NRF2 pathway activation and adjuvant chemotherapy benefit in lung squamous cell carcinoma

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Statement of 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. Since 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 (SqCC). To do so, we identified a set of genes whose expression associated with NRF2 pathway activation. Using this classifier, SqCC patients with the activated signature treated on 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 SqCC, sparing chemotherapy toxicity and identifying patients for the evaluation of alternative therapeutic strategies.
ABSTRACT

**Purpose:** Genomic profiling of lung squamous cell carcinomas (SqCC) 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 SqCC.

**Experimental Design:** Logistic regression (LR) and SAM (Significance Analysis of Microarrays) were applied to all 104 TCGA SqCC cases that had microarray gene expression and mutation data to identify genes associated with somatic NRF2 pathway alterations. The resulting signature (NRF2\textsuperscript{ACT}) was tested in 3 independent SqCC 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:** 28 genes were identified by overlap between LR (291 genes) and SAM (30 genes), and these consistently separated SqCC into 2 groups in all datasets, corresponding to putatively NRF-pathway activated and wild-type (WT) tumors. NRF2\textsuperscript{ACT} 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; NRF2\textsuperscript{ACT} HR 2.28, p=0.48; interaction p=0.15). NRF2\textsuperscript{ACT} 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 SqCC. Patients with NRF2 pathway activating somatic alterations may have reduced benefit from this therapy.
INTRODUCTION

Adjuvant platinum-based chemotherapy is a standard of care for patients with completely resected stage II-IIIA non-small cell lung cancer (NSCLC), with an absolute 5-year survival benefit of 4-15% 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 and long term toxicities, predictive biomarkers that could identify which patients do or do not benefit would be of great clinical utility.

While 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 (SqCC) and adenocarcinoma (ADC) 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 ~35% of SqCC 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 pre-clinical 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 SqCC. 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. While 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 SqCC, partially overlap with NRF2 pathway mutational status (11), no existing signature has been specifically developed a priori to identify NRF2-pathway activated SqCC. We therefore sought to define a gene expression signature for these cancers, and to test this signature as a predictor of benefit from adjuvant chemotherapy in NCIC CTG (Clinical Trials Group) JBR.10 (4).

MATERIALS AND METHODS

Patients and datasets
This study used patient data from four independent sources: The Cancer Genome Atlas (TCGA) (11); a previously published case series of resected SqCC from Raponi et al. (GSE4573) (23); a case series of resected SqCC from the University Health Network (UHN181; GSE50081) (24); and a subset of SqCC participants in the JBR.10 clinical trial (GSE14814) (9). For each contributing dataset, analysis was restricted to patients with SqCC for whom microarray gene expression data were available. For analysis of the TCGA dataset, only cases with microarray gene expression, and exome sequencing information available as of August 2012 were considered. Demographic and clinical information for SqCC patients in each dataset is summarized in Table 1.

Development of NRF2-activated (NRF2ACT) gene expression signature from TCGA
TCGA SqCC 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) (11). Logistic regression (LR; using p-value <0.001) and Significance Analysis of Microarrays (SAM) (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) vs. 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 hot-spot 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 Supplemental Table 1. Non-synonymous 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 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, Tucson, AZ). Nuclear staining intensity (0 to 3+) and the percentage of cells stained were used to calculate H-scores (intensity score x % stained) for each sample. For each antibody, cut-off 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 (Supplemental Figure S1). Interpretation was performed blinded to other variables.
NRF2 Fluorescence in situ hybridization (FISH)

Fluorescence in situ hybridization (FISH) studies were performed with the use of dual-color DNA FISH probes. Two 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 (Feb.2009 CRch37/hg19) and obtained from TCAG Genome Resource Facility (Toronto, Canada). The BAC clones were directly labeled with Spectrum Orange (BAC clones corresponding to NRF2 gene) or Spectrum Green (CEP2 BAC clones) fluorochromes using a commercially available nick translation kit according to the manufacturer's protocol (Abbott Laboratories, Abbott Park, Illinois, USA). Probes were first verified on normal blood metaphases to confirm their correct allocations. Briefly, TMA's and FFPE sections were deparaffinized in 3 changes of xylenes, dehydrated in ethanol, pretreated in citrate buffer (pH-6.8) for 50 min at 80°C followed by pepsin digestion for 18 minutes at 37°C. Slides were hybridized for 2 days 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., San Jose, USA) and the MetaSystems Isis FISH Imaging software programs v5.3 (MetaSystems, Germany) were used for capturing images. The number of red and green signals as well as their distribution were analyzed by an experienced cancer cytogenetic technician (blinded to outcomes and gene expression classification) in 50 non-overlapping tumor cell nuclei. Amplification was defined as an NRF2:CEP2 ratio ≥ 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. Cox proportional hazards model was employed 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 data set. The associations between categorical variables (NRF2, KEAP1, CUL3 mutations, NRF2, KEAP1 IHC with NRF2\textsuperscript{ACT} signature) were tested using Fisher’s Exact Test.

The set of genes associated with the NRF2 pathway alterations were selected based on logistic regression and SAM (significance analysis of microarray) (25). The alpha level for selection for the logistic regression 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 up-regulated in NRF2 or KEAP1 mutants. Statistical analyses were performed using the open source software R version 2.12.1, and the publicly available \textit{samr} package.

RESULTS

\textit{NRF2\textsuperscript{ACT} signature in TCGA SqCC}

104 unique SqCC 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. Logistic regression identified 291 genes with significant differential expression (p<0.001) between somatically altered and wild-type cases (Supplemental Table S2a). Thirty genes were found to be up-
regulated in NRF2-altered cases by SAM analysis (Supplemental S2b). A comparison of these gene lists identified 28 overlapping genes, which define our NRF2\textsuperscript{ACT} signature (Supplemental Table S2c). As predicted, re-clustering of the TCGA cases using the 28-gene set confirmed that NRF2\textsuperscript{ACT} identifies 2 major gene expression subgroups, with most NRF2-activating mutations segregating into the NRF2\textsuperscript{ACT} subgroup (Figure 1).

**NRF2\textsuperscript{ACT} as a marker of NRF2 pathway activation.**

To test the biological relevance of genes contained in the 28-gene 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=5.96x10-11; hypergeometric test), confirming that the 28- gene set contains \textit{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 SqCC, we performed hierarchical clustering using the 28-gene set, as well as NRF2 and KEAP1 mutational analysis by Sanger sequencing, in 43 SqCC from the UHN181 dataset (24). As observed in the TCGA derivation set, NRF2\textsuperscript{ACT} identified two major subgroups in the UHN181 SqCC set, with similar proportions to TCGA (Figure 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 SqCC (Supplemental Figure S2).
To evaluate whether immunohistochemical analysis of NRF2 or KEAP1 might serve as a useful surrogate of NRF2-pathway activation/mutational status, semi-quantitative IHC was performed on whole tissue sections. Representative images are shown in Figure 3, and IHC scores are indicated for each case in Figure 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 SqCC 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 SqCC surgery-alone dataset (Raponi, n=129), NRF2\textsuperscript{ACT} identified 2 subgroups based on gene expression (Figure 2b), but was not significantly associated with overall survival (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 NRF2\textsuperscript{ACT} in SqCC patients treated with adjuvant chemotherapy**

The predictive effect of NRF2\textsuperscript{ACT} was examined in the SqCC subset of JBR.10. Patients with NRF2\textsuperscript{ACT}-high tumors did not appear to benefit from adjuvant chemotherapy (HR 2.28, 95%CI 0.24–22, p=0.48), while a trend towards chemotherapy benefit, was observed in NRF2\textsuperscript{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). While 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 SqCC is of additional clinical consequence.

In an effort to apply the findings of TCGA (and the work that preceded it (13,34)) to current clinical management, we have focused on the NRF2 pathway, which is somatically activated in over one third of SqCC (11), and has a well-characterized role in chemotherapy sensitivity based on pre-clinical studies.

Using gene expression and sequencing data from TCGA we identified a 28 gene-set (NRF2\textsuperscript{ACT}) that is able to separate SqCC from multiple datasets into 2 subgroups. Application of NRF2\textsuperscript{ACT} in an adenocarcinoma dataset showed no similar discriminatory ability, indicating that this signature is specifically relevant for the SqCC 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 biological confirmation that NRF2\textsuperscript{ACT} indeed reflects transcriptional activation of this pathway. In both the TCGA derivation set and an independent SqCC 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 NRF2\textsuperscript{ACT}-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 analyse 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 (35)), gene expression could be a superior biomarker for this purpose.

In three independent datasets of SqCC patients treated with surgery alone, we observed no significant prognostic effect of our NRF2\textsuperscript{ACT} signature. However, in keeping with our hypothesis and the pre-clinical data that supported it, we observed a trend towards 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 chemo-resistance 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 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 SqCC samples for NRF2 and KEAP1. Comprehensive evaluation of mutational status, NRF2\textsuperscript{ACT} 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 association between KEAP1 staining and NRF2\textsuperscript{ACT} 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, NRF2\textsuperscript{ACT} 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 pathological evaluation. While 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 NRF2\textsuperscript{ACT} gene expression signature might serve as a biomarker of adjuvant cisplatin-based chemotherapy benefit in SqCC. 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, immunohistochemistry 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.

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REFERENCES


Table 1. Demographic and clinical characteristics of SqCC patients from three cohorts included in analyses.

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FIGURE LEGENDS

Figure 1. Re-clustering of TCGA cases using NRF2-associated 28 gene set identifies two major expression subgroups. The NRF2$^{\text{ACT}}$ subgroup (right side) contains the majority of cases with NRF2- activating somatic alterations, identified by the color-coded bar (top).

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 NRF2$^{\text{ACT}}$ 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 NRF2$^{\text{ACT}}$ subgroup ($p=0.0002$). For KEAP1, no association between gene expression class and protein expression by IHC was observed. B. Hierarchical clustering of 129 SqCC cases (Raponi) using NRF2 28-gene set identifies two major subgroups.

Figure 3. Representative images of NRF2 and KEAP1 IHC staining, showing (a) NRF2 high (3+) and (b) low (0+); and (c) KEAP1 high (3+) and (d) low (0+) examples.

Figure 4. Prognostic effect of NRF2$^{\text{ACT}}$ signature in patients treated with surgery alone. Kaplan Meier plots are shown for (A) UHN cohort (B) Raponi cohort and (C) observation (surgery alone) arm of JBR.10. No significant association between NRF2 signature status and survival was observed in any dataset.

Figure 5. Effect of adjuvant chemotherapy on survival in JBR.10 SqCC patients with NRF2$^{\text{ACT}}$- low (Left Panel) and NRF2$^{\text{ACT}}$-high (Right Panel) gene expression signature. A non-significant trend toward benefit from adjuvant chemotherapy is observed only in the NRF2-low subgroup.
Figure 1.
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Figure 4.
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