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 Culin 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,
Age, y (median, range) 64.2 (45.4–87.9)
Sex (n, %)
Female 6 (11.5) 47 (36.4) 18 (41.9)
Male 46 (88.5) 82 (63.6) 25 (58.1)
Stage (n, %)
1A 27 (20.9) 8 (18.6)
1B 25 (48.1) 46 (35.7) 19 (44.2)
2A 4 (7.7) 6 (4.7) 2 (4.7)
2B 23 (44.2) 27 (20.9) 14 (32.6)
3A 23 (44.2) 27 (20.9) 14 (32.6)
Median follow-up, y (range) 6.8 (2.9–9.3) 2.5 (0.2–12.0) 5.9 (1.4–12.0)
Died (n, %) 19 (36.5) 68 (52.7) 17 (39.5)
contain somatic alterations in TCGA from a total of 178 samples. Forty-one cases were found to have expression, mutation, and copy number data were identified (25). The alpha level for statistical analyses was performed using the open-source software R version 2.12.1 and the publicly available samr package.

**Results**

**NRF2<sup>ACT</sup> 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<sup>ACT</sup> or KEAP1 mutants. Statistical analyses were performed using the open-source software R version 2.12.1 and the publicly available samr package.

**NRF2<sup>ACT</sup> as a marker of NRF2 pathway activation**

To test the biologic relevance of genes contained in the 28-gene NRF2<sup>ACT</sup> 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<sup>-11</sup>; hypergeometric test), confirming that the 28-gene set contains bona fide NRF2 targets.

**Concordance between NRF2<sup>ACT</sup> status, NRF2/KEAP1 IHC and somatic alterations in an independent dataset**

To validate the association between the NRF2<sup>ACT</sup> 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 of 43 patients treated with surgery alone, without adjuvant chemotherapy (HR, 0.86, P = 0.79; Fig. 4A).

![NRF2 Pathway and Chemotherapy in Lung Squamous Cell Carcinoma](image)

**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<sup>ACT</sup> 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<sup>ACT</sup> 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<sup>ACT</sup> subgroup.
Similarly, in a second SCC surgery-alone dataset (Raponi; \( n = 129 \)), NRF2ACT 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 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.

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 (HR = 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.
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, KEAP1, or possibly even other events to disrupt the relationship between KEAP1 and NRF2. 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 the relationship between mutational status 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 the relationship between mutational status and NRF2 protein/transcription factor activity.

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