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Use of protein array technology to investigate receptor tyrosine kinases activated in hepatocellular carcinoma

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Abstract. Receptor tyrosine kinases (RTKs) play a role in various processes, including cell growth, differentiation, apoptosis and carcinogenesis. RTKs are activated in various types of cancers, including breast, stomach, colon, pancreas and liver cancer and hepatocellular carcinoma (HCC). In the present study, protein array technology was used to analyze the expression status of various RTKs activated in HCC. The expression of activated RTKs was examined in the HCC cell lines, Alex, HuH7, Li-7, Hep3B, HLE and HLF; in the human normal hepatocyte cell line, hNHeps; and in human HCC and adjacent non-cancerous tissues. Of the 42 different phospho-RTKs, 15 (ErbB2, ErbB3, ErbB4, FGFR2α, FGFR3, insulin R, Mer, PDGFRβ, c-Ret, ROR2, Tie, TrkA, VEGFR3, EphA1 and EphA4) were activated in some of the cancer cell lines studied. Among these, only ErbB2 was activated in all the HCC cell lines examined. Also, *in vitro* experiments were performed in subcutaneous HCC-bearing athymic nude mice to determine the therapeutic effects of inhibiting ErbB2 activation using the ErbB2-targeting drug trastuzumab. The results revealed that trastuzumab markedly suppressed the growth of HCC. These data suggest that ErbB2 is activated in HCC and that trastuzumab may play a role in the treatment of this disease. In addition, the use of protein array technology is proposed as a tool for detecting the expression of activated RTKs and identifying an effective RTK-based therapy.

Introduction

Hepatocellular carcinoma (HCC) is one of the most serious malignancies worldwide (1), particularly in Asian countries due to the high prevalence of the hepatitis virus (2). It is also the third most common cause of cancer-related mortality (3,4). Despite intensive efforts to develop novel treatment modalities for HCC, the prognosis remains poor. Thus, there is a strong demand for effective new approaches to HCC therapy.

Receptor tyrosine kinases (RTKs) are a family of 56 proteins each characterized by a transmembrane domain and a tyrosine kinase motif (5). The known RTKs consist of a ligand-binding domain at the extracellular surface, a single transmembrane segment and a cytoplasmic part harboring the protein kinase activity. They are divided into 21 families, including the epidermal, vascular endothelial and fibroblast growth factor receptor families, which are characterized by a similar structure and the potential for intrafamilial dimerization (6). Various RTKs have been implicated in intracellular signal transduction pathways involved in growth, differentiation, adhesion, migration, apoptosis and carcinogenesis (7). In regard to the relationship between human cancers and RTKs, aberrant RTK activity was initially found in various epithelial cancers, including breast (8), gastric (9), lung (10), colon (11) and esophageal cancer (12), and HCC (13,14). It is now accepted that the activation of certain RTKs plays a key role in the development of almost all types of cancer. Accordingly, a number of clinical trials with various settings and designs are currently exploring the potential of anti-RTK therapies in various cancers (15). A recent study reported that the receptor tyrosine kinase inhibitor sorafenib is effective in patients with advanced HCC (16). However, the anti-cancer effects of sorafenib under investigation for the treatment of HCC remain unknown.

The aims of this study were two-fold: i) to use protein array technology to determine the expression status of various activated RTKs in HCC; and ii) upon identifying ErbB2 as the most consistently up-regulated RTK, to investigate whether an ErbB2-targeting drug, trastuzumab, would be effective as an anti-cancer agent in an HCC xenograft model.

Materials and methods

Materials. The RayBio™ Human Phospho Array kit (catalog no. ARY 001) was purchased from RayBiotech Inc. (Norcross,

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Key words: receptor tyrosine kinases, hepatocellular carcinoma

PY-Control											PY-Control
EGFR	ErbB2	ErbB3	ErbB4	FGFR1	FGFR2α	FGFR3	FGFR4	Insulin R	IGF-1R	Axl	Dtk
Mer	HGFR	MSPR	PDGFRα	PDGFRβ	SCFR	Flt-3	M-CSFR	c-Ret	ROR1	ROR2	Tie-1
Tie-2	TrkA	TrkB	TrkC	VEGFR1	VEGFR2	VEGFR3	MuSK	EphA1	EphA2	EphA3	EphA4
EphA6	EphA7	EphB1	EphB2	EphB4	EphB6	Mouse IgG1	Mouse IgG2A	Mouse IgG2B	Goat IgG	PBS	
PY-Control											PY-Control

Figure 1. Template showing the location of a tyrosine kinase antibody spotted onto a RayBio™ Human phospho array. PY-Control, phospho-tyrosine positive control; EGFR, epidermal growth factor receptor; ErbB2, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2; ErbB3, v-erb-b2 erythroblastic leukemia viral oncogene homolog 3; ErbB4, v-erb-a erythroblastic leukemia viral oncogene homolog 4; FGFR, fibroblast growth factor receptor; Insulin R, insulin receptor; IGF-1R, insulin-like growth factor I receptor; Axl, Axl receptor tyrosine kinase; Dtk, developmental receptor tyrosine kinase; Mer, tyrosine-protein kinase Mer; HGFR, hepatocyte growth factor receptor; MSPR, macrophage stimulatory protein receptor; PDGFR, platelet-derived growth factor receptor; SCFR, stem-cell factor receptor; Flt-3, Fms-like tyrosine kinase 3; M-CSFR, macrophage colony-stimulating factor receptor; c-Ret, receptor tyrosine kinase c-ret; ROR, receptor tyrosine kinase-like orphan receptor; Tie, tyrosine kinase with immunoglobulin-like and EGF-like domains; TrkA, neurotrophic tyrosine kinase, receptor, type 1; TrkB, neurotrophic tyrosine kinase, receptor, type 2; TrkC, neurotrophic tyrosine kinase, receptor, type 3; VEGFR, vascular endothelial growth factor receptor; MuSK, muscle, skeletal, receptor tyrosine kinase; Eph, Eph receptor; PBS, phosphate-buffered saline.

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

Human tissues. Human HCC tissue samples and the adjacent hepatic tissues were obtained during surgery from 5 patients (3 male and 2 female; mean age 69.6±10.6 years; range 57-81). A total of 3 patients were positive for hepatitis C virus RNA, and 2 patients with chronic hepatitis were positive for the hepatitis B surface antigen. The histology of the adjacent hepatic tissue in the patients was F4 according to Desmet's classification (17). None of the patients had received any chemotherapy or radiotherapy prior to surgery. The use of human specimens was approved by the Human Subjects Committee of Kagawa University School of Medicine.

Cell lines. Alex, HuH7, Li-7, Hep3B, HLE and HLF cells, a kind gift from the Japanese Cancer Resource Bank (Tokyo, Japan), were used as the HCC cell lines. These cell lines were plated at a density of $1x10^5$ cells/cm^3 in plastic flasks containing Dulbecco's modified minimum essential medium (DMEM) (Gibco BRL Co., Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum, 100 μg/ml penicillin and 100 μg/ml streptomycin at 37˚C in 5% CO_2 in air. The human normal hepatocyte cell line, hNHeps, was used as the normal hepatocyte cell line.

Cell and tissue lysates. The cell lysate was prepared according to the methods previously described (9,18,19). The steps were performed at 4˚C. The protein concentration of the cell and tissue lysates was measured using a dye-binding protein assay based on the Bradford method (13,14,20).

Antibody arrays of phospho-receptor tyrosine kinases. An assay for phospho-RTK array was performed as previously described (9,11). Briefly, phospho-RTK array membranes were blocked with 5% BSA/TBS (0.01 M Tris HCl, pH 7.6) for 1 h. The membranes were subsequently incubated with ~2 ml (protein contents: 100 μg/ml) of lysate prepared from cell lines or tissues after normalization with equal amounts of protein. To remove unbound materials, the membranes were washed three times with TBS, including 0.1% v/v Tween-20 for 10 min each time, and then twice with TBS alone for 10 min each time. They 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).

In vivo anti-tumor effects of trastuzumab on hepatocellular carcinoma. Athymic 8-week-old male BALB/c-nu/nu mice, weighing 20-22 g, were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan) and kept under specific pathogen-free conditions at 24±2˚C. The animal experiments were performed with approved protocols and in accordance with the institutional recommendations for the proper care and use of laboratory animals. HuH7 and HLF HCC cells were suspended in PBS at a concentration of $5x10^7$ cells/ml, respectively, and 100 μl inoculum volumes were injected subcutaneously into the flank regions of the mice. When the tumors 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 the Her2/Neu oncogene) was administered intraperitoneally three times a week for 3 weeks. Only PBS was administered to the control group (n=10). After initiation of the trastuzumab administration, the tumor growth was monitored by the same investigators (I.G. and 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 (21). The animals were sacrificed on day 16 after treatment. The animals remained alive throughout the observation.

Statistical analysis. The results are expressed as the means ± SD. The analysis was performed using the computer-assisted StatView program (SAS Institute, Cary, NC, USA). Paired analysis between two groups was performed using the

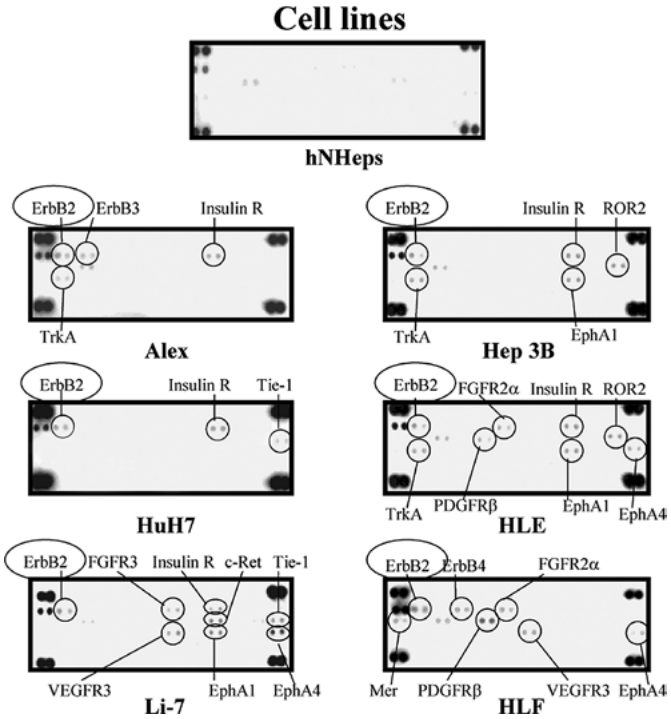

Figure 2. Representative expression in the hNHeps and HCC cell lines of various tyrosine kinases, including Alex, HuH7, Li-7, Hep3B, HLE and HLF. As compared to the hNHeps cell line, ErbB2, ErbB3, ErbB4, insulin R, ROR2, TrkA, EphA1, Tie-1, FGFR2α, FGFR3, PDGFRβ, EphA4, c-Ret, Mer and VEGFR3 were up-regulated in some of the cancer cell lines studied. The up-regulation of ErbB2 (●) was detected in all of the HCC cell lines examined in this study, while it was not detected in the hNHeps cell line.

Student's t-test. Values of $p<0.05$ were considered to indicate a significant difference between groups.

Results

Activity level of activated receptor tyrosine kinases is associated with hepatocellular carcinoma. A phospho-RTK array system was used to identify the 'key RTKs' that are associated with HCC. Using the antibody array, the expression of 42 different activated RTKs were simultaneously screened (Fig. 1). Compared to the hNHeps cell line, ErbB2, ErbB3, ErbB4, FGFR2α, FGFR3, insulin R, Mer, PDGFRβ, c-Ret, ROR2, Tie, TrkA, VEGFR3, EphA1 and EphA4 were up-regulated in some of cancer cell lines studied (Fig. 2). One of these molecules, ErbB2 (●), was up-regulated in all of the HCC cell lines examined in this study, while it was not detected in the hNHeps cell line. Also, in the cancerous tissue, ErbB2 was the only RTK up-regulated in all five tissue samples (Fig. 3). These results suggest that an ErbB2-targeting drug is a useful agent for the treatment of HCC.

In vivo anti-tumor effects of an ErbB2-targeting drug, trastuzumab. Athymic 8-week-old male BALB/c-nu/nu mice were implanted subcutaneously with HuH7 and HLF cells. When the animals developed palpable tumors, they were treated intraperitoneally with trastuzumab three times a week for 3 weeks. Animals in the control group received intraperitoneal administration of the vehicle (PBS).

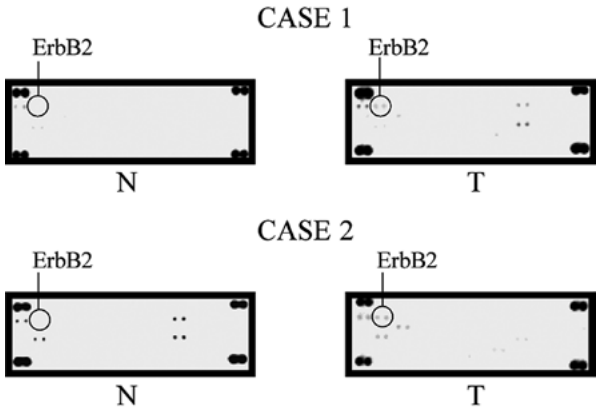

Figure 3. Representative sample results (n=2) revealing marked increase of $ERbB_2$ activation in cancerous tissue (T) when compared to pair-matched non-tumorous tissues (N).

As shown in Fig. 4A, animals in the control group that were implanted subcutaneously with HuH7 cells developed rapidly growing subcutaneous HCC while animals in the trastuzumab group exhibited significantly retarded tumor development compared to the animals in the control group (Fig. 4A). Fig. 4B shows representative images of gross HuH7 tumors from nude mice treated with either trastuzumab or vehicle.

As shown in Fig. 4C, the tumor size increased until the sixth day after implantation in the control animals implanted subcutaneously with HLF cells, but decreased gradually thereafter. By contrast, the animals in the trastuzumab group exhibited significantly retarded tumor development, and the tumors in 4/5 animals disappeared. Fig. 4D shows representative images of gross HLF tumors from the nude mice treated with either trastuzumab or the vehicle. The tumors in the trastuzumab group disappeared completely while the tumors in the control group did not disappear.

Animals in the trastuzumab group implanted with the strains HuH7 or HLE did not show substantial changes, while those in the control group showed disheveled fur and decreased body weight. The animals remained alive throughout the experiment.

Discussion

The human epidermal growth factor receptor 2 (HER2) gene, also known as ErbB2, encodes a 185-kDa transmembrane glycoprotein receptor. This receptor belongs to the ErbB family of growth factor receptors with intrinsic tyrosine kinase activity, the membranes of which exist in homodimer and heterodimer forms when activated (22).

ErbB2 is activated by ligand-binding in its extracellular region and subsequently the intracellular tyrosine kinase region is phosphorylated and sends signals to the cell to regulate numerous crucial processes (23), including growth, differentiation and carcinogenesis.

Overexpression of ErbB2 is frequently observed in a variety of tumor types (24-29), including colon, gastric, non-small cell lung, epithelial ovarian (30), endometrial carcinoma (31,32), prostate (33), urinary bladder (34) and uterine papillary serous cancers (35,36). Although overexpression of ErbB2 is rarely observed in HCC, mutation of ErbB2 is found in 11%

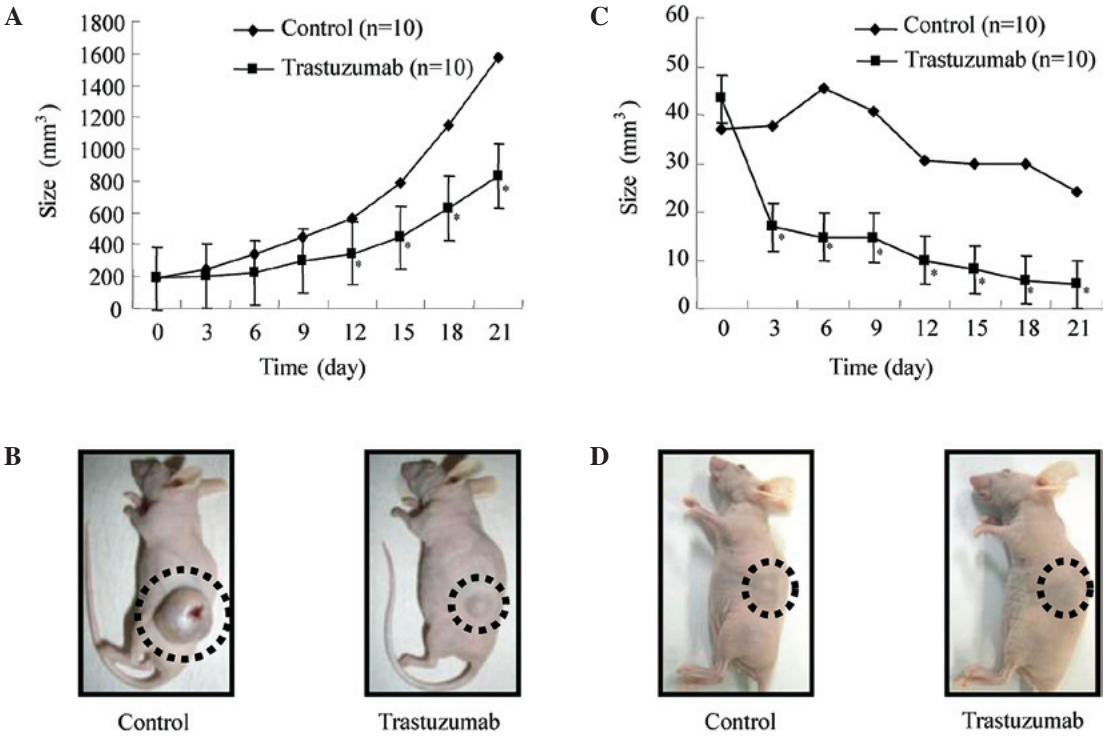

Figure 4. *In vivo* anti-tumor effects of trastuzumab on established HCC with HuH7 and HLF cells in nude mice. (A) HuH7 cells were implanted subcutaneously into the flank regions of nude mice. When tumors became palpable, 750 μg trastuzumab was injected intraperitoneally three times a week for 3 weeks. Animals in the control group developed rapidly growing subcutaneous HCC. Animals in the trastuzumab groups exhibited significantly retarded tumor development compared to the animals in the control group. Each data point represents the mean ± SD of 10 animals. $^{*}p<0.001$. (B) Representative images of gross HuH7 cell tumors from the nude mice treated with either control or trastuzumab, respectively. (C) *In vivo* tumor anti-tumor effects of trastuzumab on established HCC with HLF cells in nude mice. HLF cells were implanted subcutaneously into the flank regions of nude mice. When tumors became palpable, 750 μg trastuzumab was injected intraperitoneally three times a week for 3 weeks. The tumors decreased gradually from the sixth day both in the control and the trastuzumab groups. However, animals in the trastuzumab groups exhibited significantly retarded tumor development compared to animals in the control group. Each data point represents the mean ± SD of 10 animals. $^{*}p<0.001$. (D) Representative images of gross HLF cell tumors from nude mice treated with either control or trastuzumab, respectively. The tumors in the control group did not disappear, while the tumors in the trastuzumab group disappeared completely.

of cases (37). Therefore, ErbB2 is targeted using antibodies directed against the extracellular domain in various types of human cancers, including HCC. These strategies have been successful in the area of breast cancer (25). However, there is currently no evidence supporting a potential role for trastuzumab in HCC comparable to its role in breast cancer. Thus, in the present study we examined the possibility of an anti-tumor effect of trastuzumab in HCC.

In the present study, we first identified the 'key RTKs' associated with HCC by studying 42 activated phospho-RTKs, using the phospho-RTK array system (Fig. 1). As a result, ErbB2 was found to be activated in all six of the HCC cell lines examined (Fig. 2), and in all cancerous samples (Fig. 3). Next, we determined that the inhibition of ErbB2 by trastuzumab retarded the tumor development of HCC cells (HuH7 and HLF). These data suggest that an ErbB2-targeting drug will aid in the treatment of HCC.

Our studies demonstrated that ErbB2, ErbB3, ErbB4, insulin R, ROR2, TrkA, EphA1, Tie-1, FGFR2α, FGFR3, PDGFRβ, EphA4, c-Ret, VEGFR3 and Mer were up-regulated in some of the cancer cell lines studied. Overexpression of ErbB2, ErbB3, TrkA, EphA1, Tie-1, FGFR2, FGFR3, PDGFR, Ret and VEGFR3 was previously reported in HCC (38-46). These previous reports support our results on the various RTKs activated in HCC derived from the protein array in this study. In summary, our results suggest that protein arrays aid in studying the expression of activated RTKs in various tissues, including malignant tissues. Furthermore, these results suggest that the immunological inhibition of ErbB3, ErbB4, insulin R, ROR2, TrkA, EphA1, Tie-1, FGFR2α, FGFR3, PDGFRβ, EphA4, c-Ret, VEGFR3 and Mer in addition to ErbB2 also have an anti-tumor effect for certain cases of HCC.

In conclusion, the ErbB2-targeting drug trastuzumab may aid in the treatment of HCC. In addition, the present results suggest that protein arrays are useful for detecting the expression of activated RTKs and developing efficient RTK-targeted therapies for HCC.

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