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Head & Neck – Original Research Article

Mutational Profiles of Persistent/Recurrent Laryngeal Squamous Cell Carcinoma

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Abstract

Background: We sought to describe targeted DNA sequencing data of persistent/recurrent LSCC and to compare gene-specific alteration frequencies with that of primary, untreated LSCC specimens from TCGA.

Methods: The tumors of twenty-one patients with persistent/recurrent LSCC were subjected to targeted DNA sequencing using the Ion AmpliSeq Comprehensive Cancer Panel. Gene-specific alteration frequencies were compared (Chi-Square test) to primary, untreated LSCC sequencing data from TCGA using the cBioPortal platform.

Results: Persistent/recurrent LSCC was characterized by a high rate of inactivating alterations in *TP53* (38.1 %) and *CDKN2A* (33 %), amplification events of *CCND1* (19.1 %), and *ERBB2* (14.3 %), and *NOTCH1* (19.1 %) mutations. Comparison of primary vs. persistent/recurrent LSCC revealed significant differences in alteration frequencies of eight critical genes: *BAP1, CDKN2A, DCUN1D1*, *MSH2*, *MTOR*, *PIK3CA*, *TET2*, and *TP53.*

Conclusions: Our results provide preliminary support for a distinct mutational profile of persistent/recurrent LSCC that requires validation in larger cohorts.

Introduction

Laryngeal squamous cell carcinoma (LSCC) remains a significant source of morbidity and mortality, with 4,000 deaths annually in the United States.[1] Organ preservation radiotherapy (RT) and chemoradiotherapy (CRT) have become standard of care for many primary LSCC. However, disease recurrence remains a difficult issue, with five-year disease-free survival (DFS) rates for advanced LSCC after initial RT or CRT[2] ranging from 30 – 60 %.[3,4] For patients with persistent/recurrent LSCC after initial treatment, prognosis is guarded.[5-7] Our best data on the genomic landscape of LSCC comes from previously-untreated patients within TCGA. Thus, a better understanding of genetic drivers of persistent/recurrent LSCC to provide prognostic biomarkers and targets for novel therapeutics are needed in this patient cohort.[8]

Analysis of primary LSCC specimens in recent whole-exome sequencing (WES) studies by Stransky et al (n = 15)[9] and the Cancer Genome Atlas Network (n = 117)[10] has identified high rates of *TP53* mutations, activating *PIK3CA* alterations, *CCND1* and *EGFR* amplification,

CDKN2A deletion, and *NOTCH1* aberrations in primary LSCC. *ERBB2* amplification has similarly emerged as a potential oncologic driver in a smaller subset of LSCC.[11] Importantly, these studies did not specifically look into persistent/recurrent tumor mutational profiles after RT/CRT.

The biology of recurrent head and neck squamous cell carcinoma is not well characterized, though recent sequencing efforts have revealed unique, and occasionally targetable, mutational signatures in a handful of these tumors.[12,13] There is significant rationale for distinct mutational signatures in recurrent LSCC given the poor initial clinical response of these tumors, the potential for de novo mutations due to RT/CRT, and tumor evolution and subclonal proliferation. As such, a better understanding of molecular alterations underlying these cancers is paramount to advancing precision oncology paradigms in this cohort.

We hypothesized that the mutational landscape in persistent/recurrent LSCC will be distinct from primary, untreated LSCC. Herein, we report mutational profiles of persistent/recurrent LSCC in the largest patient cohort to date and illustrate important differences in mutational rates of LSCC driver genes by comparison to TCGA database of primary, untreated LSCC.

Materials and Methods

Our study employed a convenient sample of patients with biopsy-proven persistent/recurrent LSCC (n = 21) who were treated at the University of Michigan Hospital and Health Systems between 2000 – 2012. Clinical (e.g. demographics, tobacco and alcohol history, tumor sub-site, AJCC stage, treatment) and survival data were collected and summarized.

Formalin-fixed, paraffin-embedded (FFPE) specimens were obtained from the University of Michigan pathology archive under an IRB-approved protocol (HUM00080561). Tumor cores and adjacent normal tissue were taken from FFPE blocks, and genomic DNA was isolated using the Qiagen Allprep DNA/RNA FFPE kit (Qiagen, Hilden, Germany) and quantified using a Qubit as previously described.[14]

Amplicon-based DNA sequencing of genes relevant to LSCC biology was performed on the Ion Torrent Personal Genome Machine, utilizing the Ion AmpliSeq Comprehensive Cancer Panel (Thermo Fisher Scientific, Waltham, MA), as previously described.[11,15] Single-nucleotide variants (SNV) and indels were called using the Torrent Variant Caller, annotated using Annovar, and filtered for candidate non-synonymous somatic variants.[16] Gene-level copy number variants (CNV) were determined by taking the mean of the coverage-weighted per-probe ratios, as described.[17] Per the manufacturer's specifications, the Ion AmpliSeq Comprehensive Cancer Panel yields ≥ 94 % exome sequencing coverage at an average read depth of 350x.[11,15]

Whole exome sequencing data of primary LSCC (n = 117) specimens was retrieved from the TCGA provisional dataset using the cBioPortal platform.[18,19] Chi-Square test ($\alpha = 0.05$) using GraphPad Prism (GraphPad Software, La Jolla, California, USA) was employed for comparison of mutational rates in persistent/recurrent vs. primary LSCC.

Results

Clinical characteristics of the cohort are summarized in **Table 1**. All included patients were male with significant tobacco use history. The mean (range) age of included patients at diagnosis was 59 (40 – 77) years. Early- and late-stage initial tumors were equally represented in our cohort. Primary treatment consisted of CRT (n = 10), RT (n = 9), and partial or total laryngectomy with adjuvant RT (n = 2). The mean (range) disease-free interval (DFI) was 29 (3 – 150) months. Sites of LSCC recurrence included local (n = 18), regional (n = 1), local and distant (n = 1), and distant (n = 1). Despite surgical salvage in 17 (81 %) patients, nine (43 %) died of LSCC.

Targeted DNA sequencing of persistent/recurrent LSCC specimens revealed a high frequency of alterations also present in primary LSCC, including inactivating *TP53* (38.1 %) and *CDKN2A* (33 %) mutations and complex *NOTCH1* (19.1 %) alterations **(Figure 1)**. Potentially targetable amplifications of *CCND1* (19.1 %), *ERBB2* (14.3 %), and *PIK3CA* (9.5 %) were also seen. Our modest sample size precludes definitive statistical analyses of the effect of specific genetic

alterations on survival in our cohort. However, we are able to highlight mutational profiles of individual patients with persistent/recurrent LSCC who recurred and ultimately died of disease, thus nominating such genetic alterations for further study and potential therapeutic targeting.

Patient number four recurred locally 28 months after CRT for stage III primary disease, ultimately dying of recurrent LSCC 16 months after salvage laryngectomy. His recurrent tumor harbored missense mutations in *BRCA2* and *MSH2*. Patient number six experienced a late, unresectable local recurrence, 63 months after CRT for stage IV disease, and ultimately died 4 months later. His recurrent tumor was characterized by *CDKN2A* deletion and amplification of *PIK3CA* and *SOX2*. Patient number ten recurred locally 13 months after RT for stage I disease and died of recurrent disease one year after salvage laryngectomy. His recurrent LSCC harbored nonsense *NOTCH1* alteration and *TP53* missense mutation. Finally, patient number 14 recurred distantly four months after CRT for stage IV disease, dying three months after initiation of palliative RT. His tumor harbored multiple deleterious alterations, including inactivating missense mutations in *ATM*, *NOTCH1*, and *TP53*, as well as *ERBB2* amplification and *CDKN2A* deletion.

We next compared the alteration frequency of 52 genes in the Ion AmpliSeq Comprehensive Cancer Panel between our persistent/recurrent LSCC cohort and the primary, untreated TCGA cohort (n = 117) **(Figure 2, Supplemental Table S1)**. Primary LSCC harbored a significantly

higher rate of characteristic *CDKN2A*, *PIK3CA*, and *TP53* alterations. Amplification of *DCUN1D1,* a candidate oncogene at the 3q chromosomal locus implicated in squamous cell carcinogenesis, was also seen more frequently in primary LSCC **(Figure 2).**[20] Conversely, the PI3K pathway effector *MTOR*, the critical DNA mismatch repair gene *MSH2*, and the *BRCA1*-associated protein *BAP1* harbored complex mutational patterns at a higher frequency in persistent/recurrent LSCC.

Discussion

Disease recurrence after definitive initial treatment for LSCC is a therapeutic challenge due to constraints imparted by previous therapies, considerable morbidity of salvage surgery, and potential acquired resistance of disease to chemotherapy and radiation. Prognosis for recurrent LSCC has stagnated in recent decades, with median survival after recurrence of 22 months for patients eligible for salvage surgery or re-irradiation and less than 12 months in those administered palliative chemotherapy.[21] The complex genetic alterations driving LSCC recurrence, progression, and treatment resistance need to be better characterized to guide development of precision therapeutics in the bench-to-bedside paradigm.

Biologically, cancer recurrence is preceded by a complex, branched evolution of subclonal populations within the primary tumor that persist under selective treatment pressures and propagate with further accumulation of deleterious mutations.[22] Hedberg et al performed WES of

eight primary tumor-metachronous recurrence pairs of head and neck squamous cell carcinoma (HNSCC).[12] The authors showed that nearly 40 % of somatic variants identified in the recurrent cancers were new and absent in the primary tumor, including amplification of *CCND1* and non-synonymous mutations of *DDR2* (discoidin domain receptor tyrosine kinase 2). *DDR2* alterations promote cellular migration, invasion, and metastasis in HNSCC[23] and confer sensitivity to the SRC-family kinase (SFK) inhibitor dasatinib in other cancers.[24] Implementation of routine sequencing panels in persistent/recurrent LSCC management may therefore exploit unique biology of tumor recurrence and nominate novel therapeutic options in this patient cohort.

To date, ours is the largest targeted sequencing cohort of persistent/recurrent LSCC, allowing for comparison to primary LSCC mutational profiles and nomination of specific genetic aberrations for further study. Because of our modest sample size, we were unable to derive statistical models for predicting survival outcomes in persistent/recurrent LSCC on the basis of specific genetic perturbations. However, it is evident that the recurrent tumors of patients with particularly poor outcomes harbor multiple complex, and potentially targetable, genomic alterations that are attractive candidates for further study.

Surprisingly, we found higher rates of deleterious alterations in *CDKN2A*, *PIK3CA*, and *TP53* in primary, untreated LSCC **(Figure 2).** One possibility for this observation is the existence of a sampling bias within our cohort such that alteration frequencies for these crucial genes in

persistent/recurrent LSCC are falsely lowered. These findings require validation in larger sequencing cohorts. However, our preliminary data suggest that LSCC recurrence and progression may rely on more complex, alternative patterns of dysregulation in cellular growth and cell-cycle control pathways relative to primary LSCC. This unique pathogenetic complexity of persistent/recurrent LSCC likely manifests clinically as resistance to conventional chemotherapy and targeted agents and disease progression and early mortality.

The histone modifier *BAP1* and the DNA mismatch repair gene *MSH2* were both more frequently altered in persistent/recurrent LSCC. *BAP1* was recently shown to induce resistance to RT in HNSCC by modulating homologous recombination mechanisms.[25] Defects in *MSH2* promote microsatellite instability and accumulation of deleterious mutations by interference with nucleotide mismatch repair processes.[26] Aberrations in cellular DNA damage repair pathways may therefore be central to pathogenesis of persistent/recurrent LSCC and are attractive targets for potential therapeutic modulation.

Ultimately, confirmation of unique mutational profiles of primary vs. persistent/recurrent LSCC suggested by our study and other authors is needed in larger cohorts. Similarly, how genomic aberrations in LSCC interact with other aspects of tumor biology (e.g. immune microenvironment) to promote disease recurrence and progression requires further elucidation to improve outcomes in this population.[27]

Our study has several limitations worth noting. Our persistent/recurrent LSCC samples were sequenced from FFPE on a targeted exome, commercially-available sequencing platform. This is in contrast to TCGA, which employed whole-genome and whole-exome sequencing of fresh-frozen primary tumors. Our comparison of mutational rates in primary vs. persistent/recurrent LSCC need to be interpreted in this light. However, several recent studies have demonstrated high (i.e. > 90 %) concordance rates in detection of SNVs and CNVs in coding genomic regions between matched fresh-frozen and FFPE tumor samples.[28-30] Additionally, comparison of amplicon-based, targeted exome sequencing to TCGA dataset is a well-established practice in the HNSCC literature.[11, 13] Thus, we believe that these differences in DNA sequencing methodology do not significantly diminish the validity of our comparisons and conclusions. Second, the relatively small number of persistent/recurrent LSCC samples in our study limits our ability to draw sophisticated statistical conclusions regarding the impact of specific genomic alterations on survival in persistent/recurrent LSCC. Finally, due to limited availability of archived tissue from our patients' primary tumors, we were unable to directly compare gene alteration frequencies in primary vs. persistent/recurrent LSCC within individual patients.

Future studies should prioritize whole-exome and transcriptomic profiling of larger patient cohorts enriched for both locoregional and distant recurrences of LSCC to fully capture the unique mutational profiles of persistent/recurrent LSCC. As in Hedberg et al,[12] continued efforts

to characterize pathogenetic mechanisms of disease recurrence by DNA sequencing of matched primary and recurrent LSCC specimens from individual patients is paramount.

Conclusions

Targeted DNA sequencing of persistent/recurrent LSCC reveals important differences in gene-specific alteration frequencies of these tumors compared to primary, untreated LSCC from TCGA. Our results provide preliminary support for a distinct mutational profile of persistent/recurrent LSCC that requires confirmation in larger cohorts. These data may be leveraged in future precision oncology initiatives to inform patient and mutation-centric targeted interventions in this patient population.[31]

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Table & Figure Captions

TABLE 1. Clinical characteristics of patients with persistent/recurrent LSCC.

* Patient underwent salvage laryngectomy and thorascopic wedge resection of solitary lung metastasis

† Time from date of recurrence to most recent clinical follow-up or death from any cause

FIGURE 1. Mutational profiles of persistent/recurrent LSCC. Each column represents one patient/sequenced tumor. Genes altered in ≥ 1 patient/sequenced tumor shown.

FIGURE 2. Frequency of alterations in eight critical genes differs in primary vs. persistent/recurrent LSCC. P value for comparison (Chi-Square) listed above each gene.

Patient No.	Gender	Age	Smoking Status	Pack Years	Primary Stage	Initial Treatment	Recurrence Site	DFI (months)	Recurrence Treatment	Survival Months†	Died of Disease
1	M	72	Former	40	III	CRT	Larynx	10	Surgery	104	No
2	M	48	Current	70	IV	RT	Larynx	4	Surgery	37	No
3	M	64	Former	120	II	CRT	Larynx	21	Surgery	87	No
4	M	62	Former	20	III	CRT	Larynx	28	Surgery	16	Yes
5	M	59	Current	80	IV	Surgery	Neoglottis, Lung	118	Surgery*	102	No
6	M	68	Former	20	IV	CRT	Larynx	63	None	4	Yes
7	M	65	Former	63	I	RT	Larynx	9	Surgery	98	No
8	M	52	Former	50	IV	CRT	Larynx	10	Surgery	67	Yes
9	M	68	Current	80	II	CRT	Larynx	4	Surgery	9	No
10	M	58	Current	30	I	RT	Larynx	13	Surgery	12	Yes
11	M	40	Current	20	II	RT	Larynx	71	Surgery	55	No
12	M	52	Current	40	IV	CRT	Tongue Base	50	Surgery	7	Yes
13	M	57	Former	40	II	RT	Larynx	3	Surgery	114	No
14	M	61	Current	45	IV	CRT	Diffuse	4	Palliative RT	3	Yes
15	M	63	Former	180	IV	CRT	Larynx	9	Surgery	80	No
16	M	75	Former	30	I	RT	Larynx	16	Surgery	52	No
17	M	50	Former	30	III	RT	Larynx	3	Surgery	10	No
18	M	53	Current	35	IV	CRT	Neck	9	Surgery	6	Yes
19	M	51	Former	35	I	RT	Larynx	10	Surgery	88	No
20	M	43	Current	20	II	Surgery	Larynx	150	RT	44	Yes
21	M	77	Former	30	I	RT	Larynx	8	Surgery	44	Yes