Y-box binding protein-1 (YB-1) contributes to both HER2/ErbB2 expression and lapatinib sensitivity in human gastric cancer cells

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Running title: YB-1-HER2 axis in gastric cancer cells

Keywords: Gastric cancer, HER2, lapatinib, trastuzumab, YB-1.
Financial Support: This work is supported by the 3rd Term Comprehensive Control Research for Cancer from the Ministry of Health, Labour and Welfare, Japan and by Grant-in-Aid for Challenging Exploratory Research from Japan Society for the Promotion of Science (JSPS), KAKENHI Grant Number 24650646 (M. Ku.).

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Disclosure of Conflict of Interest:
The authors declare there is no conflict of interest.

Word count: 4471 words
YB-1-HER2 axis in gastric cancer cells

Total number of figures: 5

Total number of table: 1

Total number of supplementary table: 1
Abstract

Gene amplification of \textit{HER2/ErbB2} occurs in gastric cancer, and the therapeutic efficacy of the HER2-targeted antibody, trastuzumab, has recently been improved against HER2-positive advanced stomach cancer. Here we examined whether Y-box-binding protein-1 (YB-1) could selectively control \textit{HER2} gene expression and cellular sensitivity to epidermal growth factor receptor (EGFR) family protein-targeted drugs in human gastric cancer cells. \textit{HER2} expression was specifically downregulated by YB-1 silencing using its cognate siRNA, whereas there was less change in the expression of EGFR and HER3. A chromatin immunoprecipitation assay revealed the specific binding of YB-1 to its consensus sequence on the 5′-regulatory region of \textit{HER2}. YB-1 knockdown induced drug resistance to lapatinib, a dual EGFR and HER2 kinase inhibitor, and also to erlotinib, an EGFR kinase inhibitor. Moreover, phosphorylation of protein kinase B (Akt) was not markedly affected by lapatinib or erlotinib when YB-1 was silenced. Nuclear YB-1 expression was significantly ($p=0.026$) associated with \textit{HER2} expression, but not with EGFR or HER3, in patients with gastric cancer ($n=111$). The YB-1-HER2 axis might therefore be useful for the further development of personalized therapeutics against gastric cancer by HER2-targeted drugs.


**Introduction**

Overexpression of the HER2 is predictive for overall survival and disease-free survival in node-positive breast cancer (1, 2). The humanized anti-HER2 antibody trastuzumab improves the therapeutic efficacy of HER2-positive breast cancer (3-5). HER2 overexpression is also predictive of poor prognosis in patients with gastric cancer (6-10). A trastuzumab phase III study in HER2-positive advanced gastric cancer (the ToGA trial) reported a survival benefit when trastuzumab was added to chemotherapy in HER2-overexpressing gastric cancer patients (11), and trastuzumab was approved for treatment of HER2-positive metastatic gastric and gastroesophageal junction cancer. On the other hand, small-molecule tyrosine kinase inhibitors that simultaneously target HER2 and other EGFR family proteins have been developed as an alternative anti-HER2 therapeutic strategy. Lapatinib (Tykerb) is a potent ATP-competitive dual kinase inhibitor that targets both inhibits EGFR and HER2 (12), and demonstrates anti-proliferative activity against human HER2-amplified breast cancer cell lines in culture (12). It also shows improved therapeutic efficacy in trastuzumab-refractory breast cancer patients (13, 14). Consistent with the hypothesis that HER2-directed therapy is clinically effective against HER2-amplified gastric cancer, lapatinib selectively inhibits the proliferation of HER2-amplified human gastric cancer cells, and
this effect is synergistic with trastuzumab both in vitro and in vivo (15).

The Y-box binding protein-1 (YB-1) containing a conserved cold-shock domain is a candidate regulatory molecule for HER2 expression in human cancer cells (16, 17). YB-1 also plays a key role in embryogenesis and differentiation (18-20), and cell proliferation and malignant transformation (18, 19, 21, 22). YB-1 expression is also significantly correlated with the acquirement of global drug resistance to various anticancer agents in human cancers (23). YB-1 knockdown inhibits the cell proliferation of human breast cancer, and suppresses the expression of various cell cycle/DNA replication-related genes including cell-division control protein 6 homolog (CDC6) (24-26). Astanehe et al. demonstrated that YB-1 activation mediated drug resistance to trastuzumab through the mitogen-activated protein kinase-interacting kinase in breast cancer cells, suggesting a critical role for YB-1 in the acquirement of drug resistance to trastuzumab (27). YB-1 was initially reported to interact with the Y-box element in the 5′ regulatory element of the EGFR and HER2 genes (28), and it had a critical role in the expression of both genes as shown by the effects of its knockdown in breast (29-31) and lung (32, 33) cancer cells in culture. HER2 expression is more specifically susceptible to YB-1-dependent regulation than EGFR in cultured breast and lung cancer cells (31, 32, 34). Consistent with these in vitro studies, nuclear YB-1 expression was more
closely correlated with HER2 than EGFR expression in patients with breast (31) and lung (32) cancer.

HER2 could play a pivotal role in the tumor growth of both breast and gastric cancer, and its expression is related to the therapeutic efficacy of molecular-targeted drugs such as trastuzumab and lapatinib in gastric cancer cells (11, 15). However, trastuzumab alone has only marginal cytotoxicity against various cancer cell lines in vitro. Here we asked whether YB-1 plays a specific regulatory role in the expression of HER2 in gastric cancer, and whether the YB-1-HER2 axis modulates cellular sensitivity to lapatinib and erlotinib. The association of YB-1 and HER2 expression in patients with gastric cancer was also considered.
Materials and Methods

Cell lines and culture, and reagents

The human gastric cancer cell lines MKN7, MKN28, MKN45, MKN74, KATO3, and NUGC3 were purchased from Health Science Research Resources Bank (Osaka, Japan). The human duodenal cancer cell line, AZ521, was also purchased from Health Science Research Resources Bank. NCI-N87 was purchased from the American Type Culture Collection, and SNU216 was from the Korean Cell Line Bank (Seoul, Korea). 44As3 was kindly provided by Dr. K. Yanagihara (National Cancer Center Research Institute, Japan) (35). All cell lines were purchased more than 6 months ago and were not further tested or authenticated by the authors. And these cell lines were cultured in RPMI-1640 supplemented with 10% FBS in a humidified atmosphere containing 5% CO₂ at 37 °C.

Erlotinib was kindly provided by F. Hoffmann-La Roche Ltd. (Basel, Switzerland), cisplatin was by Bristol-Myers Squibb K.K (New York, NY), and CPT-11 was by Yakult Honsha Co., Ltd. (Tokyo, Japan). Lapatinib was purchased from Toronto Research Chemicals (Toronto, Canada), and SU11274 and anti-phospho-EGFR (pEGFR) antibodies were from Calbiochem (San Diego, CA). Anti-HER2 and anti-phospho-HER2 (pHER2) antibodies were purchased from Upstate Biotechnology
(Lake Placid, NY). Anti-EGFR, anti-phospho-Met (pMet), anti-phospho-HER3 (pHER3), anti-extracellular-signal-regulated kinase 1/2 (Erk1/2), anti-phospho-Erk1/2 (pErk1/2), anti-protein kinase B (Akt), anti-phospho-Akt (pAkt), and anti-CDC6 antibodies were purchased from Cell Signaling Technology (Beverly, MA), anti-Met and anti-HER3 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), anti-α-tubulin antibody was purchased from Sigma-Aldrich (St Louis, MO), anti-GAPDH antibody was from Trevigen (Gaithersburg, MD). Anti-YB-1 antibody (st1968) was generated by immunization of New Zealand white rabbits with synthetic YB-1 peptide (carboxy-terminal amino acids 299-313) as described previously (36). This antibody could detect both cytoplasmic and nuclear YB-1. Anti-YB-1 antibody (EPITOMICS) was purchased from EPITOMICS (Burlingame, CA). The siRNA corresponding to YB-1 (5’-GGUUCCCACCUUACUACAU-3’) was purchased from QIAGEN Inc. (Valencia, CA), corresponding to HER2 (5’-AAACGUGUCUGUGUUGUAGGUGACC-3’), HER3 (5’-GGCAGUGUAUAAUCUACUCCACUA-3’) were purchased from Invitrogen, and corresponding to EGFR (5’-GAGGAAAUAUGUACUACGA-3’) was purchased from Sigma-Aldrich. Cells were transfected with siRNA duplexes using Lipofectamine RNAiMAX and Opti-MEM (Invitrogen) according to the manufacturer's
recommendations.

**Western blot analysis**

Cells were rinsed in ice-cold PBS and lysed in Triton X-100 buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 50 mmol/L NaF, 1% Triton X-100, and 10% glycerol containing 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 10 µg/mL aprotinin, 10 µg/mL leupeptin, and 1 mmol/L sodium orthovanadate). Cell lysates were separated by SDS-PAGE and transferred to Immobilon membranes (Millipore Corp., Bedford, MA). After transfer, membranes were incubated in blocking solution, probed with various antibodies, washed, and visualized by using horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Piscataway, NJ) and enhanced chemiluminescence reagents (Amersham, Arlington Heights, IL).

**Quantitative reverse transcription polymerase chain reaction (qRT-PCR)**

Forty-eight hours after siRNA transfection, total RNA was isolated from cell cultures by using ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan), according to the manufacturer’s instructions. The RNA concentration was assessed by spectrophotometry at 260 nm. RNA was reverse-transcribed from random hexamers using AMV reverse
transcriptase (Promega, Madison, WI), and qRT-PCR was performed by using the Real-Time PCR system 7300 (Applied Biosystems, Foster City, CA).

**Chromatin immunoprecipitation (ChIP) assay**

The ChIP assay was performed using the EZ ChIP kit (Millipore Corp.), according to the manufacturer’s recommendations. Briefly, soluble chromatin from $1 \times 10^6$ cells was incubated with 1 µg anti-YB-1 antibody. Purified DNA was dissolved in 50 µl H$_2$O, and 4 µl DNA was used for PCR analysis (40 cycles) with the following primer pairs: HER2#1, 5′-GCTCCAAATTGCCTTTCCA-3′ (forward) and 5′-CAGTTCCGCTTTGAAGACG-3′ (reverse); HER2#2, 5′-AAAAGGCTCATGTCCCCGTG-3′ (forward) and 5′-GGTGTACCGCGACTGCGTAC-3′ (reverse); and HER2#3, 5′-AGGGGCTCCAATAGAA-3′ (forward) and 5′-AATTTGGGAGGAGACAG-3′ (reverse). PCR products were analyzed on 1% agarose gels and stained with ethidium bromide.

**Cell proliferation assay**

Cells ($5 \times 10^3$) were seeded in 24-well plates, and the cell numbers in each well
were counted by a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, Fullerton, CA) at 5 days after siRNA transfection. Results were expressed as the mean ± SD of triplicate wells.

**Immunohistochemistry (IHC)**

Tissue sections were taken from 111 gastric cancer patients who underwent radical surgery at the Department of Surgery, Kurume University Hospital, Japan, between 2001 and 2004. Patient characteristics are summarized in Table 1. The 4-μm tissue sections were deparaffinized, and the slides were heated in a cell conditioning solution buffer for 60 min at 100 °C. The sections were immunohistochemically stained with anti-YB-1 antibody (st1968), anti-CDC6, anti-HER2, anti-HER3, and anti-EGFR antibodies by using the BenchMark XT (IHC Automated System, Tucson, AZ) and ChemMate ENVISION method (Dako Corporation, Carpinteria, CA). Samples were viewed by using a BX51 fluorescence microscope (Olympus, Tokyo, Japan), and the extent of YB-1 staining was classified according to the percentage of cells with strongly stained nuclei (≥10% indicated that a gland was positive). All IHC studies were evaluated by two experienced observers who were blind to the conditions of the patients.
Statistical analysis

The associations between YB-1 and clinicopathological findings were tested by Fisher’s exact test or the Chi-squared test depending on the type of data. $p<0.05$ was regarded as significant unless otherwise indicated.
Results

Effects of lapatinib and erlotinib on the phosphorylation of EGFR family proteins and their downstream signaling molecules in human gastric cancer cell lines

We initially examined protein expression levels of HER2 and other growth-factor receptors in 10 human gastric cancer cell lines using western blot analysis. As shown in Fig. 1A, HER2 expression was the highest in NCI-N87, whereas MKN7 and SNU216 showed relatively high HER2 expression, which is consistent with the previous studies (15, 37). HER2 was expressed at lower levels in MKN45, AZ521, and KATO3 cells, whereas Met levels were the highest in MKN45 cells (Fig. 1A).

Cellular sensitivity to anticancer drugs targeting growth-factor receptors and cytotoxic drugs, such as cisplatin and CPT-11, was compared among 10 cell lines, and IC_{50} values for each drug were presented based on dose-response curves for various drugs (Supplementary Table S1). Of the 10 cell lines, NCI-N87 and SNU216 were highly susceptible to lapatinib, whereas MKN45 was the most sensitive to SU11274. By contrast, all 10 cell lines showed similar drug sensitivities to cisplatin and CPT-11 (Supplementary Table S1). Trastuzumab had only a slight cytotoxic effect on NCI-N87 (data not shown), but not on the other cell lines.

We further examined the effect of erlotinib and lapatinib on activation of EGFR
family proteins and downstream signaling molecules, Akt and Erk, in SNU216, MKN45, and NCI-N87 cells (Fig. 1B, C). Western blot analysis showed that 10μM erlotinib blocked the phosphorylation of EGFR, HER2, and Akt in SNU216 and NCI-N87 (Fig. 1B). By contrast, 10μM erlotinib only slightly, if any, inhibited phosphorylation of HER2, HER3, and Akt in MKN45. On the other hand, phosphorylation of EGFR, HER2, HER3, Akt and Erk was inhibited by lapatinib at 1 and 10μM in SNU216 and NCI-N87, but not in MKN45 (Fig. 1C). Akt and Erk phosphorylation was more sensitive to the inhibitory effects of lapatinib and erlotinib in SNU216 and NCI-N87 cells than MKN45, suggesting that EGFR and its family proteins are more closely linked to cell growth and survival in the former two cell lines.

YB-1 knockdown suppresses HER2 expression in gastric cancer cells

We next examined the effect of YB-1 knockdown on the expression of EGFR family proteins in MKN45, SNU216, and NCI-N87 cell lines (Fig. 2A, B). YB-1 siRNA suppressed HER2 expression in all three cell lines, whereas there was almost no or much less suppression of EGFR. HER3 expression was not at all affected by YB-1 knockdown. To determine whether HER2 mRNA levels were affected by YB-1 knockdown, we used qRT-PCR in YB-1 siRNA-treated cells. HER2, but not EGFR nor
HER3, mRNA was decreased following treatment with YB-1 siRNA (Fig. 2C), which was consistent with the western blot analysis.

Y-box-like elements are located in the 5′-regulatory promoter region of HER2 (28, 29). As HER2 has two transcription variants (NM_001005862 and NM_004448), we analyzed the binding of YB-1 to the two putative promoters (1 and 2) of HER2. Of the three putative YB-1-responsive elements in promoters 1 and 2, YB-1 bound directly to the #2 and #3 elements, but not to the #1 element (Fig. 2D).

YB-1 knockdown alters sensitivity to lapatinib and erlotinib

We next examined whether YB-1 knockdown altered sensitivity to lapatinib and erlotinib in MKN45, SNU216, and NCI-N87 cells (Fig. 3). We previously demonstrated that YB-1 knockdown confers drug resistance to cisplatin, which is a DNA-damaging anticancer agent, in both cancer cells and normal cells (19, 36, 38). We also examined the effect of YB-1 knockdown on sensitivity to cisplatin in gastric cancer cells. There was no marked change in sensitivity to erlotinib and lapatinib when YB-1 was silenced in MKN45 (Fig. 3A). By contrast, treatment with YB-1 siRNA resulted in varying degrees of decreased sensitivity to both erlotinib and lapatinib in the two gastric cancer cell lines, SNU216 and NCI-N87 (Fig. 3 B, C), suggesting that the suppression
of HER2 expression by YB-1 knockdown is closely associated with sensitivity to the above mentioned drugs. Of the three cell lines, YB-1 knockdown enhanced sensitivity to cisplatin only in MKN45 cells.

We further examined the effects of erlotinib and lapatinib on the phosphorylation of Akt, Erk1/2, EGFR, HER2, and HER3 in a gastric cancer cells treated with or without YB-1 siRNA. Treatment of NCI-N87 cells with YB-1 siRNA suppressed HER2 expression without affecting EGFR and HER3 expression (Fig. 4A, C). Erlotinib inhibited the phosphorylation of EGFR, HER3, Akt, and Erk both with and without YB-1 siRNA (Fig. 4A). HER2 phosphorylation was only slightly inhibited by erlotinib, whereas Erk and Akt phosphorylation was highly susceptible to its inhibition. Quantitative analysis showed that Akt phosphorylation was relatively less sensitive to erlotinib under YB-1 knockdown condition (Fig. 4B). Phosphorylation of EGFR, HER3, Akt, and Erk was suppressed by lapatinib both with and without YB-1 knockdown (Fig. 4C). Quantitative analysis showed that Akt phosphorylation was relatively less sensitive to lapatinib under YB-1 knockdown compared with under control conditions (Fig. 4D).

**Correlation of YB-1 with HER2 expression in gastric cancer**

Use of a specific YB-1 antibody recognizing YB-1 in both the nucleus and
YB-1-HER2 axis in gastric cancer cells

cytoplasm allowed us to perform IHC of surgically resected gastric tumors. Fig. 5A shows typical nuclear YB-1 immunostaining in gastric cancer. IHC profiles of YB-1 nuclear expression were summarized by dividing all samples into five groups (0-4%, 5-9%, 10-29%, 30-39%, and >40%) (Table 1). Of all 111 samples, approximately 60% showed relatively low expression (0-4% and 5-9%). We used a cut-off value of 10% to divide the samples into decreased (<10%) and increased (≥10%) expression, which were evaluated as negative and positive, respectively. Fig. 5B shows representative images for EGFR, HER2, HER3, and CDC6 expression. Fig. 5C shows the typical IHC images of both nuclear YB-1 expression and HER2 expression in cancer cells of the serial sections. Two clinical samples (case 1 and 2) showed higher expression of HER2 with nuclear YB-1 expression, but the other two samples (case 3 and 4) did not show apparent expression of HER2 without nuclear YB-1 expression.

We classified gastric cancer samples into high (2+, 3+) and low (0, 1+) expression of EGFR, HER2, HER3, and CDC6. Consistent with the association of YB-1 with CDC6 in breast cancer patients (26), nuclear YB-1 expression was found to be significantly associated with CDC6 expression (p<0.0027) in gastric cancer in the present study (Table 1). Of the three EGFR family proteins, HER2 expression was significantly (p=0.026) correlated with YB-1. By contrast, EGFR expression was closely,
but not significantly ($p=0.097$), associated with YB-1. There was no association of nuclear YB-1 expression with HER3 expression.
Discussion

The present study demonstrated that YB-1 knockdown specifically suppressed the expression of HER2 and induced drug resistance to lapatinib in gastric cancer cells. Lapatinib has previously been shown to be effective against HER2-positive human breast and gastric cancer cells in vitro and in vivo (12, 15, 39). Higher expression of YB-1 in the nucleus of cancer cells was significantly correlated with HER2, but not EGFR or HER3, expression in gastric cancer (Table 1). And nuclear YB-1 expression was found to be significantly associated with poor prognosis (p<0.01) and with metastasis to the lymph node and peritoneum (p<0.005) in gastric cancer patients (unpublished data). Recent study by Wu et al. has also demonstrated the close association of high YB-1 expression with liver metastasis and poor survival in advanced gastric cancer (40).

Of the three cell lines used in this study, SNU216 and NCI-N87 cells showed higher HER2 expression than MKN45 cells. In particular, NCI-N87 cells expressed the highest level of HER2 expression and showed constitutive activation of both EGFR and HER2. It was highly susceptible to the cytotoxic effect of lapatinib, and SNU216 cells showed similar cellular sensitivity to lapatinib as NCI-N87 cells (Supplementary Table S1). The phosphorylation of EGFR, HER2 and HER3 was similarly suppressed by lapatinib in both SNU216 as NCI-N87 cells (Fig. 1C). Treatment of SNU216 and
YB-1-HER2 axis in gastric cancer cells

NCI-N87 cells with lapatinib thus disrupts the heterodimer formation of EGFR/HER2 and HER2/HER3, and inactivates their downstream signaling pathways involving Akt and Erk.

Akt and Erk activation was markedly blocked by erlotinib or lapatinib in SNU216 and NCI-N87 cells (Fig. 1B, C), suggesting a close association of EGFR family proteins, including HER2, with cell growth and survival. Following YB-1 knockdown, Akt phosphorylation was less susceptible to the inhibitory effects of lapatinib or erlotinib in NCI-N87 cells when compared with under control conditions (Fig. 4). Together with these findings, our present study suggests that YB-1 knockdown-induced drug resistance to lapatinib is mainly caused by decreased HER2 expression without affecting EGFR and/or HER3 expression. HER2 might play essential roles in the determination of sensitivity to EGFR-targeted drugs in gastric cancer cells as seen in lung cancer cells (41-44). The close link between YB-1 and HER2 might therefore limit sensitivity to EGFR-targeted drugs not only in lung cancer cells but also in gastric cancer cells.

The Met gene is amplified in MKN45 cells (Fig. 1A), which are highly susceptible to the cytotoxic effects of SU11274, a Met-targeted drug (Supplementary Table S1). Therefore, Met driven signaling pathway in MKN45 cells limit cytotoxicity
YB-1-HER2 axis in gastric cancer cells

to EGFR or HER2 targeted drugs (42). YB-1 knockdown decreased HER2 expression without affecting Met expression (data not shown). Consistent with these findings, YB-1 knockdown did not affect sensitivity to lapatinib or erlotinib in MKN45 cells (Fig. 3).

The ChIP assay in the present study revealed an interaction of YB-1 with Y-boxes in the promoter region of HER2. Previously, Wu et al. used microarray analysis to establish a close correlation between YB-1 and EGFR or HER2 expression in tumor samples from breast cancer patients (29), and this was also observed by IHC analysis. We previously demonstrated the selective association of YB-1 and HER2 expression in human breast cancer cell lines (31) as well as in human lung cancer cells both in vitro and in vivo (32). These studies suggest that YB-1 expression selectively controls the expression of HER2, rather than EGFR and HER3, in human gastric cancer cells and breast and lung cancer cells.

Finally, one could ask whether the association of nuclear YB-1 expression and HER2 expression is differentially affected by tumor types, including breast cancer (31), lung cancer (32) and gastric cancer (cf. this study). YB-1 knockdown reduced expression of HER2 in 2 of 4 breast cancer cell lines (31), 2 of 5 lung cancer cell lines (32), and 3 of 3 gastric cancer cell lines (Fig. 2), respectively in vitro. In patients with
negative nuclear YB-1 expression, frequencies of relatively higher HER2 expression (+2 and +3) were less than 10% for breast cancer (4/43 = 9.3%) and lung cancer (adenocarcinoma) (2/47 = 4.3%), and relatively high for gastric cancer (12/64 = 18.8%). Although there is such a difference in baseline frequencies, frequencies of high HER2 expression in patients with positive nuclear YB-1 expression were found to be about 20% higher than those in patients with negative nuclear YB-1 expression consistently in these three different tumor types (10/30 = 33.3% versus 9.3% for breast cancer; 4/19 = 21.1% versus 4.3% for lung cancer; and 18/47 = 38.3% versus 18.8% for gastric cancer). Taken together, nuclear YB-1 expression may have similar effects on HER2 expression in breast, lung and gastric cancer.

In conclusion, HER2 and HER3 appear to play an important role in the activation of human gastric cancer cell-signaling pathways for survival and proliferation. YB-1 specifically controls the expression of HER2, rather than EGFR and HER3, affecting drug sensitivity to HER2- and/or EGFR-targeted anticancer drugs in gastric cancer cells. As YB-1 nuclear expression is significantly correlated with HER2 expression in human gastric cancer, the YB-1-HER2 axis could contribute to the further development of personalized therapeutics by EGFR-targeted drugs against human gastric cancer.
Acknowledgements

We thank Drs. I. Okamoto and K. Nakagawa at Kinki University for fruitful discussion. We also thank E. Kajita at Kyushu University for preparing this manuscript.

Grant Support

This work is supported by the 3rd Term Comprehensive Control Research for Cancer from the Ministry of Health, Labour and Welfare, Japan and by Grant-in-Aid for Challenging Exploratory Research from Japan Society for the Promotion of Science (JSPS), KAKENHI Grant Number 24650646 (M. Ku).
References


YB-1-HER2 axis in gastric cancer cells


YB-1-HER2 axis in gastric cancer cells


YB-1-HER2 axis in gastric cancer cells

Table 1. Correlation between nuclear YB-1 expression and expression of three EGFR family proteins and CDC6 in gastric cancer patients (n=111)\textsuperscript{a}

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\textsuperscript{a} Determined by Fisher’s exact test
YB-1-HER2 axis in gastric cancer cells

Figure legends

Figure 1. Expression of EGFR family proteins and effects of erlotinib and lapatinib in gastric cancer cell lines. (A) Western blots showing the expression of EGFR, pEGFR, HER2, pHER2, HER3, pHER3, Met, pMet, Erk, pErk, Akt, and pAkt proteins and α-tubulin as a loading control in 10 human gastric cancer cell lines. The inhibitory effect of erlotinib (B), and lapatinib (C), on the expression of pEGFR, pHER2, pAkt, and pErk in MKN45, SNU216, and NCI-N87 cells. Cells were treated with erlotinib or lapatinib for 6 hours, then analyzed by western blotting.

Figure 2. Effect of YB-1 knockdown on EGFR, HER2, HER3, Akt, pAkt, Erk and pErk expression. (A) MKN45, SNU216, and NCI-N87 cells were treated with YB-1 siRNAs for 48 hours, and western blotting was used to analyze the expression of EGFR, HER2, HER3, Akt, pAkt, Erk, and pErk. (B) Quantitative analysis of the effect of YB-1 knockdown on expression of EGFR, HER2 and HER3 by YB-1 siRNA (as indicated in Fig. 2A) when normalized in the absence of the siRNA. (C) Effect of YB-1 siRNA on EGFR, HER2, and HER3 mRNA levels as determined by qRT-PCR in MKN45 cells. Each bar is an average ± SD of triplicate experiments, *p<0.01. (D) Schematic representation of potential YB-1 binding sites and primer locations used for ChIP assay.
Figure 3. Effect of YB-1 knockdown on sensitivity to erlotinib, lapatinib, and cisplatin in MKN45, SNU216, and NCI-N87 cells. MKN45 (A), SNU216 (B), and NCI-N87 (C) cells were treated with YB-1 siRNA for 48 hours, exposed to drugs, and a cell-proliferation assay was carried out. Each value is an average ± SD of triplicate dishes. Values expressed as % normalized to the value with no drugs (100%).

Figure 4. Effect of erlotinib and lapatinib on EGFR, pEGFR, HER2, pHER2, HER3, pHER3, Akt, pAkt, Erk, and pErk expression in the presence or absence of YB-1 siRNA in NCI-N87 cells. (A) Effect of erlotinib on expression of growth-factor receptors and their downstream-regulatory molecules with or without YB-1 siRNA treatment for 48 hours, followed by erlotinib exposure and western blot analysis. (B) Quantitative analysis of the effect of erlotinib on Akt and Erk phosphorylation in the presence of YB-1 siRNA. Akt and Erk phosphorylation in the presence of YB-1 siRNA is normalized by pAkt and pErk protein levels, respectively, in the absence of erlotinib. (C) Effect of lapatinib on expression of growth-factor receptors and their downstream-regulatory molecules with or without YB-1 siRNA, followed by lapatinib exposure and western blot analysis. (D) Quantitative analysis of the effect of lapatinib
on Akt and Erk phosphorylation. Akt and Erk phosphorylation in the presence of YB-1 siRNA is normalized by pAkt and pErk protein levels in the absence of the drug.

Figure 5. IHC images for expression of nuclear YB-1, EGFR, HER2, HER3 and CDC6 in gastric tumor specimens. (A) Representative images of nuclear YB-1 expression. Positive (2 cases on the left) and negative (2 cases on the right) cases are presented. (B) Representative images for low (0, +1) and high (2+, 3+) expression of EGFR, HER2, HER3, and CDC6 in various tumor samples. (C) Representative images of HER2 and nuclear YB-1 expression in the serial section. Of 4 clinical samples of the serial section, 2 cases (case 1 and 2) show higher expression of both HER2 (3+) and nuclear YB-1, and the other 2 cases (case 3 and case 4) show no apparent expression of both HER2 (0, +1) and nuclear YB-1 in cancer cells.
Figure 2

A

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<th>siYB-1(nM)</th>
<th>MKN45</th>
<th>SNU216</th>
<th>NCI-N87</th>
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C

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D

![Enzyme digestion diagram](#)
Figure 3

A  MKN45

- si Control
- si YB-1

B  SNU216

C  NCI-N87

% of control

Erlotinib (μM)

Cisplatin (μM)

Lapatinib (μM)
Figure 4

A

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<th>Erlotinib (μM)</th>
<th>Cont siRNA</th>
<th>YB-1 siRNA</th>
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B

![Graph B](image)

C

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D

![Graph D](image)
Figure 5

A  Nuclear YB-1

<table>
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<th>Positive</th>
<th>Negative</th>
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</table>

B  High (2+, 3+)  Low (0, 1+)

| EGFR | HER2 | HER3 | CDC6 |

C  Case 1  Case 2  Case 3  Case 4

| Nuclear YB-1 | | | |
| - | + | - | - |

| HER2 | | | |
| + | + | - | - |
Y-box binding protein-1 (YB-1) contributes to both HER2/ErbB2 expression and lapatinib sensitivity in human gastric cancer cells

Tomohiro Shibata, Hitoshi Kan, Yuichi Murakami, et al.

Mol Cancer Ther Published OnlineFirst February 27, 2013.

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