

Nrf2 and Keap1 Abnormalities in Non–Small Cell Lung Carcinoma and Association with Clinicopathologic Features

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Abstract

Purpose: To understand the role of nuclear factor erythroid-2–related factor 2 (Nrf2) and Kelch-like ECH-associated protein 1 (Keap1) in non–small cell lung cancer (NSCLC), we studied their expression in a large series of tumors with annotated clinicopathologic data, including response to platinum-based adjuvant chemotherapy.

Experimental Design: We determined the immunohistochemical expression of nuclear Nrf2 and cytoplasmic Keap1 in 304 NSCLCs and its association with patients' clinicopathologic characteristics, and in 89 tumors from patients who received neoadjuvant ($n = 26$) or adjuvant platinum-based chemotherapy ($n = 63$). We evaluated *NFE2L2* and *KEAP1* mutations in 31 tumor specimens.

Results: We detected nuclear Nrf2 expression in 26% of NSCLCs; it was significantly more common in squamous cell carcinomas (38%) than in adenocarcinomas (18%; $P < 0.0001$). Low or absent Keap1 expression was detected in 56% of NSCLCs; it was significantly more common in adenocarcinomas (62%) than in squamous cell carcinomas (46%; $P = 0.0057$). In NSCLC, mutations of *NFE2L2* and *KEAP1* were very uncommon (2 of 29 and 1 of 31 cases, respectively). In multivariate analysis, Nrf2 expression was associated with worse overall survival [$P = 0.0139$; hazard ratio (HR), 1.75] in NSCLC patients, and low or absent Keap1 expression was associated with worse overall survival ($P = 0.0181$; HR, 2.09) in squamous cell carcinoma. In univariate analysis, nuclear Nrf2 expression was associated with worse recurrence-free survival in squamous cell carcinoma patients who received adjuvant treatment ($P = 0.0410$; HR, 3.37).

Conclusions: Increased expression of Nrf2 and decreased expression of Keap1 are common abnormalities in NSCLC and are associated with a poor outcome. Nuclear expression of Nrf2 in malignant lung cancer cells may play a role in resistance to platinum-based treatment in squamous cell carcinoma. *Clin Cancer Res*; 16(14); 3743–53. ©2010 AACR.

Lung cancer is the most common cause of cancer-related death in the world (1). Non–small cell lung carcinoma (NSCLC), adenocarcinoma, and squamous cell carcinoma are the most common histologic types (85%; ref. 2). De-

spite intensive research, the prognosis of lung cancer patients remains poor, with a 15% 5-year overall survival (OS) rate (1). For patients with early-stage disease, surgery is the standard treatment (2). Adjuvant chemotherapy has been found to be beneficial for some patients with stage II to IIIA NSCLC (3), whereas for patients with stage IIIB and IV disease, chemotherapy is the standard frontline treatment (3, 4). A combination of drugs that includes a platinum agent is the most common regimen administered for NSCLC (3, 4). However, most tumors either fail to respond due to intrinsic resistance or else develop drug-acquired resistance after an initial response to therapy (3).

Nuclear factor erythroid-2–related factor 2 (Nrf2) is a transcription factor that has been suggested to be associated with cancer development and progression, including in NSCLC (5–8). Nrf2 enables the adaptation of normal cells to oxidants and electrophiles generated by harmful exogenous agents and to reactive oxygen species and their secondary metabolites (9). Under homeostatic conditions, Nrf2 is principally repressed by Kelch-like

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Translational Relevance

Nuclear factor erythroid-2-related factor 2 (Nrf2) is a transcription factor associated with chemotherapy resistance and tumor growth, which is repressed by Kelch-like ECH-associated protein 1 (Keap1). We tested the hypothesis that the abnormal expression of these two proteins correlated with non-small cell lung cancer (NSCLC) patients' outcome and response to adjuvant chemotherapy. We showed that increased Nrf2 expression and decreased Keap1 expression are common abnormalities in NSCLC and are associated with clinical outcome. In our study, abnormal expression of Nrf2 and Keap1 proteins was more common than that of the corresponding gene mutations, suggesting that other mechanisms are involved in the activation of *NFE2L2* and inactivation of *KEAP1*. Nrf2 expression may play a role in response to adjuvant platinum-based chemotherapy in patients with squamous cell carcinoma. Identifying patients with abnormal Nrf2 expression may be important for selection for chemotherapy in NSCLC.

ECH-associated protein 1 (Keap1), which functions as an intracellular redox sensor, targeting Nrf2 for proteasomal degradation. Under oxidant or xenobiotic stress, Keap1 releases Nrf2, which translocates to the nucleus and activates antioxidant response elements and xenobiotics element genes [including NAD(P)H dehydrogenase quinone 1 (NQO1)], resulting in the protein expression of growth factors and receptors, drug efflux pumps, drug-metabolizing enzymes, heat shock proteins, and various transcription factors (5, 9, 10).

One of the mechanisms involved in NSCLC is the nuclear translocation of Nrf2 due to loss of Keap1 expression by biallelic inactivation of the gene by mutation and loss of heterozygosity, or promoter methylation (5, 8, 11–13). An alternative mechanism of Nrf2 activation is mutation of the gene *NFE2L2*, which affects the region of exon 2 that codes the Keap1-binding site of Nrf2 (12); these mutations have been detected in 8% to 11% of NSCLCs, mainly squamous cell carcinoma tumors (12, 14). It has been suggested that abnormalities of the Nrf2/Keap1 pathway that lead to nuclear Nrf2 expression in tumors are an important mechanism to induce platinum-based chemotherapy resistance by promoting tumor cell survival and increasing proliferation (5–8). Recently, it was shown that the inhibition of Nrf2 expression using small interfering RNA augmented carboplatin-induced tumor growth inhibition in a NSCLC xenograft mouse model (8).

To date, no comprehensive analysis has been done for Nrf2 and Keap1 expression and associated genetic abnormalities in NSCLC, and no studies have determined the relationship between Nrf2 expression and clinical outcome after treatment with platinum-based adjuvant che-

motherapy. Therefore, in this retrospective study, we characterized the expression of these two proteins in a large series of NSCLC tissue specimens with annotated clinicopathologic characteristics, including outcome, determined the frequency of exon 2 *NFE2L2* and exon 2 to 5 *KEAP1* mutations, and evaluated the relationship between nuclear Nrf2 expression and outcome in patients treated with platinum-based adjuvant chemotherapy. Because in NSCLC the presence of epidermal growth factor receptor (*EGFR*) and *KRAS* mutation has been associated with the response of the tumor to chemotherapy (15), we also investigated in adenocarcinoma tumors the association between both mutation status and Nrf2 and Keap1 protein expressions.

Materials and Methods

Nrf2 and Keap1 Western blot analysis in cell lines

The human NSCLC cell lines A549 and H460 (with known downregulation of Keap1 protein; ref. 5), H1993, and an SV40-transformed human bronchial epithelial cell line (BEAS2B) were evaluated for nuclear and cytoplasmic expression of Nrf2 and Keap1 proteins by Western blot analysis. Nuclear and cytoplasmic protein extracts of these cell lines were obtained using NE-PER nuclear extraction reagents (Pierce). The lung cancer cell lines were provided by Dr. John D. Minna's laboratory, and they were authenticated by testing them using the PowerPlex^R 2.1 system (Promega). Protein concentrations were estimated using the Bradford assay (Bio-Rad). For Western blot analysis, 25 µg of cell line protein from nuclear and cytoplasmic extracts were loaded in each lane, run on a NuPAGE 4% to 12% Bis-Tris gel (Invitrogen), and transferred onto a nitrocellulose membrane. After being blocked with 5% nonfat milk, the blots were exposed to rabbit primary antibody against Nrf2 (dilution 1:500, clone H300; Santa Cruz Biotechnology) and Keap1 (dilution 1:600; Proteintech), followed by anti-rabbit secondary antibody. The signals were detected using SuperSignal West Pico chemiluminescent substrate (Pierce). β-Actin and poly(ADP-ribose) polymerase (dilution 1:100; Cell Signaling Technology) were used as the controls. The Western blot analysis was done in triplicate.

Case selection for immunohistochemical analysis

To determine the expression of Nrf2 and Keap1 in primary NSCLCs, we selected archived, formalin-fixed, paraffin-embedded (FFPE) tumor tissue samples from surgically resected lung cancer specimens from the Lung Cancer Specialized Program of Research Excellence Tissue Bank at The University of Texas M.D. Anderson Cancer Center. This study was approved by the M.D. Anderson Cancer Center institutional review board. Tumor tissues were histologically analyzed and classified using the 2004 WHO classification system (16). These samples were used to evaluate the immunohistochemical expression of Nrf2 and Keap1 in both a tissue microarray (TMA) and whole tissue sections.

For the TMA, we used 304 tumor tissue samples collected between 1997 and 2003, including 190 adenocarcinomas and 114 squamous cell carcinomas. The cases were selected based on the availability of FFPE tissue blocks with enough tumor tissue for TMA construction. These samples were placed in a TMA using three 1-mm-diameter cores that included tissue from the center, intermediate, and peripheral areas of the tumor, as previously described (17). Detailed clinicopathologic information, including demographics, performance status (based on Eastern Cooperative Oncology Group scale), smoking history (never, former, or current), pathologic tumor-node-metastasis stage (I-IV; Table 1), recurrence-free survival (RFS), and OS duration, were available for most cases. To determine the heterogeneity of nuclear Nrf2 expression in NSCLC tissues, we evaluated whole tumor tissue sections from 36 tumors, including 18 adenocarcinomas and 18 squamous cell carcinomas; 19 of these cases expressed nuclear Nrf2. Thirty of these cases were also examined for the expression of NQO1. The whole tumor histology sections consisted of 1- to 2-cm-diameter tumor specimens with adjacent normal lung tissue.

To determine the expression of Nrf2 and Keap1 proteins in NSCLC after chemotherapy, we evaluated 26 tumor tissues from patients who had undergone neoadjuvant platinum-based chemotherapy. The chemotherapy regimens included carboplatin with paclitaxel ($n = 21$) or cisplatin with etoposide ($n = 4$) or docetaxel ($n = 1$). We also determined the association between Nrf2 and

Keap1 expression and histologic parameters associated with chemotherapy effects in tumor tissues, including the percentages of tumor necrosis, fibrosis, and viable malignant cells.

To determine the relationship between nuclear Nrf2 expression and outcome after adjuvant chemotherapy, we selected 122 NSCLC tumors, 63 from patients who had undergone adjuvant platinum-based chemotherapy, and a similar number of patients who had not undergone any adjuvant therapy ($n = 59$; Supplementary Table S1). The chemotherapy regimens included carboplatin with docetaxel ($n = 9$), gemcitabine ($n = 9$), paclitaxel ($n = 32$), or cisplatin ($n = 1$), either alone or with pemetrexed ($n = 4$), docetaxel ($n = 7$), or etoposide ($n = 1$).

Immunohistochemical analysis of cell lines and tissue specimens

An immunohistochemical analysis was done using commercially available antibodies against Nrf2 (dilution 1:200, clone H300; Santa Cruz Biotechnology), Keap1 (dilution 1:25; Proteintech), and NQO1 (dilution 1:1,000, clone A180; Novus Biologicals). Immunohistochemical staining was done using an automated stainer (Dako, Inc.) with 5- μ m-thick sections from FFPE tissues. Tissue sections were deparaffinized and hydrated, and antigen retrieval was done in pH 6.0 citrate buffer in a decoaking chamber (121°C \times 30 seconds, 90°C \times 10 seconds) and washed on Tris buffer. Peroxide blocking was done at ambient temperature for 30 minutes with 3% H₂O₂ in distilled water for Nrf2 and methanol for Keap1 and NQO1. The slides were incubated with primary antibody at ambient temperature and washed with Tris buffer, followed by incubation with biotin-labeled secondary antibody for 15 minutes and streptavidin peroxidase for 15 minutes (LSAB system, Dako) for Nrf2 and EnVision Dual Link System-HRP (Dako) for 30 minutes for Keap1 and NQO1. Staining was developed with 0.5% 3,3'-diaminobenzidine, freshly prepared with imidazole-HCl buffer (pH 7.5) containing hydrogen peroxide and an antimicrobial agent (Dako) for 5 minutes, and then counterstained with hematoxylin, dehydrated, and mounted.

To determine the association between Nrf2 and Keap1 expression on Western blot and immunohistochemical analyses, we prepared FFPE cell pellets from four cell lines (A549, H460, H1993, and BEAS2B). For the immunohistochemical analysis, the pellets were used as positive controls. As negative controls, we used positive control sections, replacing the primary antibody with universal negative control anti-rabbit (Dako).

Immunohistochemical expression was quantified jointly by two pathologists (L.M.S. and I.I.W.). Nuclear Nrf2, cytoplasmic Keap1, and cytoplasmic NQO1 expressions were quantified using a four-value intensity score (0, 1+, 2+, or 3+) and the percentage (0-100%) of the extent of reactivity. An immunohistochemical expression score was obtained by multiplying the intensity and reactivity extension values (range, 0-300), and these expression scores were used to determine expression levels. Positive

Table 1. Clinicopathologic features of NSCLCs evaluated for Nrf2 and Keap1 expression in TMA

Characteristic*	Total ($n = 304$)	Adenocarcinoma ($n = 190$)	Squamous cell carcinoma ($n = 114$)
Age, mean (y)	66	65	68
Sex			
Female	157	113	44
Male	147	77	70
Pathologic stage			
I	191	127	64
II	58	24	34
III	46	32	14
IV	9	7	2
Smoking history [†]			
Current/former	253	144	109
Never	50	46	4

*Eastern Cooperative Oncology Group performance status of the patients was 0 and 1.

[†]Smoking history was not available for one patient.

nuclear Nrf2 expression was defined as a score >0. Low or absent cytoplasmic Keap1 expression was defined as a score <150, which represents the mean of expression for all NSCLC TMA cases. High cytoplasmic NQO1 expression was defined as a score >130, which represents the median expression for NSCLCs evaluated using whole tissue sections.

EGFR and KRAS mutation analyses

Exons 18 to 21 of *EGFR* and exon 1 of *KRAS* were PCR amplified using intron-based primers, as described previously (18, 19), and DNA specimens were extracted from microdissected FFPE tissue. All PCR products were directly sequenced using the Prism Dye Terminator Cycle Sequencing method (Applied Biosystems). All sequence variants were confirmed by independent PCR amplifications from at least two independent microdissections and sequenced in both directions, as reported previously (18, 19).

NFE2L2 and KEAP1 mutation analysis in tumor specimens

To determine the mutation status of *NFE2L2* (exon 2) and *KEAP1* (exons 2-5) genes, we selected 31 NSCLC tumors from the TMA set for which DNA extracted from fresh tumor tissue was available. The cases included 20 tumors (9 adenocarcinomas and 11 squamous cell carcinomas) with nuclear Nrf2 expression on immunohistochemical analysis and 11 (8 adenocarcinomas and 3 squamous cell carcinomas) without nuclear Nrf2 expression. The mutation analysis was done using direct sequencing after PCR amplification of *NFE2L2* and *KEAP1* genes. For *NFE2L2*, intron-based PCR primers (forward, 5'-CCACCATCAACAGTGGCATA-3'; reverse, 5'-AGGCAAAGCTGGAACCTAAA-3') for exon 2 were designed using Primer3 software (<http://frodo.wi.mit.edu/>) and synthesized by Sigma-Aldrich. The PCR cycling conditions were 94°C (15 minutes) for 1 cycle; 94°C (30 seconds), 58°C (45 seconds), and 72°C (1 minute) for 45 cycles; and a final extension of 72°C (5 minutes). For the *KEAP1* gene, we analyzed exons 2 to 5 using intron-based PCR primer sequences, as previously described (5). All PCR products were directly sequenced using the Applied Biosystems Prism Dye Terminator Cycle Sequencing method. All sequence variants were confirmed by independent PCR amplifications and sequenced in both directions.

Statistical analysis

The clinicopathologic data were summarized using descriptive statistics and frequency tabulations. Wilcoxon rank-sum and Kruskal-Wallis tests were used to compare biomarker expression among different prognostic factor levels. We determined the association between 5-year RFS and OS rates and Nrf2 and Keap1 expression in NSCLC patients with stage I or II disease who had not undergone adjuvant or neoadjuvant chemotherapy and in patients with stage I to IIIB disease who had and had not undergone platinum-based adjuvant therapy. RFS was defined as the time from surgery to recurrence or

the end of the study, and OS was defined as the time from surgery to death or the end of the study. The cutoff for nuclear Nrf2 expression was a score >0, which was defined as positive staining; and the cutoff for cytoplasmic Keap1 was 150, which represents the mean score. Survival curves were estimated using the Kaplan-Meier method. Univariate and multivariate Cox proportional hazards models were used to assess the effects of covariates on RFS and OS rates. Two-sided *P* values of <0.05 were considered statistically significant. All analyses were conducted using SAS (version 9.1) and S-plus (version 8.0) software.

Results

Nrf2 and Keap1 protein expression in cell lines by Western blot analysis and validation of immunohistochemical results

We found on Western blot analysis that Nrf2 protein levels were higher in the nucleus of NSCLC cell lines A549 and H460 than in the cytoplasm (Supplementary Fig. S1). The BEAS2B bronchial epithelial cell line had a significantly lower Nrf2 expression level, both in the nucleus and in the cytoplasm, than did the malignant cell lines. These findings are consistent with previously reported data (5). In contrast, BEAS2B cells had significantly higher Keap1 expression levels in the nucleus and cytoplasm than did the NSCLC cell lines evaluated, and in all cells, Keap1 was expressed mostly in the cytoplasm (Supplementary Fig. S1). The cell line H1993 had a similar Nrf2 and Keap1 expression pattern to that of BEAS2B on Western blot analysis, which is consistent with H1993 cell line being heterozygous for *KEAP1* mutation (5). We evaluated the immunohistochemical expression of Nrf2 and Keap1 in FFPE cell pellets and found similar expression patterns to those found on Western blot analysis (Supplementary Fig. S1). In FFPE tumor specimens, in malignant tumor cells, we found both nuclear and cytoplasmic expression of Nrf2 and exclusively cytoplasmic expression of Keap1 (Fig. 1). For the study of the expression of these markers in NSCLC TMAs and whole section tissue specimens, we focused on nuclear Nrf2 expression because this is the subcellular location where it is considered to be biologically active (5), and on cytoplasmic Keap1 expression because it was the only expression detected in malignant tumor cells in the FFPE tissue specimens.

Nrf2 and Keap1 immunohistochemical expression in NSCLC TMA and association with clinicopathologic and genetic features

We determined nuclear Nrf2 and cytoplasmic Keap1 protein expression in 304 tumors in TMAs using levels and scores of expression. Positive nuclear Nrf2 expression (score > 0) was detected in 26% of NSCLCs, and the frequency of positive cases was significantly (*P* < 0.001) higher in squamous cell carcinomas (38%) than in adenocarcinomas (18%; Table 2). In most positive tumors (49 of 77, 64%), the Nrf2 nuclear expression was mild and, in

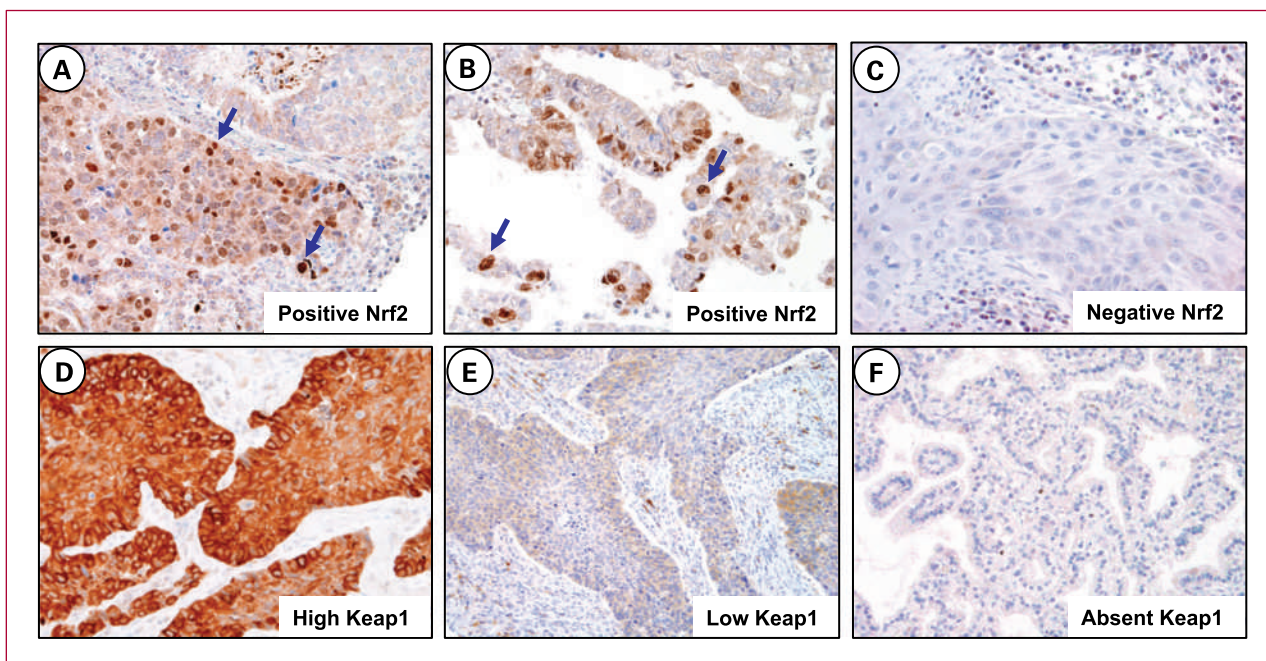


Fig. 1. Microphotographs showing positive (blue arrows) and negative nuclear Nrf2 expression and absent, low, and high cytoplasmic Keap1 expression in NSCLC tumor tissue specimens: squamous cell carcinoma (A and C-E) and adenocarcinoma (B and F). Magnifications, $\times 100$ (A, B, and D-F) and $\times 200$ (C).

the remaining cases, was moderate to strong. On the other hand, negative or low levels of cytoplasmic Keap1 expression (score < 150) were observed in 56% of NSCLCs. The frequency of low or negative Keap1 expression was significantly higher in adenocarcinomas (62%) than in squamous cell carcinomas (46%; $P = 0.0057$). Overall, the expression of nuclear Nrf2 was statistically associated ($P = 0.0041$; $r = 0.17$) with higher cytoplasmic Keap1 expression in all NSCLCs. However, we identified a subset (39 of 295, 13%) of tumors with positive Nrf2 and low or absent Keap1 expression, including squamous cell carcinomas (19 of 111, 17%) and adenocarcinomas (20 of 184, 11%). Nrf2 and Keap1 expression was not associated with sex, smoking history, or pathologic tumor stage (stage I or II versus III or IV; data not shown).

Among adenocarcinomas, *EGFR* and *KRAS* mutations were detected in 23 of 172 (13%) and 28 of 171 (16%) cases, respectively. Interestingly, no *EGFR*-mutant adenocarcinomas expressed Nrf2, whereas 21% of *EGFR* wild-type tumors expressed nuclear Nrf2; this difference was statistically significant ($P = 0.009$). Although *EGFR* mutations were significantly higher in tumors from nonsmokers (13 of 40, 33%) compared with smokers (10 of 130, 8%), the distribution of smoking status of the 23 patients with *EGFR* mutation and lack of Nrf2 expression was similar: 10 (43%) smokers and 13 nonsmokers (57%). There was no association between cytoplasmic Keap1 expression and *EGFR* mutation status. No relationship was found between the expression of these two markers and tumors with *KRAS* mutation.

Table 2. Nuclear Nrf2 expression according to NSCLC histology type and chemotherapy treatment

Sample type	Adenocarcinoma	Squamous cell carcinoma	NSCLC	P^*
	Positive Nrf2 [†] /total (%)	Positive Nrf2 [†] /total (%)	Positive Nrf2 [†] /total (%)	
TMA	34/188 (18%)	43/112 (38%)	77/300 (26%)	< 0.0001
Whole sections				
Neoadjuvant treated	4/20 (20%)	3/6 (50%)	7/26 (27%)	0.2929
Adjuvant treated	13/35 (37%)	14/28 (50%)	27/63 (43%)	0.3055
No adjuvant treated	10/27 (37%)	10/32 (31%)	20/59 (34%)	0.6399

* P value comparison of the frequencies of marker expression.

[†]Positive Nrf2, score > 0 .

***NFE2L2* and *KEAP1* mutation analysis**

In NSCLC tumor, we studied mutation status of both genes at sites (*NFE2L2*, exon 2; *KEAP1*, exons 2-5) to be previously reported as mutated in lung cancer (5, 12, 13). To correlate mutation status of both genes with activation of Nrf2, we selected 20 tumors with DNA extracted from fresh tissue available and with nuclear Nrf2 immunohistochemical expression; as controls, we used 11 tumors lacking nuclear Nrf2 expression. *NFE2L2* mutation was found in 2 of 29 tumors successfully examined. Both mutations were located in codon 28 (ATA to ACA; substitution of isoleucine for threonine) and codon 79 (GAG to CAG; substitution of glutamic acid for glutamine) of exon 2. These two cases corresponded to squamous cell carcinomas with nuclear Nrf2 expression and high cytoplasmic Keap1 expression. *KEAP1* mutation (exons 2-5) was detected in only 1 of 31 tumors examined. This was a nonsense mutation (TAC to TAA; substitution of tyrosine for stop codon) in codon 537 (exon 5) in a squamous cell carcinoma with nuclear Nrf2 expression and low cytoplasmic Keap1 expression.

Nrf2, Keap1, and NQO1 immunohistochemical expression in NSCLC tumors and corresponding normal adjacent tissues using whole tissue section analysis

To determine the heterogeneity of nuclear Nrf2 and cytoplasmic Keap1 expression in tumor tissues, we evaluated the expression of both markers using whole histologic sections obtained from 36 NSCLCs (18 adenocarcinomas and 18 squamous cell carcinomas) included in the TMA, 19 of which were Nrf2 positive and 17 of which were negative. Nuclear Nrf2 expression was highly heterogeneous throughout the positive tumors, with 14 (78%) expressing nuclear Nrf2 in 5% to 30% of malignant cells. Of interest, only 1 of the 17 tumors that was Nrf2 negative on the TMA analysis was positive on whole histologic section examination.

To determine the biological effect of Nrf2 expression in NSCLC malignant cells, we studied the correlation of expression of nuclear Nrf2 with the immunohistochemical protein expression of NQO1, a gene transcriptionally regulated by Nrf2 (5, 10), using a subset ($n = 30$) of NSCLC with whole tissue sections available. Of interest, 12 of 16 (75%) nuclear Nrf2-positive cases expressed high levels of cytoplasmic NQO1, whereas only 3 of 12 nuclear Nrf2-negative tumors expressed this protein at high levels. Similarly, the tumors expressing nuclear Nrf2 had a significantly ($P = 0.0211$) higher NQO1 expression score (mean, 176.3) compared with the Nrf2-negative tumors (mean, 92.9).

On the other hand, we found that regardless of the intensity of expression, cytoplasmic Keap1 was homogeneously expressed throughout the tumors. Only 4 of 36 (11%) tumors showed small distinct areas of malignant cells (defined as 10-30% of the tumor section) lacking cytoplasmic Keap1 expression.

Nrf2 expression was detected in the nucleus of normal bronchial epithelial adjacent tumors in 6 of 127 (5%)

chemotherapy-naive NSCLCs on whole histologic section, including 19 cases with Nrf2-positive tumors. The six cases with nuclear Nrf2 expression in normal epithelium did not express the marker in their corresponding tumors. As expected, Keap1 cytoplasmic expression was found in normal bronchial epithelium in all 25 chemotherapy-naive NSCLCs evaluated. Similar cytoplasmic Keap1 expression scores were detected in the tumors (mean score, 126.0; SD, 81.8) and corresponding normal bronchial epithelia (mean score, 130.0; SD, 59.2).

Association between Nrf2 and Keap1 expression and NSCLC patient outcome using TMA specimens

We determined the association between nuclear Nrf2 and cytoplasmic Keap1 expression and RFS and OS rates in patients with stage I and II NSCLC who had not undergone neoadjuvant or adjuvant treatment. In patients with NSCLC ($n = 235$), positive nuclear Nrf2 expression (score > 0) was associated with worse 5-year RFS and OS on univariate analysis and a worse OS on multivariate Cox model analysis [$P = 0.0139$; hazard ratio (HR), 1.75; 95% confidence interval (95% CI), 1.12-2.73] when adjusted for age at surgery, smoking history, and pathologic stage (Table 3; Fig. 2A and B). No association was found between nuclear Nrf2 expression and outcome by histologic tumor type (Supplementary Fig. S2).

No association was found between Keap1 expression and patients' outcome for all NSCLCs (Supplementary Fig. S3). Then, we examined the effect of Keap1 in the outcome of patients by individual histologic tumor types. We found that negative and low cytoplasmic Keap1 expression (score < 150) was associated with worse 5-year RFS and OS in patients with squamous cell carcinomas on univariate analysis and a worse OS on multivariate Cox model analysis ($P = 0.0181$; HR, 2.09; 95% CI, 1.13-3.84) when adjusted for age at surgery and pathologic stage (Table 3; Fig. 2C and D). No association was found between Keap1 expression and outcome in patients with adenocarcinoma (Supplementary Fig. S3).

The subset (39 of 295, 13%) of NSCLC tumors with both positive Nrf2 and low or absent Keap1 expression was significantly associated with worse OS ($P = 0.0111$; HR, 1.97; 95% CI, 1.17-3.31) and RFS ($P = 0.0325$; HR, 1.69; 95% CI, 1.04-2.73) on the univariate and multivariate analysis when adjusted for age at surgery, pathologic stage, and smoking history (Table 3; Fig. 2E and F).

Nrf2 and Keap1 expression in neoadjuvant therapy-treated NSCLC

To investigate if neoadjuvant platinum-based chemotherapy leads to increased nuclear Nrf2 expression in NSCLC tumors, we determined the expression of Nrf2 and Keap1 in 26 surgically resected tumors from patients who had undergone platinum-based chemotherapy before surgery. Nuclear Nrf2 expression (score > 0) was found in 7 (27%) tumors (Table 2), but negative or low Keap1 expression (score < 150) was detected in 18 (69%). The

Table 3. Multivariate Cox model of 5-y OS and RFS in patients with NSCLC and squamous cell carcinoma

Variable	HR (95% CI)	P
OS, Nrf2 expression, NSCLC		
Age at surgery (per 1-y increase)	1.067 (1.043-1.092)	<0.0001
Stage (II vs I)	2.096 (1.332-3.299)	0.0015
Smoking (current and former vs never)	2.476 (1.069-5.736)	0.0331
Nuclear Nrf2 (positive vs negative)	1.747 (1.120-2.726)	0.0139
OS, Keap1 expression, squamous cell carcinoma		
Age at surgery (per 1-y increase)	1.054 (1.020-1.090)	0.0020
Stage (II vs I)	1.876 (1.033-3.409)	0.0389
Cytoplasmic Keap1 (score <150 vs ≥150)	2.087 (1.134-3.841)	0.0181
OS, Nrf2 and Keap1 expression, NSCLC		
Age at surgery (per 1-y increase)	1.063 (1.039-1.087)	<0.0001
Stage (II vs I)	2.093 (1.332-3.290)	0.0014
Smoking (current and former vs never)	2.639 (1.134-6.142)	0.0244
Nrf2/Keap1 status (positive Nrf2 and Keap1 <150 vs other)	1.966 (1.167-3.313)	0.0111
RFS, Nrf2 and Keap1 expression, NSCLC		
Age at surgery (per 1-y increase)	1.043 (1.023-1.064)	<0.0001
Stage (II vs I)	1.962 (1.296-2.970)	0.0014
Smoking (current and former vs never)	2.115 (1.064-4.203)	0.0325
Nrf2/Keap1 status (positive Nrf2 and Keap1 <150 vs other)	1.688 (1.045-2.727)	0.0325

expression of these markers was not associated with the histologic effects of chemotherapy in tumor tissues, including percentages of tumor necrosis, fibrosis, and viable malignant cells (data not shown).

Association between Nrf2 expression and outcome in patients treated with adjuvant chemotherapy

To evaluate the role of Nrf2 in response to platinum-based chemotherapy, we determined nuclear Nrf2 expression in whole histologic sections of 122 surgically resected NSCLC tumors (stages I-IIIB) and the RFS and OS rates. These samples were obtained from 63 patients who had undergone adjuvant chemotherapy (35 adenocarcinomas and 28 squamous cell carcinomas) and 59 patients who had not undergone adjuvant treatment (27 adenocarcinomas and 32 squamous cell carcinomas). Overall nuclear Nrf2 expression was detected in 47 (39%) of these NSCLCs (Table 2).

Because a significant difference was observed in the nuclear Nrf2 expression frequency between squamous cell carcinomas and adenocarcinomas examined in the TMA specimens, we evaluated the predictive effect of Nrf2 expression in adjuvant-treated patients in both tumor histologies separately. In patients with squamous cell carcinoma who had undergone adjuvant treatment, nuclear Nrf2 expression (score > 0) was associated with worse RFS on univariate Cox model analysis ($P = 0.0410$; HR, 3.37; 95% CI, 1.05-10.81; Supplementary Fig. S4); however, this association was not significant on multivariate analysis ($P = 0.0618$; HR, 3.11; 95% CI, 0.95-10.20). In the same tumor type, nuclear Nrf2

showed a trend toward worse OS in patients who received adjuvant chemotherapy; this association was not significant on univariate Cox model analysis ($P = 0.0590$; HR, 3.52; 95% CI, 0.95-13.04) and multivariate analysis ($P = 0.092$). In patients with squamous cell carcinomas who had not undergone adjuvant treatment, Nrf2 expression was not associated with the RFS or OS rate (Supplementary Fig. S4).

In patients with adenocarcinoma who had not undergone adjuvant therapy, Nrf2 was statistically associated with RFS ($P = 0.0092$) in univariate analysis, although no association was found between Nrf2 expression and the RFS or OS rate on the multivariate analysis. Of interest, in patients with adenocarcinoma, the nuclear expression of Nrf2 did not associate with outcome in patients who received adjuvant chemotherapy.

Discussion

In lung cancer, Nrf2 activation in malignant cells has been associated with tumor progression and chemotherapy resistance (8, 11, 20–22). High levels of nuclear Nrf2 facilitate cancer cell growth and cell survival as a result of the transactivation of cytoprotective genes (8, 20, 21). In NSCLC, the overexpression of nuclear Nrf2 is principally attributable to genetic and epigenetic alterations and the loss of function of its repressor, Keap1 (5, 21, 23). In NSCLC, *KEAP1* mutations at exons 2 to 6 have been detected in 50% ($n = 12$) of cell lines (5) and in 8% ($n = 65$) and 19% ($n = 54$) of tumors (5, 21). Promoter hypermethylation of *KEAP1* was found in all lung cancer cell lines (three

of three) and tumor tissues (five of five) evaluated (11). In addition, mutations of the Nrf2 gene *NFE2L2*, which affect the region that codes for Keap1-binding sites, have been proposed as an alternative mechanism of Nrf2 activation in lung cancer (12), but *NFE2L2* mutations have only been found in 2% (2 of 85) of lung cancer cell lines and in 8% to 11% of lung cancer tumor specimens, principally in patients with a smoking history and squamous cell carcinoma histologic tumor type (12, 14). Despite all these recent findings, the characteristics of NSCLC tumors with Nrf2 activation and loss of Keap1 expression and the role

of Nrf2 and Keap1 in the response to platinum-based chemotherapy are not well understood.

We did a comprehensive immunohistochemical analysis of Nrf2 and Keap1 expression in FFPE NSCLC tumors. We used a validated method in which we tested both antibodies in a panel of NSCLC cell lines with known Nrf2 and Keap1 expression (5) by Western blot analysis and immunohistochemical analysis of FFPE cell pellets. The protein expression pattern and subcellular localization of Nrf2 and Keap1 on Western blot analysis in our cell lines was consistent with previously reported data (5), and the

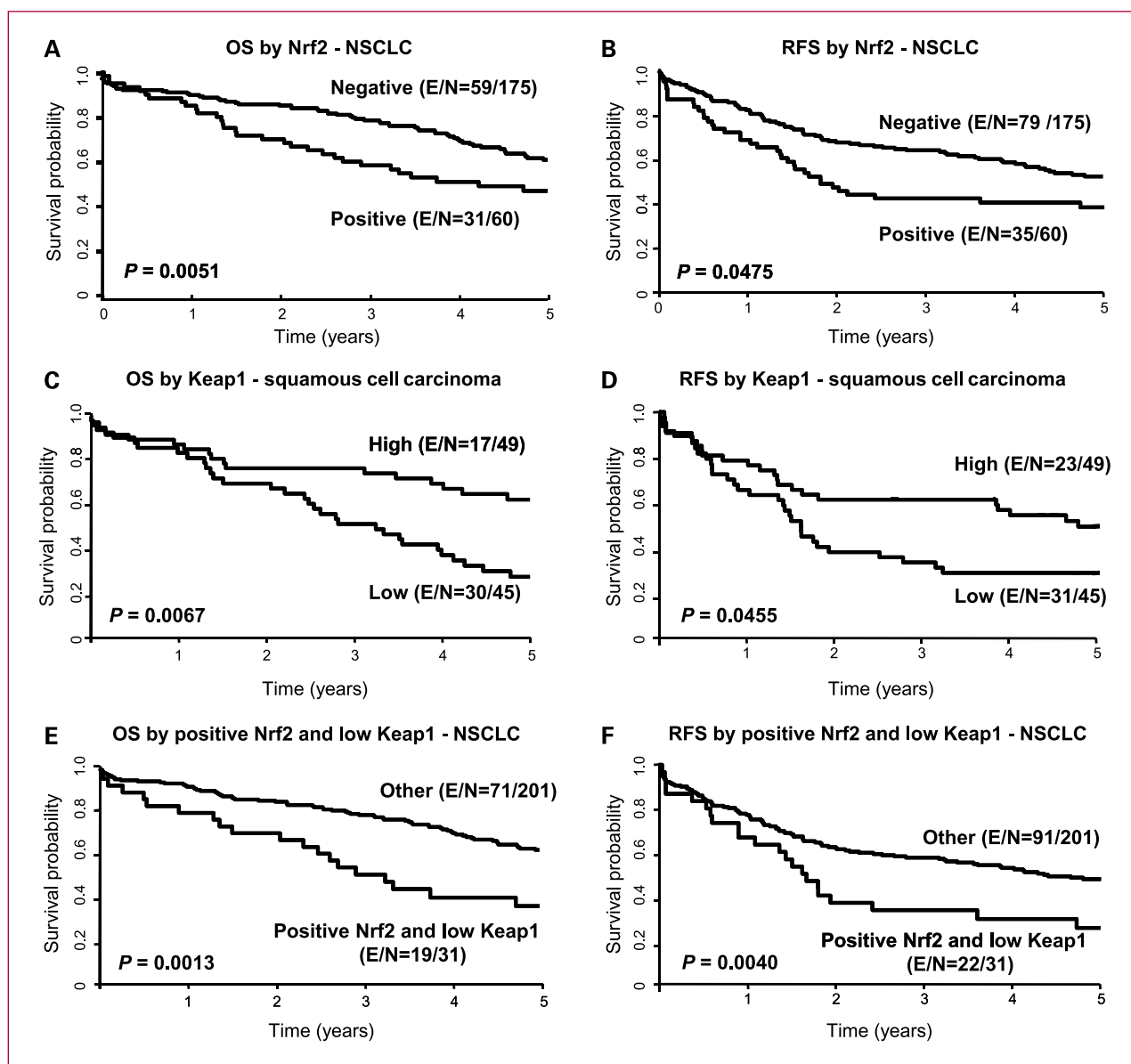


Fig. 2. Five-year OS (A) and RFS (B) rated by nuclear Nrf2 protein expression in all patients with NSCLC. Five-year OS (C) and RFS (D) rated by cytoplasmic Keap1 protein expression in patients with squamous cell carcinoma. Five-year OS (E) and RFS (F) rated by nuclear Nrf2 and low cytoplasmic Keap1 protein expression in all patients with NSCLC. E, events; N, total number of cases.

expression found on Western blot analysis was consistent with that in cell line pellets.

In our study of TMA specimens, nuclear Nrf2 was expressed in a subset of NSCLCs (26%), more commonly in squamous cell carcinomas (38%) than in adenocarcinomas (18%). To our knowledge, only one report exists of the immunohistochemical expression of Nrf2 in lung cancer; evaluations of whole histologic sections from stage I NSCLCs revealed that 62% (55 of 89) of tumors expressed nuclear Nrf2 (24). Although we were not able to obtain detailed information from this report (24), this study seems to differ significantly from ours in the immunohistochemical method used to evaluate Nrf2 expression in tissue specimens, including the quantification method. One intriguing observation of our study is that the frequency of nuclear Nrf2 expression was significantly higher in *EGFR* wild-type (21%) adenocarcinomas than in mutant (0%) tumors, and this association was independent of patients' smoking history. Although the number of *EGFR*-mutant tumors evaluated was relatively small, this finding is of potential interest. To the best of our knowledge, this association has not been previously reported, and it warrants further study. Our observation concurs with the finding that *EGFR* mutations associate with better survival in advanced NSCLC patients treated with chemotherapy (carboplatin and paclitaxel) with and without an *EGFR* tyrosine kinase inhibitor (erlotinib; ref. 15). We speculate that the lack of nuclear Nrf2 expression in *EGFR*-mutant NSCLCs may contribute to the benefit of administering platinum-based chemotherapy.

Nrf2 can be activated by several mechanisms, including mutations of the gene (*NFE2L2*) affecting the Keap1-binding site (12, 14). In our NSCLC samples, exon 2 *NFE2L2* mutations, which code for the Keap1-binding site region of the Nrf2 protein, were infrequently detected, occurring in 2 of 29 (7%) tumors examined. Interestingly, both tumors were squamous cell carcinomas that expressed nuclear Nrf2 and had high Keap1 cytoplasmic expression levels. Other *NFE2L2* mutation analyses in lung cancer have been conducted; they reported that 8% to 11% of NSCLCs had mutations on exon 2, including both mutations detected in our study, and they associated with the squamous cell carcinoma histology of the tumors and patients' smoking history (12, 14).

Our analysis of whole histologic NSCLC sections showed that nuclear Nrf2 expression in tumor tissues was heterogeneous and involved a small percentage (5–30%) of malignant cells. Despite the heterogeneity of nuclear Nrf2 expression in tumor cells, we found an association between Nrf2 expression in whole histologic sections and corresponding TMA tissue cores in 36 cases examined. The correlation observed between the expressions of nuclear Nrf2 with cytoplasmic NQO1 protein in a subset of our NSCLC suggests that Nrf2 is biologically active in the nucleus of the malignant cells. *NQO1* gene is transcriptionally regulated by Nrf2, and as expected, higher levels of NQO1 protein expression were observed in tumors showing nuclear Nrf2 compared with tumor

lacking nuclear Nrf2 (5, 10). Nuclear Nrf2 expression has been reported at different frequencies (range, 54–92%) in other epithelial tumors, including squamous cell carcinoma of the head and neck (25) and gallbladder carcinoma (13).

Keap1 is the principal cytoplasmic repressor of Nrf2 (23, 26–28). Ours is the first reported study to determine the frequency of low or absent cytoplasmic Keap1 expression in NSCLC and its association with the clinicopathologic characteristics of the tumors. Low or absent Keap1 expression was common in NSCLC (56%), mainly in adenocarcinomas. However, we identified only 1 *KEAP1* mutation (exons 2–5) in 31 tumors examined, including 20 with nuclear Nrf2 expression, suggesting that *KEAP1* mutation is not the main mechanism of protein loss or reduction. Our findings differ from those of previous publications that reported *KEAP1* mutations in 8% and 19% of two NSCLC cohorts, predominantly adenocarcinomas (26% and 30%; refs. 5, 21). Our findings of a positively significant correlation between the expression of nuclear Nrf2 and cytoplasmic Keap1, and that only 13% of tumors in our study had low or absent Keap1 and nuclear Nrf2 expression, suggest that other mechanisms, not associated with Keap1 inactivation, promote Nrf2 nuclear localization and subsequent activation. There are few alternative mechanisms proposed to activate Nrf2, including phosphorylation of Nrf2 protein by several protein kinases, including protein kinase C, extracellular signal-regulated kinase, c-Jun NH₂-terminal kinase, and phosphatidylinositol 3-kinase (7). Additionally, there is recent evidence that the presence of certain protein motifs determines Nrf2 subcellular localization (29). The Nrf2 protein has the nuclear export signal motif, which transports the protein from the nucleus to the cytoplasm, as well as the nuclear localization signal motif, which transports the protein from the cytoplasm to the nucleus. It has been suggested that the net result of these two driving forces is important to regulate Nrf2 subcellular localization independently of its interaction with Keap1 (29).

Nuclear Nrf2 expression in all NSCLC patients, low or absent Keap1 expression in patients with squamous cell carcinoma, as well as the subset of NSCLC with both nuclear Nrf2 and low or absent Keap1 were associated with poor outcome. Regardless of the mechanism that leads to nuclear Nrf2 activation in tumor cells, there is evidence that this phenomenon promotes cell survival in malignant cells (8, 12, 13, 21) and may explain the low RFS and OS rates in our NSCLC patients who had undergone surgical resection with curative intent. Our finding of poor survival in patients with low or absent Keap1 expression suggests that inactivation of this putative tumor suppressor gene affects the growth and progression of tumors by mechanisms that are not mediated by Nrf2. One of those unknown mechanisms could involve other Keap1-binding proteins that have antiapoptotic and proliferative functions (30–32), including phosphoglycerate mutase family member 5 (31), the nuclear oncoprotein prothymosin α (30), and fetal Alz-50 reactive clone 1 protein (32). The

association between Keap1 and patient outcome has not been previously reported in human epithelial tumors, except in one study of renal cell carcinoma that showed that Keap1 overexpression was associated with more advanced tumor stage and poor OS (33).

It has been suggested that abnormalities in the Nrf2/Keap1 pathway that lead to Nrf2 overexpression in tumors induce platinum-based chemotherapy resistance by promoting tumor cell survival and increasing proliferation (5–8). In NSCLC cell lines, the upregulation of the downstream genes of Nrf2 plays a critical role in platinum chemoresistance, mainly because of increased transcription of the genes involved in drug efflux systems and of antioxidant proteins, including glutathione, thioredoxins, and NQO1 (20, 24, 34). Furthermore, studies in *in vitro* and *in vivo* NSCLC models have shown that inhibition of Nrf2 expression by RNA interference suppressed tumor growth and induced sensitivity to platinum-based chemotherapy drugs (8, 20). We hypothesized that neoadjuvant platinum-based chemotherapy would lead to increased nuclear Nrf2 expression in NSCLC tumors; however, we found similar nuclear Nrf2 expression in both chemotherapy-naïve and chemotherapy-treated tumors, including those with pathologic characteristics that are associated with no response to neoadjuvant chemotherapy.

We observed a trend toward an association between nuclear Nrf2 expression and worse RFS and OS in patients with squamous cell carcinomas who had undergone surgery and received adjuvant platinum-based chemotherapy. Interestingly, this phenomenon was not observed in adenocarcinoma patients. These findings suggest that, as has been shown in *in vitro* and *in vivo* studies using NSCLC cell lines (8, 20, 22, 34), nuclear Nrf2 expression in malignant lung cancer cells may play a role in chemotherapy resistance in squamous cell carcinoma subtype. However, these observations need to be studied further in a larger number of cases as part of prospectively conducted clinical trials. Importantly, the role of Nrf2 expression as a potential

predictive marker associated with resistance to platinum-based chemotherapy needs to be addressed in NSCLC patients with advanced (metastatic) tumors in which a more direct correlation between Nrf2 expression and response to chemotherapy can be established.

In summary, increased Nrf2 expression and decreased expression of Keap1 are common abnormalities in surgically resected NSCLCs and are associated with clinical outcome. In our study, abnormal expression of Nrf2 and Keap1 proteins was more common than that of the corresponding gene mutations, suggesting that other mechanisms are involved in the activation of *NFE2L2* and inactivation of *KEAP1*. Nrf2 expression may play a role in response to adjuvant platinum-based chemotherapy in patients with squamous cell carcinoma. Identifying patients with abnormal Nrf2 expression may be important for selection for chemotherapy in NSCLC, and our data suggest that Nrf2 expression could be added to the list of potential molecular markers to be tested to personalize treatment of NSCLC when platinum-based chemotherapeutic agents are used.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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