

Expression of CD24 is associated with HER2 expression and supports HER2-Akt signaling in HER2-positive breast cancer cells

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Key words

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Human epidermal growth factor receptor 2 (HER2)-positive breast cancer is treated with HER2-targeted agents, such as trastuzumab and lapatinib, that suppress signaling by phosphatidylinositol 3-kinase (PI3K)-Akt and MAPK pathways. However, resistance to HER2-targeted therapy remains a major clinical problem. Overexpression of CD24 has been detected in many cancers and is associated with a poor prognosis in women with breast cancer. HER2-positive breast tumors are predominantly positive for CD24, suggesting that the expression of the two molecules is related. To investigate the relation between HER2 and CD24, we overexpressed HER2 in breast cancer cells that were triple-negative for the estrogen receptor, progesterone receptor and HER2. We found that expression of CD24 was increased by stable overexpression of HER2. Flow cytometry thus revealed that the percentage of CD24-positive cells was markedly higher in the HER2-positive fraction than in the HER2-negative fraction. Knockdown of CD24 in breast cancer cells positive for endogenous HER2 downregulated HER2 expression, whereas knockdown of HER2 did not affect the expression of CD24. Knockdown of CD24 also suppressed the phosphorylation of Akt, which functions downstream of HER2 and PI3K to promote cell survival. Moreover, knockdown of CD24 increased the sensitivity of HER2-positive breast cancer cells to lapatinib treatment. Our results thus indicate that CD24 supports both the expression of HER2 and the consequent activation of PI3K-Akt signaling. Furthermore, CD24 may contribute to resistance to HER2-targeted therapy and, therefore, is itself a potential therapeutic target in HER2-positive breast cancer.

The human epidermal growth factor receptor 2 (HER2) gene is amplified in 20% to 30% of invasive breast cancers, and such amplification is associated with a poor patient prognosis and a high rate of metastasis.^(1,2) The standard treatment for HER2-positive breast cancer is the combination of chemotherapy plus a HER2-targeted agent such as trastuzumab, lapatinib or pertuzumab, all of which suppress signaling downstream of HER2 mediated by phosphatidylinositol 3-kinase (PI3K)-Akt and MAPK pathways.⁽³⁾ The combination of trastuzumab, a humanized monoclonal antibody to HER2, with adjuvant chemotherapy has been found to significantly improve disease-free and overall survival rates in individuals with early-stage HER2-overexpressing breast cancer.^(4–6) However, approximately 15% of patients relapse after adjuvant chemotherapy combined with trastuzumab,⁽⁷⁾ and >70% of patients with HER2-positive metastatic breast cancer show resistance to HER2-targeted therapy within 1 year.^(8,9) Indicators that identify such resistant tumors are thus needed to optimize treatment strategies.

Cancer stem cells (CSC) have been proposed to constitute a subpopulation of undifferentiated tumor cells that are resistant to both radiotherapy and chemotherapy.⁽¹⁰⁾ Aldehyde dehydrogenase 1 (ALDH1) is a functional marker for human breast CSC. Overexpression of HER2 was found to increase the size

of the ALDH1-positive subpopulation of breast cancer cells, which exhibited increased capacities for invasiveness and tumorigenesis, whereas exposure to trastuzumab reduced the ALDH1-positive cell fraction in some HER2-positive breast cancer cell lines.⁽¹¹⁾ Moreover, a PI3K inhibitor reduced the size of the ALDH1-positive subpopulation in trastuzumab-resistant HER2-positive cell lines,⁽¹¹⁾ suggesting that aberrant activation of the PI3K-Akt signaling pathway is associated with resistance to HER2-targeted therapy.

CD24 is a glycosylphosphatidylinositol-anchored membrane protein and a ligand for P-selectin, an adhesion receptor expressed on normal endothelial cells and platelets.⁽¹²⁾ Although CD24 has been identified as a marker of differentiated normal mammary epithelial cells,⁽¹³⁾ its overexpression in breast cancer is associated with a poor patient prognosis.⁽¹⁴⁾ In addition, HER2-positive breast cancer has a predominantly CD24-positive status.⁽¹⁵⁾ Therefore, we wondered whether co-expression of CD24 with HER2 might be associated with therapeutic resistance of HER2-positive cells.

To investigate the relevance of CD24 expression in HER2-positive breast cancer, we established HER2-expressing cell lines by introducing an expression vector for HER2 into MDA-MB-231-Luc, a human breast cancer cell line that is

triple-negative for the estrogen receptor, the progesterone receptor and HER2, and which has been engineered to express luciferase. We found a positive association between HER2 and CD24 expression, and that expression of CD24 supports the HER2-PI3K-Akt signaling pathway in HER2-positive breast cancer cells.

Materials and Methods

Transfection with plasmid DNA. MDA-MB-231-Luc-D3H2LN (231-Luc) cells were transfected with either an expression plasmid for wild-type HER2 (16257 [Addgene, Cambridge, MA, USA]) or a control plasmid (18964, Luciferase-pcDNA3 [Addgene]) with the use of the Lipofectamine 2000 reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA). 231-Luc cells stably expressing HER2 were selected by exposure of the transfected cells to neomycin (G418, 1200 $\mu\text{g}/\text{mL}$) and were sorted with the use of a MoFlo flow cytometer (Beckman Coulter, Brea, CA, USA).

Flow cytometry. For sorting of HER2-expressing 231-Luc cells, transfected and selected cells were stained with FITC-conjugated antibodies to HER2 (Becton Dickinson, Franklin Lakes, NJ, USA) for 30 min at 4°C in the dark and were then processed with the MoFlo instrument to yield the HER2-60 line. We obtained the HER2-90 line from HER2-60 by repeating the cell sorting described above. For triple-staining of HER2, CD24 and CD44, nonconfluent cell cultures were exposed to cell dissociation buffer (Gibco, Life Technologies, Carlsbad, CA, USA), and the resulting single cells were counted and then stained with FITC-conjugated antibodies to HER2 (Becton Dickinson), phycoerythrin-conjugated antibodies to CD24 (BD Pharmingen, Tokyo, Japan) and allophycocyanin-conjugated antibodies to CD44 (BD Pharmingen). A total of 500 000 cells was incubated with the antibodies for 30 min at 4°C in the dark. The cells were counterstained with propidium iodide and then analyzed with a FACSCalibur (BD Biosciences, Tokyo, Japan) or Gallios (Becton Dickinson) instrument. The subpopulation of cells positive for ALDH enzymatic activity was detected with the use of an ALDEFLUOR kit (Stem Cell Technologies, Tokyo, Japan). All flow cytometry data were analyzed with Flowjo software (Tree Star, Ashland, OR, USA).

Immunoblot analysis. Immunoblot analysis was performed as previously described⁽¹⁶⁾ with primary antibodies as follows: anti-HER2 (#2242), anti-phosphorylated HER2 (#2249), anti-Akt (#9272), anti-phosphorylated Akt (#4060), anti-Erk1/2 (#9102), anti-phosphorylated Erk1/2 (#4370), anti-EGFR (#4267), anti-phosphorylated EGFR (#3777), anti-phosphorylated HER3 (#4791) (all of which were obtained from Cell Signaling Technology, Danvers, MA, USA), anti-HER3 (M7297, DAKO, Glostrup, Denmark) and anti- α -tubulin (Sigma-Aldrich, St. Louis, MO, USA). Immune complexes were detected with HRP-conjugated secondary antibodies, a chemiluminescence detection system (Perkin-Elmer, Waltham, MA, USA) and an LAS-3000 instrument (Fujifilm, Tokyo, Japan).

Quantitative RT-PCR analysis. Reverse transcription and real-time PCR analysis were performed as previously described⁽¹⁶⁾ with the PCR primers listed in Table S1.

Immunohistochemistry. Immunohistochemical staining was performed as previously described with rabbit polyclonal antibodies to HER2 (Cell Signaling Technology).⁽¹⁶⁾

Transfection with siRNA. Negative control (GAPD), HER2 and CD24 siRNA SMART pools were obtained from Dharma-

con (Thermo Fisher Scientific, Waltham, MA, USA). Cells were transfected with the use of the Lipofectamine RNAiMAX reagent (Invitrogen).

Other methods. Cell culture, assay of cell proliferation, tumorsphere formation, xenograft transplantation, bioluminescence imaging and statistical analysis are described in the Data S1.

Results

Ectopic expression of human epidermal growth factor receptor 2 upregulates CD24 in triple-negative breast cancer cells. To determine the functional relevance of CD24 expression in HER2-positive breast cancer cells, we introduced an expression vector for HER2 into MDA-MB-231-Luc-D3H2LN (231-Luc) cells, which are triple-negative breast cancer cells engineered to express luciferase. We obtained two lines of 231-Luc cells, designated HER2-60 and HER2-90, in which the subpopulation of HER2-positive cells constituted 63.2% and 92.4% of all cells, respectively (Fig. 1a). We assessed the expression of HER2 and activation of HER2 signaling in these cell lines by immunoblot analysis (Fig. 1b). The phosphorylation levels of HER2 and Akt were increased in a manner dependent on the proportion of HER2-positive cells, whereas the phosphorylation of Erk1/2 was not affected by ectopic expression of HER2. We also confirmed that the abundance of *HER2* mRNA was increased correspondingly in HER2-60 and HER2-90 cells compared with 231-Luc cells (Fig. 1c). There was no significant difference in cell proliferation among 231-Luc, HER2-60 and HER2-90 lines under either adherent or nonadherent conditions (Fig. S1).

We next examined the expression of CD44 and CD24 in the three cell lines, given that the CD44⁺CD24⁻ fraction is thought to constitute a stem-progenitor cell subpopulation in human breast cancer.⁽¹³⁾ The CD44⁺CD24⁻ fraction constituted 93.6% of 231-Luc cells but only 71.2% and 45.7% of HER2-60 and HER2-90 cells, respectively (Fig. 1d, left panels). The mean fluorescence intensity for CD24 was 4.50 in 231-Luc cells, whereas it was increased to 8.70 and 11.7 in HER2-60 and HER2-90 cells, respectively (Fig. 1d, right panels). Given that CD44 expression levels did not differ substantially among the three cell lines, we compared the expression level of CD24 between the HER2-positive fraction and the HER2-negative fraction. The percentage of CD24-positive cells was 54.4% and 63.4% in the HER2-positive fraction and 7.33% and 2.65% in the HER2-negative fraction for HER2-60 and HER2-90 cells, respectively (Fig. 1e). The expression of CD24 was thus higher in the HER2-positive fraction than in the HER2-negative fraction, suggesting that the expression of CD24 is positively associated with that of HER2.

Effects of human epidermal growth factor receptor 2 overexpression on cancer stem cells properties. To determine if HER2 overexpression might affect CSC properties, we examined sphere formations of HER2-90 and 231-Luc cells. The numbers of spheres formed with a diameter of >100 μm were counted. HER2-90 cells formed significantly fewer spheres than did 231-Luc cells (Fig. 2a,b). Cells were also cultured using 1% methylcellulose containing a medium to avoid cell aggregation. In contrast to 231-Luc cells, HER2-90 cells did not form sphere-like colonies in the presence of methylcellulose (Fig. 2a,b). An examination of ALDH1 activity, a functional marker for breast cancer CSC,⁽¹⁷⁾ revealed that overexpression of HER2 decreased the proportion of ALDEFLUOR-positive CSC (Fig. 2c). To determine the ability of 231-Luc and

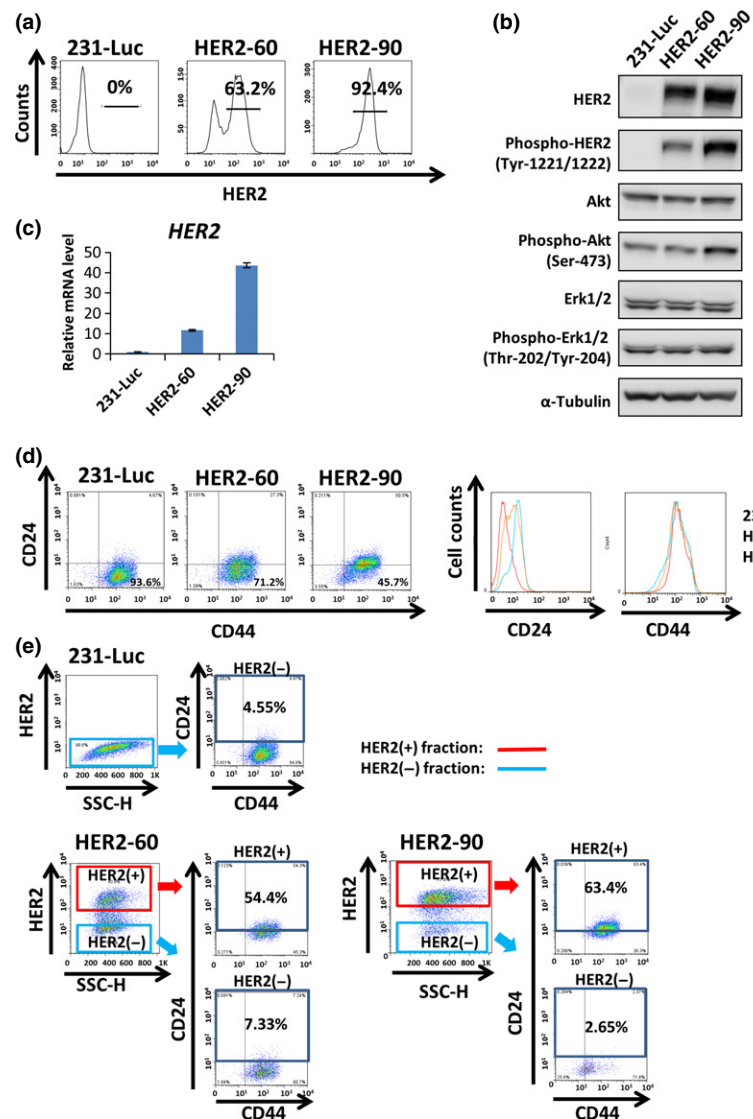


Fig. 1. Ectopic expression of human epidermal growth factor receptor 2 (HER2) upregulates CD24 in triple-negative breast cancer cells. (a) Flow cytometric analysis of HER2 expression in 231-Luc, HER2-60 and HER2-90 cells. The percentage of HER2-expressing cells is indicated. (b) Immunoblot analysis of total and phosphorylated forms of HER2, Akt and Erk1/2 in 231-Luc, HER2-60 and HER2-90 cells. (c) Quantitative RT-PCR analysis of *HER2* mRNA in 231-Luc, HER2-60 and HER2-90 cells. Data are normalized by the amount of *hypoxanthine phosphoribosyltransferase 1 (HPRT1)* mRNA, expressed relative to the normalized value for 231-Luc cells, and presented as means \pm SD from triplicate experiments. (d) Flow cytometric analysis of CD44 and CD24 expression in 231-Luc, HER2-60 and HER2-90 cells. The percentage of CD44⁺CD24⁻ cells is indicated in the right lower panel. (e) Comparison of the expression of CD24 between the HER2-positive fraction (gated in red) and the HER2-negative fraction (gated in blue) for 231-Luc, HER2-60 and HER2-90 cells. Cells were stained for HER2, CD44 and CD24 and then gated based on the expression level of HER2. The percentage of CD24-positive cells in each fraction is indicated. SSC-H, side scatter pulse height.

HER2-90 cells to initiate tumors, we injected cells into a mammary fat pad of female NOD/SCID mice, which revealed that there was no difference in the frequency of tumor initiation (Fig. 2d). These results suggested that overexpression of HER2 decreased representative CSC properties, such as the sphere-forming ability and ALDH1 activity, and did not affect the tumorigenicity of 231-Luc triple-negative breast cancer cells.

Given that the epithelial-mesenchymal transition (EMT) is thought to contribute to the aggressiveness and metastatic ability of cancer and has been associated with characteristics of CSC,⁽¹⁸⁾ we measured the expression levels of EMT-related genes by quantitative RT-PCR analysis (Fig. 2e). The amounts of mRNA for the EMT transcription factors *Snail*, *Slug*, *ZEB1* and *ZEB2* did not differ between the two cell lines. We also determined the expression of embryonic stem (ES) cell-related genes (Fig. 2f), given that an ES cell-like gene signature has been associated with a poorly differentiated state of tumors and that expression of ES cell-associated transcription factors (*SOX2*, *Nanog* and *OCT3/4*) contributes to stem cell-like phenotypes.⁽¹⁹⁾ We found that the amounts of *Nanog* and *OCT3/4* mRNA were significantly increased in HER2-90 cells compared with 231-Luc cells. All those find-

ings suggest that HER2 may contribute to the stem-like characteristics of breast cancer cells but other factors are required to regulate CSC functions.

Effect of human epidermal growth factor receptor 2 overexpression on tumor growth in an orthotopic xenograft model. We injected 231-Luc, HER2-60 or HER2-90 cells (2×10^5) into a mammary fat pad of female nude mice in order to examine tumor growth in an orthotopic xenograft model. Tumors formed by HER2-60 or HER2-90 cells tended to be larger than those formed by 231-Luc cells, although the differences were not statistically significant (Fig. 3a,b). Immunohistochemical analysis of formalin-fixed tumor tissue revealed the proportionate overexpression of HER2 in tumors derived from HER2-60 or HER2-90 cells, whereas HER2 immunoreactivity was not detected in 231-Luc tumors (Fig. 3c).

Positive relation between human epidermal growth factor receptor 2 and CD24 expression among human epidermal growth factor receptor 2-positive breast cancer cell lines. We next examined the expression of HER2, CD24 and CD44 in five HER2-positive breast cancer cell lines: BT-474, HCC202, SKBR3, ZR-75-1 and HCC1569. Flow cytometry showed that the CD44⁺CD24⁻ subpopulation constituted 2.65%, 0.057%, 0.143%, 1.42% and 91.7% of cells in these lines, respectively

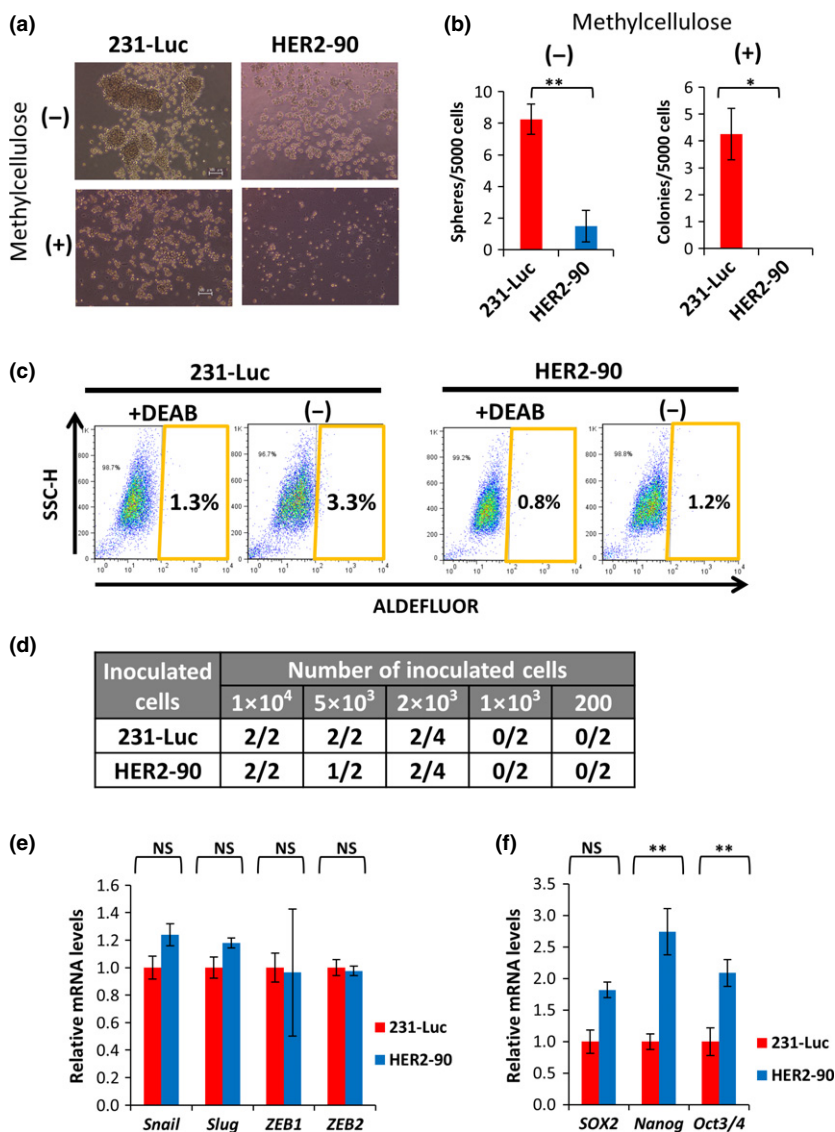


Fig. 2. Effects of overexpression of human epidermal growth factor receptor 2 (HER2) on cancer stem cell (CSC) properties. (a) Tumorsphere formation by 231-Luc and HER2-90 cells. Tumorspheres were photographed after culture of the cells for 6 days. Scale bar, 100 μ m. (b) Quantitation of tumorsphere formation by 231-Luc and HER2-90 cells. The number of tumorspheres with a diameter of >100 μ m was counted after cell culture for 6 days. Data are means \pm SD for triplicate experiments. $**P < 0.01$ (Student's *t* test). (c) Aldehyde dehydrogenase (ALDH) activity of 231-Luc and HER2-90 cells as determined with an ALDEFLUOR kit. Cells were incubated with the ALDEFLUOR substrate in the absence or presence of the specific ALDH inhibitor diethylaminobenzaldehyde (DEAB) and then analyzed by flow cytometry. The percentage of ALDEFLUOR-positive cells is indicated. (d) Comparison of tumor-initiating activity between 231-Luc and HER2-90 cells. Tumor formation was evaluated 8 weeks after injection of the indicated numbers of cells into a mammary fat pad of NOD/SCID mice. (e,f) Quantitative RT-PCR analysis of mRNA derived from epithelial-mesenchymal transition-related (e) or ES cell-related (f) genes in 231-Luc and HER2-90 cells. Data are normalized by the amount of *HPRT1* mRNA, expressed relative to the corresponding normalized value for 231-Luc cells, and are presented as means \pm SD for triplicate experiments. $*P < 0.05$, $**P < 0.01$ (Student's *t*-test). NS, not significant.

(Fig. 4a). Next, we measured CD24 expression levels in the top 20% fraction of HER2-high cells and the bottom 20% fraction of HER2-low/- cells of each cell line (Fig. 4b). For BT-474, which has an amplification of *HER2* gene, the CD24-positive subpopulation was 97.1% in the HER2-high fraction but 73.4% in the HER2-low/- fraction. For ZR-75-1, which has a normal level of HER2, the proportion of CD24-positive cells was 97.2% in the HER2-high fraction but only 27.4% in the HER2-low/- fraction (Fig. 4c). A similar pattern was apparent for the other three *HER2*-amplified cell lines. The expression of CD24 was thus higher in HER2-high cells than in HER2-low/- cells for all five breast cancer cell lines.

Knockdown of CD24 downregulates human epidermal growth factor receptor 2 expression and Akt phosphorylation. We examined whether CD24 might affect HER2 expression with the use of RNA interference in the HER2-positive cell line BT-474. The abundance of *CD24* and *HER2* mRNA was significantly reduced by transfection of the cells with corresponding targeted siRNA (Fig. 5a). Flow cytometry analysis revealed that the percentage of CD24-expressing cells was also markedly reduced by knockdown of CD24 (from 98.9% for cells transfected with a control siRNA to 56.2%). The HER2-nega-

tive fraction was increased by transfection with the CD24 siRNA (from 71.3% to 59.8%) (Fig. 5b). In contrast, knockdown of HER2 did not affect the proportion of cells expressing CD24 (98.9% for the control siRNA versus 98.1% for the *HER2* siRNA) (Fig. 5c). These results imply that CD24 plays a role in maintenance of HER2-positive cells.

Western blot analysis also showed that the knockdown of CD24 by siRNA decreased the expression and the phosphorylation of HER2 in the HER2-positive cell lines, BT-474, HCC202, and HCC1569 (Fig. 6a). However, in 231-Luc derived cells, HER2-60 and HER2-90, the exogenous HER2 expression driven by the cytomegalovirus (CMV) promoter was not affected by siRNA-mediated CD24 depletion (Fig. 6b).

In Figure 1(d), we show that the stable overexpression of HER2 promoted CD24 expression. To further investigate the hierarchical relationship between CD24 and HER2, we performed transient expression of HER2 or CD24 in 231-Luc cells for 48 h and analyzed HER2 and CD24 expression by using flowcytometry (Fig. S2). Contrary to the stable expression, the transient expression of HER2 did not significantly affect the HER2⁺CD24⁺ fractions. Furthermore, the transient

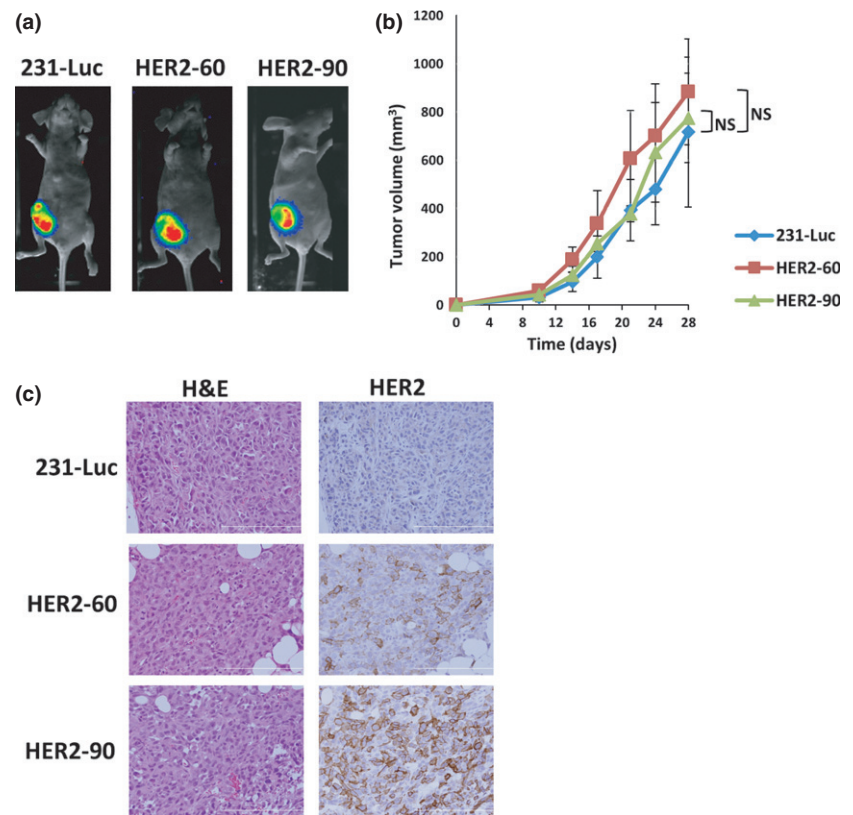


Fig. 3. Effect of overexpression of human epidermal growth factor receptor 2 (HER2) on tumor growth in an orthotopic xenograft model. (a) Bioluminescence imaging of nude mice injected with 231-Luc, HER2-60 or HER2-90 cells in a mammary fat pad at 4 weeks after cell injection. (b) Volume of orthotopic tumors determined at the indicated times after cell injection as in (a). Data are means \pm SD for 8 to 10 mice per group. NS, not significant for comparison of 231-Luc cells with HER2-60 or HER2-90 cells (Student's *t*-test). (c) H&E staining and immunohistochemical analysis of HER2 expression in formalin-fixed, paraffin-embedded sections of orthotopic tumors formed at 3 to 4 weeks after cell injection. Scale bars, 200 μ m.

expression of CD24 also did not alter the population of HER2⁺CD24⁺ cells. These results indicate that the hierarchical relationship between HER2 and CD24 is not simple and that the transient expression system may not be appropriate to clarify their association.

Given that an ectopic expression of HER2 increases the level of Akt phosphorylation (Fig. 1b), we next examined if the knockdown of CD24 might suppress Akt phosphorylation in HER2-expressing cells. We found that the phosphorylation of Akt was, indeed, downregulated by CD24 depletion in both the HER2-expressing cell lines BT-474, HCC202 and HCC1569 (Fig. 6a), and in HER2-60 and HER2-90 cells (Fig. 6b). Together, these results suggest that CD24 supports the activation of HER2 and thereby promotes Akt signaling.

We also examined the expression levels of other HER family proteins, such as HER1/EGFR and HER3. Knockdown of CD24 decreased the phosphorylation of EGFR and the expression of HER3 in BT-474, HCC202 and HCC1569 cells (Fig. 6a). In contrast, knockdown of CD24 increased the phosphorylation of EGFR in HER-60 and HER-90 cells, in which HER3 were not detected (Fig. 6b).

Knockdown of CD24 sensitizes cells to human epidermal growth factor receptor 2-targeted therapy. Finally, we examined whether knockdown of CD24 might increase the sensitivity of breast cancer cells to HER2-targeted therapy. Treatment of CD24-depleted or control BT-474 cells with lapatinib, a dual tyrosine kinase inhibitor for both the epidermal growth factor receptor and HER2, revealed that knockdown of CD24, indeed, enhanced the antiproliferative and death-promoting effects of lapatinib (Fig. 7a). We also found that HER2-90 cells are resistant to lapatinib (Fig. 7b), but that knockdown of CD24 increased the sensitivity of these cells to the antiproliferative and death-promoting effects of this agent (Fig. 7c). These data

thus suggested that the expression of CD24 supports HER2-dependent cancer cell survival.

Discussion

We found that a stable overexpression of ectopic HER2 increases the expression of CD24 in breast cancer cells, and that a knockdown of CD24 reduces the expression of endogenous HER2. In addition, we found that the phosphorylation (activation) of Akt, a downstream mediator of HER2 signaling, promotes cell survival. Our results indicate that CD24 plays an important role in regulating HER2-initiated survival signaling in breast cancer cells.

CD24 has previously been linked to the expression of HER2 in breast cancer. The expression of CD24 was detected in 84.6% of human breast cancer specimens and was associated with shortened patient survival,⁽¹⁴⁾ and an inhibitor of signal transducer and an activator of transcription 3 (STAT3) was shown to downregulate the expression of both CD24 and HER2 in breast cancer cells.⁽²⁰⁾ These findings support the notion that CD24 contributes to the regulation of HER2 signaling, but the underlying molecular mechanisms of the functional interaction between these two proteins remain to be elucidated.

The knockdown of CD24 decreased the expression of HER2 and the phosphorylation of Akt, whereas the knockdown of HER2 did not affect the expression of CD24 in BT474 cells (Fig. 5b,c), implying that CD24 is upstream of HER2. However, the stable HER2 overexpression increased CD24 expression in 231-Luc cells originally derived from triple-negative breast cancer cells (Fig. 1d). To further clarify the hierarchical relationship between HER2 and CD24, we attempted to determine whether or not the overexpression of

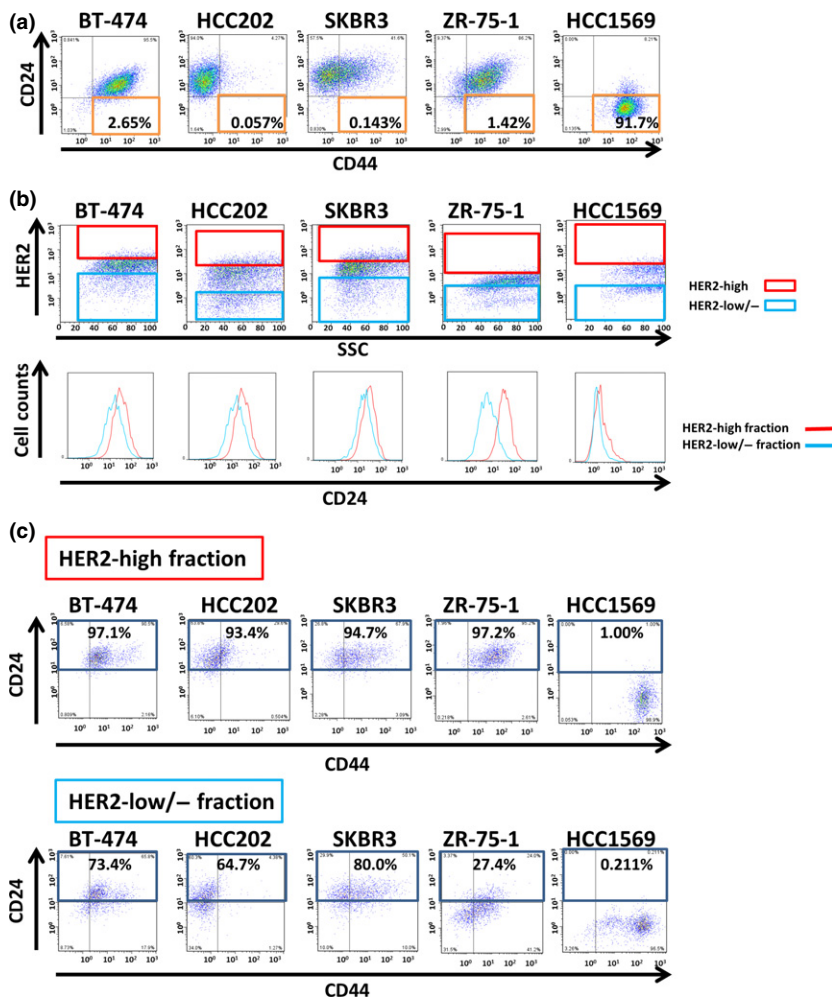


Fig. 4. Positive relation between human epidermal growth factor receptor 2 (HER2) and CD24 expression among HER2-expressing breast cancer cell lines. (a) Flow cytometric analysis of CD44 and CD24 expression in HER2-positive breast cancer cell lines. The percentage of CD44⁺CD24⁻ cells is indicated in the right lower panel. (b) Cells were stained with antibodies to HER2, to CD44 and to CD24 and then gated on the basis of HER2 expression. The top 20% fraction of HER2-high cells (red boxes) and the bottom 20% fraction of HER2-low/- cells (blue boxes) were gated in each cell line. The histograms show the expression level of CD24 in these two fractions for each cell line. (c) Comparison of the expression of CD44 and CD24 between the HER2-high and HER2-low/- fractions of each cell line isolated as in (b). The percentage of CD24-positive cells is shown.

CD24 affects HER2 expression. 231-Luc cells were transfected with the CD24-expressing plasmid and HER2 expression was analyzed by flowcytometry. However, the HER2⁺CD24⁺ fraction was not increased by overexpression of CD24 (Fig. S2, 1.19% in control and 0.853% in CD24 expressing cells). Furthermore, the transient expression of HER2 did not affect the HER2⁺CD24⁺ fraction (Fig. S2, 1.19% in control and 2.23% in HER2 expressing cells). Accordingly, the relationship between HER2 and CD24 seems to be complicated. However, all our data suggest that HER2-positive cells have advantages in survival under the co-expression of CD24 through the phosphorylation of Akt. In other words, HER2⁺CD24⁺ cells might be selected with an advantage in growth and survival.

The knockdown of CD24 reduced HER2 expression in endogenous HER2-expressing cells, BT-474, HCC202 and HCC1569 cells (Fig. 6a). However, in ectopic HER2-expressing cells, HER2-60 and HER2-90 cells, which were engineered to express HER2 under CMV promoter, CD24 depletion did not affect their HER2 expression (Fig. 6b). These results suggest that CD24 is involved in the transcriptional machineries of HER2 expression. Expression of HER2 was previously shown to be induced by radiation in a manner dependent on the transactivation activity of nuclear factor- κ B (NF- κ B),⁽²¹⁾ a transcription factor that plays key roles in cell proliferation, survival and differentiation. In addition, an inhibitor of NF- κ B was recently shown to

downregulate the expression of CD24 in mammary epithelial cells.⁽²²⁾ These observations suggest that NF- κ B signaling might play a role in the association between CD24 and HER2 expression.

Given that the CD44⁺CD24⁻ subpopulation is thought to constitute CSC in breast cancer,⁽¹³⁾ the absence of CD24 expression may be involved in CSC characteristics. However, there has been substantial evidence suggesting that CD24 has conflicting biological roles in tumors and metastasis. We here show that CD24 promotes HER2-Akt signaling in breast cancer. Others have shown that CD24 positively regulates MAPK signaling and thereby promotes colorectal cancer cell proliferation.⁽²³⁾ In addition, a monoclonal antibody to CD24 was found to suppress MAPK signaling in colorectal and pancreatic cancer.⁽²⁴⁾ CD24 is expressed on hematopoietic cells such as B cells, T cells and neutrophils,^(25,26) and is known to be a ligand for P-selectin. It has been shown that overexpression of CD24 stabilizes the kinase-active form of c-src and FAK in breast cancer cells, increasing proliferation and migration *in vitro*,⁽²⁷⁾ and that CD24 supports the rolling of breast cancer cells on the vascular endothelium in a P-selectin-dependent manner,⁽²⁸⁾ leading to the extravasation of cancer cells from vessels. CD24 has also been implicated as playing a role in the downstream of hedgehog pathway that is often active in CSC of glioblastoma.⁽²⁹⁾ Furthermore, in pancreatic cancers, CD24 positive cells (CD44⁺/CD24⁺/ESA⁺) have been shown to exhibit

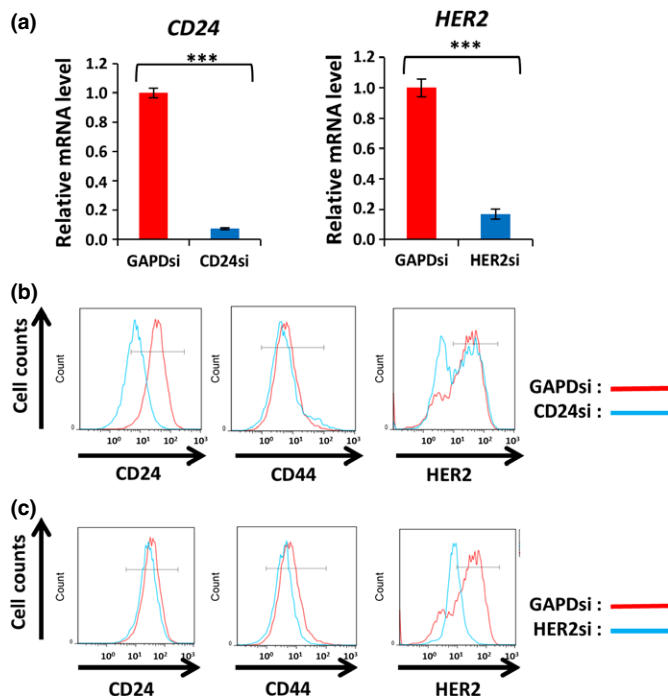


Fig. 5. Knockdown of CD24 downregulates human epidermal growth factor receptor 2 (HER2) expression in BT-474 cells. (a) Quantitative RT-PCR analysis of *CD24* and *HER2* mRNA in cells transfected with CD24, HER2 or control (GAPD) siRNA, as indicated, for 48 h. Data are normalized by the amount of *HPRT1* mRNA, expressed relative to the corresponding normalized value for cells transfected with GAPDsi, and are presented as means \pm SD for triplicate experiments. *** $P < 0.001$ (Student's *t* test). (b) Flow cytometric analysis of CD24, CD44 and HER2 expression in cells transfected with CD24 or control siRNA. (c) Flow cytometric analysis of CD24, CD44 and HER2 expression in cells transfected with HER2 or control siRNA.

CSC traits.⁽³⁰⁾ CD24 thus supports cancer progression and CSC characteristics in a manner dependent on cancer type and the corresponding activated survival pathway.

Recently, HER2 has been considered to play a functional role in CSC based on the remarkable clinical efficacy of anti-HER2 agents, which may be related to their ability to target the CSC population.⁽³¹⁾ However, our findings suggest that the expression of HER2 is not necessarily related to CSC properties. We investigated various CSC characteristics, such as sphere forming ability, ALDH1 activity, tumorigenicity, EMT properties and expression of ES cell-related genes in HER2-negative and positive cells and found that some results suggest that HER2-expressing cells are CSC-like but others do not. Therefore, HER2-positivity alone may not be sufficient to determine breast cancer CSC.

We suggest that CD24 expression promotes the resistance of HER2-positive cancer cells to HER2-targeted therapy. Indeed, CD24 has been previously linked to such resistance. The nonreceptor tyrosine kinase Src is thus hyperactivated in various trastuzumab-resistant HER2-expressing breast cancer cell lines,⁽³²⁾ and CD24 activates Src and promotes Src-mediated phosphorylation of FAK, leading to the binding of CD24 to fibronectin in an integrin-dependent manner.⁽³³⁾ These observations suggest that Src activity in HER2-positive cancer cells is enhanced by CD24 expression. Both FAK and Src are activated downstream of β_1 -integrin in lapatinib-resistant HER2-positive breast cancer cells, and inhibition of β_1 -integrin signaling has been found to overcome lapatinib resistance.⁽³⁴⁾ In addition, our results now suggest that aberrant activation of the HER2-PI3K-Akt pathway by CD24 contributes to lapatinib resistance. In fact, the knockdown of CD24 decreased the phosphorylation of Akt in 231-Luc cells without HER2 expression (Fig. 6b), suggesting that there are direct mechanisms through which CD24 regulates aberrant activation of Akt signaling pathway independent of activation of HER2.

Finally, we found that knockdown of CD24 sensitized breast cancer cells to HER2-targeted therapy. Given that depletion of CD24 increased the size of the HER2-negative cell subpopulation and suppressed Akt phosphorylation, we suggest that CD24 positively regulates HER2-Akt signaling and thereby promotes resistance to HER2-targeted therapy

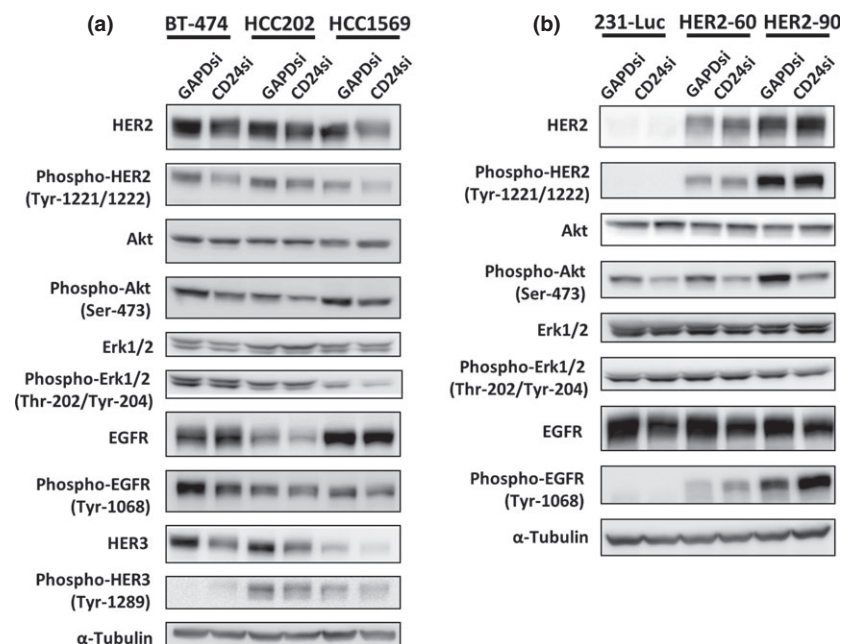


Fig. 6. Knockdown of CD24 suppresses Akt phosphorylation in human epidermal growth factor receptor 2 (HER2)-expressing cells. The abundance of total or phosphorylated forms of HER2, Akt, Erk1/2, EGFR and HER3 was examined by immunoblot analysis in BT-474, HCC202 and HCC1569 cells (a) as well as in 231-Luc, HER2-60 and HER2-90 cells (b) that had been transfected with control (GAPD) or CD24 siRNAs for 48 h.

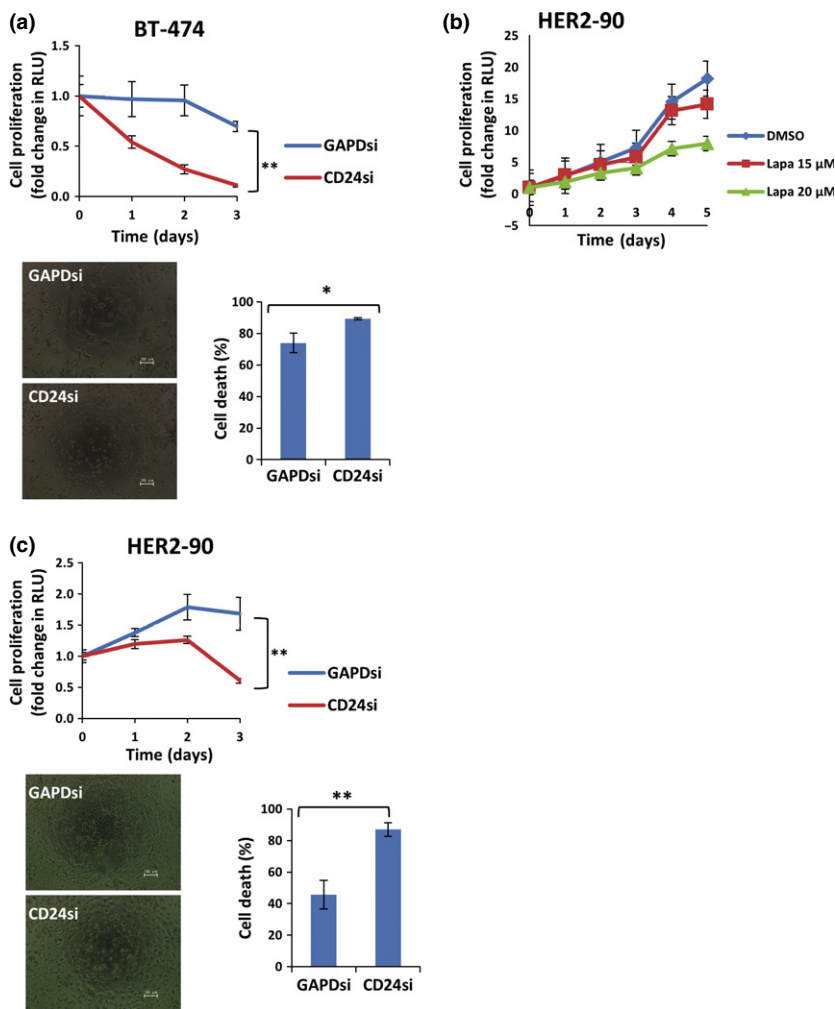


Fig. 7. Knockdown of CD24 sensitizes human epidermal growth factor receptor 2 (HER2)-positive breast cancer cells to lapatinib treatment. (a) BT-474 cells were transfected with control (GAPD) or CD24 siRNA for 24 h and then exposed to 0.5 μ M lapatinib for 48 h for cell death assay or for the indicated times, after which cell viability was determined with an Cell titer Glo assay kit (top panel) and cell death was evaluated by staining with trypan blue (bottom panels). Cell proliferation data are expressed as fold change in relative luminescence units (RLU) relative to time 0, and all quantitative data are means \pm SD from triplicate experiments. * $P < 0.05$, ** $P < 0.01$ (Student's *t*-test). Scale bars, 100 μ m. (b) HER2-90 cells were incubated with the indicated concentrations of lapatinib or with DMSO vehicle for the indicated times, after which cell viability was determined with an assay kit as in (a). (c) HER2-90 cells were transfected with control (GAPD) or CD24 siRNAs for 24 h and then treated with 15 μ M lapatinib for 48 h, after which cell viability and cell death were determined as in (a).

and consequent tumor progression. Therapeutic targeting of CD24 might thus be expected to enhance the efficacy of HER2-targeted agents.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Data S1. Materials and Methods.

Table S1. Primers for quantitative RT-PCR analysis.

Fig. S1. Cell proliferation assays for 231-Luc, HER2-60 and HER2-90 cells.

Fig. S2. Flow cytometric analysis of CD24 and HER2 expression in HER2-transfected or CD24-transfected 231-Luc cells.