HER2 As Therapeutic Target for Overcoming ATP-Binding Cassette Transporter–Mediated Chemoresistance in Small Cell Lung Cancer

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Abstract
Small cell lung cancer (SCLC) easily acquires multidrug resistance after successful initial therapy. Overexpression of ATP-binding cassette (ABC) transporters is important for the multidrug resistance. Among them, ABCB1 and ABCG2 are known to be upregulated in chemoresistant SCLC cells. We found that human epidermal growth factor receptor 2 (HER2) expressions are also upregulated in chemoresistant SBC-3/ETP, SBC-3/SN-38, and SBC-3/CDDP cells, compared with chemosensitive SBC-3 cells. Lapatinib, a tyrosine kinase inhibitor of HER2, could not suppress proliferation of these HER2-positive SCLC cells alone but successfully restored chemosensitivity to etoposide and SN-38 with a clinically applicable concentration. The reversal effect of lapatinib was thought to be caused by inhibition of drug efflux pump functions of ABC transporters, although lapatinib itself has been reported to be a substrate for them. Moreover, knocking down of HER2 by an short interfering RNA weakened the effect of lapatinib on ABCB1, indicating the involvement of HER2 in the inhibitory mechanisms. Notably, we showed that caveolin-1 and Src play key roles in modulating ABCB1 function via HER2 inactivation. In SBC-3/ETP cells, dephosphorylation of HER2 by lapatinib activates Src and successively leads to increased caveolin-1 phosphorylation. Through this process, caveolin-1 dissociates from HER2 and strengthens association with ABCB1, and finally impairs the pump functions. Furthermore, we showed that treatment by lapatinib in combination with etoposide or irinotecan significantly suppresses the growth of subcutaneous SBC-3/ETP and SBC-3/SN-38 tumors in mice, respectively. Collectively, these results indicate that combination therapy with lapatinib and cytotoxic agents could conquer ABC transporter–mediated chemoresistance especially in HER2-positive SCLC.

Introduction
Lung cancer is the leading cause of cancer-related deaths worldwide. Approximately 15% of all histologic types consist of small cell lung cancer (SCLC), the type bearing poorest outcome. The extreme aggressiveness of SCLC is due to its early and widespread metastases and development of multidrug resistance (MDR) to chemotherapy (1, 2). The current front-line standard chemotherapy regimen for SCLC, etoposide or irinotecan plus cisplatin, is active in most SCLC cases, but the disease recurs shortly after the first successful treatment with MDR phenotype (2, 3). In recent years, molecular target therapy has brought about a breakthrough and progress in the treatment of non-SCLC. For instance, gefitinib and erlotinib, selective tyrosine kinase inhibitors (TKI) against human epidermal growth factor receptor 1 (HER1), also known as EGF receptor (EGFR), have presented survival benefit especially in EGFR-activating mutation–positive non-SCLC (4, 5). They specifically and strongly block EGFR-mediated proliferation and survival signals in EGFR mutation–positive cells (6). However, no favorable therapeutic strategy has been established in recurrent SCLC to date. Development of novel strategy to overcome MDR that confers significant survival benefit is urgently desired in SCLC.

It is well known that ATP-binding cassette (ABC) transporters play a crucial role in MDR of SCLC. Among them, ABC subfamily B member 1 (ABCB1), also known as P-glycoprotein (Pgp), and ABC subfamily G member 2 (ABCG2), also known as breast cancer resistance protein (BCRP), have been reported in SCLC to cause resistance against 2 key chemotherapeutic drugs, etoposide and...
irinotecan, respectively. EGFR-TKIs, gefitinib and erlotinib, have been shown to inhibit ABCB1 and ABCG2 functions and contribute to reverse MDR in breast cancer cells and SCLC cells, regardless of EGFR expression. The direct functional inhibition of ABC transporters by competitive binding of EGFR-TKIs to their ATP-binding sites is the most conceivable inhibitory mechanism (7–9).

HER2, also known as ErbB2, belongs to the HER family of receptor tyrosine kinases (RTK). It can transduce cellular proliferative and survival signals as a homodimer without ligand stimulation or a heterodimer with another HER family members formed by ligand stimulation (10). In breast cancer, HER2 is overexpressed in about 30% of cases and its overexpression correlates with poor outcome (11, 12). Lapatinib, a dual TKI of both EGFR and HER2, is clinically applicable in combination with capecitabine in trastuzumab-refractory patients with HER2-positive metastatic breast cancer (13). Moreover, like EGFR-TKIs, lapatinib has also been reported to inhibit the functions of ABC transporters in breast cancer cells (14). In SCLC, HER2 expression is also reported to be an independent negative prognostic factor of extensive disease (15–17). However, no studies have yet been conducted to assess whether HER2 can be a therapeutic target in SCLC. From these views, lapatinib is expected to not only induce apoptosis of HER2-positive chemoresistant SCLC cells but also restore chemosensitivity of ABC transporters–positive MDR SCLC.

In the present study, we investigated the therapeutic potential of lapatinib toward MDR SCLC. We have shown that lapatinib can restore the chemosensitivity of HER2- and ABC transporter–positive cheromoreistant SCLC cells by dysfunctioning ABC transporters within a clinically applicable concentration. We also have elucidated the roles of Src and caveolin-1 in the signal transduction between the inhibition of HER2 and the dysfunction of ABCB1.

Materials and Methods

Cell lines and cell culture

SCLC cell lines (SBC-1 and SBC-2) were obtained from the Japanese Collection of Research Biresources, Tokyo, Japan. SBC-3 (18), its chemoresistant sublines SBC-3/CDDP (19), SBC-3/ETP (20), and SBC-3/SN-38 (21), and SBC-5 (22) were kindly provided by Dr. K. Kiura (Okayama University, Okayama, Japan). SCLC cell lines NCI-H69, NCI-N231, NCI-H46, a non-SCLC cell line HCC827, and a breast carcinoma cell line SK-BR-3 were purchased from American Type Culture Collection. Chemoresistant sublines H69/CDDP (23) and H69/VP (24) were obtained from National Cancer Center, Tokyo, Japan. OC-10, Smk, and CADO LC6 were provided by Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan. O5-1, O52RA, and O53RS were established in our laboratory and their biologic properties were previously characterized (25). All of lung cancer cell lines and SK-BR-3 were maintained in RPMI-1640 and McCoy’s 5A medium, respectively, supplemented with 10% heat-inactivated FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL). All cell lines were periodically authenticated by morphologic inspection and tested negative every 6 months for mycoplasma contamination using PCR ELISA Kit (Roche Applied Science).

Antibodies

Mouse monoclonal antibodies (mAb) against HER2 (191924), ABCB1 (JSB-1), and phospho-tyrosine (PY99) were purchased from R&D Systems, Abcam, and Santa Cruz Biotechnology, respectively. Rabbit mAbs against caveolin-1 (D46G3) and phospho-HER2 (6B12) were commercially available from Cell Signaling Technology. We also obtained rabbit polyclonal Abs (pAb) against HER2, phospho-caveolin-1 (Tyr 14), phospho-Src (Tyr 416), phospho-Src (Tyr 527), and Src from Cell Signaling Technology; ABCB1 and ABCG2 from GeneTex; and EGFR from Santa Cruz Biotechnology. Goat pAb against actin was also purchased from Santa Cruz Biotechnology. Mouse IgG2b isotype control (Ancell) and horseradish peroxidase–conjugated goat antirabbit or antimouse IgG (BioRad) were also used.

Reagents

Nippon Kayaku Co. supplied us with cisplatin (CDDP; C2H2N2Cl2Pt) and etoposide (VP-16; C23H27O13). Irinotecan (CPT-11; C25H22N4O6) and its active form, SN-38 (C22H20N2O5), were provided by Yakult Co. Lapatinib (C29H26ClFN4O4S.2C7H8O3S) and gefitinib (C21H22ClFN4O3) were purchased from BioVision and Santa Cruz Biotechnology, respectively. Structures of these chemicals are shown in Fig. 1. Rhodamine 123, Hoechst 33342, and Cell Counting Kit-8 (CCK-8) were purchased from Sigma Chemical, Invitrogen, and Dojindo, respectively.

Flow cytometry

Cells (2 × 10⁵) were incubated with 0.5 μg of mouse antihuman HER2 mAb or isotype control mouse IgG2b for 30 minutes at 4°C followed by labeling with fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (Invitrogen). Stained cells were analyzed by FACSort (Becton Dickinson).

Immunoprecipitation and immunoblotting

Cells were untreated or treated with lapatinib and then lysed in lysis buffer. For immunoprecipitation, after pre-clearing with 20 μl protein G sepharose bead (GE Healthcare; slurry), whole-cell lysates were incubated with antihuman caveolin-1 or ABCB1 Ab (diluted 1:100 to 1:200) overnight at 4°C with gentle agitation. The immunoprecipitates were washed 3 times in lysis buffer and denatured before carrying out SDS-PAGE. For immunoblotting, whole-cell lysates or immunoprecipitates were separated in a 5% to 20% gradient gel (Wako) by SDS-PAGE, thereafter transferred to polyvinylidene difluoride membranes. The membranes were incubated with the proper

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primary Abs (diluted 1:250 to 1:500) overnight at 4°C followed by appropriate horseradish peroxidase-conjugated secondary Abs (diluted 1:5,000 to 1:10,000) for 1 hour at room temperature. Immunoreactive bands were visualized using a chemiluminescent technique with ECL Plus Western Blotting Detection Reagents (GE Healthcare).

**Drug sensitivity assay**

Cells (5 x 10^5 cells per well) were plated onto 96-well tissue culture–treated plates (Corning Costar) and treated with serially diluted cytotoxic compounds in serum-containing medium for 72 hours. The relative number of viable cells was quantified using CCK-8 following the instruction manual (26).

**ABC transporter function analysis**

To assess ABCB1- and ABCG2-inhibitory effect of lapatinib, flow cytometric analysis using rhodamine 123 and Hoechst 33342 was conducted as previously described (27, 28). Briefly, cells (1 x 10^6/mL) were treated with 0.1, 1, or 10 μmol/L of lapatinib for 15 minutes, then rhodamine or Hoechst was added to final concentration of 0.1 μmol/L or 4 μg/mL, respectively. After incubation for 1 hour at 37°C for staining, intracellular dye accumulations were analyzed by FACSort or FACSArria (Becton Dickinson).

**HER2 knockdown analysis**

Cells were transfected with either 30 pmol/L of short interfering RNA (siRNA) against human ERBB2 (SHF27B-0846) or control RNAs (S30C-0126; B-Bridge International) using Lipofectamine RNMiMAX (Invitrogen). After 48-hour incubation, cells were used for experiments.

**Treatment of chemoresistant SCLC xenografts**

Male, 6- to 8-week-old, BALB/cA Jcl nu/nu mice were obtained from CLEA Japan. SBC-3/ETP cells (2 x 10^6) or SBC-3/SN-38 cells (1 x 10^6) were injected subcutaneously in the flank of mice. When the tumor volume reached approximately 400 to 800 mm^3, mice were randomized into the following 4 groups: (A) vehicle; (B) chemotherapy; (C) lapatinib; and (D) chemotherapy plus lapatinib.

Treatment schedules were as follows: etoposide or irinotecan was intraperitoneally administered at a dose of 20 mg/kg to mice bearing SBC-3/ETP xenograft on days 1 to 3 or SBC-3/SN-38 xenograft on days 1, 5, and 9, respectively. Lapatinib was orally given at a dose of 100 mg/kg 1 hour before chemotherapy. Tumor volume (V) was calculated according to the following equation: V (mm^3) = length x (width)^2./2.

**Statistical analysis**

All the studies for statistical evaluation were conducted in triplicate in each experiment and repeated at least 3 times. Mean ± SD values were calculated and differences were evaluated by 2-sided Student t test. P < 0.05 was considered statistically significant.

**Results**

**HER2 is preferentially expressed in Japanese SCLC**

Because HER2 has been reported to be a negative prognostic factor in ED-SCLC (15–17), we consider HER2 as a key target molecule in treating SCLC. We first assessed the expression of HER2 in SCLC cell lines by fluorescence-activated cell sorting (FACS) and immunoblotting (Fig. 2A and B). Consistent with the previous findings, the expression of HER2 was detected in none of 3 SCLC cell lines (0%) of Caucasian origin (H69, H446, and N231). In contrast, 6 of 10 cell lines (60%) of Japanese origin (SBC-3, SBC-5, OS3R5, Smk, OC-10, and CADO LC6) expressed various amount of HER2. The higher frequency of HER2 expression in SCLC cell lines from Japanese patients suggests the ethnic difference between Japanese and Caucasians.

**HER2 is upregulated in SBC-3–derived chemoresistant cells**

We next examined whether cytotoxic chemotherapy affects HER2 expression by comparing chemosensitive H69 and SBC-3 cells with chemoresistant SCLC cells, H69/CDDP, H69/VP, SBC-3/CDDP, SBC-3/ETP, and SBC-3/SN-38. These chemoresistant SCLC cells were established from their parental cells by long-term exposure to respective cytotoxic agents. The expression of HER2 was upregulated in all SBC-3–derived chemoresistant cells compared with that of parental cells but not detected in any of H69 and H69-derived chemoresistant cells (Fig. 2C and D). This finding raises a possibility that

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Figure 1. Chemical structures of lapatinib, gefitinib, etoposide (VP–16), cisplatin (CDDP), irinotecan (CPT–11), and SN–38.
upregulation of HER2 is involved in MDR mechanism of SCLC.

**Lapatinib restores chemosensitivity in ABC transporter–positive SCLC cells**

We then investigated whether HER2 could be a direct therapeutic target for SCLC. The cytotoxic effects of lapatinib on SCLC cells were examined using a CCK-8 cytotoxicity assay. A phase I pharmacokinetic study has shown that peak plasma concentration of lapatinib is 2.43 μg/mL (¼ 2.58 mmol/L; ref. 29). Consistently, lapatinib alone could not affect the viability of HER2-positive SCLC cells up to this concentration (Supplementary Fig. S1). Next, we examined the cytotoxic effects of combination therapy with lapatinib and cytotoxic agents. Lapatinib at 1 μmol/L, which is a clinically achievable concentration, reversed drug resistance in HER2-positive cells, considerably in SBC-3/ETP (fold reversal = 10.2; P = 0.0076) and almost completely in SBC-3/SN-38 (fold reversal = 15.5; P = 0.0024), respectively. HER2-negative H69/VP cells were also restored the sensitivity to etoposide slightly by lapatinib (fold reversal = 3.3; P = 0.0175).

Because lapatinib has been reported to inhibit ABC transporter functions and restore chemosensitivity in breast carcinoma (14), we evaluated the expression

![Figure 2. HER2 is preferentially expressed in Japanese SCLC and upregulated in SBC-3–derived chemoresistant cells. A and B, HER2 expression in 13 SCLC cell lines (H69, H446, and N231 are Caucasian origin. SBC-1, SBC-2, SBC-3, SBC-5, OS-1, OS2RA, OS3R5, Smk, OC-10, and CADO LC6 are Japanese origin). C and D, comparison of HER2 expression between parental SBC-3 and chemoresistant SBC-3 sublines. In FACS analysis (A and C), cells were stained with 5 μg/mL of either anti-HER2 mouse mAb or isotype-matched control antibody and presented as histogram of HER2-stained cells (black shaded) overlaid with control cells (solid line). In immunoblotting analysis (B and D), 30 μg of total protein from whole-cell lysate was separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Immunodetection of HER2 and actin was conducted using anti-HER2 mouse mAb (diluted 1:500) and anti-actin goat pAb (diluted 1:800), respectively. Actin was used as an internal control. Each experiment was carried out at least twice and representative results are shown.](https://www.aacrjournals.org/Mol-Cancer-Ther/article-pdf/11/4/832/5425608/mct-11-0884.pdf)
Figure 3. Lapatinib restores chemosensitivity in ABC transporter–positive SCLC cells. A, after cells were treated with serially diluted concentration of indicated drugs for 72 hours with or without 1 µmol/L of lapatinib, relative number of viable cells was quantified by CCK-8 assay. Experiments were repeated at least 3 times in triplicates. Points, the mean percentage of viable cells; bars, SD. B, expression of ABCB1 and ABCG2 in parental and chemoresistant SCLC cells. Each lane contains 30 µg of total protein from whole-cell lysate. Anti-ABCB1 rabbit pAb (diluted 1:500) and ABCG2 rabbit pAb (diluted 1:500) were used for immunodetection. C, expression status of ABCB1 and ABCG2 during lapatinib treatment. After cells were treated with various concentrations of lapatinib up to 72 hours, ABCB1 in SBC-3/ETP and ABCG2 in SBC-3/SN-38 cells were examined by immunoblotting as mentioned above and are shown with actin as internal control.
of ABCB1 and ABCG2 in chemoresistant SCLC cells by immunoblotting. As shown in Fig. 3B, overexpression of ABC transporters was detected exclusively in the cells, ABCB1 in H69/VP and SBC-3/ETP cells, and ABCG2 in SBC-3/SN-38 cells, in which lapatinib could restore the chemosensitivity. In contrast, no detectable ABC transporters were expressed in H69/CDDP and SBC-3/CDDP cells in which lapatinib did not show the reversal effect. We also confirmed that lapatinib does not downregulate the expression of ABCB1 and ABCG2 up to 72 hours (Fig. 3C). These results imply that lapatinib reverses the chemoresistance by inhibiting ABC transporter functions without affecting their expression, and the reversal effect in ABCB1-positive cells has a dependency on HER2 expression.

**Lapatinib suppresses ABCB1 functions through HER2 inactivation**

Because lapatinib restored the sensitivity to etoposide of ABCB1-positive cells more effectively in HER2-positive SBC-3/ETP cells than in HER2-negative H69/VP cells (Fig. 3A), we investigated whether HER2 is involved in the lapatinib-mediated inhibition of ABCB1 functions. We then conducted a rhodamine 123 flow cytometric assay to examine whether lapatinib inhibits the efflux pump function of ABCB1 differentially depending on HER2 expression status. Intracellular accumulation of rhodamine was less in etoposide-resistant SBC-3/ETP and H69/VP cells than the respective parental SBC-3 and H69 cells without lapatinib treatment. However, lapatinib increased the accumulation of rhodamine in both SBC-3/ETP and H69/VP cells but not in their parental cells (Fig. 4A and B). Notably, this accumulation was observed even at a lower concentration of lapatinib (1 μmol/L) in HER2-positive SBC-3/ETP cells. In contrast, in HER2-negative H69/VP cells, the accumulation was observed only with a higher concentration (10 μmol/L), which is far beyond the maximum plasma concentration in humans. Because SBC-3/ETP cells express not only HER2 but also EGFR (Supplementary Fig. S2), we tested whether gefitinib, a selective EGFR-TKI, could exert similar effects. Gefitinib could accumulate rhodamine in SBC-3/ETP cells only with a higher concentration (10 μmol/L) but not with a maximum clinical plasma concentration in humans (<1.0 μmol/L; ref. 30; Fig. 4C).

To determine the involvement of HER2 in the functional regulation of ABCB1 by lapatinib, we knocked down HER2 in SBC-3/ETP cells using siRNA technique. FACS and immunoblotting analysis confirmed that the expression of HER2 was successfully suppressed, whereas no change was seen in the expression of ABCB1 (Supplementary Fig. S3). Lapatinib-induced increase in accumulation of rhodamine was considerably attenuated in HER2 knocked down SBC-3/ETP cells (Fig. 4D). The values of mean fluorescence intensity of intracellular rhodamine in cells transfected with control siRNA versus HER2-targeting siRNA at various concentrations of lapatinib were 5.4 versus 2.1 at 0.1 μmol/L, 75 versus 48 at 1 μmol/L, and 201 versus 127 at 10 μmol/L, respectively. These results indicate that lapatinib can suppress ABCB1 functions partly through inactivation of HER2.

**Caveolin-1 and Src are involved in the regulation of ABCB1 functions by HER2**

Caveolin-1 is a major component and an integral membrane protein of caveolae, which is known to interact with ABCB1 (31). In addition, overexpression of Src is reported to induce caveolin-1 phosphorylation and also negatively modulate ABCB1 functions (32). We therefore focused on caveolin-1 and Src to explore the mechanisms involved in lapatinib-mediated suppression of ABCB1 functions in HER2-positive SCLC cells. In SBC-3/ETP cells, HER2 was dephosphorylated by lapatinib, whereas Src phosphorylation levels were decreased at the 527th tyrosine residue in the C-terminal tail and increased at the 416th tyrosine residue in the activation loop. Moreover, caveolin-1 was further phosphorylated at the 14th tyrosine residue. Finally, Src enzyme activity was upregulated along with inactivation of HER2 by lapatinib, which might successively bring about further phosphorylation of caveolin-1 and lead to the functional suppression of ABCB1 (Fig. 5A).

Immunoprecipitation study showed that caveolin-1 primarily associated with HER2. However, once SBC-3/ETP cells were exposed to lapatinib, interaction between caveolin-1 and HER2 was abated, whereas interaction between caveolin-1 and ABCB1 was enhanced along with phosphorylation of caveolin-1. In contrast, direct association of HER2 with ABCB1 was not detected regardless of lapatinib treatment (Fig. 5B). Lapatinib-mediated molecular events involved in the suppression of ABCB1 function via inactivation of HER2 are schematized in Fig. 5C.

**Lapatinib suppresses ABCG2 functions independently of HER2 inactivation**

We further investigated whether lapatinib inhibits ABCG2 functions directly or by way of HER2 inactivation in ABCG2-positive SBC-3/SN-38 cells. Intracellular Hoechst accumulation was dramatically increased by lapatinib dose dependently and reached plateau at 1 μmol/L in SBC-3/SN-38 cells but not in ABCG2-negative SBC-3 cells, indicating that treatment with 1 μmol/L of lapatinib completely inhibited ABCG2 functions in SBC-3/SN-38 cells comparable with the parental SBC-3 cells (Fig. 6A). This inhibitory effect on ABCG2 functions, unlike in case of ABCB1, was hardly ever affected by knocking down of HER2 (Fig. 6B). These findings suggest that lapatinib inhibits ABCG2 functions independently of HER2 inactivation, which is different from the HER2-dependent inhibitory mechanism of ABCB1.
Combination therapy with lapatinib and cytotoxic drugs suppresses the growth of ABC transporter–positive SCLC tumors in vivo

To examine whether combination therapy of lapatinib with cytotoxic agents could reverse MDR in vivo, we established SBC-3/ETP and SBC-3/SN-38 xenograft models and subsequently treated them with or without lapatinib. There were no significant differences in tumor size between animals treated with vehicle, cytotoxic agent (etoposide or irinotecan), or lapatinib alone. However, in both xenografts, the lapatinib combination therapy with either etoposide or irinotecan significantly inhibited tumor growth compared with the treatment with only vehicle, cytotoxic agent, or lapatinib ($P < 0.02$ in SBC-3/ETP xenograft, $P < 0.0002$ in SBC-3/SN-38 xenograft; Fig. 7A and B). In addition, no mortality or serious decrease in body weight was observed at the doses tested. These results indicate that the combination therapy with lapatinib and cytotoxic agent is promising to overcome MDR SCLC in vivo.

Discussion

We have investigated the participation of HER2 in ABC transporter–mediated chemoresistance in SCLC, and a series of molecular mechanisms on how lapatinib-triggered HER2 inactivation leads to ABCB1 functional inhibition. As a result, we have presented that clinically applicable low dose of lapatinib could reverse
chemoresistance of SCLC cells that overexpress ABC transporters and that the reversal effect by lapatinib is more prominent in HER2-positive cells than in HER2-negative cells. These findings imply that combination therapy of lapatinib and cytotoxic agent would be effective for chemoresistant SCLC.

Some studies have identified HER2 expression as a negative prognostic factor of SCLC, especially in extensive diseases in Caucasians (15, 16). HER2 overexpression has been reported to be about 10% to 30% in Western patients with SCLC (17) and 10% (1 of 11) of SCLC cell lines established from Caucasian patients (33). However, here we have found that HER2 is expressed more frequently in SCLC cell lines of Japanese origin (60%) than Caucasian origin (0%), showing the possibility of ethnic differences of HER2 expression in

Figure 5. Caveolin-1 and Src are involved in the regulation of ABCB1 functions by HER2. A, SBC-3/ETP cells were treated with 1 μmol/L lapatinib up to 120 minutes. Tyrosine (Tyr) phosphorylation and expression of HER2, Src, and caveolin-1 (Cav-1) in whole-cell lysates were examined by immunoblotting. Each lane contains 30 μg of total protein, and antibody dilution used to detect each protein was as follows: anti-phospho-HER2 rabbit mAb (1:250), anti-HER2 rabbit pAb (1:500), anti-phospho-Src (Tyr 416 and Tyr 527) rabbit pAb (1:500), anti-Src rabbit pAb (1:500), anti-phospho-Cav-1 (1:250), and anti-Cav-1 (1:500). B, SBC-3 cells were treated with 1 μmol/L lapatinib up to 120 minutes, lysed in lysis buffer, and cell lysates were immunoprecipitated (IP) with anti-ABCB1 mouse mAb (diluted 1:50) and anti-Cav-1 rabbit mAb (diluted 1:200). Immunoprecipitates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Immunodetection of ABCB1, Cav-1, and phospho-tyrosine was done with anti-ABCB1 rabbit pAb (1:500), anti-Cav-1 rabbit mAb (1:500), and anti-phospho-tyrosine mouse mAb (1:500), respectively. Representative blots from 3 independent experiments with similar results are shown. C, molecular events on how lapatinib (Lap) acts as an inhibitor of ABCB1, despite being its substrate, in the presence of etoposide (VP-16) are schematized. In addition to direct inhibition (light blue curved line), indirect inhibitory pathway (pink curved line) through suppressing HER2 signal transduction is presumed to exist on the findings in A and B. Lapatinib as a substrate for ABCB1 is not shown in this schema. IB, immunoblotting; P, phosphate.
SCLC. On the basis of the clinical evidence that EGFR-TKI-sensitive mutations are detected more frequently in East Asian patients with non-SCLC than in Caucasians (6, 34), we consider ethnic difference as an important factor for molecular targeting therapy. We also showed that HER2 is upregulated after acquiring MDR phenotype in a HER2-positive SCLC cell line. In this context, it would be clinically important to determine whether targeting HER2 therapy is effective in SCLC.

Targeting cell surface RTKs is an attractive strategy for cancer treatment. In fact, several clinical trials targeting RTKs such as c-Kit and EGFR by TKIs have been conducted in SCLC but eventuated in disappointing results (35, 36). We here tested the antitumor activity of targeting HER2 by lapatinib as monotherapy at clinically achievable concentration in vitro. However, we could not observe satisfactory effects (Supplementary Fig. S1). A possible reason why inhibition of one specific RTK was not effective for SCLC is that SCLC cells lack addiction to a specific RTK signaling pathway. Indeed, we have detected the presence of multiple RTKs, such as HER2, EGFR, insulin-like growth factor-1 receptor, c-Kit, and c-Met, in each SCLC cell line (data not shown). SCLC cells thus probably use a variety of growth factor/RTK signals to proliferate and survive. Therefore, additional effects apart from its direct inhibitory effect on RTKs are needed for TKIs to exert expected results in SCLC.

Figure 6. Lapatinib suppresses ABCG2 functions independent of HER2 inactivation. Cells were treated with serially diluted lapatinib for 15 minutes and thereafter exposed to 4 μg/mL of Hoechst 33342 for 1 hour at 37°C. Thereafter, intracellular accumulation of Hoechst, as the inhibitory index of ABCG2 functions, was analyzed by FACS Aria. A, comparison of intracellular Hoechst accumulation between ABCG2-negative SBC-3 cells and ABCG2-positive SBC-3/SN-38 cells in the presence (0.1 or 1 μmol/L) or absence of lapatinib. B, comparison of intracellular Hoechst accumulation between control siRNA–transfected SBC-3/SN-38 cells and HER2 siRNA–transfected SBC-3/SN-38 cells at each concentration of lapatinib. These experiments were repeated at least twice with similar results, and representative data are shown. RNAi, RNA interference.
HER2 Involvement in ABC Transporter–Mediated MDR in SCLC

Figure 7. Combination therapy with lapatinib and cytotoxic drugs suppresses the growth of ABC transporter–positive SCLC tumors in vivo. SCLC cells were injected subcutaneously in the flank of athymic nude mice. When the tumor volume reached approximately 400 to 800 mm³, mice were randomized into 4 treatment groups (n = 9–11 for each). A, etoposide (VP-16) was i.p. administered into mice bearing SBC-3/ETP tumors at a dose of 20 mg/kg on days 1 to 3. B, irinotecan was i.p. administered into mice bearing SBC-3/SN-38 tumors at a dose of 20 mg/kg on days 1, 5, and 9. Lapatinib was orally given at a dose of 20 mg/kg on days 1 to 3. B, irinotecan was i.p. administered into mice bearing SBC-3/ETP tumors at a dose of 20 mg/kg on days 1, 5, and 9. Lapatinib was orally given at a dose of 100 mg/kg 1 hour before every cytotoxic drug administration. Tumor volume (V) was calculated according to the following equation: V(mm³) = length × width²/2. There was no significant difference (N.S.) in tumor size between animals treated with vehicle, cytotoxic drug, or lapatinib alone. However, in both xenografts, lapatinib combination therapy significantly suppressed the tumor growth compared to cytotoxic drug alone (P < 0.02 in A and P < 0.0002 in B). Points, mean tumor volumes; bars, SD; arrows, drug administration.

Recently, it has been reported that many anticancer TKIs are substrates for ABC transporters and also act as inhibitors of them to increase intracellular accumulation of coexisting other substrates such as cytotoxic drugs (37–39). HER-TKIs (e.g., gefitinib, erlotinib, and lapatinib) have been shown to have potentials to restore sensitivity in chemoresistant cancer cells by inhibiting ABC transporter functions (7–9, 14). These studies proposed that HER-TKIs directly inhibit drug efflux pump functions because the inhibitory effects were observed regardless of HER expression. However, in our present study, the inhibitory effects of lapatinib on ABCB1 were observed with a lower concentration in HER2-positive SBC-3/ETP cells than in HER2-negative Hi69/VP cells (Figs. 3A and 4A and B), and the effect was considerably attenuated in HER2 knocked down SBC-3/ETP cells (Fig. 4D). Our results suggest that lapatinib not only inhibits ABCB1 functions directly but also indirectly through HER2 inactivation. Contrastively, inhibitory effect of lapatinib on ABCG2 functions is far stronger than on ABCB1 and 1 μmol/L of lapatinib is enough to overcome ABCG2-mediated MDR in SCLC (Figs. 3A and 6A and B).

We here have highlighted the involvement of HER2 in the regulation of ABC transporter functions. This study is the first to elucidate the mechanism behind lapatinib’s reversal of ABCB1-mediated MDR, partly through HER2 inactivation. We focused on caveolin-1 as a molecule which intervenes between HER2 and ABCB1. Caveolin-1 is recognized as a negative regulator of EGFR and HER2 (40, 41). Moreover, a recent work showed that overexpression of caveolin-1 decreased the transport activity of ABCB1 (31). In another study, downregulation of caveolin-1 by siRNA was shown to reduce the interaction with ABCB1 and augment its activity (32). In HER2-positive SBC-3/ETP cells, lapatinib suppresses HER2 tyrosine kinase activity, which causes caveolin-1 phosphorylation and dissociation from HER2, perhaps because caveolin-1 no longer needs to downregulate HER2. On the contrary, Src enzyme activity is upregulated. This is a key phenomenon to explain how interaction between caveolin-1 and ABCB1 enhances to inhibit ABCB1 functions. Barakat and colleagues reported that Src overexpression induced caveolin-1 phosphorylation and downregulated ABCB1 functions through increasing interaction between caveolin-1 and ABCB1 in rat brain endothelial cells (32). In addition, Hawkins and colleagues showed that YEEIP, a Src kinase–activating peptide, induced caveolin-1 phosphorylation and led to downregulate ABCB1 activity in rat brain capillaries (42). Our findings presented in Fig. 5 support these observations. Namely, lapatinib treatment induces caveolin-1 phosphorylation through activating Src enzyme, then enhances interaction between caveolin-1 and ABCB1, and finally inhibits ABCB1 activity. Thus, we have crystallized the molecular regulation system between HER2 and ABCB1 and that is why relative low concentration of lapatinib can restore chemosensitivity in HER2-positive SCLC cells.

In the clinical setting, relapse with brain metastases is the major problem in SCLC. Systemic chemotherapy alone is less effective for asymptomatic brain metastases than expected (43), for which blockade of drug penetration into the brain by the blood–brain barrier (BBB) is believed to be the main reason. ABCB1 is known to be abundantly expressed on the BBB and excrete its substrates. Polli and colleagues showed that the concentration of orally administered lapatinib in the brain was far lower than in the plasma in rat because this compound served not only as an inhibitor but also as a substrate of ABCB1 (38). They further showed that ABCB1 and ABCG2 on the BBB synergistically hindered lapatinib from penetrating into the central nervous system (45). On the other hand, the BBB is thought to have been already disrupted in advanced SCLC with radiologically recognizable brain metastases (46).
Moreover, Taskar and colleagues reported that the concentration of lapatinib in metastatic brain tumors was markedly higher than normal brain tissues in mice that received intracardiac injection of breast cancer cells (47). Lapatinib, like other TKIs, serves as a substrate of ABC transporters and is excreted from cells at lower concentrations. However, it functions as an inhibitor of ABC transporters at higher doses in turn (39). Therefore, in relapsed SCLC, lapatinib is expected to penetrate into metastatic brain tumors and reach the concentration enough to restore the chemosensitivity.

Collectively, our present study showed that an ethnic difference might exist in HER2 expression in SCLC. We are now conducting histologic study of HER2 status in Japanese patients with SCLC. We also proved that HER2 is involved in the inhibitory effect of lapatinib on ABC transporters. Lapatinib combination therapy is promising to bring about better prognosis by overcoming MDR and is valuable for future clinical applications in patients with SCLC who develop chemoresistance, especially in patients with HER2-positive SCLC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

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Conception and design: T. Minami, T. Kijima
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Minami, T. Kijima
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Minami, T. Kijima, R. Takahashi, A. Kumanogoh

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