Targeted molecular characterization of external auditory canal squamous cell carcinomas

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Abstract
Hypothesis: Squamous cell carcinomas (SCC) of the external auditory canal (EAC) may harbor unique genomic alterations that may explain aggressive behavior and differentiate these tumors from cutaneous SCCs of other subsites.

Background: EAC SCCs arise in a non-ultraviolet-exposed region of the head and neck, are often locally aggressive and may metastasize to lymph nodes or distant sites. The genomic alterations underlying cutaneous SCC of other sites are well-documented; however, mutational profiles of EAC SCC are less well characterized and may contribute to the unique anatomic site, high rates of recurrence and tumor spread. We performed targeted sequencing of a cohort of primary EAC SCCs to identify recurring and potentially targetable genomic alterations.

Methods: Genomic DNA was extracted from formalin-fixed paraffin-embedded specimens of 7 EAC SCCs and subjected to targeted DNA sequencing using a 227-gene panel. Somatic alterations and gene copy number alterations were annotated using our validated, in-house bioinformatics pipelines.

Results: In our EAC SCCs, we found recurrent alterations in TP53 and genes of receptor tyrosine kinase (eg, EGFR, FGFR) and PI3K pathways (eg, PIK3CA), similar to cutaneous SCCs of other head and neck sites. We also observed a high frequency of telomerase reverse transcriptase amplification and DNA methyltransferase 1 alterations, both of which are rarely observed in cutaneous SCCs of other sites.

Conclusion: These data represent the first step toward precise molecular characterization of EAC SCCs that may lead to an enhanced understanding of tumor biology and modernized precision medicine approaches for unique tumors.

Level of Evidence: NA

Keywords
DNMT1, external auditory canal (EAC), PI3K, squamous cell carcinoma (SCC), TERT
Primary squamous cell carcinoma (SCC) of the external auditory canal (EAC) is rare. Typically, cutaneous SCC in the periauricular region arises from the skin of the pinna or adjacent scalp. Overall, primary cutaneous malignancies of the EAC proper are rare, and comprise approximately <1% of all head and neck (HN) malignancies annually. Cutaneous malignancies of the EAC proper are rare, and comprise approximately <1% of all head and neck (HN) malignancies annually. (6/1 000 000).1,2 Patients with primary EAC SCC may present with a variety of symptoms including otalgia, bloody otorrhea, sudden or progressive hearing loss, facial weakness or paralysis, vertigo or chronic imbalance and even gross tumor burden or metastasis within the parotid bed, ipsilateral neck or emanating from the meatus of the EAC.3 Because these tumors sporadically arise in non-ultraviolet-exposed regions of the body, early detection can be challenging. Moreover, EAC SCC may not necessarily present in those with fair skin or light complexion typically considered at risk for cutaneous malignancies. While highly aggressive, primary EAC SCC does not typically metastasize to distant sites unless there is local spread to the adjacent external ear/pinna, parotid gland, or postauricular lymph nodes.

Surgical resection is the primary means of treatment of EAC SCC. Lateral temporal bone resection (LTBR) with or without concurrent parotidectomy and selective neck dissection (SND) is designed to remove the primary tumor en bloc as well as to microscopically stage the disease and determine the need for postoperative chemo- and/or radiation therapy.4,6 While the histologic diagnosis of EAC SCC is straightforward, precise identification of genomic features that may contribute to the particularly aggressive nature of these tumors is unclear. Molecular markers may improve prognostic significance and when combined with surgical staging (Pittsburgh classification), may provide insight into optimal management of these rare and aggressive tumors.7 Given the location of the tumor and the aggressive and rapid growth rates, neoplasms may already be advanced at the time of eventual diagnosis.8 Despite advances in the management of temporal bone malignancies, staging and prognostic predictors for tumors remain elusive.

While rare in prevalence, mounting evidence has identified several genes that are mutated that may relate to the aggressive nature of EAC SCC. For example, mutation and expression dysregulation in CDKN2A/p16, TP53, epidermal growth factor receptor (EGFR), pSTAT3, and relaxin-2 (RLN2) have been reported in EAC SCC.7 As such, overexpression of p53 and EGFR may be important biomarkers for identifying EAC SCC with high-risk features including lymph node metastasis. Recently, Wei et al.9 sequenced a single whole exome of EAC SCC and identified several significantly mutated cancer genes including CTNNB1 and vascular endothelial growth factor receptor 2 (VEGFR-2). VEGFR-2 functions as the main mediator of VEGF-induced angiogenesis in a variety of cancers. Overexpression of this gene in EAC SCC might serve as a potential target for therapy.

In the present study, we screened gene mutation profiles of seven primary EAC SCC tumors following LTBR. DNA extracted from formalin-fixed paraffin-embedded (FFPE) samples was subjected to targeted sequencing of an internally designed, 227-gene panel comprising genes mutated at >1% frequency in the HN SCC Cancer Genome Atlas Network (HNSCC TCGA) cohort.9 We chose to sequence these 227 genes owing to their prognostic and potentially therapeutic significance in both mucosal and cutaneous SCCs of the HN.10

Overall, our analysis identified several novel molecular alterations in our EAC SCC tumors, many of which are commonly seen in other SCCs found in the HN. The recurrent alterations disrupted TP53, NOTCH, receptor tyrosine kinase, and PI3K pathways, which are established drivers of SCCs. Of note, we observed a high frequency of telomerase reverse transcriptase (hTERT) amplifications and DNMT1 alterations suggesting a more prevalent role for these genes in EAC SCCs than SCCs of other HN subsites. The data extend the understanding of gene mutation in carcinogenesis and identify clinically relevant targets in EAC SCC.

2 | MATERIALS AND METHODS

We retrospectively queried the electronic medical record for patients meeting the following inclusion criteria: (a) pathologically confirmed cutaneous SCC of the EAC proper; (b) primary, untreated disease; (c) surgical management with LTBR ± superficial parotidectomy and/or SND followed by adjuvant therapy as indicated; and (d) FFPE specimens containing adequate tumor and adjacent normal tissue for targeted, next-generation sequencing. In total, seven patients met these criteria, and demographics, clinical characteristics, and survival data are shown in Table 1. EAC SCC tumors were staged according to the Pittsburgh Staging System.11,12 Participants provided informed consent and this study was approved by the University of Michigan IRB (HUM00080561).

2.1 | Clinical material and targeted exome sequencing

FFPE tissue blocks and matched hematoxylin and eosin-stained slides for all seven patients were assessed by an expert HN pathologist for area of highest-quality tumor and matched, adjacent normal tissue. Punch cores of tumor and adjacent normal were taken, followed by genomic DNA isolation and purification (DNAstorm FFPE Kit, Cell Data Sciences Cat# CD502) and quantification with Qubit (Qiagen), as described.13,14 DNA that met our quality control standards was used to prepare sequencing libraries with the Rubicon Genomics ThruPLEX kit (Cat# R400674) according to the manufacturer’s protocol. We performed a custom capture using a bait set manufactured by Nextera consisting of 227 genes, which represented genes mutated at >1% frequency in the original HNSCC TCGA project9 and high-density probes for HPV16 using the Nextera Rapid Capture Custom Enrichment Kit (Catalogue #: FC-140-1008). The genes targeted in this panel are listed in Supplemental Table 1. Custom capture libraries were then pooled and sequenced at an average of 96 targeted exomes per lane on an Illumina HiSeq 4000 platform with paired-end 150-nt sequencing.
2.2 | Targeted exome variant calling

The quality of the sequencing reads was first determined using FastQC v.0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/, RRID:SCR_014583). Trim galore v0.4.5 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/, RRID:SCR_011847) was used to trim reads containing sequencing adapters and it was not deemed necessary to trim the reads further as previously described by our team.15,16 The processed reads were then aligned to the hg19 reference genome using BWA v0.7.15 (http://bio-bwa.sourceforge.net/, RRID:SCR_010910). We used PicardTools v2.4.1 (http://broadinstitute.github.io/picard/, RRID:SCR_006525) to sort, deduplicate, and index the mapped reads. Base quality score recalibration was then performed using GATK v3.6 (https://software.broadinstitute.org/gatk/, RRID:SCR_001876) to generate clean aligned reads for variant calling. For each tumor-normal pair in the set, we used Samtools v1.9 (http://htslib.org/, RRID:SCR_002105) to create pileup files. Next, Varscan v2.4.1 (http://tvap.genome.wustl.edu/tools/varscan/, RRID:SCR_006849) was used to call variants from these mpileup files using the somatic mode of the variant caller. Variant calls were annotated using Goldex Helix Varseq v2.1.0 (http://goldenhelix.com/, RRID:SCR_012191). This was followed by filtering the variants in the introns and intergenic regions. Variants with a minimum of five reads supporting the alternate allele in the tumor samples were considered as potential positives, as described.17

2.3 | Copy number analysis

Copy number (CN) estimation calls from the preprocessed tumor-normal BAM files were made using Aberration Detection in Tumour Exome (ADTEx) v.2.0 (http://sourceforge.net/projects/adtex/, RRID:SCR_012059) as described.13 The software assigns five CN states from 0 to 4 based on its estimated CN. State 0 represents a homozygous deletion, state 1 corresponds to a heterozygous deletion, normal CN is denoted by state 2, while states 3 and 4 correspond to a copy gain and amplification, respectively. Representative Manhattan plots for each chromosome of each tumor/normal pair were made and a R script v3.4.0 (http://www.r-project.org/, RRID:SCR_001905) was used to annotate genes associated with each change.

3 | RESULTS

3.1 | Sequencing characteristics

From this cohort, we successfully performed targeted capture sequencing on our seven EAC SCC samples using our custom gene panel. Sequencing yielded an average of 6 704 111 total mapped reads per tumor, of which an average of 91.3% were uniquely mapped to the human genome (Supplemental Table 2). Of the 227 genes analyzed in each tumor, we identified an average of 8.7 somatic...
mutations and 1.5 INDELs per tumor (Figure 1) as well as several CN alterations to recurrently altered pathways, which are highlighted below (Figure 2).

3.2 | TP53 pathway alterations

Disruptive TP53 molecular alterations are common in both mucosal and cutaneous SCC of the HN, occurring in >70% of HPV-negative disease.9,10,18 We similarly observed a high rate of TP53 alterations with individually, EAC-SCC4 harboring a TP53 stop-gain Trp53Ter (Chr17:7579528, C to T), EAC-SCC5 containing a TP53 Glu294Ter (Chr17:7577058, C to A), and EAC-SCC6 containing a TP53 Trp146Ter (Chr17:7578492, CC to TT). Similarly, we observed a single TP53 copy loss in three of the tumors including EAC-SCC2, CN = 1, EAC-SCC3, CN = 1, and EAC-SCC6, CN = 1. Interestingly, EAC-SCC4 had three copies of TP53 (although one copy was mutated), but we also observed an MDM4 amplification in this tumor. This suggests that in this particular case the effects of the TP53 amplification event may have been offset by amplification of the negative regulator of p53 protein expression. Nonetheless, TP53 disruptive alterations are likely an important oncogenic event for the pathogenesis of EAC tumors.

3.3 | Receptor tyrosine kinase alterations

The EGFR is a well-established oncogene in mucosal and cutaneous HN SCC and has been found to be overexpressed in >80% of tumors at the protein level, albeit by unknown genetic mechanisms. Here, we found that EAC-SCC4 harbored an EGFR Asp994Asn (Chr7:55268914, G to A) and several additional tumors harbored EGFR amplifications (EAC-SCC3, CN = 4; EAC-SCC4, CN = 3; EAC-SCC6, CN = 3; and EAC-SCC7, CN = 3), supporting a pivotal role for EGFR in EAC SCC. Additionally, we identified several additional receptor tyrosine kinases with recurrent CN amplifications in our sample set, including FGFR1 (EAC-SCC5, CN = 4; and EAC-SCC7, CN = 3), FGFR3 (EAC-SCC2, CN = 1, and EAC-SCC3, CN = 3), FLT3 (EAC-SCC2, CN = 3) and FLT4 (EAC-SCC3, CN = 3; and EAC-SCC4, CN = 3). These data are consistent with SCCs of other HN anatomic sites supporting a role for FGF/FGFR and FLT3/4 signaling in a subset of tumors.

3.4 | PI3K/RAS/RAF pathway alterations

Activating alterations to the PI3K signaling pathway are the most common activating oncogenic pathway in the mucosal HNSCC project, and thus we hypothesized that EAC tumors would contain a similarly high frequency of activating PI3K pathway alterations. Indeed, we observed CN alterations predicted to activate the pathway in several tumors including BRAF gain (EAC-SCC7, CN = 3), NRAS gain (EAC-SCC4, CN = 3), PTEN loss (EAC-SCC2, CN = 1), PIK3CA gain (EAC-SCC4, CN = 3; EAC-SCC7, CN = 3), PIK3CB gain (EAC-SCC6, CN = 3; and EAC-SCC7, CN = 3), PIK3CD gain (EAC-SCC4, CN = 3), and PIK3CG gain (EAC-SCC3, CN = 3; EAC-SCC6, CN = 3; and EAC-SCC7, CN = 3). Additionally, we observed several mutations of unknown consequence in this pathway including PIK3R1 Glu212Asp (Chr5:67576357, A to T) in EAC-SCC5, PIK3CG Pro262Ser (Chr7:106508790, C to T) in EAC-SCC6 and YAP1 Arg58Cys (Chr11:101981751, C to T) in EAC-SCC3.

3.5 | Notch pathway alterations

The Notch pathway has been reported to be both activated and inactivated in mucosal and cutaneous HN SCC.9,10,18 In the tumors profiled in this study, we surprisingly found three tumors with NOTCH gene amplifications (EAC-SCC4, NOTCH2, CN = 3; EAC-SCC5, NOTCH3, CN = 3; and EAC-SCC6, NOTCH4, CN = 3).
NOTCH1, CN = 3; and EAC-SCC6, NOTCH1, CN = 4) suggesting an activated state of NOTCH signaling in these tumors. We also identified three tumors with NOTCH mutations of unknown consequence including EAC-SCC3 which contained a NOTCH2 5' UTR insertion (NM_024408.3:c-29_-21dupCGGCGGCGG), EAC-SCC6 with a NOTCH2 Ser1467Phe (Chr1:120468039, G to A), EAC-SCC7, which contained a NOTCH3 Gly1154Glu (Chr19:15290093, C to T) and NOTCH4 Gly1151Ser (Chr6:32170157 C to T). Collectively, these data demonstrates the importance of NOTCH signaling in EAC SCC and suggests that genetic alterations that activate NOTCH signaling may be prevalent in this disease.

### 3.6 | TERT alterations

Immortalization requires disruption of molecular processes to maintain telomere ends, which often occurs through de-regulation of TERT. While TERT amplification is a relatively rare event in mucosal and cutaneous SCC, occurring in only 7% of samples in the HNSCC TCGA project, we identified three EAC tumors with TERT amplifications: EAC-SCC3, CN = 3, EAC-SCC4, CN = 3, and EAC-SCC6, CN = 4, suggesting that this genomic event may be common in this disease.

### 3.7 | DNMT1 alterations

DNA methyltransferase 1 (DNMT1) is altered in <3% of mucosal and cutaneous HN SCC. The gene is known to play a pivotal role in maintenance of the tumor epigenome. Here, surprisingly, we found that three of the tumors we profiled contained alterations in DNMT1, including a copy loss in EAC-SCC2 (CN = 1), and somatic mutations in EAC-SCC5, which contained a Asp329Asn (Chr19:10273366, C to T), and EAC-SCC6, which had Pro325Leu (Chr19:10273377, G to A). The mutations occur prior to the cytosine specific DNA methyltransferase replication foci domain (amino acids: 400-533) in the protein but are adjacent to two mutations identified in this gene in the HNSCC TCGA data suggesting a functional role for these alterations. Despite this, given the relatively low frequency of DNMT1 alterations in mucosal and cutaneous SCC of other HN subsites, this gene has not yet been explored as a driver of HNSCC pathogenesis but may play a critical role in EAC tumors.

### 4 | DISCUSSION

In this study, we sought to characterize the recurring molecular alterations underlying EAC SCC carcinogenesis and progression. We hypothesized that these tumors may harbor unique mutations that explain their genesis in an ultraviolet-shielded anatomic location and their local aggressiveness and propensity for spread. Overall, our analysis identified several potentially targetable genetic alterations in EAC SCCs. These included disruptions to TP53, Notch and PI3K pathways, and receptor tyrosine kinase (eg, EGFR, FGFR) signaling characteristic of cutaneous SCCs of other sites. TERT upregulation, through amplification, promotor mutation, and/or epigenetic modification, is a known driver of cellular immortalization and malignant transformation via stabilization of chromosomal telomeres. DNMT1 inactivation, as seen in three of our tumors, is similarly emerging as a critical event in chromatin instability and epigenetic dysregulation across several human malignancies. TERT
amplification and DNMT1 inactivation are quite rare in mucosal and cutaneous SCCs of other HN subsites, suggesting a potentially unique tumor biology of EAC SCCs frequently relying on telomere maintenance and epigenetic dysregulation for carcinogenesis and progression. Validation of the alteration frequency of TERT and DNMT1 in larger EAC SCC cohorts is crucial, especially as targeted therapies directed at these pathways begin to enter into clinical trials.

Much like cutaneous and mucosal SCCs of other HN sites, EAC SCCs demonstrate recurring and complex alterations in Notch, PI3K/Ras/Raf, and EGFR signaling. Notch pathway signaling is complex and variably upregulated or downregulated in a context-dependent fashion in cutaneous and mucosal SCCs. In our cohort, NOTCH receptor amplification occurred in three of seven tumors, suggesting that upregulation of oncogenic Notch signaling may predominate in EAC SCCs. Similarly, we saw nearly universal amplification of PI3K/Ras/Raf pathway signaling in our tumors, solidifying these oncogenes as prime candidates for development of targeted therapies in EAC SCCs.

At present, there is no universally accepted staging system for SCCs of the EAC and temporal bone. The Pittsburgh classification and the Stell and McCormick staging systems are the two most widely applied, yet their discriminatory and prognostic capacity and clinical utility are limited. Additionally, these systems are based solely on clinical and radiographic variables. Genetic biomarkers predictive of invasion, metastasis and treatment response and prognostic of survival, would likely greatly improve staging of EAC SCCs. While our modest cohort limits correlations of specific genetic alterations with staging and survival, it does posit several specific alterations for future validation as predictive and prognostic biomarkers for these tumors.

The main limitation of our study is the small number of tumors sequenced. The role of specific genetic alterations showed herein (eg, TERT amplification, DNMT1 mutation, Notch upregulation) should be validated and further explored in larger cohorts that may span multiple treatment centers given the rarity of these tumors. Importantly, our preliminary data supports the use of therapies targeting common drivers in EAC SCCs, such as EGFR inhibitors (ie, cetuximab), PI3K inhibitors and emerging therapeutic approaches for NOTCH-disrupted tumors. The relatively small size of the tumors prevented more comprehensive exome/transcriptome sequencing approaches, thus motivating our decision to focus on a smaller subset of SCC related genes for this analysis. Despite these limitations, we developed the genetics data related to this rare tumor subset, which motivates further evaluation of existing and emerging therapeutics targeting common SCC-related genes in this subset of disease. In the future, precision guided approaches targeting these common alterations may have clinical benefit for patients with this debilitating disease.

These data represent an initial step in the molecular characterization of EAC SCCs that may lead to modernized precision medicine approaches for this disease.

5 | CONCLUSION

EAC SCC is a rare, but aggressive disease which poses challenges in diagnosis, tumor staging, and treatment. Molecular characterization of these tumors is an initial step toward identifying driver mutations and potential molecular treatment targets. This investigation identified disruptions to TP53, Notch and PI3K pathways, receptor tyrosine kinase signaling, TERT amplifications, and alterations to DNMT1, which may be prevalent and potentially targetable in EAC SCC. Further investigation with larger tumor cohorts and across institutions is an important next step.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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