Cancer Stem Cell Biomarkers in *EGFR*-Mutation–Positive Non–Small-Cell Lung Cancer

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Abstract

Epidermal growth factor receptor (EGFR) signaling deregulation promotes cancer stem cell (CSC) enrichment in non–small-cell lung cancer (NSCLC). In vitro experiments showed that inhibition of EGFR, signal transducer and activator of transcription 3 (STAT3), and Src decreased the CSC subpopulation. High expression of aldehyde dehydrogenase (ALDH) 1 isoforms and target hairy and enhancer of split 1 (HES1) was predictive of worse outcome to EGFR inhibition in *EGFR*-mutation–positive NSCLC patients. ALDH1, HES1, and B-cell–specific Moloney murine leukemia virus integration site 1 (Bmi-1) could be useful as biomarkers to monitor clinical progression, and the use of STAT3 and Src inhibitors could be useful to inhibit the CSC subpopulation induced by EGFR inhibitor treatment.

Introduction: Epidermal growth factor receptor (EGFR) pathway deregulation promotes the acquisition of stemlike properties in non–small-cell lung cancer. EGFR inhibition through NOTCH enriches lung cancer stem cells (CSCs). Src through Yes-associated protein 1 (YAP1) activates NOTCH. Signal transduction and activator of transcription 3 (STAT3) activation occurs upon EGFR blockade and regulates the generation of CSCs. **Patients and Methods:** Using the Aldefluor assay kit, we investigated the enrichment of aldehyde dehydrogenase (ALDH)-positive cells in *EGFR*-mutation–positive cells treated with gefitinib, afatinib, and osimertinib. Western blot analysis was performed to evaluate changes in CSC marker expression upon EGFR blockade. We performed gene expression analysis in a cohort of *EGFR*-mutation–positive non–small-cell lung cancer patients. We evaluated the association of gene expression with treatment outcomes. **Results:** The cell subpopulation surviving EGFR inhibition had high ALDH activity and elevated CSC marker expression. Concurrent inhibition of EGFR, STAT3, and Src diminished the CSC subpopulation in an *EGFR*-mutation–positive cellular model. In a cohort of 64 *EGFR*-mutation–positive patients, 2 ALDH1 isoforms and the NOTCH target hairy and enhancer of split 1 (HES1), when highly expressed, were predictive of worse outcome to EGFR blockade. The gene expression of B-cell–specific Moloney murine leukemia virus integration site 1 (Bmi-1) that maintains the self-renewal of stem cells was also related to treatment outcome. **Conclusion:** Single EGFR inhibitors increase the population of CSCs. Combinatory therapy targeting STAT3 and Src may be of potential benefit. ALDH1, HES1, and Bmi-1 are essential biomarkers in the initial assessment of *EGFR*-mutation–positive patients.

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Introduction

In several human cancers, cancer stem cells (CSCs) are highly relevant and are associated with tumor progression, genetic instability, and drug resistance.[1] These cells possess self-renewal capacity and are involved in tumor maintenance, metastases, and resistance to antitumor compounds,[1-4] including targeted therapies. Aldehyde dehydrogenase 1 (ALDH1), a detoxifying enzyme responsible for the oxidation of intracellular aldehydes, has been identified as a CSC marker in non–small-cell lung cancer (NSCLC)[5] and is related to resistance to therapies.[6] B-lymphoma Moloney murine leukemia virus insertion region 1 (Bmi-1), a well-known epithelial–mesenchymal transition (EMT)-inducing transcription factor, is also involved in the maintenance of CSCs.[7] Moreover, the NOTCH signaling pathway is essential for the tumorigenicity of CSCs.[8] We have shown that epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) induce the activation of signal transducer and activator of transcription 3 (STAT3) in *EGFR*-mutation–positive cells.[9,10] The EGFR TKI afatinib induced STAT3 activation and increased the $ALDH^+$ cell population in the *EGFR*-mutation–positive PC9 cell line.[10] Still, when we combined afatinib with a STAT3 inhibitor in vitro, we were not able to avoid, either the enrichment in the $ALDH^+$ subpopulation, or the increase of the expression of the NOTCH target hairy and enhancer of split 1 (HES1), events that occur upon single EGFR blockade.[10] This is because, independent of STAT3, the interleukin-6 signaling pathway activates the Yes-associated protein 1 (YAP1), and then NOTCH, through direct[11] association with the Src-family kinase Yes.[12] Erlotinib induces elevated ALDH activity in *EGFR*-mutation–positive NSCLC cells by activation of the NOTCH3 signaling pathway.[6]

In previous work, we have shown that combined inhibition of EGFR, STAT3, and Src-YAP1 is more effective than single EGFR blockade or double combinations in culture and in vivo.[9,11] In the current study, we investigated the effect of EGFR blockade on ALDH activity and the expression of CSCs and EMT markers. In a cohort of *EGFR*-mutation–positive NSCLC patients treated with EGFR TKIs, we explored the messenger RNA (mRNA) expression of HES1, ALDH1A1, ALDH1A3, and Bmi-1 as adverse effectors in *EGFR*-mutation–positive NSCLC patients.

Patients and Methods

Study Oversight and Sample Collection

Clinical data were assessed in accordance with the protocol approved by the institutional review board of Germans Trias i Pujol Hospital, Badalona, and were deidentified for patient confidentiality. We studied remaining material from pretreatment tumors from a cohort of *EGFR*-mutation–positive NSCLC patients from hospitals in Spain and Colombia.[9,11]

Cell Lines

Human lung adenocarcinoma PC9 cells, harboring *EGFR* exon 19 deletion (E746-A750), were provided by Hoffmann-La Roche (Basel, Switzerland). Human lung adenocarcinoma H1975 cells, harboring both sensitizing L858R and resistant T790M mutation, were purchased from the American Type Culture Collection. Five osimertinib-resistant cell lines were generated by treating PC9 cells with increasing concentrations of osimertinib. Sequencing analyses revealed that all 5 cell lines retained the *EGFR* exon 19 deletion. The half-maximal inhibitory concentration (IC_{50}) for osimertinib of parental PC9 cells was in the nanomolar range compared to 3.1 to 3.7 μM in the resistant cell lines. All cell lines were maintained in RPMI 1640 medium supplemented with 1% penicillin/streptomycin/glutamine (Gibco; Thermo Fisher Scientific, Waltham, MA) and 10% fetal bovine serum (Gibco) in 5% CO_2, 37°C cell culture incubator, and were routinely evaluated for mycoplasma contamination as previously described.[9,11] Media and supplements were obtained from Life Technologies (Gaithersburg, MD).

Chemical and Reagents

Gefitinib, TPCA-1 (2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide), and AZD0530 (Src-family kinase inhibitor; saracatinib) were purchased from Tocris Bioscience (Bristol, UK), Sigma-Aldrich (St Louis, MO), and Selleck Chemicals (Houston, TX), respectively. Osimertinib and afatinib were purchased from Selleck Chemicals. Drugs were prepared in dimethyl sulfoxide at a concentration of 10 to 100 mmol/L stock solutions and were stored at −20°C. Further dilutions were made in culture medium to final concentration before use. The following antibodies were purchased from Cell Signaling Technology (Beverly, MA, U,S): EGFR (Cat#4267); phospho-EGFR (Tyr 1068) (Cat#3777); STAT3 (Cat#9139); phospho-STAT3 (Tyr705) 24 (Cat#9145); protein kinase B (AKT; Cat#9272); phospho-AKT (Ser473) (Cat#9271); extracellular signal-regulated kinase (ERK) 1/2 (Cat#9102); phospho- 25 ERK 1/2 (Thr202/Tyr204) (Cat#9101); NOTCH3 (Cat#5276); Bmi-1 (Cat#6964); and HES1 (Cat#11988). Other antibodies were ALDH1A3 (Cat#AP7847a) from Abgent (San Diego, CA), CD44 (Cat#ab2212) from Abcam (Cambridge, UK), and α-tubulin from Sigma-Aldrich. IRDye 800CW goat anti-rabbit and IRDye 800CW goat anti-mouse antibodies were purchased from LI-COR Biosciences (Lincoln, NE).

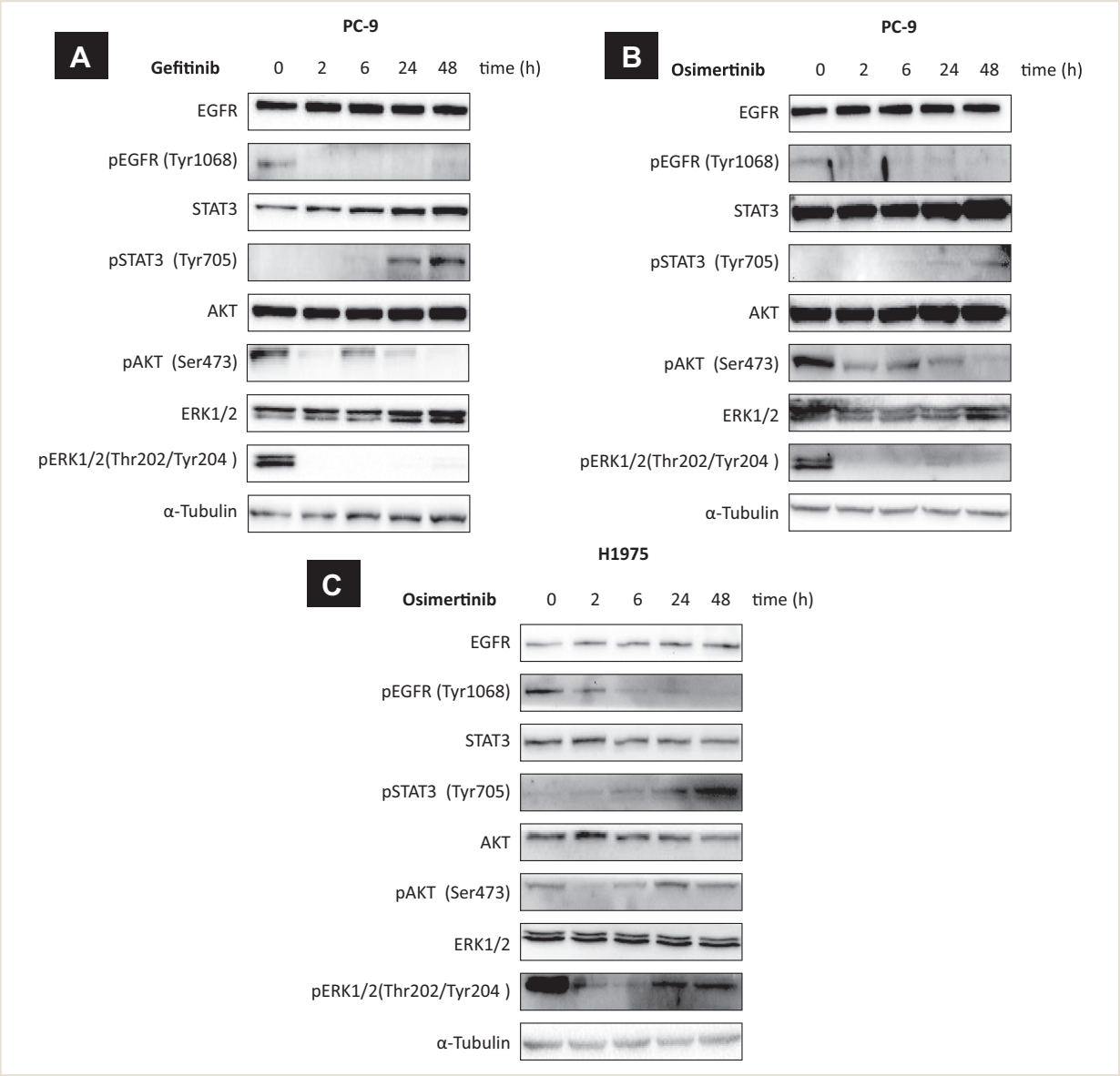
Aldefluor Assay and Flow Cytometry

The Aldefluor assay kit (STEMCELL Technologies, Vancouver, BC, Canada) was used to determine the profile of cells with high and low ALDH activity. The assay was performed according to the manufacturer's instructions, with certain modifications. Briefly, 2×10^6 cells were suspended in Aldefluor assay buffer and divided into 2 groups. One group was pretreated for 10 minutes with the ALDH-specific inhibitor diethylaminobenzaldehyde (DEAB), and then both groups were incubated with ALDH enzyme substrate BODIPY-aminoacetaldehyde for 30 minutes at 37°C. Cells were then centrifuged and resuspended in a fresh Aldefluor assay buffer to remove the unutilized substrate. The fluorescence intensity of stained cells was analyzed using a FACS canto II (BD Biosciences, San Jose, CA) flow cytometer. For the analysis, DEAB-treated sample was used as a negative control. The ALDH activity of a sample was determined to be high or low on the basis of fluorescence intensity above or below the threshold defined by the reaction with DEAB.

Western Blot Test

For Western blot assays, cells were cultured in cell culture flasks and left untreated or treated as indicated in each experiment. Cells were lysed in ice-cold radioimmunoprecipitation assay buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1

Figure 1 Effect of EGFR TKIs on STAT3 Activation in *EGFR*-Mutation–Positive Cells. PC9 Cells Were Cultured in Presence of (A) Gefitinib (40 nM) or (B) Osimertinib (0.17 μM), and H1975 Cells Were Cultured in Presence of (C) Osimertinib (0.17 μM) at Indicated Time Intervals; Western blot Analysis for various Signaling Molecules Was Performed. α-Tubulin Was Used as Housekeeping Protein. Independent Experiments Were Conducted at Least Twice



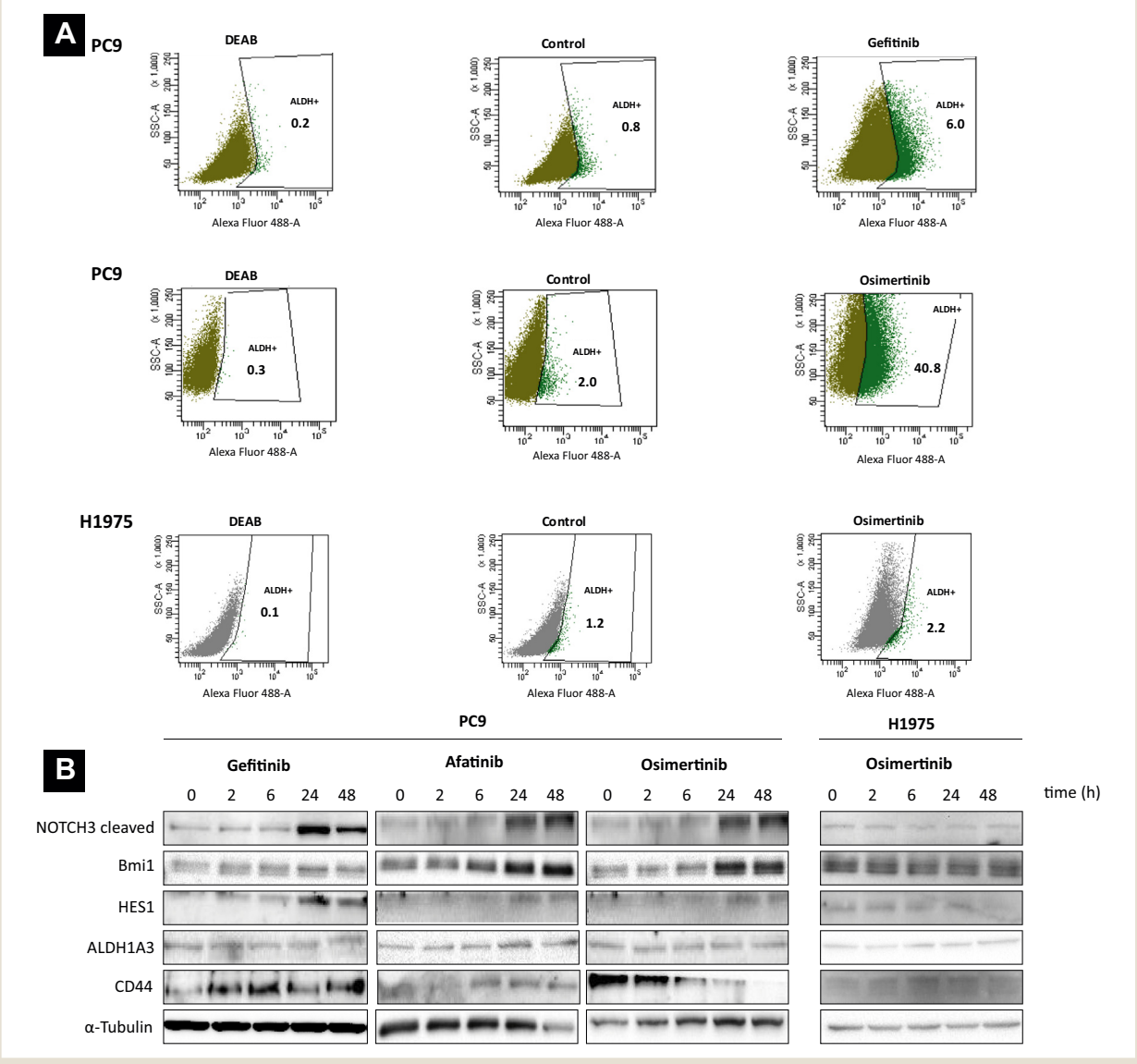
Abbreviations: EGFR = epidermal growth factor receptor; STAT3 = signal transducer and activator of transcription 3; TKI = tyrosine kinase inhibitor.

mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, 1 mM PMSF)]. After incubating for 20 minutes at 4°C, the samples were centrifuged, and the supernatant was kept at −80°C. Protein concentration was determined by bicinchoninic acid protein assay. Equal amounts of protein from each cell lysate (30 μg per lane) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, New Bedford, MA). The membranes were blocked in Tris-buffered saline containing 5% fat-free dry milk and then probed with primary antibodies at 4°C overnight. After washing, the membrane was incubated with horseradish peroxidase–conjugated secondary antibodies for 2 hours at room temperature. Specific proteins were visualized with enhanced chemiluminescence detection reagent according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL) and analyzed by Chemidoc MP Imaging System (Bio-Rad, Hercules, CA). α-Tubulin was used as an internal control to confirm equal gel loading.

Gene Expression Analyses

All analyses of tumor samples were carried out at the ISO 15189–certified Pangaea Oncology laboratory located in the Quiron Dexeus University Hospital (Barcelona, Spain). RNA was isolated from

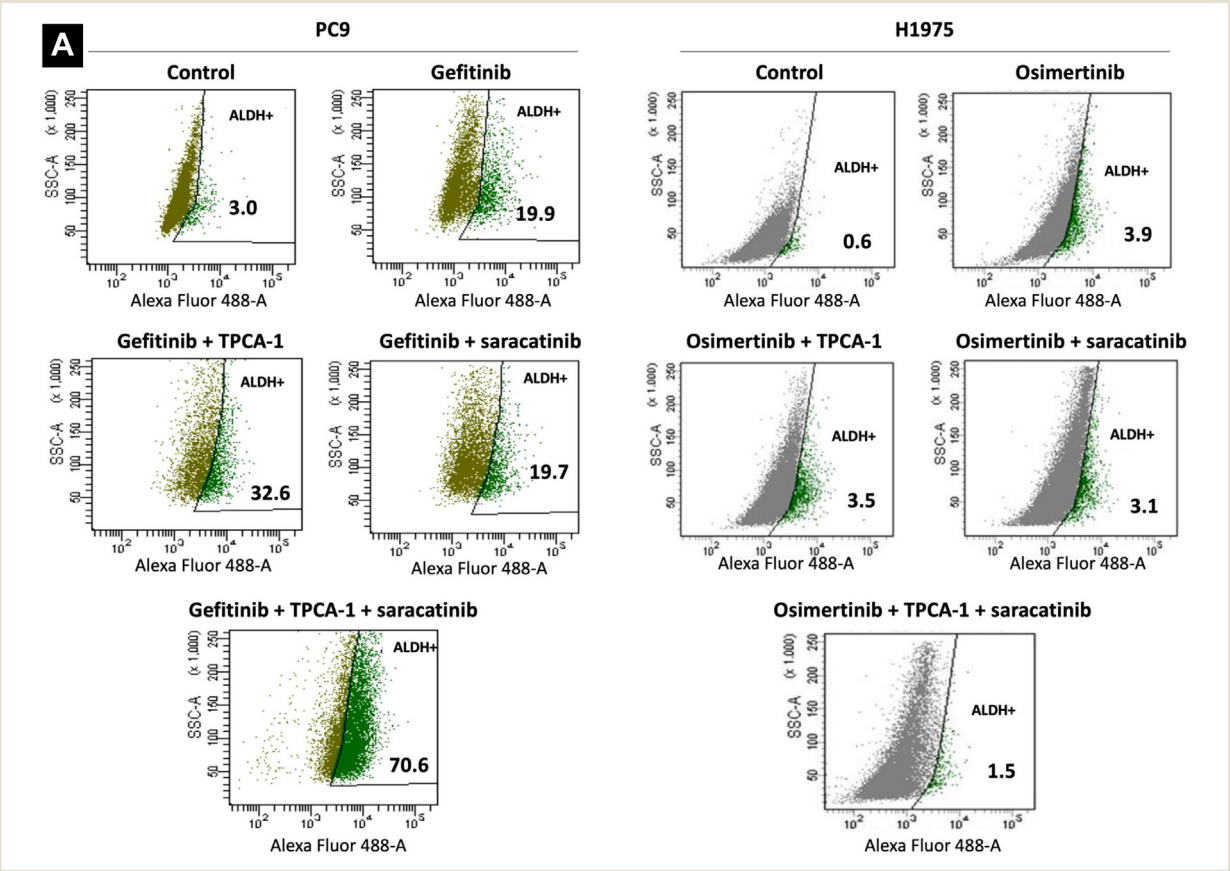
Figure 2 Effect of EGFR Blockade EGFR TKIs on ALDH-Activated Cell Subpopulation and CSC Protein Marker Expression. (A) PC9 and H1975 Cells Were Treated With EGFR TKIs (Gefitinib, 40 nM; Osimertinib 0.17 μM; Afatinib, 3.1 nM) for 7 Days. Identification of ALDH Positivity Was Made Using Aldefluor assay. Cells Were Separated by Flow Cytometry. (B) PC9 and H1975 Cells Were Treated With EGFR TKIs as in Figure 3A for Indicated Time Intervals; Western Blot Analysis for CSC Markers Was Performed. α-Tubulin Was Used as Housekeeping Protein. Independent Experiments Were Conducted at Least Twice



Abbreviations: ALDH = aldehyde dehydrogenase; CSC = cancer stem cell; EGFR = epidermal growth factor receptor; TKI = tyrosine kinase inhibitor.

the tumor tissue specimens in accordance with a proprietary procedure (European patent EP1945764-B1), as previously described.[11,13] The primer and probe sets were designed using Primer Express 3.0 Software (Applied Biosystems; Thermo Fisher Scientific) according to their Ref Seq (http://www.ncbi.nlm.nih.gov/LocusLink). Gene-specific primers are as follows: β-actin, forward: 5′-TGAGCGCGGCTACAGCTT-3′ and reverse: 5′-TCCTTAATGTCACGCACGATTT-3′; HES1, forward: 5′-GGACATTCTGGAAATGACAGTGAA-3′ and reverse: 5′-CAGCACACTTGGGTCTGTGC-3′; ALDH1A1, forward: 5′-TGCAACTGAGGAGGAGCTCTG-3′ and reverse: 5′-CTTCAC TGCCTTGTCAACATCC-3′. Gene expression of ALDH1A3 and Bmi-1 were analyzed with Hs00167476_m1 and Hs00995536_m1 (Applied Biosystems), respectively. Quantification of gene expression was performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Quantification of gene expression was calculated according to the comparative cycle threshold (C_t) method. Final results were determined as follows: $2^{-(\Delta Ct\text{sample}-\Delta Ct\text{calibrator})}$, where ΔC_t values of the calibrator and sample are determined by subtracting the C_t value of the target gene from the value of the endogenous gene (β-actin). Commercial RNA controls were used as calibrators (liver and lung; Stratagene,

Figure 3 Effect of EGFR TKI Alone and Combined With STAT3 and Src Inhibitors on ALDH Subpopulation and CSC Protein Marker Expression. (A) Measurement of ALDH$^+$ Subpopulation of PC9 and H1975 Cell Lines Treated for 7 days With Indicated Drugs (Gefitinib, 40 nM; Osimertinib, 0.17 μM; Saracatinib, 10 μM; and TPCA-1, 4 nM) Using Aldefluor Assay and Separation by Flow Cytometry. (B) Protein Expression of CSC Markers Was Analyzed by Western Blot Test in PC9 and H1975 Cell Lines Treated for 24 Hours With Gefitinib (40 nM), Osimertinib (0.17 μM), Saracatinib (10 μM), and TPCA-1 (4 nM) in Combination or as Single Agent, as Indicated. (C) Endogenous Protein Levels in 5 OR Cell Lines Compared to Parental Cell Line PC9. α-Tubulin Was Used as Housekeeping Protein. Independent Experiments Were Conducted at Least Twice



Abbreviations: ALDH = aldehyde dehydrogenase; CSC = cancer stem cell; EGFR = epidermal growth factor receptor; OR = osimertinib resistant; STAT3 = signal transducer and activator of transcription 3; TKI = tyrosine kinase inhibitor.

La Jolla, CA). In all quantitative experiments, a sample was considered not evaluable when the standard deviation of the C_t values was > 0.30 in 2 independent analyses. As a result, the number of evaluable samples varied among the genes examined.

Statistical Analysis

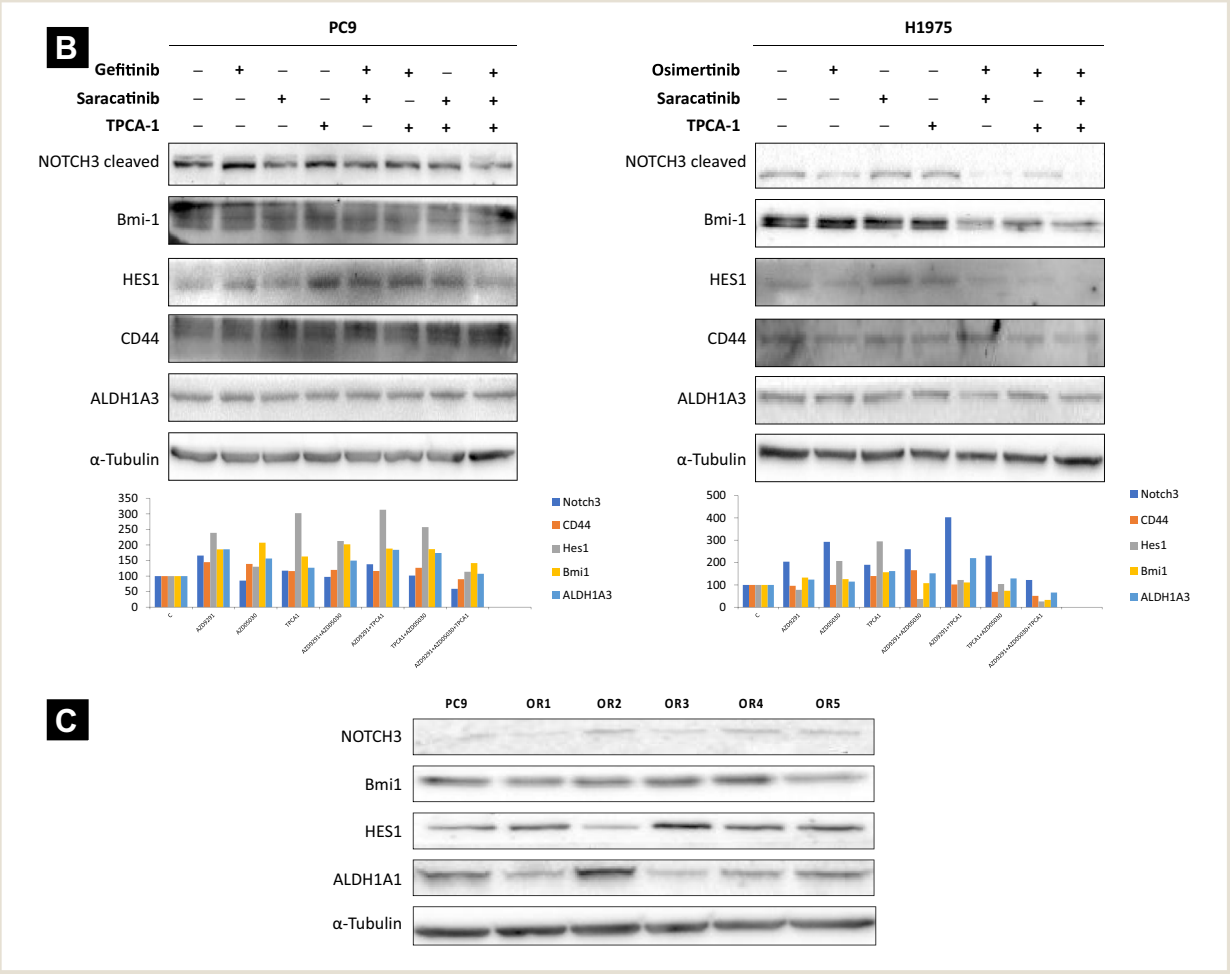
The primary end point of the clinical part of the study was to examine the potential effects of gene messenger RNA (mRNA) expression levels on survival. Progression-free survival (PFS) and overall survival (OS) were estimated by the Kaplan-Meier method and compared by a nonparametric log-rank test. In addition to analyzing gene expression as a continuous variable, expression levels were divided into 2 groups according to the median relative expression. A multivariate Cox proportional hazard model was applied with treatment and potential risk factors as covariates, obtaining hazard ratios (HRs) and their 95% confidence interval (CI). Each analysis was performed with the use of a 2-sided 5% significance level and a 95% CI. Association between biomarkers was assessed by Pearson correlation analysis. Statistical analyses were performed by SAS 9.4 software (SAS Institute, Cary, NC).

Results

Effect of Single EGFR Inhibition on CSC Markers in EGFR*-Mutation–Positive Cells*

We initially extended our previously published findings in which STAT3 is activated on treatment with EGFR TKIs in *EGFR*-mutation–positive cells,[9-11] with a time-response Western blot experiment. As expected, gefitinib suppressed EGFR, AKT, and ERK1/2 phosphorylation in PC9 cells, but at 6 hours, and more clearly at 24 and 48 hours, it stimulated STAT3 phosphorylation on the critical tyrosine residue 705 (Figure 1A). Similar results were obtained when PC9 cells were treated with osimertinib (Figure 1B).

Figure 3 Continued



Western blot experiments of protein extracts of H1975 cells treated with osimertinib showed that the drug inhibits EGFR, AKT, and ERK1/2 and increases STAT3 phosphorylation (Figure 1C).

After confirming our previous findings, and knowing that STAT3 activation increases the $ALDH^+$ cell subpopulation in NSCLC,[14] we then explored the effect of EGFR TKIs on CSC marker expression. We reported that afatinib increases the $ALDH^+$ subpopulation in PC9 cells.[10] The same occurred when we applied the Aldefluor assay in PC9 and H1975 cells treated with gefitinib or osimertinib. Gefitinib and osimertinib in PC9 and osimertinib in H1975 cells clearly increased the $ALDH^+$ subpopulation (Figure 2A). Specifically, in the untreated PC9 cell line existed 0.8% to 2% of $ALDH^+$ cells, compared to 6% and 40.8% in the gefitinib- and osimertinib-treated cells, respectively. Similarly, in the untreated H1975 cell line existed 1.2% of $ALDH^+$ cells, compared to 2.2% in the osimertinib-treated cell line (Figure 2A).

Next, we analyzed the effect of gefitinib, afatinib, and osimertinib on the expression of several CSC- and EMT-related markers in both the PC9 and H1975 cell lines. The IC_{50} of gefitinib, afatinib, and osimertinib in PC9 and H1975 cells has previously been reported.[10,11] Western blot time-response experiments revealed an increase in the expression of most of the CSC and EMT markers analyzed, such as NOTCH3, CD44, Bmi-1, and HES1. CD44 expression was decreased in PC9 cells treated with osimertinib (Figure 2B). Our results indicate that first-, second-, and third-generation EGFR TKIs activate resistance pathways, such as STAT3, and select for a CSC-enriched cell population.

Effect of Combined EGFR, STAT3, and Src-YAP1 Inhibition on CSC Markers in EGFR*-Mutation–Positive Cells*

Our previous work clearly demonstrated that single EGFR inhibition in *EGFR*-mutation–positive cells causes an imbalance in parallel and downstream signaling pathways that ultimately bypass the *EGFR* blockade.[9-11] Cotargeting EGFR, STAT3, and Src-YAP1 causes significant tumor growth inhibition compared to single gefitinib, afatinib, or osimertinib.[9-11] Here we tried to see the effect of this combination on CSCs by both Aldefluor assay and Western blot analysis. In the PC9 cell line, gefitinib increased the $ALDH^+$ subpopulation, but we were not able to reverse this phenomenon with the double combination of gefitinib and a STAT3 (TPCA-1) or Src inhibitor (saracatinib or AZD05030). To our surprise, the

Table 1 Cox Regression Model of PFS Univariate Analysis With HES1, Bmi-1, ALDH1A1, and ALDH1A3 mRNA Expression as Continuous Variables

Survival	Biomarker	N	Contrast	*P*	HR (95% CI)
PFS	HES1	41	HES1 as continuous variable	.0104	1.49 (1.10-2.03)
	Bmi-1	42	Bmi-1 as continuous variable	.0003	1.61 (1.25-2.07)
	ALDH1A1	37	ALDH1A1 as continuous variable	.0196	2.73 (1.17-6.33)
	ALDH1A3	41	ALDH1A3 as continuous variable	.1030	1.06 (0.99-1.139)
OS	HES1	41	HES1 as continuous variable	.0658	1.37 (0.98, 1.91)
	Bmi-1	42	Bmi-1 as continuous variable	.0003	1.70 (1.27, 2.27)
	ALDH1A1	37	ALDH1A1 as continuous variable	.8487	1.13 (0.33, 3.90)
	ALDH1A3	41	ALDH1A3 as continuous variable	.3723	1.03 (0.96, 1.11)

Abbreviations: ALDH = aldehyde dehydrogenase; Bmi-1 = B-cell–specific Moloney murine leukemia virus integration site 1; CI = confidence interval; HES1 = target hairy and enhancer of split 1; HR = hazard ratio; mRNA = messenger RNA; OS = overall survival; PFS = progression-free survival.

triple combination of gefitinib, TPCA-1, and Src inhibitor further increased the $ALDH^+$ subpopulation (Figure 3A). In contrast, in the H1975 cell line, we observed a decrease of the $ALDH^+$ subpopulation when osimertinib was combined with TPCA-1 or saracatinib. This decrease was even clearer when we treated H1975 cells with the triple combination (Figure 3A). Similarly, by Western blot analysis, we observed decreased levels of cleaved intracellular NOTCH3, Bmi-1, and HES1 with the triple combination in the H1975 cell line, but not in the PC9 cell line (with the exception of cleaved intracellular NOTCH3) (Figure 3B). Thus, combined EGFR, STAT3, and Src-YAP1 inhibition increased the $ALDH^+$ subpopulation in PC9 cells, while a significant decrease was observed when using the triple-drug combination in H1975 cells. Furthermore, CSC marker protein levels were decreased after treatment with the triple-drug combination in H1975 cells but not in PC9 cells. These results indicate the heterogeneity of resistance mechanisms in different tumor types. Finally, to delineate the CSC phenotype of cells with acquired resistance to EGFR TKIs, we investigated by Western blot analysis the expression levels of CSC markers in osimertinib-resistant PC9 cells and compared them to the parental PC9. HES1 up-regulation was the most common molecular event, observed in 4 of the 5 osimertinib-resistant clones, while NOTCH3 and ALDH1A1 were also found in some of the resistant cell lines (Figure 3C).

CSC Biomarkers Related to Clinical Outcome to EGFR Inhibition

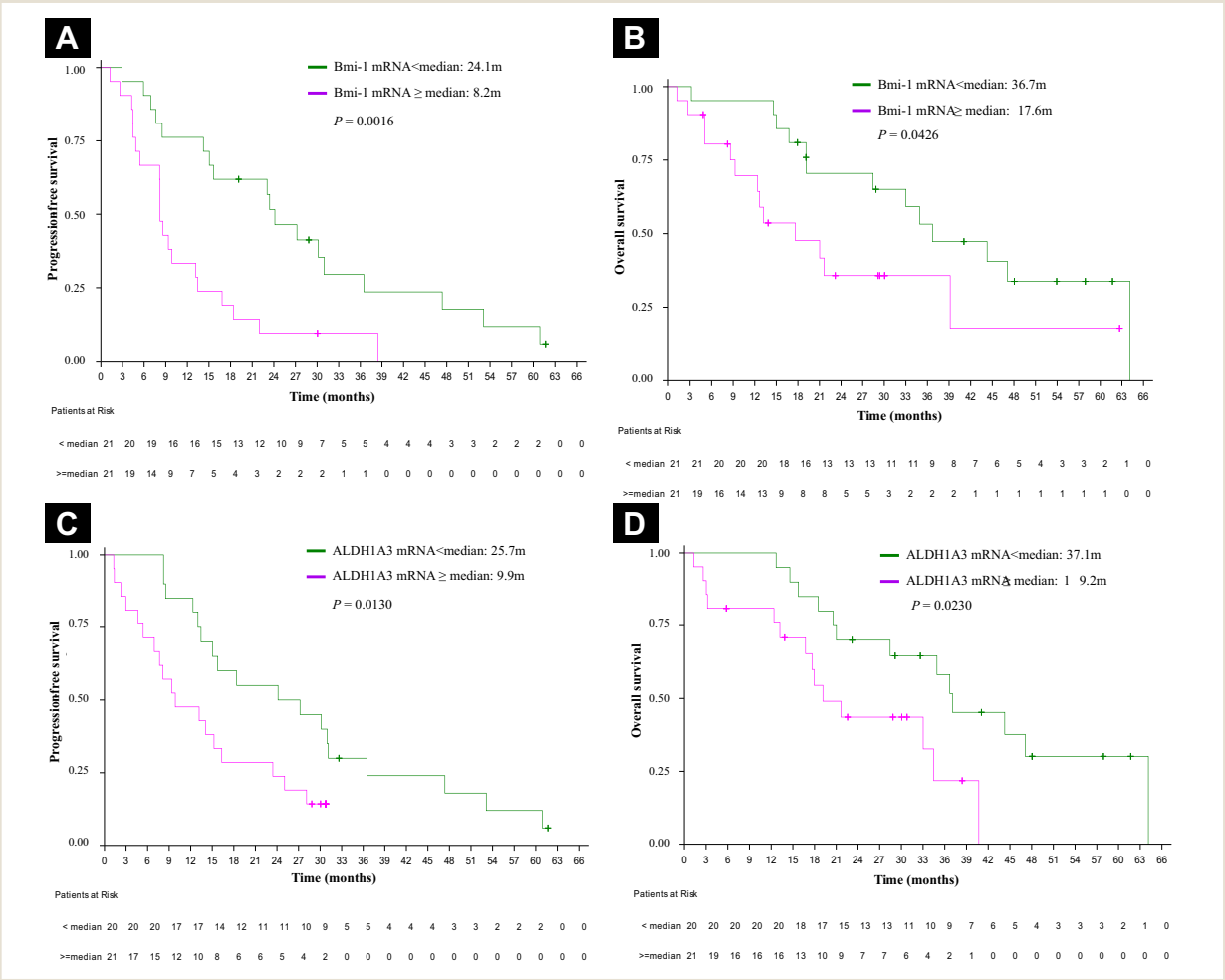
To test whether our findings were clinically relevant, we investigated the relationship between HES1, Bmi-1, ALDH1A1, and ALDH1A3 mRNA expression with clinical outcome in *EGFR*-mutation–positive NSCLC clinical specimens.[9-11] The baseline characteristics of the 64 *EGFR*-mutation–positive NSCLC patients included in this analysis have been previously reported.[9-11] A significant correlation was found between Bmi-1 and ALDH1A1 ($r = 0.52$; $P = .0060$), as well as between Bmi-1 and HES1 ($r = 0.56$; $P = .0012$). Firstly, HES1, Bmi-1, ALDH1A1, and ALDH1A3 were assessed as continuous variables and then as binary (high vs. low) variables. As continuous variables, HES1, Bmi-1, and ALDH1A1 were independent predictors of PFS benefit, while for OS, the evidence was equivocal, and only Bmi-1 mRNA expression remained significant (Table 1).

In an exploratory dichotomous model using the median expression as a cutoff point, the population was divided into 2 groups for each of the biomarkers. Marked survival benefit was observed in the low Bmi-1 (HR for disease progression = 0.34; 95% CI, 0.17-0.68; $P = .025$; HR for death = 0.44; 95% CI, 0.19 0.99; $P = .0479$) (Figure 4A, B) and ALDH1A3 (HR for disease progression = 0.40; 95% CI, 0.19-0.85; $P = .0163$; HR for death = 0.40; 95% CI, 0.17-0.90; $P = .0276$) groups (Figure 4C, D). The low HES1 (HR for disease progression = 0.40; 95% CI, 0.19-0.83; $P = .0142$; HR for death = 0.54; 95% CI, 0.24-1.19; $P = .1273$) and ALDH1A1 (HR for disease progression = 0.29; 95% CI, 0.13-0.66; $P = .030$; HR for death = 0.73; 95% CI, 0.32-1.67; $P = .4552$) groups showed PFS but not OS benefit (Figure 5). These findings provide further support for the CSC-based *EGFR* TKI resistance that our study highlights.

Discussion

Previous studies have shown that in lung cancer, CSCs are responsible for aggressive disease behavior and resistance to therapy.[14-17] This distinct population of tumor cells is capable of continuous self-renewal and differentiation through several signaling pathways, including NOTCH, Hedgehog, and Wnt.[18] STAT3, integrin/focal adhesion kinase, and Src help cancer cells in maintaining stem cell properties.[10,19] Therefore, first the identification of CSC-specific markers, and second, the development of targeted therapeutics for CSCs elimination, remain a significant challenge. In the present study, we reconfirmed that in 2 *EGFR*-mutation–positive cell lines, first-, second-, and third-generation EGFR TKIs induce STAT3 phosphorylation,[9,10] and increase the fraction of preexisting $ALDH^+$ cells and the expression of CSCs markers. We also show that acquired resistance to osimertinib is related with a CSC phenotype. The same EGFR pathway promotes acquisition of stem cell properties in head and neck squamous-cell carcinoma,[20,21] while EGFR blockade enriches and selects for CSCs in *EGFR*-mutation–positive cells.[6,10,22] A correlation between $ALDH^+$ cells and STAT3 activation has also been reported in pancreatic cells.[23,24] Pharmacologic inhibition of STAT3 decreases the number and the size of lung CSCs.[23] In the present work we have not explored the effect of silencing CSC markers on sensitivity to EGFR TKIs. However, there is previous evidence that the knockdown of CSCs augments the sensitivity to cancer therapeutics, including EGFR

Figure 4 PFS and OS by Expression of Bmi-1 and ALDH1A3 in Cohort of *EGFR*-Mutation–Positive NSCLC Patients. (A) Median PFS was 24.1 Months (95% CI, 14.2-36.5) for 21 Patients With Low Bmi-1 and 8.2 Months (95% CI, 4.8-13.2) for 21 Patients With High Bmi-1 mRNA Expression; $P = .0016$. (B) Median OS was 36.7 Months (95% CI, 19.2-64.1) for 21 Patients With Low Bmi-1 and 17.6 Months (95% CI, 8.6-39.2) for 21 Patients With High Bmi-1 mRNA Expression; $P = .0426$. (C) Median PFS was 25.7 Months (95% CI, 13.0-36.5) for 20 Patients With Low ALDH1A3 and 9.9 Months (95% CI, 5.4-16.3) for 21 Patients With High ALDH1A3 mRNA Expression; $P = .0130$. (D) Median OS was 37.1 Months (95% CI, 20.6-64.1) for 20 Patients With Low ALDH1A3 and 19.2 Months (95% CI, 13.2-34.5) for 21 Patients With High ALDH1A3 mRNA Expression; $P = .0230$

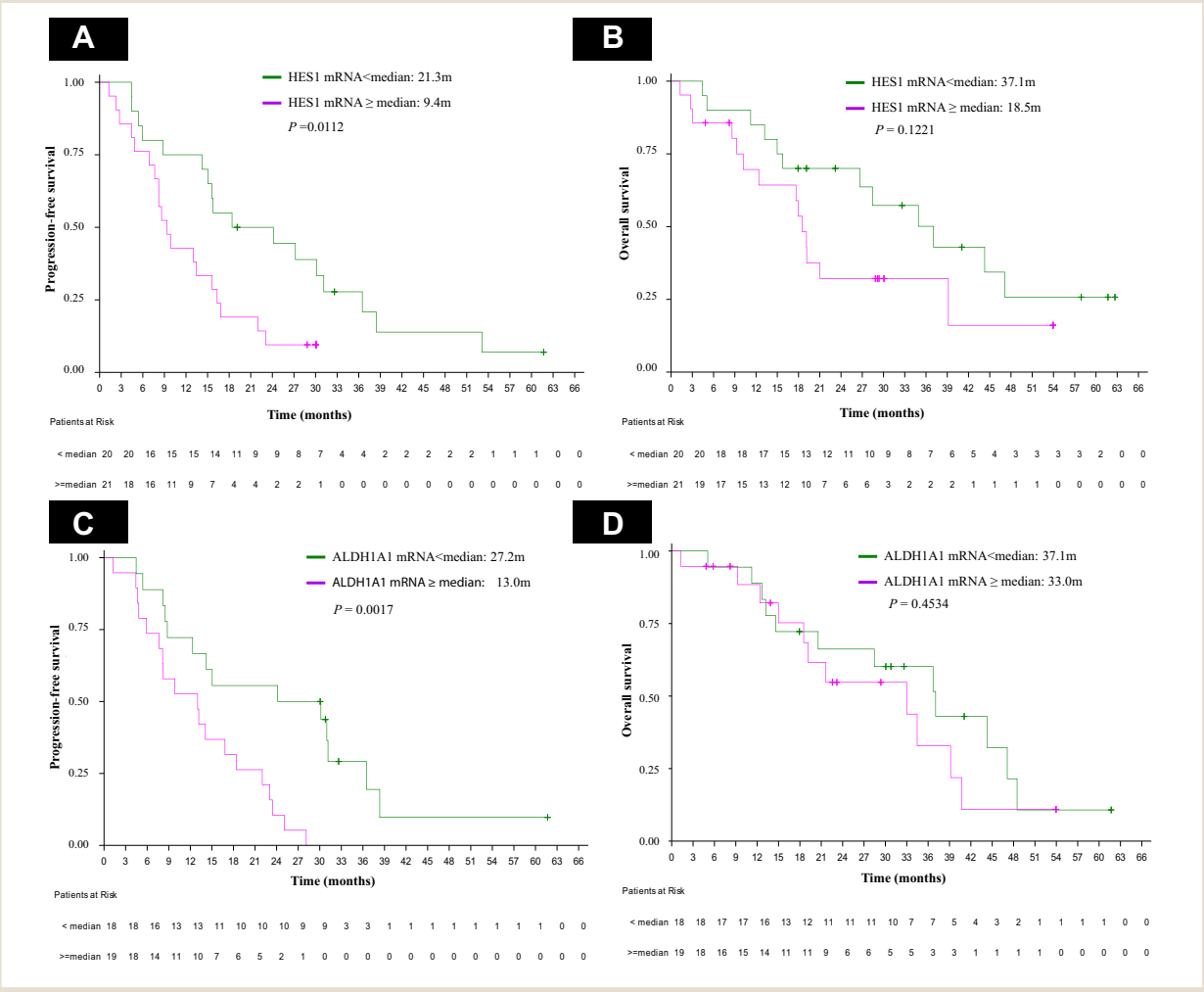


Abbreviations: ALDH = aldehyde dehydrogenase; Bmi-1 = B-cell–specific Moloney murine leukemia virus integration site 1; CI = confidence interval; EGFR = epidermal growth factor receptor; mRNA = messenger RNA; NSCLC = non–small-cell lung cancer; OS = overall survival; PFS = progression-free survival.

TKIs. Increased side-population and self-renewal capabilities were reported in gefitinib-resistant cells.[4] Oct-4 has been also related to resistance to gefitinib in cell lines and samples of patients.[25] Sphere PC9 cells with expression of CSC markers, like CD133, CD44, Oct-4, and ABCG2, were resistant to erlotinib compared to parental cells, with no sphere phenotype.[26] Finally, resistance to the second-generation EGFR TKI afatinib has been related with a CSC phenotype, including increased expression of ALDH1.[27]

We found that combined EGFR, STAT3, and Src inhibition may overcome the deleterious effect of single EGFR inhibition. In the PC9 cell line, we previously found that the triple combination of gefitinib, TPCA-1, and saracatinib is highly synergistic,[9] but now we see that it cannot eliminate the $ALDH^+$ cells. In contrast, in the H1975 model, osimertinib, with a STAT3 and/or Src inhibitor, efficiently decreased the osimertinib-induced $ALDH^+$ cells. Our model is depicted in Figure 6. Several markers have been described as identifiers of human lung CSCs, such as CD133,[28] ALDH1A1,[29] and ALDH1A3.[14] ALDH activity is dependent on NOTCH signaling.[6,29] The EMT-inducing transcription factor Bmi-1 is also involved in initiation and maintenance of CSCs.[30] In breast cancer, Bmi-1 is related with resistance to tamoxifen and down-regulates immune surveillance.[31] It also attenuates DNA damage–induced G_2/M checkpoint activation.[32] Consistent

Figure 5 PFS and OS by Expression of HES1 and ALDH1A1 in Cohort of *EGFR*-Mutation–Positive NSCLC Patients. (A) Median PFS was 21.3 Months (95% CI, 8.8-31.1) for 20 Patients With Low HES1 and 9.4 Months (95% CI, 6.9-15.6) for 21 Patients With High HES1 mRNA Expression; $P = .0112$. (B) Median OS Was 37.1 Months (95% CI, 15.1 to NA) for 20 Patients With Low HES1 and 18.5 Months (95% CI, 9.3-39.2) for 21 Patients With High HES1 mRNA Expression; $P = .1221$. (C) Median PFS was 27.2 Months (95% CI, 8.8-36.5) for 18 Patients With Low ALDH1A1 and 13.0 Months (95% CI, 5.9-18.4) for 19 Patients With High ALDH1A1 mRNA Expression; $P = .0017$. (D) Median OS Was 37.1 Months (95% CI, 14.6-47.1) for 18 Patients With Low ALDH1A1 and 33.0 Months (95% CI, 15.0-39.2) for 19 Patients With High ALDH1A1 mRNA Expression; $P = .4534$

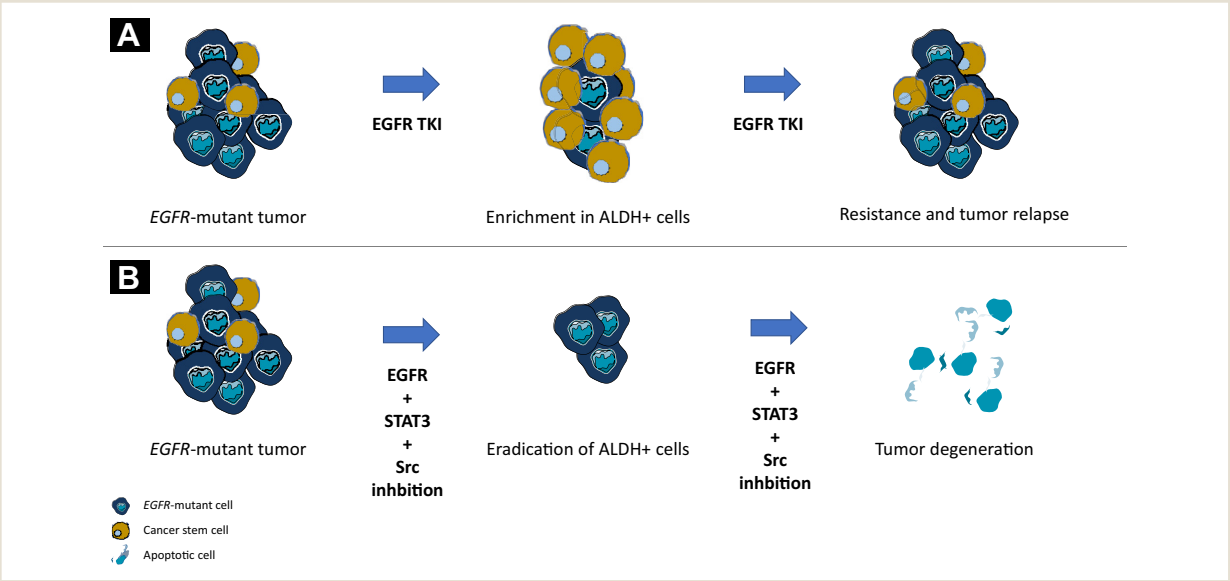


Abbreviations: ALDH = aldehyde dehydrogenase; EGFR = epidermal growth factor receptor; HES1 = hairy and enhancer of split 1; NSCLC = non–small-cell lung cancer; OS = overall survival; PFS = progression-free survival.

with Aldefluor assay results, the triple-drug combination was able to decrease the expression of all investigated CSC markers in H1975 cells, while in the PC9 cells only expression of cleaved intracellular NOTCH3 was lowered (Figure 3B). Our results indicate heterogeneous parallel and downstream signaling pathways in these cell lines, reflecting the differences in the tumor resistance mechanism in patients. In a patient cohort of *EGFR*-mutation–positive NSCLC, we found that CSC-marker mRNA expression can predict survival in EGFR TKI-treated patients. The 4 markers investigated, HES1, Bmi-1, and 2 ALDH1A isoforms, when highly expressed, were shown to be indicators of worse outcome to EGFR TKIs. Importantly, these findings confirm the relevance of CSC presence after EGFR TKI treatment and their correlation with development of therapy resistance. It can be concluded that different tumors initiate distinct resistance pathways, stressing the importance of mutation analysis and personalized treatment plans. Furthermore, this study highlights the importance of CSCs in resistance to EGFR TKI treatments and presents an opportunity to stratify patients based on mRNA expression of prognostic CSC markers.

Further research should focus on developing new (combination) therapies to eliminate CSCs, although caution should be taken when treating different tumor types because of heterogeneous resistance pathways.

Figure 6 Model. (A) CSCs are Thought to be Subset of *EGFR*-Mutation–Positive Cancer Cells That are Resistant to Single EGFR Blockade and Finally Lead to Relapse of Disease. (B) Concomitant EGFR, STAT3, and Src Blockade May Improve Clinical Outcomes by Reducing Portion of CSCs Most Likely to Persist Through Single EGFR Inhibition and Lead to Tumor Regression



Abbreviations: CSC = cancer stem cell; EGFR = epidermal growth factor receptor; STAT3 = signal transducer and activator of transcription 3.

Clinical Practice Points

- Single therapy with EGFR TKIs for *EGFR*-mutation–positive NSCLC patients is insufficient and activates parallel signaling pathways that ultimately cause resistance.
- In culture, we demonstrated the enrichment in ALDH$^+$ cells upon application of gefitinib, afatinib, or osimertinib in *EGFR*-mutation–positive models. By Western blot analysis, we confirmed the increase in several CSC markers when the cells are treated with single EGFR TKIs.
- The combination of first-, second-, or third-generation EGFR TKIs with a STAT3 and Src inhibitor prevents the increase of ALDH$^+$ cells and CSC markers in one *EGFR*-mutation–positive model.
- The baseline evaluation of CSC markers, like ALDH1A1, ALDH1A3, Bmi-1, and HES1, predicted shorter PFS and OS in *EGFR*-mutation–positive patients treated with *EGFR* TKIs.
- Combination therapy may be of benefit for the poor prognostic subgroup of *EGFR*-mutation–positive NSCLC patients defined in our study.

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Disclosure

The authors have stated that they have no conflict of interest.

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