The use of protein array to identify targetable receptor tyrosine kinases for treatment of human colon cancer

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Abstract. Several studies have reported that activated receptor tyrosine kinases (RTKs) are highly expressed in colon cancer and may promote tumor growth and survival. However, there is little information available as to the function and signaling of RTKs in colon cancers. In the present study, we performed protein array technology to determine the expression status of various RTKs that are activated in colon cancer compared to normal colonic cells and tissues. Of the 42 different phospho-RTKs, 5 (ErbB2, FGFR1, FGFR2a, FGFR3 and MSPR) were activated in Caco-2, SW480, WiDr, Lovo colon cancer cell lines and cancerous tissues. In order to determine the effect of inhibition of RTKs, especially ErbB2, athymic nude mice bearing xenograft tumors were treated with the ErbB2-targeting drug trastuzumab alone, or in combination with 5-Fluorouracil (5-FU). Similar to the treatment of 5-FU alone, trastuzumab suppressed the growth of colon cancer. Combination therapy of trastuzumab and 5-FU inhibited tumor growth significantly compared to the treatment of 5-FU alone or trastuzumab alone. In addition, xenograft tumors were also analyzed by phospho-MAPK protein array. The activity of Akt3/PKBγ was inhibited with 5-FU alone and trastuzumab, indicating that trastuzumab may inhibit colon cancer growth through ErbB2-Akt3/PKBγ signaling. These data demonstrate that ErbB2 could be an important candidate for colon cancer therapy and the addition of trastuzumab to 5-FU therapy might augment the clinical response in colon cancer patients. Therefore, the analysis of phospho-RTK expression by protein array as a useful tool might identify novel therapies for individual patients with colon cancer.

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Introduction

Colorectal cancer is the fourth leading cause of death in all cancers and is responsible for approximately 500 deaths per 100,000 persons per year in the world (1). Despite the increasing emphasis on screening and prevention, at least 5% of patients will present with locally unresectable disease with tumors fixed to critical structures or organs not amenable nor appropriate for radical resection (2). In such patients, incomplete resection alone (3) or radiation therapy alone (4), results in few long-term survivors. Therefore, there is a strong demand for new curative approaches to colon cancer therapy.

The protein tyrosine kinases are a large and diverse multi-gene family found only in Metazoans. Their principle function involves the regulation of multicellular aspects of the organism. Cell to cell signaling concerning growth, differentiation, adhesion, motility and death, are frequently transmitted through tyrosine kinases. To date, of the 90 tyrosine kinases, 58 are receptor tyrosine kinases (RTKs) characterized by a transmembrane domain and a tyrosine kinase motif (5,6). RTKs, divided into 21 families, are membrane-bound proteins consisting of a ligand-binding domain at the extracellular surface, a single transmembrane segment and a cytoplasmic region harboring the protein kinase activity. The best known examples are the epidermal growth factor receptor (EGFR) family, the vascular endothelial growth factor receptor (VGEFR) subtypes, the fibroblast growth factor receptor (FGFR) family and the platelet-derived growth factor receptor (PDGF) family (7).

A relationship between human cancer and RTK activity has been established. Aberrant RTK activity has been detected in various human cancers including colon cancer (8-10). Therefore, instead of conventional chemotherapy, various RTK inhibitors are used for patients in the advanced stages of epithelial cancer and several clinical trials are exploring various designs to bring out the potential of RTK-targeting drugs (11,12).

HER2 (human epidermal growth factor receptor) is the human ErbB2 homologue and a member of the ErbB family of receptors. There are 4 known members of the ErbB

family, namely epidermal growth factor receptor (EGFR), HER2, HER3, HER4, that are expressed in various human tissues. These receptors are often deregulated in neoplasms and can promote proliferation, migration, angiogenesis, stromal invasion and resistance to apoptosis (13). HER2 overexpression has been previously reported in up to 85% of colon cancers; however, estimates vary depending on the methods used (14,15). Few studies have shown that inhibition of HER2 signaling may have a new approach for the treatment of colon cancer (16).

Trastuzumab (Herceptin) targets ErbB2 and is used for treating ErbB2-overexpressing breast cancers. Initially, it was thought that engagement of HER2 by trastuzumab led to an increase in HER2 endocytosis or degradation rate (17). HER2 endocytosis was analyzed, and it was concluded that the monoclonal antibodies do not affect HER2 internalization or the rate of endocytosis (18,19). In addition, trastuzumab does not block the association of ErbB2 with ligand-activated-ErbB3 or EGFR (20). In cancer cells, trastuzumab disrupts the ligand-independent ErbB2/ErbB3/PI3K complexes and blocks Akt signaling (21,22), which ultimately leads to an increase in the CDK2 inhibitor p27 (23,24). Moreover, trastuzumab is shown to have no effect on the activation status of HER2 or to cause a rapid increase or decrease of HER2 phosphorylation (23,25,26). The aim of the present study was: i) to determine which RTKs are activated in colon cancer *in vitro* and *in vivo*; and ii) to investigate whether a RTK targeting drug candidate, trastuzumab is an effective agent in a colon cancer xenograft model.

Materials and methods

Cell culture and reagents. Caco-2, SW480, WiDr, Lovo colon cancer cell lines and CCD 841 CoN a normal colon cell line were obtained as a kind gift of the Japanese Cancer Resource Bank (Tokyo, Japan), and used as the colon cancer cell lines. These cells were plated at a density of $1x10^5$ cell/cm^3 in plastic flasks of Dulbecco's modified minimum essential medium (DMEM) (Gibco BRL Co., Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 mg/ml) and 100 mg/ml streptomycin at 37˚C in 5% CO_2 in air.

Patients. Three tissue samples of colon cancer were obtained from surgery (1 male and 2 females; mean age, 63.7±2.5 years; range, 61-66 years, all samples were classified as Dukes B). Tissues were frozen immediately at -70˚C. None of the patients had received any chemotherapy or radiotherapy before surgery. Informed consent was obtained from each patient prior to participation and the experimental protocol was approved beforehand by the Human Subjects Committee of Kagawa University School of Medicine.

Cell lysate and tissue lysate. The lysate was prepared according to the methods described in our previous report (27-29). All steps were carried out at 4˚C. Protein concentration was measured using a dye-binding protein assay based on the Bradford method (27-29).

Materials for protein array. RayBio™ Human Phospho array kit (catalog no. ARY 001) was purchased from RayBiotech, Inc. (Norcross, GA, USA). Proteome Profiler™ Array Human Phospho-MAPK array kit was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Trastuzumab (Herceptin™) was purchased from Chugai Pharmaceutical Co. Ltd) (Tokyo, Japan).

Antibody arrays of phospho-RTK. The phospho-RTK array was performed according to the manufacturer's instructions. Briefly, phospho-RTK array membranes were blocked with 5% BSA/TBS (0.01 M Tris HCl, pH 7.6) for 1 h. Membranes were then incubated with 2 ml of lysate prepared from cell lines or tissues after normalization with equal amounts of protein. After extensive washing with TBS including 0.1% v/v Tween-20, 3 times for 10 min, and TBS alone, 2 times for 10 min, to remove unbound material, the membranes were then incubated with anti-phospho-tyrosine-HRP antibody for 2 h at room temperature. The unbound HRP antibody was washed out with TBS including 0.1% Tween-20. Finally, each array membrane was exposed to X-ray film using a chemiluminescence detection system (Amersham Life Sciences, Tokyo, Japan). Densitometric analysis was determined using a Kodak Image Station (New Haven, CT). The analysis of the immunoblots was conducted in duplicate, and all the data were reproducible.

Phospho-MAPK antibody array. Assessing the phosphorylation status of all three major families of mitogen-activated protein kinases (MAPKs), the extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK1-3) and different p38 isoforms (α/ß/δ/γ) is essential to understand the role of these signaling molecules in underlying cell function and disease. Apart from these intracellular kinases, other regulators of signal transduction, such as Akt, GSK-3 and p70 S6 kinase, are also important for mediating development and cell proliferation. In this method, capture and control antibodies have been spotted in duplicate on nitrocellulose membranes. Briefly, these membranes were blocked with 5% BSA/TBS (0.01 M Tris HCl, pH 7.6) for 1 h. Membranes were then incubated with 200 μl of lysate prepared from tumorous tissues after normalization with equal amounts of protein. After extensive washing with TBS including 0.1% v/v Tween-20, 3 times for 5 min, to remove unbound materials, the membranes were then incubated with HRP-phospho-MAPK antibody for 2 h at room temperature. Unbound HRP antibody was washed out with TBS including 0.1% Tween-20. Finally, each array membrane was exposed to X-ray film using a chemiluminescence detection system (Amersham Life Sciences, Tokyo, Japan).

In vivo antitumor effects of trastuzumab on colon cancer. Athymic 8-week-old male BALB/c-nu/nu mice, weighing 20-22 g, were purchased from Japan SLC (Hamamatsu, Japan) and kept under specific pathogen-free conditions at 24±2˚C. Animal experiments were performed with approved protocols and in accordance with the institutional recommendations for the proper care and use of laboratory animals. WiDr human colon cancer cells were suspended in PBS at a concentration of $5x10^7$ cells/ml, and 100 μl inoculum volumes were injected subcutaneously into the flank regions of athymic BALB/c-nu/nu mice. When the tumor became palpable in the treated group (n=10), 500 μl of PBS containing

Control											
EGFR	ErbB2	ErbB3	ErbB4	FGFR1	FGFR 2a	FGFR3	FGFR4	Insuli nR	IGFR-1R	Axl	Dtk
Mer	HGFR	MSPR	PDGR a	PDGRb	SCFR	Flt3	M-CSFR	c-Ret	ROR1	ROR2	Tie-1
TIE-2	TrkA	TrkB	TrkC	VEGFR 1	VEGFR 2	VEGF R3	MuSK	EphA1	EphA2	EphA3	EphA4
EphA6	EphB7	EphB1	EphB2	EphB4	EphB6	Mouse IgG1	Mouse IgG2A	Mouse IgG2B	Goat IgG	PBS	
Control											Control

Antibody arrays of phospho-RTK

Figure 1. Template demonstrating the location of tyrosine kinase antibody spotted onto the RayBio Human phospho array kit.

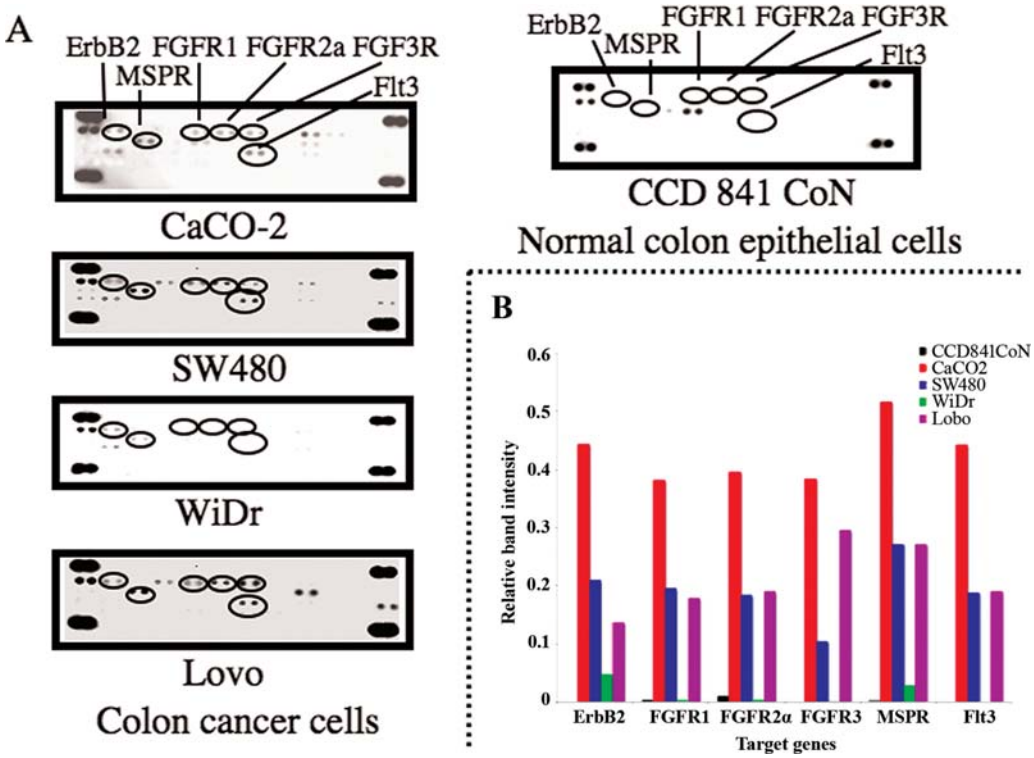

Figure 2. (A) Representative expression of various tyrosine kinases in colon cancer cell lines, CaCO-2, SW480, WiDr and Lovo and normal colon cell line, CCD841CoN. The upregulation of ErbB2 (black circle) was detected in all colon cell lines examined in this study. Especially, ErbB2 was specifically activated in all colon cancer cell lines studied in this study. The protein array methodology is described in the Materials and methods section. (B) Densitometric data on various phospho-RTK molecules in CaCO-2, SW480, WiDr and Lovo were expressed as red, blue, green and purple bars, respectively. Significantly elevated levels (>10-fold increase densitometric data) of ErbB2 and MSPR were detected in all cancer cell lines when compared with the normal colon cell line, CCD841CoN.

1.5 mg/0.5 ml trastuzumab (Herceptin, directed against the ErbB2 receptor, also known as Her2/neu oncogene), was administered intraperitoneally for three weeks, three times a week. Only PBS was administrated to the control group (n=10). After the initiation of the administration of trastuzumab, the tumor growth was monitored by the same investigators (A.M., T.M), and the tumor diameters were measured every week using a graduated caliper. Tumor growth was assessed weekly by measuring the two greatest perpendicular tumor dimensions. Tumor volume was calculated as follows: tumor volume (mm^3) = [tumor length (mm) x tumor width $(mm)^2$] / 2. All animals were sacrificed on day 14 after treatment. All animals were alive during the observation period.

Statistical analysis. Results are expressed as means ± SD. All analyses were performed using the computer-assisted StatView program (SAS Institute, Cary, NC, USA). Paired analysis between each group (trastuzumab, 5-FU, those mixed) and control groups were performed using the t-test. p=0.05 was considered to indicate a significant difference between groups.

Results

The activity level of tyrosine-activated receptor tyrosine kinases (RTKs) associated with colon cancer. Enhanced phospho-RTKs were detected by a phospho-RTKs antibody array system analyzing colon cancer cells. By using this array, the expressions of 42 different activated RTKs were simultaneously screened (Fig. 1). Compared with normal colon epithelial cells, CCD 841CoN, the RTKs activated in all the colon cancer cell lines except WiDr were ErbB2,

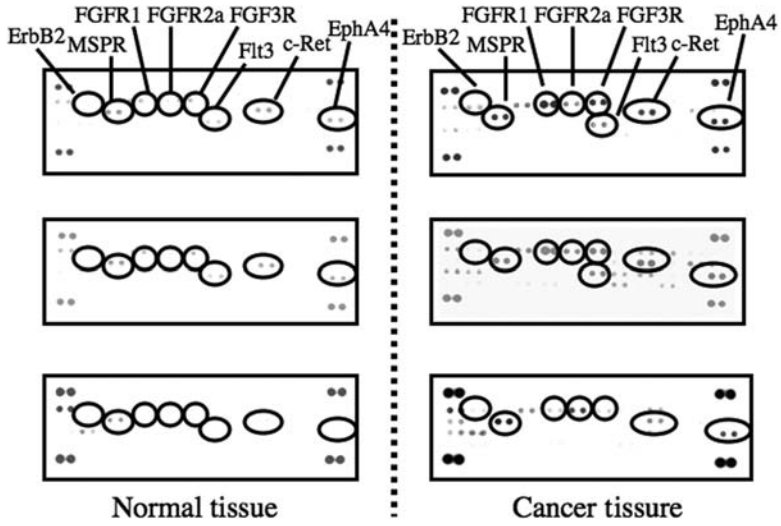

Figure 3. Representative sample results (n=3) revealing a marked increase of ErbB2, FGFR1, FGFR2a, FGFR3, c-Ret, MSPR, Flt3 and Eph-4 activation in cancerous tissue when compared with pair-matched normal colon mucosa.

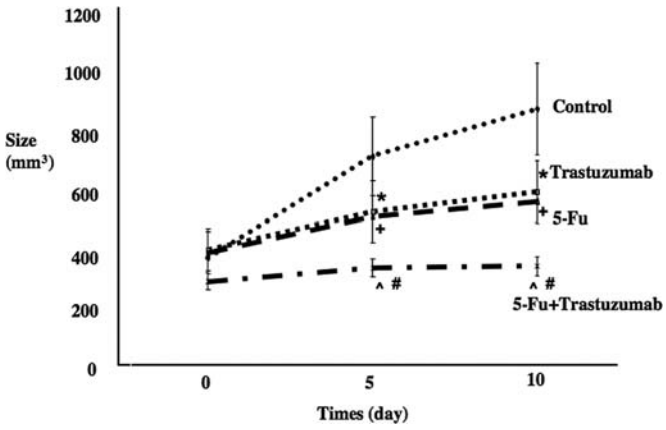

Figure 4. *In vivo* antitumor effects of trastuzumab on established colon cancer in nude mice. WiDr cells implanted subcutaneously into the flank region of nude mice. When tumors became palpable, 1.5 mg/0.5 ml of trastuzumab and 1.0 mg/0.5 ml of 5-FU were injected intraperitoneally for two weeks, three times a week. Animal in the control group developed rapidly growing subcutaneous colon cancer tumors. In contrast, animals in the trastuzumab ($^{*}p<0.05$) 5-FU ($^{+}p<0.05$), and combination therapy groups ($^{\wedge}p<0.05$) exhibited significantly retarded tumor development compared with animals in the control group. In addition, the combination group of trastuzumab and 5-FU ($^{\#}p<0.05$) significantly inhibited the growth of the tumor compared with trastuzumab alone and 5-FU alone groups. Each data point represents the mean ± SD of 10 animals. $^{*}p<0.05$.

FGFR1, FGFR2a, FGFR3, MSPR and Flt3 (Fig. 2A). Among these activated RTKs in colon cancer cell lines, ErbB2 and MSPR were also activated in all the four colon cancer cell lines, including WiDr (Fig. 2A and B).

In the case of human colon cancer tissues, ErbB2, FGFR1, FGFR2a, FGFR3, MSPR, Flt3, c-Ret and EphA4 were activated compared with the levels in normal mucosa. These activated RTKs except Flt3 were detected in all cancerous samples (three cases) used in this study (Fig. 3). These results suggest that an ErbB2-targeting drug may be a useful agent for the treatment of colon cancer.

In vivo antitumor effects of ErbB2-targeting drug, trastuzumab. Athymic 8-week-old male BALB/c-nu/nu mice were implanted subcutaneously with WiDr. When the animals developed palpable tumors, they were treated intraperitoneally for two weeks, three times a week with drug. Animals in the control group received intraperitoneal administration of the vehicle (PBS). As shown in Fig. 4, animals in the control group developed rapidly growing subcutaneous colon cancer. In contrast, animals in the trastuzumab alone group, 5-FU alone group and those in the mixed group exhibited significantly retarded tumor development compared with animals in the control group (Fig. 4). Interestingly, the animals in the mixed group (trastuzumab and 5-FU) exhibited retarded tumor growth when compared with the animals in the trastuzumab alone and 5-FU alone group (Fig. 4). Furthermore, animals in the trastuzumab, 5-FU and those in the mixed treatment group did not exhibit any gross physical changes, while those in the control group demonstrated disheveled fur and diminished body weight. All animals remained alive throughout the experiment.

Expression of various MAPK antigens in WiDr tumor tissues. The expression of PKBγ/Akt3 in the tumorous tissue was reduced by treatment with trastuzumab, 5-FU and combination therapy as detected by phospho-MAPK protein arrays (Figs. 5, 6A and B). The human phospho-MAPK array could simultaneously screen 21 different molecules (Fig. 5). The expression ratio of PKBγ/Akt3 in trastuzumab, 5-FU and the combined groups was 1.57, 6.25 and 1.30% of the control, respectively (Fig. 6B).

Discussion

The human epidermal growth factor receptor 2 (HER2) gene, also known as ErbB-2 encodes a 185-kDa transmembrane glycoprotein receptor. This receptor belongs to the ErbB family of growth factor receptors with intrinsic tyrosine kinase activity, the members of which exist in homodimer

Ctrl (+)										Ctrl (+)
	ERK1	JNK1	JNK pan	P38γ	P38δ	RSK1	GSK-3 α/β	PKBα, Akt1	PKBβ, Akt2	
	ERK2	JNK2	-	P38α	P38β	RSK2	GSK-3β	PKBγ, Akt3	Akt pan	
		JNK3	MSK2				HSP27	P70 S6 Kinase		
	Rabbit IgG	Mouse IgG1	Mouse IgG2A	Mouse IgG2B	Goat IgG	PBS				
Ctrl (+)										

Human Phopho-MAPK Array

Figure 5. Template demonstrating the location of antibodies to phospho-MAPK antigens spotted onto the Proteome Profiler human phospho-MAPK array kit. Ctrl, Control; ERK, extracellular signal-regulated kinase, JNK; c-Jun N-terminal kinase; RSK, p90 ribosomal S6 kinase; GSK, glycogen synthase kinase; PKB/Akt, protein kinases B; MSK, mitogen- and stress-activated kinase 2, HSP27, heat shock protein 27, P70 S6 kinase, ribosomal S6 protein kinase.

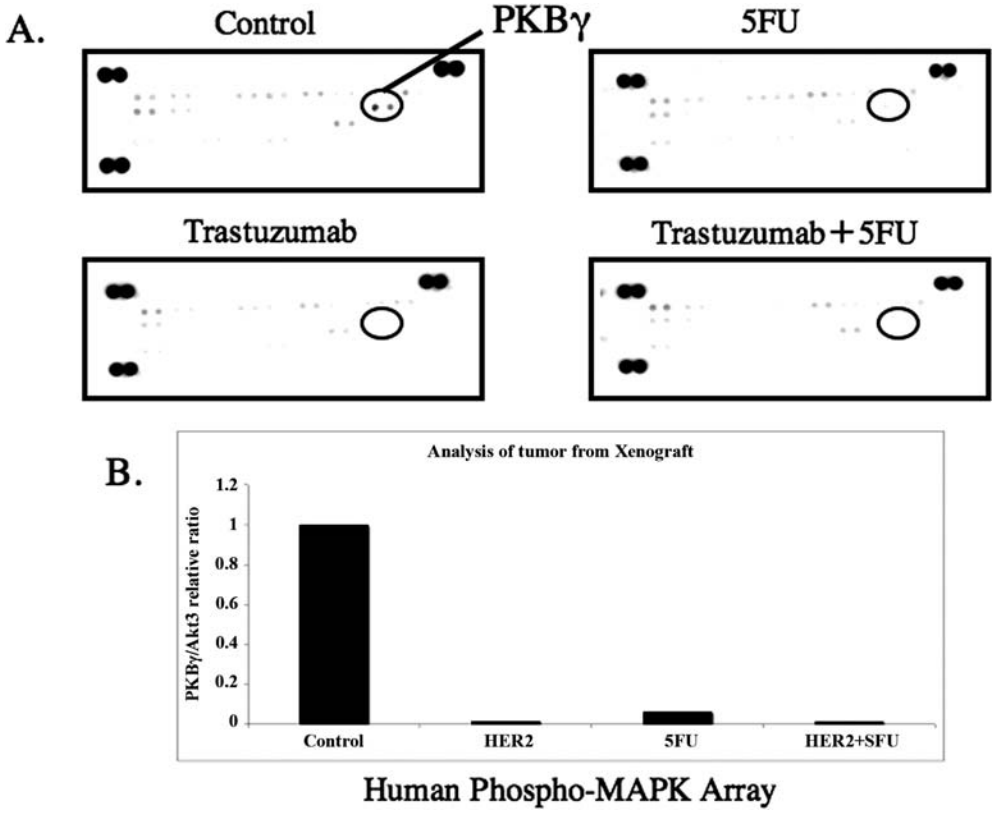

Figure 6. (A) Representative expression of various phospho-MAPK antigens in WiDr tumor tissue in nude mice treated with trastuzumab, 5-FU, combination therapy and control. Marked reduction of PKBγ/Akt3 phosphorylation was observed in the drug-added groups when compared to control. (B) The expression of PKBγ/Akt3 in the tumorous tissue of trastuzumab, 5-FU and combination therapy nude mice were 1.57, 6.25 and 1.30% of the control, respectively.

and heterodimer form when activated (29). Overexpression of HER2 has been detected in up to 25-30% of human breast cancers (30,31), and found in 11-83% of human colon cancers (14,15,32). Therefore, HER2 has been successfully targeted in breast cancer utilizing antibodies directed against the extracellular domains but the evidence supporting a potential role for trastuzumab in colorectal carcinoma has not been demonstrated (32). On the other hand, in experimental models, trastuzumab suppresses the growth of colon cancer cell lines with HER2 overexpression *in vitro* and *in vivo* (33). Several clinical trials are exploring different settings in colon cancer patients with HER2 over-expression (34). However, to date, the mechanism of the growth suppression of trastuzumab for colon cancer cells remains unknown. In the present study, we examined the relationship between the anti-tumor effect of trastuzumab with 5-FU and the expressions of phospho-MAPK molecules.

Our studies demonstrated that ErbB2 and MSPR were activated in all four of the human colon cancer cell lines and all human colon cancer tissues and ErbB2, FGFR1, FGFR2a,

FGFR3, Flt3 were detected in all of the colon cancer cell lines except WiDr. In addition, c-Ret and EphA4 were also activated in human colon cancer tissue. Overexpression of ErbB2, FGFR1, FGFR2a, c-Ret and EphA4 have already been reported in various cancers including colon cancer (14,35-39). These reports support our result on the various RTKs detected from the protein array used in this study. Furthermore, inhibition of activated RTKs actually retarded tumor growth in a xenograft model. These results suggest that there are several phenotypes of phospho-RTKs expression in colon cancer *in vitro* and *in vivo* and the immunological inhibition of ErbB2 and MSPR may have an antitumor effect for colon cancer. Therefore, the analysis of phospho-RTK expression by the protein array as a useful tool might lead to appropriate therapy for individual patients with colon cancer.

To date, there are two antitumor mechanisms proposed for the therapeutic effect of trastuzumab: a direct antiproliferative effect via the blockade of signaling pathways, down-modulation of the ErbB2 protein, and activation of apoptotic signals of the tumor cells, and an indirect antitumor effect by antibody-dependent cell-mediated cytotoxicity (40,41). Our results indicated that the expression of phospho-MAPK molecules, such as PKBγ/Akt3, was reduced by treatment with trastuzumab, indicating that trastuzumab may inhibit the expression of phospho-MAPK molecules of WiDr cells *in vivo*. These data suggest that the antitumor effect of the ErbB2-targeting drug trastuzumab may be due to the reduction of the phospho-MAPK molecule.

Recently, it has become clear that despite the specificity achieved with immunotherapy against cancers, a single modality is insufficient to eradicate such a difficult disease. Combinations of different types of immunotherapy and chemotherapy have been tested, with the suggestion of improved efficacy to spur further clinical trials (42,43). Extensive clinical trials with the combination of chemotherapy and trastuzumab have shown that the anti-breast cancer reagents doxorubicin, paclitaxel and vinorelbine all potentiate the activity of trastuzumab (44). However, to date, the effect of such combinations in colon cancer remain unknown. In the present study, we used 5-FU and trastuzumab as a new combination. Under these conditions trastuzumab effectively inhibited tumor development in the group with combination therapy compared to the group with a single drug in the xenograft model. These results suggest that the combination of trastuzumab and 5-FU might extend the possibility of new therapy for 5-FU resistant colon cancer.

In conclusion, our findings demonstrate that the expressions of PKBγ/Akt3 in colon cancer are regulated by ErbB2 activity, and suggest an additional mechanism for the contribution of the ErbB2-targeting drug trastuzumab in the inhibition of tumor proliferation through the ErbB2-PKBγ/Akt3 signaling pathway. The feasibility of utilizing protein arrays in this study suggests that its arrays might be a useful tool for detecting the expression of activated RTKs and identifying novel therapies for colon cancer.

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