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# Switching addictions between HER2 and FGFR2 in HER2-positive breast tumor cells: FGFR2 as a potential target for salvage after lapatinib failure

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ABSTRACT

Agents that target HER2 have improved the prognosis of patients with *HER2*-amplified breast cancers. However, patients who initially respond to such targeted therapy eventually develop resistance to the treatment. We have established a line of lapatinib-resistant breast cancer cells (UACC812/LR) by chronic exposure of *HER2*-amplified and lapatinib-sensitive UACC812 cells to the drug. The mechanism by which UACC812/LR acquired resistance to lapatinib was explored using comprehensive gene hybridization. The *FGFR2* gene in UACC812/LR was highly amplified, accompanied by overexpression of FGFR2 and reduced expression of HER2, and a cell proliferation assay showed that the $IC_{50}$ of PD173074, a small-molecule inhibitor of FGFR tyrosine kinase, was 10,000 times lower in UACC812/LR than in the parent cells. PD173074 decreased the phosphorylation of FGFR2 and substantially induced apoptosis in UACC812/LR, but not in the parent cells. FGFR2 appeared to be a pivotal molecule for the survival of UACC812/LR as they became independent of the HER2 pathway, suggesting that a switch of addiction from the HER2 to the FGFR2 pathway enabled cancer cells to become resistant to HER2-targeted therapy. The present study is the first to implicate FGFR in the development of resistance to lapatinib in cancer, and suggests that FGFR-targeted therapy might become a promising salvage strategy after lapatinib failure in patients with HER2-positive breast cancer.



## 1. Introduction

Breast cancer is the second most frequent malignancy worldwide, and the prognosis of patients with metastatic disease still remains very poor, despite intensive research and drug development [1]. Amplification of the human epidermal growth factor receptor 2 (HER2) gene has been detected in 20–30% of human breast cancers, driving tumor development and being associated with a poor outcome [2]. HER2 forms dimers to become active, and its dimerization partners are the epidermal growth factor receptor (EGFR), HER2 itself, and HER3 in most cases. Since EGFR is a molecule frequently expressed in HER2-positive breast cancer, interaction between EGFR and HER2 could be important for the maintenance of oncogenesis [3]. Thus, targeting HER2 and EGFR together appears to be a promising therapeutic strategy for patients with *HER2*-amplified breast cancer, and multi-targeted small-molecule inhibitors such as lapatinib, BIBW2992 and AZD8931, directed against EGFR family members, have been developed for this purpose. Lapatinib binds to the ATP binding sites of EGFR and HER2, thus inhibiting their tyrosine kinase activity [4].

Acquired resistance to HER2-targeted drugs is one of the major obstacles to further improvement of clinical outcomes in this field, and research efforts have been focused on clarifying the mechanisms by which cancer cells acquire resistance to lapatinib. Several mechanisms of resistance to trastuzumab, a humanized monoclonal antibody against HER2, have been proposed, such as the presence of a truncated form of HER2 without an extracellular domain, loss of PTEN, and *PIK3CA* mutations in pre-clinical models, and such mechanisms may also have some implications for the lapatinib resistance phenotype [5–7]. In addition, overexpression of AXL, a receptor type kinase, has been reported to be a critical player for bypassing lapatinib-elicited HER2-PI3K-Akt signaling and conferring resistance to the drug in a breast cancer cell line [8].

*Abbreviations:* FGFR2, fibroblast growth factor receptor 2; HER2, human epidermal growth factor receptor 2; TKI, tyrosine kinase inhibitor; EGFR, epidermal growth factor receptor; $IC_{50}$, median inhibitory concentration; siRNA, small interfering RNA; Erk, extracellular signal-regulated kinase; RNAi, RNA interference; CGH, comprehensive gene hybridization.

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Fibroblast growth factor receptor 2 (FGFR2) is a member of the FGFR tyrosine kinase family, and consists of 4 receptors and 23 ligands [9]. Ligand binding leads to FGFR2 dimerization, autophosphorylation, and activation of signaling components including Akt and Erk kinases. Amplification and overexpression of the *FGFR2* gene is observed in gastric cancer and breast cancer [9], and single-nucleotide polymorphisms (SNPs) of the *FGFR2* gene are associated with a higher risk of sporadic breast cancer [10]. These features suggest that *FGFR2* may have an oncogene-like character, and be capable of transforming normal cells. This gene could act as a driving force for transformation of cancer cells into a further malignant phenotype, and constitute a potential target of treatment in cancer patients whose tumors express the protein.

Here we report that subpopulations of cells with *FGFR* gene amplification play a pivotal role in development of resistance to lapatinib in HER2-positive breast cancer.

## 2. Materials and methods

### 2.1. Cell culture and reagents

A human breast cancer cell line, UACC812 was obtained from the American Type Culture Collection (Manassas, VA), and cultured under a humidified atmosphere of 5% $CO_2$ at 37 °C in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum. Gefitinib was obtained from Kemprotec Ltd. (UK). Lapatinib was obtained from Chemietek (Indianapolis, IN). PD173074 was purchased from Sigma (St. Louis, MO).

### 2.2. Generation of a lapatinib-resistant line and floating line from UACC812

The UACC812 cells were grown initially in medium containing 0.01 μM lapatinib, and the concentration was gradually increased up to 1 μM over the following 8 months to establish lapatinib-resistant cell lines (UACC812/LR).

### 2.3. Array-based comparative genomic hybridization

The Genome-wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA) was used to perform array-CGH on genomic DNA from each of the cell lines, in accordance with the manufacturer's instructions. A total of 250 ng of genomic DNA was digested with the restriction enzymes Nsp I and Sty I in independent parallel reactions (SNP6.0), ligated to the adaptor, and amplified using PCR with a universal primer and TITANIUM Taq DNA Polymerase (Clontech). The PCR products were quantified, fragmented, end-labeled, and hybridized onto a Genome-wide Human SNP Array 6.0. After washing and staining in Fluidics Station 450 (Affymetrix), the arrays were scanned to generate CEL files using the GeneArray Scanner 3000 and GeneChip Operating Software ver.1.4. In the array-CGH analysis, sample-specific changes in copy number were analyzed using Partek Genomic Suite 6.4 software (Partek Inc., St. Louis, MO).

### 2.4. Growth assay in vitro

Cells were cultured in 96-well flat-bottomed plates for 24 h before exposure to various concentrations of drugs for 72 h. TetraColor One (5 mM tetrazolium monosodium salt and 0.2 mM 1-methoxy-5-methyl phenazinium methylsulfate; Seikagaku, Tokyo, Japan) was then added to each well, and the cells were incubated for 3 h at 37 °C before measurement of absorbance at 490 nm with a Multiskan Spectrum instrument (Thermo Labsystems, Boston, MA). Absorbance values were expressed as a percentage relative to untreated cells, and the concentration of tested drugs resulting in 50% growth inhibition ($IC_{50}$) was calculated using the Prism program (GraphPad, San Diego, CA).

### 2.5. Cell death assay

After incubation, cells were harvested by trypsinization and resuspended in a solution of 1 μg/mL propidium iodide in PBS, then immediately acquired on the FL3 channel of a flow cytometer. The population of propidium iodide-positive cells was considered dead, whereas the propidium iodide-negative population was considered viable.

### 2.6. Immunoblot analysis

Cells were washed twice with ice-cold PBS and then lysed with 1× Cell Lysis Buffer (Cell Signaling Technology) containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and leupeptin (1 μg/ml). The protein concentration of cell lysates was determined with a BCA protein assay kit (Thermo Fisher Scientific), and equal amounts of protein were subjected to SDS–PAGE on a 4–12% gradient gel. The separated proteins were transferred to a PVDF membrane, which was then incubated with Blocking One solution (Nakarai Tesque, Kyoto, Japan) for 20 min at room temperature before incubation overnight at 4 °C with primary antibodies, including those against phosphorylated FGFR, phosphorylated EGFR(Y1086), phosphorylated HER2(Y1221/1222), EGFR, FGFR1, FGFR3, FGFR4, phosphorylated AKT, AKT, ERK, PARP, caspase-3 (Cell Signaling Technology, Danvers, MA), HER2 (Millipore), FGFR2 (Bek) and phosphorylated ERK (Santa Cruz Biotechnology) or β-actin (1:5000 dilution, Sigma). The membrane was then washed with PBS containing 0.05% Tween 20 before incubation for 1 h at room temperature with horseradish peroxidase-conjugated antibody against rabbit immunoglobulin G (Sigma). Immune complexes were finally detected using ECL Western blotting detection reagents (GE Healthcare, Little Chalfont, UK). The RTK array was purchased from R & D Systems (Minneapolis, MN) and used in accordance with the manufacturer's instructions.

### 2.7. Assay of phospho-FGFR2 activity

The activity of p-FGFR2 in cell lysates was measured using ELISA in accordance with the manufacturer's procedures (Human phosphor-FGFR2 Duoset; R & D Systems). The lysates were prepared as described above. All samples were run in triplicate assays. Color intensity was measured at 450 nm using a spectrophotometric plate reader. Growth factor concentrations were determined by comparison with standard curves.

### 2.8. FGFR2 gene silencing using small interfering RNA

Cells were plated at 50–60% confluence in six-well plates or 25 $cm^2$ flasks and then incubated for 24 h before transient transfection for 48 h with small interfering RNAs (siRNAs) mixed with Lipofectamine reagent (Invitrogen, Carlsbad, CA). A siRNA specific for FGFR2 mRNA and a nonspecific siRNA (control) were obtained from Nippon EGT (Toyama, Japan). The cells were then subjected to flow cytometry and immunoblot analysis.

### 2.9. Immunohistochemistry (IHC)

Paraffin-embedded tissue samples were cut at a thickness of 4 μM and examined on coated glass slides, after labeling with antibodies directed against the following using the ChemMate ENVISION method (DakoCytomation, Glostrup, Denmark). Endogenous

peroxidase activity was inhibited by incubating the slides in 3% $H_2O_2$ for 20 min. FGFR2 (C-17, Santa Cruz Biotechnology) antigen retrieval was done by microwaving for 10 min in Target Citrate Solution (pH 6.0). Each slide was incubated overnight with the antibody at 4 °C. For staining detection, the ChemMate ENVISION method was used with DAB as the chromogen. The expression of FGFR2 protein in the cell membrane and cytoplasm was investigated in detail. FGFR2 expression was classified into three categories: score 0, no staining at all; or membrane expression in <10% of cancer cells; score 1+, faint/barely perceptible partial membrane expression in ⩾10% of cancer cells; score 2+, weak to moderate expression on the entire membrane in ⩾10% of the cancer cells; score 3+, strong expression on the entire membrane in ⩾10% of cancer cells. All IHC studies were evaluated by two IHC-experienced reviewers (K.A. and J.T.) who were blind to the conditions of the patients. Consistent results were obtained in 14 out of 16 samples, and two IHC samples without consistency were subjected to scoring by a third reviewer who was also blinded to the clinical information and scores assigned previously by the other two reviewers. Then, the majority scores were employed as the final results.

### 2.10. Study population and survival analysis

All patients received lapatinib between 2009 and 2010 at Kinki University School of Medicine. Sixteen tumors from a series of 13 patients diagnosed as having HER2-positive metastatic breast cancer were collected from the files of the Pathology Department, Kinki University School of Medicine, covering the period between 2009 and 2010. The HER2 status was considered positive if the local institution reported grade 3+ staining intensity (on a scale of 0–3) by means of IHC analysis or grade 2+ staining intensity by means of IHC analysis with gene amplification on fluorescence in situ hybridization. Details of the patients' clinical characteristics, including age, hormone status, prior therapy, and tumor response were obtained from chart review by an independent reviewer who was unaware of the results of IHC analysis. Tumor responses were evaluated after chemotherapy according to the Response Evaluation Criteria for Solid Tumors (RECIST). Four sites of metastasis were included. Any material that had been poorly fixed and/or had low cellularity was rejected. Paraffin-embedded tissues were obtained, and histologic examination of slides stained with hematoxylin-eosin and saffron was carried out by a specialist. All patients provided written informed consent for collection of their tissue material and clinical data for research purposes, and the tissue procurement protocol was approved by the institutional review board.

Progression-free survival was defined as the time between the onset of chemotherapy and the date when disease progression began. Patients without progression were regarded as censored at the date of the last follow-up. Curves for progression-free survival were estimated by the Kaplan–Meier method, and differences in survival functions were compared by the log-rank test.

All tests were two-sided, and differences at $P < 0.05$ were considered statistically significant. All the statistical analyses were conducted using JMP version 8 software (SAS Institute Inc., Cary, NC).

### 2.11. Fluorescence in situ hybridization

The gene copy number per cell for *HER2* was determined by fluorescence in situ hybridization (FISH) with the use of *HER2/neu* (17q11.2–q12) Spectrum Orange and CEP17 (chromosome 17 centromere) Spectrum Green probes (Vysis; Abbott, Des Plaines, IL). Gene amplification was defined as a mean *HER2*/chromosome 17 copy number ratio of >2.

### 2.12. Statistics

Experimental values were expressed ±SE. Statistical comparison of mean values was done using Student's *t* test.

## 3. Results

### 3.1. Establishment of lapatinib-resistant breast cancer cells

The UACC812 cells were grown initially in medium containing 0.01 μM lapatinib, and the concentration was gradually increased to 1 μM over the following 8 months to establish lapatinib-resistant cell lines (UACC812/LR). Cell growth assays were performed for the UACC812 cells and the UACC812/LR cells with various doses of lapatinib and gefitinib, as indicated in Fig. 1A, and the $IC_{50}$ values were determined (Fig. 1A inset). UACC812/LR cells were resistant to lapatinib and gefinitib in comparison with the parent cells, the $IC_{50}$ values for lapatinib (4.2433 ± 0.5066 μM) and gefitinib (11.1300 ± 0.5474 μM) being 42 times and 6 times higher than those in UACC812 (0.1006 ± 0.0053 μM and 1.9223 ± 0.3744 μM), respectively, but no differences were seen between the two cell lines in terms of the $IC_{50}$ values for cisplatin and 5-FU (Fig. 1A inset), suggesting that chronic exposure of the UACC812 cells to lapatinib had induced resistance specific to EGFR or HER2 inhibitors.

### 3.2. FGFR2 gene amplification in UACC812/LR

To overview the chromosomal divergences between the parent cell line and its derivative, comprehensive gene hybridization (CGH) analyses were performed as described in Section 2. This revealed that the UACC812/LR cells harbored an amplification of the fibroblast growth factor receptor 2 (FGFR2) gene, the gene copy number in UACC812/LR being approximately 20 times that in UACC812 (Fig. 1B). Lysates of the parent and the derivative cells were subjected to Western blotting-based high-throughput analysis for expression of various receptor type kinases (RTK), and a dramatic increase in the expression of FGFR2 was observed in UACC812/LR relative to UACC812 (Fig. 1C). In contrast, the expression of HER2 was reduced in UACC812/LR in comparison to the parent cells (Fig. 1C upper panel). HER2-FISH analysis revealed that the *HER2* gene amplification was present in UACC812 cells, but not in UACC812/LR cells (Fig. 1C lower panel). To examine the role of FGFR2 in the growth of the parent cells and their derivative, an FGFR-TKI, PD173074, was utilized, and cell growth assays were performed for UACC812 and UACC812/LR treated with various concentrations of PD173074. UACC812/LR was more sensitive than UACC812 to PD173074, the $IC_{50}$ (0.00121 ± 0.0034 μM) being 10,000 times lower than that for the parent cells (10.3373 ± 1.6629 μM), indicating that UACC812/LR cells had acquired dependency on the FGFR2 pathway, whereas FGFR2 played no role in the cell growth of UACC812 (Fig. 1D and inset).

### 3.3. UACC812/LR shows high phosphorylation of FGFG2 and undergoes apoptosis upon exposure to a FGFR tyrosine kinase inhibitor

To further evaluate the findings of the RTK arrays and cell growth assays, Western blotting was performed for biochemical profiling of these cell lines. Overexpression of phosphorylated FGFR2 (p-FGFR2) and native FGFR2, and downregulation of p-HER2 and p-EGFR in UACC812/LR cells relative to the parent cells were observed (Fig. 2A). The two cell lines were treated with lapatinib (1 μM) or PD173074 (0.1 and 1 μM) for 24 h, and the cell lysates were then subjected to Western blot analysis. The basal levels of p-EGFR, p-HER2 and native HER expression were

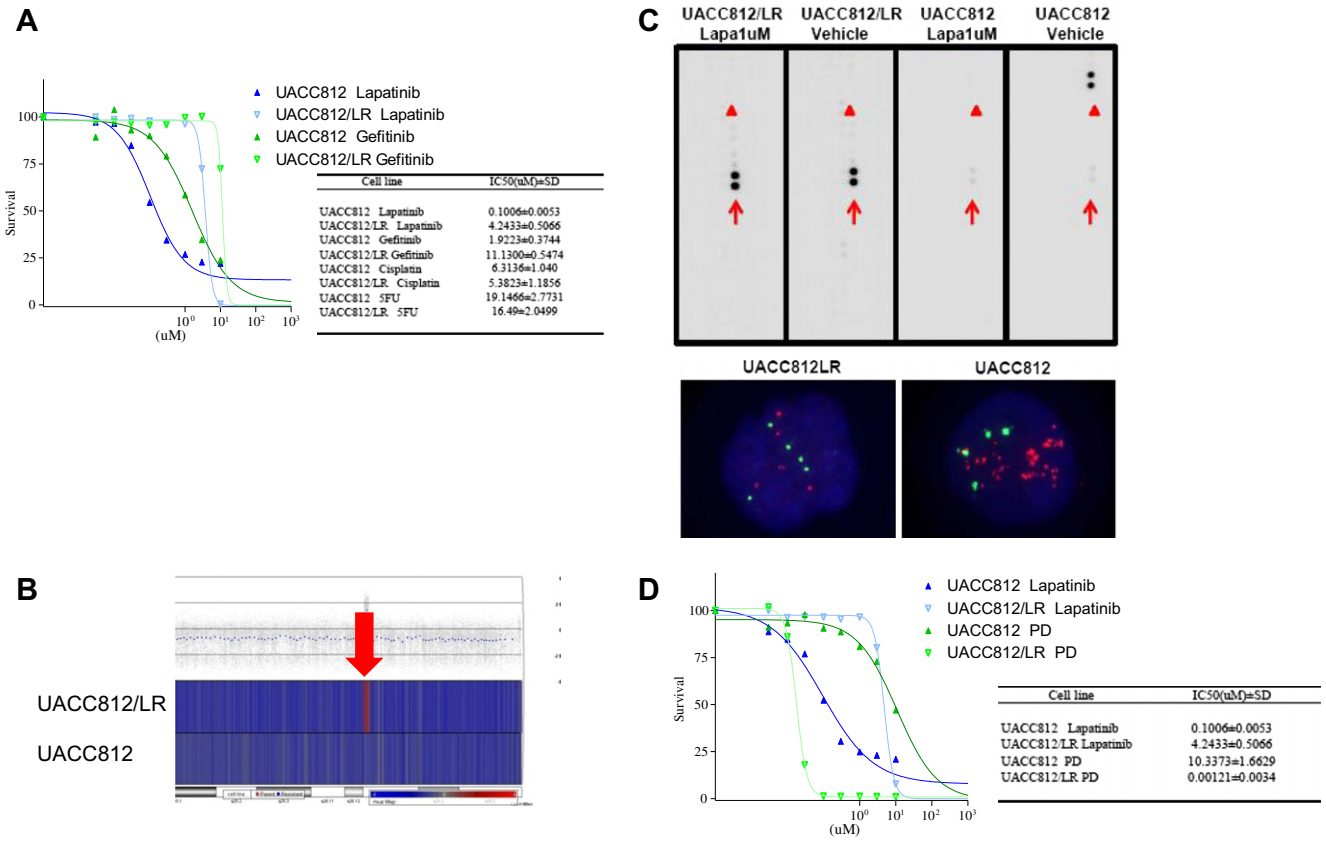


**Fig. 1.** Lapatinib-resistant cancer cells harbor *FGFR2* gene amplification, and are preferentially sensitive to an FGFR-TKI. (A) Cell growth assays were performed using UACC812 cells and their derivative, UACC812/LR cells, treated with lapatinib, gefitinib, cisplatin or 5-FU for 72 h, and $IC_{50}$ values are shown in the inset. Results represent the mean ± SE of three experiments performed in triplicate. (B) *FGFR2* gene amplification detected in UACC812/LR cells. Comprehensive gene hybridization analysis revealed that the gene on chromosome 10q26 was highly amplified in UACC812/LR relative to its parent cell line. (C) Lysates from UACC812 cells and UACC812/LR cells treated with vehicle or 1 μM lapatinib for 6 h were subjected to Western blotting-based high-throughput analysis for RTKs. Arrows and arrowheads indicate signals for FGFR2 and HER2, respectively. Left column: UACC812/LR cells treated with 1 μM lapatinib; Second column from left: UACC812/LR treated with vehicle; Second column from right: UACC812 cells treated with 1 μM lapatinib; Right column: UACC812 cells treated with vehicle. Images of HER2-FISH analyses in UACC812 and UACC812/LR are shown. (D) Cell growth assays were performed using UACC812 cells and UACC812/LR cells with lapatinib or PD173074 at various doses for 72 h. $IC_{50}$ values are shown in the inset. Results represent the mean ± SE of three experiments performed in triplicate.

decreased, and those of p-FGFR and native FGFR2 were dramatically increased in UACC812/LR cells (Fig. 2A). Lapatinib inhibited the expression of p-HER2 and p-EGFR accompanied by downregulation of p-Akt and p-Erk in UACC812 cells, but no inhibition of the phosphorylation of these signal components was observed in UACC812/LR (Fig. 2A). On the other hand, PD173074 did not affect the level of phosphorylated Akt or Erk in the parent cells, but inhibited that of p-FGFR along with p-Akt and p-Erk in UACC812/LR cells (Fig. 2A). Cleaved poly (ADP-ribose) polymerase (PARP) and caspase 3 as markers of apoptosis were increased in UACC812 and UACC812/LR after treatment with lapanib or PD173074, respectively, suggesting that the parent cells and their derivative were dependent on the different pathways for survival (Fig. 2A). Since a pan-antibody against p-FGFR was utilized in the Westen blotting to detect the pharmacological activity of PD173074, we further examined p-FGFR2 in an ELISA assay using a specific antibody against p-FGFR2 in UACC812 and UACC812/LR cells treated with lapatinib or PD173074 (Fig. 2B). We found that the basal level of p-FGFR2 was increased in UACC812/LR relative to the parent cells, and that phosphorylation was inhibited by PD173074 but not by lapatinib. Induction of cell death with lapatinib and/or pharmacological or genetic abrogation of FGFR2 was then measured in UACC812 and UACC812/LR cells (Fig. 2D). Cell death was induced in UACC812 cells treated with lapatinib but not in those treated with PD173074 (Fig. 2D left panel) or si-RNA for FGFR2 (Fig. 2D right panel). There were no increases in the percentage of cell death induced by lapatinib upon addition of PD173074 or si-RNA for FGFR2 in UACC812 cells or UACC812/LR (Fig. 2D left panel and right panel). Nonetheless, PD173074 and si-RNA for FGFR2 dramatically induced cell death in UACC812/LR cells (Fig. 2D left panel and right panel). Validation of the biochemical effects of si-RNA treatment on FGFR2 is shown in Fig. 2C. Overexpression of FGFR2 was observed in UACC812/LR cells relative to UACC812 cells, and treatment with si-RNA for FGFR2 reduced the expression of FGFR2, accompanied by inhibition of p-Akt and p-Erk in UACC812/LR cells (Fig. 2C). Cleaved PARP and caspase 3 were induced in UACC812 cells and UACC812/LR cells treated with lapatinib and/or si-RNA for FGFR2, respectively (Fig. 2C). Together, these findings suggested that UACC812/LR cells had become addicted to the FGFR2 pathway for survival in the absence of the activated HER2 pathway during the development of resistance to lapatinib.

### *3.4. High expression of FGFR2 in tumor specimens is associated with poor response to lapatinib*

To further evaluate the role of FGFR2 in a clinical setting, we examined tissue specimens obtained from 13 consecutive patients with metastatic HER2-positive breast cancer treated with lapatinib between 2009 and 2010 at our institution. The median age of the patients was 60 years (35–69 years) and the median follow-up time after administration of lapatinib was 275 days (42–358 days). All the patients had been treated with lapatinib, and the

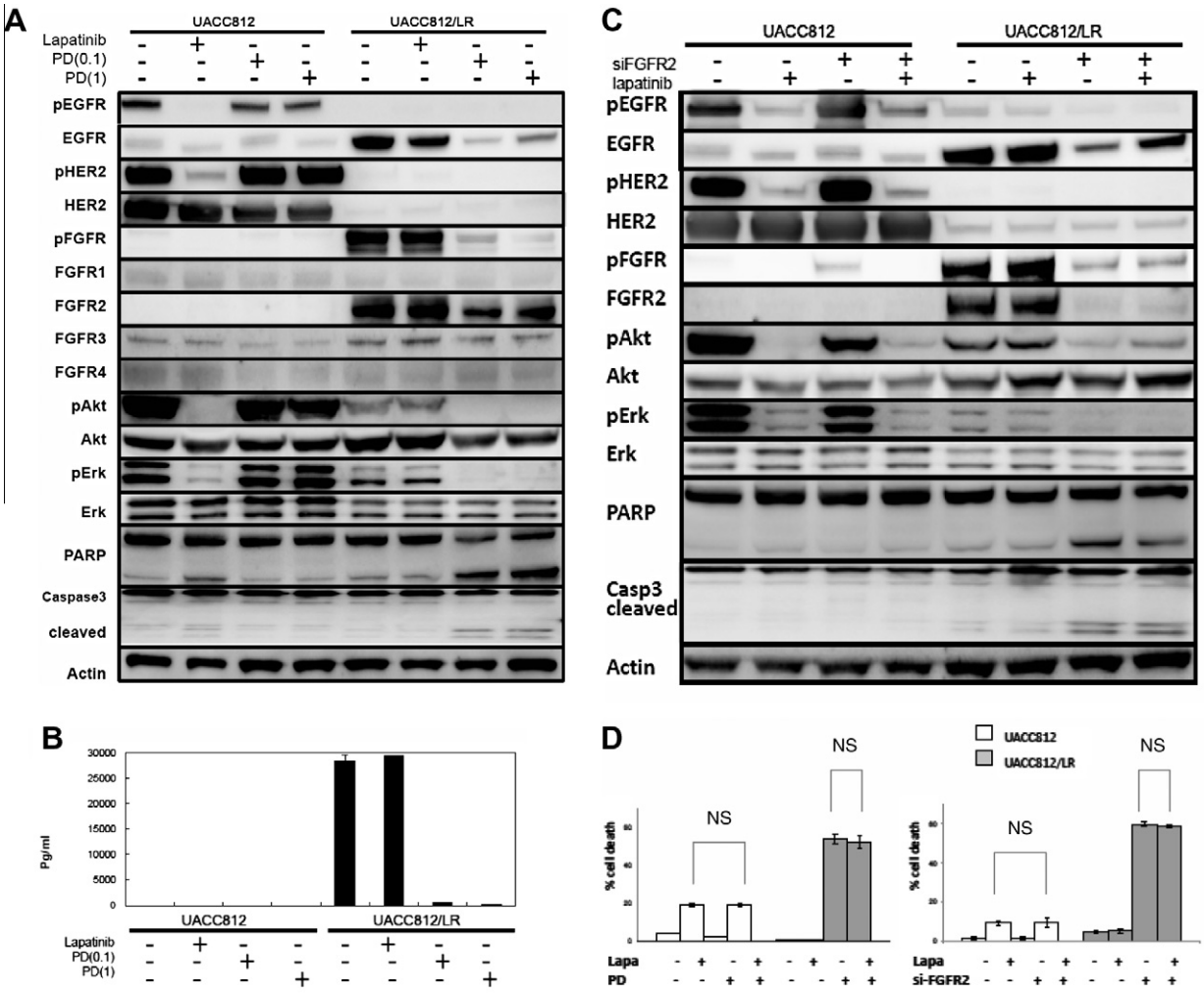


**Fig. 2.** FGFR2 is active in the lapatinib-resistant cell line, UACC812/LR, but not in the parental cells. (A) UACC812 cells and UACC812/LR cells were treated with 1 μM lapatinib, or 0.1 or 1 μM PD173074 for 24 h, as described in Section 2. Lysates were subjected to Western blotting with the indicated antibodies. (B) Phospho-FGFR2 levels were measured in UACC812 cells and UACC812/LR cells treated with vehicle, 1 μM lapatinib, or 0.1 or 1 μM PD173074 using an ELISA-based assay. Results represent the mean ± SE of three experiments performed in triplicate. (C) UACC812 cells and UACC812/LR cells were treated with 1 μM lapatinib and/or si-RNA for FGFR2 for 24 h after completion of transfection. Lysates were subjected to Western blotting with the indicated antibodies. (D) UACC812 cells and UACC812/LR cells were treated with 1 μM lapatinib and/or 0.1 μM PD173074 for 48 h (left panel) or with 1 μM lapatinib and/or si-RNA for FGFR2 for 48 h after completion of transfection (right panel). The cells were harvested and subjected to flow cytometry analysis to assess the extent of cell death. Results represent the mean ± SE of three experiments performed in triplicate. NS, not statistically significant.

**Table 1**
Clinicopathological features of HER2 positive MBC.

| Patients # | Age | Primary hormone receptor status | Prior therapy | Response* | FGFR2 expression |
|---|---|---|---|---|---|
| 1 | 52 | ER+, PgR+ | H, T, A | SD | 0 |
| 2 | 53 | ER+, PgR− | H, A | SD | 1+ |
| 3 | 47 | ER+, PgR− | H, T, A | PR | 1+ |
| 4 | 60 | ER+, PgR− | H, T | SD | 1+ |
| 5 | 69 | ER−, PgR− | H, T, A | PD | 2+ |
| 6 | 67 | ER−, PgR− | H | NE | 1+ |
| 7 | 52 | ER+, PgR− | H, T | PD | 0 |
| 8 | 47 | ER−, PgR− | H, T, A | SD | 1+ |
| 9 | 35 | ER+, PgR+ | H , T, A | PR | 1+ |
| 10 | 65 | ER−, PgR− | H, T, A | NE | 0 |
| 11 | 61 | ER−, PgR− | H , T, A | PD | 2+ |
| 12 | 69 | ER+, PgR+ | H, T, A | PD | 2+ |
| 13 | 59 | ER−, PgR− | H, T | PR | 0 |

MBC, metastatic breast cancer; ER, estrogen receptor; PgR, progesterone receptor; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable; H, Herceptin; T, Taxanes; A, Anthracycline.
* Response to lapatinib-containing regimens.

clinicopathological features including IHC scores of FGFR2 in tumor specimens are summarized in Table 1. Time to progression (TTP) while receiving the treatment was plotted using Kaplan–Meier curves stratified by FGFR2 expression (score 0, 1 vs. 2, 3, Fig. 3), and the patients with FGFR2-overexpressing tumors had significantly poor survival ($P = 0.0082$), suggesting that FGFR2 may play at least a partial role in the development of resistance to lapatinib, probably through selection of FGFR2-overexpressing tumor cells.

## 4. Discussion

Several models have been proposed to account for the clinical resistance to HER2-targeted therapies including *PIK3CA* gene mutation and *AXL gene amplification* [5–8]. Gene sequence analyses revealed that UACC812 and UACC812/LR did not harbor *PIK3CA* gene mutation (data not shown), and Western blotting showed that UACC812/LR cells do not express AXL (data not shown), indicating that these two molecules are not causative factors for acquired resistance to lapatinib in UACC812/LR. Instead, the

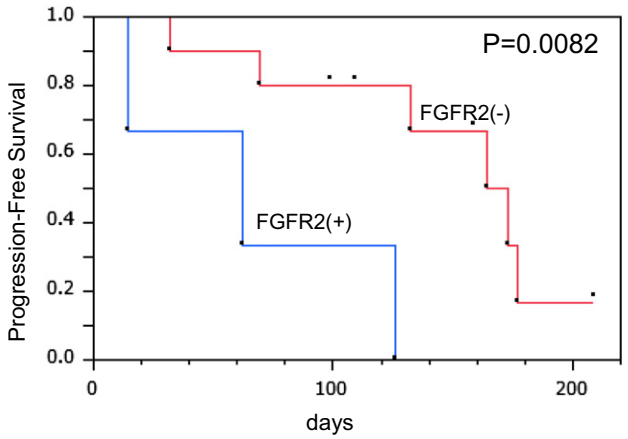


**Fig. 3.** Kaplan–Meier curves illustrating associations between protein expression and progression-free survival since the start of lapatinib. Survival curves are plotted as graphs according to FGFR2 expression level. The *P*-value was calculated using Log-rank test.

present model revealed amplification of *FGFR2* in lapatinib-resistant cells, and activation of FGFR2 substantially contributed to survival of the cells.

There is compelling evidence for deregulated FGF signaling in the pathogenesis of many cancers that originate from different tissue types. Aberrant FGF signaling can promote tumor development by directly driving cancer cell proliferation and survival. The underlying mechanism driving FGF signaling is largely tumor-specific, and can be attributed to genomic *FGFR* alterations that drive ligand-independent receptor signaling. Mutations of *FGFR2*, which are frequently extracellular, have been described in 12% of endometrial carcinomas [11]. *FGFR2*-mutant endometrial cancer cell lines are highly sensitive to FGFR tyrosine kinase inhibitors, suggesting oncogenic addiction of the cancer cells to the activated form of mutant FGFR [12]. In this study, gene sequencing analysis of *FGFR2* in UACC812 cells and UACC812/LR cells revealed no mutations, but gene amplification was noted in UACC812/LR in comparison with UACC812. Amplifications of *FGFR2* have been reported in approximately 10% of gastric cancers, and have been associated with poor prognosis [13]. Gastric cancer cell lines with *FGFR2* amplifications show ligand-independent signaling and are highly sensitive to FGFR inhibitors [13]. Inhibition of FGFR2 substantially induced cell death in UACC812 cells/LR harboring gene amplification of *FGFR2*, but not in UACC812 cells, and this was consistent with the above reports.

UACC812/LR cells showed loss of *HER2* amplification after chronic exposure to lapatinib, being reminiscent of clinical observations. Loss of *HER2* amplification following trastuzumab-based neoadjuvant systemic therapy has been reported in patients with residual breast cancers [14]. One third of patients with significant residual disease showed loss of *HER2* amplification, and this change was associated with poor relapse-free survival. We speculate that these residual tumors after trastuzumab-based therapy may have harbored alternative driving genes to support further tumor development under selection pressure with trastuzumab, and our model using UACC812/LR cells recapitulated the clinical loss of HER2 resulting from HER2-targeted therapies, although lapatinib was used instead of trastuzumab in this study.

Together, these findings suggest that FGFR2 may be a key molecule in the development of resistance to lapatinib in HER2-positive breast cancer through selection of cells with a growth advantage and improved survival, and that FGFR-targeted therapy may be a promising strategy for breast cancer patients in whom treatment with lapatinib has failed. Further clinical studies using a larger set of tumor specimens should be performed to confirm our findings in a small set of clinical samples, and development of FGFR-targeted therapy is warranted to clarify the role of FGFR in resistance to HER2-targeted medicines.

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