Studies on Biological Characteristics of Cancer Cells after Acquiring Resistance to Molecular-Targeted Therapy

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Contents

Abstract

Vascular endothelial growth factor (VEGF)-neutralizing antibody and an epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) have become increasingly important for treating cancer. However, almost all patients develop progressive disease (PD) during the therapy.

It was demonstrated that second-line chemotherapy together with anti-VEGF antibody after PD on first-line therapy including anti-VEGF antibody showed clinical benefits. In my study, to investigate the mechanisms underlying the effect of continued administration of anti-VEGF antibody plus capecitabine even after resistance to anti-VEGF antibody was acquired, I established the anti-VEGF antibody PD model using HT-29 cancer cell. The combination therapy exhibited significantly stronger antitumor and anti-angiogenic activities than did each monotherapy in PD model. Capecitabine treatment increased the intratumoral VEGF level compared with the control group; however, the combination with anti-VEGF antibody neutralized VEGF. Among angiogenic factors other than VEGF, intratumoral galectin-3, which reportedly promotes angiogenesis both dependent on and independently of VEGF, was decreased in the capecitabine group and the combination group compared with the control group. These results suggested that capecitabine has a dual mode of action: namely, inhibition of tumor cell growth and inhibition of galectin-3 production by tumor cells.

Next, to demonstrate the clinical relevance of EGFR-TKI treatment after PD, I investigated whether continuous administration of an EGFR-TKI in combination with chemotherapy has a useful effect on PD development during EGFR-TKI treatment. For this purpose, I examined the antitumor effect of combination therapy in EGFR-TKI-resistant tumor xenograft models: EBC-1, H1975, HCC827TR3, and HPAC. As a result, the combination therapy showed a significantly higher antitumor activity compared with chemotherapy alone in all xenograft models except H1975. Furthermore, EGFR-TKI alone suppressed the EGFR phosphorylation in HPAC tumors and the two NSCLC cell lines other than H1975. Therefore, when an EGFR-TKI continues to inhibit EGFR phosphorylation, combination therapy with an EGFR-TKI can be considered effective, even in EGFR-TKI-resistant tumor xenograft models.

In these studies, I demonstrated that tumors continue to express the target molecule after acquiring resistance to therapy, and combining targeted therapy with chemotherapy exerted strong antitumor activity, even after resistance to the targeted therapy was acquired. I thus suggest the clinical relevance of using targeted therapy in a combination therapy beyond PD.

General Introduction

Cancer is a deadly disease in which abnormal cells grow without control, metastasize to other organs, and ultimately cause organ failure by invading the surrounding tissues. With the number of cancer patients increasing every year, the question of how to treat cancer is one of the major issues still to be resolved in modern medicine. Although surgery, radiation therapy, and drug therapy comprise the treatment options available for cancer patients, in the metastasis phase the only treatment option is drug therapy. To improve the efficacy of drug therapy, a variety of targeted therapies have been developed focusing on several proteins that are genetically overexpressed on the surface or activated in the cytoplasm of cancer cells, such as c-KIT receptor, and mTOR, and the epidermal growth factor receptor (EGFR) family (1-3).

EGF was discovered 30 years ago and its role in cell growth has been elucidated. EGFR is a transmembrane glycoprotein with an extracellular EGF-binding domain and an intracellular domain possessing intrinsic tyrosine kinase activity (4-6). Binding by the EGF ligand activates the receptor's tyrosine kinase, initiating cascades of intracellular signaling. EGFR is the major contributor in a complex signaling cascade that modulates growth, signaling, differentiation, adhesion, migration, and survival of cancer cells. High levels of EGFR expression have been reported in a wide range of human malignancies (7-9), and members of the EGFR family are among the most often targeted proteins. EGFR-tyrosine kinase inhibitor (EGFR-TKI) reversibly binds to the intracellular domain of EGFR. This blocks autophosphorylation of EGFR with subsequent inhibition of the downstream signaling pathways which promote cell proliferation. Clinical results have demonstrated that EGFR-TKI monotherapy or combination therapy with chemotherapy showed a survival benefit for patients with NSCLC or pancreatic cancer, respectively (10, 11). However, most of these patients developed progressive disease (PD) during such therapies.

Mechanisms of acquired resistance to EGFR-TKI, which result in continued signaling in the presence of EGFR-TKI, can be classified into two broad categories: resistant EGFR mutations, and activation of bypass pathways or downstream pathways (12). In the former case, an EGFR mutation restores the binding pocket’s affinity for adenosine triphosphate, rendering the competitive inhibition of adenosine triphosphate by TKI ineffective (13). The high rate of T790M resistance after first- and second-generation TKI therapy has prompted the development of third-generation EGFR-TKI. In the latter case, the signaling that was inhibited by EGFR-TKI therapy is restored by bypass pathways activated through compensative utilization of other related

receptors or circumvention of the EGFR, or both. For example, MET amplification that results in high-level Met receptor expression can enhance heterodimerization with HER3 (ErbB3) to activate downstream PI3K signaling despite inhibition of EGFR activation (14). As with MET, HER2 (ErbB2) amplification leads to parallel signaling that bypasses the effects of EGFR-TKI (15). Upregulation of insulin-like growth factor 1 receptor has also been shown to confer resistance to EGFR-TKI (16, 17). However, blockades of these receptors have not been effective in clinical trials (18).

In another approach, therapy has been developed to target not the tumor cells but the tumor stromal cells. Angiogenesis is one of the fundamental causes of tumor progression (19). Folkman has suggested that tumor growth is angiogenesis-dependent and proposed that anti-angiogenesis might be an effective therapeutic strategy to treat cancer (20, 21). As tumor blood vessels are mainly made from endothelial cells, an important factor in tumor angiogenesis and growth is vascular endothelial growth factor (VEGF) (22-25). VEGF is a heparin binding growth factor, and is a dimeric protein composed of two identical subunits (26). Six subtypes of VEGF, VEGF-A, -B, -C, -D, -E, and -F (27-31) and three receptors, VEGFR-1, -2, and -3 (32, 33), are known. Each VEGF subtype binds to multiple VEGFR. Activation of VEGFR-1 results in angiogenesis and migration of hematopoietic cells, that of VEGFR-2 in angiogenesis and increased permeability (34), and that of VEGFR-3 in lymphatic formation (35).

It has been reported that VEGF is overexpressed in most human tumors (23, 36), which results in disorganization of the tumor microvasculature and lack of the conventional hierarchy of blood vessels, making arterioles, capillaries, and venules unidentifiable (37-40). In the clinical setting, anti-angiogenic therapy targeting VEGF has been demonstrated to be effective for several kinds of cancers, including colorectal cancer (41-43).

Because endothelial cells are genetically stable, it is generally considered that refractoriness to combination therapy with chemotherapeutic agents plus anti-VEGF antibody is mostly caused by resistance to the chemotherapeutic agents rather than to anti-VEGF antibody (44, 45). In this context, second-line therapies including anti-VEGF antibody (bevacizumab beyond progression therapy; BBP therapy) were intensively studied and were found to demonstrate clinical benefits in patients with metastatic colorectal and breast cancer (46, 47). However, VEGF-independent angiogenesis has been reported as a mechanism of resistance to anti-VEGF therapy (48). Therefore, resistance to anti-VEGF antibody cannot be excluded as a mechanism of resistance to combination therapy with chemotherapeutic agents plus anti-VEGF antibody.

In Chapter I of this study, I hypothesized that BBP therapy is effective even after a tumor acquires resistance to anti-VEGF antibody, and I investigated the effective mechanism of BBP therapy. I used a xenograft model of a human colon cancer cell line with acquired resistance to anti-VEGF antibody to investigate the antitumor effect of the combination of chemotherapy plus anti-VEGF antibody and its mechanism of action in terms of angiogenic factors produced by stromal cells and tumor cells.

In Chapter II of this study, I analyzed the characteristics of EGFR-TKI-resistant tumor cells and investigated the antitumor activity of combination therapies of EGFR-TKI with various cytotoxic chemotherapies, using not only an *in vivo* EGFR-TKI PD xenograft model but also EGFR-TKI-resistant tumor cell xenografts, to show the clinical relevance of continuing EGFR-TKI treatment after development of PD.

Chapter I.

Biological characters of cancer cells after acquiring resistance to anti-VEGF antibody

Introduction

Bevacizumab, a humanized anti-VEGF monoclonal antibody, is usually administered in combination with chemotherapeutic agents. Although these combination therapies improve overall survival and progression-free survival in colorectal cancer patients, almost all responders ultimately develop disease progression (PD). Therapies including bevacizumab after PD (bevacizumab beyond progression therapy; BBP therapy) were found to demonstrate clinical benefits in patients with metastatic colorectal and breast cancer (46, 47).

Capeciatbine is an oral fluoropyrimidine drug widely used. It is converted first to 5'-deoxy-5-fluorocytidine by carboxylesterase located in the liver, then to 5'-deoxy-5-fluouridine by cytidine deaminase expressed in the liver and various solid tumors, and finally to 5-FU by thymidine phosphorylase highly expressed in many tumors. It has been reported that capecitabine and 5-FU inhibited angiogenesis in colon and gastric cancer models by suppressing the secretion of angiogenic factors from tumor cells (49, 50). A clinical study has reported that the combination therapy of bevacizumab with chemotherapy involved in capecitabine improves progression free survival compared with chemotherapy alone in several kinds of cancers including colorectal cancer. And this combination was used for 26% of patients in the metastatic colorectal cancer BBP therapy trial and 60% of patients in the breast cancer BBP therapy trial (46, 47).

In this study, I used a xenograft model of a human colon cancer cell line with acquired resistance to bevacizumab (hereafter, bevacizumab PD model) to investigate the antitumor effect of the combination of capecitabine plus bevacizumab and its mechanism of action in terms of angiogenic factors produced by stromal cells and tumor cells.

Materials and Methods

Reagents

Bevacizumab and capecitabine were provided by F. Hoffmann-La Roche (Basel, Switzerland) as a liquid and fine powder, respectively. Human immunoglobulin G (HuIgG) was purchased from MP Biomedicals (Solon, OH, USA). Capecitabine was dissolved in 40 mM citrate buffer (pH 6.0) containing 5% gum arabic (capecitabine vehicle).

Cells

Five human colorectal cancer cell lines (COLO 205, HCT-8, HCT-116, LS411N, and HT-29), and two mouse cancer cell lines (B16-F1 and LLC1) were used in this study. All cancer cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). COLO 205 and LS411N were maintained in RPMI-1640 supplemented with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/l glucose, 1.5 g/l sodium bicarbonate, and 10% FBS at 37°C under 5% CO_2. HCT-116 and HT-29 were maintained in McCoy's 5A supplemented with 10% FBS at 37°C under 5% CO_2. HCT-8 was maintained in RPMI-1640 supplemented with 1 mM sodium pyruvate and 10% horse serum at 37°C under 5% CO_2. B16-F1 and LLC1 were maintained in D-MEM supplemented with 10% FBS at 37°C under 5% CO_2.

Animals

Five-week-old male BALB/c-nu (CAnN.Cg-Foxn1 <nu>/CrlCrlj) mice were obtained from Charles River Laboratories Japan (Yokohama, Japan). All animals were allowed to acclimatize and recover from shipping-related stress for at least 4 days prior to the study. The health of the mice was monitored by daily observation. The animals were allowed free access to chlorinated water and irradiated food, and the animals were kept under a controlled light-dark cycle (12 h–12 h). All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Chugai Pharmaceutical Co., Ltd.

In vivo tumor growth inhibition by bevacizumab

Each mouse was inoculated subcutaneously into the right flank with 5×10^6 cells/mouse of human colorectal cancer cell line (either COLO 205, HCT-8, HCT 116, LS411N or HT-29). Several weeks after tumor inoculation, mice were randomly allocated to control and treatment groups. Bevacizumab (5 mg/kg) was administered

intraperitoneally (i.p.) once a week for 3 weeks. To evaluate the antitumor activity of the test agents, tumor volume (TV) was measured twice a week. The tumor volume was estimated from the equation $TV = ab^2/2$, where a, and b are tumor length and width, respectively. Tumor volume ratios were calculated by dividing mean tumor volume on day 8 by mean tumor volume on day 1 and mean tumor volume on day 22 by mean tumor volume on day 15 in both the bevacizumab and control groups.

Combination of bevacizumab plus chemotherapy in the bevacizumab PD model

Mice inoculated with HT-29 cells that had been treated with bevacizumab (5 mg/kg) on days 1 and 8 (1st treatment) were randomly allocated to control IgG plus capecitabine vehicle (control), bevacizumab, capecitabine, and bevacizumab plus capecitabine groups on day 29. Bevacizumab (5 mg/kg) was administered i.p. Once a week and capecitabine (359 mg/kg) was administered orally (p.o.) every day for 3 weeks (2nd treatment). To evaluate the antitumor activity and the tolerability of the test agents, TV and body weight was measured twice a week.

Quantification of microvessel density in tumor tissues

HT-29 tumor tissues were resected from the bevacizumab PD model on day 50, and microvessel density (MVD) was evaluated immunohistochemically by using a monoclonal anti-mouse CD31 antibody (rat anti-mouse CD31 monoclonal antibody, clone MEC 13.3; BD Biosciences, Franklin Lakes, NJ, USA). Immunohistochemical staining was performed as described previously using 5-μm-thick sections from freshly frozen tissues (51). MVD (%) was calculated from the ratio of the CD31-positive staining area to the total observation area. Fields excluding necrotic areas were analyzed. Positive staining areas were calculated by using imaging analysis software (Definiens Tissue Studio; Definiens, Munich, Germany).

Flow cytometric analysis of tumor-infiltrating cells

HT-29 tumors were collected from xenografted mice on day 26 after inoculation. Single cell suspensions were prepared by mincing the tumors, followed by treating with a Tumor Dissociation kit for human tumor tissue (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) in a gentleMACS Dissociator (Miltenyi Biotec). The cells were stained with antibodies to mouse CD11b (PerCP/Cy5.5), Gr1 (APC), and CD45 (FITC) (BioLegend, San Diego, CA, USA) at 1 μg/ml and analyzed by using FACSAria (Becton-Dickinson) and FlowJo software (Tree Star, Ashland, OR, USA).

Measurement of angiogenesis-related proteins

HT-29 tumor tissues collected on day 50 from the bevacizumab PD model were homogenized with cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). The supernatant after centrifugation (14,000 × g, 5 min, 4°C) was used for the assays. The protein concentration of the supernatant was quantified using a Direct Detect spectrometer (Merck Millipore, Frankfurter, Germany). The relative expression profiles of human and murine angiogenesis-related proteins were analyzed by membrane-based antibody array (Proteome Profiler Angiogenesis Array kit; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. The human angiogenesis array simultaneously detect the relative levels of 55 angiogenesis-related proteins (Activin A, ADAMTS-1, Angiogenin, Angiopoietin-1, Angiopoietin-2, Angiostatin/Plasminogen, Amphiregulin, Artemin, Tissue Factor/Factor III, CXCL16, DPPIV/CD26, EGF, EG-VEGF, Endoglin/CD105, Endostatin/Collagen XVIII, Endothelin-1, FGF acidic, FGF basic, FGF-4, FGF-7/KGF, GDNF, GM-CSF, HB-EGF, HGF, IGFBP-1, IGFBP-2, IGFBP-3, IL-1β, CXCL8/IL-8, TGF-β1, Leptin, CCL2/MCP-1, CCL3/MIP-1α, MMP-8, MMP-9, NRG1-β1, Pentraxin 3, PD-ECGF, PDGF-AA, PDGF-AB/PDGF-BB, Persephin, CXCL4/PF4, PlGF, Prolactin, Serpin B5/Maspin, Serpin E1/PAI-1, Serpin F1/PEDF, TIMP-1, TIMP-4, Thrombospondin-1, Thrombospondin-2, uPA, Vasohibin, VEGF, VEGF-C). The murine angiogenesis array simultaneously detects the relative levels of 53 angiogenesis-related proteins (ADAMTS1, Amphiregulin, Angiogenin, Angiopoietin-1, Angiopoietin-3, CCL2/MCP-1, CCL3/MIP-1α, CXCL1, CXCL10, SDF-1, CXCL16, CXCL4, IGFBP-10, DLL4, DPPIV, EGF, Endoglin, Endostatin, FGF acidic, FGF basic, FGF-7/KGF, Fractalkine, GM-CSF, HB-EGF, HGF, IGFBP-1, IGFBP-2, IGFBP-3, IL-1α, IL-1β, IL-10, Leptin, MMP-3, MMP-8, MMP-9, IGFBP-9, Osteopontin, PD-ECGF, PDGF-AA, PDGF-AB/BB, Pentraxin-3, PlGF-2, Prolactin, Proliferin, Serpin E1/PAI-1, Serpin F1/PEDF, Thrombospondin-2, TIMP-1, TIMP-4, Coagulation Factor III, VEGF and VEGF-B). The array was hybridized with 280 μg of total protein. The concentrations of human VEGF, galectin-1, and galectin-3 were measured by a Quantikine ELISA kit (R&D Systems).

***In vitro* cell treatment with 5-FU**

HT-29 cells were seeded on 24-well plates at 4×10^4 cells/well and were incubated overnight at 37°C under 5% CO_2. The cells were then treated with 5-fluorouracil (5-FU) at 0.4, 2, or 20 μM for 24 h.

Statistical analysis

The Wilcoxon test and Dunnett's test were used, with $P<0.05$ considered statistically significant. Statistical analyses were carried out using the SAS preclinical package (SAS Institute, Cary, NC, USA).

Results

Establishing the bevacizumab PD model

The antitumor activity of bevacizumab monotherapy was examined in five human colorectal cancer xenograft models in which tumors produced VEGF (Table 1). Bevacizumab exhibited significant antitumor activity in all of the five models when evaluated on day 22. To analyze in detail the mode of antitumor activity of bevacizumab during the treatment, tumor volume ratio was calculated separately in the early phase (days 1–8) and in the late phase (days 15–22). Acquisition of resistance to bevacizumab was evaluated by comparing the differences in tumor volume ratio in the early and late phases of treatment. In the COLO 205 and LS411N models, bevacizumab exhibited a significant antitumor activity in both the early and late phase. In the HCT-8 and HCT 116 models, bevacizumab exhibited a significant antitumor activity only in the late phase. Of note, in the HT-29 model, bevacizumab exhibited significant antitumor activity in the early phase but not in the late phase (Fig. 1) indicating that the HT-29 model became non-sensitive to bevacizumab during treatment.

Effect of combination therapy with bevacizumab plus capecitabine on tumor growth and MVD in the bevacizumab PD model

The HT-29 xenografted mice treated with bevacizumab (the bevacizumab PD model mice) were then treated with either control IgG plus capecitabine vehicle, bevacizumab, capecitabine, or bevacizumab plus capecitabine. As was expected, bevacizumab alone did not exhibit any significant antitumor effect, indicating that the HT-29 tumors pretreated with bevacizumab were refractory to bevacizumab. Capecitabine alone exhibited a significant antitumor effect versus control. The combination of bevacizumab plus capecitabine exhibited a stronger antitumor effect than capecitabine alone (Fig. 2A). No difference in MVD was observed in tumor tissues from the control, bevacizumab, and capecitabine groups. However, a significant decrease in MVD was observed in the combination group compared with the groups treated with each drug alone (Fig. 2B).

Intratumoral stromal cell-derived angiogenic factors

To investigate whether stromal cell-derived angiogenic factors are involved in sensitivity to combination therapy with bevacizumab, I examined the change in expression of murine angiogenesis-related factors in tumors from each treatment group. VEGF, bFGF, HGF, PDGF-AA, PDGF-BB, angiopoietin-1 (Ang-1), and δ-like ligand 4

(DLL4) were not detected. Although SDF-1 and MCP-1 were detected, none of the treatment groups showed any difference in these angiogenic factors (Fig. 3).

Tumor cell-derived angiogenic factors

Expression of human angiogenesis-related factors in tumors from each treatment group was examined by Proteome Profiler Angiogenesis Array. VEGF, bFGF, and IL-8 were detected, whereas PDGF-AA, PDGF-BB, VEGF-C, HGF, PlGF, angiopoietin-1 (Ang-1), and angiopoietin-2 (Ang-2) were not detected regardless of the treatment (Fig. 4A). bFGF and IL-8 were similar in the capecitabine group, bevacizumab group, and combination group (Fig. 4A). VEGF level was significantly increased by capecitabine treatment compared with the control group, while VEGF was unsurprisingly neutralized by the bevacizumab-containing treatments when measured by ELISA (Fig. 4B). Galectin-3 levels were significantly decreased in the capecitabine and combination groups compared with the control group, whereas no significant difference was observed in galectin-1 levels (Fig. 4C). When the HT-29 cells were treated *in vitro* with 5-FU, an active metabolite of capecitabine, production of galectin-3 per cell was inhibited in a dose-dependent manner (Fig. 4D). These data suggested that capecitabine inhibited galectin-3 production by HT-29 cells via a mechanism independent of cell growth inhibition.

Discussion

In colorectal cancer, it was unclear whether maintenance with a combination of bevacizumab plus a chemotherapeutic agent would benefit patients who had acquired resistance to bevacizumab (52). In this study, it was found that the combination of bevacizumab plus capecitabine exhibited a strong antitumor effect on xenografted human colorectal cancer that had acquired resistance to bevacizumab in vivo. The mechanism of action of the combination of bevacizumab plus capecitabine was analyzed in terms of tumor angiogenesis.

It previously was reported that the molecular targeted drugs trastuzumab exhibited antitumor effects when administered with chemotherapeutic agents after the tumor had acquired resistance to monotherapy with each if the tumor kept expressing the target molecules (53). If a tumor continues to express VEGF, combining bevacizumab with second-line chemotherapy after resistance has been acquired, may be an appropriate therapy. In order to establish a colorectal cancer xenograft model that develops resistance to bevacizumab, five human colorectal cancer xenografts which produced VEGF (Table 1) were treated with bevacizumab. Bevacizumab was given as the sole regimen to avoid development of resistance to chemotherapeutic agents. As a result, a bevacizumab PD model was established using the HT-29 xenograft model and was used in the following experiments.

I investigated the antitumor effect of the combination of bevacizumab plus capecitabine by using the HT-29 PD model. Of note, the combination of bevacizumab plus capecitabine exhibited a significantly stronger antitumor effect than capecitabine alone (Fig. 2A). These data suggested that it is important to continue to inhibit VEGF by bevacizumab in combination with an appropriate chemotherapeutic agent even after the tumor has acquired resistance to bevacizumab per se. Indeed, the combination group showed a significant reduction in MVD, while the groups receiving bevacizumab or capecitabine alone did not at this stage (Fig. 2B). These results suggested that a synergistic anti-angiogenic effect was one of the mechanisms underlying the stronger antitumor effect of the combination treatment.

To examine the mechanism through which the combination treatment exerted an anti-angiogenic effect in the bevacizumab PD model, I analyzed the effect of capecitabine on the production of several angiogenic factors by both tumor cells and stromal cells. Since it has been reported that the infiltration of $CD11b^{+}/Gr1^{+}$ cells into tumors directly or indirectly produces other angiogenic factors causing resistance to anti-VEGF treatment (54), I analyzed stromal cell-derived angiogenic factors and the

number of $CD11b^+/Gr1^+$ cells in the tumor in the HT-29 PD model. However, the number of $CD11b^+/Gr1^+$ cells was as few as in the anti-VEGF antibody sensitive tumor B16-F1 (data not shown). It has also been reported that bFGF, Ang-1, DLL4, HGF, SDF-1, MCP-1, PDGF-AA, and PDGF-BB from stromal cells are implicated in the development of resistance to VEGF inhibition (55-58). MCP-1 and SDF-1 were detected in the HT-29 PD model, but the levels were similar in the capecitabine, bevacizumab, and combination groups (Fig. 3). There were no obvious differences in any of the other murine angiogenic factors detected in the capecitabine, bevacizumab, or combination groups (Fig. 3). Therefore, the mechanism of action of the combination of bevacizumab plus capecitabine in this model could not be explained by the stromal cell-derived angiogenic factors tested.

Next, I investigated whether tumor cell-derived angiogenic factors were involved in the anti-angiogenic effect of the combination therapy. I examined bFGF, IL-8, PDGF-AA, PDGF-BB, VEGF-C, Ang-1, Ang-2, HGF, and PlGF, which are reported to be angiogenic factors for tumors (59-61). PDGF-AA, PDGF-BB, VEGF-C, Ang-1, Ang-2, HGF, and PlGF were not detected in the HT-29 PD model. bFGF and IL-8 were detected but their levels were similar in the capecitabine, bevacizumab and combination groups (Fig. 4A). There were no obvious differences in any of the other human angiogenic factors detected in the capecitabine, bevacizumab, or combination groups (Fig. 4A). Therefore, the anti-angiogenic effect of the combination therapy in this model could not be explained by the factors tested.

Recently, galectins, members of a family of carbohydrate-binding proteins with multiple functions, were reported to be potent prognostic marker in colorectal cancer and to promote angiogenesis (62, 63). Galectin-1 maintains angiogenesis in anti-VEGF-refractory tumors (64), and galectin-3 has been shown as an important mediator of VEGF- as well as FGF-mediated angiogenic response (65-67). In light of these findings, I investigated the intratumoral levels of human galectin-1, galectin-3, and VEGF in each treatment group. No significant change was observed in galectin-1 level. However, galectin-3 level was significantly decreased in the capecitabine and combination groups compared with level in the control group (Fig. 4C). In addition, 5-FU, an active metabolite of capecitabine, directly inhibited galectin-3 production by HT-29 cells in vitro (Fig. 4D). Since VEGF- and bFGF-mediated angiogenesis has been reported to be greatly reduced by galectin-3 inhibitors such as dominant negative galectin-3 or in galectin-3 knockdown cells in vitro and also in $Gal3^{-/-}$ mice (67), the decrease in intratumor galectin-3 level by capecitabine in my model was suggested to contribute, at least in part, to synergistic inhibition of angiogenesis in combination with

anti-VEGF antibody. Despite the reduction of galectin-3, no difference in MVD was observed between tumor tissues in the control and capecitabine groups. It has been reported that conventional 5-FU treatment may promote tumor angiogenesis by increasing the production of VEGF (49, 50). In contrast with galectin-3 levels, VEGF levels indeed increased in the capecitabine treatment group in my model (Fig. 4B). These bilateral effects of capecitabine on the production of angiogenic factors may be a reason for the apparent absence of anti-angiogenic effect by capecitabine alone. However, by neutralization of VEGF through concurrent administration of bevacizumab, a synergistic anti-angiogenic effect emerged (Fig. 4B). These data raise the possibility that the inhibition of galectin-3 production by capecitabine and the neutralization of VEGF by bevacizumab were one of the mechanisms underlying the effect of combination treatment.

Regarding the development of resistance to bevacizumab in the HT-29 model, I investigated whether galectin-3 plays a role in acquisition of resistance. However, galectin-3 levels in the tumors did not change before and after developing PD (data not shown). Galectin-3 interacts with Mgat5-modified N-glycans on cytokine receptors such as EGFR, IGFR, PDGFR, and bFGFR, and activates downstream signal transduction pathways (68). Therefore, alteration in the expression of any of these receptors may cause galectin-3-induced resistance to bevacizumab. Thus, further investigations are required to clarify the mechanisms of resistance to bevacizumab in this model.

In this study, I indicated that combination therapy with bevacizumab plus capecitabine could exert strong antitumor activity, even after resistance to bevacizumab was acquired, by recovering the anti-angiogenic effect, thus suggesting the clinical relevance of treatment with bevacizumab in combination therapy beyond PD.

Chapter II

Biological characters of cancer cells after acquiring resistance to EGFR-TKI

Introduction

Erlotinib is an oral, small molecule tyrosine kinase inhibitor that reversibly binds to the intracellular domain of EGFR. This blocks autophosphorylation of EGFR with subsequent inhibition of the downstream signaling pathways which promote cell proliferation. Erlotinib is used to treat metastatic NSCLC and pancreatic cancer in many countries. Clinical results have demonstrated that erlotinib monotherapy or combination therapy with gemcitabine showed a survival benefit for NSCLC or pancreatic cancer, respectively (10, 11). However, most of these patients developed progressive disease (PD) during such therapies and it is usually considered best to switch to chemotherapy after developing PD. It is reported that the major mechanisms of erlotinib resistance are gatekeeper mutation (T790M) of EGFR and c-Met amplification in tumor cells (14, 69). On the other hand, it is reported that the tumor cells express active EGFR even after acquiring resistance to erlotinib (14, 70). Considering that EGFR overexpression is a factor of poor prognosis, discontinuing erlotinib treatment after PD has developed may be an inappropriate option and combining erlotinib with the next stage of chemotherapy may be an appropriate therapy. I have previously reported that the combination of docetaxel with erlotinib showed a synergistic effect in NSCLC cell lines in vivo irrespective of EGFR or K-RAS mutation status (71).

In this study, I investigated the antitumor effect of combination therapies of erlotinib with various chemotherapeutic agents, docetaxel, irinotecan, and gemcitabine, using erlotinib-resistant tumor cell xenografts as well as an in vivo erlotinib PD xenograft model, to show the clinical relevance of continuing erlotinib treatment after development of PD.

Materials and Methods

Reagents

Erlotinib was provided by F. Hoffman-La Roche (Basel, Switzerland) as a fine powder and was dissolved in distilled water containing 6% (w/v) Captisol (CyDex Pharmaceuticals, KS, USA) and diluted with saline for in vivo experiments. Erlotinib was dissolved in DMSO for in vitro experiments. Docetaxel was synthesized by Kanto Chemical Co., Inc. (Tokyo, Japan) as a fine powder and was dissolved in saline containing 2.5% (v/v) polysorbate 80 (Sigma-Aldrich Co., USA) and 2.5% (v/v) ethanol for in vivo experiments. Irinotecan was purchased from Daiichi Sankyo Pharmaceutical Co., Ltd. (Tokyo, Japan) as an aqueous solution and diluted with saline.

Cells

Human non-small cell lung cancer (NSCLC) cell lines, HCC827 (exon 19 deletion EGFR) and H1975 (T790M mutation in EGFR), and human pancreatic cancer cell line, HPAC (wild-type EGFR), were obtained from the American Type Culture Collection. Human NSCLC cell line, EBC-1 (c-Met-amplification) was obtained from the RIKEN BRC (Ibaraki, Japan). Erlotinib-resistant cell line HCC827TR3 was established in-house by exposing HCC827 cells to increasing concentrations of erlotinib in vitro. The HCC827, HCC827TR3 and H1975 cells were maintained at 37°C under 5% CO_2 in RPMI-1640 medium (Sigma-Aldrich Co.) containing 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, and 4.5 g/l glucose. The HPAC cell line was maintained in DMEM: Ham's F12 combined medium (1:1) (Invitrogen, USA) containing 5% FBS, 2 μg/ml insulin, 5 μg/ml transferrin, 40 ng/ml hydrocortisone, and 10 ng/ml EGF. The EBC-1 was maintained in EMEM (Sigma-Aldrich Co.) containing 10% FBS.

Animals

Male 5-week-old BALB-nu/nu mice (CAnN.Cg-Foxn1<nu>/CrlCrlj nu/nu) were obtained from Charles River Japan (Kanagawa, Japan). All animals were allowed to acclimatize and recover from shipping-related stress for 1 week prior to the study. The health of the mice was monitored by daily observation. Chlorinated water and irradiated food were provided ad libitum, and the animals were kept in a controlled light-dark cycle (12 h-12 h). The protocol was reviewed by the Institutional Animal Care and Use Committee of Chugai Pharmaceutical Co., Ltd., and all mouse experiments were performed in accordance with the Guidelines for the Accommodation and Care of Laboratory Animals promulgated in Chugai Pharmaceutical Co., Ltd.

Evaluation of antitumor activity

Study 1

HCC827TR3, EBC-1, H1975 xenograft models and treatment. A suspension of tumor cells (5×10^6 cells/mouse) was inoculated subcutaneously into the right flank of mice. Tumors were allowed to reach 0.1–0.3 cm^3 in size, mice were randomly allocated to the control group, erlotinib group, chemotherapy group and combination of erlotinib with chemotherapy group and these were treated with vehicle of erlotinib and vehicle of chemotherapy, erlotinib and vehicle of chemotherapy, vehicle of erlotinib and chemotherapy, or erlotinib and chemotherapy, respectively. Erlotinib was administered orally (p.o.) once a day from Day 2. Docetaxel was administered intravenously (i.v.) once in 3 weeks (Day 1). Irinotecan was administered intravenously (i.v.) once in 2 weeks (Day 1). To evaluate the antitumor effect and tolerability, tumor volume and body weight were measured twice a week. The tumor volume (V) was estimated from the equation $V = ab^2/2$, where a, and b were tumor length and width, respectively.

Study 2

Establishment of *in vivo* erlotinib PD model and treatment. To establish an in vivo erlotinib PD model, a suspension of HPAC cells (5×10^6 cells/mouse) was inoculated subcutaneously into the right flank of the mice. Tumors were allowed to reach 0.1–0.3 cm^3 in size, mice were randomly allocated to control and erlotinib groups. Erlotinib was administered orally (p.o.) once a day starting from Day 1 to Day 18.

After establishment of PD during erlotinib treatment was confirmed, mice were re-randomized and allocated to the control group, erlotinib group, gemcitabine group, and combination of gemcitabine with erlotinib group and these were treated with vehicle of erlotinib and vehicle of gemcitabine, erlotinib and vehicle of gemcitabine, vehicle of erlotinib and gemcitabine, or erlotinib and gemcitabine, respectively. Erlotinib was administered orally (p.o.) on Days 21–25, 28–32, 35–40. Gemcitabine was administered i.v. once a week (on Days 20, 27 and 34). To evaluate the antitumor effect and tolerability, tumor volume and body weight were measured twice a week. The tumor volume (V) was estimated from the equation $V = ab^2/2$, where a, and b were tumor length and width, respectively.

Western blotting

Cells (HCC827, HCC827TR3, EBC-1 and H1975) were seeded into 6-well plates at a concentration of 5×10^5 cells per well and were preincubated overnight. Then, erlotinib was added and incubation continued for 2 h. Cells were stimulated with 100 ng/ml of EGF (Invitrogen) for the last 15 min of the incubation. HPAC tumor tissues of the in

vivo PD model were pulverized in liquid nitrogen. Cellular total protein was prepared from cell lysates and the pulverized frozen tumors. Proteins (100 μg each of HPAC, EBC-1 and H1975; 5 μg each of HCC827 and HCC827TR3) were electrophoresed on SDS-PAGE with 7.5% gel and transferred onto PVDF membranes (GE Healthcare Japan, Tokyo, Japan). The membranes were blocked with a blocking buffer (Thermo Fisher Scientific, Kanagawa, Japan), immunoblotted with primary antibody against EGFR (Santa Cruz Biotechnology Inc., CA, USA), pY1068 pEGFR (Cell Signaling Technology Inc.) and GAPDH (Santa Cruz Biotechnology Inc.). The protein-antibody complex was detected by chemiluminescence (GE Healthcare Japan).

Cell proliferation assay

Cells were seeded at a density of 1000 or 3000 cells/well in 96-well plates and were preincubated overnight. The cells were then treated with erlotinib for 96 h. Cell proliferation was evaluated by Cell Counting Kit-8 (Dojindo, Kumamoto, Japan).

Statistical analysis

Statistical analysis to evaluate the antitumor activity was performed using the Mann-Whitney U test. For in vitro experiments, Student's t-test was used. Differences were considered to be significant at $P \leq 0.05$. Statistical analysis was carried out using the SAS preclinical package (SAS Institute, Inc., Tokyo, Japan).

Results

Erlotinib sensitivity, EGFR expression and effect of erlotinib on phosphorylation of EGFR and downstream signaling molecules in erlotinib-resistant NSCLC cells

First, I examined the growth inhibition of tumor cells, namely HCC827, HCC827TR3, EBC-1 and H1975. HCC827TR3 was 1000 times more resistant to erlotinib than parental HCC827 (Fig. 5A) *in vitro*. I found that the mechanism of erlotinib resistance of HCC827TR3 was neither c-Met amplification nor T790M mutation in EGFR (data not shown). Almost no growth inhibition was observed in EBC-1 and H1975 cells up to 3 μmol/l of erlotinib (Fig. 5B). Next, I examined EGFR expression in the tumor cells and the effect of erlotinib on the phosphorylation of EGFR, as well as its major downstream signal molecules such as Akt, ERK, Stat3, by Western blotting. All of the cell lines expressed EGFR and phosphorylated EGFR (Fig. 5C). The EGFR phosphorylation was completely suppressed by erlotinib in HCC827, HCC827TR3 and EBC-1, although erlotinib did not inhibit the proliferation of HCC827TR3 and EBC-1. On the other hand, erlotinib did not suppress the phosphorylation of EGFR in H1975 cells (Fig. 5C). Erlotinib suppressed the phosphorylation of Akt and ERK in HCC827 cells. However, out of the three erlotinib-resistant cell lines, only a slight inhibition of ERK phosphorylation in HCC827TR3 was observed (Fig. 5C).

Antitumor effect of combination therapy of chemotherapeutic agents with erlotinib in erlotinib-resistant tumor xenografts

Because EGFR phosphorylation was suppressed by erlotinib in the erlotinib-resistant cells (EBC-1, HCC827TR3), it may be of value to administer erlotinib concurrently with a chemotherapeutic agent when treating erlotinib-resistant tumors. Therefore, I next examined the antitumor activity of combination therapy of a chemotherapeutic agent with erlotinib against these erlotinib-resistant cell lines in xenografts.

First, I examined the antitumor effect of docetaxel monotherapy and docetaxel + erlotinib therapy using the HCC827TR3 xenograft model. In this model, erlotinib monotherapy did not show any antitumor effect even at a dose of 25 mg/kg, which was higher than the effective dose for parental HCC827 xenograft model (Fig. 6A). However, docetaxel in combination with erlotinib showed a significantly higher antitumor activity compared with docetaxel monotherapy (Fig. 6B). A similar result was obtained in the combination therapy of irinotecan with erlotinib in the same xenograft model (Fig. 6C). In the EBC-1 xenograft model, similarly, significantly higher

antitumor effect was obtained in the combination therapy of docetaxel (5 mg/kg) with erlotinib (75 mg/kg) compared to docetaxel monotherapy whereas erlotinib did not show any antitumor effect at the same dose (Fig. 7). Namely, the combination therapy of chemotherapeutic agent with erlotinib showed a significantly higher antitumor effect compared with chemotherapy alone while erlotinib monotherapy showed no effect in HCC827TR3 or EBC-1 xenografts. On the other hand, no significant effect was seen between docetaxel monotherapy (5 mg/kg) and combination of docetaxel (5 mg/kg) with erlotinib (75 mg/kg) in the H1975 xenograft model (Fig. 8).

Establishment of *in vivo* erlotinib-resistant model and antitumor activity of gemcitabine in combination with erlotinib

To mimic the clinical PD phenomenon and examine the effect of combination therapy of docetaxel with erlotinib, I established an in vivo erlotinib-resistant model using EGFR-positive pancreatic cancer cell line HPAC. The HPAC cells were subcutaneously inoculated into BALB/c-nu/nu mice, and erlotinib (75 mg/kg) was administered p.o. once a day for 18 days. In this model, erlotinib significantly inhibited tumor growth up to 5 days after the start of administration (Fig. 9A). Subsequently, however, the tumor growth inhibition effect by erlotinib disappeared, even though erlotinib was continuously administered (Fig. 9A). Fig. 9B shows the constant tumor volume ratio of erlotinib group to vehicle group after around Day 8. On Day 20, the mice in the erlotinib group were randomly allocated to 4 groups, namely, vehicle group, erlotinib group, gemcitabine group, and gemcitabine + erlotinib group. Although EGFR protein remained positive and its phosphorylation had been substantially reduced by erlotinib by Day 21 (Fig. 9C), the erlotinib group did not show significant tumor growth inhibition compared with the vehicle group (Fig. 9D). This indicated that the HPAC tumors had become resistant to erlotinib. Using this model, I examined the antitumor activity of combination therapy of gemcitabine (25 mg/kg) with erlotinib (75 mg/kg). The results indicated that the combination therapy showed a significant antitumor effect compared with gemcitabine monotherapy (Fig. 9D) even though erlotinib monotherapy showed no tumor inhibitory effect.

Discussion

By using two types of tumor models, I was able to investigate the mechanism by which NSCLC and pancreatic cancer become resistant to erlotinib. Although EBC-1 and H1975 show amplification of c-Met and mutation of T790M, respectively, HCC827TR3, which was established in-house, has neither. In the HCC827TR3 cells, neither EGFR down-regulation nor reduction of EGFR phosphorylation was observed (Fig. 5C). The fact that EGFR phosphorylation was inhibited by erlotinib in HCC827TR3 cells but the PI3K pathway was not inhibited and the Ras-ERK/MAPK pathway only partially inhibited (Fig. 5C) indicates that the resistance mechanism may be the activation of these pathways by protein kinase(s) other than c-MET. (Fig. 10)

Erlotinib completely inhibited EGFR phosphorylation in EBC-1 and HCC827TR3 cells but not in H1975 cells (Fig. 5C). This coincides well with the previous reports (14, 69, 70) which state that, in cells with c-Met amplification, erlotinib resistance is activated in the cell growth signaling pathway through heterodimer formation of MET and HER3 molecules. Thus, EGFR remains intact in c-Met amplification cells such as EBC-1, and erlotinib is able to inhibit EGFR phosphorylation. In the case of HCC827TR3, although the precise mechanism of resistance is not yet clear, it would seem that EGFR phosphorylation was inhibited by a similar mechanism. On the other hand, erlotinib could not inhibit EGFR phosphorylation in H1975 cells because the T790M mutation in EGFR causes a conformation change at the ATP binding pocket, thus decreasing the affinity between erlotinib and EGFR.

Since all of the erlotinib-resistant cell lines express EGFR, I examined the antitumor effect of combination therapy of erlotinib with docetaxel or irinotecan. In these models, erlotinib monotherapy did not show significant antitumor effect compared with the control group (Figs. 6A, 7 and 8). Interestingly, however, combination therapy of docetaxel with erlotinib showed a synergistic effect in HCC827TR3 (Fig. 6B) and EBC-1 (Fig. 7) xenografts. A similar result was obtained in HCC827TR3 xenografts using irinotecan as a chemotherapeutic agent (Fig. 6C). These results may indicate that the chemotherapeutic agent used in the combination therapy need not be restricted to a specific drug. On the other hand, no significant increase of antitumor effect of combination therapy compared with docetaxel monotherapy was observed in H1975 xenografts (Fig. 8). These results coincide well with the report of Okabe et al in which gefitinib and S-1 were used in combination in H1975 and HCC827GR5 xenografts (72). Since EGFR phosphorylation was completely inhibited by erlotinib in HCC827TR3 cells and EBC-1 cells but not in H1975 cells (Fig. 5C), it is possible that inhibition of

EGFR phosphorylation is prerequisite for the combination therapy to be effective. EGFR phosphorylation activates signal transduction pathways, such as PI3K and Ras-ERK/MAPK, and erlotinib inhibits these pathways. However, the role of erlotinib in combination therapy in erlotinib-resistant xenograft models may be inhibition of signal pathway(s) other than the PI3K or Ras-ERK/MAPK pathways, because erlotinib monotherapy did not show any antitumor effect in HCC827TR3 and EBC-1 xenografts. In the H1975 xenograft model, erlotinib failed to inhibit EGFR phosphorylation (Fig. 5C) hence the antitumor effect of combination therapy was not enhanced. Okabe et al reported that the combination effect of S-1 with gefitinib was attributed to the down-regulation of thymidylate synthase (TS) by gefitinib and the mechanism could work even after the tumor cells became resistant to gefitinib (72). I consider that similar mechanisms are involved in my system, although the target molecules have so far not been specified. In the case of EBC-1, combination therapy using docetaxel was expected to reduce c-MET in cells, but this was not observed (data not shown). It was reported that erlotinib restores the effect of chemotherapeutic agents through direct inhibition of PgP or BCRP (73, 74). However, this is unlikely because verapamil, a PgP or BCRP inhibitor, did not restore the sensitivity to docetaxel in HCC827TR3 cells (data not shown).

In my HPAC in vivo model which mimics PD in clinical therapy, the combination therapy of gemcitabine with erlotinib showed significantly strong antitumor effect compared with gemcitabine monotherapy (Fig. 9D). EGFR expression and phosphorylated EGFR were detected in the tumors of the control group after PD had developed. Surprisingly, phosphorylation of EGFR was completely inhibited in the tumors of the erlotinib group (Fig. 9C). These results indicate the usefulness of the combination therapy of a chemotherapeutic agent with erlotinib against in vivo-induced erlotinib-resistant tumors.

Erlotinib is currently approved for the treatment of NSCLC and pancreatic cancer. In the present study, I showed that combination therapy of a chemotherapeutic agent with erlotinib is efficacious against two erlotinib-resistant NSCLC cell lines (EBC-1, HCCC827TR3) and one pancreatic cancer cell line (HPAC) which had become erlotinib resistant, suggesting that this form of treatment would be useful against NSCLC and pancreatic cancer which developed PD. Erlotinib has been reported to have an excellent benefit for patients with NSCLC harboring mtEGFR and to prolong the overall survival of patients with NSCLC harboring wtEGFR (11). The combination therapy may be effective regardless of the EGFR mutation status because it was effective on both HCC827TR3 (mtEGFR) and HPAC (wtEGFR) cells.

The results suggest that combination therapy of a chemotherapeutic agent with erlotinib showed stronger antitumor effect compared with chemotherapy alone against erlotinib-resistant tumors in that erlotinib inhibited the phosphorylation of EGFR in the tumor. It may be possible to obtain evidence for the suitability of the combination therapy by monitoring the EGFR phosphorylation level in tumors after PD has developed following erlotinib treatment. However, this test cannot distinguish tumors which had intrinsically low EGFR phosphorylation and, to solve the problem, it may be necessary to test the EGFR phosphorylation level before the start of erlotinib therapy. In the present study, docetaxel, irinotecan and gemcitabine were used as chemotherapeutic agents. Whether or not similar results can be obtained with other chemotherapeutic agents is an issue for future research. If a patient goes into PD during combination therapy, a possible treatment modality may be to change the chemotherapeutic agent while continuing erlotinib.

General Discussion

In cancer treatment, chemotherapy have been used for a long time. However, they cause severe side-effects because their activity is not specific to tumors. The specific action of targeted therapy, which stops the action of molecules that are key to the growth of cancer cells, differs from traditional chemotherapy, which affects all fast-growing cells. Therefore many targeted agents have been developed recently to limit toxicity and to improve the efficacy of cancer therapy.

There are two main types of targeted therapy. The first type targets tumor cells by attacking molecules expressed on the surface or activated in the cytoplasm of cancer cells (1-3). The second type targets the tumor microenvironment by attacking tumor angiogenesis and the antitumor immune response. It has long been recognized that construction of blood vessels in the tumors is necessary to supply sufficient nutrition and oxygen for tumor cells to proliferate. VEGF is an important factor in tumor angiogenesis and growth (22-25), but VEGF-independent angiogenesis has been reported as a mechanism of resistance to anti-VEGF therapy (48). In order to establish a colorectal cancer xenograft model that develops resistance to anti-VEGF antibody, five human colorectal cancer xenografts were treated with anti-VEGF antibody. Although at first anti-VEGF antibody exhibited significant antitumor activity in all of the five models, in the HT-29 model anti-VEGF antibody exhibited significant antitumor activity in the early phase but not in the late phase, indicating that the HT-29 model became non-sensitive to anti-VEGF antibody during treatment. Also, no difference was observed in the tumor vessel density of HT-29 tumor tissue from the control and bevacizumab groups. These facts indicate that HT-29 tumor has VEGF-independent angiogenesis. However, even in the presence of VEGF-independent angiogenesis, HT-29 continues to express VEGF.

It was previously reported that the molecular targeted drug trastuzumab exhibited antitumor effects when administered with chemotherapeutic agents after the tumor had acquired resistance to monotherapy, if the tumor kept expressing the target molecule (53). Similarly, when a tumor continues to express VEGF, combining an anti-VEGF antibody with second-line chemotherapy after resistance has been acquired, may be an appropriate therapy. Therefore, I hypothesized that BBP therapy is effective even after a tumor acquires resistance to anti-VEGF antibody, and I used the HT-29 PD model to investigate the antitumor effect of the combination of an anti-VEGF antibody plus capecitabine. Of note, the combination of anti-VEGF antibody plus capecitabine exhibited a significantly stronger antitumor effect than capecitabine alone. These data

suggested that it is important to continue to inhibit VEGF by an anti-VEGF antibody in combination with an appropriate chemotherapeutic agent, even after the tumor has acquired resistance to the anti-VEGF antibody per se. Next, I used the same model to investigate the mechanism by which the combination of anti-VEGF antibody plus capecitabine achieved this antitumor effect. The results showed that tumor vessel density was significantly reduced in the combination group, whereas it was not reduced in the groups receiving anti-VEGF antibody or capecitabine alone at this stage. In addition, the level of galectin-3, which is known as an important mediator of angiogenic response, was significantly decreased in the capecitabine and combination groups compared with the control group. In contrast to galectin-3 levels, VEGF levels increased in the capecitabine treatment group in our model, and this bilateral effect of capecitabine on the production of angiogenic factors may be a reason for the apparent absence of anti-angiogenic effect by capecitabine alone. Nevertheless, by neutralization of VEGF through concurrent administration of bevacizumab, a synergistic anti-angiogenic effect emerged. These data raise the possibility that the inhibition of galectin-3 production by capecitabine and the neutralization of VEGF by bevacizumab formed the mechanisms underlying the effect of combination treatment.

EGFR genetically overexpressed on the surface of cancer cells, and EGFR is the major contributors of a complex signaling cascade that modulates growth and survival of cancer cells. EGFR-TKI reversibly binds to the intracellular domain of EGFR. This blocks autophosphorylation of EGFR with subsequent inhibition of the downstream signaling pathways which promote cell proliferation. Two mechanisms of acquired resistance to EGFR-TKI, i) resistant EGFR mutations, and ii) activation of bypass pathways or downstream pathways, have been suggested in the literature.

To analyze the characteristics of EGFR-TKI-resistant tumor, I used models with EGFR-TKI-resistant tumors which continue to express EGFR. Because EGFR phosphorylation was completely inhibited by EGFR-TKI in HCC827TR3 cells, EBC-1 cells, and HPAC in vivo resistant-tumor, but not in H1975 cells, it is possible that inhibition of EGFR phosphorylation is prerequisite for the combination therapy to be effective. Therefore, I examined the antitumor effect of combining chemotherapy with EGFR-TKI. In these models, EGFR-TKI monotherapy did not show significant antitumor effect compared with the control group, but interestingly, combination therapy of chemotherapeutic agent showed a synergistic effect in the HCC827TR3 and EBC-1 models. These results may indicate that the chemotherapeutic agent used in the combination therapy need not be restricted to a specific drug. On the other hand, no significant increase in the antitumor effect of combination therapy compared with

chemotherapeutic agent monotherapy was observed in the H1975 model. Although an EGFR-TKI inhibits the signal transduction pathways activated by EGFR phosphorylation, such as PI3K and Ras-ERK/MAPK, the role of EGFR-TKI in combination therapy in EGFR-TKI-resistant tumor may be to inhibit signal pathway(s) other than the PI3K or Ras-ERK/MAPK pathways, because EGFR-TKI monotherapy did not show any antitumor effect in the HCC827TR3 and EBC-1 models. It may be possible to obtain evidence for the suitability of the combination therapy by monitoring the EGFR phosphorylation level in tumors after PD has developed following EGFR-TKI treatment.

In these studies, I showed that tumors continue to express the target molecule after acquiring resistance to therapy, and combining VEGF- or EGFR-targeted therapy with chemotherapy exerted strong antitumor activity, even after resistance to the targeted therapy was acquired, by inhibiting the effect of the target molecules. These results indicate that VEGF- or EGFR-independent tumor depended on these molecules again through chemotherapy treatment. Tumor cell may have instability not only in drug sensitivity but also in drug resistance. And chemotherapy may enhance the instability in drug resistance and reverse the resistance.

It has been reported that after resistance has been acquired to other targeted drugs, tumors continue to express the target molecule (75, 76). Therefore, targeted therapy in a combination with chemotherapy after PD may be applicable in many other targeted therapies.

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Table

Table 1.

VEGF expression levels in tumor tissue of colorectal cancer xenograft models.

Tumor type	VEGF (pg per mg protein)
HCT-8	1669
COLO 205	1778
HCT 116	2539
HT-29	3132
LS411N	3540

Figures

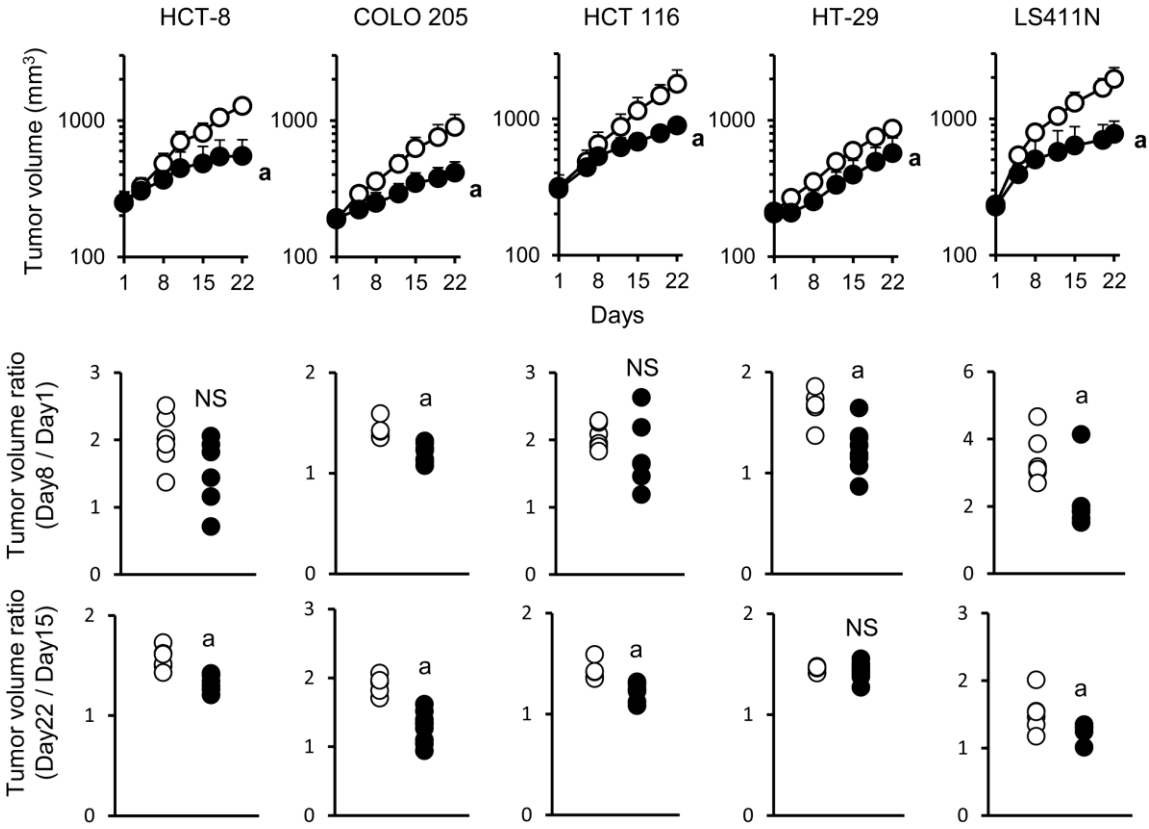

Figure 1.

Bevacizumab shows antitumor activity in the early phase but not in the late phase in HT-29 xenograft model. Mice were randomized into 2 groups (n=5–10/group). Bevacizumab and control human IgG were intraperitoneally administered once a week. Data points show mean ± SD of tumor volume. Control, open circles; bevacizumab, closed circles. a: $P<0.05$ versus control IgG group by Wilcoxon test.

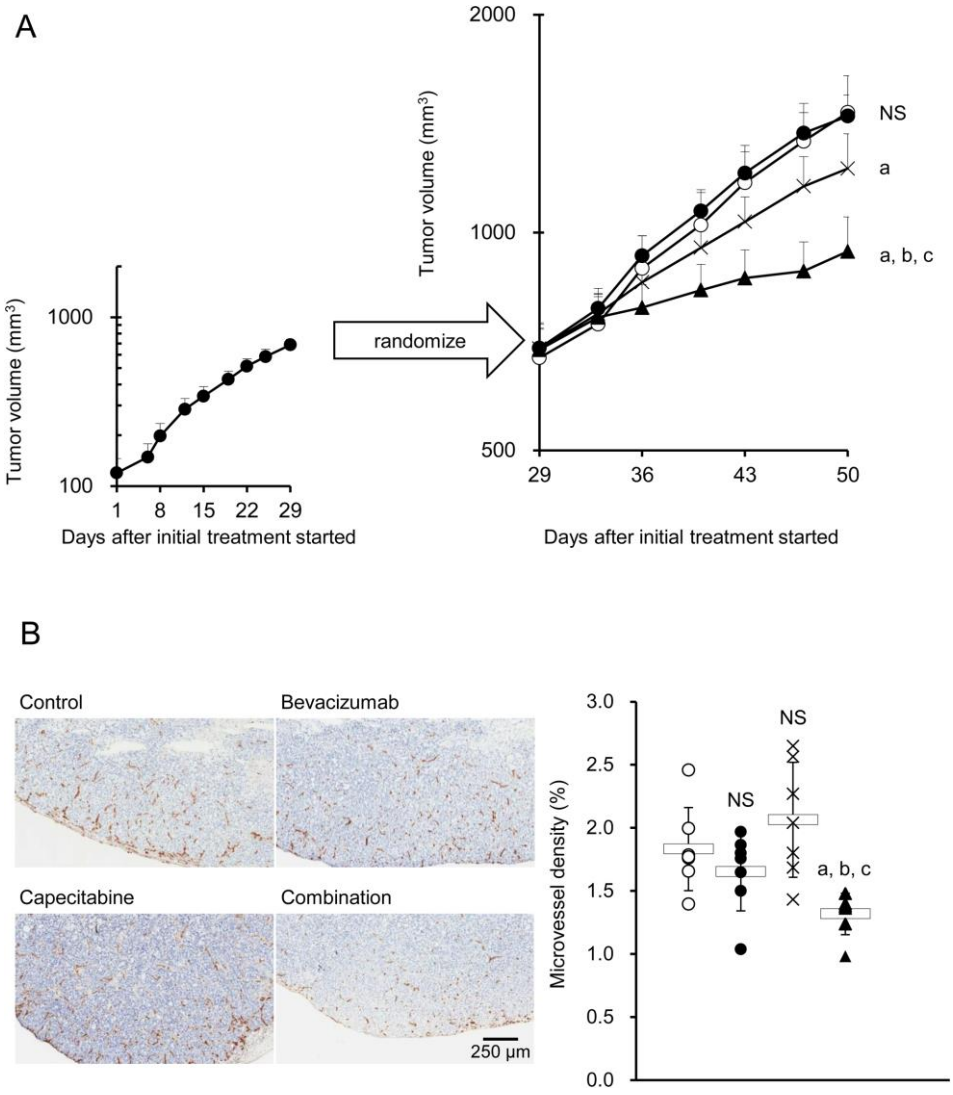

Figure 2.

Combination therapy with bevacizumab plus capecitabine inhibits tumor growth and tumor angiogenesis in the HT-29 xenograft bevacizumab PD model. Antitumor activity (A) and anti-angiogenic activity (B) of combination therapy with capecitabine plus bevacizumab in the HT-29 xenograft model unresponsive to bevacizumab. Mice that had been treated with bevacizumab on days 1 and 8 were randomly allocated to control, bevacizumab, capecitabine, and bevacizumab plus capecitabine groups on day 29 (n=7/group). CD31 immunostaining in tumor tissue at day 50 (n=7/group). Data points show mean ± SD of tumor volume. Control, open circles; bevacizumab, closed circles; capecitabine, cross marks; combination group, closed triangles. The box-and-whisker plots show mean ± SD. a: $P<0.05$ versus control group; b: $P<0.05$ versus capecitabine group; c: $P<0.05$ versus bevacizumab group by Wilcoxon test.

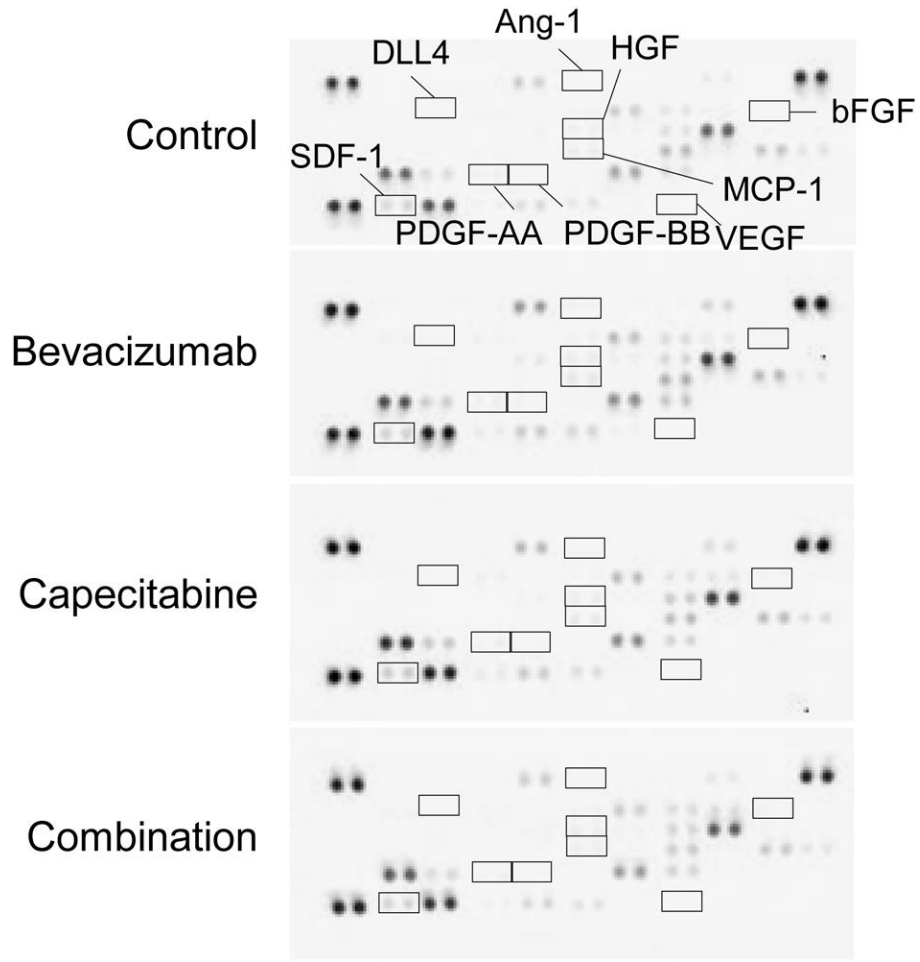

Figure 3.

Combination therapy did not change the expression of stromal cell-derived angiogenic factors in tumor tissues. Changes in stromal cell-derived angiogenic factors. Membranes-based antibody arrays were reacted with homogenized tumor tissue harvested from the control, bevacizumab, capecitabine, and combination group on day 50.

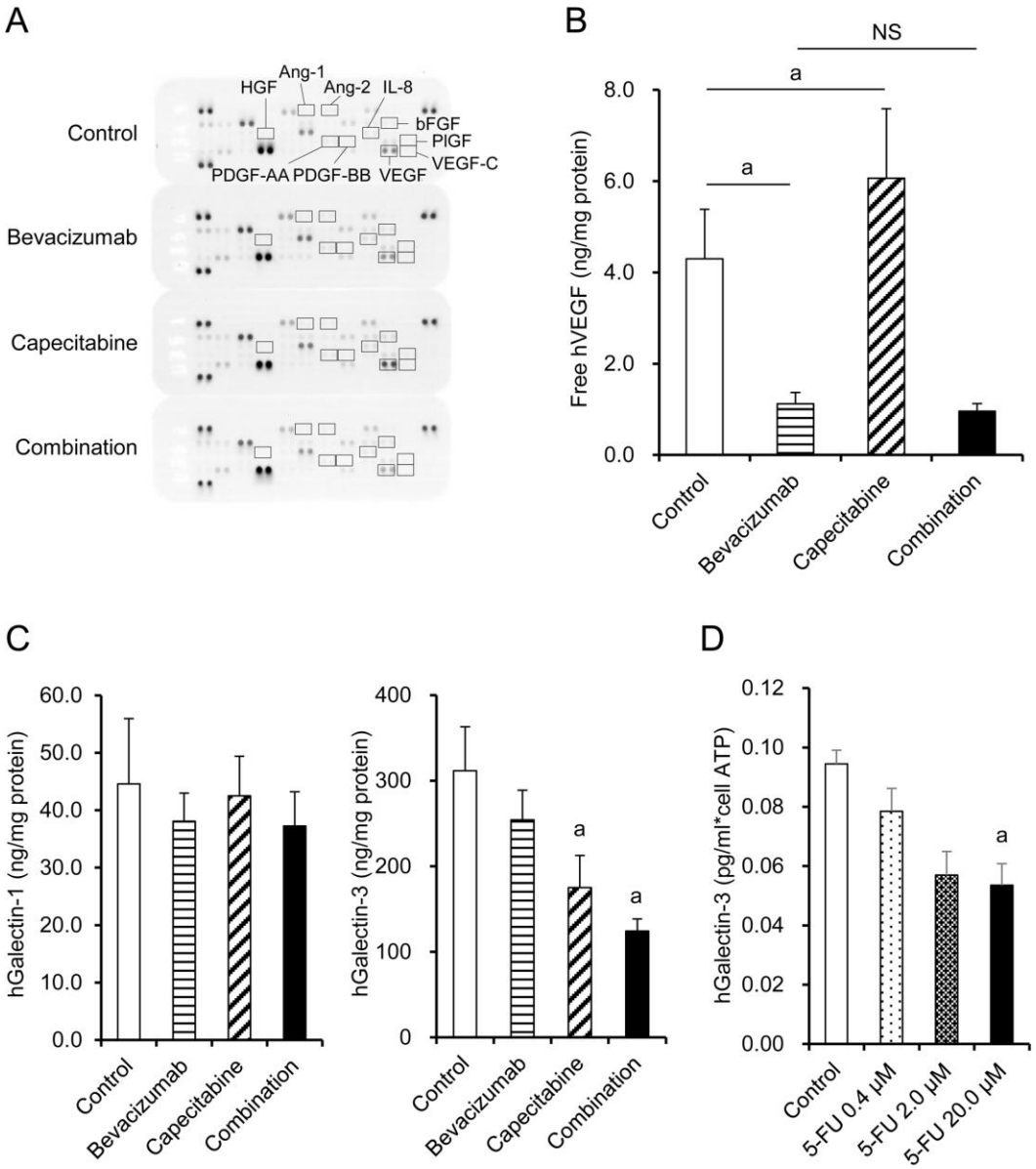

Figure 4.

Combination therapy suppresses tumor cell-derived VEGF and galectin-3 in tumor tissues. (A) Membranes-based antibody arrays were reacted with tumor tissue homogenates from the control group, bevacizumab group, capecitabine group, and combination group on day 50. (B) Levels of free VEGF in the tumor tissues (n=7). (C) Levels of galectin-1 and galectin-3 in the tumor tissues (n=7). The control group (open), bevacizumab group (horizontal striped), capecitabine group (hatched), and combination group (closed). Columns show mean ± SD. a: $P<0.05$ versus control by Wilcoxon test. (D) Downregulation of galectin-3 expression by 5-FU in in vitro culture. Culture supernatant was collected and subjected to ELISA (n=3). Columns show mean ± SD. a: $P<0.05$ versus control by Dunnett's test.

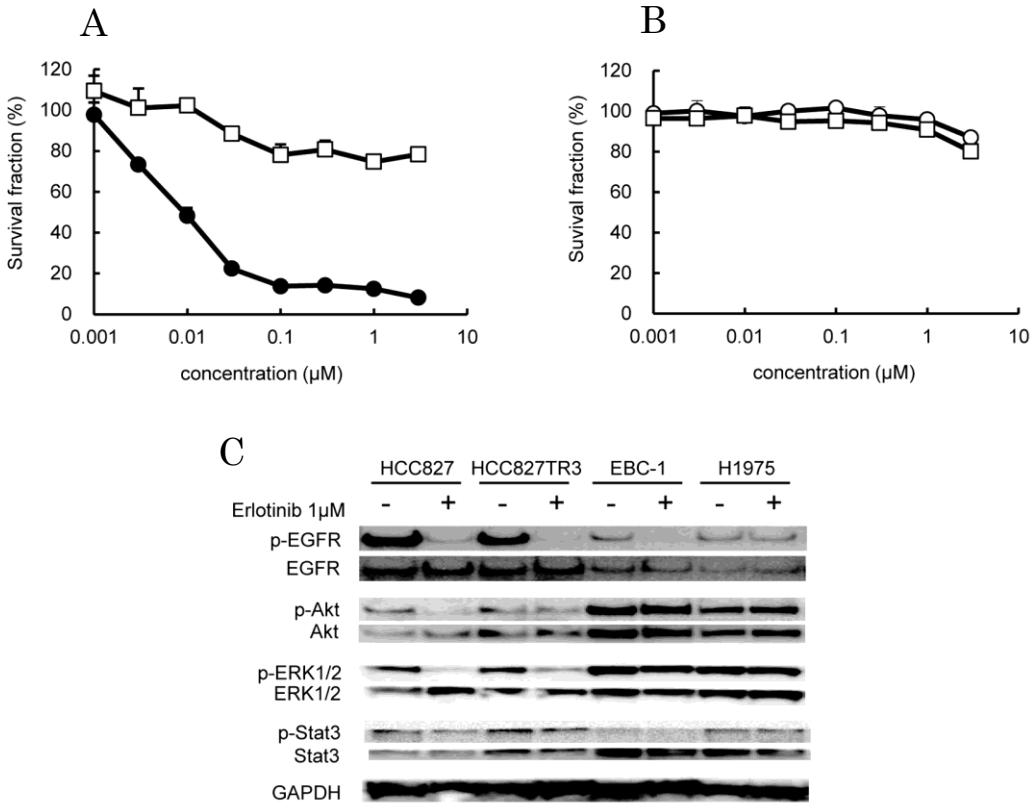

Figure 5.

Erlotinib sensitivity, EGFR expression and effect of erlotinib on phosphorylation of EGFR and downstream signaling molecules in cancer cell lines in vitro. (A) Growth inhibition of erlotinib in parental HCC827 (●) and resistant HCC827TR3 (□). (B) Growth inhibition by erlotinib in EBC-1 (○) and H1975 (□). (C) Expression of EGFR and inhibition of phosphorylation in signal transduction molecules by erlotinib in NSCLC cell lines.

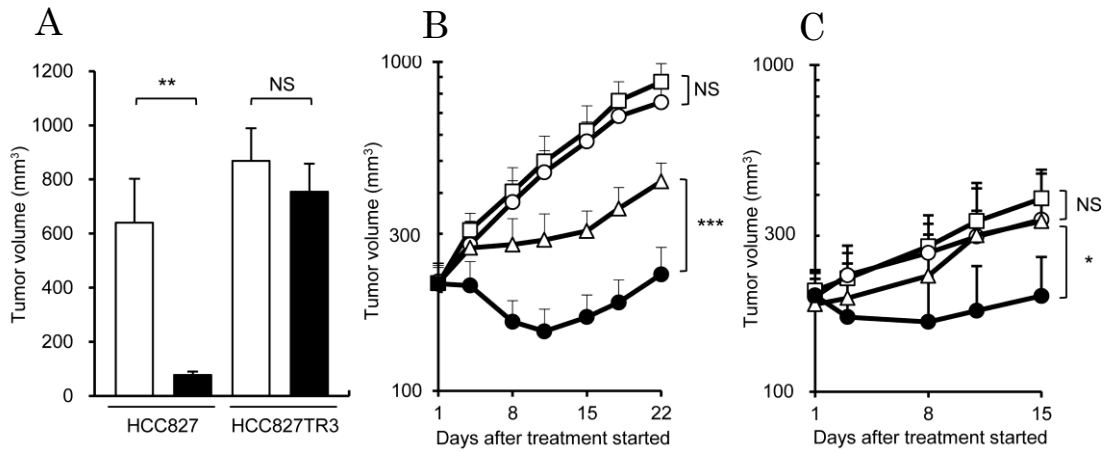

Figure 6.

Antitumor effect in parental HCC827 and resistant HCC827TR3 xenograft models. (A) Erlotinib monotherapy at Day 22. □, control; ■, erlotinib 15 mg/kg (HCC827), 25 mg/kg (HCC827TR3), n=5/group. (B) Combination therapy of docetaxel with erlotinib in HCC827TR3 xenograft model. □, control; ○, erlotinib 25 mg/kg; △, docetaxel 20 mg/kg; ●, combination, n=7/group. (C) Combination therapy of irinotecan with erlotinib in HCC827TR3 xenograft model. □, control; ○, erlotinib 25 mg/kg; △, irinotecan 60 mg/kg; ●, combination, n=5/group. Statistically significant differences are shown. NS, not significant; *P≤0.05, **P≤0.01, ***P≤0.001 by Wilcoxon test.

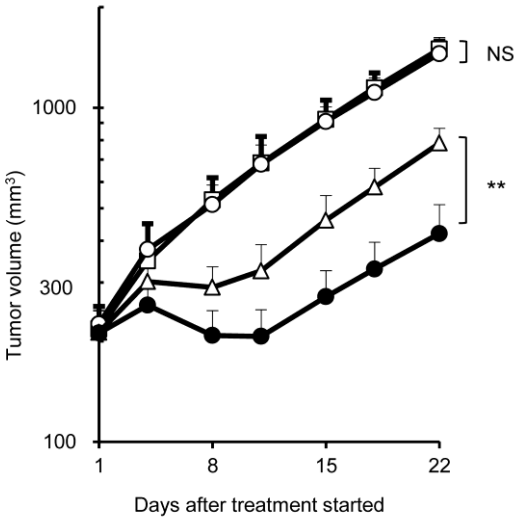

Figure 7.

Antitumor effect of docetaxel in combination with erlotinib in EBC-1 xenograft model. □, control; ○, erlotinib 75 mg/kg; △, docetaxel 5 mg/kg; ●, combination, n=6/group. Statistically significant differences are shown. NS, not significant; **P≤0.01 by Wilcoxon test.

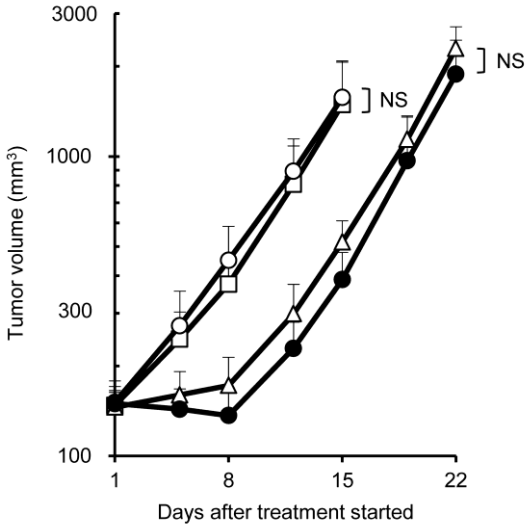

Figure 8.

Antitumor effect of docetaxel in combination with erlotinib in H1975 xenograft model. □, control; ○, erlotinib 75 mg/kg; △, docetaxel 5 mg/kg; ●, combination, n=7/group. Statistically significant differences are shown. NS, not significant.

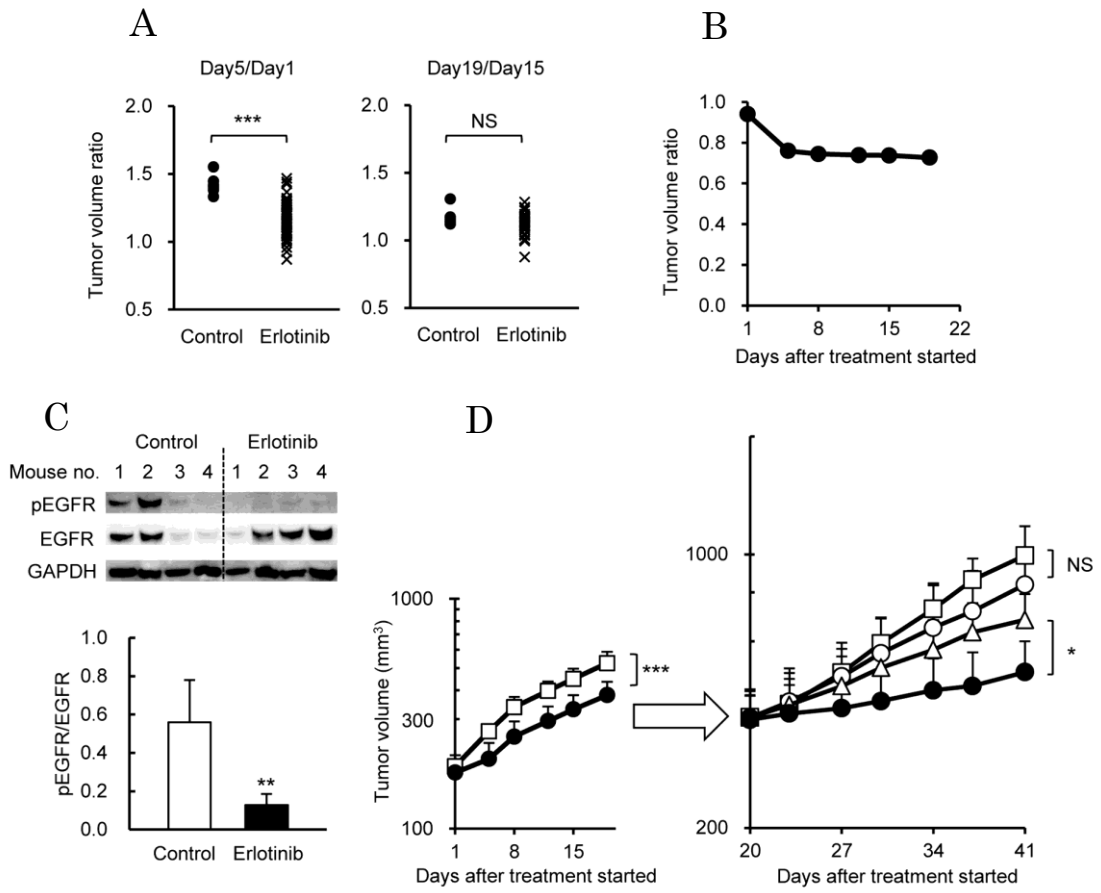

Figure 9.

Establishment of an in vivo erlotinib-resistant model and antitumor activity of gemcitabine in combination with erlotinib. (A) Ratio between tumor volume and that of 4 days previously. Appearance of the progressive tumor during 19 days of treatment with erlotinib is shown. Mice were allocated to groups of 6 mice for control and 70 mice for erlotinib treatment. Erlotinib was administered p.o. qd for 19 days. ●, control; ×, erlotinib 75 mg/kg. (B) Time course of the tumor volume ratio of erlotinib group to vehicle group. (C) Expression and phosphorylation of EGFR after acquiring resistance. Tumor tissues after acquiring resistance to erlotinib treatment were collected on Day 21. (D) Antitumor activity of gemcitabine in combination with erlotinib. On Day 20 of erlotinib treatment, mice in the erlotinib group were randomly allocated to 4 groups (n=6/group). □, control; ○, erlotinib 75 mg/kg; △, gemcitabine 25 mg/kg; ●, combination. Statistically significant differences are shown. NS, not significant. ***P≤0.001, *P≤0.05 by Wilcoxon test.

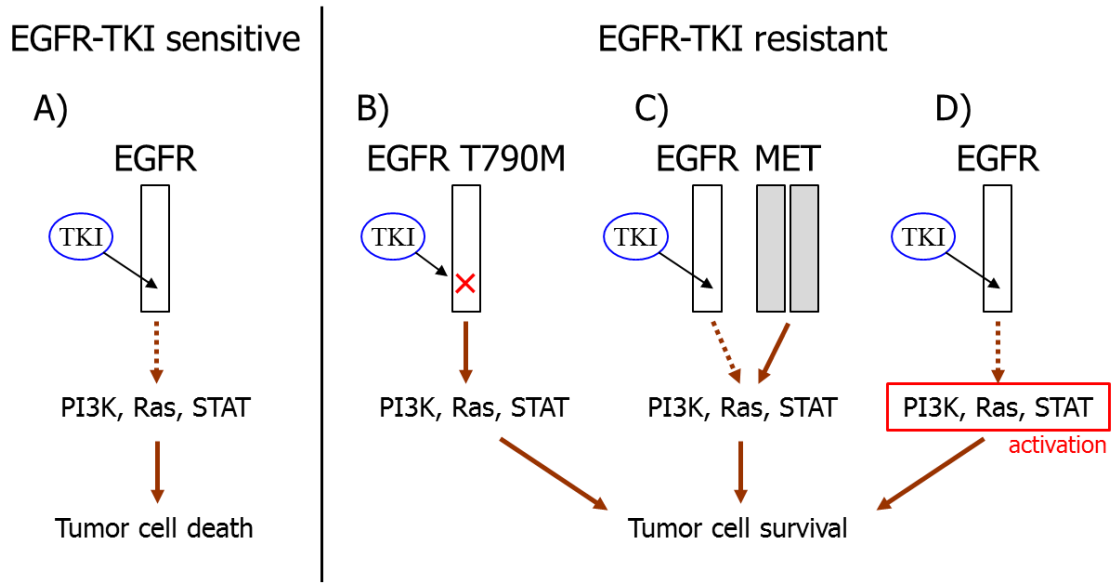

Figure 10.

Mechanisms of EGFR resistance to EGFR-TKI. (A) EGFR-TKI inhibits phosphorylation of EGFR and leads to apoptosis of cells. (B) T790M mutation prevents binding of EGFR-TKI to EGFR, resulting in cell survival. (C) Amplification of MET activates the downstream signaling pathways. (D) Activating mutation of the downstream signaling pathways leads to survival of cells.