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Research Article **Genetics** **Oncology**

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RESEARCH ARTICLE

Improved outcomes in PI3K-pathway-altered metastatic HPV oropharyngeal cancer

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Authorship note: RIH and LEMC are co–senior authors and contributed equally to this work.

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Introduction

While a national decline in smoking has led to decreased rates of tobacco-associated head and neck cancer in the United States, cancers arising in the oropharynx (base of tongue and tonsil) are increasing (1, 2). Causally related to human papillomavirus (HPV) infection, it is now well established that HPV-associated oropharyngeal cancer (OPC) often presents in young men without traditional risk factors (3). And while treatment algorithms historically did not account for HPV, prognostic outcomes in general are much more favorable when compared with non-HPV head and neck cancers (4–7). A separate staging system has been employed (8) and treatment paradigms are now shifting to recognize this unique biologic entity, with standard approaches incorporating radiation, chemotherapy, and/or surgery aimed at deintensification (9) to minimize treatment toxicity but preserve excellent outcomes in this generally curable population.

Previous reports have characterized HPV OPC distant failures as often encompassing atypical anatomic sites and yielding a more disseminated phenotype (10, 11). It is now appreciated that the natural oncologic history of advanced HPV OPC is distinct, where distant metastases are the dominant pattern of failure, and time to distant failure can often be measured in years. In addition, compared with their non-HPV counterparts, patients with metastatic HPV OPC often experience improved survival, as has been established in the curable disease setting (12, 13).

In 2015, The Cancer Genome Atlas (TCGA) reported genome-wide profiling results of head and neck cancer, of which 36 patients (15%) were HPV positive (14). Activating mutations in *PIK3CA*, loss of *TRAF3*, and amplifications in *E2F1* all pointed towards NF-κB and cell cycle pathway alterations as integral to HPV OPC oncogenesis — separate from tobacco-associated cancers, which often demonstrate *TP53* loss and inactivating mutations in *CDKN2A*. Around the same time, Seiwert and colleagues reported massively parallel sequencing data on 51 HPV OPC patients showing enrichment for somatic alterations in

both *PIK3CA* and *NOTCH1*, as well as in fibroblast growth factor receptor (*FGFR*) genes (15). While these efforts have begun to define the molecular landscape of HPV OPC, these samples were selected largely from cohorts treated with definitive locoregional therapies.

Roughly 10%–15% of HPV OPC patients develop distant, metastatic disease either at presentation or recurrence and are deemed incurable (16). The molecular landscape that defines this HPV subgroup remains unexplored: What genomic alterations distinguish curable and noncurable HPV OPC tumors? Do molecular biomarkers exist that could identify those likely to develop distant disease, or predict the timing and location of metastatic involvement? Here, we present the largest molecularly characterized series of metastatic HPV OPC to begin to understand molecular predictors with clinical and prognostic significance.

Results

Clinical characteristics of the cohort. Among the entire cohort, nearly all patients (50/52, 96%) were middle-aged (median age 56) men and most (29/52, 56%) were self-reported never smokers (Table 1). Fifty patients (96%) had evidence of p16 overexpression by IHC with positive confirmatory testing (PCR or ISH) in 41 (79%) cases. Both p16-negative cases in the cohort had positive confirmatory PCR results for HPV. All patients had evidence of metastatic disease: 25 (48%) with solely pulmonary metastases and 27 (52%) with at least one extrapulmonary site of metastatic disease (Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.122799DS1). No clinically significant differences were observed between those patients with pulmonary versus extrapulmonary sites of metastatic disease with regards to clinical features.

Survival outcomes. With a median follow-up time of 12 months, there were 28 deaths among the cohort. Median overall survival (OS) was 60 months (95% CI: 47.5–72.3) for the entire HPV metastatic cohort, with a 1-year and 5-year OS of 96% and 47%, respectively (Figure 1). Median time to recurrent disease (TTR) was 12 months (range 0–75) for the entire cohort, which was similar regardless of metastatic site of disease (11.5 pulmonary [P] vs. 14.0 extrapulmonary [EP], $P = 0.92$). Among clinical variables, younger age at initial diagnosis of HPV OPC was associated with a decreased risk of death (hazard ratio [HR] 0.94, 95% CI: 0.85–0.96, $P < 0.01$). In addition, pulmonary involvement as the sole site of distant metastatic spread portended worse outcomes (HR 5.54, 95% CI: 2.44–8.15, $P = 0.02$) (Supplemental Table 2). Similarly, metastatic HPV OPC patients with any degree of extrapulmonary disease demonstrated improved survival (HR 0.37, 95% CI: 0.20–0.88, $P = 0.02$). However, a history of smoking was not associated with worse outcomes in either multiple regression or survival analyses at this cohort size.

Molecular insights. Forty-two (81%) patients had targeted massively parallel sequencing data available for analysis: 16 (38%) samples were obtained from a site of locoregionally recurrent or persistent disease, whereas 26 (62%) were obtained from metastatic foci. We first determined total mutational burden (TMB) among the metastatic HPV OPC cohort and its impact on outcomes. Normalized TMB ranged from 0 to 26.5 mutations/Mb (median 5.3). Median TMB was similar regardless of metastatic site(s) of involvement (6.1 P vs. 5 EP, $P = 0.53$). As expected, smokers had higher TMBs (6.8 smokers vs. 3.7 nonsmokers, $P = 0.02$). In multiple regression analysis a higher TMB correlated with a trend towards improved outcomes (HR 0.88, 95% CI: 0.78–0.93, $P = 0.04$). Additionally, patients with a TMB less than 5 mutations/Mb had a median OS of 26 months compared with 47 months for those with greater than 10 mutations/Mb ($P = 0.02$).

We next sought to characterize the tumor mutational landscape unique to metastatic HPV OPC. Nonexclusive somatic alterations in *KMT2D* (19%) and *PIK3CA* (17%; all E542K or E545K amino acid substitutions resulting in activating mutations) were most frequent among our metastatic HPV cohort (Figure 2). When comparing commonly mutated genes among HPV$^+$ oropharyngeal cancers as reported in TCGA–Pan Cancer Atlas (TCGA-PCA) ($n = 72$) and the University of Chicago ($n = 51$) cohorts there was mutational overlap with a dominance of *PIK3CA* alterations among all 3 cohorts (17%–28%), but an important finding emerged; *PRKDC* alterations occurred at a higher frequency among metastatic HPV OPC patients compared with all sequenced HPV OPC patients (14% vs. 2%, $P = 9.7 \times 10^{-4}$), although this did not reach statistical significance using Bonferroni's correction for multiple testing.

With an overall similar molecular landscape observed between metastatic and nonmetastatic HPV patients, we further sought to investigate genomic biomarkers among individuals based on their pattern of metastatic spread (Figure 3). Patients with pulmonary-only metastases ($n = 20$) had similar mutational profiles compared with all patients in the cohort, with an increased frequency of *KMT2D* alterations (30%) ($P = 0.08$). Of interest,

Table 1. Demographics, clinical and survival characteristics of HPV⁺ oropharyngeal cancer patients with metastatic disease

Characteristic	All (%)[A], *n* = 52	Pulmonary Metastases (%), *n* = 25	Extrapulmonary Metastases (%), *n* = 27	*P* value[B]
Age (median, yr)	56 (32–75)	56 (43–75)	56 (32–72)	–
Gender				
Male	50 (96)	23 (92)	27 (100)	0.486
Female	2 (4)	2 (8)	0	
Smoking history				
Never or <10 pack-year	29 (56)	15 (60)	14 (52)	0.349
Former or current (≥10 pack-year)	23 (44)	10 (40)	13 (48)	
ECOG[C] performance status				
0 to 1	36 (69)	15 (60)	21 (78)	0.165
2 or greater	16 (31)	10 (40)	6 (22)	
Primary site of disease				
Tonsil	26 (50)	15 (60)	11 (41)	0.110
Base of tongue	20 (38)	6 (24)	14 (52)	
Unknown	6 (12)	4 (16)	2 (7)	
Initial staging at diagnosis[D]				
Stage I, II	1 (2)	1 (4)	0	0.934
Stage III, IV	51 (98)	24 (96)	27 (100)	
HPV status[E]				
p16⁺ by immunohistochemistry	50 (96)	24 (96)	26 (96)	–
Confirmatory PCR or ISH⁺	41 (79)	19 (76)	22 (81)	
Initial treatment regimen				
Surgery	12 (23)	11 (44)	1 (4)	0.619
Surgery + radiation	1 (2)	1 (4)	0	
Surgery + CRT	1 (2)	0	1 (4)	
Definitive CRT	31 (60)	11 (44)	20 (74)	
Chemotherapy	7 (13)	2 (8)	5 (18)	
Site of recurrence or metastases[F]				
Pulmonary	25 (48)		–	
Extrapulmonary	27 (52)			
Targeted sequencing data[G]				
From the primary tumor	16 (38)	7 (44)	9 (56)	–
From a metastatic focus	26 (62)	13 (50)	13 (50)	
Survival outcomes				
Number of deaths	28 (54)	14	14	
Median OS (95% CI)	60.0 (47.5–72.3)	29.0 (24.4–33.4)	61.0 (58.7–68.6)	0.02
1-year OS (95% CI)	96% (91.9–98.1)	96% (78.1–98.2)	95% (86.9–99.2)	
5-year OS (95% CI)	47% (38.9–51.6)	26% (13.2–28.3)	56% (42.1–63.5)	
Time to recurrence (range)	12.0 (0–75)	11.5 (3–75)	14.0 (0–52)	0.92
Median follow-up (range)	12.0 (0–62)	11.5 (0–44)	13.5 (0–62)	

[A]Except for age. [B]$P < 0.05$ (Fisher's exact test for categorical variables, log-rank testing based on hazard ratios for survival data, and Mann-Whitney test for time to recurrence). [C]Eastern Cooperative Oncology Group. [D]American Joint Committee on Cancer (AJCC) 7th edition staging. [E]HPV status was determined by p16 immunohistochemistry and confirmatory PCR or ISH testing (2 patients were negative for p16 IHC but had positive confirmatory testing). [F]Eight patients with pulmonary and 6 patients with extrapulmonary metastases also had evidence of coexisting locoregional disease. [G]Total of 42 patients. HPV, human papillomavirus; PCR, polymerase chain reaction; ISH, in situ hybridization; CRT, concurrent chemoradiation; OS, overall survival.

CYLD mutations were observed only in metastatic foci biopsied among the pulmonary-only cohort — a gene encoding a protein involved in ubiquitination and regulation of nuclear factor-κB (NF-κB). In the extrapulmonary metastatic subgroup ($n = 22$), mutational frequencies of genes were, in general, similar compared with the entire cohort.

We next examined whether the presence of certain somatic alterations appeared to impact survival or recurrence (Figure 4). We separated metastatic HPV patients by length of survival from diagnosis (less than 2 years, 2–5 years, or more than 5 years) and sought to identify any outliers in mutational frequency; *PIK3CA* mutations occurred in 33% of patients living 5 or more years from diagnosis. Similarly,

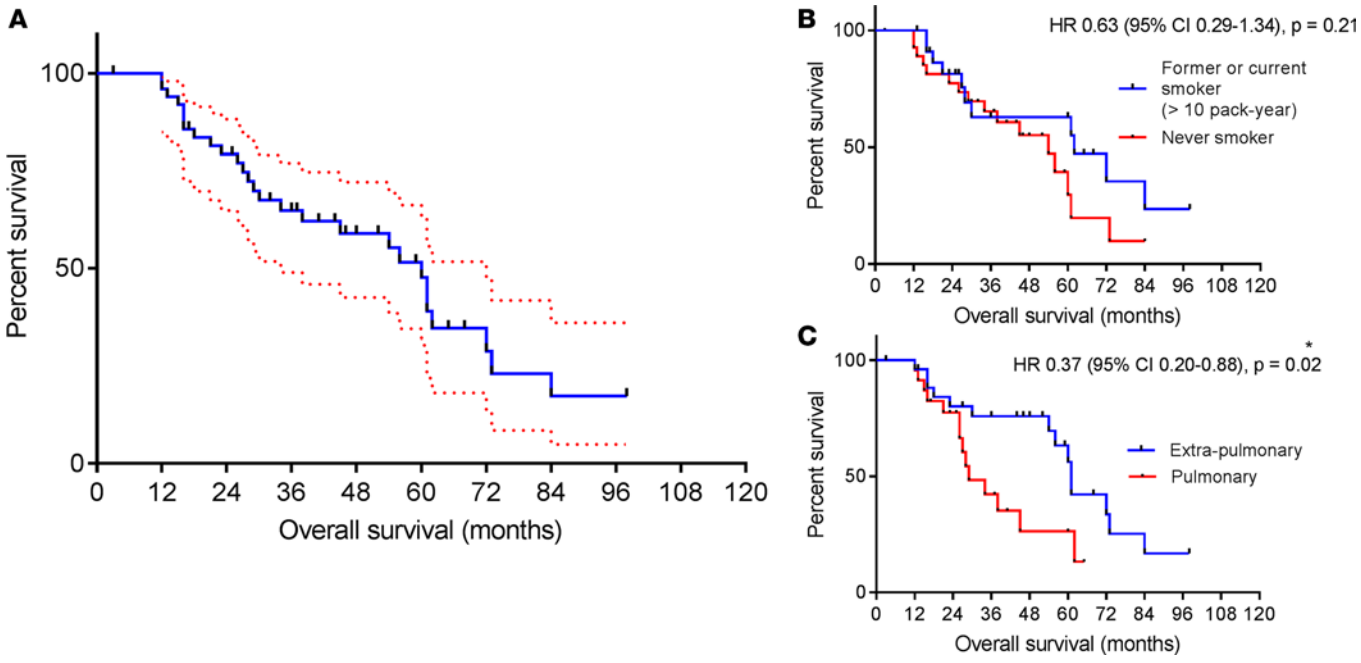

Figure 1. Survival outcomes in patients with metastatic HPV-associated oropharyngeal cancer. (**A**) Overall survival (in months) among n = 52 patients with metastatic oropharyngeal cancer causally related to human papillomavirus (HPV). Dotted lines represent 95% confidence intervals. Overall survival among metastatic HPV-associated oropharyngeal cancer patients based on (**B**) smoking history, and (**C**) metastatic site of disease. The pulmonary subgroup represents those with lung involvement as their only known site of distant disease, while the extrapulmonary subgroup signifies the patient had at least one site of involvement outside the lungs. HR, hazard ratio; CI, confidence interval. *P < 0.05, log-rank testing.

MTOR mutations were only observed in patients experiencing survival beyond 5 years (22%). Of note, the total number of PI3K signaling alterations between our metastatic HPV cohort and prior studies was similar (16/42 vs. 25/87, P = 0.28). In fact, survival was significantly improved in patients with any aberration in the PI3K pathway (HR 0.26, 95% CI: 0.10–0.69, P < 0.01).

A median of 40 gene-level copy-number alteration events occurred per sample (range: 0–185), with a median of 12.3% of the interrogated genome being copy-number altered (range: 0%–39.1%) among the cohort. Among our sequenced metastatic HPV$^+$ patients, recurrent copy-number alterations included single-copy deletions of *ATM*, *KMT2A*, *SDHD* (on 11q), and amplifications of *SOX2* (on 3q) — with each occurring in 60% of sequenced cases (Figure 5). Copy-number events were similar in patients regardless of site(s) of distant disease (36 P vs. 42 EP, P = 0.98), as was the percentage of the genome that was copy-number altered (14% P vs. 11% EP, P = 0.21). In comparison to the TCGA-PCA data among HPV$^+$ patients there was no difference in percentage of the genome that was copy-number altered (22.1% vs. 16.5%, sequenced HPV$^+$ metastatic cohort vs. TCGA-PCA HPV$^+$ patients, respectively; P = 0.54). Eighteen (18/42, 43%) patients had *PIK3CA* gene amplification, but only 1 of these cases was concurrently PI3K pathway mutated. No recurrent rearrangements were identified among the cohort, although 6 cases had evidence of structural variation in individual gene loci.

We then screened our HPV$^+$ cohort for established mutational signatures (17–19); none had evidence of mismatch-repair (MMR) deficiency (or high homopolymer indel counts), although APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide–like), and tobacco signatures were detected in a few cases.

Discussion

With a rising incidence in North America and newer generations composed of fewer smokers, attention has shifted towards HPV OPC (2, 20) — a distinct clinical and biologic entity among head and neck cancers. In an era of molecular characterization of cancer and large-scale sequencing efforts to understand genomic drivers and nominate actionable therapeutic targets, the molecular features that distinguish curable HPV OPC tumors from those destined for metastatic spread remain elusive. To that end, we present the largest clinically annotated cohort to date of metastatic HPV OPC patients with genomic profiling data integrated with survival

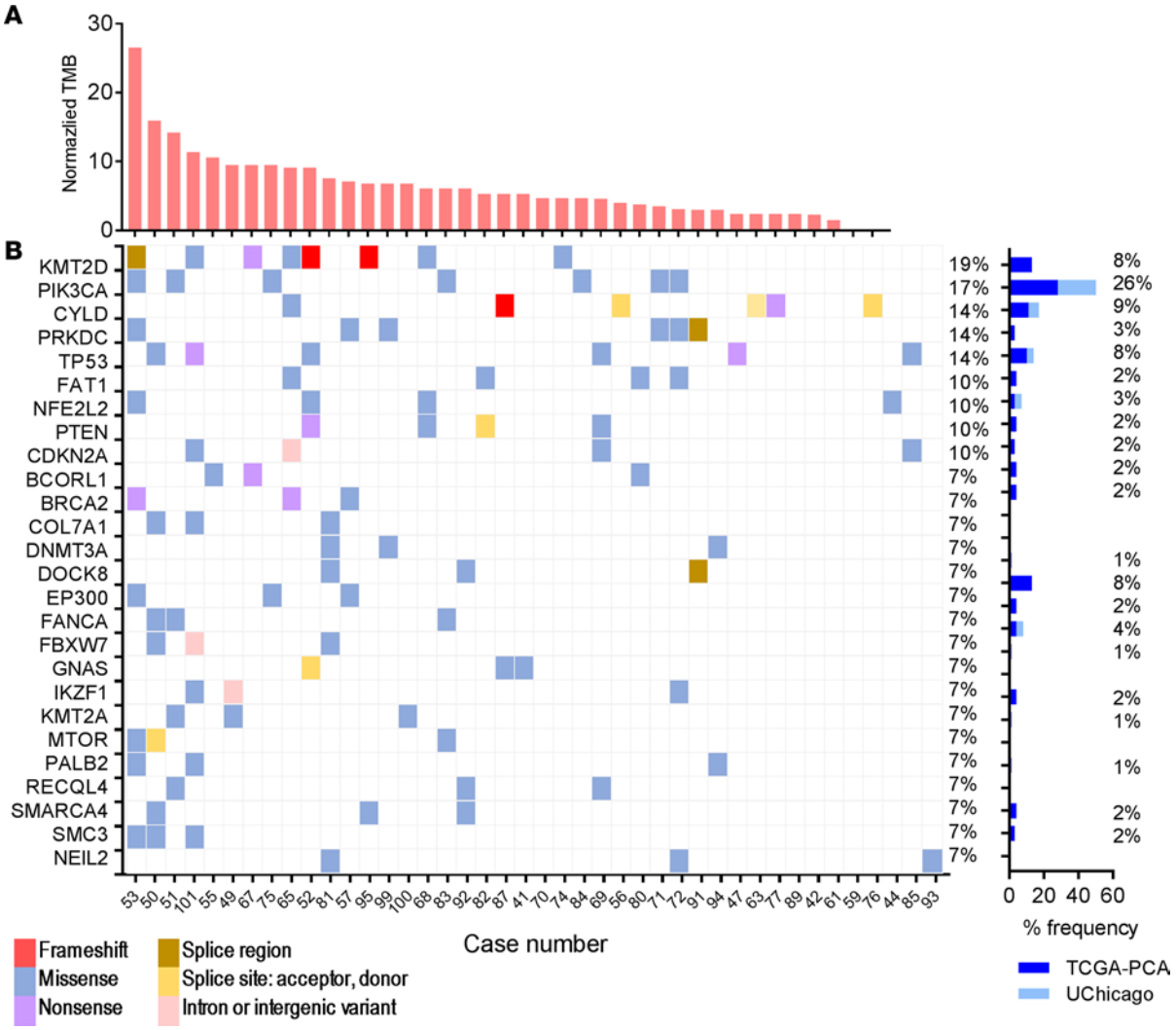

Figure 2. Mutational landscape of metastatic HPV-associated oropharyngeal cancer. (**A**) Normalized total mutational burden (TMB) including all nonsynonymous gene alterations per sample (n = 39). (**B**) Mutational plot showing the most frequently mutated genes (top to bottom, >5% frequency) with gene frequency listed at right (%) among 42 metastatic HPV$^+$ oropharyngeal tumors. The vertical bar graph shows mutational frequency compared with whole-exome sequencing results among n = 72 patients in the The Cancer Genome Atlas–Pan Cancer Atlas (TCGA-PCA) and n = 51 in the University of Chicago cohorts with nonmetastatic HPV-associated head and neck cancer. Gene frequency (%) is also shown among both comparator cohorts. Significance evaluated by Bonferroni-corrected 2-sided χ^2 test.

outcomes. The demographics of our metastatic HPV OPC cohort are as expected, given the epidemiology of this increasingly recognized entity: largely affecting middle-aged men often lacking conventional risk factors.

The generally favorable prognosis of HPV OPC has been documented over the last decade, yet upwards of 25% of HPV OPC tumors will recur within 2 years and up to 36% at 8 years (21). However, it has been observed that HPV remains a favorable prognostic association even when disease progression ensues (5). Fakhry and colleagues reported on 105 p16$^+$ OPC patients with disease progression following definitive therapy showing improved 2-year OS compared with non-HPV patients (55% vs. 28%, $P < 0.001$). We show superior outcomes in our metastatic HPV OPC cohort with 2-year OS at 79% (95% CI: 65%–88%), but the Fakhry cohort included more patients (55%) with locoregionally persistent disease. Further corroborating our findings, retrospective analyses of 2 large Eastern Cooperative Oncology Group (ECOG) trials, 1395 and 3301, showed improved median OS among patients with recurrent, metastatic HPV-associated disease compared with their non-HPV counterparts (12.9 vs. 6.7 months, $P = 0.014$) (13). Our median OS of 60 months and excellent 1- to 2-year survival outcomes may reflect that our population was managed at an academic center (64% of our cohort was treated on protocol during their disease management; 78% received an immune checkpoint inhibitor on or off protocol).

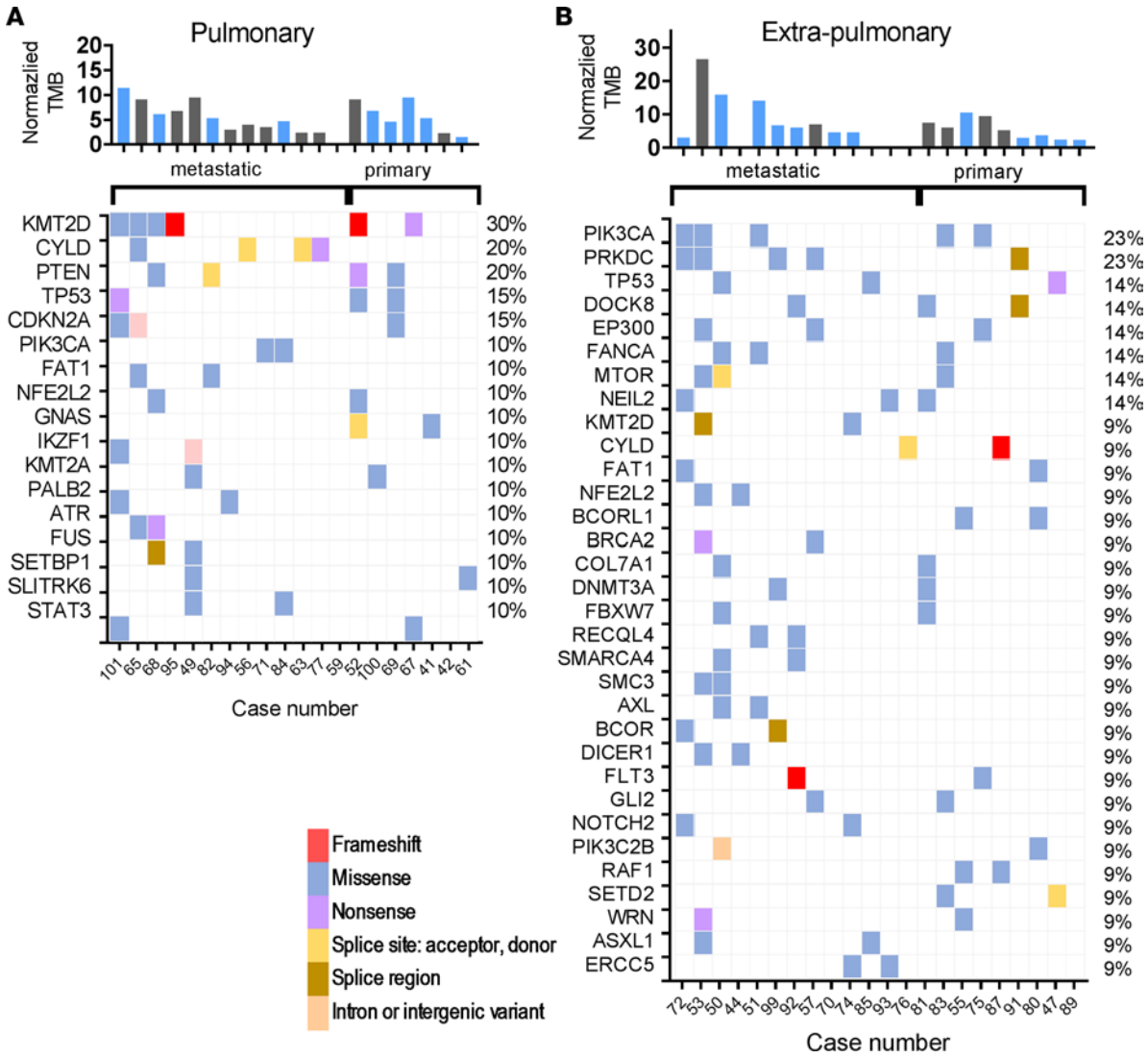

Figure 3. Mutational landscape in metastatic HPV-associated oropharyngeal cancer by site of disease. (**A**) Mutational plot showing the most frequently altered genes (top to bottom) among metastatic HPV$^+$ tumors with only pulmonary metastases (n = 20), and in those with (**B**) extrapulmonary sites of metastatic disease (n = 22). Normalized total mutational burden (TMB) is shown in the bar graph above. Smoking status is depicted by colored shading in the bar graph: blue represents never smokers, dark gray represents former (>10 pack-years) or current smokers. Each column represents an individual patient sample and they are arranged according to where the biopsy material for massively parallel sequencing was obtained (primary site of disease or metastatic focus).

Many have proposed that metastatic HPV OPC involves distinct anatomic locations and is associated with a delayed time to the development of distant metastases (22). We show a relative balance between pulmonary and extrapulmonary sites (hepatic, 10; osseous, 13; dermal or soft tissue, 1; central nervous system, 4; other, 6) of metastatic spread with no clinically significant differences identified among these subgroups. Our reported median TTR of 12 months is in line with prior studies showing usual progression within the first 1–3 years after completing therapy (4–6, 23). We clarify further that the site of metastatic spread appears to be independent of time to progression. Importantly, we did observe that pulmonary involvement as the sole site of metastatic spread predicted worse outcomes (HR 5.54, P = 0.02), which likely relates to the tendency for vital pulmonary function to progressively decline earlier in the natural history of disease. Conversely, our metastatic HPV subgroup with extrapulmonary disease experienced improved median survival (61 vs. 29 months, P = 0.02).

In evaluating the molecular landscape of metastatic HPV tumors, we first assessed TMB, a measure of total mutation events in the coded genome, as it holds significant promise as a biomarker for response to immunotherapy (24). Our own work has recently shown that TMB is lower among virally mediated recurrent head

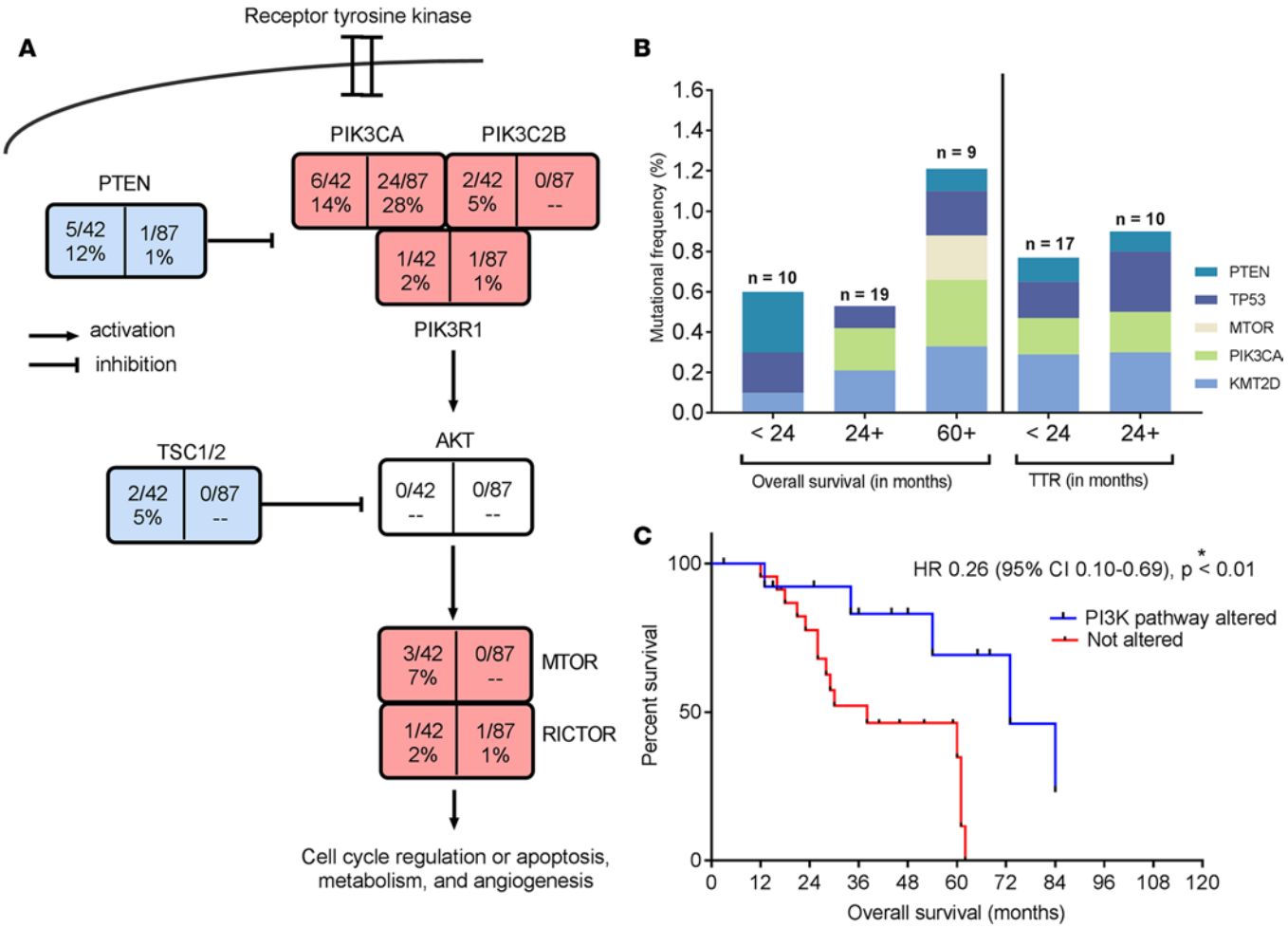

Figure 4. Key signaling pathway deregulation and survival outcomes in metastatic HPV-associated oropharyngeal cancer. (**A**) Mutational signaling of the PI3K/MTOR/AKT pathway and its dysregulation in HPV⁺ oropharyngeal carcinoma. Each box represents the mutational frequency of gene(s) associated with a particular pathway protein. Somatic alteration frequencies among a metastatic HPV⁺ oropharyngeal cancer cohort (left, *n* = 42) and the TCGA and University of Chicago HPV⁺ oropharyngeal cancer cohorts (right, *n* = 87) are shown. Blue shading represents a dominance of inactivating mutations and red shading, activating mutations. (**B**) Key mutational frequencies among subgroups arranged by overall survival (OS) and time to recurrence (TTR), in months. Mutational frequencies (%) are not mutually exclusive and therefore columns total greater than 100% in some cases. (**C**) OS among metastatic HPV⁺ oropharyngeal cancer patients separated by PI3K pathway alteration status. **P* < 0.05, log-rank testing.

and neck tumors compared with non-HPV tumors (4.7 vs. 8.2 mutations/Mb, $P < 0.01$). In our current study restricted to metastatic HPV patients, median TMB was similar (5.3 mutations/Mb) regardless of metastatic disease site(s). One possible explanation is that the overall lower TMB in HPV⁺ patients reflects a dominance of virus-specific proteins. Of particular interest was the finding in multivariate analysis that higher TMB correlated with improved survival (HR 0.88, $P = 0.04$). The association between high TMB and improved outcomes in the current study may reflect immunogenic potential — whereby high TMB facilitates neoantigen recognition and a resulting immunologic response to attack the tumor. But independent of TMB, HPV⁺ patients appear to have higher response rates to immune checkpoint blockade (24–26).

When comparing the mutational landscape of our metastatic HPV cohort to existing genomically characterized series of patients ($n = 123$) with a diagnosis of HPV OPC (14, 15), we found overall similar mutational profiling results — and it should be noted that 62% of our cohort biopsies were from metastatic foci. There was a trend towards more frequent *PRKDC* alterations — a protein kinase associated with chromosomal instability by regulating DNA double-strand-break repair pathways (27) —among our metastatic HPV⁺ cohort, perhaps suggesting that pharmacological inhibitors of the enzyme poly-ADP ribose polymerase (PARP) might be useful in this disease. In further separating metastatic HPV⁺ patients with pulmonary-only and extrapulmonary disease, mutational profiling again revealed similar results. We did

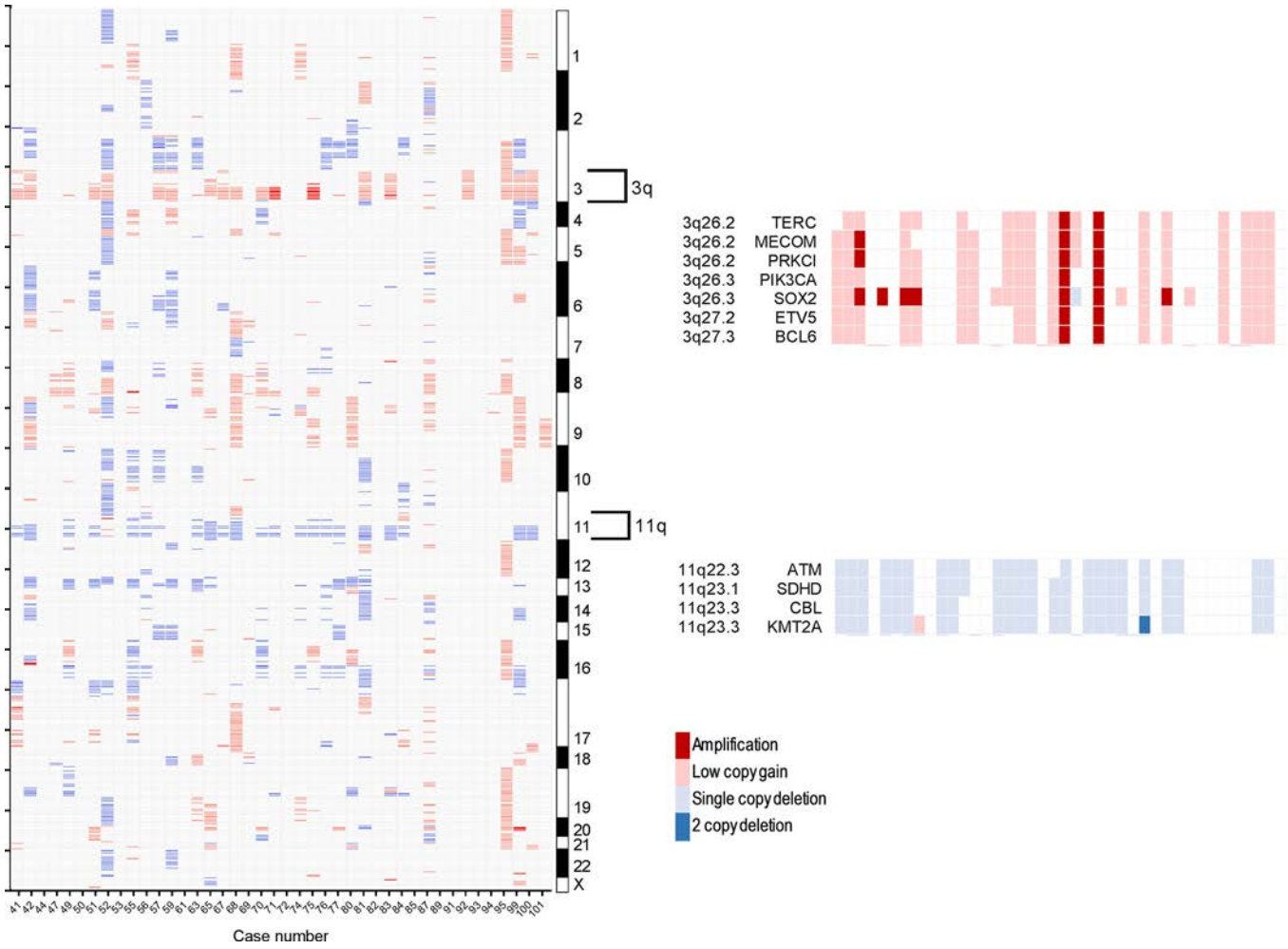

Figure 5. Copy-number alterations in metastatic HPV-associated oropharyngeal cancer. Copy-number alterations among metastatic HPV$^+$ oropharyngeal tumors (n = 42) arranged by chromosomal band loci (left). Each column represents an individual tumor and corresponding chromosomal gene loci are arranged from top to bottom. Color shading indicates areas of amplification (red) or low copy gain (pink) versus single (light blue) and 2-copy (dark blue) gene deletion. Shown to the right in more detail are regions of recurrent alterations in 3q and 11q with their corresponding gene and genetic loci depicted.

observe higher rates of *PRKDC* alteration among patients with extrapulmonary metastatic foci. Although speculative, this gene encodes DNA-dependent protein kinase, catalytic subunit (DNA-PKcs), which is important in DNA damage signaling and repair. Partner proteins of DNA-PKcs have been shown to be involved in cell-cell adhesion, thus supporting their role in cancer metastasis (28). DNA-PKcs controls protein secretion within the tumor environment, which may promote angiogenesis (29). Subsequently, hepatic and bone metastases were common in *PRKDC*-altered patients in our cohort, metastatic sites that are rich in vascular networks. Perhaps more interesting was the observation that tumors with any alteration in the PI3K pathway were associated with improved survival ($P < 0.01$). Importantly, none of these patients were treated with a PI3K pathway inhibitor on or off protocol in the metastatic setting. These findings would suggest that not only are PI3K pathway alterations more common in HPV-related oropharyngeal tumors, but their presence affords prognostic benefit in the metastatic setting. This finding is notable given that PI3K pathway activity has been linked to mechanisms of radioresistance in the upfront disease setting (30), and others have shown an association between PI3K pathway aberrations and advanced staging (31). This becomes all the more relevant in that this pathway has actionable therapeutic targets. A recent study reported on the use of paclitaxel with an oral pan-PI3K inhibitor (buparlisib) or placebo in previously treated, advanced or metastatic head and neck squamous cell carcinoma patients (32). Acknowledging the limited sample size of 17 (17/158, 11%) patients with HPV$^+$ disease in the paclitaxel-buparlisib arm, there was no significant benefit in terms of response or survival in this subgroup.

Our study has some notable limitations; capturing disease heterogeneity with tumor biopsies is always a challenge given that not all patients have matched primary and metastatic biopsies from the same time point in their disease course. That said, more than 60% of our cohort yielded metastatic disease site biopsies and we show comparable genomic findings between sites of disease. Matched primary and metastatic tumor sites in the same patient would be ideal for comparison, but were not routinely available. Using targeted next-generation sequencing as opposed to whole-exome analysis has obvious limitations, but our institutional panel has proven informational and representative in multiple prior genome-wide analyses. Finally, we acknowledge that our findings are largely observational and warrant further validation.

In conclusion, we present the largest genomically profiled cohort of metastatic HPV OPC patients to date, with over 80% of the population having sequencing data available for review. We show favorable survival outcomes compared with historical non-HPV, advanced head and neck cancer populations and further associate pulmonary-only metastases with worse outcomes. We demonstrate subtle differences in the mutational landscape between curable and metastatic HPV OPC populations, with a trend towards more frequent DNA repair protein alterations in the latter. Moreover, we found that PI3K pathway alterations are associated with improved survival among metastatic HPV OPC patients. These findings taken together provide what we believe are novel molecular insights for this important HPV^+ subgroup, and have significant implications for therapeutic intervention.

Methods

Study cohort. Fifty-two patients with metastatic HPV OPC who received treatment at the Dana-Farber/Harvard Cancer Center (DF/HCC) from 2011 to 2018 were identified retrospectively following institutional review board approval. HPV status was confirmed in all patients using p16 IHC followed by confirmatory testing with either PCR or in situ hybridization (ISH). Carcinoma of unknown primary (CUP) patients with HPV confirmed cervical neck adenopathy were permitted (n = 6). Among 52 patients, 42 (81%) had fresh or archival tumor material available for targeted massively parallel sequencing. Biopsy material was obtained from either the primary tumor (prior to initial therapy but before documented metastatic spread) or from a metastatic focus. Patient demographics, clinicopathologic features, and treatment outcomes were recorded.

OS was determined from the date of initial diagnosis of HPV OPC to death from any cause, otherwise censored at date of last known follow-up. Duration of response (time to recurrence) was defined as time from documentation of tumor response to disease progression at the primary site or diagnosis of distant metastatic spread, whichever occurred first.

Targeted massively parallel sequencing. All sequenced patients separately consented to our institutional Cancer Research Study (33), and molecular testing was performed in a CLIA-certified laboratory. Hematoxylin and eosin–stained slides were reviewed by a pathologist to identify areas of greater than 20% tumor for molecular analysis. As previously described, DNA was isolated using standard methods with a kit (Qiagen) followed by quantification (Qubit dsDNA detection, Invitrogen). DNA (50–200 ng) was fragmented ultrasonically (Covaris), size selected, and quantified. Dual-indexed sequencing libraries were prepared using KAPA HTP library preparation kits (Roche) on 50 ng DNA. Libraries were prepared and hybridized to a custom biotinylated RNA bait set (Agilent SureSelect) targeting the full coding regions of 300 genes plus selected intronic regions of 35 genes (OncoPanel version 2 ; ref. 34); or targeting 447 genes and selected intronic regions of 60 genes (OncoPanel version 3; ref. 24). Hybrid-capture libraries were sequenced on an Illumina HiSeq 2500 using 2 × 100 paired-end reads.

The mean sequencing coverage for cases was 318× unique, high-quality, mapped reads per sample (range 80× to 640×; 50× minimum required to pass quality control thresholds). Genomic data (vcf files) are included in a publicly available database (AACR Project GENIE). Users can access the data directly via cbioportal (http://www.cbioportal.org/genie), or download data from Sage Bionetworks (https://www.synapse.org).

As described previously (32, 34), a custom bioinformatics pipeline was used for data analysis. In brief, pooled sequencing reads were deconvoluted and sorted using Picard tools, followed by alignment using BWA to reference sequence b37 edition from the Human Genome Reference Consortium. Localized realignment around indel sites and recalibration of quality scores was performed using the Genome Analysis Toolkit (GATK, version 3.3.0). Mutation analysis for single-nucleotide variants was performed using MuTect v. 1 0.27200; variant calls were annotated by Variant Effect Predictor (VEP) v79. Paired germline samples were not sequenced; for each sequencing run, non-neoplastic samples were included as controls. Variants identified in these control samples were filtered as sequencing artifacts. Additional informatics

steps were taken to identify common SNPs that were not filtered by the analysis pipeline: SNPs present at greater than 0.1% frequency in Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP) (http://evs.gs.washington.edu/EVS/, accessed May 30, 2013) or present in dbSNP were filtered; variants that were also present twice or more in COSMIC were rescued for review. For copy-number analysis, a custom internal R-based tool (RobustCNV) was used to calculate the fractional coverage of genomic intervals compared with the median fractional coverage obtained in a panel of 152 FFPE normal samples. Structural variant analysis was performed using BreaKmer (35) followed by review of reads in integrative genomics viewer (IGV). TMB was calculated by determining the number of nonsynonymous somatic mutations that occur per megabase of exonic sequence data across all genes on the panel.

Statistics. Fisher's exact test was utilized to compare categorical data for the likelihood of observed differences with respect to clinical features between metastatic subgroups. Multiple regression analysis utilized the Cox proportional hazards model (if $n \geq 15$ patients were available in each clinical subgroup) and proportional hazards assumption testing was verified. Pearson's χ^2 test was used to compare categorical data for the likelihood of observed differences with regards to the presence of individual mutations among metastatic subgroups. Spearman's ρ was used to evaluate correlation. A Mann-Whitney U test to compare ranks (or Kruskal-Wallis test or 1-way ANOVA on ranks for multiple comparisons) was utilized to analyze continuous genomic data among HPV subgroups. All statistical tests used a significance cutoff of less than 0.05 and were 2-sided. Bonferroni's correction ($\alpha = [0.05/k]$) was applied to adjust the α value, accounting for multiple testing among genomic subgroups. Kaplan-Meier statistics were applied using log-rank testing to evaluate outcome data. Data were analyzed using Stata/IC (version 14.2).

Study approval. Written informed consent for existing institutional review board-approved protocols (DF/HCC) was received from all participants prior to inclusion in the study.

Author contributions

GJH, RIH, and LEM conceived and designed the project. GJH, AK, NGC, JHL, RU, and LEM conducted experiments and acquired data. GJH, AK, PS, RU, and LEM analyzed and interpreted the data. GJH, AK, and LEM drafted the manuscript. All authors reviewed and revised the manuscript.

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