Steroid Receptor Coactivator-3 Expression in Lung Cancer and Its Role in the Regulation of Cancer Cell Survival and Proliferation

Di Cai1,2, David S. Shames1, Maria Gabriela Raso7, Yang Xie6, Young H. Kim10, Jonathan R. Pollack10, Luc Girard1, James P. Sullivan1, Boning Gao1, Michael Peyton1, Meera Nanjundan11, Lauren Byers8, John Heymach8, Gordon Mills9, Adi F. Gazdar1, Ignacio Wistuba2, Thomas Kodadek2, and John D. Minna1,3,4,5

Abstract

Steroid receptor coactivator-3 (SRC-3) is a histone acetyltransferase and nuclear hormone receptor coactivator, located on 20q12, which is amplified in several epithelial cancers and well studied in breast cancer. However, its possible role in lung cancer pathogenesis is unknown. We found SRC-3 to be overexpressed in 27% of non–small cell lung cancer (NSCLC) patients (n = 311) by immunohistochemistry, which correlated with poor disease-free (P = 0.0015) and overall (P = 0.0008) survival. Twenty-seven percent of NSCLC’s exhibited SRC-3 gene amplification, and we found that lung cancer cell lines expressed higher levels of SRC-3 than did immortalized human bronchial epithelial cells (HBEC), which in turn expressed higher levels of SRC-3 than did cultured primary human HBECs. Small interfering RNA-mediated downregulation of SRC-3 in high-expressing, but not in low-expressing, lung cancer cells significantly inhibited tumor cell growth and induced apoptosis. Finally, we found that SRC-3 expression is inversely correlated with gefitinib sensitivity and that SRC-3 knockdown results in epidermal growth factor receptor tyrosine kinase inhibitor–resistant lung cancers becoming more sensitive to gefitinib. Taken together, these data suggest that SRC-3 may be an important oncogene and therapeutic target for lung cancer. Cancer Res; 70(16); OF1–9. ©2010 AACR.

Introduction

Steroid receptor coactivator-3 (SRC-3; AIB1/ACTR/RAC3/p/CIP1) is a member of the p160 SRC family. SRC-3 has histone acetyltransferase activity and interacts with multiple nuclear receptors and transcription factors to regulate the expression of their target genes, including estrogen receptor (ER), progesterone receptor (PR), E2F1, NF-kB, and activator protein-1 (AP-1). SRC-3 also plays a role in epidermal growth factor receptor (EGFR) signaling (2).

SRC-3 has been implicated in the development of many human cancers. Amplification and overexpression of SRC-3 is detected in 5% to 10% of ovarian and 30% to 60% of breast cancers (3). SRC-3 overexpression in breast cancer is associated with high levels of human epidermal growth factor receptor 2 (HER2/neu) and EGFR, tamoxifen resistance, and poor disease-free survival, suggesting that there may be cross-talk between the SRC-3, HER2/neu, and EGFR signaling pathways in the genesis and progression of some breast tumors (4, 5). SRC-3 is also amplified and overexpressed in many other types of cancer, including prostate cancer (6), ER- and PR-negative breast cancer (7), and gastric cancer and colorectal carcinoma (1). Increased SRC-3 expression is also observed during pancreatic cancer (8) and esophageal tumor progression (9). However, a comprehensive profiling of SRC-3 gene and protein expression levels in lung cancers is lacking, as is knowledge of the function of SRC-3 in lung cancer cell survival and proliferation.

In this study, we show that SRC-3 is overexpressed in a subset of lung cancers, which correlates with poor disease-free and overall survival and, in some cases, is associated with DNA amplification. Knockdown of SRC-3 in lung cancer leads to reduced cell growth, decreased anchorage-independent colony formation ability, and increased apoptosis in non–small cell lung cancer (NSCLC) cell lines with high endogenous levels of SRC-3. In addition, we show that SRC-3 knockdown can potentiate the effect of gefitinib in EGFR tyrosine kinase inhibitor (TKI)–resistant cells.
Materials and Methods

Case selection and tissue microarray construction

We obtained archived, formalin-fixed, paraffin-embedded (FFPE) tissues from surgically resected (with curative intent) NSCLC specimens (lobectomies and pneumonectomies) containing tumor and adjacent normal epithelium tissues from the Lung Cancer Specialized Program of Research Excellence Tissue Bank at The University of Texas M.D. Anderson Cancer Center (Houston, Texas), under a protocol approved by the institutional review board. The tissues had been collected from 1997 to 2001. The tissue specimens were histologically examined and classified using the 2004 WHO classification system (10). We selected 311 NSCLC tissue samples (188 adenocarcinomas and 123 squamous cell carcinomas) for our tissue microarrays (TMA). TMAs were constructed using triplicate 1-mm-diameter cores per tumor, and each core included central, intermediate, and peripheral tumor tissues. Detailed clinical and pathologic information, including demographics, smoking history (never and ever smokers), smoking status (never, former, and current), clinical and pathologic tumor-node-metastasis (TNM) stage, overall survival duration, and time to recurrence, was available for most cases. Patients who had smoked at least 100 cigarettes in their lifetime were defined as smokers, and smokers who quit smoking at least 12 months before their lung cancer diagnosis were defined as former smokers. Tumors were pathologic TNM stages I to IV according to the revised International System for Staging Lung Cancer.

Immunohistochemical staining, evaluation, and statistical analysis of clinical correlation

Using anti–SRC-3/AIB-1 mouse monoclonal antibody from BD Transduction Laboratories (6), immunohistochemical staining was performed as follows: Five-micrometer FFPE tissue sections were deparaffinized, hydrated, heated in a steamer for 10 minutes with 10 mmol/L sodium citrate (pH 6.0) for antigen retrieval, and washed in Tris buffer. Peroxide blocking was done with 3% H2O2 in methanol at room temperature for 15 minutes, followed by 10% fetal bovine serum (FBS) in Tris-buffered saline-T for 30 minutes. The slides were incubated with primary antibody at 4°C for 90 minutes, washed with PBS, and incubated with biotin-labeled secondary antibody (Envision Dual Link +, DAKO) for 30 minutes. Staining for the slides was developed with 0.05% 3′,3-diaminobenzidine tetrahydrochloride, which had been freshly prepared in 0.05 mol/L Tris buffer at pH 7.6 containing 0.024% H2O2, and then the slides were counterstained with hematoxylin, dehydrated, and mounted. FFPE A549 cells were used as a positive control. For the negative control, we used the same specimens used for the positive controls but replaced the primary antibody with PBS. For this antibody, we performed titration experiments using a relatively wide range of antibody concentrations (1:50, 1:100, 1:200, and 1:500), including the concentration suggested by the manufacturer. One observer (M.G.R.) quantified the immunohistochemical expression using light microscopy (magnification, ×20). Both nuclear and cytoplasmic expressions were quantified using a four-value intensity score (0, 1+, 2+, and 3+) and the percentage (0–100%) of reactivity. We defined the intensity categories as follows: 0, no appreciable staining; 1+, barely detectable staining in epithelial cells compared with the stromal cells 2+, readily appreciable staining; and 3+, dark brown staining of cells. Next, an expression score was obtained by multiplying the intensity and reactivity percent values (theoretical range, 0–300). There were three cores of tissue per case in the TMA. On each core, the score was obtained as described earlier. The average of the three cores was used as the score for this case.

Array comparative genomic hybridization

Comparative genomic hybridization (CGH) on cDNA microarrays was carried out as previously described (11). Briefly, 4 μg of tumor and normal sex-matched reference genomic DNA were random primer labeled with Cy5 and Cy3, respectively, then hybridized to a cDNA microarray (Stanford microarray core) containing ~39,000 cDNAs representing ~26,000 mapped genes/expressed sequence tags. Hybridized arrays were scanned on a GenePix scanner (Axon Instruments), and fluorescence ratios extracted using SpotReader software (Niles). Normalized log 2 ratios were then mapped onto the genome using the National Center for Biotechnology Information genome assembly (Build 36). A preliminary report of these data (focused on TITF1 amplification) was recently published (12). Copy number at the SRC-3 locus was determined with the cghFLasso algorithm using a false discovery rate of 0.001 (13).

Cell lines and cell culture

All cells were maintained in RPMI 1640 supplemented with 5% FBS (Invitrogen). Most of these cell lines were established by John D. Minna and Adi Gazdar at the National Cancer Institute and the Hamon Cancer Center for Therapeutic Oncology at University of Texas Southwestern Medical Center (14, 15). The other cell lines were obtained from the American Type Culture Collection (ATCC). All of the cell lines have been DNA fingerprinted for provenance using the PowerPlex 1.2 kit (Promega). The DNA fingerprints were all confirmed to be same as in the DNA fingerprint library maintained by ATCC or the Minna/Gazdar lab (which is the primary source of the lines). The lines were also tested to be free of Mycoplasma by e-Mycoto kit (Boca Scientific).

Reverse transcription

Total RNAs were isolated using Trizol reagent (Invitrogen) following the manufacturer’s instruction. First-strand cDNA was reverse transcribed with 3 μg of mRNA using the SuperScript II first-strand synthesis system (Invitrogen). The final volume was 20 μL.
Quantitative PCR

TaqMan Gene Expression Assays for the SRC-3/NCoA3 gene and the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were purchased from Applied Biosystems. To generate the standard curve, serial dilution of MCF-7 cDNAs was run in triplicate for both SRC-3 and GAPDH. Efficiency for both primer and probe is almost as high as 2 ($E = 10^{(-1/slope)} = 10^{(-1/-3.3)} = 2$) (data not shown), making the $\Delta \Delta Ct$ method appropriate for use in data analysis.

For the SRC-3 expression in 33 tested cell lines, all the cDNAs were run for both SRC-3 and GAPDH in triplicate. Threshold and baseline were set up similarly for both relative standard curve generation and comparative Ct generation for the whole panel. In each group of cells [i.e., NSCLC versus small cell lung cancer (SCLC) versus human bronchial epithelial cells (HBEC)], the cell line that expresses the lowest level of SRC-3 in the group is defined as 1, and the other lines are relative levels to the lowest one, using [relative expression level = $100 \times 2^{-\Delta \Delta Ct}$] for calculation.

Immunoblot analysis

All cells were maintained in RPMI 1640 supplemented with 5% FBS (Invitrogen); cells were grown to 80% confluency before lysis. Cell lysates were prepared with 1% SDS–containing lysis buffer followed by boiling. Cell extracts with equal amounts of proteins were analyzed by immunoblotting. The SRC-3/AIB1 antibody (BD Transduction Laboratories) was used at 1:2,000 dilution. Antibodies for cleaved caspase-7 (1:1,000) and phospho-SRC-3 (Thr24; 1:1,000) were from Cell Signaling Technology and antibody for GAPDH was from Santa Cruz. Western blot band intensity is quantified using ImageJ following the program instruction.

Reverse-phase protein array

Protein lysates were prepared as previously described (16). Reverse-phase protein array (RPPA) was produced and analyzed as previously described, with slight modifications (17). RPPA data were quantified using the SuperCurve method, which detects changes in protein level with MicroVigene software (VigeneTech) and an R package developed in-house (18).

Small interfering RNA transfection

Human SRC-3 SMARTpool small interfering RNA (siRNA) and nonspecific control siRNA were obtained from Dharmacon Research, Inc. siRNAs were transfected with Dharmafect 2 (Dharmacon) into H1299, Dharmafect 4 into H1819, Dharmafect 3 into H2073, and Dharmafect 1 into A549 at 50 nmol/L following the reverse transfection protocol.

Liquid colony formation assay

Forty-eight hours after siRNA treatment, cells were harvested and counted and single cells were seeded (500–2,000 cells per well) in triplicate into six-well plates. Cells were grown for 11, 12, or 14 days (for H1299, H1819, and H2073, respectively); colonies were stained with methylene blue (0.5% in 70% isopropyl alcohol) or crystal violet for 1 hour at room temperature and counted.

EGFR inhibitor treatment

Clinical-grade gefitinib and cetuximab were obtained from the University of Texas Southwestern Medical Center pharmacy. Gefitinib was dissolved in DMSO to prepare a 10 mmol/L stock drug solution, which was stored at 4°C. The NSCLC cell line H1819 was treated with SRC-3-specific siRNA, nontargeting siRNA pool, or transfection reagent only 24 hours after 100 nmol/L gefitinib was added to the medium; 72 hours later, cells were harvested for Annexin V staining. Treated H1819 cells were trypsinized, counted, and then resuspended in 1× binding buffer (BD Pharmingen; 1 × 10⁶ cells/mL). Annexin V-FITC (5 μL) and propidium iodide (5 μL; BD Pharmingen) were added; the solution was incubated in the dark at room temperature for 15 minutes; and staining was quenched by adding 400 μL of 1× binding buffer to 100 μL of cells. Flow cytometry analysis was done, with the percentage of Annexin V–positive cells normalized to the transfection reagent-only treatment.

Results

SRC-3 expression is highly variable in NSCLCs, and patients whose tumors have high SRC-3 expression have inferior prognosis

Using a specific anti–SRC-3 antibody, we performed semi-quantitative immunohistochemistry on a TMA of 311 clinically annotated NSCLCs and found a wide range of tumor SRC-3 expression (nuclear staining; Fig. 1; see also Materials and Methods): 85 NSCLCs (27%) expressed SRC-3 (staining score ≥10), 144 (46%) showed no SRC-3 staining, whereas 84 (27%) exhibited a SRC-3 nuclear staining score between 0 and 10. Kaplan-Meier analysis was performed on the 215-patient subset for which clinical data including survival were available. As shown in Fig. 1C, patients whose tumors have high SRC-3 levels by immunohistochemistry have significantly shorter overall ($P = 0.0008$, log-rank test) and progression-free ($P = 0.0015$, log-rank test) survival time than patients with low SRC-3 levels. The median overall survival time was 54 months for the high SRC-3 expression group and 98 months for the low SRC-3 expression group (Fig. 1C). After adjusting for the effects of histology, gender, race, tobacco stage, and lymph node status in the multivariate proportional hazard survival model, high SRC-3 was still significantly associated with poor overall (hazard ratio, 2.02; $P = 0.0007$) and progression-free survival (hazard ratio, 1.92; $P = 0.0011$; Table 1). Importantly, the expression of SRC-3 did not correlate with EGFR mutation ($P = 0.365$, $t$ test) or KRAS mutation ($P = 0.256$, $t$ test; Supplementary Table S1).

SRC-3 copy number alterations and mRNA and protein levels in NSCLC and breast cancer lines

Analysis of SRC-3 (at 20q12) DNA copy number alterations by array-based CGH in a panel of lung and breast cancer cell lines revealed SRC-3 copy number gain in 14 of 55 (25%) NSCLC lines, 8 of 23 (35%) SCLC lines, and 15 of 31 (48%) breast cancer cell lines, whereas 4 of 55 (7%) NSCLC lines, 1 of 23 (4%) of SCLC, and 1 of 31 (3%) breast cancer cell lines showed copy number loss at this locus (Supplementary Table S2).
SRC-3 mRNA expression analysis by quantitative reverse transcription-PCR (RT-PCR) of 16 NSCLC, 11 HBEC lines immortalized by expression of cyclin-dependent kinase 4 and telomerase (HBEC-KT), and 6 primary, unimmortalized HBEC lines (HBEC-UI) showed that >30% of NSCLCs overexpress SRC-3 at least 3- to 4-fold higher than the average for the HBECs. Overall, there was a >20-fold variation in the mRNA level of SRC-3 expression within NSCLCs and the average

Figure 1. NSCLCs have variable immunohistochemical expression of SRC-3, but patients whose tumors have high SRC-3 expression have inferior survival. A, representative immunohistochemical images showing low and high SRC-3 expression in NSCLCs. B, distribution of SRC-3 nuclear expression in 311 clinically localized (see Materials and Methods) NSCLCs. C, Kaplan-Meier analysis of disease-free survival (left) and overall survival (right) with high (red; nuclear staining index >1.6667) or low (blue; index ≤1.6667) SRC-3 expression.
SRC-3 expression level of NSCLCs was greater than that of the immortalized HBECs, which in turn was greater than that of the nonimmortalized HBECs. The means and 95% confidence intervals of mRNA expression of SRC-3 for NSCLC, HBEC-KT, and HBEC-UI are 12.6 [7.3–17.8], 9.0 [6.2, 11.8], and 3.6 [3.0, 4.3], respectively. The overall P value of comparing three groups is 0.078 based on the ANOVA test; therefore, the differences seen in mRNA expression of SRC-3 between NSCLC, and HBEC-UI are 12.6 [7.3–17.8], 9.0 [6.2, 11.8], and 3.6 [3.0, 4.3], respectively. The overall P value of comparing three groups is 0.078 based on the ANOVA test; therefore, the differences seen in mRNA expression of SRC-3 between NSCLC,

### Table 1. Multivariate survival analysis of the clinical correlation with SRC-3 expression in lung cancer patients

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<td>0.5278</td>
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**NOTE:** (A) Multivariate analysis for disease-free survival. (B) Multivariate analysis for overall survival. Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval.
HBEC-KT, and HBEC-UI are significant (Fig. 2A). The NSCLC lines (HCC44, HCC15, and H28) with SRC-3 expression level lower than that in HBEcs were those with SRC-3 DNA copy number loss. In addition, SRC-3 protein expression was highly variable in HBEC and NSCLC lines as analyzed by Western blotting (Fig. 2B).

The function of SRC-3 is regulated by protein phosphorylation, and among the eight mapped phosphorylation sites in SRC-3, modification of Thr24 is crucial for the interaction with ER, androgen receptor, and NF-κB to achieve maximum transcriptional coactivation activity (19). Phosphorylation at this site is also important for the transforming ability of SRC-3 (19). Using an anti–phospho-SRC-3 (Thr24) specific antibody, we found the phosphorylation level of SRC-3 to be highly variable among the lung cancer cell and HBEC lines (Fig. 2B). For example, HBEC13KT and H2073 show no detectable pSRC-3, although they express modest amounts of total protein. On the other hand, H1299 has the highest pSRC-3/SRC-3 ratio, although it does not express the highest level of total SRC-3 protein.

Inhibition of SRC-3 expression in high-expressing NSCLC cells significantly decreases cell growth and elicits a proapoptotic response

To investigate whether the cells expressing high levels of SRC-3 rely on it for survival and proliferation, a pool of four siRNA oligonucleotides targeting SRC-3 was transfected into H1299, which expresses significant levels of both total and phosphorylated (Thr24) SRC-3. The cell viability and liquid colony formation were found to be decreased by more than 70% when SRC-3 was knocked down (Fig. 3). Cleaved caspase-7 expression was detected 72 hours after siRNA transfection and was sustained up to 96 hours, indicating that SRC-3 knockdown in H1299 cells is associated with activation of the apoptosis pathway (Fig. 3A). The SMARTpool siRNAs targeting SRC-3 had been deconvoluted into the individual targeting sequences, and the degree of SRC-3 knockdown is correlated with the degree of effect observed (Supplementary Fig. S1). Note that the control siRNA oligo itself was somewhat toxic and rendered a modest effect (~20% reduction) on cell viability, yet showed no effect on the caspase activation. Contrary to the results in H1299 cells, H1819 and H2073 NSCLCs (expressing medium and low SRC-3 levels, respectively) showed only modest or no effect on growth and liquid colony formation after SRC-3 knockdown (Supplementary Fig. S2). These results suggest that only NSCLCs expressing high levels of SRC-3 (or pSRC-3) show dependence on continued SRC-3 expression for survival and growth.

SRC-3 knockdown sensitizes NSCLC to EGFR antagonists

Several studies have revealed a relationship between SRC-3 expression level and EGFR family pathway signaling in breast
cancer (4, 5). Thus, we wished to know if SRC-3 expression in NSCLC correlates with the response to EGFR-targeted chemotherapy. We examined the expression of 160 proteins potentially involved in signaling and oncogenesis by RPPAs using validated antibodies and correlated protein levels with in vitro sensitivity and resistance to erlotinib, gefitinib, and cetuximab in 48 NSCLC lines using an MTS proliferation assay (20). High EGFR protein expression (often associated with the presence of EGFR oncogenic mutations and amplification) correlated with cetuximab, erlotinib, and gefitinib sensitivity (Supplementary Table S3), whereas high SRC-3 protein expression correlated with resistance to these drugs (Fig. 4A). This result led us to question if SRC-3 knockdown would sensitize NSCLCs with wild-type EGFR to EGFR TKIs. H1819 is a NSCLC cell line expressing moderate levels of SRC-3 and with wild-type EGFR but shows phosphorylation of EGFR, HER2, ErbB3, and downstream effectors such as AKT and P44/42 extracellular signal–regulated kinase, indicating that the EGFR pathway is active (Supplementary Fig. S3; ref. 20). H1819 cells are resistant to gefitinib (IC50, 15 μmol/L), and knocking down SRC-3 decreases their proliferation (Fig. 4B). Whereas neither of these treatments alone elicited apoptosis (with gefitinib given at 0.1 μmol/L, a concentration that is within the range of achievable serum levels in patients), combined siRNA SRC-3 knockdown and 0.1 μmol/L gefitinib led to induction of apoptosis detected by Annexin V staining (Fig. 4B). Also, cells treated with SRC-3–targeted siRNA and gefitinib were less well attached, rounded up, and showed a “spider web”–like appearance when compared with cells treated with SRC-3 siRNA or gefitinib alone (not shown).

Discussion

Studies of SRC-3 in lung cancer have revealed DNA copy number increases and great variation in expression levels, with ~25% of NSCLCs dramatically overexpressing this gene compared with other NSCLCs, normal lung epithelium, and immortalized HBECs. Patients whose tumor showed SRC-3 overexpression also had inferior survival compared with those with low or no SRC-3 expression. Increased expression was also associated with SRC-3 DNA copy number increase. This frequency of high SRC-3 expression is comparable to that found in breast, ovarian, and prostate cancers, all of which can become dependent on SRC-3 for proliferation (3, 6, 7). In addition, NSCLCs varied in their levels of pSRC-3 expression, and the variations in SRC-3 DNA copy number and mRNA, protein, and phosphoprotein levels suggest that there is regulation of SRC-3 at the transcriptional, translational, and posttranslational levels. For example, a recent study identified a ubiquitin ligase, CHIP, which can directly target SRC-3 for ubiquitinylination and degradation, and these changes inhibit the anchorage-independent cell growth and the metastatic potential of cancer cells (21). This is consistent with our general finding that siRNA-mediated SRC-3 knockdown
inhibited cell growth and colony formation in NSCLCs expressing high levels of SRC-3 and pSRC-3, whereas little or no effects of SRC-3 knockdown were seen in NSCLCs with low endogenous SRC-3 levels. It is true that in the A549 cell line, which expresses the highest level of the protein, knockdown of SRC-3 resulted in only a 20% reduction in cell viability. However, we were unsuccessful in achieving efficient knockdown in this line; the maximum knockdown efficiency was 50% using up to 100 nmol/L siRNAs, which still left a significant amount of SRC-3 protein in the cells.

Finally, we discovered a correlation between SRC-3 expression levels and resistance to EGFR TKIs gefitinib and erlotinib. SRC-3 knockdown led to dramatic sensitization of NSCLC with wild-type EGFR to EGFR TKI targeted therapy. The mechanism by which SRC-3 knockdown leads to gefitinib sensitization remains to be elucidated. One possibility is that SRC-3 might be crucial for the activation of

Figure 4. SRC-3 protein expression correlates with EGFR TKI resistance, and SRC-3 knockdown sensitizes NSCLC to EGFR antagonist. A, RPPA correlation analysis with drug sensitivity suggests a relationship between high SRC-3 expression and EGFR TKI resistance. For a panel of 48 NSCLC lines, the expression levels of a panel of proteins were quantified using RPPA and correlated (Pearson values determined) with the IC50 values (MTS assay) for gefitinib, erlotinib, and cetuximab. Red, a correlation of increased IC50 values (resistance) with increased expression of the particular protein; green, a correlation of decreased IC50 values (sensitivity) with increased expression of a protein. Color brightness level indicates the level of the Pearson correlation, with bright red levels found with SRC-3 representing R values of >0.7 of protein with IC50 values. B, SRC-3 knockdown combined with 0.1 μmol/L gefitinib treatment induces apoptosis (measured by Annexin V staining by fluorescence-activated cell sorting) in H1819 cells.
other receptor tyrosine kinases, such as insulin-like growth factor-I (IGF-I) receptor, in lung cancers that can bypass EGFR to activate critical downstream signaling pathways. For example, SRC-3 is known to mediate IGF-I–induced phenotypic changes in human breast cancer cells (22) and SRC-3 deficiency affects breast cancer initiation and progression in mice (23). After this article was submitted for review, an article was published which showed that overexpression and amplification of SRC-3 was found in 48.3% and 8.2% of NSCLCs, respectively, and that overexpression of SRC-3 negatively affects the survival of surgically resected NSCLC patients (24). This article provides an independent validation of our findings. The difference in overexpression and amplification frequencies is most likely due to the fact that an Asian population was the subject of their experiments, whereas a Caucasian population was the subject of ours.

Taken together, our results suggest that SRC-3 is an important new oncogene and potential therapeutic target for lung cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Di Cai, David S. Shames, Maria Gabriela Raso, et al.


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