Dual Tyrosine Kinase Inhibitor for Focal Adhesion Kinase and Insulin-like Growth Factor-I Receptor Exhibits Anticancer Effect in Esophageal Adenocarcinoma \textit{In vitro} and \textit{In vivo}

Nobuyuki Watanabe,¹ Munenori Takaoka,¹ Kazufumi Sakurama,¹ Yasuko Tomono,³ Shinji Hatakeyama,⁵ Osamu Ohmori,⁹ Takayuki Motoki,¹ Yasuhiro Shirakawa,¹ Tomoki Yamatsuji,¹ Minoru Haisa,⁴ Junji Matsuoka,¹ David G. Beer,⁶ Hitoshi Nagatsu,² Noriaki Tanaka,³ and Yoshio Naomoto¹

Abstract

\textbf{Purpose:} Focal adhesion kinase (FAK) regulates integrin and growth factor – mediated signaling pathways to enhance cell migration, proliferation, and survival, and its up-regulation correlates malignant grade and poor outcome in several types of cancer. In this study, we aimed to raise a potential therapeutic strategy using a FAK inhibitor for Barrett’s esophageal adenocarcinoma.

\textbf{Experimental Design:} The expression status of FAK in clinical Barrett’s esophageal adenocarcinoma tissues was determined by immunohistochemistry. Cultured esophageal adenocarcinoma cells were treated with TAE226, a specific FAK inhibitor with an additional effect of inhibiting insulin-like growth factor-I receptor (IGF-IR), to assess its anticancer effect \textit{in vitro}. Western blot was carried out to explore a participating signaling pathway for TAE226-induced cell death. Furthermore, TAE226 was orally administered to s.c. xenograft animals to investigate its anticancer effect \textit{in vivo}.

\textbf{Results:} Strong expression of FAK was found in 94.0\% of Barrett’s esophageal adenocarcinoma compared with 17.9\% of Barrett’s epithelia, suggesting that FAK might play a critical role in the progression of Barrett’s esophageal adenocarcinoma. When esophageal adenocarcinoma cells were treated with TAE226, cell proliferation and migration were greatly inhibited with an apparent structural change of actin fiber and a loss of cell adhesion. The activities of FAK, IGF-IR, and AKT were suppressed by TAE226 and subsequent dephosphorylation of BAD at Ser136 occurred, resulting in caspase-mediated apoptosis. \textit{In vivo} tumor volume was significantly reduced by oral administration of TAE226.

\textbf{Conclusions:} These results suggest that TAE226, a dual tyrosine kinase inhibitor for FAK and IGF-IR, could become a new remedy for Barrett’s esophageal adenocarcinoma.

Focal adhesion kinase (FAK) is a nonreceptor type tyrosine kinase composed of ~1,030 amino acids that regulates integrin and growth factor signaling pathways, thereby playing an important role in cell proliferation, differentiation, and apoptosis (1–5). It is known that FAK is activated from integrin and growth factor receptors by autophosphorylation at Tyr192 (6), followed by subsequent activation of other functional phosphorylation sites to advance the signals to downstream pathways, such as AKT (7–11). Based on these facts, FAK is thought to play a critical role in malignant behavior including proliferation, survival, and invasion (12–16). Indeed, there are several studies reporting that FAK is overexpressed in human carcinomas including breast, thyroid, ovarian, head and neck, liver, pancreas, lung, and colon cancers and that the expression status of FAK in cancers is closely related with tumor progressiveness and clinical outcome (17–25). However, there are still many questions about detailed FAK functions with surrounding molecules and its downstream signaling.

Among gastrointestinal malignancies, there is only one report that FAK overexpression seems to be involved in tumor invasiveness and lymph node metastasis in squamous cell carcinoma of the esophagus (26), whereas no detailed information is available about FAK expression and function in Barrett’s esophageal adenocarcinoma. Barrett’s esophagus is an acquired condition in which the normal squamous cell epithelium of the esophagus is replaced by a metaplastic columnar epithelium due to chronic gastroesophageal reflux.
Barrett’s esophagus is associated with a predisposition to adenocarcinoma of the distal esophagus or the gastric cardia and carries about 30 to 125 times higher risk than the general population (27). Barrett’s esophageal adenocarcinoma, which has an increasing incidence in the western world (28), is an extremely aggressive tumor with a 5-year survival rate of <25% (27). Carcinomas that arise in the setting of Barrett’s esophagus are thought to develop as part of the metaplasia-dysplasia-carcinoma sequence (27–29). Alterations in tumor suppressor genes, among which are p53 and p16, are early events in this neoplastic sequence, followed by a loss of cell cycle checkpoints and the development of tetraploidy and aneuploidy (29). These gene alterations are believed to invite additional genetic changes, resulting in carcinogenesis. Alternative expression of FAK is likely to happen during the multistep carcinogenesis of Barrett’s esophageal adenocarcinoma as well and may potentiate tumor progression and invasiveness.

Therefore, we hypothesized that FAK may be a critical molecule for cell proliferation and survival in Barrett’s esophageal adenocarcinoma. To gain insight on the potential role of FAK in Barrett’s carcinogenesis, we assessed the expression status of FAK in clinical tissues of Barrett’s epithelium as well as in esophageal adenocarcinoma. Then we determined how the inhibition of FAK signaling affects cell growth, focal adhesion, and migration using a specific small-molecule inhibitor against FAK, named TAE226. This is a novel ATP-competitive tyrosine kinase small-molecule inhibitor designed to target FAK (30) with an additional inhibitory effect on the tyrosine kinase activity of insulin-like growth factor 1 (IGF-I) receptor (IGF-IR; ref. 31). IGF-IR is one of the major receptor tyrosine kinases to activate several key molecules for cell proliferation and survival and is often up-regulated in malignancies, including Barrett’s esophageal adenocarcinoma (32). TAE226 has been reported to show a certain antitumor effect on brain tumors (30, 31), although its anticancer effect on other types of malignancies is unknown and the detailed mechanism of the effect needs to be elucidated. Our specific aims in this study are the following: (a) to determine whether FAK may play a role in Barrett’s esophageal adenocarcinoma; (b) to apply TAE226 as a novel therapeutic strategy for gastrointestinal malignancies; and (c) to explore the effectiveness and machinery of cell growth suppression by the inhibitor, especially dissecting the apoptosis pathway.

**Materials and Methods**

**Cell lines and culture conditions.** Barrett’s esophageal adenocarcinoma cell lines (SEG-1, FLO-1, and BIC-1) were used in this study. These cell lines were derived from surgically resected tissues of human Barrett’s esophageal adenocarcinoma (generously provided by Dr. David G. Beer, University of Michigan, Ann Arbor, MI). SEG-1 cells were cultured in DMEM, and FLO-1 and BIC-1 cells were cultured in high-glucose DMEM, with l-glutamine (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Gemini Biologicals), 100 units/ml penicillin G sodium, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Life Technologies). and maintained in a monolayer culture at 37°C in humidified air with 5% CO2. Cellular morphology was observed through a phase-contrast microscope during culture and experiments.

**Reagents.** TAE226, a dual tyrosine kinase inhibitor for FAK and IGF-IR, was synthesized and provided by Novartis Pharma AG through a materials transfer agreement with Okayama University. Stock solutions of the compound were reconstituted with DMSO (Sigma Chemical Co.) and diluted with culture media before use. The final DMSO concentration in all cultures was 0.025%. Reconstituent human IGF-1 was purchased from Life Technologies and reconstituted with double-distilled water to make 1 mg/mL stock solution. Reconstituted solution was further diluted with culture medium to a working concentration.

**Immunohistochemistry.** Surgically resected tissues from patients with Barrett’s esophageal adenocarcinoma were used for immunohistochemical study following procedures described previously (33). All of the clinical samples were obtained from patients who fully gave informed consent. Briefly, deparaffinized tissue sections were immersed in methanol containing 0.03% hydrogen peroxide to block endogenous peroxide activity. An autoclave pretreatment in Avidin DH-biotinylated horseradish peroxidase complex (Nichirei). Peroxide staining was done for 2 to 5 min using a solution of 3,3’-diaminobenzidine tetrahydrochloride in 50 mmol/L Tris-HCl (pH 7.5) containing 0.001% hydrogen peroxide. The sections were counterstained with 0.1% methylgreen or hematoxylin. The percentage of positive-stained tumor cells was calculated and categorized into three groups: -, 0% to 10% positive; +, 10% to 50%; and ++, >50%.

**Trypan blue exclusion assay.** SEG-1, FLO-1, and BIC-1 cells (1.0 × 10⁶ per well) were plated directly in 24-well dishes with a culture medium. Subconfluent cells were treated with different concentrations of TAE226 (0, 0.037, 0.11, 0.33, 1.0, and 3.0 μmol/L) for 48 h. After treatment, cells were harvested with trypsin, stained with trypan blue, and counted manually with a hemacytometer. Dose-effect plots were created to calculate the IC₅₀ of TAE226 for each cell line using Calcsyn software (Biosoft).

**Scratch assay.** The ability of cell migration was determined by the scratch assay. SEG-1 and FLO-1 cells were grown to confluence on six-well tissue culture dishes and a single scratch was made in the confluent monolayer using a sterile 200-μL pipette tip. The monolayer was washed with PBS and then complete medium containing either 1.0 μmol/L TAE226 or DMSO alone was added. Serial photographs of the same scratched section were taken after 24 and 48 h. The number of cells that had migrated over the margins of the wounds was counted after 48 h.

**Immunofluorescence staining.** For indirect immunofluorescence staining, SEG-1 cells grown on Lab-Tek 8-well permeabon chamber slides (Nunc) were fixed with 4% paraformaldehyde in PBS for 20 min and then permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Cells were incubated with a blocking solution (1% bovine serum albumin in PBS) for 30 min and then with primary antibodies of Affinity-purified mouse anti-human phosphorylated-FAK (Tyr977; BD Biosciences) diluted in 1% bovine serum albumin-PBS for 90 min at room temperature. After three washings with PBS, the cells were incubated in the presence of secondary antibodies of rabbit anti-mouse IgG antibody labeled with FITC for 1 h. For actin staining, fixed cells were incubated with rhodamine-conjugated phalloidin (Chemicon) for 1 h at room temperature. After being washed twice with PBS, cells were counterstained with1,000 4’6-diamidino-2-phenylindole diluted to 1:100 with PBS for 5 min at room temperature. The slides were mounted with Vectashield (Vector Laboratories, Inc.) and examined under a confocal inverted fluorescence microscope (Zeiss Axiosvert 200M) and digitally processed with Zeiss LSM510META 3.2 software.

**Apoptosis assay.** A terminal deoxynucleotidyltransferase–mediated UTP nick end-labeling assay (TUNEL assay) was done to determine drug-induced apoptosis using the MBESTAIN Apoptosis Kit II (MBL International Co.) according to the manufacturer’s protocol. Briefly, SEG-1 cells (1.0 × 10⁶ per well) were seeded directly in Lab-Tek 8-well permeabon chamber slides and were treated with 1.0 μmol/L TAE226 or...
FAK as a Target for Esophageal Adenocarcinoma Treatment

Fig. 1. Expression of FAK in Barrett’s epithelium and esophageal adenocarcinoma. Surgically resected tissues of Barrett’s epithelium (A) and Barrett’s esophageal adenocarcinoma (B) were used for immunohistochemistry. Each picture was taken at a magnification of ×100 and positively staining cells were counted. C, the population of each group based on the expression status of FAK in different tissue types (squamous epithelium, gastric epithelium, Barrett’s epithelium, and Barrett’s esophageal adenocarcinoma) is shown in the histogram.

with an equivalent volume of the diluent DMSO (final concentration, 0.005%) as a control for 0 to 24 h (0, 1, 6, and 24 h). Apoptotic cells were assessed at the end of the experiment with a fluorescence microscope according to the percentage of TUNEL-positive cells.

Cell cycle analysis by flow cytometry. For cell cycle analysis, SEG-1 cells were plated in six-well tissue culture plates. Subconfluent cells were treated with 1.0 μmol/L TAE226; harvested at different time points of 0, 1, 6, and 24 h; and stained with 20 μg/mL propidium iodide. The DNA content was analyzed with a fluorescence-activated cell sorter (FACScan, Becton Dickinson and Company) using CellQuest software (BD Biosciences).

Western blotting. Cells were collected by trypsinization and washed twice in cold saline. Cells were then dissolved in a protein lysis buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.5% Triton X-100, and protease inhibitors [0.2 mmol/L phenylmethylsulfonyl fluoride, 0.2 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL pepstatin, and 1 μg/mL aprotinin] on ice for 30 min, and the collected cells were scraped into a microtube. After sonication and centrifugation at 15,000 rpm for 4°C for 15 min, the supernatant was collected for protein determination and Western blotting analysis. The concentration of protein lysates was measured with the bicinchoninic acid protein assay kit (Pierce). Equal amounts (20 μg) of protein were electrophoresed under reducing conditions in 5% to 20% (w/v) gradient polyacrylamide gels (ATTO). Proteins were then transferred onto GVHP filter membranes (Nihon Millipore) and incubated with primary antibodies at 4°C overnight, followed by incubation with peroxidase-linked secondary antibodies at room temperature for 1 h. For signal detection, a chemiluminescence Western system (Amersham ECM Plus Western Blotting Detection Reagents, GE Healthcare) was used for signal detection.

The antibodies used for Western blotting were the following: affinity-purified mouse anti-human phosphorylated-FAK (Tyr935; BD Biosciences), affinity-purified mouse anti-human total FAK (BD Biosciences), affinity-purified rabbit anti-human phosphorylated-IGF-IR (Cell Signaling Technology, Inc.), affinity-purified rabbit anti-human total IGF-IR (Santa Cruz Biotechnology), affinity-purified rabbit anti-human phosphorylated-AKT (Ser473; Cell Signaling Technology), affinity-purified rabbit anti-human total AKT (Cell Signaling Technology), affinity-purified rabbit anti-human phosphorylated extracellular signal–regulated kinase (ERK) 1/2 (Cell Signaling Technology), affinity-purified rabbit anti-human total extracellular signal–regulated kinase (Cell Signaling Technology), affinity-purified rabbit anti-human phosphorylated-BAD (Ser136; Cell Signaling Technology), affinity-purified rabbit anti-human phosphorylated-BAD (Ser112; Cell Signaling Technology), affinity-purified rabbit anti-human phosphorylated-BAD (Ser155; Cell Signaling Technology), affinity-purified rabbit anti-human phosphatidyl-inositols, horseradish peroxidase–conjugated rabbit anti-mouse IgG (DakoCytomation), and goat anti-rabbit IgG (American Qualex Antibodies).

Animal experiments. BALB/cA.CaC1-8mu 8-week-old female mice derived from C.B-17/lcr (Clea) were used in this study. All mice were maintained under pathogen-free conditions. SEG-1 cells (3.0 × 106) mixed with Matrigel (BD Biosciences; 100 μL) were s.c. inoculated (n = 6 each; day 0). From days 1 to 14, TAE226 (30 and 60 mg/kg) or methylcellulose as a vehicle was orally administered once a day and the width and length of each s.c. tumor were measured to calculate tumor volume. On day 15, all of the mice were sacrificed to take the tumors out and each tumor was weighed. Tumor volume (V) was calculated as (length × width)2/2, as described elsewhere. Note that the final concentration of methylcellulose and TAE226 used in these experiments did not affect any organs or life span.

Statistical analysis. Student’s t test was used to compare data between two groups. Data represent the mean ± SD. P < 0.05 was considered statistically significant.

Results

The overexpression of FAK in Barrett’s esophageal adenocarcinoma. First, we determined the expression status of FAK in
Barrett's esophageal adenocarcinoma by immunohistochemical staining. We used 42 surgically resected tissues from patients with Barrett's esophageal adenocarcinoma and stained them with anti-FAK antibody. We observed the following parts in those tissues: 308 areas of Barrett's epithelia, 168 areas of adenocarcinoma, 93 areas of squamous epithelia, and 56 areas of gastric epithelia. We categorized them into the three groups defined in Materials and Methods. As shown in Fig. 1, FAK expression was increased in cancerous parts compared with noncancerous areas, and the strong expression (>50% positive-staining cells per area) was observed in 94.0% of Barrett's esophageal adenocarcinoma, compared with 17.9% of Barrett's epithelia. This tendency seemed to be obvious and we would say that, like other malignancies, the expression of FAK is also up-regulated in Barrett's esophageal adenocarcinoma as well. Interestingly, squamous epithelia revealed relatively higher percentage of the positive staining (43.0% for FAK+ and 47.3% for FAK++) than columnar epithelia such as gastric mucosa, whose staining status of FAK was quite similar with that of Barrett's epithelia (Fig. 1B), indicating that the expressions of FAK may differ among histologic configurations.

Inhibition of FAK by TAE226 suppresses cell growth and migration in esophageal adenocarcinoma cell lines. Because cultured adherent cells are quite dependent on adhesion to maintain their growth, it is easily hypothesized that the inhibition of FAK, which plays a critical role in integrin-mediated cell adhesion as well as in advancing signaling from integrin to its downstream effectors, should affect cell proliferation. To determine an inhibitory effect on cell growth by the chemical compound and to optimize its concentration for further experiments, the IC50 was measured to examine the effect of TAE226 in esophageal adenocarcinoma cell lines. All of three esophageal adenocarcinoma cell lines (SEG-1, FLO-1, and BIC-1) were treated with different concentrations of TAE226 for 48 hours and their susceptibility to TAE226 was assessed by the trypan blue exclusion method. The IC50 of SEG-1 cells was 0.47 µmol/L and those of FLO-1 and BIC-1 were 1.03 and 1.29 µmol/L, respectively (Fig. 2A-C). SEG-1 cells seemed to be the most sensitive to TAE226 among the three esophageal adenocarcinoma cell lines. TAE226 at 3 µmol/L seemed to be lethal to all of the cell lines because more than 90% of cells were dead within 48 hours (Fig. 2A-C).
Second, we determined the inhibitory effect on cell migration by the FAK inhibitor. Monolayer cultures of SEG-1 cells were scratched with a micropipette tip at the time of full confluence (time 0) and then kept at 37°C for another 48 hours with TAE226 or its vehicle (DMSO). Figure 2D and E shows microscopic observations of the scratch assay. Certain areas within the scratched defect were occupied by migrated cells with DMSO treatment (332 ± 7 cells per area), whereas TAE226 treatment significantly decreased the number of cells that migrated to the defect (59 ± 1 cells per area), suggesting that FAK inhibition by the compound can negatively affect cell mobility. Cell migration of FLO-1 cells was also suppressed by TAE226 treatment at a concentration of 3 μmol/L (73 ± 26 versus 41 ± 11 cells per area; P < 0.05).

TAE226-treated SEG-1 cells displayed a structural change of actin fiber and a loss of cell adhesion with inhibited FAK activity. When observed under a microscope, TAE226-treated cells displayed an unhealthy morphology such as round, flattened, and burst shapes with a disordered nuclear structure and a cell density that was strongly decreased by the treatment (Fig. 3A). Some cells looked as if they were shrinking with easy detachment from the surface of the plastic plates, assuming that cell adhesion may be weakened by the inhibition of FAK.

To further explore the structural changes of the cell cultures from the inhibition of FAK, cells were stained with antibodies for actin, phosphorylated FAK, and 4′6′-diamidino-2-phenylindole, and immunofluorescent signals were observed by confocal microscopy. As shown in Fig. 3B and C, cells with FAK activity keep the actin fiber structure intact, accompanied by FAK phosphorylation and localization at the end of the actin fiber, assuming that activated FAK is located at focal adhesion points to maintain cytoplasmic structure. When cells were treated with TAE226, the actin fiber structure was dramatically destroyed and the signal of phosphorylated FAK was abolished.

**FAK inhibition leads to apoptosis.** To clarify whether the inhibition of FAK leads to cell death, the distribution of the cell cycle was analyzed by flow cytometry (fluorescence-activated cell sorting analysis). As shown in Fig. 4A and B, TAE226 treatment increased the population of the sub-G₀ phase. We did not see any obvious changes of cell distribution in the G₁ or G₂-M phase. Based on the results of the fluorescence-activated

![Fig. 3. Structural change of SEG-1 cells by TAE226. A, SEG-1 cells treated with either TAE226 (1 or 3 μmol/L) or DMSO were monitored under a phase-contrast microscope and representative pictures (×200) were taken at the different time points indicated. Immunofluorescent stainings for phosphorylated-FAK (green), actin (red), and 4′6′-diamidino-2-phenylindole (blue) are shown in SEG-1 cells treated with vehicle only (B) and with 1 μmol/L TAE226 (C). The cells were serially dissected under a confocal microscope at a magnification of ×400.](image-url)
cell sorting analysis, we speculate that the increase in cell numbers in the sub-G0 phase represents increasing cell death by the FAK inhibitor. The next question was whether cells undergo apoptosis with TAE226 treatment. To answer this question, TUNEL staining was carried out (Fig. 4C). TAE226 treatment apparently increased TUNEL-positive cells within 24 hours compared with DMSO treatment (Fig. 4D), suggesting that the inhibition of FAK activity by TAE226 leads the esophageal adenocarcinoma cells to apoptosis.

**TAE226 inhibits FAK and IGF-IR activity and suppresses their downstream signaling.** To explore which signaling pathway is involved in TAE226-induced apoptosis, we dissected out downstream molecules of FAK by Western blot. TAE226 inhibited phosphorylation of FAK in a dose- and time-dependent manner (Fig. 5A and B). AKT activation was also suppressed, whereas the inhibition of ERK activity was modest. Phosphorylation of BAD, which is a key modulator for cell survival and is regulated by AKT and mitogen-activated protein kinase, was inhibited at Ser136 but not at Ser112, suggesting that suppression of FAK activity by TAE226 caused an inhibition in AKT phosphorylation, which prevents it from being able to phosphorylate BAD at Ser136, thereby promoting apoptosis. We looked further downstream along the apoptosis pathway. Caspase-3, a key molecule through caspase-dependent apoptosis, which is also regulated by BAD, was cleaved by TAE226 treatment. These results support our initial finding that the inhibition of FAK by TAE226 indeed leads to apoptosis through the AKT-BAD-caspase pathway.

---

**Fig. 4.** TAE226-induced apoptosis in esophageal adenocarcinoma cells. SEG-1 cells were treated with 1 μmol/L of TAE226 or DMSO and applied for cell cycle analysis by flow cytometry (A), and the cell distribution at each phase is drawn in histograms at the different time points indicated (B). C. TUNEL staining was done to assess TAE226-induced apoptosis in SEG-1 cells and the percentage of TUNEL-positive cells is displayed in histograms (D). White and black columns, percentage of cells with DMSO and TAE226, respectively.

**Cancer Therapy: Preclinical**

www.aacrjournals.org ClinCancerRes 2008;14(14) July 15, 2008 4636

It is reported that TAE226 also has a certain inhibitory effect on IGF-IR (~25-fold less than on FAK; ref. 31). AKT is well known as a main downstream molecule of receptor tyrosine kinase such as IGF-IR. It is possible that the inhibition of IGF-IR may also contribute to TAE226-induced apoptosis through the AKT-BAD-caspase pathway. We saw a faint expression of IGF-IR phosphorylation in regular culture conditions without the inhibitor and its activity was shut down by the addition of TAE226, although the effect looked unclear. Therefore, the cells were treated with IGF to stimulate the receptor and to see specific inhibition of IGF-IR signaling by TAE226. As shown in Fig. 5C, 100 ng/mL IGF reasonably activated the receptor and its downstream effectors, whereas TAE226 suppressed IGF-induced activation of IGF-IR and AKT. In summary, TAE226 has a strong inhibitory effect on cell proliferation and migration in esophageal adenocarcinoma cells and leads the cells to apoptosis through the AKT-BAD-caspase pathway by inhibiting both FAK and IGF-IR signaling.

Oral administration of TAE226 suppresses s.c. tumor growth in vivo. We confirmed a potent inhibitory effect on cell growth and migration of esophageal adenocarcinoma cells in vitro. For the next step, we inoculated SEG-1 cells s.c. in nude mice (day 0). The animals were treated with an oral administration of either TAE226 (30 or 60 mg/kg) or methylcellulose as a vehicle. The daily administration started the day after inoculation and continued for 2 weeks (days 1-14).

**Fig. 5.** Western blotting analysis for TAE226-induced apoptosis. A, the activation of FAK, IGF-IR, and their downstream molecules was suppressed by TAE226 treatment in a dose-dependent manner. B, the inhibition of the cells survival pathway with 1 μmol/L TAE226 was prolonged for at least 24 h. C, TAE226 displayed a certain effect on the inhibition of IGF-IR signaling, which is specifically activated by IGF-I.
Tumor volume was measured twice a week during the treatment and the tumors were taken out of the mice and weighed at day 15. Both tumor volume and weight were significantly decreased in mice that had TAE226 treatment compared with mice treated with vehicle only (Fig. 6; Supplementary table). No obvious side effects occurred during the treatment and there is not a statistical significance of tumor regression between the two different doses (30 and 60 mg/kg/daily).

Discussion

Here we first report that our immunohistochemical analysis using clinical samples revealed the up-regulation of FAK in Barrett’s esophageal adenocarcinoma (Fig. 1), suggesting that FAK might play a critical role for cancer progression in Barrett’s esophageal adenocarcinoma as it reportedly does in other types of malignancies (17–25). Because FAK is known as a key molecule for cell proliferation, migration, and invasion during cancer progression (1–5), targeting FAK can potentially be a good practice for cancer therapy. Indeed, a number of chemical compounds have recently been developed to target certain cancer-specific functions without apparent side effects (34, 35). TAE226 is one such small-molecule inhibitor specifically targeting FAK (30, 31). Thus, we determined that the inhibition of FAK by TAE226 can be a potential therapeutic strategy against adenocarcinoma of the esophagus, which is known as one of the most aggressive cancers (27–29).

TAE226 treatment displayed significant inhibitory effects on cancer proliferation and migration in vitro, and esophageal adenocarcinoma cells underwent apoptosis by the FAK inhibitor. TAE226 causes an inhibition in AKT phosphorylation, which prevents it from being able to phosphorylate BAD at Ser136, thereby promoting apoptosis (Fig. 5A and B). Because phosphorylation of BAD by Akt is known to keep it sequestered in 14-3-3 protein and to prevent the association between BAD and antiapoptotic Bcl-2 family proteins (36, 37), this is the first report to explore a major mechanism of anticancer effect by TAE226 that leads cancer cells to apoptosis through the AKT-BAD-caspase pathway.

TAE226 treatment also impaired the morphologic structure of the cytoplasm through the invalidation of actin fibers that stabilize focal adhesions via FAK (Fig. 3). This observation supports the principal of the inhibitor that should abolish the ability of focal adhesion to modify the downstream signaling pathways related to cell proliferation and migration (30).

Another importance of our findings is that TAE226 treatment of s.c. xenograft animals revealed a remarkable reduction of tumor volume by oral administration without any apparent side effects (Fig. 6; Supplementary table). It should be noted that a maximum of 100 mg/kg/daily administration of TAE226 for 14 days was done and no complication was found.7 We did not see a statistical significance of tumor regression by TAE226 in vivo, suggesting that a low dose of TAE226 could be sufficient in certain types of cancers. These data encourage us to advance FAK targeting as a novel cancer therapy with an eye toward clinical application.

TAE226 was originally synthesized as a specific inhibitor for FAK, and it is reported that the drug also has a certain inhibitory effect on IGF-IR activity, which is also well known as a major intracellular pathway for cell proliferation and survival, although this effect is ~25-fold less than the effect on FAK (31). As we showed, TAE226 displayed a suppression of IGF-IR activation by IGF-I in SEG-1 cells (Fig. 5C), indicating that it should not be neglected that any anticancer effect on Barrett’s esophageal adenocarcinoma by TAE226 may be partially mediated through IGF-IR signaling. It is very meaningful that a single chemical compound can inhibit two major signaling pathways for cancer progression because a crucial issue that may often be faced in the clinical field is that targeting one particular molecule only is not sufficient enough to control cancer progression because most malignancies can be regulated by multiple signaling pathways (38) and that they can easily acquire a resistance to certain drugs through genetic alterations (39, 40). To overcome these problems, it is necessary to synthesize novel drugs that show multiple inhibitory mechanisms, such as TAE226, whose effect is mainly to inhibit

---

7 Personal observation.
activation of FAK and IGF-IR. Another strategy to gain the maximum effect for cancer therapy is a combination of two or more drugs (e.g., 5-fluorouracil and cisplatin). It is potentially intriguing to combine TAE226 with other compound(s) to test its synergistic effect on inhibiting cancer progression.

In this study, SEG-1 cells displayed the highest susceptibility to TAE226 compared with the other cells (Fig. 2A-C). This difference may have occurred due to the intrinsic background. It is reported that SEG-1 cells retain an intact function of wild-type p53 whereas FLO-1 and BIC-1 cells have acquired a p53 mutation (41). p53 status may partially affect the response to anticancer drugs as described previously (42–44).

In conclusion, we found that FAK is up-regulated in Barrett’s esophageal adenocarcinoma and that the inhibition of FAK by TAE226 led esophageal adenocarcinoma cells to apoptosis through the AKT-BAD-caspase pathway and displayed significant anticancer effects in vitro and in vivo. Targeting FAK can be a novel strategy for cancer therapy, and TAE226, which has a specific inhibition of FAK along with a certain inhibitory effect on IGF-IR activity, might be a potential candidate for clinical application.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We thank Drs. Michael Veth (Institute of Pathology, Klinikum Bayreuth, Bayreuth, Germany) and Kaiyo Takubo (Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan) for providing clinical tissues for immunohistochemistry, Dr. Hitoshi Nagatsuka for performing the immunohistochemistry, Tae Yamashiri for technical assistance, and Dr. Motowo Nakajima for his useful advice.

References

Clinical Cancer Research

Dual Tyrosine Kinase Inhibitor for Focal Adhesion Kinase and Insulin-like Growth Factor-I Receptor Exhibits Anticancer Effect in Esophageal Adenocarcinoma In vitro and In vivo

Nobuyuki Watanabe, Munenori Takaoka, Kazufumi Sakurama, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/14/14/4631

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2008/07/15/14.14.4631.DC1

Cited articles
This article cites 44 articles, 13 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/14/14/4631.full#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/14/14/4631.full#related-urls

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

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

Permissions
To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/14/14/4631. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.