Activated Epidermal Growth Factor Receptor – Stat-3 Signaling Promotes Tumor Survival *In vivo* in Non – Small Cell Lung Cancer

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Abstract

Purpose: Signal transducers and activators of transcription 3 (Stat3), a member of the STAT family of transcription factors, regulates multiple oncogenic pathways, including pathways regulating tumor cell survival. We evaluated Stat3 activation in early stage non – small cell lung cancers (NSCLC) and how this relates to upstream epidermal growth factor receptor (EGFR) activation, tumor apoptosis, and prognosis.

Experimental Design: High-density tissue microarrays using tissues from 176 surgically resected NSCLC were evaluated for expression of phosphorylated Stat3 (pStat3) and epidermal growth factor receptor (pEGFR) along with tumor apoptosis. Using NSCLC cell lines, we evaluated how pStat3 expression relates to EGFR mutations and sensitivity of cells to gefitinib.

Results: We identified nuclear pStat3 expression in 54% of tumors. pStat3 expression was correlated with smaller tumors ($P < 0.0001$) and with limited smoking history ($P = 0.02$). We identified a trend toward higher pStat3 expression in adenocarcinomas compared with other tumor histology ($P = 0.09$). No relationship was found between pStat3 and prognosis following surgical resection. Importantly, we found a strong positive correlation between pEGFR expression and pStat3 expression ($P < 0.0001$) and an inverse correlation between pStat3 and apoptosis ($P = 0.01$) consistent with less apoptosis in tumors expressing high amounts of pStat3. Cell lines with mutant EGFR have increased levels of pStat3 compared with cell lines without mutant EGFR and this correlates with their sensitivity to gefitinib. Finally, antisense-mediated knockdown of Stat3 induces apoptosis in EGFR mutant lung cancer cells.

Conclusions: Early-stage NSCLC tumors have activated EGFR-Stat3 signaling with low apoptosis. Our findings suggest that pStat3 expression may be helpful in identifying patients appropriate for treatment with EGFR tyrosine kinase inhibitors.

Non – small cell lung cancer (NSCLC) continues to be a disease characterized by late stage of presentation coupled with intrinsic resistance to cytotoxic chemotherapy. The epidermal growth factor receptor (EGFR) is frequently overexpressed in NSCLC and can regulate NSCLC growth and survival. Gefitinib and erlotinib are small molecule inhibitors of EGFR tyrosine kinase activity that act by inhibiting EGFR autophosphorylation and subsequently inhibit NSCLC tumor growth (1, 2). Gefitinib or erlotinib monotherapy in patients who received prior platinum therapy resulted in objective responses of ~10% and stable disease in nearly 30% of patients (3, 4). More recently, erlotinib was shown to extend survival for previously treated patients with NSCLC (5). No added benefit of EGFR agents was found when combined with chemotherapy despite preclinical evidence suggesting otherwise (6 – 8).

Mutations in the tyrosine kinase domain of EGFR have been described that correlate with clinical responses to gefitinib (9 – 11). These mutations result in EGFR proteins demonstrating increased sensitivity to gefitinib and erlotinib. Cell lines harboring mutant EGFR molecules are dependent on EGFR for survival because inhibition of EGFR using gefitinib, RNA interference knockdown, or EGFR antibodies results in apoptosis (12 – 14). Importantly, mutant EGFR proteins selectively activate Akt and signal transducers and activators of transcription (STAT) pathways that are important in NSCLC cell survival (12, 13).

A key pathway downstream of EGFR is the STAT pathway. Originally identified as key components linking cytokine signals to transcriptional events in cells, STAT proteins, especially Stat3 and Stat5, play a major role in tumorigenesis (15). Stat3 forms dimers when activated by tyrosine kinase signals and translocates to the nucleus to regulate expression of genes by binding to elements within promoters (16). Stat3 regulates a number of pathways important in tumorigenesis,

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including cell cycle progression, apoptosis, tumor angiogenesis, invasion and metastasis, and tumor cell evasion of the immune system (15, 17, 18). One critical role of Stat3 is protecting cells against apoptosis through the transcriptional up-regulation of survival genes, such as *Bcl-xL*, *Bcl-2*, *Mcl-1*, and *survivin* (19–22).

Because of the important role of Stat3 in tumor growth and survival, we evaluated activated Stat3 signaling in early-stage NSCLC tumors and correlated activated Stat3 with clinical characteristics as well as upstream EGFR signaling and tumor cell apoptosis. Using NSCLC cell lines, we evaluated how activated Stat3 expression relates to EGFR mutations and sensitivity of cells to gefitinib as well as addressed the role of Stat3 in promoting survival in lung cancer cells harboring EGFR mutations.

Patients and Methods

Tissue arrays and immunohistochemistry. The tissue arrays from stage I NSCLC have been described (23). The samples collected for our tissue microarray construction met the following requirements: (*a*) diagnosis of stage I NSCLC without preoperative radiation or chemotherapy, (*b*) surgically resected specimens formalin fixed and embedded in paraffin block, and (*c*) adequate tumor tissue in size for at least three tissue cores. Immunostaining for phosphorylated Stat3 (pStat3) was done as previously described using a rabbit anti-human polyclonal antibody (phosphotyrosine-Stat3 705; Cell Signaling Technology, Beverly, MA); as negative controls, rabbit immunoglobulins (Vector, Burlingame, CA) were used as a primary antibody (24). Immunostaining for pEGFR was done using a rabbit anti-human polyclonal antibody (phosphotyrosine-EGFR 845, Cell Signaling Technology; ref. 23).

Immunohistochemical staining of the slides was reviewed and scored. For pStat3, the score system included counting the percentage of nuclear pStat3-stained tumor cells and estimating the intensity of nuclear pStat3-stained tumor cells in a semiquantitative manner. Because transcriptional activity of Stat3 requires nuclear localization to regulate gene expression, we chose nuclear staining instead of cytoplasmic staining (16). For pEGFR, the score system included counting the percentage of positively stained tumor cells and estimating the intensity staining in a semiquantitative manner. Intensity was classified as 0 to 3 (0 = no staining, 1 = weak staining, 2 = medium, and 3 = strong staining). For triplicate samples, mean values of the percentage of staining, the intensity, and a composite score (percentage multiplied by intensity, range 0-300) were derived.

Tumor apoptosis was evaluated by detecting apoptotic cells and apoptotic bodies using *in situ* labeling with an ApopTag Plus Peroxidase *In situ* Apoptosis Detection kit (Chemicon International, Temecula, CA). This technique detects DNA fragmentation due to apoptosis at the single cell level. The 3′-OH termini generated by DNA end nicking are labeled with modified nucleotides by terminal deoxynucleotidyl transferase, which more selectively detects apoptotic cells rather than necrotic cells. The incorporation of these modified nucleotides is assayed by a specific antibody for immunohistochemical analysis. The scoring system for ApopTag involved counting the percentage of positively stained tumor cells, estimating the intensity staining in a semiquantitative manner as before, generating a composite score and taking the mean value from the three cores.

Correlation coefficients between the expression of pStat3 and demographic data were calculated according to Spearman. The Wilcoxon rank sum test was used to test for significant associations between dichotomous variables and pStat3 expression, and the Kruskal-Wallis test was used for variables with more than two categories. Overall and disease-free survival probabilities were estimated using the Kaplan-Meier method and log-rank testing was used to determine the level of significance between survival curves.

Cell lines and cell culture. Human NSCLC cell lines were purchased from American Type Culture Collection (ATCC; Manassas, VA). H3255 cells were provided by Dr. Pasi Janne and grown as previously described (25). All other cells were grown in RPMI 1640 supplemented with 2 mmol/L L-glutamine (Santa Cruz Biotechnology, Santa Cruz, CA) and 5% bovine calf serum (Hyclone). Subconfluent cells were prepared for protein extracts as detailed below. Cell numbers were determined by counting with a hemocytometer. Gefitinib (ZD1839) was provided by Astra Zeneca (Wilmington, DE).

Cytotoxicity assays. Cytotoxicity assays [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were done according the recommendations of the manufacturer (Roche, Indianapolis, IN). Cells were counted and 5×10^4 cells were placed into single wells in a 96-well plate. Cells were grown as above and exposed to indicated agents as described. Cell viability was assessed following 72 hours. Data presented represents two separate experiments with eight data points per condition. Data were expressed as mean of eight data points.

Protein expression analysis. Cell lysates were normalized for total protein content (50 μg) and subjected to SDS-PAGE as described before (26). Primary antibodies used in these studies consisted of Stat3 (Transduction Laboratories; BD Biosciences, Mississauga, Ontario, Canada), pStat3 Y705 (Cell Signaling Technology), cleaved poly(ADP-ribose) polymerase (Cell Signaling Technology), Mcl-1 (Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-2 (DAKO, Carpinteria, CA), Bcl-xL (PharMingen, San Diego, CA), and β-actin (Sigma-Aldrich, Milwaukee, WI). Detection of proteins was accomplished using horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence purchased through Amersham Biosciences (Pittsburgh, PA).

Antisense oligonucleotides. Antisense Stat3 oligonucleotides, the control mismatch oligonucleotides, and the transfection method had been previously described (26). Cells were transfected using LipofectAMINE 2000 per the recommendations of the manufacturer.

Results

We characterized patterns of activated Stat3 using high-density tissue microarrays using surgical resected NSCLC. Primary NSCLC from 176 patients were arrayed in triplicate. We used a phosphotyrosine 705–Stat3 antibody to investigate the levels and cytologic locations of activated Stat3 (termed pStat3) in tumor specimens (24). Tyrosine 705 on Stat3 is phosphorylated by upstream tyrosine kinases, such as EGFR, and this allows for dimer formation, translocation to the nucleus, and DNA binding. Because transcriptional activity of Stat3 requires nuclear localization to regulate gene expression, we evaluated nuclear staining (16). For each tumor, the mean composite nuclear pStat3 score (intensity multiplied by percentage of tumor cells stained in the three cores) was recorded and correlated with clinical and pathologic variables. A description of the patient characteristics is shown in Table 1. A representative figure for pStat3 staining is shown in Fig. 1. Results of pStat3 staining and correlations with patient characteristics are shown in Table 2.

These results show nuclear pStat3 expression in 54% of tumors (composite score range 0-222). We found higher pStat3 expression in patients with limited smoking history ($P = 0.02$) and in smaller tumors ($P < 0.0001$). We identified a trend toward higher pStat3 expression in tumors with bronchioloalveolar carcinoma or adenocarcinoma histology compared with either squamous cell or large cell histology ($P = 0.09$). No relationship was identified between pStat3 and either overall or disease-free survival following surgical resection (Fig. 2).

We next evaluated how activated EGFR correlates with pStat3. We previously found expression of pEGFR in 51% of tumor tissues with higher expression in patients with low smoking history and in smaller tumors (23). Consistent with laboratory

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Table 1. Patient demographics

	n (%)
Patient characteristics	
Total	176
Age (range)	45-84
Age (median)	69
Men	97 (55.1)
Women	79 (44.9)
White	170 (96.6)
Other race	6 (3.4)
Active smoker	42 (23.9)
Former smoker	92 (52.3)
Lifelong nonsmoker	26 (14.8)
Unknown smoking	16 (9.1)
Histology	
Adenocarcinoma	69 (39.2)
Bronchioloalveolar carcinoma	26 (14.8)
Squamous	58 (33.0)
Large cell	23 (13.1)
Pathologic stage	
IA	72 (40.9)
IB	94 (53.4)
>I	10 (5.7)
Follow-up	
Follow-up	0-146 mo
Median follow-up	37 mo
Total alive	96 (54.5)
Total dead	80 (45.5)

Abbreviation: pStage, pathologic stage.

studies showing EGFR signaling can activate Stat3, we identified a very strong correlation between pEGFR expression and pStat3 expression in this patient cohort (Spearman's $\rho = 0.55$, $P < 0.0001$; refs. 15, 26). Based on published reports showing that Stat3 provides an antiapoptotic signal, we correlated pStat3 expression and apoptosis within the primary tumor (15, 26). Tumor apoptosis was evaluated by detecting apoptotic cells and apoptotic bodies using *in situ* labeling with the ApopTag Detection kit. This technique detects DNA fragmentation due to apoptosis at the single-cell level. Consistent with the known role of Stat3 in promoting tumor cell survival, we found a negative correlation between pStat3 and tumor apoptosis (Spearman's $\rho = -0.19$, $P = 0.01$) consistent with less apoptosis in tumors expressing higher amounts of pStat3.

We also evaluated how the level of Stat3 activation correlates with sensitivity of NSCLC cell lines to death induced by gefitinib. Based on our previous work and those describing EGFR mutations, we postulated that cell lines harboring mutant EGFR would possess high levels of constitutively activated Stat3 (12, 26). H3255, H1650, and H1975 cell lines that contain mutations of EGFR, along with H460, H358, H1299, and A549 NSCLC cell lines with wild-type EGFR, were exposed to increasing concentrations of gefitinib and cell viability was assayed. Whole cell proteins from untreated cells were evaluated for pStat3 and total Stat3. As shown in Fig. 3A, mutant EGFR cells are sensitive to gefitinib with an approximate IC_{50} of 100 nmol/L, whereas wild-type cell lines are resistant to gefitinib (IC_{50} > 10 μmol/L). The mutation status and sensitivity of cell lines to gefitinib correlates with the level of whole cell pStat3 activity because H3255, H1650, and H1975 cells have markedly higher levels of pStat3 compared with the other cell lines (Fig. 3B).

We next evaluated how inhibition of EGFR tyrosine kinase activity by gefitinib affects downstream Stat3 activity. NSCLC cells with distinct EGFR mutations were exposed to increasing doses of gefitinib for 24 hours and total proteins were evaluated for pStat3 and total Stat3 (Fig. 3C). In H3255 cells with the L858R mutation, we find that inhibition of EGFR tyrosine kinase by gefitinib results in a complete inhibition of pStat3. This suggests that loss of Stat3 activity contributes to gefitinib-mediated apoptosis. However, in H1650 cells with the L746-p753 deletion mutation or in gefitinib-sensitive H1975 cells, gefitinib has no effect on pStat3. We confirmed that gefitinib inhibits EGFR function because pTyr 1068 EGFR is down-regulated in all the cells tested (data not shown). Therefore, despite enhanced levels of pStat3 in cells harboring EGFR mutation, the ability of gefitinib to down-regulate Stat3 activity seems to be cell line dependent. Our results may suggest that Stat3 activation in EGFR mutant cells results from altered scaffolding function of EGFR cooperating with a separate tyrosine kinase that is responsible for Stat3 activation.

To determine if Stat3 plays a role in survival in EGFR mutant cells, we used antisense Stat3 oligonucleotides to down-regulate Stat3 as previously described (26). H1650 and H1975 cells exposed to antisense Stat3 show decreased Stat3 protein levels as well as cleavage of poly(ADP-ribose) polymerase indicative of apoptosis (Fig. 3D). Similar to our previous results, no

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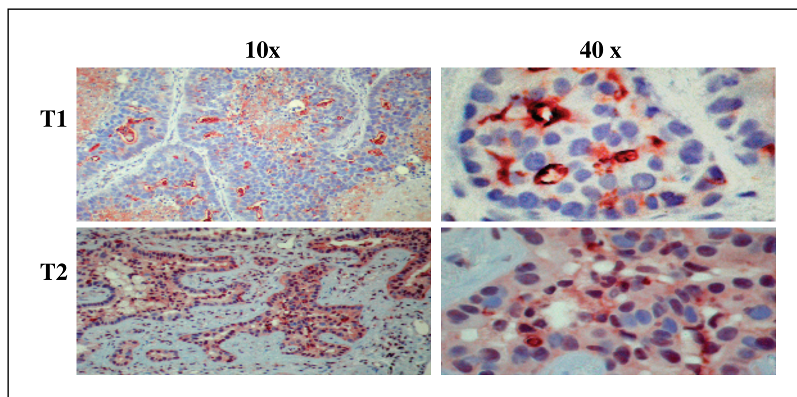

Fig. 1. Representative pStat3 expression in NSCLC. Representative example of pStat3 expression in NSCLC tumors demonstrating the absence of nuclear expression in negative tumor (*T1*) and the presence of nuclear pStat3 expression in positive tumor (*T2*).

Table 2. Correlation of pStat3 with patient characteristics

	pStat3 positive	pStat3 negative	Percentage pStat3 positive
Age			
P	0.20		
Sex			
Male	49	48	51%
Female	45	34	57%
P	0.27		
Tumor size			
P	<0.0001		
Stage			
IA	44	28	61%
IB	44	50	47%
P	0.11		
Pack-year smoking			
P	0.02		
Histology			
Adeno/BAC	58	37	61%
Large	11	12	48%
Squamous	25	33	43%
P	0.09		

NOTE: Percentages of pStat3-positive and pStat3-negative patients are shown based on sex, stage, and histology.
Abbreviations: Adeno, adenocarcinoma; BAC, bronchioloalveolar carcinoma.

apoptosis was observed in H1299 cells despite Stat3 knockdown (26). Levels of the Bcl-2 family proteins Bcl-xL, Mcl-1, and Bcl-2 were reduced in cells treated with antisense Stat3 consistent with the known role of Stat3 in regulating these proteins (Fig. 3E; ref. 15). Taken together with our previous findings, our results suggest that Stat3 communicates a survival signal from mutant EGFR to downstream Bcl-2 survival proteins (26).

Discussion

We found nearly 50% of early stage NSCLC tumors have activated EGFR-Stat3 signaling with subsequent suppression of apoptosis. These findings extend results in cell culture models demonstrating that EGFR signaling enhances Stat3 activity and Stat3 promotes survival in tumor specimens from NSCLC patients (26). pStat3 expression was found more often in patients with limited smoking history and we identified a trend toward higher pStat3 expression in adenocarcinoma/bronchioloalveolar carcinoma tumors. In our patient cohort consisting mainly of stage I tumors, expression of pStat3 had no apparent influence on prognosis following surgery. Similar results have been found by other investigators (27). Interestingly, cell lines harboring EGFR mutations that are sensitive to gefitinib have abundant pStat3 expression above that of cell lines without EGFR mutations but we have not addressed this in tumor specimens.

Our results may be helpful in identifying patients appropriate for EGFR targeting agents. Substantial data now exists that mutations in the tyrosine kinase domain of EGFR are associated with increased sensitivity to EGFR tyrosine kinase inhibitors erlotinib and gefitinib (9 – 11, 28). A recent paper found that 14% of patients with NSCLC in the United States have EGFR tyrosine kinase domain mutations that are more common in never smokers, adenocarcinoma histology, and in females (29). These findings provide a molecular mechanism for the previous finding that patients with bronchioloalveolar subtype of adenocarcinoma who were lifelong nonsmokers were more likely to respond to gefitinib (30). The rate of EGFR mutation closely matches the response rates to single agent gefitinib or erlotinib (~10%) and in conjunction with the above *in vitro* findings suggests that mutational status can accurately predict response to EGFR tyrosine kinase inhibitor (3, 4). Despite these findings, mutation in EGFR was not found to correlate with response to erlotinib and other have suggested that EGFR or HER2 gene amplification predicts gefitinib response (31 – 34).

Although both EGFR mutations and EGFR gene amplification have been shown to predict sensitivity to EGFR tyrosine kinase inhibitor, identifying patients who may clinically benefit from EGFR tyrosine kinase inhibitor, other than through overt tumor response, remains unclear. EGFR tyrosine kinase inhibitor are known to result in disease stabilization in ~30% to 40% of patients and a hypothetical analysis suggests that survival rates are improved in erlotinib-treated patients when responders are eliminated from the survival analysis (3 – 5, 35). These findings suggest the need for other biomarkers, possibly pStat3, that can provide additional information to predict clinical benefit for EGFR targeting agents (11). Although one simple approach would be to assay for activated EGFR using phosphospecific antibodies, this is problematic because multiple tyrosine residues on EGFR can be phosphorylated. The identification of pStat3 expression may identify tumors with upstream EGFR signaling that is physiologically important for tumor cell growth and/or survival. Simply put,

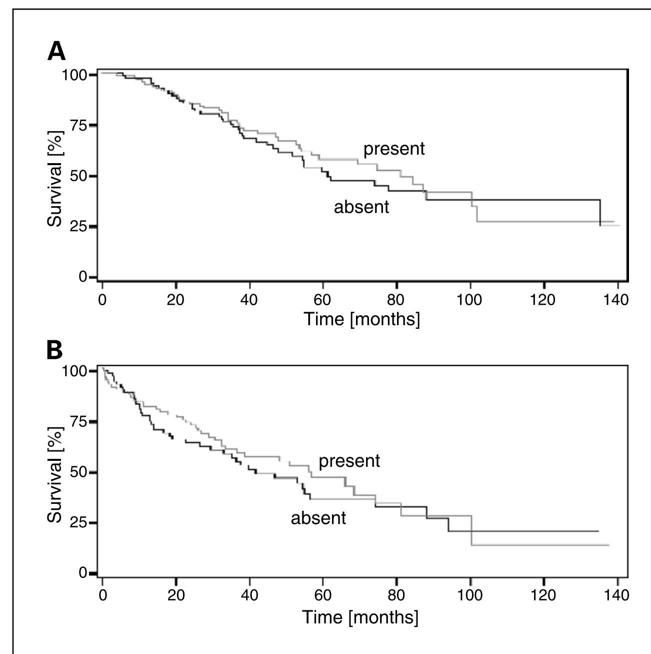

Fig. 2. Survival curves for pStat3. Overall (*A*) and disease-free survival (*B*) curves for a patient cohort dichotomized between the presence or absence of pStat3 expression (*P* = 0.59 and 0.44, respectively).

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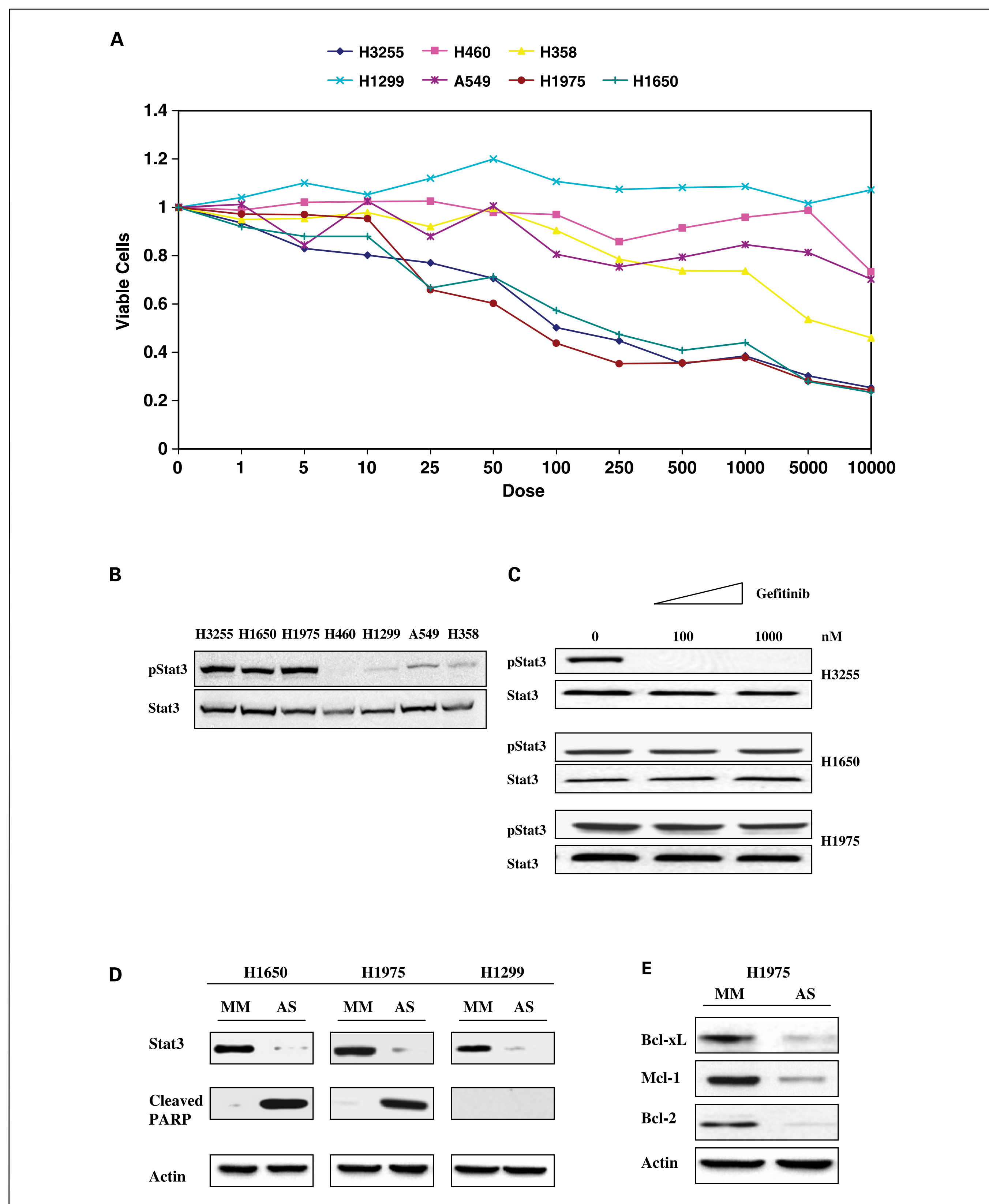

Fig. 3. Mutant EGFR cell lines possess high levels of activated Stat3 and this correlates with their sensitivity to gefitinib. *A,* cell lines were exposed to increasing doses of gefitinib (nmol/L) and cell viability was assayed after 72 hours. *B,* proteins from untreated cell lines were evaluated for pStat3 and total Stat3 levels using Western analysis. *C,* cells were treated with indicated concentration of gefitinib for 24 hours and proteins were evaluated for pStat3 and total Stat3 levels using Western analysis. *D,* cells were treated with either antisense Stat3 or mismatch control oligonucleotides for 24 hours, and total Stat3 and cleaved poly(ADP-ribose) polymerase (*PARP*) were evaluated by Western analysis. *E,* proteins from either antisense Stat3 (*AS*) or mismatch-treated H1975 (*MM*) cells were probed with antibodies specific for Bcl-xL, Mcl-1, and Bcl-2.

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pStat3 expression may behave as a sensor of upstream EGFR signaling, either through mutation, gene amplification, coexpression of other Erb family members, or other unknown mechanisms (14). This is supported by our previous studies in NSCLC cell lines showing that EGF can activate Stat3 as well as reports that transformation of lung epithelial cells by ErbB-2 induces Stat3 activation (26, 36). Interestingly, our finding that nearly 50% of NSCLC show pStat3 expression closely matches the combined rates of objective tumor response and disease stabilization found with gefitinib or erlotinib (3, 4). Finally, ~10% to 15% of patients responding to EGFR targeting agents do not have identifiable EGFR mutations (28). The ability of pStat3 to act as a sensor of EGFR activity may identify this subset of patients that lack EGFR mutation but nonetheless are dependent on EGFR for survival. It remains unclear if a certain threshold of pStat3 expression is required to predict sensitivity or resistance to EGFR targeting agents and further studies are required to evaluate this possibility as well as the above hypotheses.

We were initially surprised to find that gefitinib reduced pStat3 in only one of the three EGFR mutant cells tested (H3255). This result suggests that the simple model of mutant EGFR directly leading to enhanced Stat3 phosphorylation may be incorrect. Although Stat3 activation can result from simple interaction between receptor or nonreceptor tyrosine kinase molecules, it can also result from a multimeric complex interaction between receptors with intrinsic tyrosine kinase activity in cooperation with Src and Janus-activated kinase proteins. Adding to the complexity, this process can be cell type–specific as well as depend on the particular oncogenic signals involved. Zhang et al. (37) previously reported data, suggesting a model whereby Stat3 is activated by Src and Janus-activated kinase cooperating on a growth factor receptor. In this model, Src activates Jak1 that in turn phosphorylates the cytoplasmic tail of a membrane receptor. The receptor phosphotyrosines act as docking sites for recruited Stat3 that is activated by Src proteins. Importantly, the intrinsic tyrosine kinase activity of the receptor was not required, suggesting that the receptor serves as a scaffold for recruiting Stat3 that is activated through other protein tyrosine kinases. This model has important relevance to studying mutant EGFR proteins by suggesting that recruitment of oncoproteins such as Src or Janus-activated kinases may be involved in mediating Stat3 activation and this process may be independent of intrinsic tyrosine kinase activity of the receptor proteins.

Our results suggest that approximately one half of all stage I NSCLC tumors may be dependent on continuous signaling from the EGFR-Stat3 pathway for growth and/or survival and agents targeting this pathway may have better efficacy in this subset of NSCLC compared with advanced stage patients. Expression patterns of pStat3 in more advanced stage patients also remains to be defined. Our data also suggests that a therapeutic opportunity exists to suppress tumor cell growth through Stat3 inhibitors appropriately used in patients with Stat3-positive NSCLC tumors (15, 38). Finally, one could predict that tumors with activated Stat3 are inherently more resistant to chemotherapy through enhanced activation of survival pathways such as enhanced Bcl-xL expression (15). Therefore, Stat3 activation may be a molecular marker in lung cancer that predicts tumor dependency on upstream EGFR and relative resistance to cytotoxic chemotherapy via regulation of survival pathways. Confirmation of these hypotheses will require prospective testing in clinical trials.

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