-112 was used as a tumor marker in this study,, since SCC-112 expression was detected in esophageal carcinoma but not in the normal counterpart tissues [55]. In this study, FGFR3IIIc was detected in the esophageal carcinoma cells which also expressed SCC-112. However, it is yet unclear to have the functional relationship between FGFR3IIIc and SCC-112.

In the in vitro study, our results showed that the overexpression of FGFR3IIIc, but not that of FGFR3IIIb, enhanced cell proliferation in EC-GI-10 cells. Conversely, the knockdown of endogenous FGFR3 by siRNA reduced cell proliferation beyond that observed in the cells treated with sicontrol. FGF2 was endogenously expressed in EC-GI-10 cells, suggesting that the enhanced expression of FGFR3IIIc was beneficial for cancer cell proliferation due to a FGFR3IIIc-specific FGF (such as FGF2) binding via an autocrine pathway. In ESCC patients, FGF2 was expressed in both NCM and ESCC, suggesting that the upregulated expression of FGFR3IIIc triggered the carcinogenesis of esophageal cancer and malignant progression.

AZD4547, an FGFR-specific kinase inhibitor, inhibits activated FGFRs, suppressing tumor growth by inhibition of FGFRs signaling. Inhibitory

concentration (IC_{50} values) of the compound in cells are represented as 12 nmol/L to FGFR1, 2 nmol/L to FGFR2, 40 nmol/L to FGFR3 [33]. In this study, AZD4547 suppressed the enhanced cell proliferation of EC-GI-10 cells by overexpressed FGFR3IIIc, indicating that FGFR3IIIc transduces signals to stimulate cell proliferation in the cells.

In conclusion, FGFR3IIIc has the potential to be not only an early stage tumor marker, but also a molecular target for ESCC therapy. However, the number of esophageal cancer samples examined here was limited, further studies are required to validate this conclusion.

7. General discussion

This study demonstrated that FGFR3IIIc isoform, but not FGFR3IIIb isoform, was upregulated in the early stage of ESCC, but the mechanism of FGFR3IIIc upregulation was not clear. As described in General introduction, the FGFRIIIb isoform is predominantly expressed in epithelial and the FGFRIIIc isoform is predominantly expressed in mesenchymal, suggesting that FGFR3IIIc expression may be upregulated by epithelial-mesenchymal transition (EMT). Twist has been identified as a promoting factor for EMT [63]. The upregulation of Twist was associated with neoplastic transformation, and its expression was upregulated in ESCC [64]. However, it is not clear whether Twist regulates alternative splicing of FGFR3. In this study, the expression of FGFR2IIIc in EC was not upregulated. Therefore, the involvement of Twist in FGFR3IIIc upregulation in EC seems unlikely. In the immunohistochemical analysis, FGFR3IIIc positive cells expressed SCC-112. SCC-112 interacts p63, which is an important transcription factor that regulates epidermal differentiation [61], suggesting that SCC-112 changed the gene expression in the FGFR3IIIc positive cells. Changing of the gene expression has the potential to affect expression of some factors, which regulate the splicing factor for FGFR3IIIc.

Although aberrant FGFRs containing mutation, amplification, and translocation have been found in 6 to 40% of several cancer patients, those cancers is a molecular target for cancer therapy [30, 31]. In this study, 75% of ESCC patients expressed FGFR3IIIc isoform. The incidence of FGFR3IIIc expression in ESCC is higher than that of aberrant FGFRs in several cancers, suggesting that FGFR3IIIc isoform is sufficient to be a target for ESCC therapy containing personalized medicine.

In RT-PCR analysis, the expression level of FGFR2IIIc in EC appears to decrease compared with NCM. Reduced FGFR2 expression has been reported in several cancers [65, 66]. Mice that specifically lack FGFR2IIIb in keratinocytes are sensitive to carcinogenic insults to their skin [66]. FGFR2IIIb expression in human salivary adenocarcinoma cells inhibits the cell proliferation and induces apoptosis [65]. Forced expression of either FGFR2IIIb or FGFR2IIIc in thyroid epithelial cancer cells each diminished invasive behavior in vitro and reduced tumor growth and metastasis in vivo [67], although they were expressed in fibroblasts, in which they both stimulated cell growth. Thus their functions in growth-promoting or growth-suppressive functions are depend on cell context in which they are expressed. Therefore, in the present study, the decreased

incidence of FGFR2IIIc expression in EC compared to NCM may suggest a tumor suppressive effect of FGFR2IIIc in normal esophageal epithelial cells.

In this study, the enhanced FGFR3IIIc expression in ESCC in vitro promoted cell proliferation, suggesting that endogenous FGF (such as FGF2) may create an autocrine pathway. FGFs are produced in either epithelial or mesenchymal tissue and generally activate receptors of the opposite tissue specificity. Pathological states can result from a breakdown in binding specificity, as is common in cancer in which FGFs are overexpressed [12]. As described in General introduction, it has been reported that FGF2 was expressed in esophageal cancer (Barclay et al. 2005). Indeed, in this study, FGF2 was shown to be expressed in esophageal carcinoma. Moreover, it has also been reported that expression of FGF18, another FGFR3IIIc specific ligand, in tumor fibroblasts at esophageal cancer is upregulated compare to nontumorous fibroblasts of esophagus [68]. In this study,, expression of FGF18 in parental EC-GI-10 cells was not detected by RT-PCR analysis (data not shown). In vivo, there might be a possibility that esophageal carcinoma cells expressing FGFR3IIIc may undergo cell proliferation by receiving exogenous FGF18 from tumor activated fibroblasts invaded into the tumor microenvironment. On the other hand, expression of the other FGFs in esophageal carcinoma has not been clarified yet. Trapping FGF ligands is a molecular target for cancer therapy. FP-1039 is a soluble FGFR1 receptor, which was constructed with additional of the human IgG1 Fc region to the extracellular ligand-binding domain of FGFR1IIIc receptor splice isoform. FP-1039 is evaluated in phase Ib clinical trials for non small cell lung cancer and mesothelioma. FP-1039 blocks the activity of multiple FGF ligands with binding to FGF1, FGF2, FGF3, FGF4, FGF5, FGF6, FGF8, FGF9, FGF16, FGF17, FGF18, FGF20, and FGF23, resulting that FP-1039 inhibits tumor cell proliferation and blocks angiogenesis [69]. Therefore, analyzing expression of FGFs in esophageal carcinoma has the potential to find a new target for ESCC therapy.

In conclusion, the expression of FGFR3IIIc isoform was enhanced in ESCC, and FGFR3IIIc expression may contribute to enhance cell proliferation in ESCC, leading to tumor progression. Thus, FGFR3IIIc expression in early stage ESCC has important implications for the prevention and treatment of ESCC.

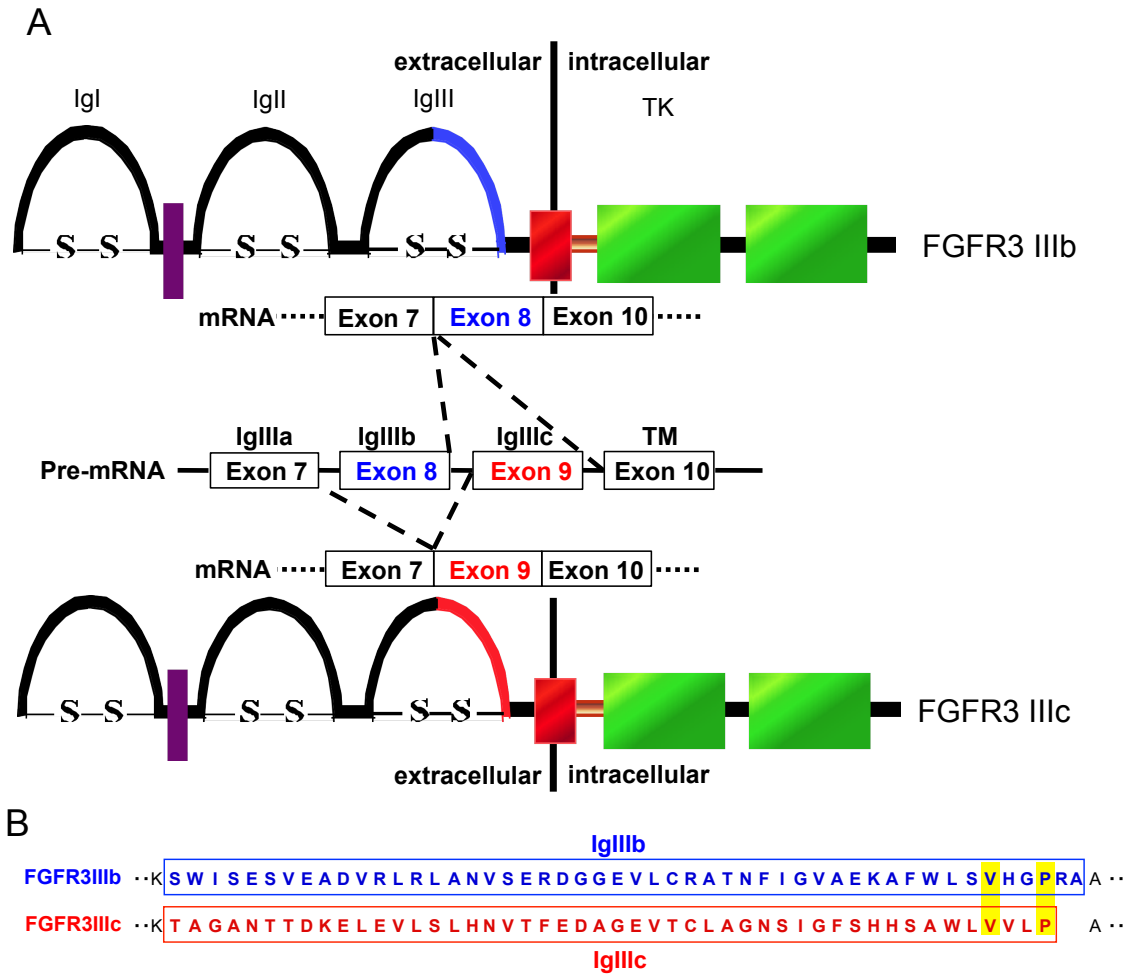


Fig. 1 The schematic drawing of FGFR3 isoforms and alternative splicing of the transcripts. (A) The FGFR3 gene has three exons for the IgIIIa, IIIb, and IIIc domains. FGFR3IIIb isoform is generated by alternative splicing between exon 8 and exon 10 which encode the C-terminal half of IgIIIb and the transmembrane, respectively. On the other hand, FGFR3IIIc isoform is generated by alternative splicing between exon 7 and exon 9, which encode the C-terminal half of IgIIIa and the another C-terminal half of IgIIIc, respectively. (B) Amino acid sequence of IgIIIb and IgIIIc. The same amino acid in IgIIIb and IgIIIc are highlighted in yellow.

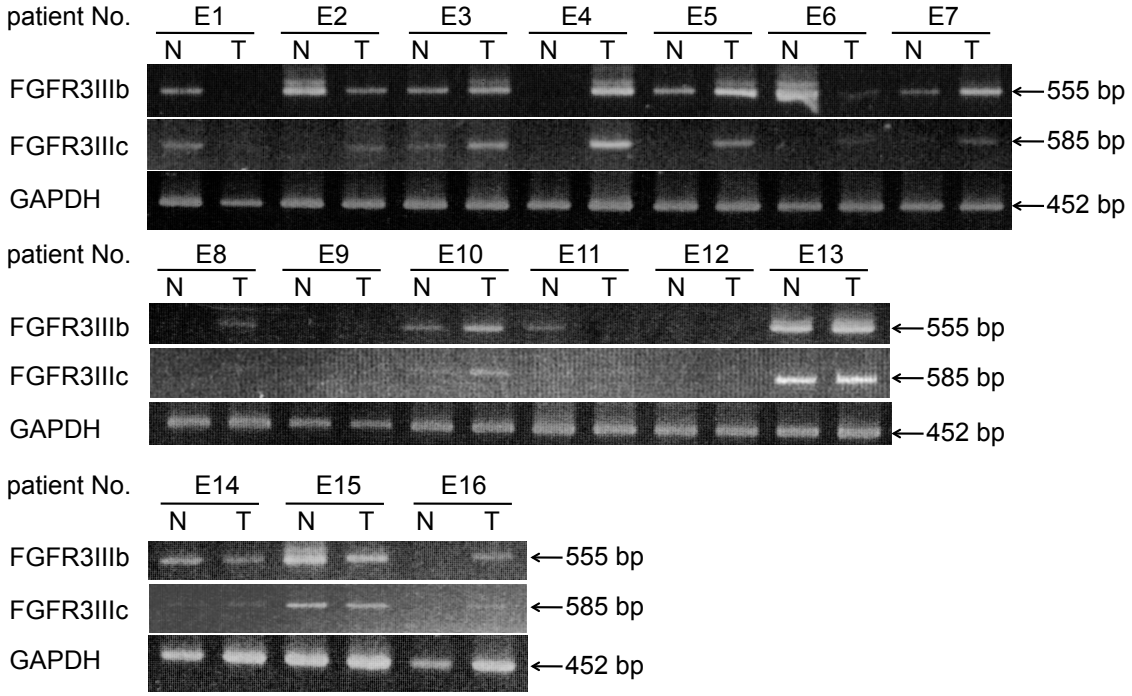


Fig. 2 Gene expression of FGFR3 isoforms in EC and NCM. Total RNAs were extracted from the specimens of EC patients. The gene expression of FGFR3 in 16 patients (E1 to E16) was analyzed by RT-PCR. FGFR3IIIc expression was clearly detected in the EC (T) of 12 patients and in the NCM (N) of 6 patients. FGFR3IIIb expression was clearly detected in the EC (T) of 12 patients and in the NCM (N) of 11 patients. GAPDH, glyceraldehyde-3-phosphate dehydrogenase

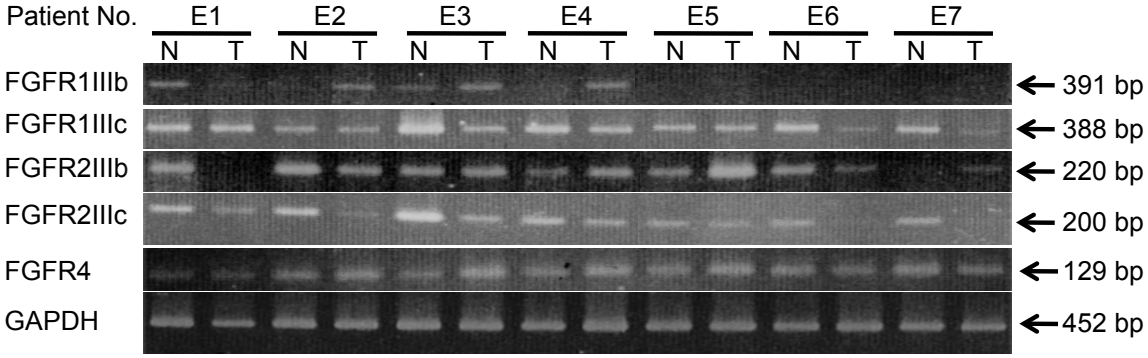


Fig. 3 Gene expression of FGFR1, FGFR2, and FGFR4 isoforms in EC and NCM. FGFR1IIIb, FGFR1IIIc, FGFR2IIIb, FGFR2IIIc, and FGFR4 isoforms were expressed in ESCC and NCM. The incidence of FGFR1IIIb, FGFR1IIIc, FGFR2IIIb, FGFR2IIIc, and FGFR4 expression in EC was not higher than that in NCM.

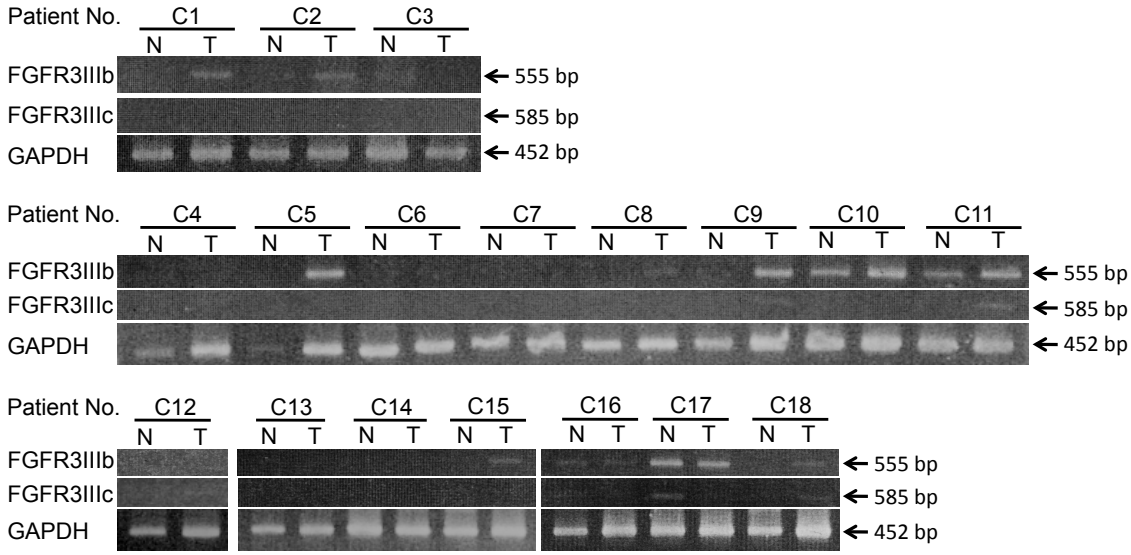


Fig. 4 Gene expression of FGFR3 isoforms in 18 colorectal cancer patients (C1 to C18). FGFR3IIIc expression was clearly detected in the colorectal cancer (T) of 4 patients and in the NCM (N) of 1 patient (C17). FGFR3IIIb expression was clearly detected in the colorectal cancer (T) of 11 patients and in the NCM (N) of 5 patients.

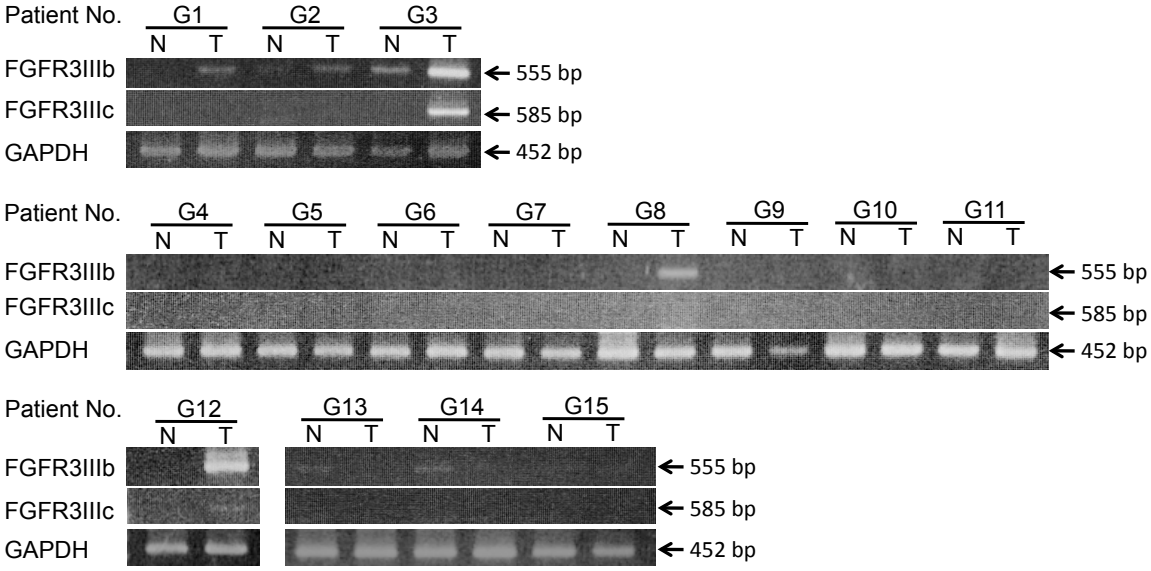


Fig. 5 Gene expression of FGFR3 isoforms in 15 gastric cancer patients (G1 to G15). FGFR3IIIc expression was clearly detected in the gastric cancer (T) of 2 patients and was not detected in the NCM (N). FGFR3IIIb expression was clearly detected in the gastric cancer (T) of 5 patients and in the NCM (N) of 4 patients.

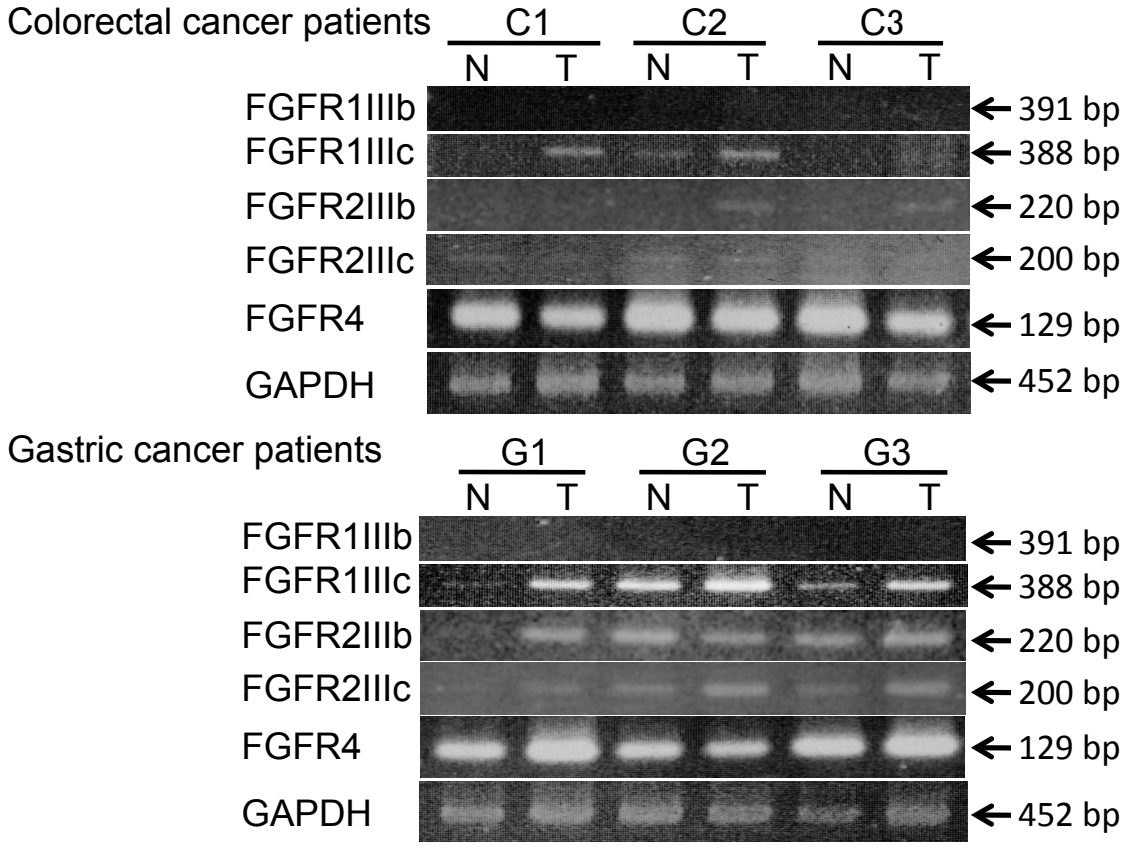
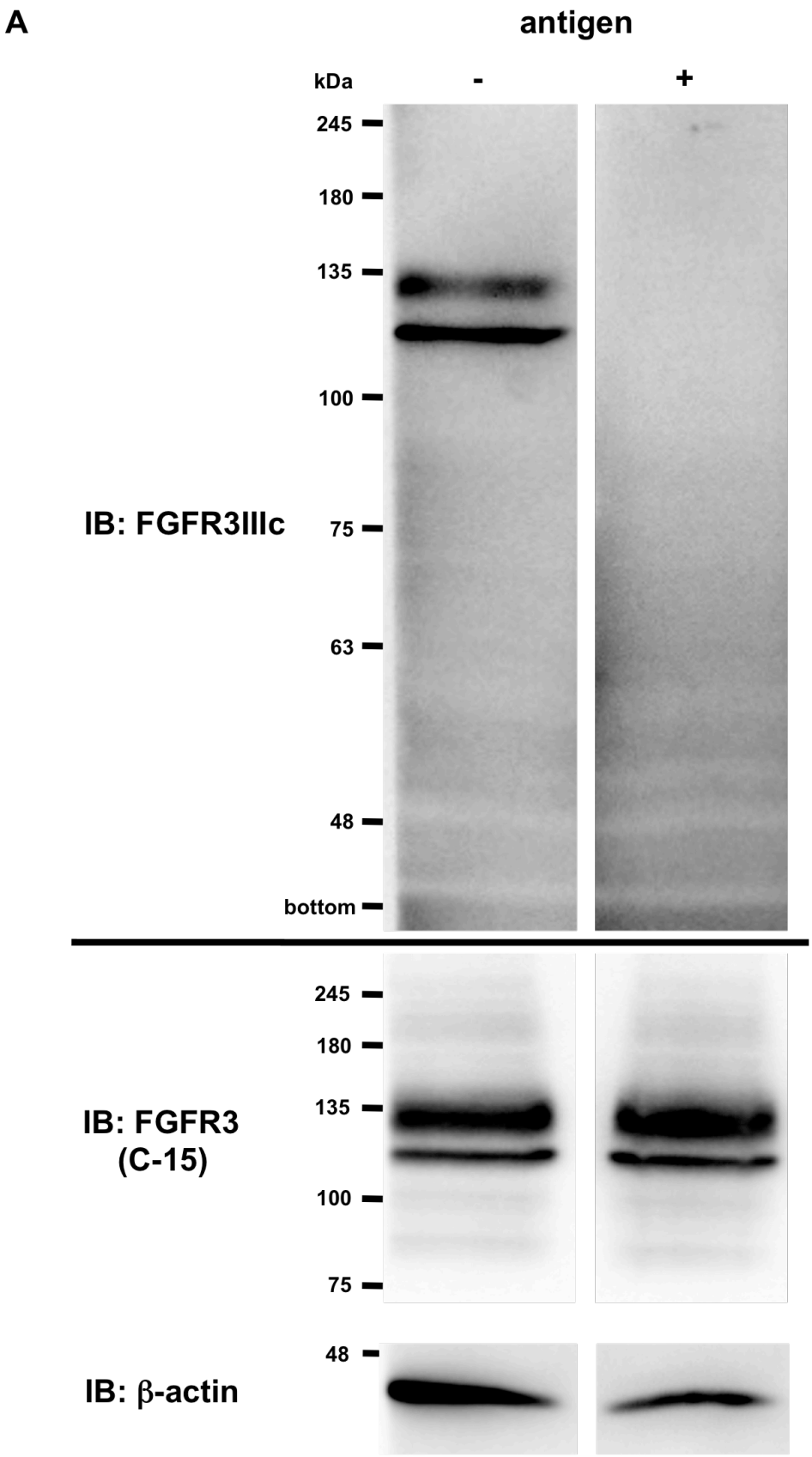


Fig. 6 Gene expression of FGFR1, FGFR2, and FGFR4 isoforms in 3 colorectal cancer patients (C1 to C3) and 3 gastric cancer patients (G1 to G3). The gene expression of FGFR1, FGFR2, and FGFR4 isoforms in colorectal cancer patients and gastric cancer patients was analyzed by RT-PCR. The incidence of FGFR1IIIb, FGFR1IIIc, FGFR2IIIb, FGFR2IIIc, and FGFR4 expression in colorectal cancer (T) and gastric cancer patients was not higher than that in NCM. GAPDH, glyceraldehyde-3-phosphate dehydrogenase


A
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kDa
-
+
245
180
135
100
75
63
48
bottom
IB: FGFR3IIIc
245
180
135
100
75
IB: FGFR3
(C-15)
48
IB: β-actin

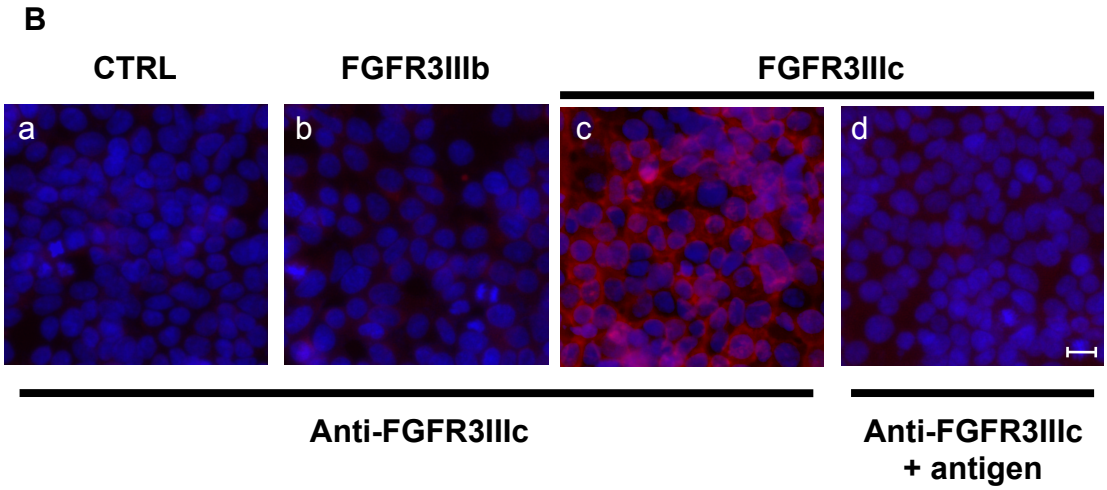
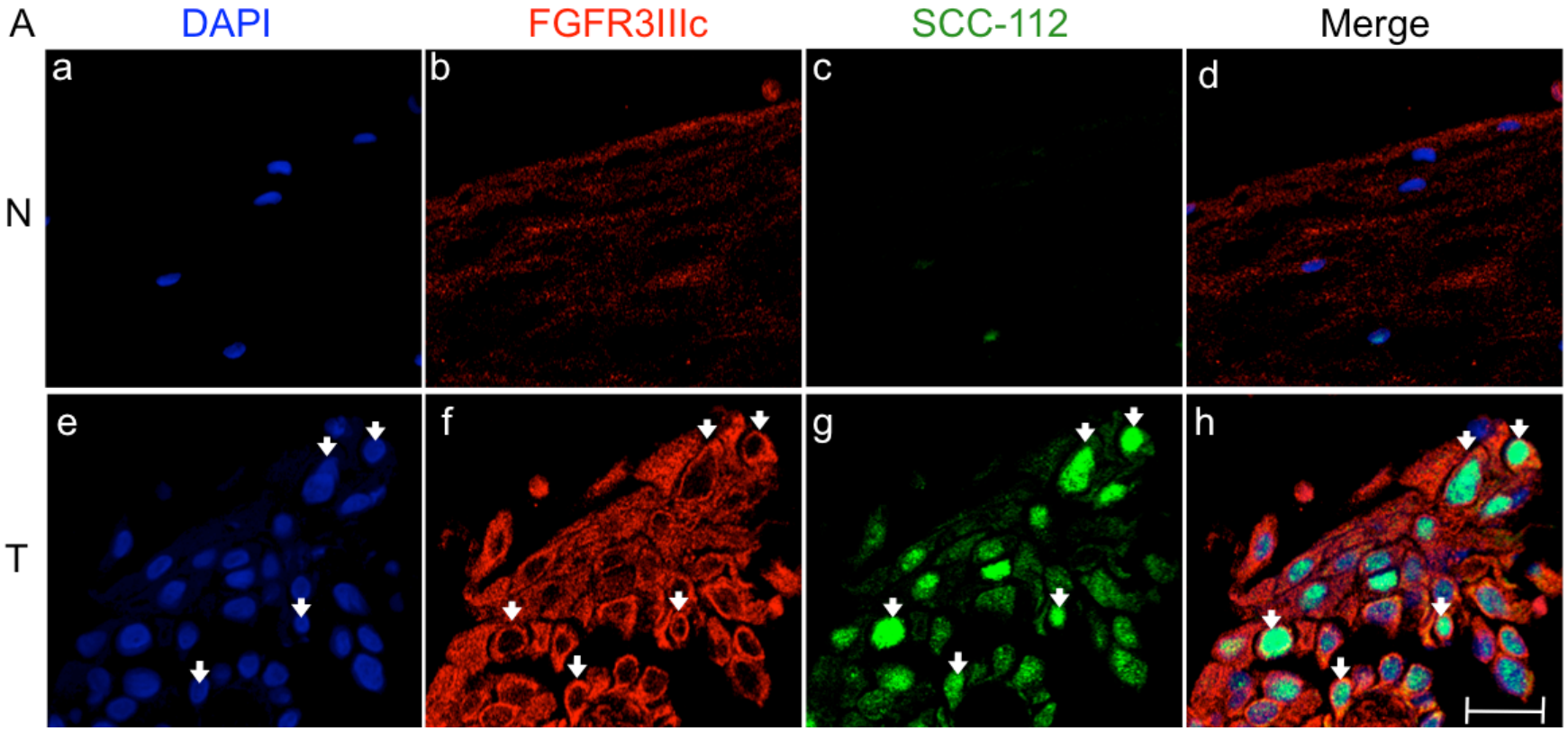


Fig. 7 Anti-FGFR3IIIc antibody recognizes FGFR3IIIc isoform, but not FGFR3IIIb. (A) Western blot analysis was performed using cell lysate of FGFR3IIIc-overexpressed EC-GI-10 cells. Upper panel: FGFR3IIIc isoform was detected by anti-FGFR3IIIc antibody (-), whereas FGFR3IIIc isoform was not detected by the anti-FGFR3IIIc antibody pre-absorbed with the antigen, recombinant human FGFR3IIIc Fc chimera (+). Lower pannel: FGFR3 (anti-FGFR3 antibody (C-15)) and β-actin were detected equally between those lanes. (B) Immunofluorescence of FGFR3IIIc expression in HEK293T cells (a: CTRL), FGFR3IIIb-overexpressed HEK293T cells (b: FGFR3IIIb), FGFR3IIIc-overexpressed HEK293T cells (c, d: FGFR3IIIc). FGFR3IIIc-overexpressed HEK293T cells were stained by anti-FGFR3IIIc antibody (c, Red), but were not stained by the pre-absorbed anti-FGFR3IIIc antibody with the antigen (d: Anti-FGFR3IIIc + antigen). HEK293T and FGFR3IIIb-overexpressed HEK293T cells were also not stained by anti-FGFR3IIIc antibody (a, b: Anti-FGFR3IIIc). (DAPI: Blue). Bar = 20 μm.


A
DAPI
FGFR3IIIc
SCC-112
Merge
N
T
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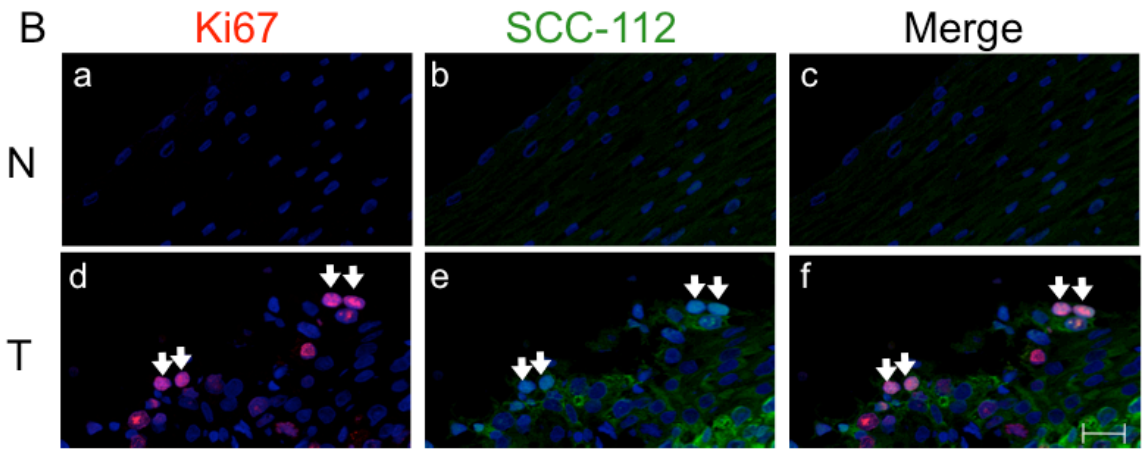
h


B
Ki67
SCC-112
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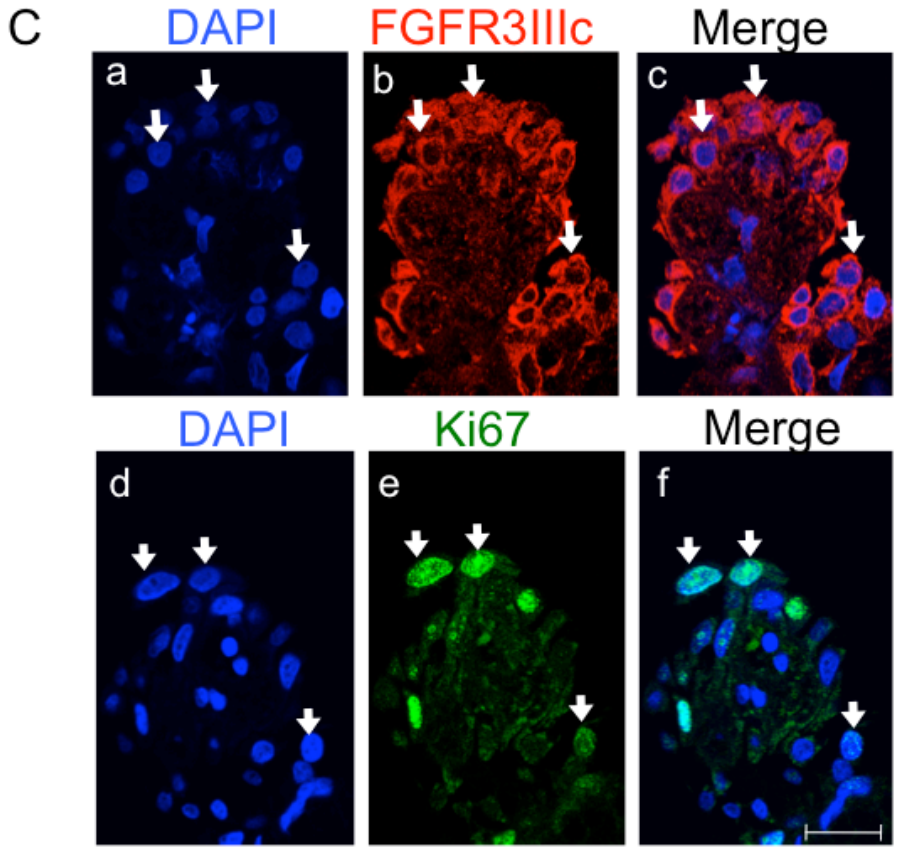
C
DAPI
FGFR3IIIc
Merge
DAPI
Ki67
Merge
a
b
c
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e
f

Fig. 8 Enhanced expression of FGFR3IIIc was associated with proliferating esophageal carcinoma cells. (A) Immunofluorescence staining of DAPI (a, e), FGFR3IIIc (b, f) and SCC-112 (c, g) in NCM (N) and ESCC (T), which was diagnosed as stage 0. In confocal microscopic images, the strong staining of FGFR3IIIc (red) was observed in esophageal carcinoma cells (f), but not in normal esophageal epithelium cells (b). FGFR3IIIc-positive cells in ESCC were consistent with SCC-112-positive cells (green) (f, g, h: white arrows). Nuclei were stained with DAPI (blue). (B) Immunofluorescence staining of Ki-67 (red, a, d) and SCC-112 (green, b, e) in NCM (N) and ESCC (T). The strong staining of Ki-67 was observed in esophageal carcinoma cells, and Ki67-positive cells were consistent with SCC-112-positive cells in ESCC (d, e, f: white arrows). On the other hand, staining of Ki-67 and SCC-112 were not observed in NCM (a, b, c). (C) Immunofluorescence staining of DAPI (a, d), FGFR3IIIc (b), Ki-67 (e) and merged images (c, f) in ESCC. The expression of FGFR3IIIc was detected in the same cells, which also expressed Ki-67 in the consecutive sections (c, f: white arrows). Bar = 20 μm.

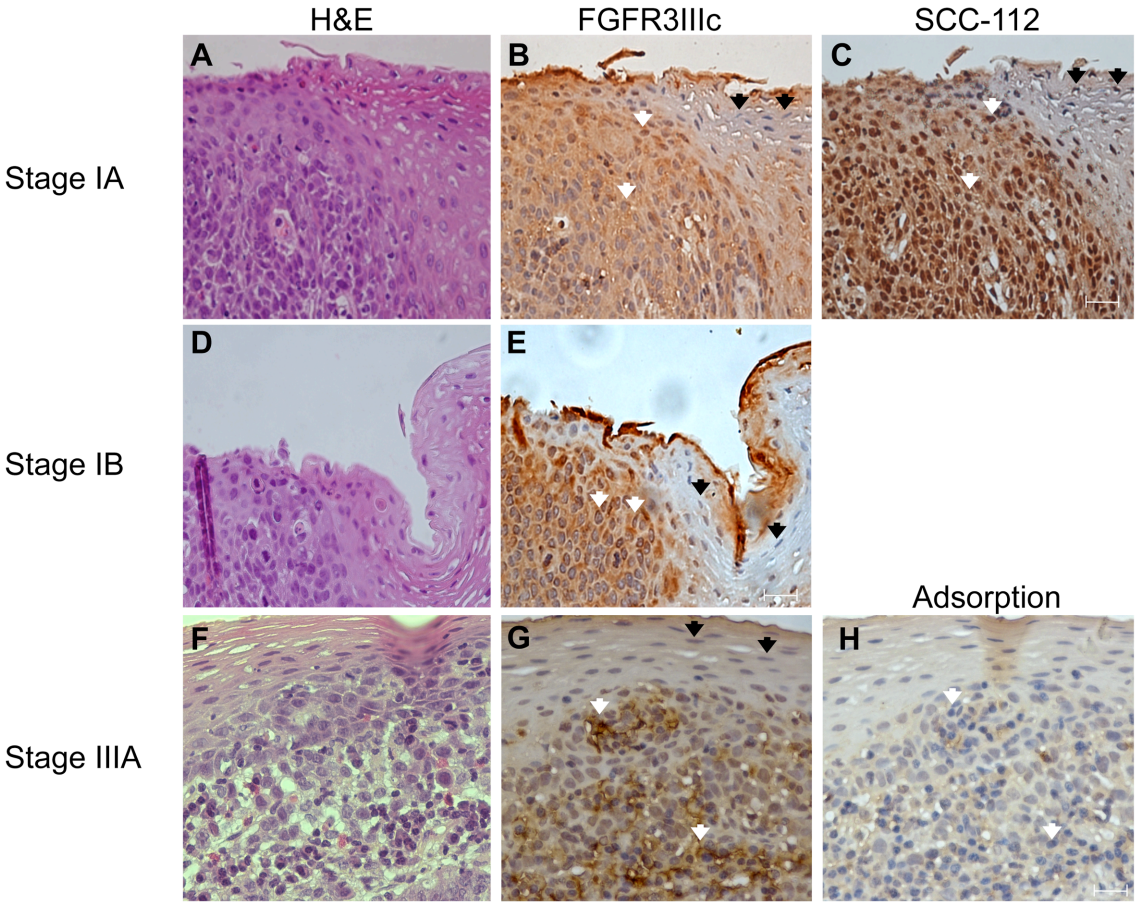


Fig. 9 Expression of FGFR3IIIc was enhanced in esophageal carcinoma cells diagnosed as stage IA (A, B, and C), stage IB (D and E), stage IIIA (F, G and H), but not in normal esophageal epithelium cells. FGFR3IIIc was not stained by anti-FGFR3IIIc antibody after pre-absorbing recombinant human FGFR3IIIc Fc chimera (H). Hematoxylin and eosin staining (H&E stain, A, D, and F), FGFR3IIIc (B, E, and G), and SCC-112 (C). Strong staining for FGFR3IIIc was observed in esophageal carcinoma cells (B, E, and G: white arrows), and FGFR3IIIc expression was clearly restricted in the carcinoma area with a clear border. Normal esophageal epithelium cells did not express FGFR3IIIc (B, E, and G: black arrows). FGFR3IIIc-positive cells were consistent with SCC-112-positive cells at nuclei in the consecutive sections (C: white arrows). Bar = 20 μm.

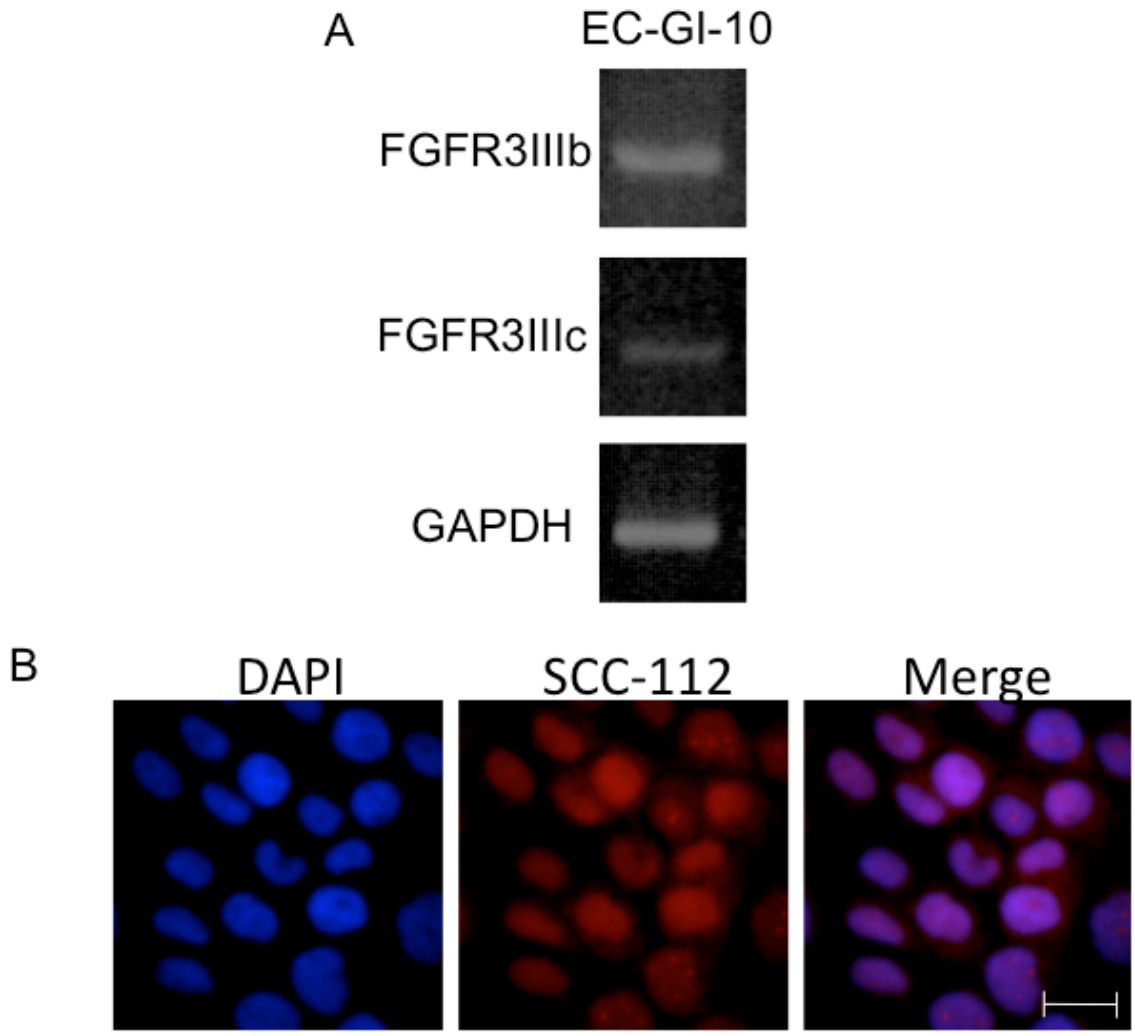
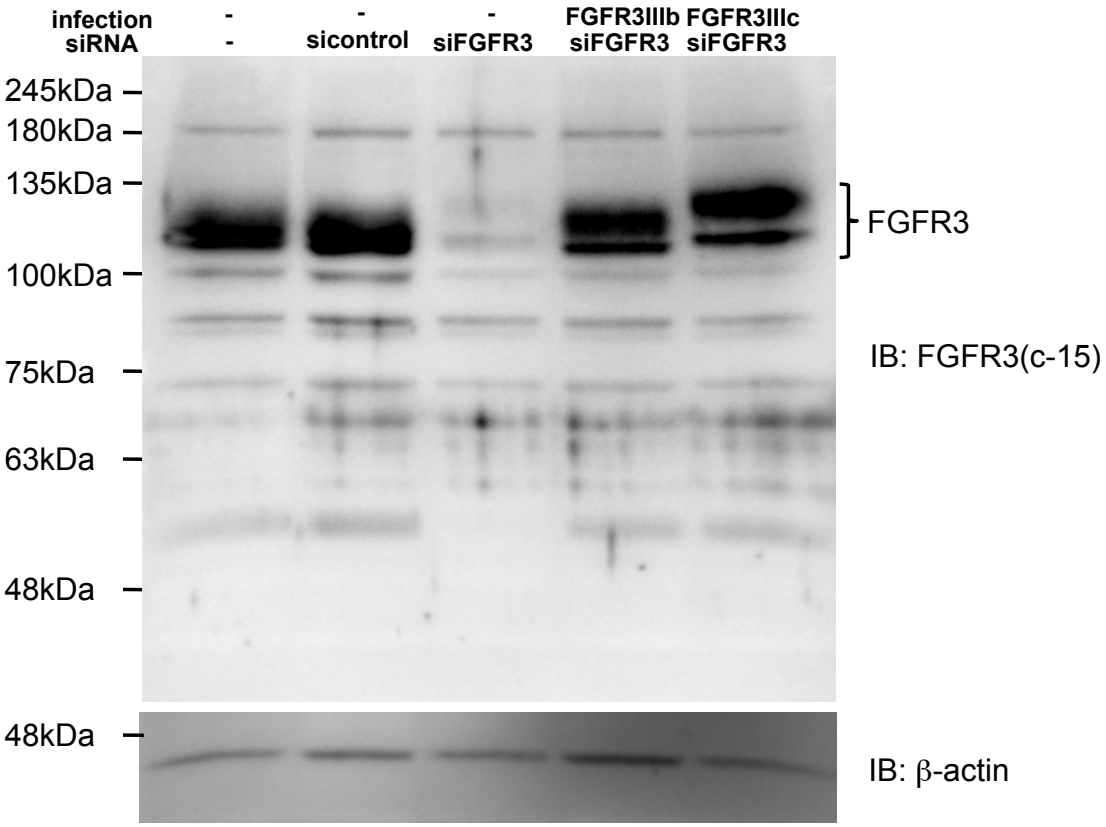
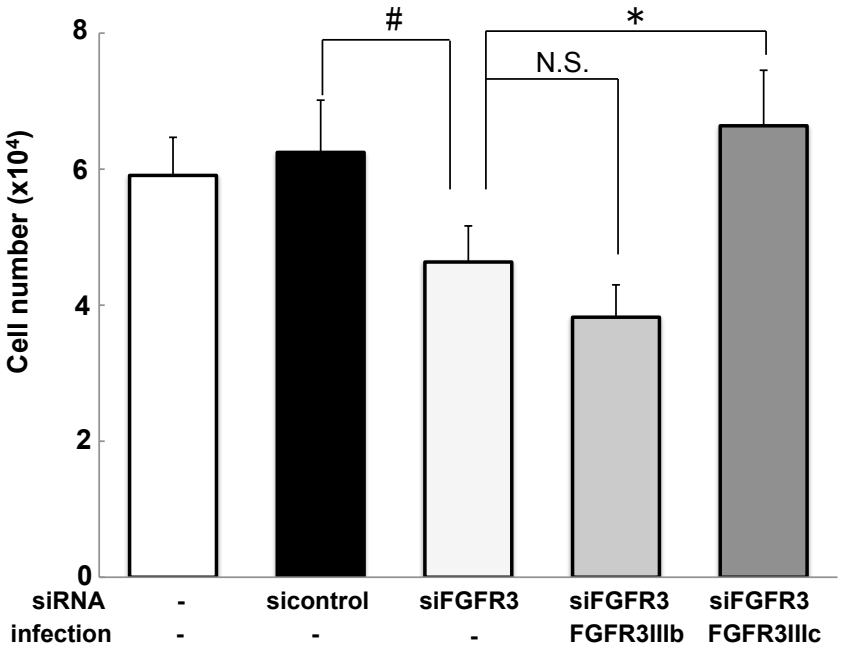


Fig. 10 Gene expression of FGFR3IIIb and FGFR3IIIc in parental EC-GI-10 cells by RT-PCR analysis and expression of SCC-112 in parental EC-GI-10 cells by immunofluorescence. (A) FGFR3IIIb and FGFR3IIIc were endogenously expressed in parental EC-GI-10 cells. (B) Immunofluorescence staining of SCC-112 (red) was observed at nuclei (DAPI: blue) in EC-GI-10 cells. Bar = 20 μm.

A


infection
siRNA
-
-
-
sicontrol
-
siFGFR3
FGFR3IIIb
siFGFR3
FGFR3IIIc
siFGFR3
245kDa
180kDa
135kDa
100kDa
75kDa
63kDa
48kDa
FGFR3
IB: FGFR3(c-15)
48kDa
IB: β-actin

B


#
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Cell number (x10⁴)
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siRNA
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FGFR3IIIb
siFGFR3
FGFR3IIIc

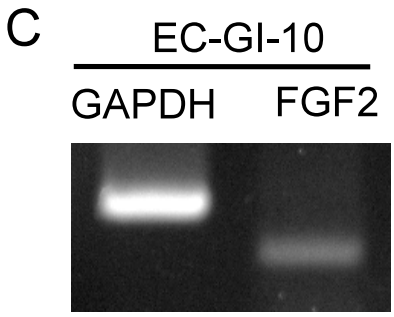

Fig. 11 Enhanced expression of FGFR3IIIc accelerated cell proliferation. (A) Western blotting showed the FGFR3 expression levels of the parental, sicontrol, siFGFR3, FGFR3IIIb, and FGFR3IIIc cells. β-actin was expressed equally among those cells. (B) Cell proliferation was significantly weaker (by 25%) in EC-GI-10 cells treated with FGFR3 siRNA (siFGFR3) than in EC-GI-10 cells treated with sicontrol (sicontrol) after 6 culture days in DMEM containing 1% FBS. The cell proliferation of FGFR3IIIc-overexpressed siFGFR3-EC-GI-10 cells (FGFR3IIIc) was significantly stronger than that of siFGFR3-EC-GI-10 cells (by 1.4-fold), whereas the overexpression of FGFR3IIIb (FGFR3IIIb) was not. The parental EC-GI-10 cells were not treated with siRNA (-) and not infected (-). Results are shown as the mean ± S.D. and are representative of 3 independent experiments, each in triplicate (#, $p<0.05$ versus the sicontrol cells and *, $p<0.05$ versus the siFGFR3 cells, t-test, N.S., not significant). (C) RT-PCR showed that FGF2 was endogenously expressed in parental EC-GI-10 cells.

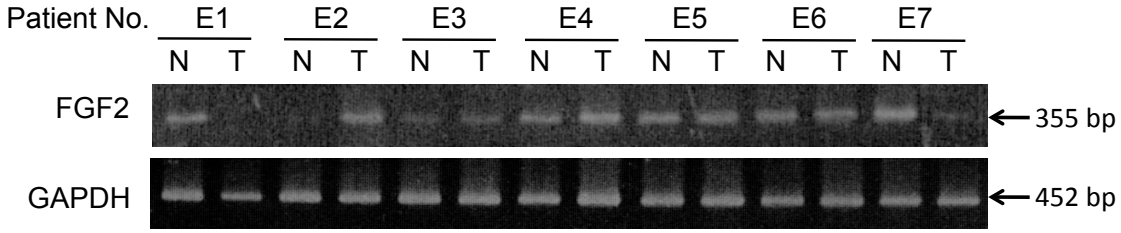

Fig. 12 Gene expression of FGF2 in 7 patients (E1 to E7) by RT-PCR. No significant difference was observed in FGF2 expression between ESCC (T, 6/7, 86%) and NCM (N, 6/7, 86%).

Table 1 The stage profile of 16 esophageal carcinoma patients whose specimens were analyzed by RT-PCR

Variables		No. of patients	
		N	%
Gender	Male	14§	87.5
	Female	2	12.5
Stage	I	5	31.3
	II	1	6.3
	III	9§	56.2
	IV	1	6.3

§: Two patients were diagnosed as adenocarcinoma.

Table 2 Clinicopathologic features in 16 EC patients

Patient No.	Gender	Differentiation	pStage	Chemotherapy	pT (Invasion depth)	pN	Relapse-free survival (months)	Cause of death	Recurrence
E1	Male	Spindle cell carcinoma	I	No	T1b(SM)	0	22	EC	Yes
E2	Male	Well differentiated	III	No	T3（Ad)	1	7	EC	Yes
E3	Male	Moderately	III	Yes	T3（Ad)	16	13	EC	Yes
E4	Female	Moderately	III	Yes	T3（Ad)	1	176	-	-
E5	Male	Well	III	Yes	T2（MP)	4	7	EC	Yes
E6	Male	Poorly	III	Yes	T3（Ad)	1	75	AML	No
E7	Male	Well	II	Yes	T3（Ad)	0	158	-	No
E8	Male	Moderately	III	Yes	T4（Lung)	1	0	EC	NR
E9	Male	Poorly	III	Yes	T1b(SM)	6	16	EC	Yes
E10	Male	Moderately	I	No	T1b(SM)	0	10	-	Yes
E11	Male	Moderately	I	Yes	T2（MP)	0	152	-	No
E12	Male	Adenocarcinoma	III	Yes	T2（SS)	5	15	PC	?
E13	Female	Well	I	Yes	T2（MP)	0	6	EC	Yes
E14	Male	Poorly	I	Yes	T1b(SM)	0	137	-	-
E15	Male	Poorly	IV	Yes	T4(pancreas)	3	0	PB	No
E16	Male	Adenocarcinoma	III	Yes	T3(Ad)	3	17	EC	Yes

SM: Submucosa, MP: Muscularis propria, Ad: Adventitia, SS: Subserosa, AML: Acute myelogenous leukemia
EC: Esophageal cancer, PC: Pancreatic cancer, PB: Postoperative bleeding, NR: noncurative resection
pT: Pathological tumor, pN: Pathological lymph nodes, The pN shows number of lymph node metastasis.
Relapse-free survival shows the period from surgery to recurrence.

Table 3 RT-PCR analysis and immunohistochemical analysis of FGFR3IIIc expression in esophageal squamous cell carcinoma

Analysis	patient No.	FGFR3IIIc expression (EC or ESCC/NCM or N)
RT-PCR analysis	E1	-/+
	E2	+/-
	E3	+/+
	E4	+/-
	E5	+/-
	E6	+/-
	E7	+/-
	E8	+/-
	E9	-/-
	E10	+/+
	E11	-/-
	E12	-/-
	E13	+/+
	E14	+/+
	E15	+/+
	E16	+/-
Immunohistochemical analysis	A1 (Fig 8)	+/-
	A2 (Fig 9A, B, C)	+/-
	A3 (Fig 9D, E)	+/-
	A4 (Fig 9F, G)	+/-
	A5 (not shown)	+/-
	A6 (not shown)	+ §

EC: Esophageal carcinoma, NCM: non-cancerous mucosa, ESCC: Esophageal squamous cell carcinoma area, N: Normal esophageal epithelium area, +: positive expression or staining, -: negative expression or staining.

§: N did not appear in this patient sample.

Table 4 PCR primer sequences

Gene Name		Sequence	PCR products
FGFR1IIIb	forward	5'-ACCTGACCACAGAATTGGAGGCTAC-3'	391 bp
	reverse	5'-ATTGAACAGGGTCAGCACCTCCGCATCCGAGCTATTAATTCCCGA-3'	
FGFR1IIIc	forward	5'-ACCTGACCACAGAATTGGAGGCTAC-3'	388 bp
	reverse	5'-ATGAACACCTCCATTTCCTTGTCGGTGGTATTAACTCCAGCAGT-3'.	
FGFR2IIIb	forward	5'-AAGCTGGACTGCCTGCAAATGCCT-3'	220 bp
	reverse	5'-TCCGTCACATTGAACAGAGCC-3'	
FGFR2IIIc	forward	5'-AAGCTGGACTGCCTGCAAATGCCT-3'	200 bp
	reverse	5'-CTCAATCTCTTTGTCCGTGGTG-3'	
FGFR3IIIb	forward	5'-CTGTCGAGCCACCAATTTCATAGGC-3'	555 bp
	reverse	5'-GACAGGTCCTTGTCAGTGGCATC-3'	
FGFR3IIIc	forward	5'-CTTGCACAACGTCACCTTTGAG-3'	585 bp
	reverse	5'-GACAGGTCCTTGTCAGTGGCATC-3'	
FGFR4	forward	5'-CCAACGCATGGAGAAGAAACTGCAT-3'	129 bp
	reverse	5'-TTCTCCCCATGGAAGGCCTGT-3'	
FGF2	forward	5'-CTTCTTCCTGCGCATCCATCC-3'	355 bp
	reverse	5'-TCAGCTCTTAGCAGACATTGG-3'	
GAPDH	forward	5'-ACCACAGTCCATGCCATCAC-3'	452 bp
	reverse	5'-TCCACCACCCTGTTGCTGTA-3'	

Table 5 FGFR3IIIb and FGFR3IIIc expression in esophageal carcinoma (EC) and non-cancerous mucosa (NCM).

		FGFR3IIIb expression		FGFR3IIIc expression	
		NCM	EC	NCM	EC
Stage of ESCC patients (n=14)	I	5/5 (100%)	3/5 (60%)	4/5 (80%)	3/5 (60%)
	II	1/1 (100%)	1/1 (100%)	0/1 (0%)	1/1 (100%)
	III	4/7 (57%)	6/7 (86%)	1/7 (14%)	6/7 (86%)
	IV	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)
Stage of EAC patients (n=2)	III	0/2 (0%)	1/2 (50%)	0/2 (0%)	1/2 (50%)
Incidence of all patients (n=16)		11/16 (69%)	12/16 (75%) (p=1)	6/16 (38%)	12/16 (75%) (p=0.073)

ESCC: Esophageal squamous cell carcinoma, EAC: Adenocarcinoma

Table 6 Expression of FGFR1, FGFR2 and FGFR4 isoforms in 7 EC patients.

FGFR isoforms		NCM	EC
FGFR1	IIIb	3/7 (43 %)	4/7 (57 %) p=1.000
	IIIc	7/7 (100 %)	7/7 (100 %) p=1.000
FGFR2	IIIb	6/7 (86 %)	6/7 (86 %) p=1.000
	IIIc	7/7 (100 %)	5/7 (71 %) p=0.461
FGFR4		7/7 (100 %)	7/7 (100 %) p=1.000

NCM:non-cancerous mucosa, EC: esophageal carcinoma

Table 7 FGFR3IIIb and FGFR3IIIc expression in colorectal cancer or gastric cancer patients.

Cancer	FGFR3IIIb expression		FGFR3IIIc expression	
	NCM	Tumor	NCM	Tumor
Colorectal cancer	5/18 (28%)	11/18 (61%) p=0.092	1/18 (6%)	4/18 (22%) p=0.338
Gastric cancer	4/15 (27%)	5/15 (33%) p=1.000	0/15 (0%)	2/15 (13 %) p=0.482

NCM:non-cancerous mucosa

Table 8 Expression of FGFR1, FGFR2 and FGFR4 isoforms in 3 colorectal cancer patients and 3 gastric cancer patients.

Cancer	FGFR isoforms		NCM	Tumor
Colorectal cancer	FGFR1	IIIb	0/3 (0 %)	0/3 (0 %) p=1.000
		IIIc	1/3 (33 %)	2/3 (66 %) p=1.000
	FGFR2	IIIb	0/3 (0 %)	2/3 (66 %) p=0.4
		IIIc	2/3 (66 %)	1/3 (33 %) p=1.000
	FGFR4		3/3 (100 %)	3/3 (100 %) p=1.000
Gastric cancer	FGFR1	IIIb	0/3 (0 %)	0/3 (0 %) p=1.000
		IIIc	3/3 (100 %)	3/3 (100 %) p=1.000
	FGFR2	IIIb	2/3 (66 %)	3/3 (100 %) p=1.000
		IIIc	2/3 (66 %)	3/3 (100 %) p=1.000
	FGFR4		3/3 (100 %)	3/3 (100 %) p=1.000

NCM:non-cancerous mucosa

8. Acknowledgments

The present thesis is a summary of my studies from 2010 to 2015 at the Department of Biotechnology, Faculty of Engineering, Kyoto Sangyo University.

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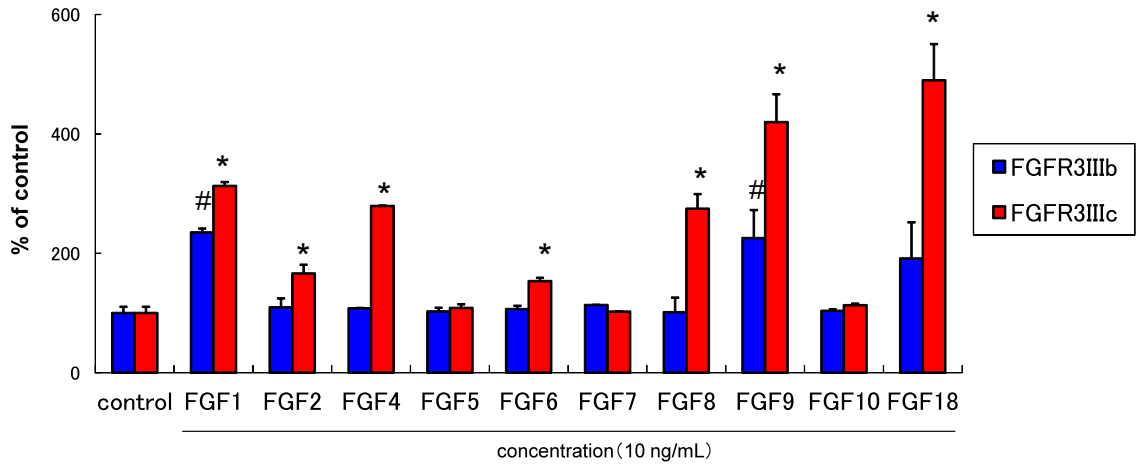


Fig. S1 Mitogenic response of BaF3 cells expressing FGFR3IIIb or FGFR3IIIc stimulated with FGFs. Cell proliferation was assayed by counting the number of BaF3 cells stably expressing FGFR3IIIb or FGFR3IIIc. Cells were washed twice with RPMI1640 containing 10% FBS but lacking IL-3. The cells were plated at 1 x 10^4 cells per well in a total volume of 500 μL medium in 24-multiwell plates. FGFs (10 ng/mL) were added in the presence of heparin (10 μg/mL). FGFs and Heparin were not added in the control. After 3 days, the viable cells were harvested and counted by a Coulter Counter (ZM type; Beckman Coulter Co.). Experiments were performed in triplicate and the results are expressed as means ± standard deviations (#, $p<0.05$ versus the control of FGFR3IIIb expressed BaF3 cells and *, $p<0.05$ versus the control of FGFR3IIIc expressed BaF3 cells, t-test). FGFR3IIIb expressed BaF3 cells significantly promoted cell proliferation with FGF1 and FGF9. On the other hand, FGFR3IIIc expressed BaF3 cells significantly promoted cell proliferation with FGF1, FGF2, FGF4, FGF6, FGF8, FGF9, and FGF18, suggesting that response to FGFs is different between FGFR3IIIb isoform and FGFR3IIIc isoform, because of different amino acid sequence between C-terminal half of IgIIIb in FGFR3IIIb isoform and that of IgIIIc in FGFR3IIIc isoform (Fig. 1B).

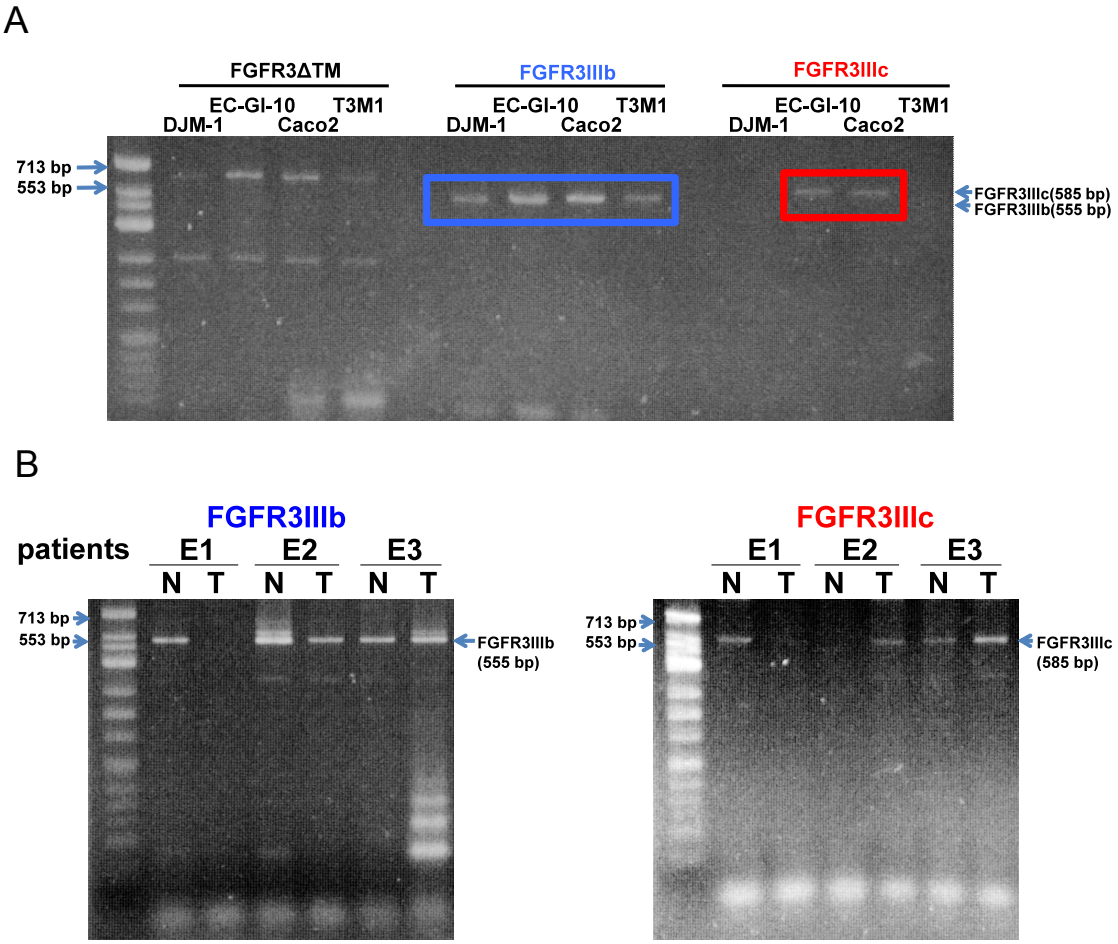


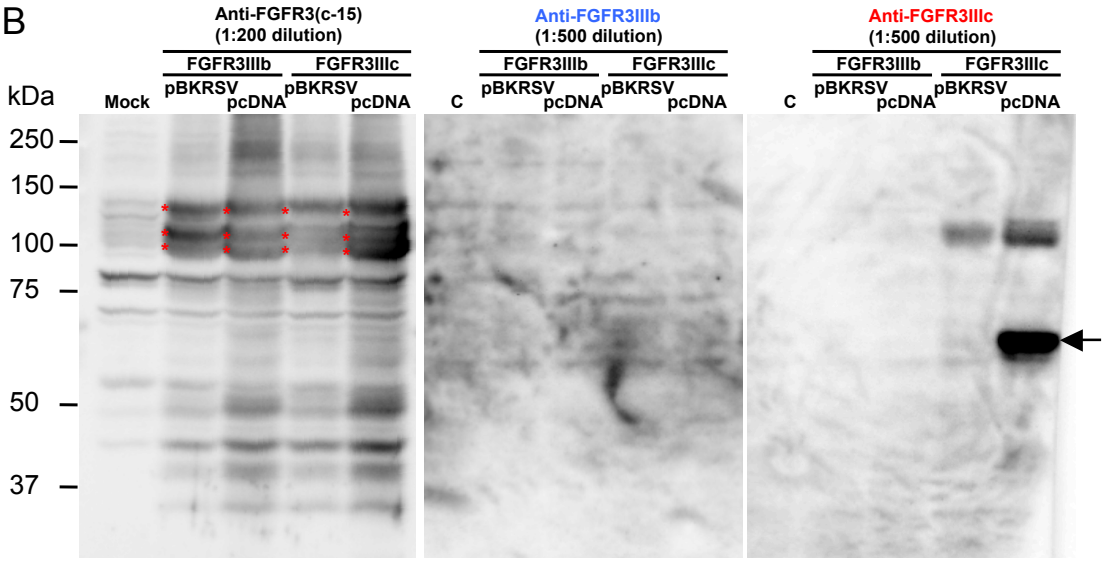
Fig. S2 RT-PCR analysis of FGFR3IIIb or FGFR3IIIc. (A) Total RNAs were extracted from various human cells with Isogen according to the procedure specified by the manufacturer. RT-PCR was then performed using the primers for FGFR3IIIb and FGFR3IIIc (see “Materials and Methods”). The products of amplification were separated on a 3.5 % agarose gel and stained with ethidium bromide. The 555 bp DNA bands indicate that FGFR3IIIb was expressed in all human cells tested here. The 585 bp DNA bands indicate that FGFR3IIIc was expressed in EC-GI-10 cells and Caco2 cells.

DJM-1 cells (skin squamous cell carcinoma), EC-GI-10 cells (esophageal squamous cell carcinoma), Caco2 cells (colorectal adenocarcinoma), and T3M1 (oral squamous cell carcinoma), (B) The 555 bp DNA bands indicate that FGFR3IIIb was expressed in NCM (N) or EC (T) from 3 EC patients (E1 to E3). The 585 bp DNA bands indicate that FGFR3IIIc was expressed in NCM (N) or EC (T) from 3 EC patients (E1 to E3) (Table 2).

A

Antibody	Company	Source	Specificity
Anti-FGFR3 (c-15)	SantaCruz (cat#sc-123)	Rabbit polyclonal	FGFR3IIIb FGFR3IIIc
Anti-FGFR3IIIb	R&D (cat#MAB1474)	Mouse monoclonal	FGFR3IIIb
Anti-FGFR3IIIc	R&D (cat#MAB7662)	Mouse monoclonal	FGFR3IIIc

B



C

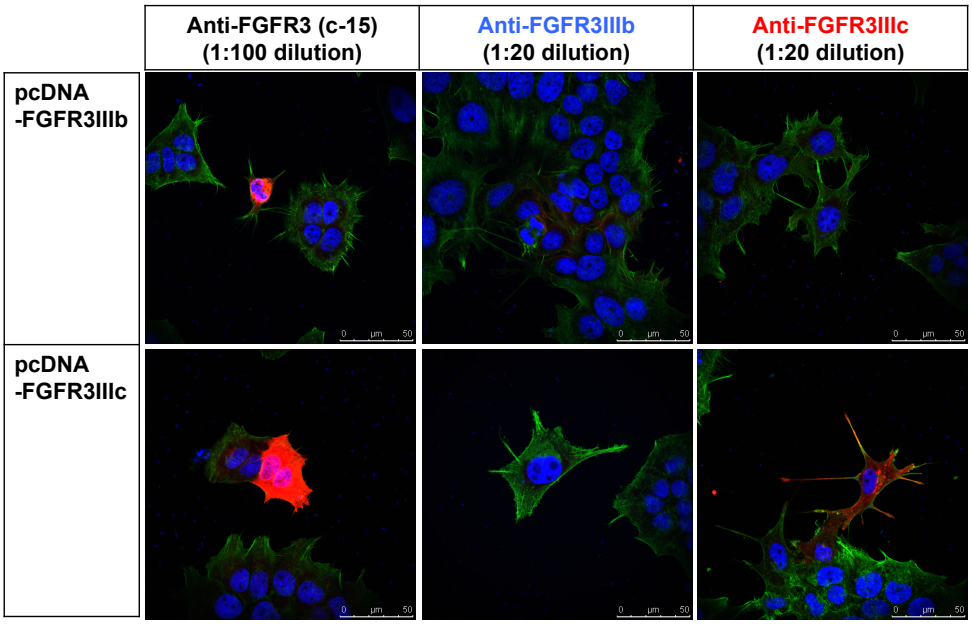


Fig. S3 Specificity of anti-FGFR3 (c-15), anti-FGFR3IIIb, and anti-FGFR3IIIc. (A) Description of three anti-FGFR antibodies. (B) Western blot analysis was performed using cell lysate from HEK293T transfected with pcDNA empty vector (Mock), pBKRSV-FGFR3IIIb or pBKRSV-FGFR3IIIc (pBKRSV), pcDNA-FGFR3IIIb or pcDNA-FGFR3IIIc (pcDNA) by FuGENE6 Transfection Reagent. Anti-FGFR3 (c-15) detected FGFR3IIIb and FGFR3IIIc isoforms (astarisk, left panel, 100～140 kDa). Anti-FGFR3IIIb did not detect either FGFR3IIIb or FGFR3IIIc isoform (center panel). Anti-FGFR3IIIc only detected FGFR3IIIc isoform (120kDa). 60 kDa band (arrow) in pcDNA-FGFR3IIIc lane is likely to be the degradation product of FGFR3IIIc isoform (C) Immunofluorescence staining of FGFR3 (red), β-actin (green), and DAPI (blue) was performed using EC-GI-10 cells transfected with pcDNA-FGFR3IIIb or pcDNA-FGFR3IIIc by FuGENE6 Transfection Reagent. Anti-FGFR3 (c-15) stained FGFR3IIIb or FGFR3IIIc overexpressed EC-GI-10 cells (upper and lower left pannel: red). Anti-FGFR3IIIb did not stain either FGFR3IIIb or FGFR3IIIc overexpressed EC-GI-10 cells (upper and lower center panel). Anti-FGFR3IIIc only stained FGFR3IIIc overexpressed EC-GI-10 cells (upper and lower right panel). Bar = 50 μm

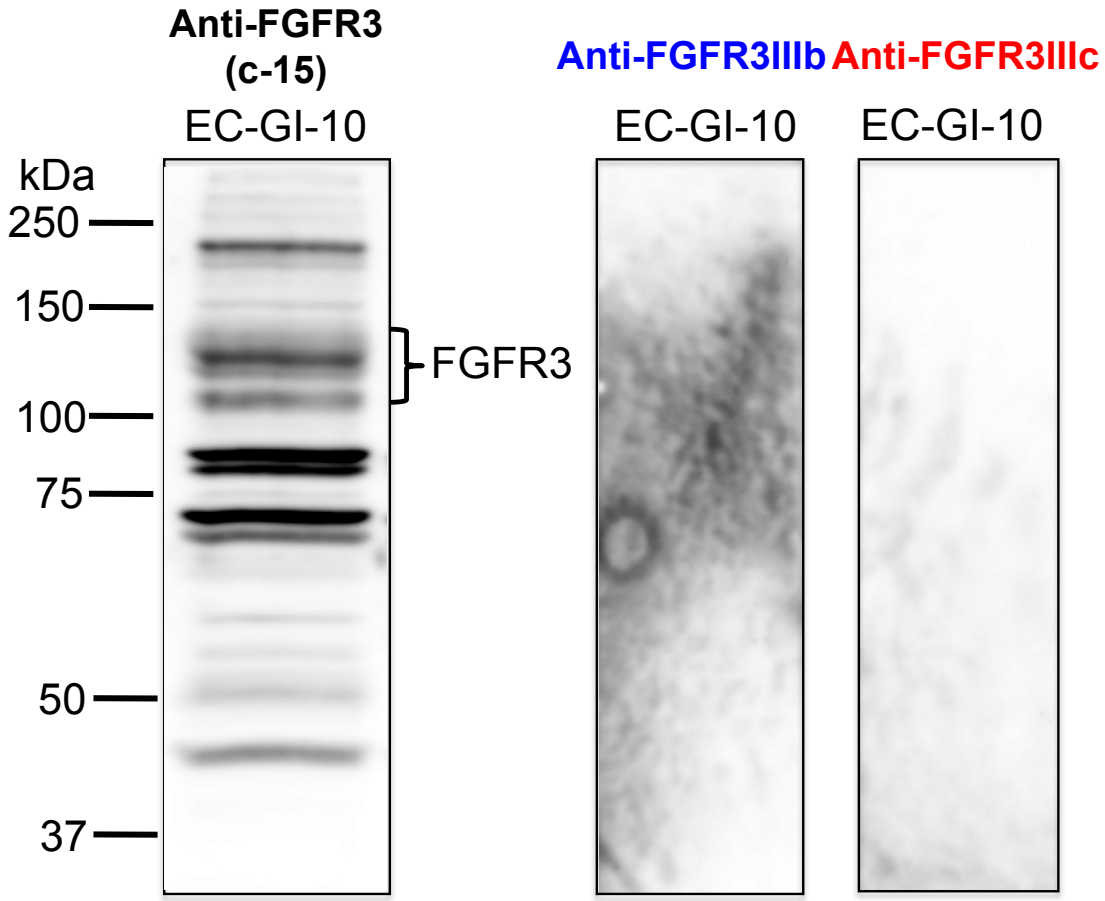
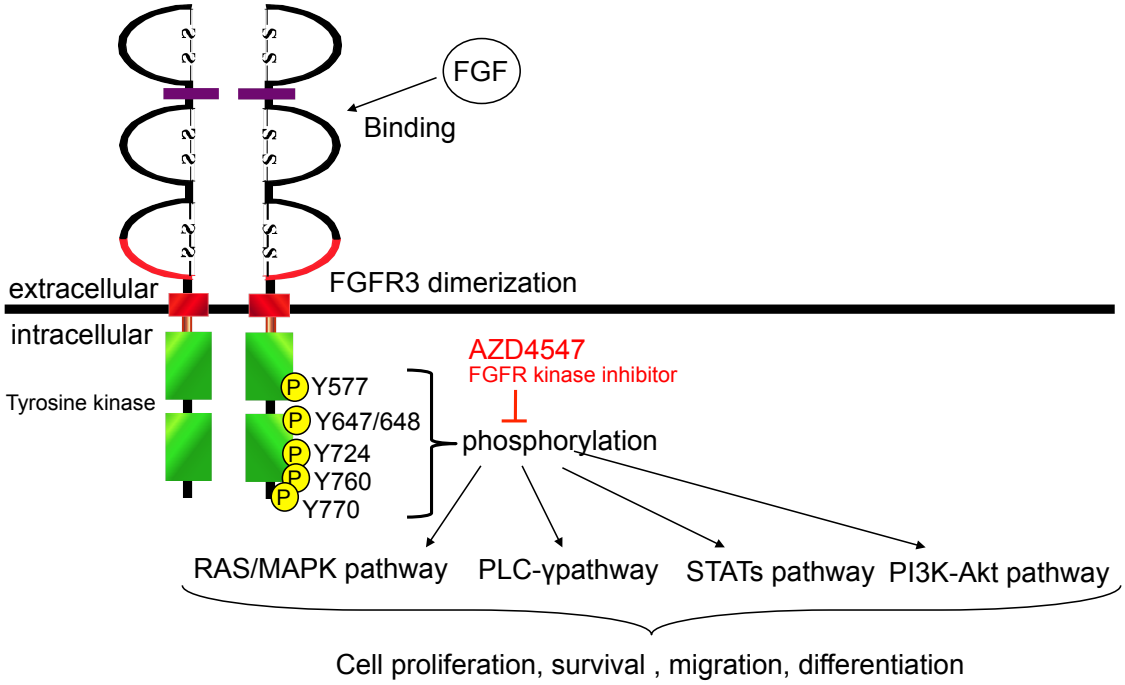


Fig. S4 Endogenous expression of FGFR3IIIb and FGFR3IIIc isoforms in EC-GI-10 cells. Western blot analysis was performed using cell lysate from parental EC-GI-10 cells. Anti-FGFR3 (c-15) (1:200 dilution) detected FGFR3 (110～140 kDa). However, anti-FGFR3IIIb (1:500 dilution) and anti FGFR3IIIc (1:500 dilution) did not detect FGFR3IIIb isoform or FGFR3IIIc isoform, respectively.

A


FGF
Binding
FGFR3 dimerization
extracellular
intracellular
Tyrosine kinase
Y577
Y647/648
Y724
Y760
Y770
AZD4547
FGFR kinase inhibitor
phosphorylation
RAS/MAPK pathway
PLC-γpathway
STATs pathway
PI3K-Akt pathway
Cell proliferation, survival , migration, differentiation

B

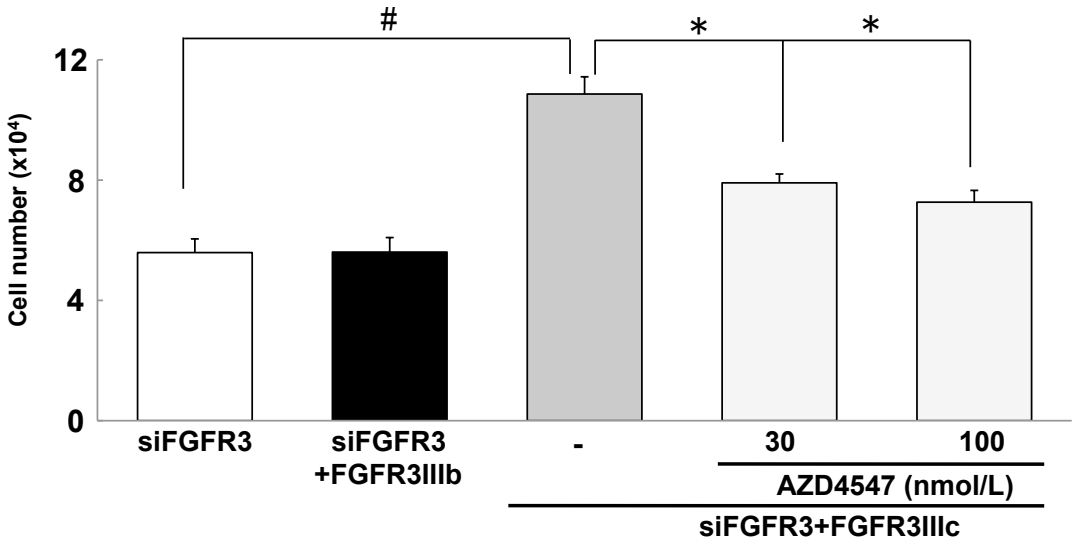

#
*
*
12
8
4
0
Cell number (x10⁴)
siFGFR3
siFGFR3
+FGFR3IIIb
-
30
100
AZD4547 (nmol/L)
siFGFR3+FGFR3IIIc

Fig. S5 FGFR3 activation and FGFR-targeting AZD4547. (A) FGF-binding to FGFR3 causes to assemble into dimers, which enables the two receptor cytoplasmic domains to cross-phosphorylate each other on multiple tyrosine residues (Y577, Y647/648, Y724, Y760, Y770). The autophosphorylated tyrosines on the intracellular domain of FGFR3 generates signals through, for example the Ras-MAPK, PI3K-Akt, STATS, and PLC-γ-PKC pathway to induce cell proliferation, survival, differentiation and migration. AZD4547, FGFR kinase inhibitor, competes for the adenosine triphosphate (ATP)-binding site and reduce the kinase activity in FGFR, suppressing cancer cell proliferation. (B) After EC-GI-10 cells were treated with siRNAs followed by lentivirus infections, these cells were plated onto a well (6 x 10^3 cells/500 μL, DMEM containing 1% FBS) in a 24 well plate. After 3 h, the cells were treated with AZD4547. The cells were collected by trypsinization after a 6-days and then the number of cells was counted. The cell proliferation of FGFR3IIIc-overexpressed siFGFR3-EC-GI-10 cells was significantly stronger than that of siFGFR3-EC-GI-10 cells (by 2-fold), whereas the overexpression of FGFR3IIIb was not. 30 nmol/L and 100 nmol/L of AZD4547 suppressed about 60% and 70% of the enhanced cell proliferation with FGFR3IIIc expression, respectively. Results are shown as the mean ± S.E. each in quintuplicate (n=5) (#, $p<0.05$ versus the siFGFR3 cells, t-test, N.S., not significant and *, $p<0.05$ versus the FGFR3IIIc cells without AZD4547 (-), Dunnett's multiple comparison test)