Use of protein array to investigate receptor tyrosine kinases activated in gastric cancer

JIAN GONG[1], ASAHIRO MORISHITA[1], KAZUTAKA KUROKOHCHI[1], JOJI TANI[1], KIYOHITO KATO[1], HISAAKI MIYOSHI[1], HIDEYUKI INOUE[1], MITSUYOSHI KOBAYASHI[1], SHI LIU[1], MASAYUKI MUROTA[1], AKEMI MURAMATSU[1], KUNIHIKO IZUISHI[2], YASUYUKI SUZUKI[2], HIROHITO YOSHIDA[1], NAOHITO UCHIDA[1], KAZUSHI DEGUCHI[1], HISAKAZU IWAMA[3], ICHIRO ISHIMARU[4] and TSUTOMU MASAKI[1]

Departments of [1]Gastroenterology and Neurology and [2]Gastroenterological Surgery, [3]Information Technology Center, Kagawa Medical University School of Medicine, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793; [4]Department of Engineering, Kagawa University, 2217-20 Hayashi-cho, Takamatsu, Kagawa 761-0396, Japan

Received July 29, 2009; Accepted September 24, 2009

DOI: 10.3892/ijo_00000480

Abstract. Our study used protein array technology to analyze the expression status of various activated receptor tyrosine kinases (RTKs) in gastric carcinoma; then, we sought to discover an effective therapeutic receptor tyrosine kinase for this disease and investigated the anti-tumor mechanism of the therapeutic RTK. In addition to the expressions of activated RTKs in human gastric cancer and adjacent normal mucosa, the expression of activated RTKs in gastric cancer cell lines, MKN74, MKN45, MKN7 and MKN1, were also studied. The RTKs activated in gastric cancer tissue are EGFR, ErbB2, FGFR1, FGFR2α insulin R, and EphA4. Among the RTKs activated in gastric cancer tissues, EGFR and ErbB2 were also activated in all gastric cell lines examined in this study. A subsequent *in vitro* experiment using subcutaneous gastric cancer-bearing athymic nude mice demonstrated that the ErbB2-targeting drug trastuzumab markedly suppressed the growth of gastric cancer. Moreover, using an angiogenesis protein array, the expressions of Ang I, FGF-α, FGF-ß TGF-ß and IL-8 in MKN74 xenograft tumors were found to be significantly reduced by treatment with trastuzumab, indicating that trastuzumab may inhibit the expression of angiogenic molecules in MKN74 cells *in vivo*. These data suggest that ErbB2 is activated in gastric cancer, and the ErbB2-targeting drug trastuzumab may be related to the reduction of Ang 1, FGFα, FGFß, TGFα and IL-8.

Introduction

Gastric cancer is now the second-leading cause of cancer-related mortality worldwide (1). Apart from potentially curative surgery, chemotherapy and radiochemotherapy may be applied at advanced stages in gastric cancer, but do not cure the disease in such cases, and the prognosis is poor. Thus, there is a strong demand for new curative approaches to gastric cancer therapy.

Receptor tyrosine kinases (RTKs) are a family of 56 proteins each characterized by a transmembrane domain and a tyrosine kinase motif (2). The known RTKs consisting of a ligand-binding domain at the extracellular surface, a single transmembrane segment, and a cytoplasmic part harboring the protein kinase activity, are divided into 21 families, such as epidermal growth factor (EGFR), vascular endothelial growth factor receptor (VEGFR), and fibroblast growth factor receptor (FGFR), characterized by a similar structure and the potential of intrafamilial dimerization (3). Various RTKs have been implicated in intracellular signal transduction including growth, differentiation, adhesion, migration, apoptosis and carcinogenesis (4). Aberrant RTK activity was initially found in various epithelial cancers, such as breast cancer, hepatocellular carcinoma, and lung cancer, as well as gastric cancer. It is considered that receptor tyrosine kinases play an important role in the development of almost all types of cancer (5-7). As a result, several clinical trials are exploring in different settings and with diverse designs the potential of anti-RTK therapies in various cancer types. However, the mechanism of the suppression of cancer growth by RTK-targeting drugs remains relatively unknown.

Here we have investigated the various RTKs that are activated in gastric cancer using protein arrays. We have shown that the immunological inhibitor for ErbB2, trastuzumab, is effective in gastric cancer, and have also examined the relationship between the growth suppression of trastuzumab and the expression of angiogenesis molecules to explore the mechanism of the anti-tumor effect of trastuzumab.

Materials and methods

Materials. RayBio™ Human Phospho Array Kit (Catalog no. ARY 001) was purchased from RayBiotech, Inc. (Norcross, GA, USA). TranSignal™ Angiogenesis Antibody Array was purchased from Panomics, Inc. (Fremont, CA, USA).

Correspondence to: Dr Tsutomu Masaki, Department of Gastroenterology and Neurology, Kagawa University School of Medicine, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan
E-mail: tmasaki@kms.ac.jp

Key words: protein array, receptor tyrosine kinases, gastric cancer

Trastuzumab (Herceptin™) was purchased from Chugai Pharmaceutical Co., Ltd) (Tokyo, Japan).

Human tissues. Human tissues samples of gastric cancer and the adjacent normal mucosa were obtained during surgery from 5 patients (4 males and 1 female; mean age, 62.4 ±9.2; range 52-72 years). None of the patients had received any chemotherapy or radiotherapy before surgery. The use of human specimens was approved by the Human Subjects Committee of Kagawa University School of Medicine.

Cell line. MKN74, MKN45, MKN7, and MKN1 cells, a kind gift of the Japanese Cancer Resource Bank (Tokyo, Japan), were used as the gastric cancer cell lines. These cells were plated at the 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 μg/ml) and 100 μg/ml streptomycin at 37˚C in 5% CO_2 in air.

Cell lysate and tissue lysate. The lysate was performed according to the methods described in our previous reports (8-10). All steps were carried out at 4˚C. Protein concentration was measured using a dye-binding protein assay based on the Bradford method.

A*ntibody arrays of phospho-RTK*. An assay for phosphor-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 materials, 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).

Angiogenesis antibody array. To assess whether molecules important in angiogenesis are regulated by the anti-tumor effect of trastuzumab, the RayBio Human Angiogenesis Antibody Array (RayBiotech, Inc., GA) was used according to the protocol. This method is a dot-blot based assay which enables detection and comparison of 19 different angiogenesis-specific cytokines. Briefly, angiogenesis protein 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 tumor 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 angiogenesis 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).

In vivo anti-tumor effects of trastuzumab on gastric 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. MKN human gastric 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 750 mg/0.5 ml trastuzumab (Herceptin, directed against the erbB2 receptor, also known as Her2/Neu oncogene), was administrated intraperitoneally for 3 weeks, 3 times a week. Only PBS was administered to the control group (n=10). After the initiation of the administration of trastuzumab, the tumor growth was monitored by the same investigators (J. Gong and T. Masaki), 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 (11). All animals were sacrificed on day 24 after treatment. All animals were alive during the observation.

Statistical analysis. Results are expressed as mean ± SD. All analyses were performed using the computer-assisted StatView program (SAS Institute, Gray, NC, USA). Paired analysis between two groups was performed using the t- test. A p=0.05 was considered to indicate a significant difference between groups.

Results

T*he activity level of tyrosine-activated receptor tyrosine kinases (RTKs) associated with gastric carcinoma*. We used a phospho-RTK array system to identify the 'key RTKs' associated with gastric carcinoma (Fig. 1). By using the antibody array, we simultaneously screened the expressions of 42 different activated RTKs (Fig. 1). The RTKs activated in gastric cancerous tissue are EGFR, ErbB2, FGFR1, FGFR2α, insulin R and EphA4 (Fig. 2A). Compared with their levels in normal mucosa, these activated RTKs were upregulated in all cancerous samples (5 cases) used in this study. Among these activated RTKs in gastric cancerous tissue, ErbB2 was also activated in all of the four gastric cancer cell lines, namely, MKN45, MKN74, MKN1 and MKN7 (Fig. 2B). These results suggest that an ErbB2-targeting drug may be a useful agent for the treatment of gastric cancer.

In vivo anti-tumor effects of ErbB2-targeting drug, trastuzumab. Athymic 8-week-old male BALB/c-nu/nu mice were implanted subcutaneously with MKN74. When the animal developed palpable tumors, they were treated intraperitoneally for 3 weeks, 3 times a week. Animals in the control group received intra-peritoneal administration of the vehicle (PBS). As shown in Fig 3A, animals in the control group developed rapidly growing subcutaneous gastric cancer. In contrast, animals in the trastuzumab groups exhibited significantly

A

A1, A2	PY-Control	C7, C8	PDGFRα	D17, D18	EphA1
A23, A24	PY-Control	C9, C10	PDGFRβ	D19, D20	EphA2
B1, B2	EGFR	C11, C12	SCFR	D21, D22	EphA3
B3, B4	ErbB2	C13, C14	Flt3	D23, D24	EphA4
B5, B6	ErbB3	C15, C16	M-CSFR	E1, E2	EphA6
B7, B8	ErbB4	C17, C18	c-Ret	E3, E4	EphA7
B9, B10	FGFR1	C19, C20	ROR1	E5, E6	EphB1
B11, B12	FGFR2α	C21, C22	ROR2	E7, E8	EphB2
B13, B14	FGFR3	C23, C24	Tie-1	E9, E10	EphB4
B15, B16	FGFR4	D1, D2	Tie-2	E11, E12	EphB6
B17, B18	Insulin R	D3, D4	TrkA	E13, E14	Mouse IgG1
B19, B20	IGFR-1R	D5, D6	TrkB	E15, E16	Mouse IgG2A
B21, B22	Axl	D7, D8	TrkC	E17, E18	Mouse IgG2B
B23, B24	Dtk	D9, D10	VEGFR1	E19, E20	Goat IgG
C1, C2	Mer	D11, D12	VEGFR2	E21, E22	PBS
C3, C4	HGFR	D13, D14	VEGFR3	F1, F2	PY-Control
C5, C6	MSPR	D15, D16	MuSK	F23, F24	PY-Control

PY-Control: Phospho-Tyrosine Positive Control

B

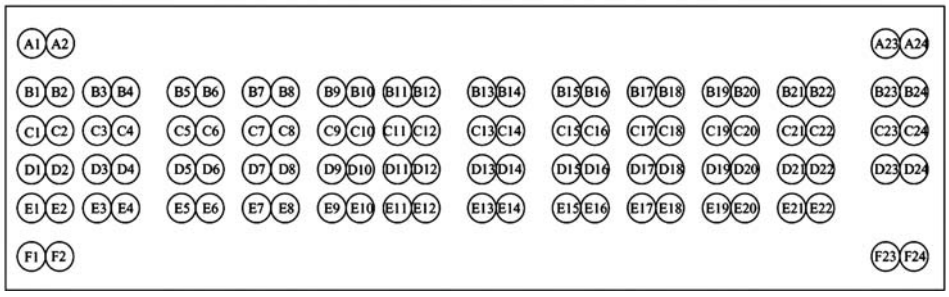

Figure 1. Template showing the location of tyrosine kinase antibody spotted onto the RayBio Human phospho array kit.

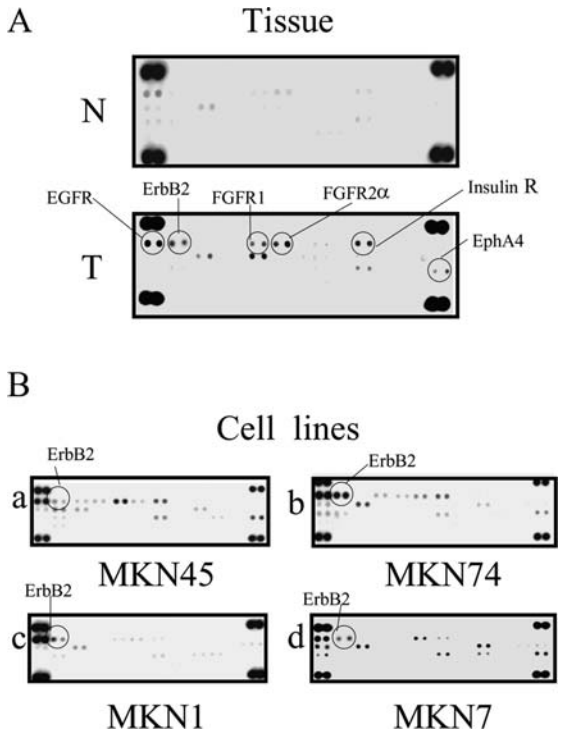

Figure 2. (A) Representative expression of various tyrosine kinases in tumor tissue and non-tumor normal mucosa from the patients with gastric cancer. Densitometric data were arbitrarily expressed as black circles for extremely high levels (>5.0-fold increase densitometric data) when compared to those found in pair-matched normal gastric mucosa. The figure shows representative sample results (n=5) revealing marked increase of EGFR, ErbB2, FGFR1, FGFR2α, insulin R, EphA4 activation (>5.0-fold increase by densitometric data) in tumor tissue when compared to pair-matched normal gastric mucosa. (B) Representative expression of various tyrosine kinases in gastric cancer cell lines, MKN45, MKN74, MKN1, and MKN7. The upregulation of ErbB2 (black circle) was detected in all gastric cell lines examined in this study. Especially, ErbB2 was most activated in MKN74. The protein array methodology is described in Materials and methods.

retarded tumor development compared with animals in the control group (Fig. 3A). Fig. 3B (a and b) are representative photographs of the gross MKN74 tumors from nude mice treated with either trastuzumab or control, respectively. Furthermore, animals in the trastuzumab group did not show any apparent changes, while those in the control group showed disheveled fur and body weight. All animals were alive during the experiment.

As shown in Fig. 3C, both ErbB2 and ErbB4 in MKN74 tumors of nude mice treated with trastuzumab (Fig. 3C,a) were not activated compared with control nude mice (Fig. 3C,b). The activity level of ErbB2 and ErbB4 in trastuzumab-treated nude mice was 4.36 and 20.67% of the control, respectively (Fig. 3D).

Expression of various angiogenesis antigens in MKN74 tumor tissues. Using various angiogenic protein arrays (Fig. 4A), the expressions of FGF-α, FGF-ß Ang 1, TGF-α and IL-8 in the tumor tissue were reduced by trastuzumab (Fig. 4B). The expression ratios of Ang 1, FGF-α, FGF-ß, IL-8 and TGF-α were 0.40, 43.87, 49.71, 57.47 and 18.21% of the control, respectively (Fig. 4C).

Discussion

ErbB-2 (also known as HER-2) encodes a receptor tyrosine kinase which plays a role as a growth regulatory protein and a cell motility factor (12). Overexpression of ErbB-2 is frequently observed in a variety of tumors (13-18). In gastric carcinoma, overexpression and/or amplification of ErbB-2 has been reported at a 6-30% incidence (19-21). These studies suggest that overexpression of ErbB-2 has been associated with poor survival in gastric carcinoma. On the other hand, in experimental models, trastuzumab suppresses the growth of

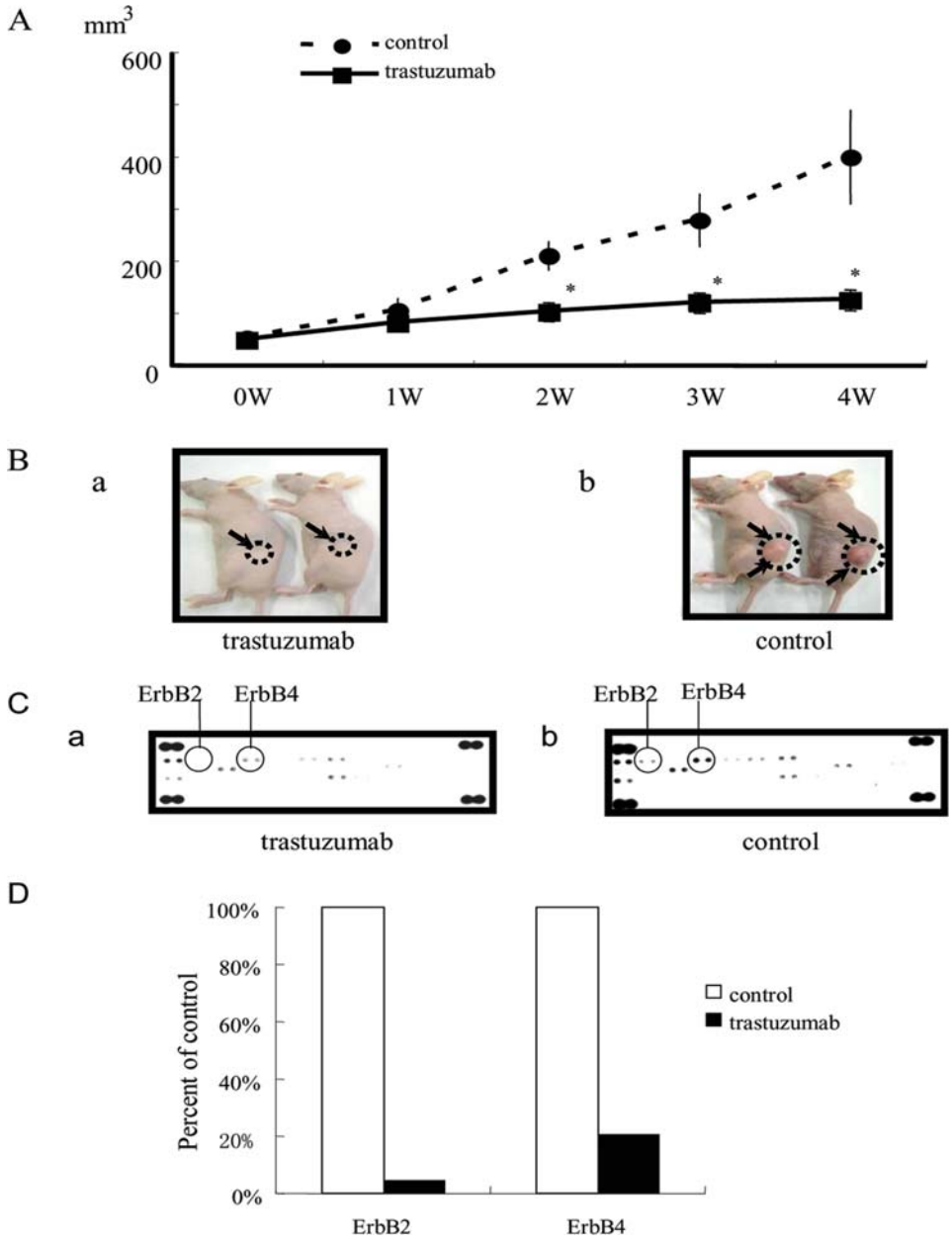

Figure 3. *In vivo* tumor anti-tumor effects of trastuzumab on established gastric cancer in nude mice. (A) MKN74 cells implanted subcutaneously into the flank regions of nude mice. When tumors became palpable, 750 μg trastuzumab was injected intraperitoneally for 3weeks, 3 times a week. Animal in the control group developed rapidly growing subcutaneous gastric cancer. In contrast, animals in the trastuzumab groups exhibited significantly retarded tumor development compared with animals in the control group. Each data point represents the mean ± SD of 9 animals. $^{*}p<0.001$. (B) (a and b) are representative photographs of the gross MKN74 tumors from nude mice treated with either trastuzumab or control, respectively. (C) Representative expression of various tyrosine kinases in MKN74 tumor tissues in nude mice treated with trastuzumab and control. Both ErbB2 and ErbB4 in the tumor tissue of trastuzumab-treated nude mice were activated as compared with control nude mice. (D) The activity level of ErbB2 and ErbB4 in trastuzumab-treated nude mice was 4.36 and 20.67% of the control, respectively.

human gastric cancer with HER2 overexpression *in vitro* and *in vivo* (22-27). As a result of these preclinical data, several clinical trials are exploring in different settings and with diverse designs the potential of anti-HER2 therapies in gastric cancer patients (22,23,26). However, to date, the mechanism of the growth suppression of trastuzumab for gastric cancer cells remains unknown. In the present study, we examined the relationship between the anti-tumor effect of trastuzumab and the expressions of angiogenic molecules.

Using the protein array, the activations of EGFR, ErbB2, FGFR1, and FGFR2α were detected in all gastric cancer tissues and cell lines studied. EphA4 was activated in all gastric cancer tissues and other cell lines except MKN1 used in the present study. Overexpression of EGFR, ErbB2, FGFR1, FGFR2α, EphA4 has been already reported in gastric cancer (26,28-31). The previous report support our results on the various RTKs activated in gastric cancer derived from the protein array used in this study. In summary, the use of protein array in this study suggests that it may be a useful tool for studying the expression of activated RTKs in various tissues, including malignant tissues. Furthermore, these results suggest that the immunological

A

	1	2	3	4	5	6	7	8
A	pos	pos	pos	pos	pos	pos	pos	pos
B	Ang	Ang	IL-1α	IL-1α	FGFα	FGFα	IFNγ	IFNγ
C	G-CSF	G-CSF	IL-1β	IL-1β	FGFβ	FGFβ	IL-12	IL-12
D	HGF	HGF	IL-6	IL-6	TNFα	TNFα	IP-10	IP-10
E	Leptin	Leptin	IL-8	IL-8	TGFα	TGFα	TIMP-1	TIMP-1
F	VEGF	VEGF	PIGF	PIGF	neg	neg	TIMP-2	TIMP-2

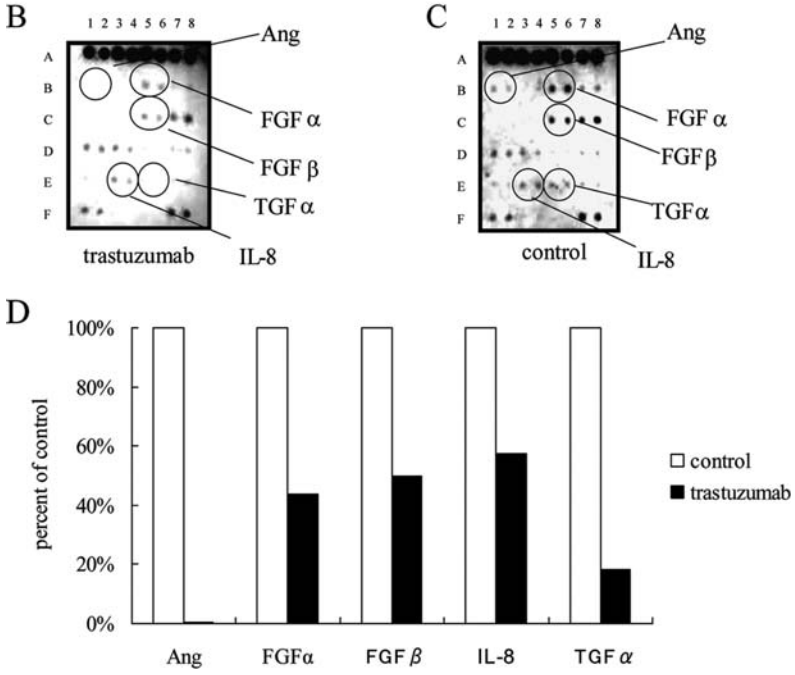

Figure 4. (A) Template showing the location of antibodies to angiogenesis antigens spotted onto the RayBio human angiogenesis antibody array kit. pos, positive control; Ang, angiopoietin; IL-1α, interleukin-1α; FGFα, fibroblast growth factor α; IFNγ, interferon γ; G-CSF, granulocyte-colony stimulating factor; IL-1ß, interleukin-1ß; FGFß, fibroblast growth factor ß; IL-12, interleukin-12; HGF, hepatocyte growth factor; IL6, interleukin-6; TNFα, tumor necrosis factor α; IP10, interferon γ-inducible 10 kDa protein; Lep, leptin; IL-8, interleukin-8; TGFα, transforming growth factor α; TIMP-1, tissue inhibitor of metalloproteinases-1; VEGF, vascular endothelial growth factor; PIGF, placental growth factor; neg, negative control; and TIMP-2, tissue inhibitor of metalloproteinases-2. (B and C) Representative expressions of various angiogenesis antigens in MKN74 tumor tissues in nude mice treated with trastuzumab and control. (D) The expressions of Ang 1, FGF-α, FGF-ß, IL-8 and TGF-α in the tumor tissue of trastuzumab treated nude mice were 0.40, 43.87, 49.71, 57.47 and 18.21% of the control, respectively.

inhibition of EGFR, ErbB2, FGFR1, FGFR2α, and EphA4 may have an anti-tumor effect for gastric cancer.

Some studies have reported that trastuzumab directed against ErbB2, among the enhanced RTKs in gastric cancer, is effective in patients with gastric cancer (22,23). However, the anti-tumor mechanism for trastuzumab has remained relatively unknown. To date, there are two anti-tumor mechanisms proposed for the therapeutic effect of trastuzumab: a direct anti-proliferative effect via a blockade of signaling pathways, down-modulation of the ErbB2 protein, and activation of apoptotic signals of the tumor cells, and an indirect anti-tumor effect by antibody-dependent cell-mediated cytotoxicity activity (32,33). In the present study, the expression of various angiogenic molecules, such as Ang 1, FGF-α, FGF-ß, IL-8 and TGF-ß was reduced by treatment with trastuzumab, indicating that trastuzumab may inhibit the expression of angiogenic molecules of MKN74 cells *in vivo*. In previous reports, treatment with trastuzumab was detected to reduce the expression of IL-8 and TGF-ß in some cancers (34-36). These data suggest that various angiogenic molecules including IL-8 and TGF-ß in gastric cancer, is regulated by ErbB2 activity, and the anti-tumor effect of the ErbB2-targeting drug trastuzumab may be due to the reduction of these angiogenic molecules.

Sequence analyses show that the asymmetric dimer interface is observed in the two other catalytically active members in the family, ErbB2 and ErbB4, suggesting that ErbB2 and ErbB4 are likely to use the same activation mechanism (37). This is confirmed by a recent structural study showing that ErbB4 also forms an asymmetric dimmer essentially identical to that of EGFR and the dimmer is important for ErbB4 activation. This previous report (37) support that the ErbB2-targeting drug trastuzumab may have inactivated not only ErbB2, but also ErbB4 in the present study.

In conclusion, our findings demonstrate that the expressions of Ang 1, FGF-α, FGF-ß, IL-8 and TGF-ß in gastric cancer

are regulated by ErbB2 activity, and they suggest an additional mechanism for the contribution of the ErbB2-targeting drug trastuzumab to the inhibition of tumor angiogenesis.

References

1. Parkin DM, Bray F, Ferlay J and Pisani P: Global cancer statistics, 2002. CA Cancer J Clin 55: 74-108, 2005.
2. Robinson DR, Wu YM and Lin SF: The protein tyrosine kinase family of the human genome. Oncogene 19: 5548-5557, 2000.
3. Becker JC, Muller-Tidow C, Serve H, Domschke W and Pohle T: Role of receptor tyrosine kinases in gastric cancer: new targets for a selective therapy. World J Gastroenterol 12: 3297-3305, 2006.
4. Hubbard SR and Till JH: Protein tyrosine kinase structure and function. Annu Rev Biochem 69: 373-398, 2000.
5. Tanner M, Hollmen M, Junttila TT, *et al*: Amplification of HER-2 in gastric carcinoma: association with Topoisomerase IIalpha gene amplification, intestinal type, poor prognosis and sensitivity to trastuzumab. Ann Oncol 16: 273-278, 2005.
6. Svensson S, Jirström K, Rydén L, Roos G, Emdin S, Ostrowski MC and Landberg G: ERK phosphorylation is linked to VEGFR2 expression and Ets-2 phosphorylation in breast cancer and is associated with tamoxifen treatment resistance and small tumours with good prognosis. Oncogene 24: 4370-4379, 2005.
7. Cao C, Albert JM, Geng L, Ivy PS, Sandler A, Johnson DH and Lu B: Vascular endothelial growth factor tyrosine kinase inhibitor AZD2171 and fractionated radiotherapy in mouse models of lung cancer. Cancer Res 66: 11409-11415, 2006.
8. Masaki T, Tokuda M, Yoshida S, *et al*: Comparison study of the expressions of myristoylated alanine-rich C kinase substrate in hepatocellular carcinoma, liver cirrhosis, chronic hepatitis, and normal liver. Int J Oncol 26: 661-671, 2005.
9. Yoshida S, Masaki T, Feng H, *et al*: Enhanced expression of adaptor molecule p46 Shc in nuclei of hepatocellular carcinoma cells: study of LEC rats. Int J Oncol 25: 1089-1096, 2004.
10. Yukimasa S, Masaki T, Yoshida S, *et al*: Enhanced expression of p46 Shc in the nucleus and p52 Shc in the cytoplasm of human gastric cancer. Int J Oncol 26: 905-911, 2005.
11. D'Incalci M, Colombo T, Ubezio P, *et al*: The combination of yondelis and cisplatin is synergistic against human tumor xenografts. Eur J Cancer 39: 1920-1926, 2003.
12. Stark A, Hulka BS, Joens S, *et al*: HER-2/neu amplification in benign breast disease and the risk of subsequent breast cancer. J Clin Oncol 18: 267-274, 2000.
13. Ginestier C, Adélaïde J, Gonçalvès A, *et al*: ERBB2 phosphorylation and trastuzumab sensitivity of breast cancer cell lines. Oncogene 26: 7163-7169, 2007.
14. Hermanova M, Lukas Z, Nenutil R, *et al*: Amplification and overexpression of HER-2/neu in invasive ductal carcinomas of the pancreas and pancreatic intraepithelial neoplasms and the relationship to the expression of p21 (WAF1/CIP1). Neoplasma 51: 77-83, 2004.
15. Friess T, Scheuer W and Hasmann M: Erlotinib antitumor activity in non-small cell lung cancer models is independent of HER1 and HER2 overexpression. Anticancer Res 26: 3505-3512, 2006.
16. Hatake K, Tokudome N and Ito Y: Tanstuzumab treatment for breast cancer. Intern Med 46: 149-150, 2007.
17. Larbouret C, Robert B, Navarro-Teulon I, *et al*: *In vivo* therapeutic synergism of anti-epidermal growth factor receptor and anti-HER2 monoclonal antibodies against pancreatic carcinomas. Clin Cancer Res 13: 3356-3362, 2007.
18. Lara PN Jr, Chee KG, Longmate J, *et al*: Trastuzumab plus docetaxel in HER-2/neu-positive prostate carcinoma: final results from the California Cancer Consortium Screening and Phase II Trial. Cancer 100: 2125-2131, 2004.
19. Shun CT, Wu MS, Lin JT, *et al*: Relationship of p53 and c-erbB-2 expression to histopathological features, Helicobacter pylori infection and prognosis in gastric cancer. Hepatogastroenterology 44: 604-609, 1997.
20. Yonemura Y, Ninomiya I, Ohoyama S, *et al*: Expression of c-erbB-2 oncoprotein in gastric carcinoma. Immunoreactivity for c-erbB-2 protein is an independent indicator of poor short-term prognosis in patients with gastric carcinoma. Cancer 67: 2914-2918, 1991.
21. David L, Seruca R, Nesland JM, *et al*: C-erbB-2 expression in primary gastric carcinomas and their metastases. Mod Pathol 5: 384-390, 1992.
22. Rebischung C, Barnoud R, Stéfani L, Faucheron JL and Mousseau M: The effectiveness of trastuzumab (Herceptin) combined with chemotherapy for gastric carcinoma with over-expression of the c-erbB-2 protein. Gastric Cancer 8: 206-208, 2005.
23. Inui T, Asakawa A, Morita Y, *et al*: HER-2 overexpression and targeted treatment by trastuzumab in a very old patient with gastric cancer. J Intern Med 260: 484-487, 2006.
24. Kim SY, Kim HP, Kim YJ, *et al*: Trastuzumab inhibits the growth of human gastric cancer cell lines with HER2 amplification synergistically with cisplatin. Int J Oncol 32: 89-95, 2008.
25. Hofmann M, Stoss O, Shi D, *et al*: Assessment of a HER2 scoring system for gastric cancer: results from a validation study. Histopathology 52: 797-805, 2008.
26. Gravalos C and Jimeno A: HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target. Ann Oncol 19: 1523-1529, 2008.
27. Marx AH, Tharun L, Muth J, *et al*: HER-2 amplification is highly homogenous in gastric cancer. Hum Pathol 40: 769-777, 2009.
28. Kim MA, Lee HS, Lee HE, Jeon YK, Yang HK and Kim WH: EGFR in gastric carcinomas: prognostic significance of protein overexpression and high gene copy number. Histopathology 52: 738-746, 2008.
29. Shin EY, Lee BH, Yang JH, *et al*: Up-regulation and co-expression of fibroblast growth factor receptors in human gastric cancer. J Cancer Res Clin Oncol 126: 519-528, 2000.
30. Zhang Y, Zhang J, Lin Y, *et al*: Role of epithelial cell fibroblast growth factor receptor substrate 2alpha in prostate development, regeneration and tumorigenesis. Development 135: 775-784, 2008.
31. Oki M, Yamamoto H, Taniguchi H, Adachi Y, Imai K and Shinomura Y: Overexpression of the receptor tyrosine kinase EphA4 in human gastric cancers. World J Gastroenterol 14: 5650-5656, 2008.
32. Sliwkowski MX, Lofgren JA, Lewis GD, Hotaling TE, Fendly BM and Fox JA: Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin). Semin Oncol 26: 60-70, 1999.
33. Cuello M, Ettenberg SA, Clark AS, *et al*: Down-regulation of the erbB-2 receptor by trastuzumab (herceptin) enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in breast and ovarian cancer cell lines that overexpress erbB-2. Cancer Res 61: 4892-4900, 2001.
34. Vazquez-Martin A, Colomer R and Menendez JA: Protein array technology to detect HER2 (erbB-2)-induced 'cytokine signature' in breast cancer. Eur J Cancer 43: 1117-1124, 2007.
35. Wen XF, Yang G, Mao W, *et al*: HER2 signaling modulates the equilibrium between pro- and antiangiogenic factors via distinct pathways: implications for HER2-targeted antibody therapy. Oncogene 25: 6986-6996, 2006.
36. Ueda Y, Wang S, Dumont N, Yi JY, Koh Y and Arteaga CL: Overexpression of HER2 (erbB2) in human breast epithelial cells unmasks transforming growth factor beta-induced cell motility. J Biol Chem 279: 24505-24513, 2004.
37. Qiu C, Tarrant MK, Choi SH, *et al*: Mechanism of activation and inhibition of the HER4/ErbB4 kinase. Structure 16: 460-467, 2008.