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.

*Clin Cancer Res; 21(11); 2499–505. © 2015 AACR.*
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, SCC 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 SCC, sparing chemotherapy toxicity and identifying patients for the evaluation of alternative therapeutic strategies.

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

TCGA SCC 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 NFE2L2, 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 (GrCh37). 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 (SCRT 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 \( \times \) % stained) for each sample. For each antibody, cutoff values were defined to categorize samples: For NRF2, samples were classified as high (\( \geq 80 \)) 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 Cgrch37/hg19) and obtained from TCAG 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

<table>
<thead>
<tr>
<th>Dataset</th>
<th>JBR10 (n = 52)</th>
<th>Raponi (n = 129)</th>
<th>UHN181 (n = 43)</th>
</tr>
</thead>
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<tr>
<td>Age, y (median, range)</td>
<td>64.2 (45.4–75.8)</td>
<td>68 (42–91)</td>
<td>69.7 (49.5–87.9)</td>
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<tr>
<td>Sex (%), n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>6 (11.5)</td>
<td>47 (36.4)</td>
<td>18 (41.9)</td>
</tr>
<tr>
<td>Male</td>
<td>46 (88.5)</td>
<td>82 (63.6)</td>
<td>25 (58.1)</td>
</tr>
<tr>
<td>Stage (%), n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A</td>
<td>27 (20.9)</td>
<td>8 (18.6)</td>
<td>27 (20.9)</td>
</tr>
<tr>
<td>1B</td>
<td>25 (48.1)</td>
<td>46 (35.7)</td>
<td>19 (44.2)</td>
</tr>
<tr>
<td>2A</td>
<td>4 (7.7)</td>
<td>6 (4.7)</td>
<td>2 (4.7)</td>
</tr>
<tr>
<td>2B</td>
<td>23 (44.2)</td>
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<td>14 (32.6)</td>
</tr>
<tr>
<td>3A</td>
<td>17 (31.2)</td>
<td></td>
<td></td>
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<tr>
<td>Median follow-up, y</td>
<td>6.8 (2.9–9.3)</td>
<td>2.5 (0.2–12.0)</td>
<td>5.9 (1.4–12.0)</td>
</tr>
<tr>
<td>Died (%), n (%)</td>
<td>19 (36.5)</td>
<td>68 (52.7)</td>
<td>17 (39.5)</td>
</tr>
</tbody>
</table>
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 JAI CV-M4+ CL progressive scan monochrome camera (JAI 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 NRF2ACT 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 NRF2ACT 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 resamples 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
NRF2ACT 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 contain somatic alterations in NRF2 (mutation or amplification), or in KEAP1 or CUL3 (mutation or deletion), and the remaining 63 cases were wild-type for these genes. LR identified 291 genes with significant differential expression (P < 0.001) between somatically altered and wild-type cases (Supplementary Table S2a). Thirty genes were found to be upregulated in NRF2-altered cases by SAM analysis (Supplementary Table S2b). A comparison of these gene lists identified 28 overlapping genes, which define our NRF2ACT signature (Supplementary Table S2c). As predicted, reclustering of the TCGA cases using the 28-gene set confirmed that NRF2ACT identifies two major gene expression subgroups, with most NRF2-activating mutations segregating into the NRF2ACT subgroup (Fig. 1).

NRF2ACT as a marker of NRF2 pathway activation
To test the biologic relevance of genes contained in the 28-gene NRF2ACT 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 × 10–11; hypergeometric test), confirming that the 28-gene set contains bona fide NRF2 targets.

Concordance between NRF2ACT status, NRF2/KEAP1 IHC and somatic alterations in an independent dataset
To validate the association between the NRF2ACT 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, NRF2ACT identified two major subgroups in the UHN181 SCC set, with similar proportions to TCGA (Fig. 2). Furthermore, the NRF2ACT-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), NRF2ACT 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/mutational status, semiquantitative 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 NRF2ACT-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 NRF2ACT or mutational status was observed.

Prognostic effect of NRF2ACT in SCC patients treated with surgery alone
No prognostic association of NRF2ACT 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 NRF2ACT was examined in the SCC 
subset of JBR.10. Patients with NRF2ACT-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 NRF2ACT-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 somat- 
ic 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" oncol- 
ogy 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 speci-

Figure 2. 
A, hierarchical clustering of UHN cases using NRF2 28-gene set identifies two major subgroups. NRF2 and KEAP1 somatic alterations (as determined by Sanger sequencing and FISH) and protein expression (IHC) are indicated by the color-coded bars above the expression heat map. The NRF2ACT subgroup is enriched for cases with alterations in NRF2 (amplification or mutation) and KEAP1 (mutation) ($P < 0.001$). High NRF2 protein expression is associated with the NRF2ACT subgroup ($P = 0.0002$). For KEAP1, no association between gene expression class and protein expression by IHC was observed. B, hierarchical clustering of 129 SCC cases (Raponi) using NRF2 28-gene set identifies two major subgroups.

Cescon et al.
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

**Figure 3.**
Representative images of NRF2 and KEAP1 IHC staining, showing (A) NRF2-high (3+) and (B) NRF2-low (0-) and (C) KEAP1-high (3+) and (D) KEAP1-low (0-) examples.

**Figure 4.**
Prognostic effect of NRF2ACT signature in patients treated with surgery alone. Kaplan–Meier plots are shown for UHN cohort (A), Raponi cohort (B), and observation (surgery alone) arm (C) of JBR.10. No significant association between NRF2 signature status and survival was observed in any dataset.
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, stream activation of NRF2 or other events could also complicate activity. Feedback regulation of KEAP1 in response to down-alterations in NRF2 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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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Sakashita, C.-Q. Zhu, F.A. Shepherd, M.-S. Tsao
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, 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

Acknowledgments

The authors gratefully acknowledge Olga Ludkovski for performing the FISH studies.

Grant Support

This work was supported by the Canadian Cancer Society grant #020527, the Ontario Premier’s Award to Dr. F.A. Shepherd, the Princess Margaret Cancer Foundation, and the Ontario Ministry of Long Term Health. Dr. S. Sakashita is supported by the Terry Fox Foundation Training Program in Molecular Pathology of Cancer at CIHR (STP 53912). Dr. F.A. Shepherd is the Scott Taylor Chair in Lung Cancer Research. Dr. M.-S. Tsao is the M. Qasim Choksi Chair in Lung Cancer Translational Research.

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Received August 25, 2014; revised February 20, 2015; accepted February 23, 2015, published OnlineFirst March 4, 2015.

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Cescon et al.
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