CD44 Facilitates Epithelial-to-Mesenchymal Transition Phenotypic Change at Acquisition of Resistance to EGFR Kinase Inhibitors in Lung Cancer

Kenichi Suda1,2, Isao Murakami3, Hui Yu1, Jihye Kim1, Aik-Choon Tan1, Hiroshi Mizuuchi4, Leslie Rozeboom1, Kim Ellison1, Christopher J. Rivard1, Tetsuya Mitsudomi2, and Fred R. Hirsch1

Abstract

Epithelial-to-mesenchymal transition (EMT) is one of the acquired resistance mechanisms to EGFR tyrosine kinase inhibitors (TKI) in lung cancers. Because EMT is related to tumor invasion, metastases, and resistance to various treatments, it is important to prevent the emergence of EMT. However, molecular mechanism(s) underlying EMT phenotypic changes, as well as biomarker(s) that predict the emergence of EMT in EGFR-mutated lung cancers, are unclear to date. Through the comparison of expression data between isogenic lung cancer cell lines that acquired resistance to EGFR-TKI (s), we identified that high CD44 expression is related to a mesenchymal phenotype and that shRNA-mediated knockdown of CD44 reversed the EMT change. High membranous CD44 expression was identified in lesions with mesenchymal phenotype that were obtained from lung cancer patients who developed acquired resistance to gefitinib or afatinib, whereas isogenic lesions without EMT change showed negative/weak staining for CD44. Immunohistochemistry for treatment-naïve lung cancer cell lines with EGFR mutations found those that acquire resistance to EGFR-TKIs via EMT (HCC4006 and H1975 cells) had strong membranous CD44 expression compared with non-EMT-transforming lines which demonstrated negative or weak staining (Fisher exact test P value = 0.036). shRNA-mediated CD44 knockdown in HCC4006 cells prevented the emergence of EMT after chronic exposure to osimertinib. These results suggest that upregulation of CD44 facilitates EMT-phenotypic change in lung cancers with EGFR mutations when treated with EGFR-TKIs. In addition, our results suggest that CD44 can be a useful biomarker to predict the emergence of EMT upon EGFR-TKI monotherapy. Mol Cancer Ther; 17(10); 2257–65. ©2018 AACR.

Introduction

Lung cancer with EGFR somatic mutation is one of the most common molecularly defined subtypes of lung cancers. Despite initial dramatic efficacy of EGFR tyrosine kinase inhibitors (TKI) in EGFR-mutant lung cancer patients (1), emergence of acquired resistance is almost inevitable at a median of 9 to 13 months (2). To understand and to overcome the mechanisms underlying the acquired resistance, a number of studies have been performed to date. These studies identified genetic changes, such as secondary mutations in the EGFR gene (T790M and other rare mutations), gene amplifications of MET or ERBB2, or genetic aberrations that activate downstream signaling of the EGFR (PTEN loss, mutations in BRAF or NRAS, or gene copy-number gains of KRAS or NRAS; refs. 3–5), and the phenotypic changes, such as small-cell lung cancer (SCLC) transformation and epithelial-to-mesenchymal transition (EMT; refs. 3–6). EMT is often observed in cell line models with EGFR mutations that were chronically treated with an EGFR-TKI monotherapy [HCC4006 (7–12) and NCI-H1975 cells (6, 13, 14)] and also in EGFR-TKI-refractory clinical specimens (6, 15). Although RBl inactivation has been identified as part of the molecular mechanisms of SCLC transformation (16), the molecular mechanism(s) underlining EMT change is still unclear to date. In addition, there is no biomarker to predict the emergence of EMT-mediated acquired resistance in clinical practice, although a few in vitro studies have suggested strategies to prevent EMT-mediated resistance using the HCC4006 cell line model (10, 12).

Materials and Methods

Cell lines, reagents, and cell proliferation assay

All TKI-resistant human lung cancer cell lines used in this study were established in our previous studies at Aichi Cancer Center Hospital, Nagoya, Japan, in 2010 (HCC827ER, HCC827EPR, and HCC4006ER cells) or at Kindai University Faculty of Medicine, Osaka-Sayama, Japan, in 2015 (HCC827CNXR-S1 and HCC827CNXR-S4 cells). The molecular mechanism(s) of resistance to TKIs for these cells are summarized in Supplementary.
Table S1 (7, 17, 18). All the TKI-naive parental cells were the kind gift of Dr. Adi F. Gazdar (Hammon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas) in 2009 or before. The identity of the parental and descendant resistant cells was confirmed by analyzing the short tandem repeat profile as reported previously (17, 18) at the establishment of the resistant cells. Mycoplasma testing was also performed using a PCR-based mycoplasma test kit (Takara), and a passage control within 20 passages was performed for all parental and resistant cells.

All cells were cultured in RPMI1640 medium supplemented with 10% FBS and 1× penicillin/streptomycin solution (Mediatech, Inc.) at 37°C and 5% CO2. A T790M-specific EGFR-TKI, osimertinib (AZD9291), and cisplatin were purchased from Selleck Chemicals. Cell proliferation was also performed using a PCR-based mycoplasma test kit (Takara), and a passage control within 20 passages was performed for all parental and resistant cells.

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We mainly used an immunohistochemistry (IHC) technique to evaluate protein expression so that we can apply the results of the current in vitro study to future analyses for clinical formalin-fixed paraffin-embedded (FFPE) specimens. FFPE cell blocks were prepared to make a cell line tissue microarray (TMA) in our recent study (14). The TMA was sectioned at a thickness of 4 μm and mounted on charged glass slides. All staining was performed on the Benchmark XT automated stainer (Ventana Medical Systems, Inc.). Antibodies against E-cadherin [anti-E-cadherin (36) Mouse Monoclonal antibody] and vimentin [anti-Vimentin (V9) Mouse Monoclonal antibody] were purchased from Ventana Medical Systems, and the antibody against variant nonspecific CD44 (HPA005785) was purchased from Sigma-Aldrich Corporation. The staining platform utilized the Ultraview development reagents (Ventana).
Medical Systems). The specimens were evaluated using the H-score assessment as previously described, and the scoring was performed by two independent investigators (K. Suda and H. Yu).

**Comprehensive comparison of cancer-related gene expression**

The HTG EdgeSeq Oncology-Biomarker (OB) Assay (HTG Molecular Diagnostics, Inc.) was performed to compare expression levels of multiple cancer-related genes as previously described (19). Parsed data for the HTG EdgeSeq OB assay panel provided raw counts of 2,545 cancer-related genes. The data were normalized by using quantile–quantile normalization that makes the library sizes equal for all samples. To evaluate the similarity of gene expression status among samples, we used Spearman rank correlation and complete linkage to generate hierarchical clustering. We also employed negative binomial to model the count distribution in the sequencing data and conditional maximum likelihood–based exact test using edgeR (20) to identify differentially expressed genes between groups. We then computed the q-value of FDR for multiple comparisons for the genes.

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**Figure 2.**

CD44 is highly expressed in HCC827CNXR-S4 cells. A, The heatmap includes significant genes from comparison between cell lines with epithelial phenotype (HCC827 and HCC827EPR cells) and a cell line with mesenchymal phenotype (HCC827CNXR-S4 cells). The expression data for HCC827ER cells and HCC827ER cells treated with osimertinib (AZD9291) were added to the heatmap for further analysis to identify gene(s) with the most importance. B, Relative expression levels of CD44 between HCC827 cells and their daughter cells. C, IHC for CD44 in HCC827, HCC827EPR, and HCC827CNXR-S4 cells, showing elevated CD44 protein expression in HCC827CNXR-S4 cells.
Antibodies and Western blot analysis
Antibodies against E-cadherin (#3195S), vimentin (#5741S), CD44 (#3570S), and β-actin (#4970S) were purchased from Cell Signaling Technology. Total cell lysates were prepared, and immunoblotting was conducted as described previously (14).

Short hairpin–mediated knockdown
Lentiviral preparations with an shRNA that specifically targets CD44 (TRCN0000296191) or a nontargeting control were purchased from the UCD Genomics Core. Transfection was performed per the established protocol using polybrene (Sigma-Aldrich Corporation), and 10 μg/mL puromycin (Invitrogen) treatment was used to select for the transfected cells.

CD44 overexpression assay
Transfection of HCC827 parental cells with a CD44 expression lentiviral vector (ccsbBroad304_05963) or a control vector (pLX304) was performed. The lentiviral vectors were purchased from the UCD Genomics Core. Transfection was performed per the established protocol using polybrene (Sigma-Aldrich Corporation), and 10 μg/mL blasticidin (Sigma-Aldrich Corporation) treatment was used to select for the transfected cells.

Clinical specimens and IHC analysis
FFPE tumor specimens were obtained at autopsy from patients with EGFR-mutant lung cancers who developed acquired resistance to gefitinib (Patient 1; identical samples of Case 3, ref. 21) or afatinib (Patient 2); the specimens were obtained in accordance with ethical guidelines (Declaration of Helsinki), and the study was approved by an Institutional Review Board. Written-informed consent from their legal guardians was obtained. All staining was performed as described in IHC analysis for cell lines.

Results
EMT status in HCC827 and isogenic resistant cell lines
First, we screened E-cadherin and vimentin expression, representing commonly used epithelial and mesenchymal markers, in HCC827 parental cells (del E746_A750) and their isogenic resistant cell lines to EGFR-TKI(s) by IHC. As shown in Fig. 1A and Supplementary Fig. S1A and S1B, HCC827 cells and HCC827EPR cells (del E746_A750 þ T790M) showed epithelial phenotype, whereas HCC827CNXR-S4 cells (del E746_A750 þ loss of amplified EGFR-mutant allele þ MET gene amplification) showed a mesenchymal phenotype. HCC827ER cells (del E746_A750 þ MET gene amplification) and HCC827EPR cells (del E746_A750 þ T790M) showed epithelial phenotype, whereas HCC827CNXR-S4 cells (del E746_A750 þ loss of amplified EGFR-mutant allele + MET gene amplification) showed a mesenchymal phenotype. Therefore, we excluded these two cell lines from the hierarchical clustering analysis. To investigate the possibility that inactivation of EGFR together with gene amplification of MET causes EMT, we employed HCC827ER cells (del E746_A750 + MET gene amplification) and HCC827CNXR-S1 cells (del E746_A750 + T790M + MET gene amplification) showed slight decrease in E-cadherin expression which is not sufficient to conclude that these cells acquired EMT phenotype. Therefore, we excluded these two cell lines from the hierarchical clustering analysis. To investigate the possibility that inactivation of EGFR together with gene amplification of MET causes EMT, we employed HCC827ER cells (del E746_A750 + MET gene amplification). We determined this was not the case, because either shRNA-mediated EGFR knockdown (Fig. 1B) or an EGFR-TKI treatment (osimertinib; Supplementary Fig. S1A and S1B) did not

Figure 3.
Knockdown of CD44 reversed EMT changes in HCC827CNXR-S4 cells. A and B, Morphologic difference between HCC827CNXR-S4 cells transfected with control shRNA (A) and anti-CD44 shRNA (B). C, The expression of E-cadherin was increased in HCC827CNXR-S4 cells after transfection with anti-CD44 shRNA. D, The expression of vimentin was increased in HCC827 parental cells after transfection with CD44-overexpressing vector.
CD44 is overexpressed in lesions with mesenchymal phenotype after acquisition of resistance to EGFR-TKIs (Patient 1). The results of IHC staining for isogenic lesions obtained from a lung adenocarcinoma patient who acquired resistance to gefitinib monotherapy are shown. The primary tumor (left) was moderately differentiated adenocarcinoma with epithelial phenotype. The resistance mechanism of the primary lesion to gefitinib was the acquisition of T790M mutation. The metastatic lesion for right adrenal gland (right) was pleomorphic carcinoma in histology with mesenchymal phenotype. Although tumor cells in the primary tumor were negative for CD44, those in the adrenal gland metastasis had high membranous staining for CD44. Other staining data for clinical specimens are available in Supplementary Figs. S2 and S3.

CD44 is highly expressed in HCC827CNXR-S4 cells

We used the HTG EdgeSeq OB panel to compare the gene expression patterns between cell lines. The hierarchical clustering (Fig. 1C) showed that HCC827 cells and their daughter cell lines with various resistance mechanisms clustered together in contrast to another EGFR-mutated lung cancer cell line (H3255). However, among the HCC827 cell line family members, we observed that HCC827CNXR-S4 cells had distinct gene expression pattern compared with parental cells and other isogenic resistant cell lines (Fig. 1C).

We then explored genes with significant difference in expression levels between epithelial phenotype cell lines (HCC827 and HCC827EPR cells) and mesenchymal phenotype cell line (HCC827CNXR-S4 cells). The cutoff value of FDR < 0.1 by exact test identified 13 upregulated and 56 downregulated genes in HCC827CNXR-S4 cells (Fig. 2A). Among these genes, we searched for candidate gene(s) which may have important roles on EMT change using the following criteria: (1) differentially expressed in HCC827CNXR-S4 cells compared with parental cells and other isogenic resistant cell lines (Fig. 1C).

From these criteria, we identified that CD44, a cell-surface glycoprotein which is related to EMT or cancer stem cell phenotype in several cancer types (22), was highly expressed in HCC827CNXR-S4 cells compared with other isogenic HCC827 cell lines (Fig. 2B and C). We also identified, through an analysis of publically available gene expression data, that a resistant cell line with an EMT phenotype which acquired resistance to gefitinib (HCC827-GR-high2 cells) harbored high CD44 expression (4.76 times compared with parental cells; ref. 8). Therefore, we focused additional efforts on evaluating CD44 related to EMT changes.

CD44 knockout reversed EMT phenotype in HCC827CNXR-S4 cells

To evaluate the role of CD44 in EMT changes of HCC827CNXR-S4 cells, we transfected lentiviral preparations with an shRNA that specifically targets CD44 as compared with a nontargeting control. shRNA-mediated knockdown of CD44 conferred parental cell-like appearance and reversion of EMT in HCC827CNXR-S4 cells (Fig. 3A–C). However, CD44 knockdown did not restore the sensitivity to EGFR-TKIs in HCC827CNXR-S4 cells (Supplementary Fig. S1C) probably due to survival signaling from amplified MET gene, whereas MET-TKI monotherapy was effective in this cell line (18).

CD44 overexpression increased vimentin expression in HCC827 parental cells

To evaluate the role of CD44 further, we transfected lentiviral preparations with an open reading frame for CD44 as compared with a nontargeting control vector. Although the forced expression of CD44 did not affect E-cadherin expression, HCC827 cells with CD44 overexpression showed higher vimentin expression compared with the cells transfected with control vector (Fig. 3D). Through a growth-inhibitory assay, we observed that CD44 overexpression did not have any impact on the sensitivity of EGFR-TKIs in HCC827 parental cells (Supplementary Fig. S1D).

CD44 is highly expressed in TKI-refractory specimens with mesenchymal phenotype

To validate our in vitro findings, we analyzed tumor specimens obtained from patients who developed TKI-refractory lesions with both epithelial phenotype and mesenchymal phenotype (isogenic lesions). In addition to the expression levels of E-cadherin, the morphology of tumor cells was distinct between these two types of lesions as mesenchymal lesions were diagnosed as pleomorphic carcinomas (Fig. 4; Supplementary Figs. S2 and S3).
Evaluating IHC staining for CD44, we found that the membranous expression of CD44 was much higher in lesions with mesenchymal phenotype than those with epithelial phenotype (Fig. 4; Supplementary Figs. S2 and S3). Although these findings are based on two cases and may not represent all EMT-mediated resistance, these data are comparable with our in vitro findings.

Innate CD44 expression status in lung cancer cells with EGFR mutations

We next evaluated CD44 expression in treatment-naive lung cancer cell lines comparing those that often acquire resistance to EGFR-TKIs by EMT (HCC4006 and NCI-H1975 cells) versus those that commonly employ other resistance mechanisms. As shown in Fig. 5, cell lines “destined” to develop EMT (HCC4006 and NCI-H1975 cells) expressed the highest levels of CD44 compared with other cell lines (Fisher exact test \( P = 0.036 \)). Data from the Cancer Cell Line Encyclopedia support the results of IHC analysis, showing that HCC4006 and NCI-H1975 cells harbored the highest expression of CD44 among lung cancer cell lines with EGFR mutations. These data support the hypothesis that CD44 expression, prior to EGFR-TKI therapy, may predict the emergence of EMT-mediated acquired resistance to EGFR-TKIs.

CD44 knockdown prevents EMT-mediated resistance to osimertinib in HCC4006 cells

To further investigate this hypothesis, we generated HCC4006 cells with shRNA-mediated CD44 knockdown (Fig. 6A) and chronically treated these cells with osimertinib as previously
described (3). As we expected, the osimertinib-resistant cells, which were designated as HCC4006 shCD44-OR cells (Fig. 6B), did not acquire EMT phenotype upon acquisition of resistance to osimertinib (Fig. 6C). The osimertinib resistance mechanism in HCC4006 shCD44-OR cells was neither secondary mutation in the \( \text{EGFR} \) exon 20 (including C797S) nor \( \text{MET} \) -mediated ones. Further experiments to identify the resistance mechanism(s) in that cell line are ongoing.

**Discussion**

Chronically treated \( \text{EGFR} \)-mutant lung cancer cell lines with \( \text{EGFR-TKIs} \) for 3 to 9 months have been widely used as \( \text{in vitro} \) models of acquired resistance to \( \text{EGFR-TKIs} \). These studies have revealed that each cell line has its “favorite” resistance mechanism, including \( \text{MET} \) gene amplification in HCC827 cells, T790M gatekeeper mutation in PC9 cells, and EMT-like change in HCC4006 and NCI-H1975 cells (2). In addition, these studies have shown that such “destiny” can be changed by upfront combination therapies which cotarget \( \text{EGFR} \) and the respective “favorite” resistance mechanism (10, 12, 17).

EMT has long been recognized as playing a major role in cancer progression and metastasis (23), as well as one of acquired resistance mechanisms to \( \text{EGFR-TKIs} \) (6, 7). Importantly, in vitro studies have shown that acquired resistant cells to \( \text{EGFR-TKIs} \) via EMT also demonstrated cross resistance to some cytotoxic agents (8, 24), which are the current gold-standard in second-line therapy in patients without detectable T790M mutations. To prevent the emergence of EMT after \( \text{EGFR-TKI} \) treatment failure, \( \text{in vitro} \) studies which used the HCC4006 cell line model have suggested combination therapies of \( \text{EGFR-TKI} \) with either the TGF\( \beta \) receptor inhibitor (10) or dasatinib (12). Both studies showed that these combination therapies led HCC4006 cells to develop T790M mutation which is treatable using osimertinib. It is of note, however, that both studies demonstrated that these combination therapies were not able to reverse EMT in HCC4006 \( \text{EGFR-TKI} \)-resistant cells after the cells once acquired mesenchymal phenotype against \( \text{EGFR-TKI} \) monotherapy. These results emphasize the importance of upfront combination therapy to prevent the emergence of EMT rather than the strategy of resistance mechanism-based second-line therapies.

However, in clinical practice, there are no pretreatment biomarkers that may predict emergence of EMT upon acquisition of resistance to \( \text{EGFR-TKI} \) monotherapies. In this study, from the comprehensive analysis of gene expression data in acquired resistant isogenic cells to \( \text{EGFR-TKIs} \) (cells with epithelial phenotype vs. those with mesenchymal phenotype), we identified that \( \text{CD44} \) expression is correlated with acquisition of EMT change, which was also confirmed in isogenic clinical specimens (lesions with epithelial phenotype vs. those with mesenchymal phenotype). In addition, we demonstrated that high \( \text{CD44} \) expression, prior to treatment, can be a biomarker that predicts the emergence of EMT.
studies have shown that CD44 was highly expressed in cell lines with EMT change which were generated to acquire resistance to ionizing radiation therapy (26) or to cisplatin (27). CD44 has multiple variants, and CD44s is one of them that has significant roles in EMT in many cancer types. Although we did not perform further variant analysis, our results suggest that the expression of variant nonspecific CD44, which is analyzable with an IHC technique, can be a biomarker to predict the emergence of EMT-mediated acquired resistance to EGFR-TKIs.

In this study, we observed that CD44 expression was upregulated in EGFR-TKI acquired resistant cells with mesenchymal phenotype and EGFR-TKI acquired resistant lesions with mesenchymal phenotype. At the same time, we also found that some of treatment-naive parental cell lines (HCC4006 and NCI-H1975 cells), which show epithelial phenotype but tend to acquire EMT upon acquisition of resistance to EGFR-TKIs, have high expression levels of CD44. These results indicate that CD44 plays an important role in the process of EMT phenotype acquisition, but high CD44 expression alone is not sufficient to convert epithelial cells to mesenchymal cells.

As described above, the blockade of TGFβ signaling or Src (one of the targets of dasatinib) pathways prevented HCC4006 cells from developing the EMT change after chronic treatment with EGFR-TKI (10, 12). In this study, we also observed that shRNA-mediated knockdown of CD44 prevented HCC4006 cells from developing the mesenchymal phenotype after chronic osimertinib exposure. Because CD44 is reported to activate TGFβ/Smad signaling through the miR-106b family (28), and CD44 is also known to activate Src-mediated signaling (29), it is possible that these phenomena in HCC4006 cell line experiments are all based on inhibition of CD44 and its downstream signaling.

In summary, we demonstrated that CD44 is correlated with mesenchymal phenotype in cell line models and clinical specimens upon acquisition of resistance to EGFR-TKIs. Our results also indicate that prior to treatment, CD44 levels can be a useful biomarker to predict the emergence of EMT when treated with EGFR-TKI monotherapies.

Disclosure of Potential Conflicts of Interest
T. Mitsudomi reports receiving other commercial research support from AstraZeneca and Chugai; has received honoraria from the speakers’ bureau of AstraZeneca, Chugai, and Roche; and is a consultant/advisory board member for AstraZeneca, Chugai, Roche, and Clovis Oncology. F.R. Hirsch reports receiving other commercial research support from Genentech/Roche, BMS, Lilly, Bayer, Amgen, and Ventana/Roche, and is a consultant/advisory board member for Genentech/Roche, Pfizer, BMS, Lilly, Merck, and Ventana/Roche. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: K. Suda, I. Murakami, K. Ellison
Development of methodology: K. Suda, K. Ellison
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Suda, I. Murakami, K. Ellison, T. Mitsudomi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Suda, I. Murakami, H. Yu, J. Kim, A.-C. Tan, C.J. Rivard
Writing, review, and/or revision of the manuscript: K. Suda, J. Kim, A.-C. Tan, H. Mizuuchi, L. Rozeboom, K. Ellison, C.J. Rivard, T. Mitsudomi, F.R. Hirsch
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Mizuuchi, L. Rozeboom, C.J. Rivard
Study supervision: K. Suda, I. Murakami, T. Mitsudomi, F.R. Hirsch

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