CD44 facilitates epithelial to mesenchymal transition phenotypic change at acquisition of resistance to EGFR kinase inhibitors in lung cancer

Kenichi Suda[1,2], Isao Murakami[3], Hui Yu[1], Jihye Kim[1], Aik-Choon Tan[1], Hiroshi Mizuuchi[4], Leslie Rozeboom[1], Kim Ellison[1], Christopher J. Rivard[1], Tetsuya Mitsudomi[2], and Fred R. Hirsch[1*]

[1]Division of Medical Oncology, University of Colorado Anschutz Medical Campus, 12801 E. 17th Ave. RC-1 South, Rm 8402J, Aurora, Colorado 80045, USA; [2]Division of Thoracic Surgery, Department of Surgery, Kindai University Faculty of Medicine, 377-2 Ohno-higashi, Osaka-Sayama; [3]Department of Respiratory Medicine, Higashi-Hiroshima Medical Center, 513 Jike, Saijo-Cho, Higashi-Hiroshima; [4]Department of Thoracic Surgery, Kitakyushu Municipal Medical Center, 1-1 Bashaku 2, Kokura-Kita, Kitakyushu, JAPAN

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*** Address for Correspondence:**

Fred R. Hirsch

Professor of Medicine and Pathology,

Pia and Fred R. Hirsch Endowed Chair

University of Colorado Cancer Center

12801 E. 17th Avenue, MS:8117, Building RC1 south, Room 8119, Aurora CO 80045

Phone: (303) 724-6858

Fax: (303) 724-3889

e-mail: fred.hirsch@ucdenver.edu

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Abstract:

Epithelial to mesenchymal transition (EMT) is one of the acquired resistance mechanisms to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) in lung cancers. Since 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 sh-RNA-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, while 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 to non-EMT transforming lines which demonstrated negative or weak staining (Fisher's 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.

Introduction

Lung cancer with *epidermal growth factor receptor* (*EGFR*) somatic mutation is one of the most common molecularly defined subtypes of lung cancers. Despite initial dramatic efficacy of EGFR tyrosine kinase inhibitors (TKIs) in *EGFR*-mutant lung cancer patients (1), emergence of acquired resistance is almost inevitable at a median of 9 – 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*) (3-5), and the phenotypic changes such as small cell lung cancer (SCLC) transformation and epithelial to mesenchymal transition (EMT) (2, 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 RB1 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 1 (7,17,18). All the TKI-naïve 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 (STR) 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, Shiga, Japan), 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% fetal bovine serum (FBS) and 1× penicillin / streptomycin solution (Mediatech, Inc., Manassas, VA) at 37°C and 5% CO_2. A T790M-specific EGFR-TKI, osimertinib (AZD9291), and cisplatin were purchased from Selleck Chemicals (Houston, TX). Cell proliferation was measured using the PrestoBlue Cell Viability Reagent (Life Technologies, Frederick, MD), according to the manufacturer's instructions as described previously (14). Experiments were repeated 2 -3 times to confirm the

results. All experiments using acquired resistance cells were performed following removal of drug(s) to avoid the direct effects of the drug(s).

TMA preparation, antibodies and immunohistochemistry (IHC) analysis for cell lines

We mainly used an 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., Tucson, AZ). Antibodies against E-cadherin (anti-E-cdherin (36) Mouse Monoclonal antibody) and vimentin (anti-Vimentin (V9) Mouse Monoclonal antibody) were purchased from Ventana Medical Systems, and the antibody against variant non-specific CD44 (HPA005785) was purchased from Sigma-Aldrich Corporation (St. Louis, MO). 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 (KS and HY).

Comprehensive comparison of cancer-related gene expression

The HTG EdgeSeq Oncology-Biomarker (OB) Assay (HTG Molecular Diagnostics, Inc., Tucson, AZ) 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 binominal to model the count distribution in the sequencing data and CML (conditional maximum likelihood)-based exact test using edgeR (20) to identify differentially expressed genes between groups. We then computed the q-value of false discovery rate for multiple comparisons for the genes.

Antibodies and western blot analysis

Antibodies against E-cadherin (#3195S), vimentin (#5741S), CD44 (#3570S), and β-actin (#4970S) were purchased from Cell Signaling Technology (Danvers, MA). Total cell lysates were prepared, and immunoblotting was conducted as described previously (14).

Short hairpin-mediated knockdown

Lentiviral preparations with a short hairpin RNA (shRNA) that specifically target CD44 (TRCN0000296191) or a non-targeting 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, Carlsbad, CA) 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 were 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, respectively, 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. 1A-B, HCC827 cells and

HCC827EPR cells (del E746_A750 + T790M) showed epithelial phenotype, whereas HCC827CNXR-S4 cells (del E746_A750 + loss of amplified *EGFR* mutant allele + *MET* gene amplification) showed a mesenchymal phenotype. 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* cause EMT, we employed HCC827ER cells (del E746_A750 + *MET* gene amplification). We determined this was not the case, since either shRNA mediated EGFR knockdown (Fig. 1B) or an EGFR-TKI treatment (osimertinib, Supplementary Fig. 1A-B) did not confer EMT in HCC827ER cells. Therefore, we further analyzed the molecular mechanism(s) which are involved in this phenotypic change in HCC827CNXR-S4 cells.

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 to 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 cut-off value of FDR (false discovery rate) < 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 to HCC827ER cells with/without osimertinib treatment, and 2) gene(s) related to EMT in the literature. 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 to parental cells) (8). Therefore we focused additional efforts on evaluating CD44 related to EMT changes.

CD44 knockdown 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 to a non-targeting 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. 1C) probably due to survival signaling from amplified *MET* gene, while 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 to a non-targeting 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. 1D).

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 were distinct between these two types of lesions as mesenchymal lesions were diagnosed as pleomorphic carcinomas (Fig. 4 and Supplementary Fig. 2 and 3). 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 and Supplementary Fig. 2 and 3). Although these findings are based on two cases and may not represent all EMT-mediated resistance, these data are comparable to our *in vitro* findings.

Innate CD44 expression status in lung cancer cells with *EGFR* mutations

We next evaluated CD44 expression in treatment-naïve lung cancer cell lines comparing those that often acquire resistance to EGFR-TKIs by EMT (HCC4006 and NCI-H1975 cells) vs. those that commonly employ other resistance mechanism(s). As shown in Fig. 5, cell lines "destined" to develop EMT (HCC4006 and NCI-H1975 cells) expressed the highest levels of CD44 compared to other cell lines (Fisher's exact test $p = 0.036$). Data from the Cancer Cell Line Encyclopedia (CCLE) supports 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 *EGFR* exon 20 (including C797S) or *MET*-mediated ones. Further experiments to identify the resistance mechanism(s) in that cell line are ongoing.

Discussion

Chronically treated *EGFR*-mutant lung cancer cell lines with EGFR-TKIs for 3 - 9 months have been widely used as *in vitro* models of acquired resistance to EGFR-TKIs. These studies have revealed that each cell line has its “favorite” resistance mechanism, including *MET* gene amplification in HCC827 cells, T790M gate-keeper 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 co-target 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 EGFR-TKIs (6, 7). Importantly, *in vitro* studies have shown that acquired resistant cells to 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 EGFR-TKI treatment failure, *in vitro* studies which used the HCC4006 cell line model have suggested combination therapies of

EGFR-TKI with either the TGFβ 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 EGFR-TKI resistant cells after the cells once acquired mesenchymal phenotype against EGFR-TKI monotherapy. These results emphasize the importance of up-front 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 pre-treatment biomarkers that may predict emergence of EMT upon acquisition of resistance to EGFR-TKI monotherapies. In this study, from the comprehensive analysis of gene expression data in acquired resistant isogenic cells to EGFR-TKIs (cells with epithelial phenotype vs. those with mesenchymal phenotype), we identified that 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 CD44 expression, prior to treatment, can be a biomarker that predicts the emergence of EMT.

CD44 is a complex / multifunctional transmembrane glycoprotein which was initially identified as a receptor for hyaluronic acid and a lymphocyte-homing receptor as well as a regulator of cell adhesion, angiogenesis, inflammation, and tumor development (22). CD44 is also reported to promote EMT in many cancer types, including colon, gastric, pancreatic, prostate, and liver cancers (22), as

well as in lung cancers (25). In addition, several *in vitro* 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 non-specific 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 naïve-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. Since 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.

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References

1. Hirsch FR, Suda K, Wiens J, Bunn PA, Jr. New and emerging targeted treatments in advanced non-small-cell lung cancer. Lancet 2016;388(10048):1012-24.
2. Suda K, Mizuuchi H, Maehara Y, Mitsudomi T. Acquired resistance mechanisms to tyrosine kinase inhibitors in lung cancer with activating epidermal growth factor receptor mutation-diversity, ductility, and destiny. Cancer Metastas Rev 2012;31(3-4):807-14.

3. Suda K, Tomizawa K, Osada H, Maehara Y, Yatabe Y, Sekido Y, et al. Conversion from the "oncogene addiction" to "drug addiction" by intensive inhibition of the EGFR and MET in lung cancer with activating EGFR mutation. Lung Cancer 2012;76(3):292-9.
4. Ohashi K, Sequist LV, Arcila ME, Moran T, Chmielecki J, Lin YL, et al. Lung cancers with acquired resistance to EGFR inhibitors occasionally harbor BRAF gene mutations but lack mutations in KRAS, NRAS, or MEK1. Proc Natl Acad Sci U S A 2012;109(31):E2127-33.
5. Eberlein CA, Stetson D, Markovets AA, Al-Kadhimi KJ, Lai Z, Fisher PR, et al. Acquired Resistance to the Mutant-Selective EGFR Inhibitor AZD9291 Is Associated with Increased Dependence on RAS Signaling in Preclinical Models. Cancer Res 2015;75(12):2489-500.
6. Sequist LV, Waltman BA, Dias-Santagata D, Digumarthy S, Turke AB, Fidias P, et al. Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. Science Transl Med 2011;3(75):75ra26.
7. Suda K, Tomizawa K, Fujii M, Murakami H, Osada H, Maehara Y, et al. Epithelial to Mesenchymal Transition in an Epidermal Growth Factor Receptor-Mutant Lung Cancer Cell Line with Acquired Resistance to Erlotinib. J Thorac Oncol 2011;6(7):1152-61.
8. Shien K, Toyooka S, Yamamoto H, Soh J, Jida M, Thu KL, et al. Acquired Resistance to EGFR Inhibitors Is Associated with a Manifestation of Stem Cell-like Properties in Cancer Cells. Cancer Res 2013;73(10):3051-61.
9. Hashida S, Yamamoto H, Shien K, Miyoshi Y, Ohtsuka T, Suzawa K, et al. Acquisition of cancer stem cell-like properties in non-small cell lung cancer with acquired resistance to afatinib. Cancer Sci 2015;106(10):1377-84.
10. Soucheray M, Capelletti M, Pulido I, Kuang Y, Paweletz CP, Becker JH, et al. Intratumoral heterogeneity in EGFR mutant NSCLC results in divergent resistance mechanisms in response to EGFR tyrosine kinase inhibition. Cancer Res 2015;75(20):4372-83.
11. Yoshida T, Song L, Bai Y, Kinose F, Li J, Ohaegbulam KC, et al. ZEB1 Mediates Acquired Resistance to the Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitors in Non-Small Cell Lung Cancer. PloS One. 2016;11(1):e0147344.
12. Sesumi Y, Suda K, Mizuuchi H, Kobayashi Y, Sato K, Chiba M, et al. Effect of dasatinib on EMT-mediated-mechanism of resistance against EGFR inhibitors in lung cancer cells. Lung Cancer 2017;104:85-90.
13. Ware KE, Hinz TK, Kleczko E, Singleton KR, Marek LA, Helfrich BA, et al. A mechanism of resistance to gefitinib mediated by cellular reprogramming and the acquisition of an FGF2-FGFR1 autocrine growth loop. Oncogenesis 2013;2:e39.
14. Suda K, Rozeboom L, Rivard C, Yu H, Ellison K, Melnick MA, et al. Therapy-induced E-cadherin downregulation alters expression of programmed death ligand-1 in lung cancer cells. Lung Cancer 2017;109:1-8.
15. Uramoto H, Shimokawa H, Hanagiri T, Kuwano M, Ono M. Expression of selected gene for acquired drug resistance to EGFR-TKI in lung adenocarcinoma. Lung Cancer 2011;73(3):361-5.

16. Niederst MJ, Sequist LV, Poirier JT, Mermel CH, Lockerman EL, Garcia AR, et al. RB loss in resistant EGFR mutant lung adenocarcinomas that transform to small-cell lung cancer. Nat Commun 2015;6:6377.
17. Suda K, Murakami I, Katayama T, Tomizawa K, Osada H, Sekido Y, et al. Reciprocal and complementary role of MET amplification and EGFR T790M mutation in acquired resistance to kinase inhibitors in lung cancer. Clin Cancer Res 2010;16(22):5489-98.
18. Mizuuchi H, Suda K, Murakami I, Sakai K, Sato K, Kobayashi Y, et al. Oncogene swap as a novel mechanism of acquired resistance to epidermal growth factor receptor-tyrosine kinase inhibitor in lung cancer. Cancer Sci 2016;107(4):461-8.
19. Suda K, Murakami I, Yu H, Kim J, Ellison K, Rivard CJ, et al. Heterogeneity in Immune Marker Expression after Acquisition of Resistance to EGFR Kinase Inhibitors: Analysis of a Case with Small Cell Lung Cancer Transformation. J Thorac Oncol. 2017;12(6):1015-20.
20. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 2010;26(1):139-40.
21. Suda K, Murakami I, Sakai K, Tomizawa K, Mizuuchi H, Sato K, et al. Heterogeneity in resistance mechanisms causes shorter duration of epidermal growth factor receptor kinase inhibitor treatment in lung cancer. Lung Cancer 2016;91:36-40.
22. Xu H, Tian Y, Yuan X, Wu H, Liu Q, Pestell RG, et al. The role of CD44 in epithelial-mesenchymal transition and cancer development. OncoTargets Ther 2015;8:3783-92.
23. De Craene B, Berx G. Regulatory networks defining EMT during cancer initiation and progression. Nat Rev Cancer 2013;13(2):97-110.
24. Mizuuchi H, Suda K, Sato K, Tomida S, Fujita Y, Koyabashi Y, et al. Collateral chemoresistance to anti-microtubule agents in a lung cancer cell line with acquired resistance to erlotinib. PloS One. 2015;19(4):e0123901.
25. Larsen JE, Nathan V, Osborne JK, Farrow RK, Deb D, Sullivan JP, et al. ZEB1 drives epithelial-to-mesenchymal transition in lung cancer. J Clin Invest 2016;126(9):3219-35.
26. Gomez-Casal R, Bhattacharya C, Ganesh N, Bailey L, Basse P, Gibson M, et al. Non-small cell lung cancer cells survived ionizing radiation treatment display cancer stem cell and epithelial-mesenchymal transition phenotypes. Mol Cancer 2013;12(1):94.
27. Barr MP, Gray SG, Hoffmann AC, Hilger RA, Thomale J, O'Flaherty JD, et al. Generation and characterisation of cisplatin-resistant non-small cell lung cancer cell lines displaying a stem-like signature. PloS One 2013;8(1):e54193.
28. Yu D, Shin HS, Lee YS, Lee YC. miR-106b modulates cancer stem cell characteristics through TGF-beta/Smad signaling in CD44-positive gastric cancer cells. Lab Invest 2014;94(12):1370-81.
29. Nam K, Oh S, Lee KM, Yoo SA, Shin I. CD44 regulates cell proliferation, migration, and invasion via modulation of c-Src transcription in human breast cancer cells. Cell Signal 2015;27(9):1882-94.

Figure legends

Figure 1. EMT status in HCC827 lung cancer cells and their EGFR-TKI resistant daughter cell lines. A, HCC827 parental cells and HCC827EPR cells (T790M mutation) demonstrated an epithelial phenotype, while HCC827CNXR-S4 cells (EGFR loss plus *MET* gene amplification) harbored a mesenchymal phenotype. B, EGFR knockdown in HCC827ER cells (*MET* gene amplification), which mimics HCC827CNXR-S4 cells, did not confer EMT changes. C, Hierarchical clustering for HTG EdgeSeq OB panel data revealed that HCC827 cells and their daughter cell lines clustered together, while HCC827CNXR-S4 cells were distinct among these cell lines.

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, Immunohistochemistry for CD44 in HCC827, HCC827EPR, and HCC827CNXR-S4 cells, showing elevated CD44 protein expression in HCC827CNXR-S4 cells.

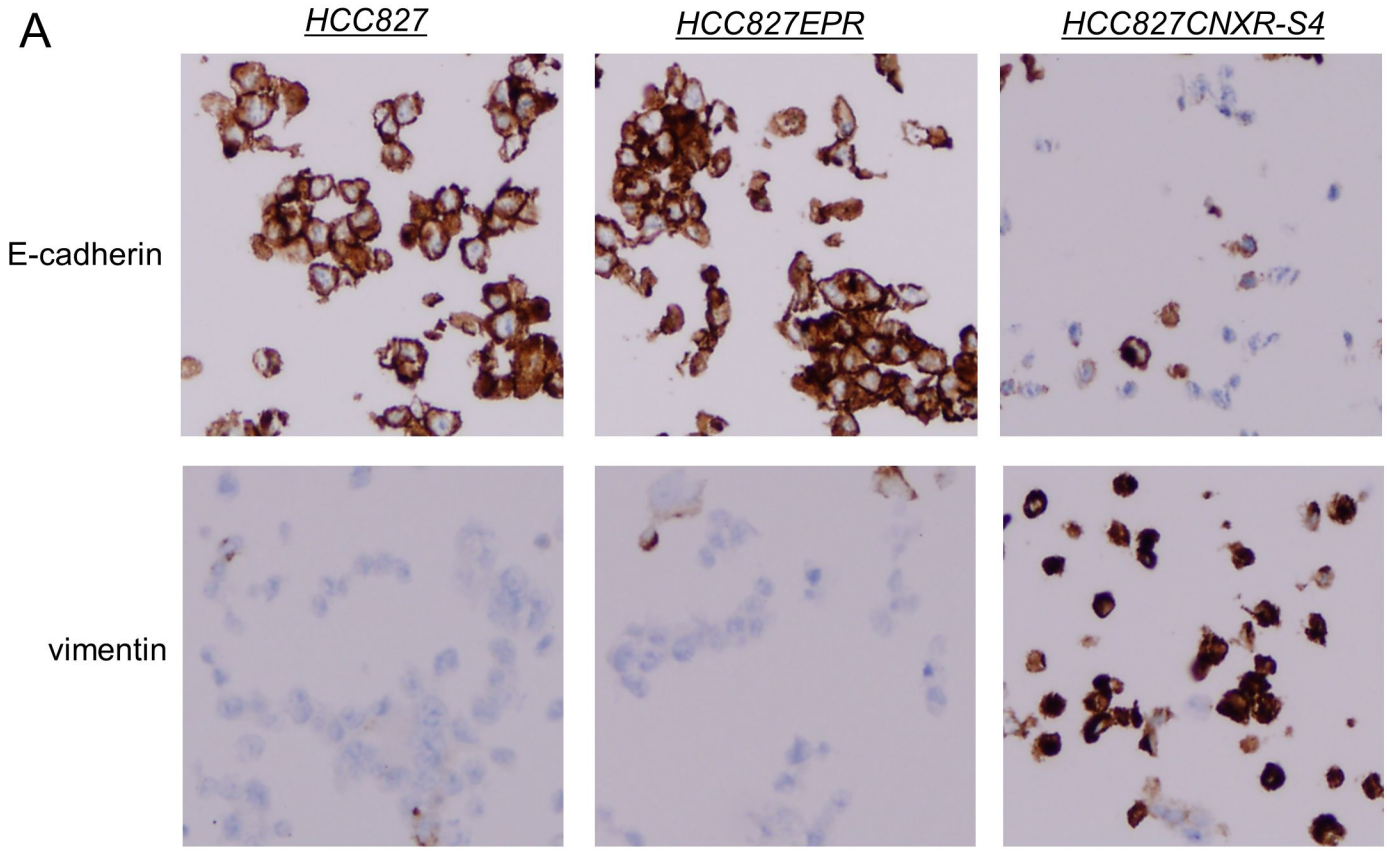
Figure 3. Knockdown of CD44 reversed EMT changes in HCC827CNXR-S4 cells. A and B, Morphological difference between HCC827CNXR-S4 cells

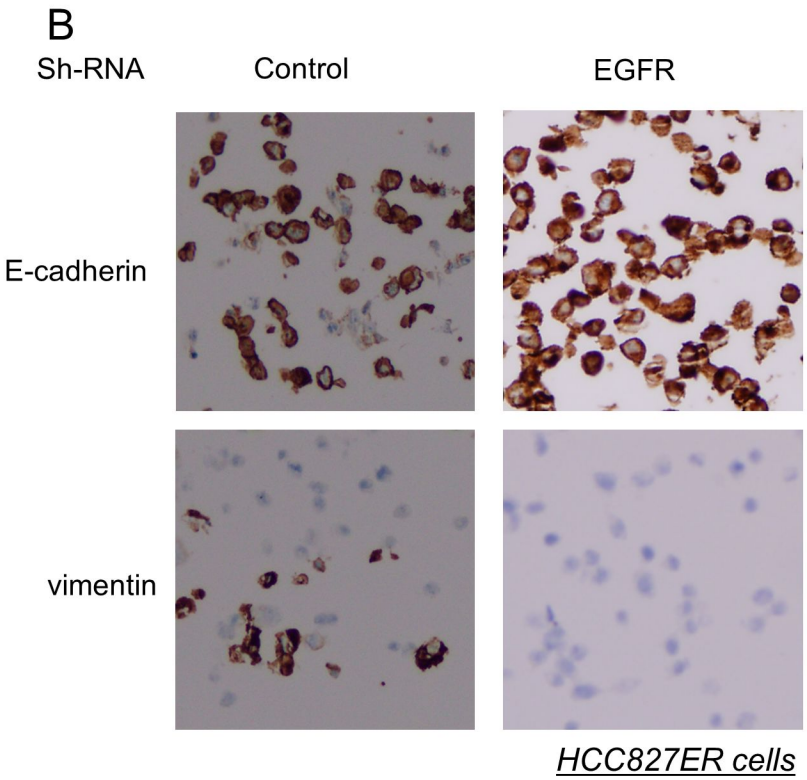
transfected with control shRNA (A) and anti-CD44 shRNA (B). C, The expression of E-cadherin was increased in HCC827CNXR-S4 cells after transfection with anti-CD44 shRNA. D, The expression of vimentin was increased in HCC827 parental cells after transfection with CD44 overexpressing vector.

Figure 4. 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. While 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 Fig. 2 and 3.

Figure 5. The results of IHC staining for CD44 in treatment-naïve lung cancer cell lines with *EGFR* mutations. The staining pattern was heterogeneous in some cell lines, however only the cell lines which often acquire resistance to EGFR-TKIs by EMT (HCC4006 and H1975 cells) showed strong (3+) expression of CD44.

Figure 6. Establishment of osimertinib resistant cells from HCC4006 cells after shRNA-mediated CD44 knockdown. A, Western blotting revealed decreased expression of CD44 in CD44 shRNA transfected cells. B, HCC4006 shCD44-OR cells showed insensitivity to osimertinib compared to HCC4006 cells transfected with CD44 shRNA (HCC4006 shCD44). HCC4006 shCD44-OR cells were established from HCC4006 shCD44 cells via chronic treatment with osimertinib. C, HCC4006 shCD44-OR cells (marked with *) harbored high E-cadherin expression level identical to HCC4006 parental cells. The data of HCC4006ER (erlotinib resistant) cells with EMT phenotypic changes that were established from HCC4006 cells via chronic treatment with erlotinib is shown for comparison.





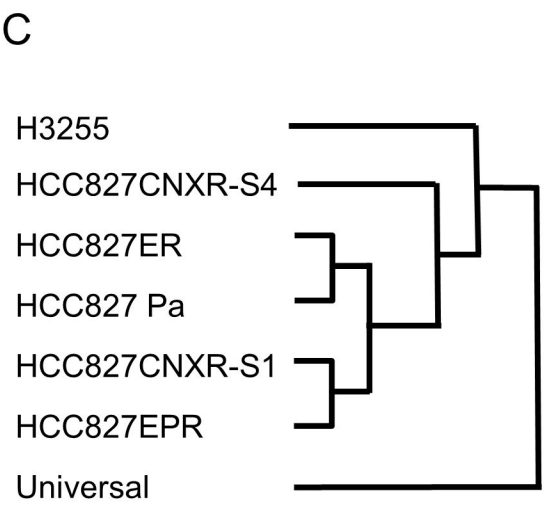
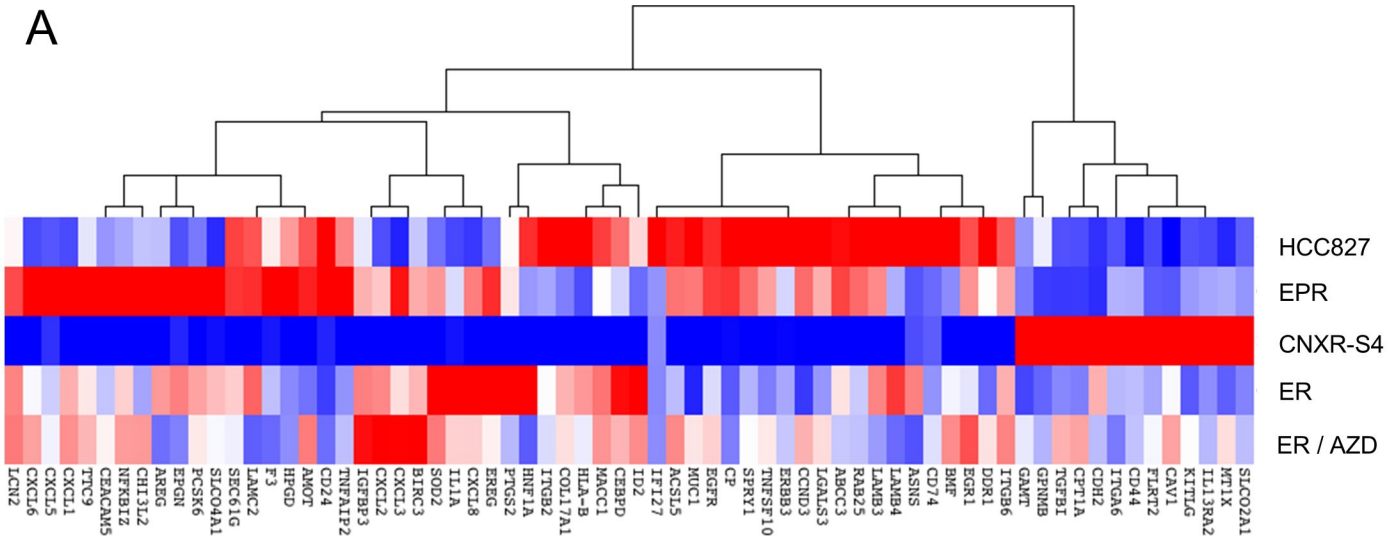
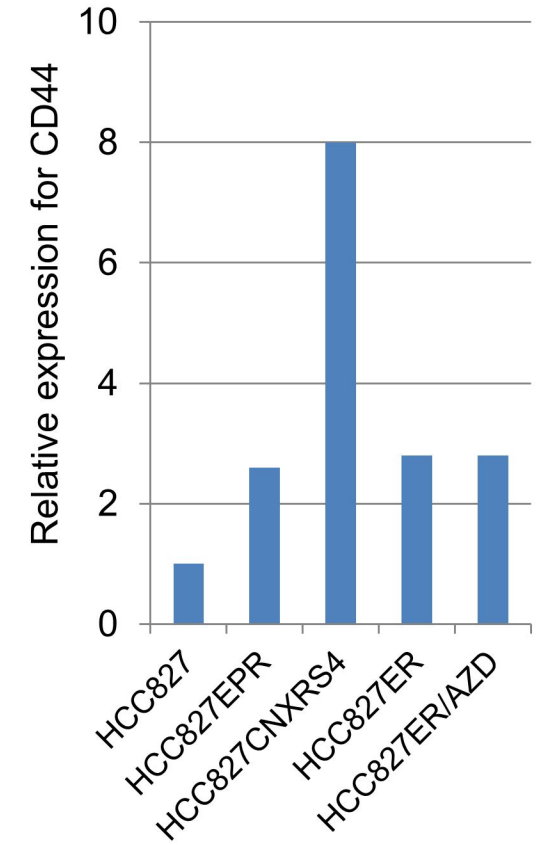


Figure 1





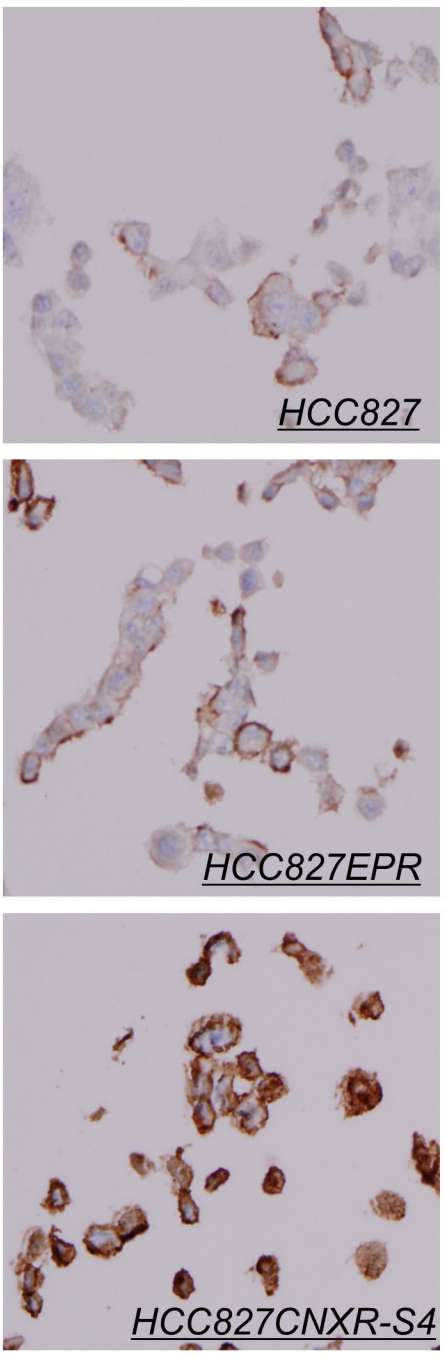
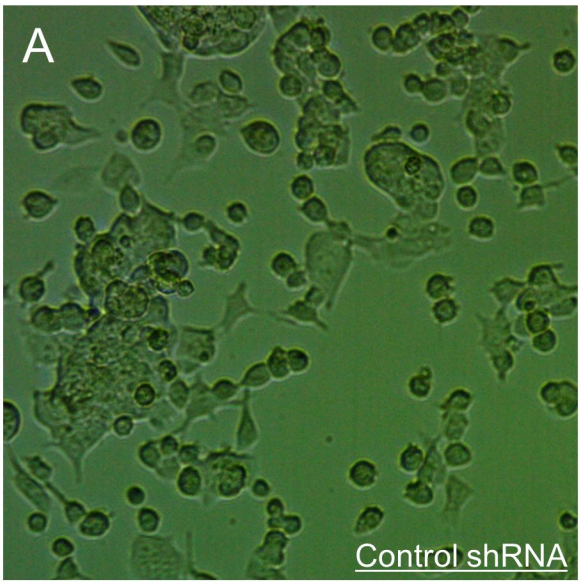
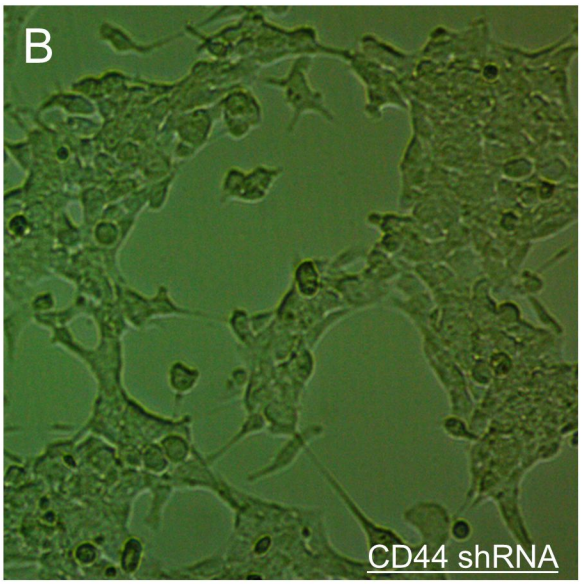
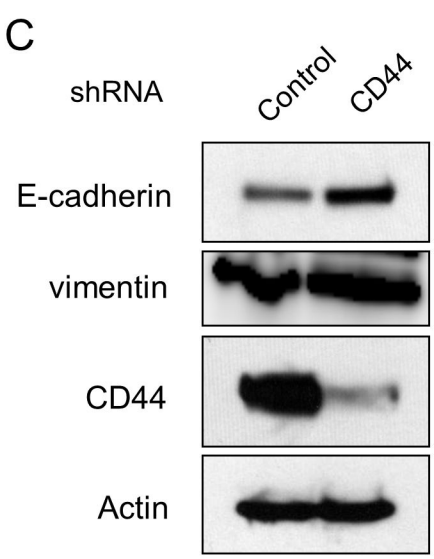


Figure 2







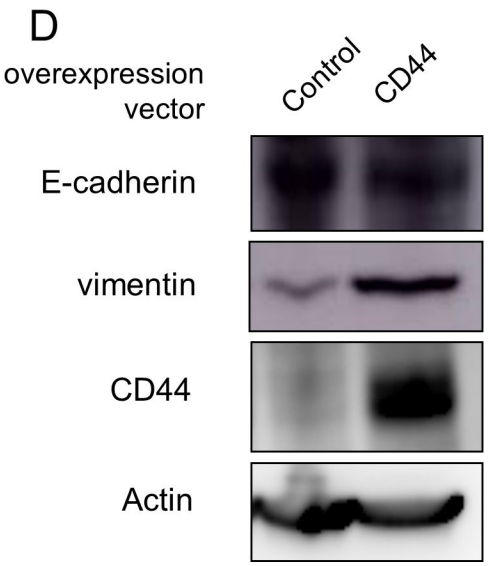


Figure 3

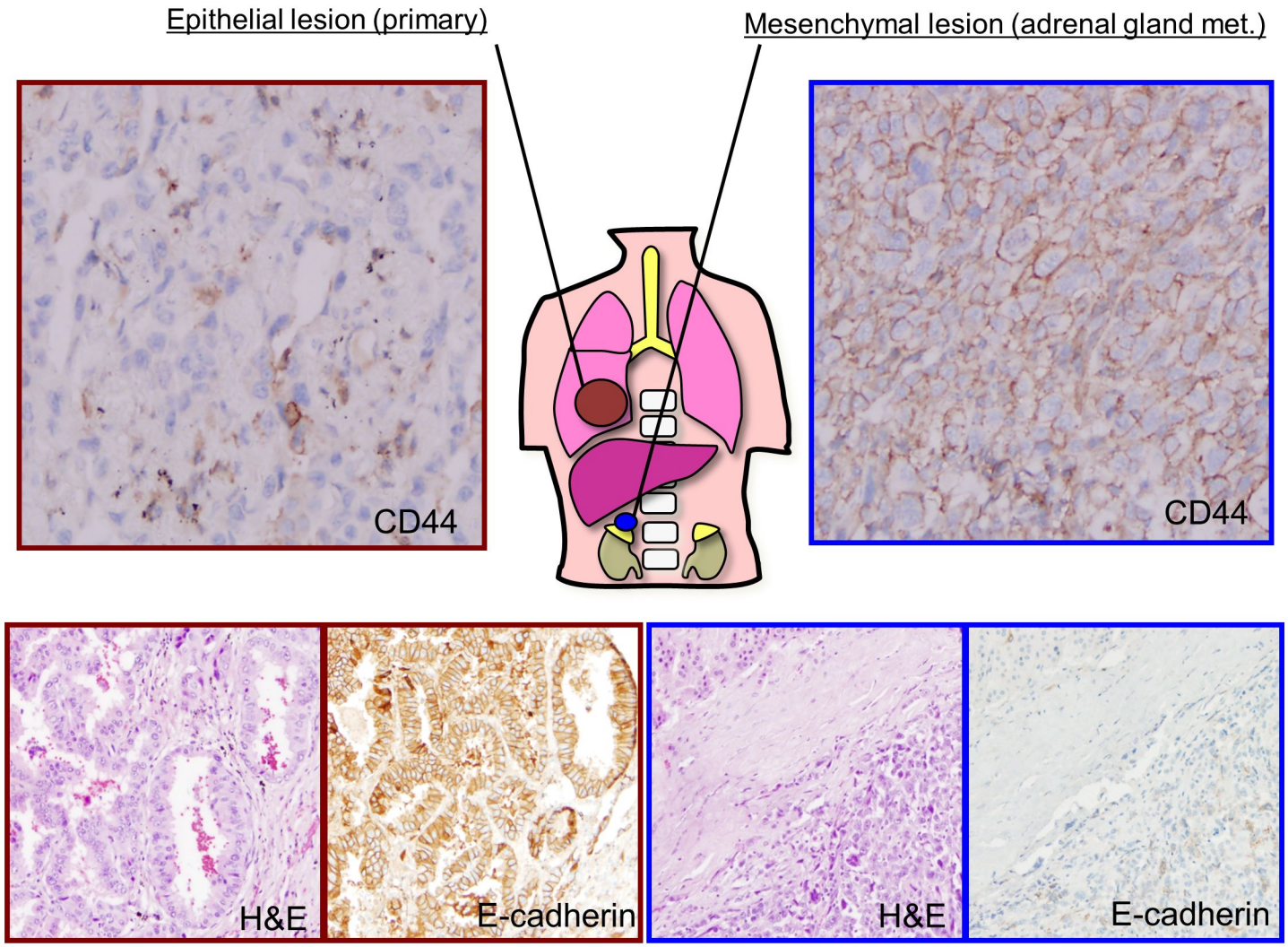


Figure 4

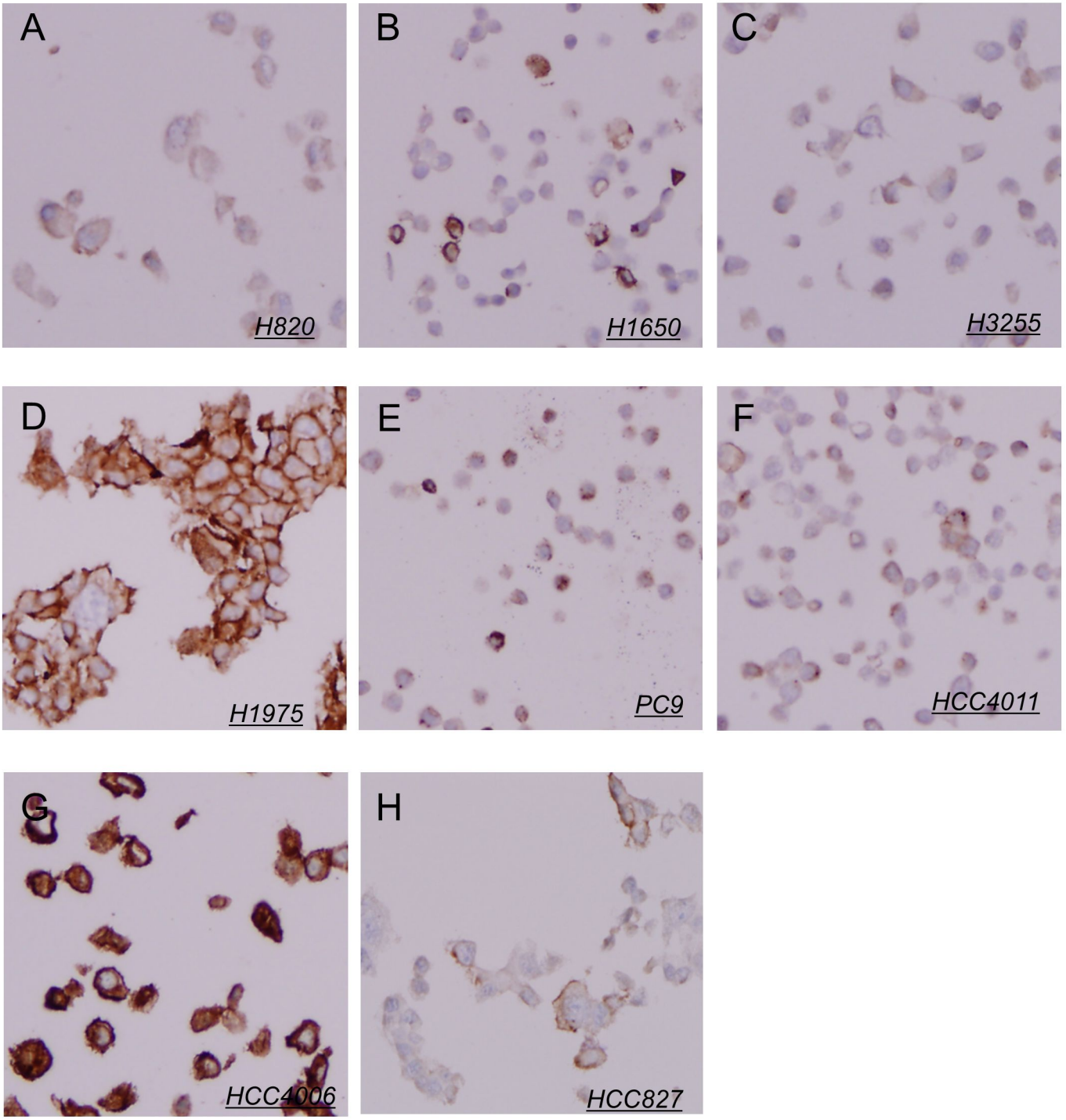


Figure 5

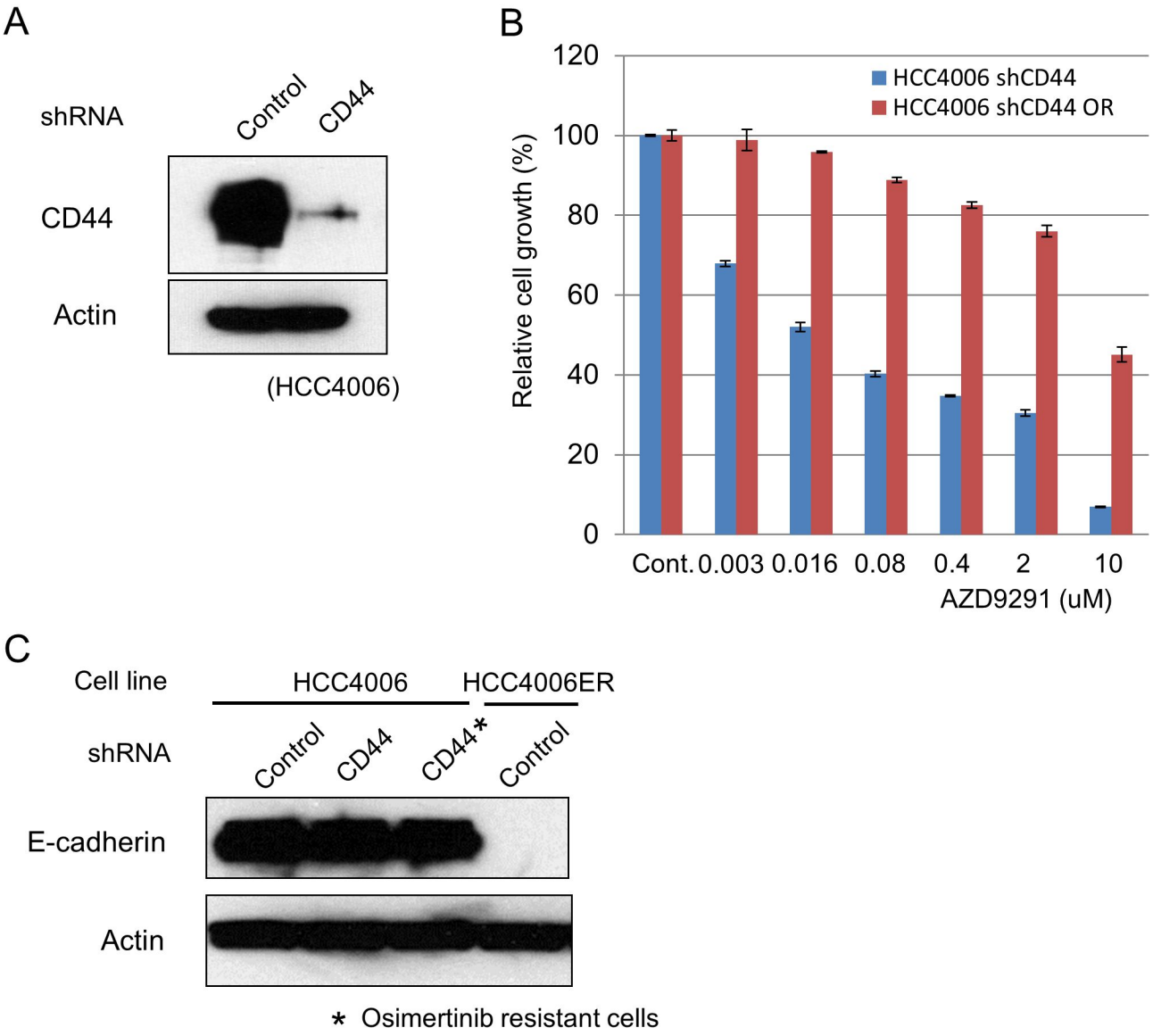


Figure 6

AACR American Association for Cancer Research

Molecular Cancer Therapeutics

CD44 facilitates epithelial to mesenchymal transition phenotypic change at acquisition of resistance to EGFR kinase inhibitors in lung cancer

Kenichi Suda, Isao Murakami, Hui Yu, et al.

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