NRF2 Pathway Activation and Adjuvant Chemotherapy Benefit in Lung Squamous Cell Carcinoma

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Abstract

Purpose: Genomic profiling of lung squamous cell carcinomas (SCC) has identified NRF2 pathway alterations, which activate oxidative response pathways, in one third of tumors. Preclinical data suggest these tumors may be resistant to platinum-based chemotherapy. We evaluated the clinical relevance of these findings and assessed whether NRF2 activation predicts benefit from adjuvant chemotherapy in SCC.

Experimental Design: Logistic regression (LR) and significance analysis of microarrays (SAM) were applied to all 104 TCGA (The Cancer Genome Atlas) SCC cases that had microarray gene expression and mutation data to identify genes associated with somatic NRF2 pathway alterations. The resulting signature (NRF2ACT) was tested in 3 independent SCC datasets to evaluate its prognostic and predictive effects. IHC and sequencing for NRF2 and KEAP1 were evaluated in one cohort (n = 43) to assess the relationship between gene expression, mutational status, and protein expression.

Results: Twenty-eight genes were identified by overlap between LR (291 genes) and SAM (30 genes), and these consistently separated SCC into 2 groups in all datasets, corresponding to putatively NRF pathway-activated and wild-type (WT) tumors. NRF2ACT was not prognostic. However, improved survival with adjuvant chemotherapy in the JBR 10-randomized trial appears limited to patients with the WT signature (HR 0.32, P = 0.16; NRF2ACT HR 2.28, P = 0.48; interaction P = 0.15). NRF2ACT was highly correlated with mutations in NRF2 and KEAP1, and with high NRF2 protein expression.

Conclusions: A gene expression signature of NRF2 pathway activation is associated with benefit from adjuvant cisplatin/vinorelbine in SCC. Patients with NRF2 pathway-activating somatic alterations may have reduced benefit from this therapy.

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Introduction

Adjuvant platinum-based chemotherapy is a standard of care for patients with completely resected stage II to IIIA non–small cell lung cancer (NSCLC), with an absolute 5-year survival benefit of 4% to 15% in several randomized trials and meta-analyses (1–7). However, as with most adjuvant therapies, only a subgroup of patients derives benefit from this intervention. Because chemotherapy causes both short-term and long-term toxicities, predictive biomarkers that could identify which patients do or do not benefit would be of great clinical utility.

Although previous efforts have evaluated potential predictive biomarkers of adjuvant chemotherapy benefit, most tested either single markers (8) or considered unstratified populations consisting of squamous cell carcinoma (SCC) and adenocarcinoma and other histologic subtypes within the spectrum of NSCLC (9, 10). Following the completion of initial large-scale efforts to characterize the genomic and molecular alterations in NSCLC by The Cancer Genome Atlas (TCGA) consortium and others, the existence of major subsets of lung cancers with shared pathway alterations has been recognized (11). This includes approximately 35% of SCC where somatic alterations resulting in activation of the NRF2 pathway via mutations or amplification of [nuclear factor (erythroid-derived 2)-like 2] NFE2L2/NRF2 or mutation or deletion of its negative regulators Kelch like-ECH-associated protein 1 (KEAP1) or Cullin 3 (CUL3) have been identified (11).

The NRF2 transcription factor is a master regulator of the antioxidant response, and dysregulation of this pathway occurs commonly in cancer (12). Several lines of preclinical and clinical investigation have suggested that NRF2 pathway activation confers resistance to chemotherapy (13–22). Furthermore, the available data indicate that mutations in this pathway define a major molecular subset of SCC. However, the critical question of whether this subset of patients derives differential benefit from adjuvant chemotherapy has not been addressed. Such an analysis requires interrogation of data from the pivotal randomized clinical trials of adjuvant chemotherapy, where gene expression but not somatic mutational data are available. Although previous studies have used groups of NRF2 target genes as a readout of NRF2 activity in NSCLC (19), and some existing gene expression signatures, including the classical expression subtype of SCC,
Translational Relevance

Adjuvant platinum-based chemotherapy improves survival in patients with non–small cell lung cancer, but benefits only a minority of those treated. Integrating emerging knowledge of molecular alterations and their cellular consequences may enable the identification of patients most likely to benefit from such treatment. Because the NRF2 pathway has been shown to alter chemosensitivity in vitro, we felt it would be important to examine the impact of recently described activating alterations on the benefit from adjuvant chemotherapy in patients with squamous cell lung carcinoma (SCLC). To do so, we identified a set of genes whose expression was associated with NRF2 pathway activation. Using this classifier, SCLC patients with the activated signature treated in the JBR.10 clinical trial did not appear to derive benefit from chemotherapy. This signature may enable more personalized approaches to the treatment of lung SCLC, sparing chemotherapy toxicity and identifying patients for the evaluation of alternative therapeutic strategies.

Development of NRF2-activated (NRF2ACT) gene expression signature from TCGA

TCGA SCLC cases with both microarray and exome sequencing data (as of August 2012) were used to define an NRF2 gene expression signature by comparing cases with or without somatic alterations in NRF2 pathway genes (mutations/amplification in NF2/2L2, mutations/deletions in KEAP1 or CUL3, as defined and identified by the TCGA analysis; ref. 11). Logistic regression (LR; using \( P < 0.001 \)) and significance analysis of microarrays (SAM; ref. 25) were applied independently to identify genes associated with somatic alterations in the NRF2 pathway. The intersection of these gene lists was used to define the NRF2 signature (NRF2ACT).

The NRF2ACT gene list was used to perform clustering analysis in the other datasets: NRF2-activated (NRF2ACT-high) versus normal/wild-type (NRF2ACT-low) subgroups were defined by their separation at the highest level of the hierarchical tree.

Mutational analysis of UHN samples

The mutational hotspot containing exon 1 of NRF2 and KEAP1 coding regions were sequenced for UHN cases by Sanger sequencing following touchdown PCR. Amplification and sequencing primers are included in Supplementary Table S1. Nonsynonymous mutations, insertions, and deletions were identified by comparison to the reference genome (GRC37). Mutation calling was performed blinded to gene expression classification and clinical outcomes.

Immunohistochemical analysis of NRF2 and KEAP1

Whole sections of formalin-fixed paraffin-embedded (FFPE) samples from UHN 181 cases were stained for NRF2 (SCBT clone H-300, 1:50 dilution) and KEAP1 (Proteintech 60027, 1:300 dilution) using a Benchmark XT autostainer (Ventana Medical Systems). Nuclear staining intensity (0 to 3+) and the percentage of cells stained were used to calculate H-scores (intensity score ×% stained) for each sample. For each antibody, cutoff values were defined to categorize samples: For NRF2, samples were classified as high (≥80) or low (<80), and for KEAP1 as high (>130), intermediate (60–130), or low (<60), based on the distributions of scores (Supplementary Fig. S1). Interpretation was performed blinded to other variables.

NRF2 fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) studies were performed with the use of dual-color DNA FISH probes. Two bacterial artificial chromosome (BAC) clones (RP11-28M17 and RP11-317C5) that hybridize to the 2q31.2 region of chromosome 2 containing the NRF2 gene and two clones (RP11-343F11 and RP11-127K18) located close to the centromeric region of chromosome 2 (2q11.2) were selected from the Human UCSC Genome Browser assembly (February 2009 GRCh37/hg19) and obtained from TCGAG Genome Resource Facility (Toronto, Canada). The BAC clones were directly labeled with Spectrum Orange (BAC clones corresponding to the NRF2 gene) or Spectrum Green (CEP2 BAC clones) fluorochromes using a commercially available nick translation kit according to the manufacturer’s protocol (Abbott Laboratories). Probes were first verified on normal blood metaphases to confirm their correct allocations. Brieﬂy, tissue microarray and FFPE sections were deparaffinized in three changes of xylene, dehydrated in ethanol, pretreated in citrate buffer (pH 6.8) for 50 minutes at 80°C, followed by pepsin digestion for 18 minutes at 37°C. Slides were hybridized for 2 days

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at 37°C, washed and counterstained with DAPI. Tissues and cells were examined and scored on an Imager M1 Zeiss microscope (Carl Zeiss Canada Limited) equipped with the appropriate filters. The IAI CV-M4+ CL progressive scan monochrome camera (IAI Inc.) and the MetaSystems Isis FISH Imaging software programs v5.3 (MetaSystems) were used for capturing images. The number of red and green signals as well as their distribution was analyzed by an experienced cancer cytogenetic technician (blinded to outcomes and gene expression classification) in 50 nonoverlapping tumor cell nuclei. Amplification was defined as an NRF2:CEP2 ratio of ≥ 2.0.

Statistical analysis
Overall survival (OS) calculated from the date of surgery (Raponi, UHN) or randomization (JBR.10) to death was the primary outcome endpoint. In JBR.10, where cause of death was known, disease-specific survival was used and non–lung cancer deaths were censored at the time they occurred (9). The survival estimates were calculated using the Kaplan–Meier method. The Cox proportional hazards model was used to test the prognostic effect of the NRF2\textsuperscript{ACT} signature as well as its predictive effect by testing its interaction with the treatment in the JBR.10 dataset. The associations between categorical variables (NRF2, KEAP1, CUL3 mutations, NRF2, KEAP1 IHC with NRF2\textsuperscript{ACT} signature) were tested using the Fisher exact test.

The set of genes associated with the NRF2 pathway alterations were selected based on LR and SAM (25). The alpha level for selection of the LR was 0.001. SAM analysis was performed using a two-class unpaired methodology to determine differential gene expression, with a set false-discovery rate of 0.05 and a call for 100 reamples to permit bootstrapping. Upon generation of a gene set, a fold-change cutoff of 2.3 was applied to reduce the number of potential probes found to be upregulated in NRF2 or KEAP1 mutants. Statistical analyses were performed using the open-source software R version 2.12.1 and the publicly available samr package.

Results
NRF2\textsuperscript{ACT} signature in TCGA SCC
A total of 104 unique SCC cases with complete microarray gene expression, mutation, and copy number data were identified in TCGA from a total of 178 samples. Forty-one cases were found to be in the NRF2\textsuperscript{ACT} set, we compared this list with a previously published NRF2 target gene set defined by transcriptional analysis and CHIP-seq in NRF2 and KEAP1 knockout mouse embryonic fibroblasts (26). This analysis revealed a highly significant enrichment (one-sided $P = 5.96 \times 10^{-11}$; hypergeometric test), confirming that the 28-gene set contains bona fide NRF2 targets.

Concordance between NRF2\textsuperscript{ACT} status, NRF2/KEAP1 IHC and somatic alterations in an independent dataset
To validate the association between the NRF2\textsuperscript{ACT} signature and somatic alterations in the NRF2 pathway in SCC, we performed hierarchical clustering using the 28-gene set, as well as NRF2 and KEAP1 mutational analysis by Sanger sequencing, in 43 SCC from the UHN181 dataset (24). As observed in the TCGA derivation set, NRF2\textsuperscript{ACT} identified two major subgroups in the UHN181 SCC set, with similar proportions to TCGA (Fig. 2). Furthermore, the NRF2\textsuperscript{ACT}-high subgroup was highly enriched for tumors with somatic alterations in NRF2 (P = 0.0001). In contrast, in the adenocarcinoma subset of UHN181 (n = 130), NRF2\textsuperscript{ACT} did not separate tumors into discrete groups, indicating that this signature is specifically relevant to SCC (Supplementary Fig. S2).

To evaluate whether IHC analysis of NRF2 or KEAP1 might serve as a useful surrogate of NRF2 pathway activation/mutation status, semi quantitative IHC was performed on whole tissue sections. Representative images are shown in Fig. 3, and IHC scores are indicated for each case in Fig. 2A. Tumors with high NRF2 expression by IHC were enriched in the NRF2\textsuperscript{ACT}-high subgroup (P = 0.0003). Furthermore, high NRF2 protein expression was associated with the presence of either NRF2 or KEAP1 alterations (P < 0.001). No association between KEAP1 protein expression and NRF2\textsuperscript{ACT} or mutational status was observed.

Prognostic effect of NRF2\textsuperscript{ACT} in SCC patients treated with surgery alone
No prognostic association of NRF2\textsuperscript{ACT} status was present in the UHN181 dataset of 43 patients treated with surgery alone, without adjuvant chemotherapy (HR, 0.86; P = 0.79; Fig. 4A).
Similarly, in a second SCC surgery-alone dataset (Raponi; \(n = 129\)), NRF2 ACT identified two subgroups based on gene expression (Fig. 2B), but was not significantly associated with OS (HR, 1.43, \(P = 0.2\); Fig. 4B). A prognostic association also was absent in the observation (no adjuvant chemotherapy) arm of JBR.10 (HR, 0.66, \(P = 0.61\); Fig. 4C).

**Predictive effect of NRF2ACT in SCC patients treated with adjuvant chemotherapy**

The predictive effect of NRF2\(^{ACT}\) in SCC patients was examined in the SCC subset of JBR.10. Patients with NRF2\(^{ACT}\)-high tumors did not appear to benefit from adjuvant chemotherapy (HR, 2.28; 95% CI, 0.24–22; \(P = 0.48\)), whereas a trend toward chemotherapy benefit was observed in NRF2\(^{ACT}\)-low patients (HR 0.32; 95% CI, 0.065–1.6; \(P = 0.16\); interaction \(P = 0.15\); Fig. 5).

**Discussion**

Considerable collaborative effort to define the recurring somatic alterations in human lung cancers has yielded remarkable insights into the molecular basis of this disease. This wealth of knowledge now enables us to delve far beyond the histologic classifications that have, until very recently, defined clinical approaches to lung cancer. The ultimate goal of these efforts is to identify actionable alterations that can be used to identify new therapeutic targets or refine treatment approaches for individual patients, thereby providing "personalized" or "precision" oncology and improving patient outcomes while reducing treatment-associated toxicities and costs.

The lung squamous cell carcinoma sequencing analysis has expanded the number of recognized putative "driver" oncogenes in this disease (11). These include a substantial number of recurrent but uncommon mutations in kinases, growth factor receptors, and related genes that might be targetable with specific small-molecule inhibitors or monoclonal antibodies, several of which currently are in clinical development (27, 28). Although it is clear that highly specific inhibitors can have dramatic efficacy when matched to tumor genotype, as in the case of EGFR-activating mutations, ALK translocations, and ROS1 rearrangements in NSCLC (29–33), the potential impact of somatic alterations on chemotherapy efficacy, which remains the standard of care for adjuvant treatment and the mainstay of therapy for SCC, is of additional clinical consequence. In an effort to apply the findings of TCGA (and the work that preceded it; refs. 13, 34) to current clinical management, we have focused on the NRF2 pathway, which is somatically activated in over one third of SCC (11) and has a well-characterized role in chemotherapy sensitivity based on preclinical studies.
Using gene expression and sequencing data from TCGA, we identified a 28-gene set (NRF2ACT) that is able to separate SCC from multiple datasets into two subgroups. Application of NRF2ACT in an adenocarcinoma dataset showed no similar discriminatory ability, indicating that this signature is specifically relevant for the SCC histology, and that interrogation of the NRF2 pathway in adenocarcinoma (where KEAP1 mutations predominate) may require a similarly dedicated approach. The substantial overlap between this gene set and an independently derived list of genes regulated by NRF2 provided strong biologic confirmation that NRF2ACT indeed reflects transcriptional activation of this pathway. In both the TCGA derivation set and an independent SCC series from UHN, where we performed mutational analysis for NRF2 pathway genes (NRF2, KEAP1), we observed a strong association between the presence of NRF2 pathway-activating somatic alterations and NRF2ACT-high expression status. The concordance of these findings supports the use of this gene expression surrogate of somatic NRF2 pathway activation to test the prognostic and predictive associations of this molecular subgroup. This provides a useful tool to analyze numerous existing datasets where gene expression, but not somatic mutational data, is available. Furthermore, as a measure of downstream effects of the NRF2 transcription factor, a gene expression–based classifier may provide a more functional measure of pathway activation than mutational status alone. Indeed, it is conceivable that by capturing the set of cancers where NRF2 transcriptional activity is upregulated (whether via somatic alteration of NRF2, KEAP1, or CUL3, or by alternate mechanisms; ref. 35), gene expression could be a superior biomarker for this purpose.

In three independent datasets of SCC patients treated with surgery alone, we observed no significant prognostic effect of our NRF2ACT signature. However, in keeping with our hypothesis and the preclinical data that supported it, we observed a trend toward
differential benefit from the addition of adjuvant chemotherapy in JBR.10. These results suggest that patients with NRF2-activating somatic alterations may not benefit from adjuvant chemotherapy, possibly due to intrinsic chemoresistance conferred by activation of the NRF2 transcriptional program. This finding is consistent with data from other cohort studies and case series in several tumor types, including NSCLC and esophageal cancer, even though different methods to assess NRF2 activation were used (14, 17). To our knowledge, ours is the first study to evaluate a predictive marker of NRF2 activation in the setting of a randomized trial with an untreated control arm.

To explore the possibility that conventional IHC staining of FFPE tissues could be used to assess NRF2 mutational/activation status, we also stained UHN SCC samples for NRF2 and KEAP1. Comprehensive evaluation of mutational status, NRF2ACT signature, and IHC revealed that NRF2 protein expression does, indeed, correlate with the presence of pathway-activating mutations and with the activated gene expression signature. These results are in keeping with the predicted effects of the somatic alterations on NRF2 stability, and the consequent transcriptional activation of target genes when protein levels of NRF2 are increased. The absence of an association between KEAP1 staining and NRF2ACT is perhaps not surprising, given the potential for somatic alterations in NRF2, CUL3, or KEAP1 itself to disrupt the relationship between KEAP1 and NRF2 protein/transcription factor activity. Feedback regulation of KEAP1 in response to downstream activation of NRF2 or other events could also complicate any expected associations. The concordance between NRF2 IHC, NRF2ACT, and NRF2-activating mutations provides a rationale for further testing of NRF2 IHC staining as a potential predictor of adjuvant chemotherapy benefit, which, if positive, could be incorporated readily into routine pathologic evaluation. Although we were unable to directly evaluate the predictive value of somatic alterations in NRF2, KEAP1, and CUL3 in JBR.10, the strong association between mutational status and gene expression class observed in the UHN validation dataset suggests that mutational status might also have potential as a predictive biomarker.

In conclusion, our results indicate that NRF2 pathway activation, as defined by the NRF2ACT gene expression signature, might serve as a biomarker of adjuvant cisplatin-based chemotherapy benefit in SCC. Validation of these findings is necessary, and this work provides a foundation for the comparative evaluation of NRF2 activation using multiple modalities (gene expression signatures, IHC, and somatic alteration profiling), all of which should be undertaken in randomized clinical trial patients and datasets. If confirmed, gene expression or mutational analysis for NRF2 pathway alterations/activation could have clinical utility in identifying patients unlikely to benefit from chemotherapy. Such patients could be spared the toxicity and costs of ineffective treatment, and instead be prioritized as candidates for trials of much-needed alternate therapeutic approaches.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.W. Cescon, D. She, S. Sakashita, C.-Q. Zhu, M. Pintilie, F.A. Shepherd, M.-S. Tsao
Writing, review, and/or revision of the manuscript: D.W. Cescon, D. She, S. Sakashita, M. Pintilie, F.A. Shepherd, M.-S. Tsao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Sakashita, F.A. Shepherd, M.-S. Tsao
Study supervision: F.A. Shepherd, M.-S. Tsao

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