Expression of fibroblast growth factor receptor 2 IIIc in human uterine cervical intraepithelial neoplasia and cervical cancer

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Abstract. Fibroblast growth factor receptors (FGFRs) 1-3 have IIIb and IIIc isoforms, and we reported that FGFR2 IIIb is highly expressed in cervical keratinizing squamous cell carcinoma (SCC). In this study, we determined the expression and roles of FGFR2 IIIc in cervical intraepithelial neoplasia (CIN) and cervical cancer. In CINs 1 and 2, FGFR2 IIIc was found to be localized at the basal to lower two-thirds of the squamous epithelium, whereas it was localized in most of the squamous epithelium, except for the superficial layer in CIN 3. In situ hybridization (ISH) analysis showed that the expression patterns of FGFR2 IIIc mRNA are similar to those of FGFR2 IIIc protein in CINs. The FGFR2 IIIc protein was detected in all invasive cervical cancer patients (29 cases) and its mRNA was found to be strongly expressed in the invasive front of cancer cell nests. FGFR2 IIIc cDNA was stably transfected into CaSki cells, which are derived from a cervical SCC. The growth rates of the CaSki cells were higher than those of Mock cells in vitro, and the CaSki cells tended to form larger subcutaneous tumors in nude mice. These findings suggest that FGFR2 IIIc plays important roles in carcinogenesis and growth of cervical cancer cells. Anti-FGFR2 IIIc therapies may represent therapeutic strategies for inhibiting the growth of CIN and cervical cancer.

Introduction

Cervical cancer is the second most common cancer among women worldwide and nearly one-third of patients who present with invasive cervical cancer die of this disease (1,2). Human papilloma virus (HPV) has been established as a causative agent of cervical carcinogenesis and the HPV DNA can be detected in 99% of patients with invasive squamous cell carcinoma (SCC) of the cervix (3). The major histological type of cervical cancer is SCC and cancer cells of this type were reported to synthesize many types of growth factor or growth factor receptors including the basic fibroblast growth factor (bFGF, FGF-2), FGF-7 (keratinocyte growth factor/KGF), FGF receptor 3 (FGFR3), platelet-derived endothelial cell growth factor (PD-ECGF), vascular endothelial growth factor (VEGF)-A, VEGF-C, hepatocyte growth factor receptor (c-Met), insulin-like growth factor (IGF)-2, IGF-1 receptor (IGF-1R) and epidermal growth factor receptor (EGFR) (4-14). Serum levels of VEGF isoforms often increase in cervical cancer patients and decrease significantly following successful treatment (15-18).

The FGF family consists of 22 members, namely, aFGF (FGF-1), bFGF (FGF-2), int-2 (FGF-3), hst/K-FGF (FGF-4), FGF-5, FGF-6, FGF-7, androgen-induced growth factor (AIGF/FGF-8), glia-activating factor (GAF/FGF-9), FGF-10, FGF-11 (FGF homologous factor-3/FHF-3), FGF-12 (FHF-1), FGF-13 (FHF-2), FGF-14 (FHF-4) and FGF-16 to FGF-23 (19). FGFs bind to four high-affinity FGF receptors (FGFR1 to FGFR4) and low-affinity heparan sulfate proteoglycans that enhance ligand presentation (20). FGFRs are single transmembrane receptors, containing extracellular, transmembrane and intracellular domains (21). The extracellular domain of FGFRs 1-3 have IIIb and IIIc isoforms, and we reported that FGFR2 IIIb is highly expressed in cervical keratinizing squamous cell carcinoma (SCC). In this study, we determined the expression and roles of FGFR2 IIIc in cervical intraepithelial neoplasia (CIN) and cervical cancer. In CINs 1 and 2, FGFR2 IIIc was found to be localized at the basal to lower two-thirds of the squamous epithelium, whereas it was localized in most of the squamous epithelium, except for the superficial layer in CIN 3. In situ hybridization (ISH) analysis showed that the expression patterns of FGFR2 IIIc mRNA are similar to those of FGFR2 IIIc protein in CINs. The FGFR2 IIIc protein was detected in all invasive cervical cancer patients (29 cases) and its mRNA was found to be strongly expressed in the invasive front of cancer cell nests. FGFR2 IIIc cDNA was stably transfected into CaSki cells, which are derived from a cervical SCC. The growth rates of the CaSki cells were higher than those of Mock cells in vitro, and the CaSki cells tended to form larger subcutaneous tumors in nude mice. These findings suggest that FGFR2 IIIc plays important roles in carcinogenesis and growth of cervical cancer cells. Anti-FGFR2 IIIc therapies may represent therapeutic strategies for inhibiting the growth of CIN and cervical cancer.

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with breast and endometrial cancers, and missense mutations or copy number gains of the FGFR2 gene occur in breast and gastric cancers (22,26-28). Therefore, FGFR2 has been noted as a molecular target for cancer treatment in patients with FGFR2 mutation or gene amplification. In cervical cancer, cDNA array analysis showed higher FGFR2 expression in malignant cervical epithelial samples than in normal epithelial samples (29). Previously, we detected the expression of FGFR2 IIIb in 86% of cervical cancer patients, and this expression may mediate the growth and differentiation of reserve cells and SCC in the cervix (5). Furthermore, FGFS-1 and 2 are reported to be expressed in cervical cancer patients (13,14). GFG-2 mRNA and its protein were also found to be highly expressed in advanced cervical cancer tissues, and the serum FGF-2 protein level was suggested to be a useful marker for the detection of primary tumors and recurrences, and monitoring of cancer therapy (13,30). However, there has been no report on the expression and role of FGFR2 IIIc in cervical cancer. In this study, we examined whether FGFR2 IIIc mRNA and protein are expressed in cervical intraepithelial neoplasia (CIN) and invasive cervical cancer tissues. FGFR2 IIIc expression was also examined in cervical cancer cell lines.

We now report that the FGFR2 IIIc expression pattern is correlated with CIN progression. FGFR2 IIIc was expressed in all the tested cancer cell lines and SCC in cervical cancer patients. Stably transfected FGFR2 IIIc in CaSki cells induced the growth of cells in vitro and in vivo.

Materials and methods

Materials. The following were purchased: recombinant human FGFR 2α(IIIb)/Fc and FGFR 2α(IIIc)/Fc chimera proteins from R&D Systems, Inc. (Minneapolis, MN, USA); horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibodies from American Qualex (San Clemente, CA, USA); rabbit polyclonal anti-human IgG and mouse monoclonal anti-Ki-67 antibodies from Dako Japan (Tokyo, Japan); Vectashield mounting medium containing 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) from Vector Laboratories, Inc. (Burlingame, CA, USA); Histofine Simple Stain MAX PO (R) or (M) kits from Nichirei (Tokyo, Japan); Matsunami Adhesive Slide Glass Ind., Ltd. (Osaka, Japan); Vectashield mounting medium (MAS) and 4-well glass bottom dishes from Matsunami (Yonezawa, Japan); Super Signal West Dura chemiluminescent substrate from Thermo Fisher Scientific, Inc. (Yonezawa, Japan); Immobilon P transfer membrane from Millipore Corporation (Billerica, MA, USA); rabbit IgG and pcDNA 3.1(-) or (+) vector from Invitrogen (Carlsbad, CA, USA); rabbit polyclonal anti-human IgG and mouse secondary antibodies from American Qualex (San Clemente, CA, USA); Alexa 488-conjugated goat anti-rabbit IgG and polyclonal anti-human IgG and mouse monoclonal anti-Ki-67 antibodies from Dako Japan (Tokyo, Japan); Immobilon P transfer membrane from Millipore (Yonezawa, Japan); Super Signal West Dura chemiluminescent substrates from Thermo Fisher Scientific, Inc. (Waltham, MA, USA); Alexa 488-conjugated goat anti-rabbit IgG and pcDNA 3.1(-) or (+) vector from Invitrogen (Carlsbad, CA, USA); RNAsin Plus and a FastPure RNA kit from Takara Bio, Inc. (Shiga, Japan); a digoxigenin (DIG) nucleic acid detection kit and a DIG RNA labeling kit from Roche Diagnostics GmbH (Mannheim, Germany); a High Capacity cDNA Reverse Transcription kit, TaqMan Universal PCR Master Mix and a TaqMan MGB probe for ribosomal eukaryotic 18S RNA from Applied Biosystems (Foster City, CA, USA); Histofine Simple Stain MAX PO (R) or (M) kits from Nichirei (Tokyo, Japan); Matsunami Adhesive Slide (MAS) and 4-well glass bottom dishes from Matsunami Glass Ind., Ltd. (Osaka, Japan); Vectashield mounting medium containing 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) from Vector Laboratories, Inc. (Burlingame, CA, USA); pRES2-EGFP vector from Clontech (Palo Alto, CA, USA); genetin from Gibco BRL (Grand Island, NY, USA); WST-8 cell counting kit from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals and reagents were purchased from Sigma Chemical Corp. (St. Louis, MO, USA).

Non-cancerous, CIN and cancerous cervical tissues. Cervical cancer samples (29 patients; mean age, 53 years; median age, 55; range, 29-72 years) were obtained from patients undergoing surgery for invasive cervical cancer at the Department of Obstetrics and Gynecology of Nippon Medical School Hospital (Bunkyo-ku, Tokyo, Japan) from 2005 to 2008 (Table I). On the basis of the classification of the International Federation of Gynecology and Obstetrics (FIGO), the stages of the patients were classified as follows: stage Ia, 5; stage Ib, 13; stage IIa, 1; stage IIb, 10. On the basis of the World Health Organization (WHO) Classification of Tumors, the patients were classified into the keratinizing type (6 patients) and non-keratinizing type (21 patients) of SCC. Fifteen samples each for CINs 1-3 were obtained by cervical biopsies at the same hospital and used for this study. For control samples, cervical biopsies from patients with chronic cervicitis (10 patients) were used. Paraffin-embedded specimens were prepared for immunohistochemical analysis as previously described (31). This study was carried out following the ethics guidelines of Nippon Medical School and the principles embodied in the Declaration of Helsinki, 1975.

Human cervical cancer cell lines. ME-180 cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). CaSki cells were obtained from RIKEN BioResource Center (Ibaraki, Japan). CaSki and ME-180 cells were grown in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 200 U/ml

Table I. Clinical features of cervical cancer patients.

<table>
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<th>Age (years)</th>
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<td>Median</td>
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<td>Mean</td>
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<td>Ia</td>
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<td>IIa</td>
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<td>IIb</td>
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<td>Keratinization</td>
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<td>Absent</td>
<td>21</td>
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<td>8</td>
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<td>Nodal metastasis</td>
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<td>Absent</td>
<td>20</td>
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<td>9</td>
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<td>HPV infection</td>
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<td>1</td>
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<td>Present</td>
<td>19</td>
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<td>Not examined</td>
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penicillin and 200 μg/ml kanamycin at 37°C under a humidified 5% CO₂ atmosphere.

**Rabbit polyclonal anti-human FGFR2 IIIc antibody.** To confirm the expression of FGFR2 IIIc at the protein level in cervical cancer, a specific antibody against human FGFR2 IIIc was prepared. The anti-FGFR2 IIIc antibody used was an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to the amino acids of the human FGFR2 IIIc protein (accession no. NM_000141). The peptide sequence ‘AAGVNTTDKEIVLYIR’ is located at the carboxyl-terminal half of the Ig loop closest to the transmembrane region that is specific for FGFR2 IIIc. To estimate the reactivity of the anti-FGFR2 IIIc antibody, FGFR 2α(IIIb)/Fc and FGFR 2α(IIIc)/Fc chimera proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred to Immobilon P transfer membranes, which were then incubated (SDS-PAGE), and the separated proteins were transferred to Immobilon P transfer membranes, which were then incubated for 10% SDS-PAGE, and the separated proteins were transferred to Immobilon P transfer membranes, which were then incubated overnight with the anti-FGFR2 IIIc antibody (1:6000 dilution) at 4°C. The membranes were washed and incubated with HRP-conjugated anti-rabbit IgG antibody for 60 min at room temperature (RT). After washing, the blot was visualized by enhanced chemiluminescence. The membrane was reblotted with rabbit polyclonal anti-human IgG antibody to confirm the equal loading.

**Immunohistochemistry.** Paraffin-embedded sections (3 μm) were subjected to immunostaining using a Histofine Simple Stain MAX PO (R) kit for FGFR2 IIIc and MAX PO(M) kit for Ki-67. After deparaffinization, the tissue sections for Ki-67 immunostaining were preheated in 10 mM citrate buffer (pH 6.0) for 15 min at 121°C in an autoclave oven. Then, endogenous peroxidase activity was blocked by incubation for 30 min with 0.3% hydrogen peroxide in methanol. The tissue sections were then incubated with the anti-FGFR2 IIIc antibody (1:200 dilution) or anti-Ki-67 antibody (1:100 dilution) in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) for 16 h at RT. Bound antibodies were detected with the Simple Stain MAX PO (R) or (M) reagent, using diaminobenzidine tetrahydrochloride as the substrate. The sections were then counterstained with Mayer's hematoxylin. Negative control tissue sections were prepared by omitting the primary antibody.

**Probe preparation.** A BamHI-EcoRI-adaptor-conjugated cDNA fragment, corresponding to nucleotides 1591-1740 of the human FGFR2 IIIc cDNA sequence (150 bp, accession no. NM_000141), was generated by PCR amplification of single-stranded cDNA that was reverse-transcribed from human fibroblast RNA as previously reported (32). The human FGFR2 IIIc cDNA fragments were subcloned into the pcDNA 3.1(-) or (+) vector and the authenticity was confirmed by sequencing. The antisense or sense probes were labeled with digoxigenin-UTP using T7 RNA polymerase from the DIG RNA-labeling kit.

**In situ hybridization (ISH) analysis.** ISH was performed as previously reported (33-35). Tissue sections were deparaffinized and incubated at RT for 20 min with 0.2 N HCl and then at 37°C for 15 min with 100 μg/ml proteinase K. The sections were then postfixed for 5 min in PBS containing 4% paraformaldehyde (PFA), and incubated twice for 15 min each with PBS containing 2 mg/ml glycine at RT and once in 50% (v/v) formamide/2X SSC for 1 h at 42°C prior to the initiation of the hybridization reaction. The hybridization buffer contained 0.6 M NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 7.6), 0.25% SDS, 200 mg/ml yeast RNA, IX Denhard's solution, 10% dextran sulfate, 40% formamide and 200 ng/ml of the indicated digoxigenin-labeled riboprobe. Hybridization was performed in a moist chamber for 16 h at 42°C. The sections were then washed sequentially with 2X SSC for 20 min at 42°C, followed by 0.2X SSC for 20 min at 42°C. For immunolological detection, the DIG nucleic acid detection was used. The sections were washed briefly with buffer 1 (100 mM Tris-HCl and 150 mM NaCl, pH 7.5), incubated with 1% (w/v) blocking reagents in buffer 1 for 60 min at RT, and with alkaline-phosphatase-conjugated polyclonal sheep anti-digoxigenin Fab fragment containing 0.2% Tween-20 at 1:2000 dilution for 60 min at RT. The sections were then washed twice for 15 min at RT with buffer 1 containing 0.2% Tween-20, equilibrated with buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂, pH 9.5) for 2 min, and incubated with a staining solution containing nitroblue tetrazolium and X-phosphate in a dark box for 1 h. After the reaction was stopped with TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), the sections were mounted in an aqueous mounting medium.

**Quantitative real-time PCR of FGFR2 IIIc in cervical cancer cell lines.** The cervical cancer cells were grown in RPMI-1640 medium with 10% FBS for 48 h. Total RNA extraction from cervical cancer cells was performed using RNAiso Plus and then purified with a FastPure RNA kit. Then, cDNA synthesis was performed using High Capacity cDNA Reverse Transcription kit following the manufacturer's protocol. Quantitative real-time PCR (Q-PCR) was performed using the ABI PRISM 7900HT Sequence Detection System. The real-time PCR primers used for FGFR2 IIIc are nucleotides (nts) 1693-1716 (5'-GGA-TAT-CCT-CTC-CTG-CAT-GGT-3') and nts 1770-1794 (5'-TGC-AGT-AAA-TGG-CTA-TCT-CCA-GGT-A-3') of the human FGFR2 IIIc cDNA (102 bp, accession no. NM_000141). As TaqMan probes, 5'-CAG-TTC-TGC-CAG-CGC-CTG-GAA-GA-3' was used for FGFR2 IIIc. The PCR reaction mixture (50 μl) contained 2 μl of template cDNA, 0.9 μM primers, 0.25 μM probe and 25 μl of TaqMan Universal PCR Master Mix. 18S rRNA, as the internal positive control, was amplified using the TaqMan MGB probe for ribosomal eukaryotic 18S rRNA. The optimized program included uracil N-glycosylase incubation at 50°C for 2 min and AmpliTaq Gold activation at 95°C for 10 min, followed by 50 cycles of amplification at 95°C for 15 sec and at 60°C for 60 sec for FGFR2 IIIc and RS-18. Results were expressed as target/RS-18 as an internal standard concentration ratio. Gene expression measurements were performed in triplicate.

**Immunofluorescence staining and confocal laser microscopy.** The same anti-FGFR2 IIIc antibody used for the immunohistochemistry was also used for immunofluorescence staining of ME-180 and CaSki cells. The cells were incubated with...
the anti-FGFR2 IIIc antibody (1:50) in PBS containing 1% BSA for 18 h at 4°C. For negative control, the cells were incubated with PBS containing 1% BSA. The cells were washed with PBS and then incubated with Alexa 488-conjugated goat anti-rabbit IgG. One hour after incubation, the cells were washed with PBS, and then mounted with Vectashield mounting medium containing DAPI. Fluorescent images were acquired using a confocal laser scanning microscope Digital Eclipse TE 2000-E (Nikon Insteck Co., Ltd., Tokyo, Japan) and a 100X immersion lens (Nikon Palm Apo VC) with blue diode (BD) and helium-neon lasers and were analyzed using the confocal microscope Digital Eclipse C1 control software EZ-C1 (version 2.30) (Nikon Insteck). The excitation wavelength for Alexa 488 was 488 nm, and the emission was selected and recorded using a 500- to 530-nm band-pass filter. In addition, the excitation wavelength for DAPI was 405 nm, and the emission was selected and recorded using a 432- to 446-nm band-pass filter.

**Construction of FGFR2 IIIc expression vector and generation of stably transfected clones.** Full-length FGFR2 IIIc cDNA fragment was ligated to the 3'-end of the human cytomegalovirus early promoter/enhancer in pIRES2-EGFP, a eukaryotic expression vector. Proper orientation of the insert was verified by DNA sequencing. CaSki cells (~1×10⁵/ml) were transiently transfected with pIRES2-EGFP, which contained a small cDNA fragment of FGFR2 IIIc. Following incubation, the cells were passaged and cultured with geneticin (500 μg/ml). Stable FGFR2 IIIc-transfected CaSki cells, empty-vector-transfected cells (Mock cells) and non-treated cells (wild-type) were incubated in RPMI-1640 medium with 10% FBS for 48 h. The morphology of the cells was observed using a phase-contrast microscope (Nikon Eclipse TE2000-U).

**Anchorage-dependent growth assays.** To examine the growth rates of FGFR2 IIIc-transfected clones, a non-radioactive cell proliferation assay was performed. The cells were cultured in RPMI-1640 medium with 10% FBS at a density of 1×10⁴ in 96-well plates followed by incubation at 37°C in a humidified 5% CO₂ atmosphere for 24, 48, 72 and 96 h. Then, the cells were incubated with WST-8 cell counting reagent for 4 h at 37°C and the optical density of the culture solution in the plate was measured using an ELISA plate reader (Bio-Rad Laboratory, Hercules, CA) at 450 nm. The experiments were performed in triplicate.

**Single-cell movement assay.** CaSki cells (5000 per well) were seeded onto a 4-well glass bottom dish and grown for 48 h. Cell movement was then monitored for 24 h using a Digital Eclipse TE 2000-E motorized inverted microscope taking pictures every 5 min. The total distance of individual cells covered within 24 h was determined using the Metamorph software 7.6 (Universal Imaging Corp., Ltd., Buckinghamshire, UK).

**In vivo tumorigenicity assay.** To assess the effect of FGFR2 IIIc expression on tumorigenicity, 3×10⁶ cells/animal were injected into 6-week-old, female, athymic (nude) mice (n=17). The CaSki cells were injected subcutaneously on both sides of the back. The animals were monitored for tumor formation thrice weekly and sacrificed 30 days after injection. Tumor volume was calculated using the formula: volume = a x b² x 0.5, where a is the longest diameter and b is the shortest.

**Statistical analysis.** All quantitative data are presented as mean ± SE and assessed using Student’s t-test and Mann-Whitney test. Computations were performed using the Stat View J version 5.0 software package (SAS Institute, Inc., Cary, NC).

**Results**

**Western blot analysis of anti-FGFR2 IIIc antibody.** To examine the FGFR2 IIIc expression at the protein level in cervical cancer, we prepared rabbit polyclonal anti-human FGFR2 IIIc antibody. FGFR2 IIIc and FGFR2 IIIb have high amino acid homology; thus, we examined the specificity of the antibody to FGFR2 IIIc using recombinant FGFR 2α(IIIc)/Fc chimera proteins (upper panel, left lane). Anti-human IgG antibody reacted with each recombinant protein on the reblotted membrane (lower panel). In contrast, the antibody did not cross-react with recombinant FGFR 2α(IIIb)/Fc chimera proteins (upper panel, right lane). Loading control of anti-human IgG antibody reacted with recombinant protein on the reblotted membrane (lower panel). Western blot analysis of anti-FGFR2 IIIc antibody. Polyclonal anti-FGFR2 IIIc antibody reacted with recombinant FGFR 2α(IIIc)/Fc chimera proteins (upper panel, right lane), while the antibody did not react with recombinant FGFR 2α(IIIb)/Fc chimera proteins (upper panel, left lane). Loading control of anti-human IgG antibody reacted with each recombinant protein on the reblotted membrane (lower panel).

**Immunohistochemical and ISH analyses of FGFR2 IIIc in non-cancerous cervical tissues.** Immunohistochemical and...
in situ hybridization analyses were performed to examine the localization of FGFR2 IIIc protein and its mRNA in cervical tissues. In non-cancerous cervical tissues, the FGFR2 IIIc protein was not or very faintly localized in squamous epithelial cells (Fig. 2A) and its mRNA was expressed in basal cells of squamous epithelium (Fig. 2B, arrows). Ki-67 was localized in parabasal cells in non-cancerous squamous epithelium (Fig. 2C). The sense probe did not detect any signals (Fig. 2D).

**Immunohistochemical and ISH analyses of FGFR2 IIIc in CIN tissues.** In CIN 1, FGFR2 IIIc and its mRNA were detected in the basal to one-third of the epithelial layers (Fig. 3A and D, respectively). Ki-67 was localized at the parabasal to lower one-third of the epithelial layers (Fig. 3G). In CIN 2, FGFR2 IIIc, its mRNA and Ki-67 were localized between the basal layer and two-thirds of the squamous epithelial layers (Fig. 3B, E and H, respectively). In CIN 3, FGFR2 IIIc, its mRNA and Ki-67 were strongly expressed in most squamous epithelial layers, except for the superficial layers (Fig. 3C, F and I, respectively). The sense probes did not yield any positive signals (Fig. 3J-L).

**Immunohistochemical and ISH analyses of FGFR2 IIIc in cervical cancer tissues.** In cervical cancer tissues, the non-
keratinizing type of cancer showed solid cancer cell proliferation and the keratinizing type of cancer showed keratinizing foci in cancer cell nests [(A) and (B), respectively]. Immunohistochemically, the FGFR2 IIIc protein was diffusely localized in cancer cell nests in the non-keratinizing type of cells, and strongly expressed in the central portions with keratinization in the keratinizing type [(C) and (D), respectively]. Moreover, the FGFR2 IIIc mRNA was strongly expressed in the invasive front of cancer cell nests [(E) and (F), arrows]. H&E, (A) and (B); immunohistochemistry, (C) and (D); in situ hybridization, (E) and (F); original magnification, (A)-(F) x200.

Expression of FGFR2 IIIc in cervical cancer cell lines. To examine whether cervical cancer cells express FGFR2 IIIc, Q-PCR was performed. FGFR2 IIIc mRNA was expressed in two cervical cancer cell lines. The expression level of FGFR2 IIIc mRNA was higher in CaSki cells than in ME-180 cells (Fig. 5). Immunofluorescent analysis showed that the FGFR2 IIIc protein is expressed in the cell membrane and cytoplasm of ME-180 and CaSki cells (Fig. 6A and B) and the FGFR2 IIIc level was higher in CaSki cells than in ME-180 cells.

Negative control without primary antibody showed no positive staining (Fig. 6C).

Stable FGFR2 IIIc-transfected CaSki cells. To examine the role of FGFR2 IIIc in CaSki cells, we prepared FGFR2 IIIc-overexpressing cervical cancer cells. A full-length FGFR2 IIIc cDNA was subcloned into pIREs2-EGFP vector and stably transfected in these cells. The expression levels of
FGFR2 IIIc mRNA in Mock and FGFR2 IIIc-transfected CaSki cells were examined by Q-PCR. FGFR2 IIIc mRNA/18S rRNA levels were high in three clones (IIIc-3, IIIc-4 and IIIc-8), whereas they were low in Mock cells (Mocks-1, -4 and -9). The expression level of FGFR2 IIIc mRNA was >500-fold higher in FGFR2 IIIc-transfected CaSki cells than in Mock cells.

Morphological alteration of FGFR2 IIIc-transfected CaSki cells. The FGFR2 IIIc-transfected CaSki cells did not show characteristic morphological alterations as compared with Mock cells (Fig. 8). The cells possessed polygonal or short spindle-like cytoplasm with branched projections.

Cell proliferation assay of FGFR2 IIIc-transfected CaSki cells in vitro. The FGFR2 IIIc-transfected CaSki and Mock cells were cultured in RPMI-1640 medium containing 10% FBS for 24, 48, 72, 96 and 120 h. Three clones of stable FGFR2 IIIc-transfected CaSki cells (Fig. 9; IIIc-3, IIIc-4 and IIIc-8) showed an increase in proliferation as compared with Mock cells (Mocks-1, 4 and 9). After 96 h, all three clones of FGFR2 IIIc-transfected cells showed significantly higher growth rates than the three Mock clones (P<0.002).

Single-cell movement assay. Single-cell movement was characterized next to determine whether FGFR2 IIIc is involved in cellular migration. Single-cell movement was monitored by time-lapse microscopy [(A) and (B), respectively]. The distances covered within 24 h by Mocks-1 and -4 and IIIc-3 and IIIc-4 cells showed no statistical difference (C).
A large number of growth factors and their receptors were reported to be expressed in CIN tissues. A significant positive correlation was found between VEGF-A, VEGF-C, VEGF-D and VEGFR-3 expressions with the different stages of cervical carcinogenesis (36). Significant differences in protein expressions for VEGF-C, VEGF-D and VEGFR-3 were found between CINs 1-2 and CIN 3, but not between CIN 3 and cervical cancer (6). CINs 2-3 exhibited higher VEGF mRNA, transforming growth factor (TGF)-β1 and TGF-β receptor 1 levels than CIN 1 (37). The expression levels of insulin-like growth factor 1 receptor (IGF-1R) and phosphorylated IGF-1 were positively correlated with the CIN grades (7). The expressions and roles of FGFR and FGFR members have not been reported. In this study, we found that FGFR2 IIIc is clearly expressed in dysplastic or neoplastic cells in CIN. FGFR2 IIIC expression patterns in CIN were similar to the expression pattern of Ki-67, one of the reliable cell proliferation markers. These findings may indicate that FGFR2 IIIC expression is closely related to the growth of neoplastic cells in CIN and carcinogenesis.

In cervical cancer tissues, FGFR2 IIIB was detected in 94% of patients with keratinizing type and 79% of patients with non-keratinizing type of cervical cancer (5). Furthermore, FGFR2 IIIB expression was detected in CaSki cells but not in ME-180 cells. CaSki cells have a large and pleomorphic cytoplasm and their histological appearance is similar to that of well-differentiated SCC, whereas ME-180 cells have a round or oval cytoplasm and a high nuclear/cytoplasm ratio similar to that of poorly differentiated SCC. In contrast, FGFR2 IIIC was expressed in all cervical cancer patients including keratinizing and non-keratinizing types and it was expressed in both CaSki and ME-180 cells. These results suggest that most patients with cervical cancer show overexpression of both FGFR2 IIIB and IIIC isoforms. The reason for the FGFR2 IIIC overexpression in cervical cancer is not yet clear, but gene amplification, mutation or spliceosome dysregulation of FGFR2 might occur in the cancer cells. HPV infection was detected in 99% of invasive SCC of the cervix, and most of our examined patients had HPV infection. Thus, further examination of the correlation between HPV infection and FGFR2 expression, including whether HPV induces FGFR2 expression in cervical cancer, is necessary (15).

FGFR2 IIIC is widely expressed in various histological types of cancer cells, including adenocarcinoma, SCC and transitional cell carcinoma. Most studies indicated that FGFR2 IIIC expression is correlated with the aggressive growth of cancer cells (38,39). In cervical cancer cases, FGFR2 IIIC mRNA was strongly expressed in the area of cancer cell infiltration in cancer tissues. The higher expression of FGFR2 IIIC in most cervical cancer cells and the strong expression in invasive fronts suggest that FGFR2 IIIC plays important roles in cancer cell growth. To the best of our knowledge, this is the first report on FGFR2 IIIC expression in cervical cancer.

To clarify whether FGFR2 IIIC contributes to the growth of cervical cancer cells, we attempted to prepare stable FGFR2 IIIC-transfected cancer cells. We were not able to successfully transfect the FGFR2 IIIC-expression vectors to ME-180 cells, but FGFR2 IIIC-transfected CaSki cells expressed high levels of FGFR2 IIIC mRNA, and the cells...
rapidly grew, both in vitro and in vivo. Single-cell movement assay was performed to determine whether FGFR2 IIIc correlates with cervical cancer cell migration, but no significant difference between FGFR2 IIIc-transfected and Mock cells was found. These findings indicate that FGFR2 IIIc directly correlates with cervical cancer cell growth, but not with cell infiltration. Further studies on the inhibition of FGFR2 IIIc expression in cancer cells, including the use of small interfering RNA (siRNA), short hairpin RNA (shRNA) and monoclonal anti-FGFR2 IIIc antibodies, are necessary to determine the growth regulation of cervical cancer. Previously, a close relationship between FGFR2 IIIc expression and epithelial-mesenchymal transition (EMT) was reported (40). In this study, no stable FGFR2 IIIc-transfected CaSk i cells showed spindle-like appearance. Further studies including expression induced cervical cancer cell growth. FGFR2 IIIc showed spindle-like appearance. Further studies including expression induced cervical cancer cell growth. FGFR2 IIIc may be a new molecular target for cervical cancer therapy.

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References