

Inhibition of ABCB1 Overcomes Cancer Stem Cell-like Properties and Acquired Resistance to MET Inhibitors in Non-Small Cell Lung Cancer

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Abstract

Patients with non-small cell lung cancer (NSCLC) *EGFR* mutations have shown a dramatic response to *EGFR* inhibitors (*EGFR*-TKI). *EGFR* T790M mutation and *MET* amplification have been recognized as major mechanisms of acquired resistance to *EGFR*-TKI. Therefore, *MET* inhibitors have recently been used in NSCLC patients in clinical trials. In this study, we tried to identify the mechanism of acquired resistance to *MET* inhibitors. We analyzed the antitumor effects of two *MET* inhibitors, PHA-665752 and crizotinib, in 10 NSCLC cell lines. EBC-1 cells with *MET* amplification were the only cells that were sensitive to both *MET* inhibitors. We established PHA-665752-resistant EBC-1 cells, namely EBC-1R cells. Activation of *KRAS*, *EGFR*, and *FGFR2* signaling was observed in EBC-1R cells by FISH and receptor tyrosine kinase phosphorylation

antibody arrays. EBC-1R cells also showed overexpression of ATP-binding cassette subfamily B member 1 (*ABCB1*) as well as phosphorylation of *MET*. EBC-1R cells grew as cell spheres that exhibited cancer stem cell-like (CSC) properties and epithelial-mesenchymal transition (EMT). The level of miR-138 that targeted *ABCB1* was decreased in EBC-1R cells. *ABCB1* siRNA and the *ABCB1* inhibitor elacridar could reduce sphere numbers and suppress EMT. Elacridar could also reverse resistance to PHA-665752 in EBC-1R cells. Our study demonstrated that *ABCB1* overexpression, which was associated with CSC properties and EMT, was involved in the acquired resistance to *MET* inhibitors. Inhibition of *ABCB1* might be a novel therapeutic strategy for NSCLC patients with acquired resistance to *MET* inhibitors. *Mol Cancer Ther*; 14(11); 2433–40. ©2015 AACR.

Introduction

Lung cancer is the most frequent cause of cancer-related death in Japan and worldwide (1). Recently, oncogenic driver mutations in non-small cell lung cancer (NSCLC) patients, such as *EGFR* mutation and anaplastic lymphoma kinase gene (*ALK*) fusion gene, have been identified (2–4). Several tyrosine kinase inhibitors (TKI) are currently approved or are under clinical development for the treatment of NSCLC. Our group and others have recently reported that first-line gefitinib treatment in advanced NSCLC patients with *EGFR* mutations improved progression-free survival (PFS) in randomized phase III studies (5, 6). Unfortunately, despite this initial and marked response, most NSCLC patients become resistant to *EGFR*-TKIs. Two major mechanisms of acquired resistance to *EGFR*-TKI were identified in patients with NSCLC (7, 8). About half of resistant tumors develop a secondary

EGFR mutation in exon20 T790M, which prevents effective inhibition by *EGFR* TKIs due to steric hindrance or an increased binding affinity for ATP (7). An additional 5% to 10% of tumors from refractory patients undergo *MET* gene amplification, which causes HER3-dependent activation of the signaling cascade downstream of *EGFR* despite its inhibition by TKIs (8).

MET is a proto-oncogene that encodes a receptor tyrosine kinase, c-MET. c-MET is the receptor for hepatocyte growth factor (HGF). The binding of HGF to c-MET leads to cellular responses, including cell proliferation, motility, migration, and invasion (9, 10). In lung cancer, *MET* can be activated by HGF stimulation (11). Our recent study demonstrated that *MET* amplification and gene copy number gains showed a short response to gefitinib treatments in lung adenocarcinoma with *EGFR* mutation (12). Recently, *MET* inhibitors have been administered to NSCLC patients who are naïve or resistant to *EGFR* TKIs in a clinical trial (13). This phase II study showed that PFS was longer in the group treated with erlotinib plus the *MET* inhibitor tivantinib than in the group treated with erlotinib alone, especially among patients with *KRAS* mutations (13). Recent studies showed mechanisms of resistance to *MET* inhibitors, including mutation in the *MET* activation loop (Y1230), bypassed *EGFR* activation, and *MET* and *KRAS* gene amplification (14, 15). However, the molecular mechanisms of the acquired resistance to *MET* inhibitors in NSCLC are not completely understood.

In this study, we aimed to identify a novel molecular mechanism for acquired resistance to *MET* inhibitors and demonstrate potential therapeutic strategies. We established *MET* inhibitor-resistant NSCLC cells (EBC-1R). EBC-1R cells

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showed overexpression of ATP-binding cassette subfamily B member 1 (ABCB1) with cancer stem cell (CSC)-like properties and epithelial–mesenchymal transition (EMT) phenotype. Inhibition of ABCB1 could overcome the stem cell–like abilities and resistance to MET inhibitors in NSCLC cells. ABCB1 may be a critical and novel therapeutic target for resistance to MET inhibitors in NSCLC cells.

Materials and Methods

Cell culture

Nine lung adenocarcinoma cell lines (A549, LC-2/ad, PC-9, PC-14, ABC-1, HCC-827, NCI-H441, NCI-H1648, and RERF-LC-MS) and one EBC-1 squamous carcinoma line were used in this study. A549 and LC-2/ad were obtained from RIKEN Cell Bank. PC-9 and PC-14 were obtained from Immuno-Biological Laboratories. ABC-1, RERF-LC-MS, and EBC-1 were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). HCC-827, NCI-H441, and NCI-H1648 were obtained from the ATCC. These cell lines were obtained from 2003 to 2011, amplified and frozen, and one aliquot of each was thawed for this project, although no authentication was done by the authors. All cells were routinely screened for the absence of mycoplasma. These cell lines were maintained in RPMI1640 medium (Gibco) or minimum essential medium eagle supplemented with 10% FBS at 37°C in a humidified incubator. EBC-1 cells were incubated with increasing concentrations of PHA-665752 by stepwise methods. Cells that survived incubation with 5 $\mu\text{mol/L}$ PHA665752 were stored for further analysis and referred to as PHA665752-resistant cells (EBC-1R).

Drugs and growth inhibition assay

The MET inhibitor PHA-665752 and the ABCB1 inhibitor elacridar were purchased from Santa Cruz Biotechnologies. The dual ALK and MET inhibitor crizotinib was obtained from Selleck Chemicals. To evaluate the sensitivity to PHA-665752 and crizotinib *in vitro*, cells were plated (5,000 cells/well) in 96-well tissue culture plates and incubated for 24 hours before being exposed to different concentrations of PHA-665752 or vehicle (DMSO). The cells were incubated with various concentrations of PHA-665752 or crizotinib at 37°C for 72 hours. After incubation at 37°C for 72 hours, MTS was added to each well and incubated at 37°C for 2 hours, after which absorbance was measured at a test wavelength of 450 nm using a microplate reader (Dynatech MR7000, Dynatech). The IC_{50} value was calculated by SigmaPlot12 (HULINKS, Inc.). Each experiment was performed independently three times. The corrected absorbance of each sample was calculated and compared with that of the untreated control.

Western blot analysis

Cells were lysed in buffer containing 50 mmol/L Tris–HCl, pH 7.6, 150 mmol/L NaCl, 0.1% SDS, 1% Nonidet P-40, and 0.5% sodium deoxycholate. Western blot analysis was performed as previously described (16). ABCB1, KRAS, and vimentin antibodies were obtained from Santa Cruz Biotechnologies. MET, p-MET, AKT, p-AKT, p-MEK, EGFR, p-EGFR, and E-cadherin antibodies were purchased from Cell Signaling Technology. β -Actin was obtained from Sigma Aldrich.

RNA extraction and microarray analysis

Total RNA was extracted using TRIzol reagent (Invitrogen) from frozen cells as previously described (17, 18). High-density oligo-

nucleotide array analysis was carried out using Affymetrix HG-U133A GeneChips (22,282 probe sets), as previously described (16). We performed human receptor tyrosine kinases (RTK) phosphorylation antibody arrays including 71 antibodies (Ray-Biotech, Inc.). Total RNA was also used for hybridization on miRNA microarray chips containing 768 probes with the TaqMan Array Human MicroRNA A + B Cards Set v3.0 (Life Technologies) on a 7900 Real Time PCR System (Applied Biosystems), as previously described (19). C_t values were provided from all miRNAs represented on the cards, and fold changes in expression were calculated using the $\Delta\Delta C_t$ method. Expression levels of MammU6 on the array card were defined as positive controls for the purpose of calculation of $\Delta\Delta C_t$.

The microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE66604 (released March 7, 2015).

Real-time quantitative reverse transcription PCR

ABCB1, *ABCG2*, *ALDH1*, and *CD44* expression levels were measured by real-time quantitative reverse transcription PCR (qRT-PCR) using TaqMan Gene Expression Assay (Applied Biosystems). GAPDH was determined as an internal control (Applied Biosystems). Expression levels of miRNAs were measured using TaqMan MicroRNA Assay (Applied Biosystems). RNU66 expression level was determined as an internal control (Applied Biosystems). Gene and miRNA expression were quantified as $2^{-\Delta\Delta C_t}$ value (20).

Oligonucleotide transfection

The miR-374a and miR-138 mimics (miR-374a mimic and miR-138 mimic) and their negative control were synthesized by Ambion. All precursors were treated with Lipofectamine 2000 transfection reagent 24 hours after seeding, according to the manufacturer's instructions (Life Technologies). The precursor complexes were transfected into cells at a final concentration of 40 nmol/L.

FISH

Gene copy numbers (GCN) and amplification of *cMET*, *KRAS*, and *EGFR* genes were examined by FISH. Tissue sections were then hybridized with *cMET*, *KRAS*, *EGFR*, *CEP7*, *CEP12*, and *D7Z1* probes (LSI Chemical Medicine Corporation). Numbers of fluorescence signals were counted independently by two investigators under an Axio Vision microscope (Carl Zeiss).

Sphere formation assays in serum-free culture

A total of 1.25×10^4 cells were plated in 24-well plates with Ultra-Low Attachment surface (Corning Inc.), and cultured in serum-free minimum essential medium Eagle (Sigma-Aldrich) with 20 ng/mL EGF and 10 ng/mL basic fibroblast growth factor (Sigma-Aldrich). The numbers of spheres exceeding 150 μm in diameter in each well were counted under a microscope after 14 days of culturing.

Statistical analysis

Data were expressed as mean (SD) of three independent experiments and evaluated with the Student *t* test.

Results

Effect of MET inhibitors on NSCLC cell lines and establishment of PHA-665752-resistant NSCLC cells

We first evaluated the antitumor effects of PHA-665752 and crizotinib in 10 NSCLC cell lines. On the basis of the IC_{50} by growth-inhibitory assays, only EBC-1 cells were sensitive ($IC_{50} < 0.1 \mu\text{mol/L}$) to both MET inhibitors (Table 1). The EBC-1 cell line had been established from a metastatic skin tumor of a patient with lung squamous cell cancer by Watanabe Y in Okayama University in 1985. EBC-1 cells have been reported to contain genomic amplification of *MET* (21). We evaluated protein expression levels of MET and EGFR signal pathway molecules in the 10 NSCLC cell lines by Western blot analysis (Fig. 1A). Total MET protein expression was increased in EBC-1, NCI-H1648, and two EGFR-mutant cell lines (PC-9 and HCC-827). MET phosphorylation (p-MET) was enhanced in only EBC-1 cells. MET protein expression status was also evaluated in 6 additional squamous cell carcinoma cell lines. EBC-1 was the only cell line that had elevated levels of both p-MET and MET among 7 squamous cell carcinoma cell lines (Supplementary Fig. S1). PHA-665752 reduced the level of p-MET in EBC-1 cells in a time-dependent manner (Fig. 1B).

Next, we established PHA-665752-resistant EBC-1R cells from EBC-1 cells by the stepwise method. After 3 months' selection, we established EBC-1R cells (Fig. 1C). EBC-1R cells showed resistance to PHA-665752 with an IC_{50} of $5 \mu\text{mol/L}$, which is an approximately 160-fold increase over the IC_{50} of the parental EBC-1 cells (Fig. 1C). EBC-1R cells were further maintained without PHA-665752 exposure for one month. The IC_{50} value of the EBC-1R cells without PHA-665752 exposure was still more than $1 \mu\text{mol/L}$.

We next evaluated the protein expression of downstream molecules in the MET and EGFR pathways in EBC-1R cells. Increased levels of p-MET, MET, KRAS, and AKT phosphorylation (p-AKT) were observed in EBC-1R cells by Western blotting (Fig. 1D). FISH analysis of *MET*, *KRAS*, and *EGFR* genes was performed to examine the mechanism of overexpression of these proteins. *MET* gene copy number gain was not increased in EBC-1R cells; however, increased copy number gains of *KRAS* and *EGFR* genes were observed in EBC-1R cells (Supplementary Table S1).

To further clarify the signaling mechanism associated with sensitivity to PHA-665752, RTKs phosphorylation expression profiles were investigated in the same set of 10 NSCLC cell lines. The phosphorylation status of four RTKs associated with PHA-665752 sensitivity is shown in Table 2 (fold change of >1.5 and <0.5). Phosphorylation of FGFR1 and FGFR2 was enhanced in EBC-1R cells. We confirmed upregulation of *FGFR2* gene expression in EBC-1R cells by qRT-PCR analysis (Fig. 1E). We also examined the effect of the FGFR-inhibitor, nintedanib, on the drug sensitivity to PHA-665752 in EBC-1 and EBC-1R cells. Nintedanib combined with PHA-665752 did not have an effect

on drug-sensitive EBC-1 cells (Supplementary Fig. S2A). In contrast, nintedanib could reverse the resistance to PHA-665752 in EBC-1R cells with overexpressed FGFR2 (Supplementary Fig. S2B). FGFR2 might contribute to the resistance to PHA-665752, probably by bypassing pathway activation (22). These findings suggested that activation of KRAS as a downstream molecule of MET and activation of EGFR and FGFR2 signaling by a MET-independent bypass pathway are partially involved in the resistance to PHA-665752.

Overexpressed ABCB1 in EBC-1R cells with stem cell-like properties and EMT

To identify genes associated with resistance to PHA-665752 in EBC-1R cells, gene expression profiles were studied in the parental EBC-1 and EBC-1R cells by cDNA microarrays. The gene encoding ABCB1 (ATP-binding cassette transporter belonging to subfamily B member 1), which belongs to the ATP-binding cassette transporter family, was among the most upregulated genes in EBC-1R cells (Table 2). ABCB1 has recently been reported to be associated with CSC-like properties (23). We confirmed that the *ABCB1* gene was significantly overexpressed in EBC-1R cells by qRT-PCR analysis (Fig. 2A). To confirm the robustness of ABCB1 overexpression in EBC-1R cells, cloned EBC-1R cells derived from a single cell by limiting dilution were used. Three independent clones also showed *ABCB1* overexpression by qRT-PCR analysis (Supplementary Fig. S3). Genes encoding other stem cell-related markers, *ABCG2* and *CD44* genes, were slightly upregulated in EBC-1R cells (Fig. 2A). We next evaluated sphere formation activities of EBC-1R cells to confirm CSC-like properties. We found that EBC-1R cells grew as cell spheres (Fig. 2B). The presence of CSC-like properties was closely related to EMT (23). Therefore, we evaluated the expression levels of EMT markers in EBC-1R cells. The level of mesenchymal cell marker vimentin was increased in EBC-1R cells, resulting in the EMT phenotype (Fig. 2C). These findings showed that EBC-1R cells exhibited CSC-like properties and EMT.

miRNAs associated with drug sensitivity to PHA-665752

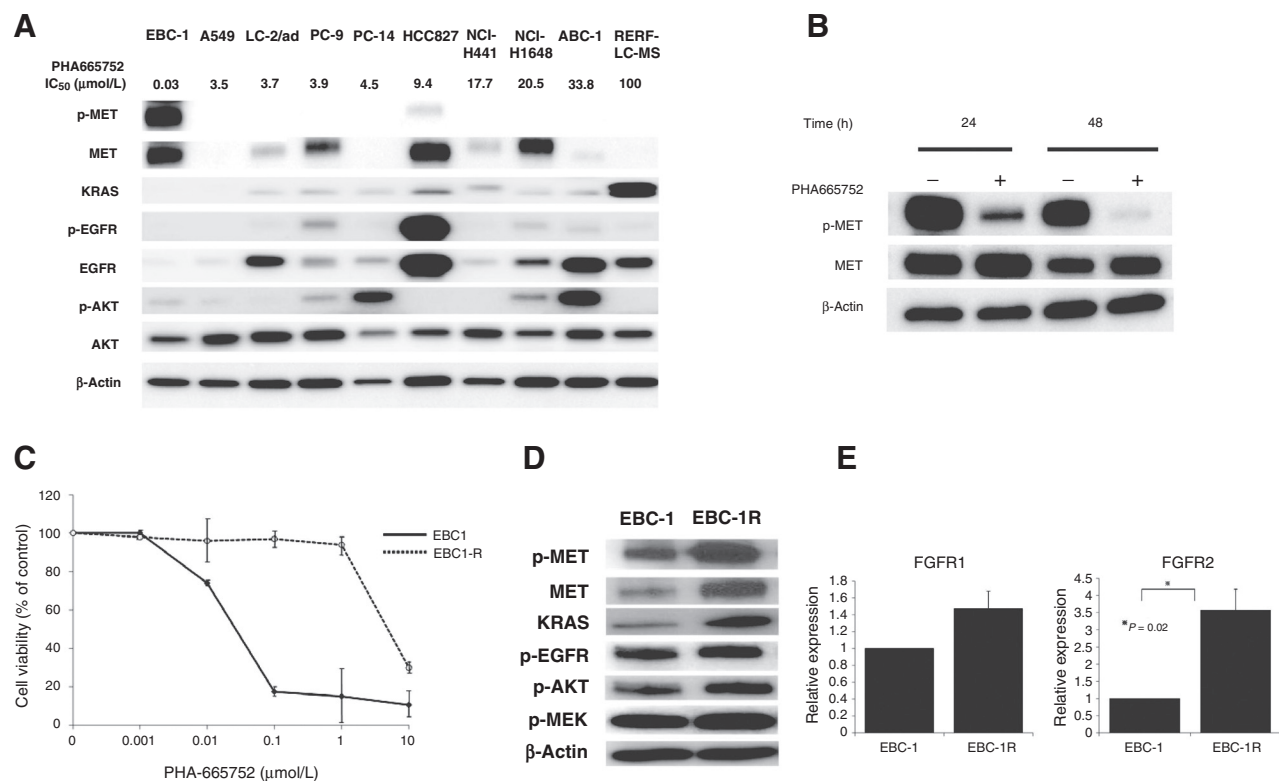
miRNAs, small noncoding RNAs that act as posttranscriptional regulators of gene expression, are involved in cancer progression and drug resistance (24–26). To identify the miRNAs that were altered in EBC-1R cells, miRNA expression profiling was performed. Twenty-three miRNAs were downregulated in EBC-1R cells (<0.5 fold changes; Supplementary Table S2). We next proceeded to identify potential targets using Target Scan 5.2 (<http://www.targetscan.org/>), a comprehensive resource of miRNA target predictions and expression profiles. We found that miR-10b, miR-130b, and miR-15a commonly target *MET*. *KRAS* could be predicted as a target of miR-504 and miR-135b. *FGFR2*

Table 1. IC_{50} values in 10 NSCLC cell lines responding to PHA665752 and crizotinib

Cell lines	EBC-1	A549	LC-2/ad	PC-9	PC-14	HCC827	NCI-H441	NCI-H1648	ABC-1	RERF-LC-MS
Pathology	SQ	AC	AC	AC	AC	AC	AC	AC	AC	AC
PHA665752	0.03	3.5	3.7	3.9	4.6	9.4	17.7	20.5	33.8	100
IC_{50} ($\mu\text{mol/L}$)										
Crizotinib	<0.01	24	4.1	3.8	26.7	19.9	12.1	19.6	20	27.9
IC_{50} ($\mu\text{mol/L}$)										
<i>MET</i> amplification	+	–	–	–	–	–	+	+	–	–
<i>KRAS</i> mutation	–	+	+	–	–	–	+	–	–	–
<i>EGFR</i> mutation	–	–	–	+	–	+	–	–	–	–

Abbreviations: AC, adenocarcinoma; SQ, squamous cell carcinoma.

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**Figure 1.**

Protein levels of molecules in the MET and EGFR signal pathways in 10 NSCLC cell lines and establishment of PHA-665752-resistant NSCLC cells. A, protein levels of p-MET, MET, KRAS, p-EGFR, EGFR, p-AKT, and AKT were examined by Western blot analysis. Significantly higher levels of p-MET and MET were found in EBC-1 cells. B, protein levels of p-MET and MET in EBC-1 cells after treatment with PHA-665752 (0.03 μmol/L) from 24 to 48 hours. The p-MET level significantly decreased after PHA-665752 treatment in a time-dependent manner. C, PHA-665752-resistant EBC-1 cells (EBC-1R). EBC-1R cells are resistant to PHA-665752 with an IC₅₀ of 5.0 μmol/L, which represents an approximately 160-fold increase compared with the IC₅₀ of the parental EBC-1 cells. Data, mean ± SD from three independent experiments. D, high protein levels of p-MET, MET, KRAS, and p-AKT in EBC-1R cells. E, *FGFR2* gene was overexpressed in EBC-1R cells on qRT-PCR.

could be predicted as a target of miR-125. Furthermore, ABCB1 could be predicted as a target of miR-374a by Target Scan and was previously reported as a target of miR-138 (27). We confirmed downregulation of miR-374a, miR-138, and miR-125 in EBC-1R cells by qRT-PCR (Fig. 3A). We next examined whether overexpression of miR-374a and miR-138 using miRNA mimics reduced the level of ABCB1. Treatment with miR-138 mimic downregulated the protein expression of ABCB1, resulting in increased E-cadherin and reduced vimentin in EBC-1 cells on Western blot analyses (Fig. 3B and C). However, overexpression of miR-374a did not result in these changes (Fig. 3B and C). These findings suggested that expression of miR-138 consequently played a key role in the resistance to PHA-665752 by targeting ABCB1 in NSCLC cells.

Downregulation of ABCB1 reverses resistance to MET inhibitors

Finally, we evaluated whether ABCB1 inhibition restores the CSC and EMT abilities, resulting in sensitivity to PHA-665752 in EBC-1R cells. ABCB1-siRNA could successfully inhibit ABCB1 expression in EBC-1R cells, resulting in induction of E-cadherin (Fig. 4A). We also found that oncosphere numbers were significantly decreased after treatment with siABCB1 (Fig. 4B). Next, we examined the effect of ABCB1 inhibitor elacridar on the CSC-property and EMT phenomenon in EBC-1R cells. ABCB1 protein

expression was knocked down by elacridar at a concentration of more than 0.5 μmol/L (Fig. 4C). Elacridar increased E-cadherin expression and reduced vimentin expression (Fig. 4C). Oncosphere numbers were significantly decreased after elacridar treatment of EBC-1R cells (Fig. 4D). We evaluated the effect of elacridar combined with PHA-665752 on the p-MET level. The p-MET was completely inhibited by treatment of elacridar with PHA-665752 (Fig. 4E). Furthermore, we evaluated whether elacridar could reverse the resistance of EBC-1R cells to PHA-665752. EBC-1 cells showed no ABCB1 expression; therefore, elacridar combined with PHA-665752 did not have an effect on drug-sensitive EBC-1 cells (Fig. 4F). In contrast, elacridar could suppress the CSC abilities and EMT, resulting in overcoming the resistance to MET inhibitors in EBC-1R cells (Fig. 4G). These results suggest that ABCB1 overexpression, which was associated with CSC and EMT, was mainly involved in the resistance to PHA-665752, and inhibition of ABCB1 is a novel therapeutic strategy for overcoming the resistance of NSCLC cells to MET inhibitors.

ABCB1 overexpression was involved in cancer progression in squamous cell carcinoma patients

We further investigated the relationship between ABCB1 protein expression and tumor progression of squamous cell carcinoma. ABCB1 expression was not found in any of the 7 squamous cell carcinoma cell lines (Supplementary Fig. S1). We also

Table 2. Differentially expressed genes and genes encoding RTKs between EBC-1 and EBC-1R cells

Gene	EBC-1R/EBC-1	Fold change	Gene	EBC-1R/EBC-1	Fold change
<i>ABCB1</i>	Up	155	<i>PRSS2</i>	Down	0.13
<i>TMEM45A</i>	Up	10	<i>CALB2</i>	Down	0.14
<i>AKRIC2</i>	Up	10	<i>FST</i>	Down	0.14
<i>HMOX1</i>	Up	9.7	<i>ENPP1</i>	Down	0.17
<i>CCDC80</i>	Up	8.3	<i>ETV1</i>	Down	0.17
<i>ABI3BP</i>	Up	7.4	<i>Let-7a2</i>	Down	0.17
<i>CCL2</i>	Up	7.1	<i>PLXNA4</i>	Down	0.18
<i>LCN2</i>	Up	7.0	<i>SLC14A1</i>	Down	0.19
<i>IL6</i>	Up	6.9	<i>BLID</i>	Down	0.19
<i>CROT</i>	Up	6.4	<i>DUSP6</i>	Down	0.19
RTK	EBC-1R/EBC-1	Fold change	RTK	EBC-1R/EBC-1	Fold change
FGFR2	Up	2.7	ROR2	Down	0.4
FGFR1	Up	1.6	MCSF2	Down	0.3

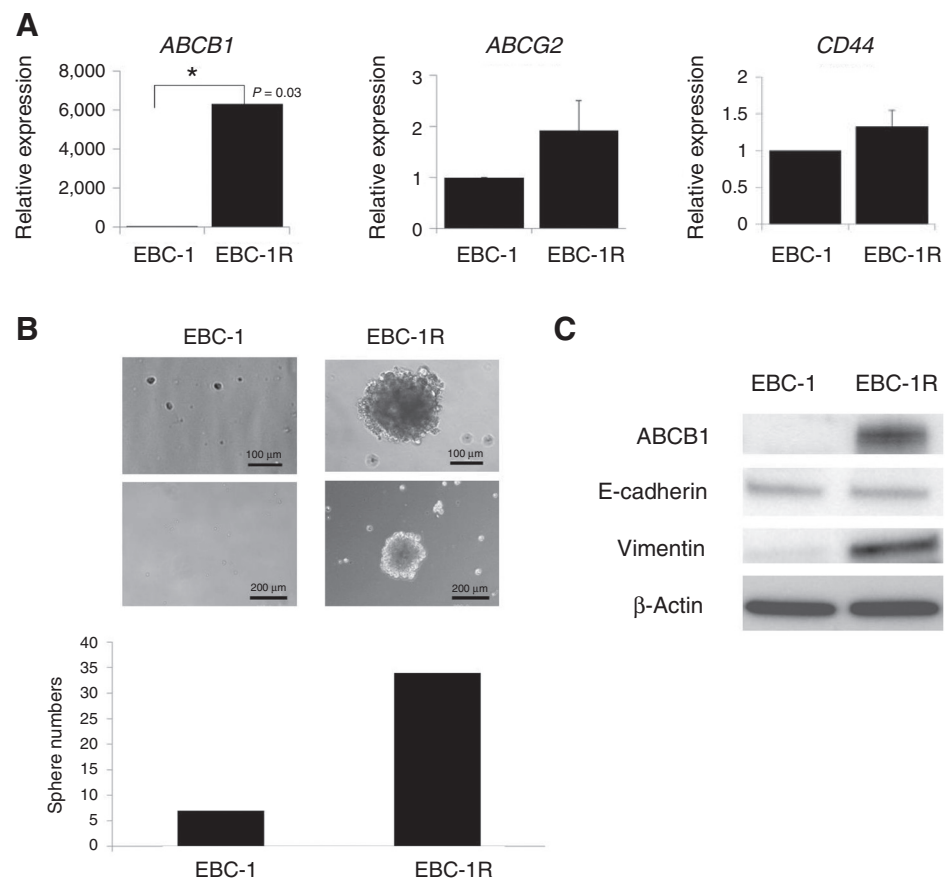
NOTE: Gene expression profiles were studied by cDNA microarrays. The expression of RTKs was studied by RTK arrays.

Abbreviations: Down, gene downregulated in EBC-1R cell line; up, gene upregulated in EBC-1R cell line.

evaluated ABCB1 protein expression in 50 squamous cell carcinoma patients with stage I or III by immunohistochemical analysis (Supplementary Fig. S4). No specimens were observed to be positive for ABCB1 in 32 stage I squamous cell carcinoma patients (Supplementary Table S3). On the other hand, 6 (33%) of 18 squamous cell carcinoma patients with stage III were positive for ABCB1 (Supplementary Table S3). These results support that ABCB1 expression was involved in tumor progression and metastasis as well as drug resistance in squamous cell carcinoma patients.

Discussion

MET gene activation is involved in resistance to anticancer agents, including EGFR-TKI, in NSCLC (8). *MET* amplification causes resistance to gefitinib by driving ERBB3-dependent activation of PI3K (8). Therefore, several MET inhibitors have been administered to EGFR-TKI-naïve or resistant NSCLC patients in clinical trials (13, 28). PFS was longer in the group treated with the MET inhibitor tivantinib combined with erlotinib than in the group treated with erlotinib alone in a phase II study (13). Another phase II trial showed that MET-positive NSCLC patients

**Figure 2.**

EBC-1R cells exhibit both CSC-like properties and EMT features. A, ABCB1, ABCG2, and CD44 gene expression levels in EBC-1 and EBC-1R cells by qRT-PCR. The ABCB1 level was significantly higher in EBC-1R cells than in EBC-1 cells. B, EBC-1R cells acquired high ability to form spheres in suspension culture in the sphere formation assay. C, the levels of ABCB1 and mesenchymal marker vimentin were increased in EBC-1R cells on Western blot analysis.

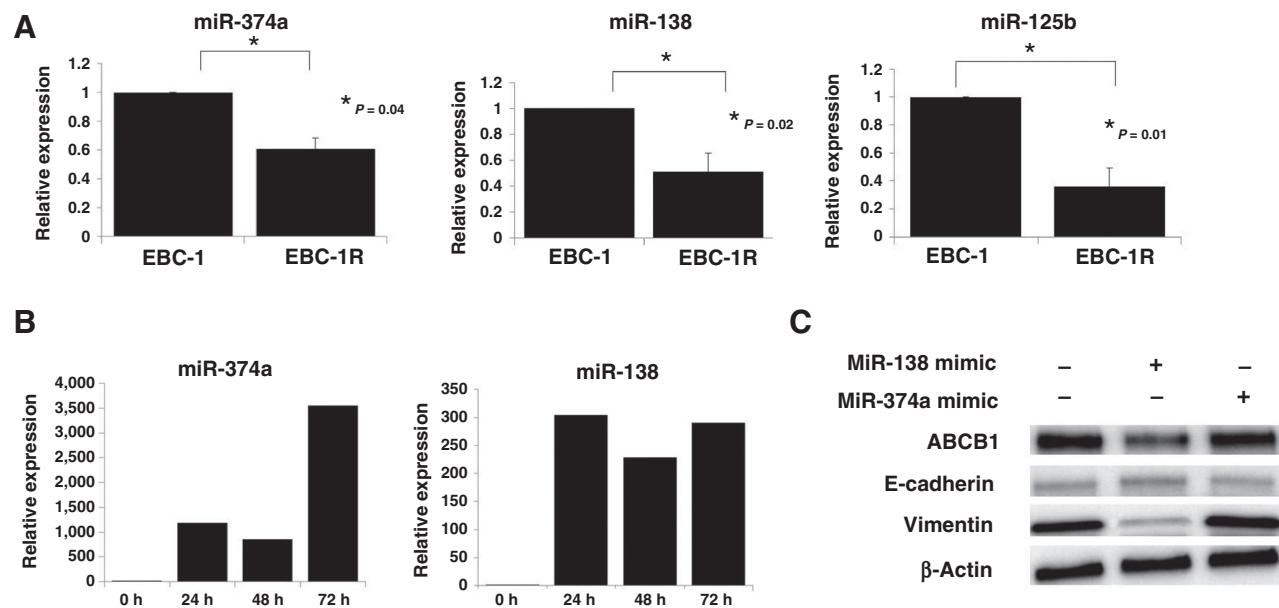


Figure 3. miR-138 was involved in the resistance of EBC-1 cells to PHA-665752. A, expression of miR-374a, miR-138, and miR-125b was decreased in EBC-1R cells on qRT-PCR. B, miR-374a or miR-138 was overexpressed after treatment with miR-374a mimic or miR-138 mimic, respectively, in EBC-1R cells from 24 to 72 hours on qRT-PCR. C, treatment of EBC-1R cells with miR-138 mimic reduced ABCB1 expression, resulting in increased E-cadherin and decreased vimentin expression on Western blotting.

by IHC showed significantly longer PFS and OS by treatment with MET monoclonal antibody onartuzumab (28). Therefore, MET inhibitors may be attractive for treating NSCLC patients with overexpressed MET. Understanding the mechanism of resistance to MET inhibitors may have a clinical benefit for NSCLC patients receiving MET inhibitors.

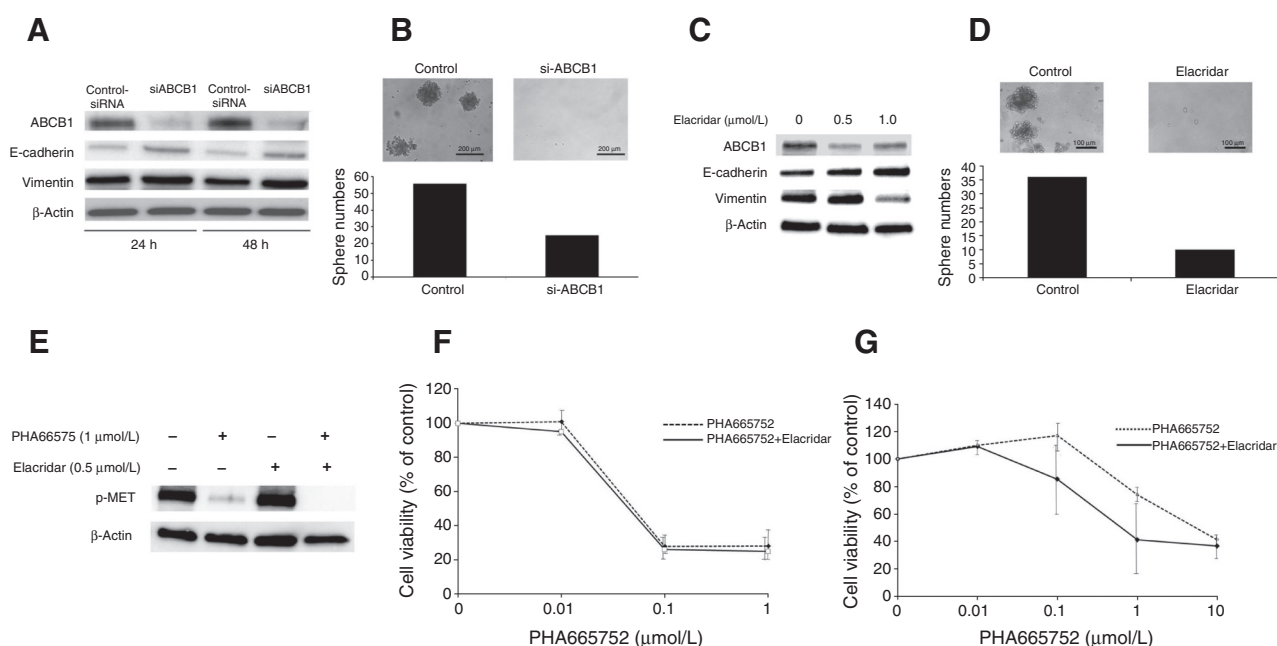
In this study, we established a MET inhibitor PHA-665752-resistant-EBC-1R cell line from the parental EBC-1 cell line. We found several acquired resistant mechanisms to MET inhibitors using EBC-1R cells. KRAS, EGFR, and FGFR2 activation were observed in EBC-1R cells. A previous study already reported that KRAS gene amplification was observed using the same EBC-1 cells as a mechanism of resistance to PHA-665752 (15). KRAS amplification may be involved in the acquired resistance to PHA-665752 as KRAS is a downstream molecule of MET. Activation of p-EGFR bypassing the need for MET signaling was observed in PHA-665752-resistant cells (14, 15). FGFR signaling alteration has been reported in several human cancers (29). EGFR and FGFR2 activation may have contributed to the resistance to PHA-665752 by a MET-independent bypass pathway in this study.

We ultimately identified that ABCB1 overexpression in EBC-1R cells was mainly involved in the acquired resistance to MET inhibitors. ABCB1 belongs to the ATP-binding cassette transporters that use the energy of ATP hydrolysis to transport substrates across cell membranes, and ABCB1 overexpression results in diminished efficacy of anticancer drugs (30). Previous studies reported that increased ABCB1 conferred resistance to chemotherapeutic agents in several cancers (31–33). ABCB1 is also closely correlated with CSC-like properties and is one of the CSC markers (23). Cells with CSC-like properties, which are characterized by the capacity for pluripotency and self-renewal, have been attracting interest as a source of cancer cells (34). The significance of CSC-like properties has been reported

in NSCLC (35, 36). EGFR-mutant NSCLC cells exhibited CSC-like properties with EMT after the failure of gefitinib treatments (23). We found that EBC-1R cells showed high levels of sphere formation and EMT phenotype. The appearance of CSC-like properties, which is associated with ABCB1 activation, may be an important mechanism of acquired resistance to MET inhibitors.

The ABCB1 inhibitor elacridar was initially developed as a multidrug reversal agent to restore sensitivity to chemotherapeutic agents in multidrug-resistant tumor cells (37). Elacridar inhibited ABCG2 as well as ABCB1 and has been used in preclinical and clinical settings (38, 39). Elacridar can also significantly increase plasma pharmacokinetics and brain distribution of several drugs, including dasatinib (40), gefitinib (41), and sunitinib (42). In this study, ABCB1 knockdown reduced the numbers of oncospheres and suppressed EMT features in EBC-1R cells. Elacridar also reversed the acquired resistance to PHA-665752 in EBC-1R cells. These findings suggest that ABCB1 overexpression, which is associated with CSC-like property and EMT features, is a critical mechanism of acquired resistance to MET inhibitors. Therefore, ABCB1 inhibition targeting the subpopulation with CSC-like property may be an attractive approach to resensitize MET inhibitor-resistant NSCLC cells to MET inhibitors. In addition, ABCB1 inhibitors may increase the concentration of MET inhibitors in EBC-1R cells, resulting in the possibility of reduced cell viability. Some portion of NSCLC cells with acquired resistance to EGFR-TKI exhibited CSC-like properties with EMT. The combination of driver gene mutation inhibitor and ABCB1 inhibition targeting CSC-like property may be an attractive strategy to NSCLC with driver gene mutation.

We found that ABCB1 expression was regulated by miR-138. miR-138 was reported to act as a tumor suppressor and serve as a therapeutic target in head and neck squamous cell carcinoma patients (43) and be involved in regulation of ABCB1

**Figure 4.**

ABCB1 inhibition overcomes the resistance to PHA-665752 in EBC-1R cells. A, RNA interference targeted to *ABCB1* resulted in marked downregulation of ABCB1 protein expression. ABCB1-siRNA increased the expression of E-cadherin from 24 to 48 hours. B, ABCB1-siRNA decreased oncosphere numbers in EBC-1R cells. C, ABCB1 inhibitor elacridar was administered to EBC-1R cells at a concentration of 0, 0.5, or 1.0 $\mu\text{mol/L}$ for 72 hours. ABCB1 expression was suppressed by elacridar at a concentration of 0.5 $\mu\text{mol/L}$. Elacridar reduced the expression of vimentin and increased the expression of E-cadherin. D, treatment with elacridar reduced oncosphere numbers. E, treatment with elacridar combined with PHA-665752 completely inhibits p-MET in EBC-1R cells. F, EBC-1 cells were incubated with various concentrations of PHA-665752 with or without 0.5 $\mu\text{mol/L}$ elacridar for 72 hours. G, EBC-1R cells were incubated with various concentrations of PHA-665752 with or without 0.5 $\mu\text{mol/L}$ elacridar for 72 hours. Each result is expressed as cell viability in the treated samples compared with the non-PHA-665752-treated sample (100%). Data, mean \pm SD from three independent experiments.

transcription (27). Therapy targeting miR-138 may be a therapeutic approach to inhibit ABCB1 in NSCLC cells.

In conclusion, ABCB1 overexpression, which was associated with CSC-like properties and EMT, may be a critical mechanism of the acquired resistance of NSCLC cells to MET inhibitors. Our study demonstrated that ABCB1 inhibition might be a novel therapeutic strategy to overcome the resistance of NSCLC cells to MET inhibitors.

Disclosure of Potential Conflicts of Interest

A. Gemma reports receiving a commercial research grant from Pfizer and has received speakers bureau honoraria from Pfizer. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: T. Sugano, M. Seike, R. Noro, A. Gemma
Development of methodology: T. Sugano, R. Noro
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Sugano, S. Nakamichi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Sugano, M. Seike, R. Noro

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Sugano, C. Soeno, M. Chiba, F. Zou, N. Nishijima, M. Matsumoto

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