ORIGINAL RESEARCH

Correlation of alterations in the *KEAP1/CUL3/NFE2L2* pathway with radiation failure in larynx squamous cell carcinoma

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Abstract

Objectives: Patients with laryngeal squamous cell carcinoma (LSCC) often fail radiation therapy (RT), when received as monotherapy or in combination with other treatment modalities. Mechanisms for RT failure are poorly understood. We hypothesized that tumors failing RT would have increased rates of somatic mutations in genes associated with radiation resistance, particularly in genes associated with the *NFE2L2* oxidative stress pathway. Using targeted exome sequencing on pretreated LSCC tumors, we retrospectively compared somatic mutation profile with clinical data and response to treatment.

Methods: Tumors were classified as either radiation-resistant (RR) or radiation-sensitive (RS). RR was defined as persistent or recurrent disease within 2 years of receiving full-dose RT. Early stage (ES) LSCC was defined as Stage I or II tumors without lymph node involvement. Eight genes associated with radiation resistance were prioritized for analysis. RT-qPCR was performed on five *NFE2L2* pathway genes.

Results: Twenty LSCC tumors were included and classified as either RR (n = 8) or RS (n = 12). No differences in individual rates of somatic mutations by genes associated with radiation resistance were identified. Higher rates of total mutational burden (TMB) and increased alterations associated with the *NFE2L2* pathway was observed in RR vs RS tumors (*P* < .05). In an analysis of only ES-LSCC patients (RR, n = 3 and

RS, n = 3), RR tumors had increased NFE2L2 somatic pathway mutations ($P = .014$) and increased *NQO1* mRNA expression ($P = .05$).

Conclusion: Increased TMB and *NFE2L2* pathway alterations were associated with radiation resistance in LSCC. *NQO1* mRNA expression may serve as a biomarker for RT response in ES-LSCC.

Level of Evidence: II1.

KEYWORDS

laryngeal squamous cell carcinoma, oxidative stress, radiation resistance

1 | INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide. Within HNSCC, laryngeal squamous cell carcinoma (LSCC) is the second most common and is diagnosed in approximately 14 000 patients each year in the United States.[1] Early stage (ES) LSCC, defined as stage I or II tumors without evidence of thyroid cartilage invasion or lymph node involvement, is generally treated with single-modality therapy by either endoscopic surgical resection or radiotherapy (RT). Both treatment modalities have demonstrated comparable outcomes with 5-year disease-specific survival rates for Stage I (90%) and Stage II (80%) disease.[2-4] Nevertheless, 10-20% of ES-LSCC patients fail RT therapy and require salvage surgical resection or palliation.[5-7] In locally advanced LSCC (stage III and IVA-B), RT is combined either with chemotherapy for laryngeal preservation, or given as adjuvant therapy with or without chemotherapy following primary surgery. Rates of local regional recurrence and overall survival remain low despite improvements in therapy.

The etiology of radiation resistance in LSCC is poorly understood and likely multifactorial. Mechanisms linked to tumor biology include DNA damage repair, detoxification of free radicals, and intratumor heterogeneity. On a genetic level, somatic mutations in multiple genes, including *TP53*, *Ki67*, *CCND1*, *RAS*, and *BCL2*, has been associated with RT resistance.[8-14] Acquired RT resistance by genetic or epigenetic changes during clinical therapy have also been purported.[15,16]

Additionally, oxidative stress, which is a well-established mechanism of cellular damage, mediated by free radical and reactive oxygen species (ROS) has been implicated in RT resistance.[17,18] In cellular homeostasis, a balance exists between formation of active ROS metabolites and their destruction by antioxidants. In a dysregulated state, oxidative stress leads to tumorigenesis and cancer progression through DNA base alterations, double-strand breaks, downregulation of tumor suppressor genes, and upregulation of proto-oncogenes.[18]

The proteins kelch-like ECH-associated protein-1 (*KEAP1*)/nuclear factor (erythroid-derived 2)-like 2 (*NFE2L2* or *NRF2*) system have a central function in cellular protection against oxidative stress. *NFE2L2* is negatively regulated by *KEAP1* via Cul3-based E3 ligase (*CUL3*). In homeostasis, *KEAP1* ubiquitates *NFE2L2* and promotes its degradation. Under conditions of oxidative stress, *KEAP1* is modified

by electrophiles or ROS and inactivated. Consequently, *NFE2L2* is stabilized, leading to its accumulation and translocation into the nucleus. *NFE2L2* induces genes regulated by the antioxidant response element (ARE), which promotes numerous cytoprotective events including detoxification, antioxidation, and anti-inflammation. Critical downstream defense enzymes include NAD(P)H quinone oxidoreductase 1 (*NQO1*) and heme oxygenase-1 (*HO1*),[19,20] Figure 1.

In The Cancer Genome Atlas (TCGA), a comprehensive genomic analysis of 279 HNSCC tumors, *KEAP1/CUL3/NFE2L2* mutations were found in 22% of HPV-negative HNSCC patients.[21] As described in multiple cancers, *NFE2L2* pathway alterations can contribute to treatment resistance. Loss of function mutations in the *KEAP1* tumor suppressor gene and activating mutations in the *KEAP1* binding domain of *NFE2L2* result in the constitutive activation of *NFE2L2*.[19,22,23] In turn, this leads to pro-tumorigenic effects, including inhibition of apoptosis, promotion of cell proliferation, and chemoresistance.[24-26] In murine models of lung squamous cell carcinoma, loss of function mutations in both *KEAP1* and *p53* resulted in increased tumor aggressiveness and resistance to radiation therapy.[27]
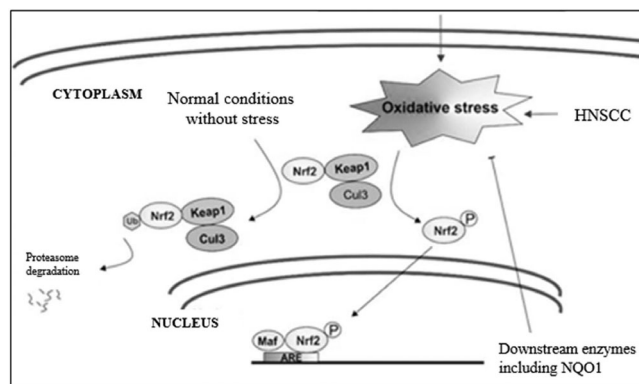
FIGURE 1 Oxidative stress pathway in normal conditions and in HNSCC. In non-stressful conditions, KEAP1 ubiquitates NFE2L2 and promotes its degradation. Under conditions of oxidative stress such as head and neck cancer (HNSCC), KEAP1 is modified and inactivated. NFE2L2 is then stabilizes, accumulates, and translocates into the nucleus. NFE2L2 induces genes regulated by the antioxidant response element (ARE). Downstream defense enzymes include NAD(P)H quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO1)

We developed two primary hypotheses based on the existing literature. First, LSCC tumors that failed radiation therapy have increased rates of somatic mutations in putative genes associated with RT resistance. Second, increased mutations in *NFE2L2*-oxidative stress pathway will lead to differential mRNA expression of downstream defense enzymes in RT resistant vs RT sensitive tumors. To evaluate these questions, we performed targeted exome DNA sequencing on pretreated LSCC tumors and correlated these findings with clinical and treatment data.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

The UNCSeq Version 8.0 involves sequencing exons of a targeted list of 247 human genes and 10 pathogen genome segments from formalin fixed paraffin embedded (FFPE) tissue and matched germline DNA. Tumor samples and outcomes data were derived from the clinical trial LCCC1108: *Development of a Tumor Molecular Analyses Program and Its Use to Support Treatment Decisions* (Institutional Review Board protocol # 16-3410), which has enrolled approximately 3000 cancer patients since opening. All studies were approved by the University of North Carolina (UNC) Institutional Review Board. All authors contributed to study design, conduct, and/or manuscript preparation and vouch for the accuracy and completeness of the data reported.

LSCC patients were eligible for study inclusion regardless of TMN stage, HPV/p16 status, or treatment received. Patient and tumor information was obtained for each study subject via chart review of electronic medical records. Demographic data including age, gender, marital status, race, smoking history, and alcohol consumption was obtained to evaluate for clinical predictors of radiation resistance. Clinical stage according to the AJCC staging system (AJCC 8th edition) and tumor grade of initial biopsy specimens were recorded.

2.2 | Definition of radiation resistance

RT was given either as monotherapy, concurrent chemoradiation (cCRT), or post-operatively. All patients received full-dose radiation treatment in the definitive setting (66-70 Gy, 2 Gy/day) or post-surgery setting (60-66 Gy, 2 Gy/day) under direct supervision of the UNC Department of Radiation Oncology. Radiation resistance was defined as persistent or recurrent disease within 2 years of receiving RT. Study patients were categorized into two groups based on retrospective evaluation of their treatment response: radiation-resistant (RR) or radiation-sensitive (RS). An a priori list of genes associated with radioresistance were prioritized for mutational evaluation including *NME1, HSBP1, RAF1, NFE2L2, KEAP1, MAGED1, BCL*-2, and *BIRC5* (Survivin).

2.3 | Next generation sequencing

2.3.1 | DNA isolation, library preparation, and sequencing

LSCC diagnosis was confirmed by a UNC surgical pathologist based on examination of H&E stained slides. Matched non-malignant tissue from each subject was also sequenced to identify somatic changes. Automated DNA extraction was obtained from FFPE tissue sections using the Promega Maxwell MDx16 instruments (Promega, Inc, Madison, WI) and then fragmented by sonication. Subsequent quality assessments were performed by ultraviolet (UV) absorbance and quantity assessments. During DNA isolation and library preparation, DNA concentration was measured by fluorometry and DNA quality was evaluated using the Agilent 2100 Bioanalyzer high sensitivity assay. DNA libraries were pooled and transferred to the UNC High Throughput Sequencing Facility (HTSF) for deep sequencing using an Illumina HiSeq2500 sequencer. Bioinformatics analysis support was offered by UNC's Lineberger Comprehensive Cancer Center Bioinformatics Department.

2.3.2 | Bioinformatics

Sequencing data were routed through an automated pipeline managed by the Lineberger Bioinformatics Core (LBC). Our current somatic workflow uses paired tumor and normal libraries to detect somatic mutations, large and small indels, structural variants, and pathogenic organisms. Raw sequence was aligned using the BWA-mem algorithm and refined using our Assembly Based ReAlignment process to allow for accurate alignment of complex sequence variation.

Copy number calls were generated with the SynthEx algorithm[28] using the tumor sequencing data and a library of 200 unmatched normal samples sequenced with the same technique. A conservative approach was taken. Thirty replicates varying parameter k (number of nearest neighbor) were done per tumor and the model with the fewest deviations from the expected copy number of 2. Sex chromosomes were excluded.

2.4 | mRNA processing

Reverse transcription reactions were performed with the VILO Reverse Transcription master mix (Life Technologies) using 0.8 μg of total RNA. Real-time RT-qPCR was performed using the QuantStudio 6 (Life Technologies) machine and TaqMan Gene Expression master mix (Thermo-Fischer). Each reaction contained 1X TaqMan Gene Expression master mix, cDNA from 20 ng of RNA, and 1X of gene specific primer/probe combinations (Life Technologies Cat. #4331182; TBP Primer ID Hs00427620_m1, CUL3 Primer ID Hs00180183_m1, HMOX1 Primer ID Hs01110250_m1, KEAP1 Primer ID Hs00202227_m1, NRF2 (NFE2L2) Primer ID

Hs00975961_g1, and NQO1 Primer ID Hs01045993_g1) in a total volume of 20 μL. PCR was performed in duplicate by cycling at 50°C for 2 minutes, 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, and annealing and extension at 60°C for 30 seconds. Values were derived using the delta-delta cT method comparing RR to RS mRNA levels with normalization to housekeeping gene expression (TBP). Values were reported as fold-change in mRNA expression of the target in question.

2.5 | Data and statistical analysis

Descriptive statistics were used to tabulate means, ranges, and frequencies. Baseline characteristics were compared between groups using the Wilcoxon Rank Sum test for continuous variables and Fisher's exact test for categorical variables. All statistical calculations were made using STATA (College Station, TX, Version 15).

3 | RESULTS

3.1 | Patient characteristics

Twenty previously untreated LSCC patients were included. Based on retrospective review of treatment response as previously defined, 12 patients (60%) were categorized as radiation sensitive (RS) while eight patients (40%) were radiation resistant (RS). Demographic characteristics were well balanced between the two groups (Table 1).

TABLE 1 Baseline characteristics for all laryngeal squamous cell carcinoma cases (n = 20) according to response to radiation therapy

	Radiation sensitive (n = 12)	Radiation resistant (n = 8)	Total (n = 20)	P-value
Sex (%)				
Female	3 (25.0)	0 (0)	3 (150)	.225
Male	9 (75.0)	8 (100.0)	17 (85.0.)	
Age (y, SD)	64.4 (7.2)	63.3 (6.7)	64.0 (6.8)	.782
Race (%)				
Black	6 (50.0)	1 (12.5)	7 (35.0)	.089
White	5 (41.7)	6 (75.0)	11 (55.0)	
Other	1 (8.3)	1 (12.5)	2 (10.0)	
T stage (%)				
1	2 (16.7)	0 (0)	2 (10.0)	.01
2	1 (8.3)	5 (62.5)	6 (30.0)	
3	4 (33.3)	1 (12.5)	5 (25.0)	
4	3 (25.0)	0 (0)	3 (15.0)	
Not reported	2 (16.7)	2 (25.0)	3 (15.0)	
N stage (%)				
0	6 (60.0)	4 (80.0)	10 (62.5)	.502
1	1 (10.0)	1 (20.0)	3 (18.8)	
2	3 (30.0)	0 (0)	3 (18.8)	
p16 status (%)				
Negative	7 (58.3)	5 (62.5)	12 (60.0)	.409
Positive	1 (8.3)	0 (0)	1 (5.0)	
Not reported	4 (3.3)	3 (37.5)	7 (35.0)	
Primary therapy (%)				
Radiation therapy (RT)	2 (16.7)	2 (25.0)	4 (20.0)	
Chemoradiation therapy	6 (50.0)	6 (75.0)	12 (60.0)	
Surgery followed by RT	4 (33.3)	0 (0)	4 (20.0)	
Salvage laryngectomy (%)				
No	12 (100)	2 (25.0)	14 (70.0)	.001
Yes	0 (0)	6 (75.0)	6 (30.0)	
Alive (%)				
No	3 (25.0)	3 (37.5)	6 (30.0)	.157
Yes	9 (75.0)	5 (62.5)	14 (70.0)	

FIGURE 2 Rates of somatic mutations in all LSCC cases. Based on targeted exome sequencing, N = 18
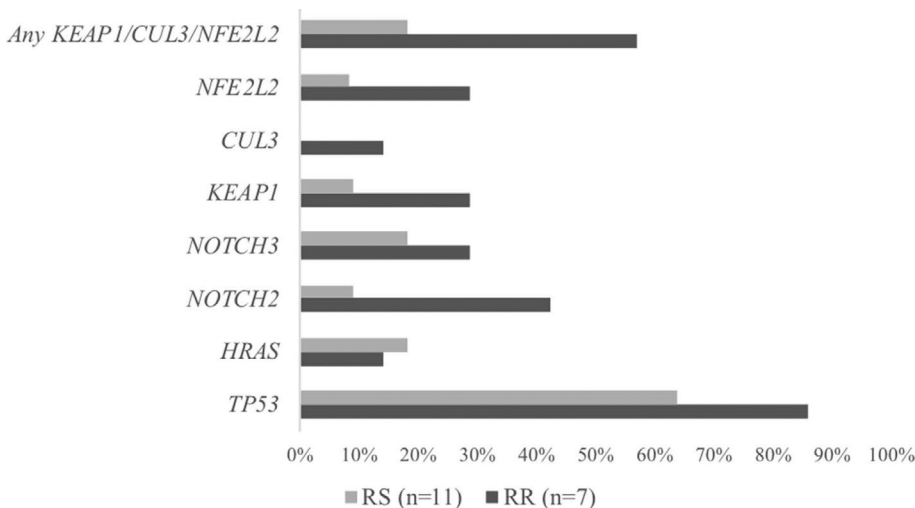

TABLE 2 Tumor mutational burden (TMB) and unique mutations by targeted exome sequencing and canonical HNSCC mutations

Unique identifier	TNM primary	TMB (per megabase)	Unique canonical mutations
Radiation resistant only (n = 7)			
RR#1	T2N1M0	11.2	4
RR#2	T2N1M0	8.8	3
RR#3	T2N0M0	10.4	4
RR#4	T3N0M0	22	6
RR#5	T2N0M0	19.8	5
RR#6	T2N0M0	101.8	13
RR#7	NR	76.6	9
Average mutation		35.8	6.3
Radiation sensitive only (n = 11)			
RS#1	T3N2M0	7.4	0
RS#2	NR	25.2	5
RS#3	T4N0M0	6.4	1
RS#4	T1aN0M0	7.2	3
RS#5	T4aN0M0	13.2	3
RS#6	T4aN1M0	12.4	4
RS#7	NR	18	4
RS#8	T3N2cM0	18	4
RS#9	T2N0M0	24.2	0
RS#10	T3N0M0	44	7
RS#11	T1N0M0	15.6	2
Average mutation		17.8	3.2

Notes: N = 18, TMB calculated per megabase. HNSCC canonical mutations calculated by individual mutation. NR = not reported.

There was no difference in smoking-pack year history. RS patients were more likely to have later T stage compared to RR patients (P = .01), with no difference in N stage and M stage. As expected LSCC, most patients with known HPV status were p16-negative; however, 7/20 (35%) patients in our cohort did not have documented p16 status. All RR patients had >20 pack year smoking histories, including two patients who were current smokers at time of initial diagnosis. Eleven out of 12 RS patients were heavy smokers. Of note, one RS patients was a non-smoker who developed early stage disease (T1N0).

3.2 | Treatment characteristics

The type of treatment received differed according to TNM stage at presentation (Table 1). Four patients (20%) received only RT, while

16 patients (80%) received either cCRT (12/16) or primary surgery followed by postoperative therapy (4/16). For those treated with concurrent chemotherapy, three patients received carboplatin/paclitaxel, one received cetuximab monotherapy, and eight received cisplatin, which was dosed either as bolus (100 mg/m^2 every 3 weeks) or weekly (40 mg/m^2). Notably, all four patients who received upfront total laryngectomy followed by postoperative radiotherapy (3/4) or chemoradiotherapy (1/4) were radiation sensitive.

All eight RR patients had disease recurrence 1-2 years following completion of their initial therapy. At time of disease recurrence, patients presented with either local (7) or locoregional (1) disease. Of note, no patients were smoking at time of recurrence. Six RR patients

FIGURE 3   Significantly increased NQO1 mRNA expression in radiation resistant ES-LSCC patients compared to radiation sensitive ES-LSCC patients. Quantitative RT-PCR was performed for *NQO1*. For each tumor, expression level is reported as relative expression of radiation resistance compared to radiation sensitive ES-LCC. Error bars represent ± SEM. *P < .05. *CUL3*, *KEAP1*, *NFE2L2*, and *HOMX1* expression was also performed (data not shown)

ultimately received salvage laryngectomies, with two patients refusing surgery and opting for palliation.

3.3 | Somatic mutational profile

All 20 patient tumors underwent targeted exome sequencing. Due to sequencing failures, two samples (one RR and one RS) were excluded from the analysis. Figure 2 shows the rate of somatic mutations based on our a priori list of genes associated with RT resistance. *TP53* was the most commonly mutated gene (72.2%; RR 6/7; RS 7/11), followed by *NOTCH3* (4/18), *NOTCH2* (4/18), and *HRAS* (3/18). Mutations in genes associated the oxidative stress pathway were *NFE2L2* (3/18), *KEAP1* (3/18), and *CUL3* (1/18). No alterations were found in *KRAS*, *RAF1*, *BCL-2*, and *BIRC5*. In the full cohort, there was no statistical difference in mutation rates by individual gene between the RS and RR groups.

Next, tumor mutational burden (TMB) was calculated in 18 LSCC patients in two ways: (1) all 247 genes in the UNCSeq panel and (2) 25 canonical HNSCC genes[29] (Table 2). Average TMB was 24.8 mutations per megabase (range 6.4-101.8 mut/mb) in all genes and 4.4 mut/mm (range 0-13 mut/mb) in canonical HSNCC genes. Upon stratification by RT response, RR tumors had higher overall TMB (35.8 vs 17.8, P = .075) and in canonical HNSCC genes (6.3 vs 3.2, P = .019) compared to RS tumors.

3.4 | Copy number changes

We then evaluated copy number changes in the panel of putative radiation resistance genes examined. One additional RR patient was excluded due to poor data quality. Of the six RR patients evaluated, three had *NFE2L2* activating copy changes, include two with multiple activating changes in the *KEAP1/NFE2L2/CUL3* pathway (five total changes in the RR group). There was no detectable difference in the overall number of sequenced bases, which were affected by copy number events in RS vs RR (16.9% vs 21.2%, P = 0.61).
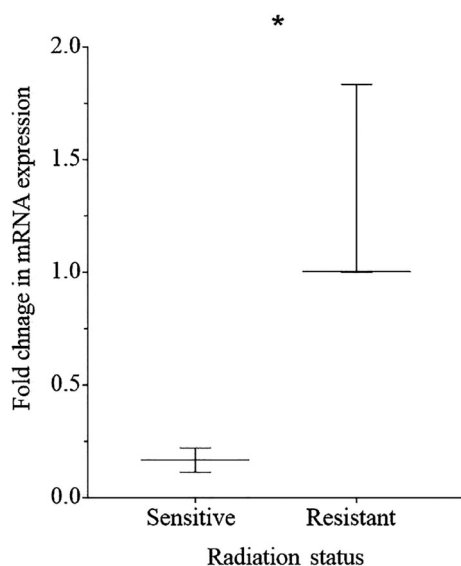
Gene name	Variable class	Summary	Protein effect	nsSNV impact
CUL3	Missense mutation	START LOSS	Deletion	HIGH
NFE2L2	Missense mutation	MISSENSE	Arg18Gly	MODERATE
NFE2L2	Missense mutation	MISSENSE	Glu63Gln	MODERATE
KEAP1	Frame shift deletion	FRAMESHIFT	Deletion	HIGH
NFE2L2	5′ flank	SILENT	Silent	MODIFIER
KEAP1	Intron	SILENT	Silent	LOW
NFE2L2	Missense mutation	MISSENSE	Glu63Gly	MODERATE
KEAP1	Intron	SILENT	Silent	LOW
KEAP1	Intron	SILENT	Silent	LOW
KEAP1	Missense mutation	MISSENSE	Ala487Pro	MODERATE
KEAP1	Silent	SILENT	Silent	LOW
KEAP1	Missense mutation	MISSENSE	Val428Gly	MODERATE

TABLE 3   Non-synonymous single nucleotide variants (nsSNVs) and expectations on loss of function

When considering all *KEAP1/CUL3/NFE2L2* loci examined, there was an increased rate of *NFE2L2* activating changes in the RR group ($P = .03$). Changes were counted as activating for *CUL3/KEAP1* loss and *NFE2L2* gain. All copy number changes in these genes were in the activating direction in the RR group, which was independently significant ($P = .04$, one-sided). The combination of TMB and activating copy number alterations by *KEAP1/CUL3/NFE2L2* loci demonstrated an overall increased probability in these three genes, when comparing the RR tumors (10/39, 25.6%) to RS tumors (3/66 4.5%, $P = .004$).

3.5 | Early stage LSCC tumors

Six patients in the study cohort were classified as ES-LSCC (3 RR and 3 RS). In the RR group, each patient had one mutation in either *CUL3*, *KEAP1* or *NFE2L2* compared to zero mutations in the RS patients ($P = .014$). RR tumors also had numerically increased rates of overall TMB and mutations in canonical HNSCC genes compared to RS tumors.

To further investigate the transcriptional effect of somatic mutations in oxidative stress pathway in the six ES-LSCC tumors, RT-qPCR was performed for *CUL3*, *KEAP1*, *NFE2L2*, *NQO1*, and *HOMX1*. There was no difference in mRNA expression levels in *NFE2L2* and *KEAP1*. However, RS tumors had higher mRNA expression of *CUL3* ($P = .03$) and lower downstream expression of *NQO1* ($P = .05$) compared to RR tumors (Figure 3). Variability of *HOMX1* expression limited interpretability.

4 | DISCUSSION

In this study, we retrospectively characterized patients according to their response to radiation therapy and compared their pretreated tumor mutational profile using targeted exome DNA sequencing. Our primary hypothesis that LSCC tumors failing RT would have increased somatic mutations in genes associated with radiation resistance was not supported. However, multiple novel observations were made. First, tumors with high TMB were associated with RT failure. The relationship of TMB and response to RT has not been previously described. Second, in the three early stage LSCC patients with radiation resistant tumors, all had mutations in the oxidative stress pathway including the genes *CUL3*, *KEAP1*, and *NFE2L2*. This contrasted with no mutations observed in radiation sensitive patients. Third, an increased rate of *NFE2L2* activating changes in the RR group was observed. Finally, in early stage LSCC tumors that were radiation resistant, increased *NQO1* mRNA expression was found. This supports the notion that increased *NFE2L2* activation resulted in increased transcriptional activity of downstream reporter genes such as *NQO1* and *HOMX1*. If validated, *NQO1* mRNA expression may serve as a canonical reporter for resistance to radiation therapy.

Mutations in *KEAP1* and *NFE2L2* have been robustly associated with increasing the transcriptional activity of *NFE2L2* in HNSCC as well as other upper aerodigestive cancers of the esophagus and lung. Further, the association of *KEAP1/NFE2L2* mutations and radiation

failure has been previously reported by Jeong et al. Forty-two patients with Stage I-III lung squamous cell carcinoma who received either RT or concurrent chemoradiotherapy underwent tumor sequencing. In patients with somatic mutations in either *KEAP1 or NFE2L2*, 70% developed local failure compared to only 18% in the wild type group ($P < .008$) at 30 months. Lung squamous cell carcinoma and laryngeal squamous cell carcinomas share similar risk factors including smoking, molecular alterations such as loss of heterogeneity and *p53* driver mutations, and morphological characteristics. Mechanistically, Jeong et al. speculated that *KEAP1/NFE2L2*-mutant tumors have increased expression of ROS scavengers and detoxification pathways, which helps confer RT resistance.[27]

We also investigated if mutations identified in the three early stage LSCC patients had been previously reported in the Catalogue of Somatic Mutations in Cancer (COSMIC). The first was a missense mutation in *NFE2L2* located on chromosome 2 (c.188A>T (p.Glu63Val). This single nucleotide variant has been reported twice in COSMIC in patients with lung squamous cell carcinoma. Second, the *CUL3* mutation (p.Met1Ile) located on chromosome 2 was a start-loss mutation, which generally occurs when the start codon is altered and results in loss of transcript translation. The third was a missense mutation in *KEAP1* (p.Ala487Pro), which is located on chromosome 19. Neither the *CUL3* or the *KEAP1* mutation have been previously reported in COSMIC and may reflect novel findings. Furthermore, we reported the non-synonymous single nucleotide variants (nsSNVs) found in all *KEAP1/NFE2L2* mutations identified in our study as well as the expectation on loss of function (Table 3). As expected, intron and silent nsSNVs were associated with a silent effect on protein function, thus resulting in low impact on functionality. However, a number of moderate and high impact variants were identified in our sample.

NFE2L2 is well known to have mutational hotspots with disrupt high affinity binding motifs ("ETGE" and "DLG") for *KEAP1*, thus circumventing regulation by the ubiquitin proteasome pathway, and activating transcription. Although the *NFE2L2* mutations identified in this study are not in these canonical hotspots, they are located in the KEAP1 binding domain, and therefore could reasonably interfere with the *KEAP1/ NFE2L2* interaction (Table 3). Bolstering this idea, the mutations surrounding but not in the "DLG" motif have been validated as *NFE2L2* activating. As a prototypical tumor suppressor, missense mutations throughout *KEAP1* have been associated with *NFE2L2* activation. Therefore, although not experimentally investigated, the KEAP1 variants detected in this study are expected to have potential to stabilize and activate *NFE2L2*. Variants in *CUL3* are weakly associated with *NFE2L2* activation in tumor data; however, biochemically *CUL3* activity is essential for the *NFE2L2* regulatory function of the *KEAP1/CUL3/RBX1* complex.

Aside from *KEAP1/NFE2L2* pathway alterations, somatic differences in other putative genes associated with radiation resistance were not identified. Therefore, alternative considerations should be explored. Clinical factors such as stage (advanced vs early), tumor site (oral cavity vs laryngeal),[30] and degree of differentiation (well-differentiated vs poor differentiated)[31] have been linked to radiation resistance. In our study, all primary tumors originated in the larynx

thus precluding comparisons with other anatomic locations. We did not capture differences in tumor differentiation status between the two groups. RS tumors in this study presented with more advanced T stage (3 or 4) compared to RR tumors, which is reassuring because any bias created from this difference should be in the direction of the null hypothesis. Furthermore, unmeasured tumor-related factors such as intratumor hypoxia and repopulation may also contribute to RT failure. Intratumor hypoxia occurs after a tumor has outgrown its own blood supply either regionally or globally.[32,33] In one HNSCC study, tumor hypoxia was found to be a negative prognostic factor, particularly for tumor pO2 levels <10 mm Hg.[33] In cell line studies, overexpression of hypoxia-inducible factor (HIF)-1 alpha was associated with increased lymph node metastasis and decreased overall survival.[34] Finally, RT can dysregulate the cell cycle and lead to accelerated stem cell division and repopulation, which is the regrowth of tumor cells following the initiation of therapy.[35,36] Our study did not measure for evaluate for these markers.

Our study has important limitations. First, the sample size was small and not statistically powered to calculate differences in individual somatic mutations by gene based on RT response. Second, there was heterogeneity in the treatment received by both early and advanced stage patients. Only four patients received RT monotherapy, while the remaining received multimodality therapy. Therefore, we are unable to conclude if treatment failure was solely due RT or as a result of resistance to chemotherapy or surgery. Interestingly, all RR patients had local disease recurrence despite receiving NCCN-guideline based therapy, which suggests in-field failure of radiation therapy. Finally, we were unable to adjust for potential confounders with a multivariable analysis given the small sample size. However, it is reassuring that potential confounders such as age, sex, and treatment type were relatively similar across RS and RR groups.

5 | CONCLUSION

In summary, this study adds to the growing body of literature highlighting the important role of the oxidative stress pathway in HNSCC. Specifically, pretreatment alterations in the *CUL3/KEAP1/NFE2L2* oxidative stress pathway may be associated with primary radiation resistance in patients with LSCC. To validate our findings, we plan to prospectively evaluate early stage LSCC patients receiving only radiation therapy and following treatment response based on the *KEAP1/NFE2L2* mutational status. We will also further evaluate if pretreatment mRNA *NQO1* may be a predictive biomarker for radiation therapy.

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