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Targeted Molecular Characterization of External Auditory Canal Squamous Cell Carcinomas

Running Title: Targeted Sequencing of EAC SCC

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Abstract (250 words)

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 (FFPE) specimens of seven 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 (e.g., *EGFR, FGFR)* and PI3K pathways (e.g., *PIK3CA*), similar to cutaneous SCCs of other head and neck sites. We also observed a high frequency of *TERT* (telomerase reverse transcriptase) amplification and *DNMT1* (DNA Methyltransferase 1) alterations, both of which are rarely observed in cutaneous SCCs of other sites.

Conclusion

This data represents 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.

Keywords: External auditory canal (EAC), squamous cell carcinoma (SCC), PI3K, TERT, DNMT1

Level of Evidence: NA

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Introduction

Primary squamous cell carcinoma (SCC) of the external auditory canal (EAC) is rare. Typically, cutaneous SCC in the peri-auricular 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 (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 post auricular 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 is designed to remove the primary tumor *en bloc* as well as to microscopically stage the disease and determine the need for post-operative 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

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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. Over-expression 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 head and neck 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 head and neck[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 head and neck. 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 *TERT* amplifications and *DNMT1* alterations suggesting a more prevalent role for these genes in EAC SCCs than SCCs of other head and neck subsites. The data extend the understanding of gene mutation in carcinogenesis and identify clinically relevant targets in EAC SCC.

Materials and Methods

We retrospectively queried the electronic medical record for patients meeting the following inclusion criteria: (1) pathologically confirmed cutaneous SCC of the EAC proper; (2) primary, untreated disease; (3) surgical management with LTBR ± superficial parotidectomy (SP) and/or selective neck dissection (SND) followed by adjuvant therapy as indicated; and (4) FFPE specimens containing adequate tumor and adjacent normal tissue for targeted, next-generation sequencing. In total, seven patients met these criteria, and

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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).

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 head and neck 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 project[9] 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.

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, de-duplicate, 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,

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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 5 reads supporting the alternate allele in the tumor samples were considered as potential positives, as described[17].

Copy Number Analysis

Copy number estimation calls from the pre-processed 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 copy number states from 0 to 4 based on its estimated copy number. State 0 represents a homozygous deletion, state 1 corresponds to a heterozygous deletion, normal copy number 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.

Results

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 copy number (CN) alterations to recurrently altered pathways, which are highlighted below (Figure 2).

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TP53 Pathway Alterations

Disruptive *TP53* molecular alterations are common in both mucosal and cutaneous SCC of the head and neck, 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.

Receptor Tyrosine Kinase Alterations

The epidermal growth factor receptor (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.

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

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high frequency of activating PI3K pathway alterations. Indeed, we observed copy number 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.

Notch Pathway Alterations

The Notch pathway has been reported to be both activated and inactivated in mucosal and cutaneous HNSCC[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, *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, this 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.

TERT Alterations

Immortalization requires disruption of molecular processes to maintain telomere ends, which often occurs through de-regulation of *TERT*[19]. 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.

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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[20]. 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.

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 (e.g. *EGFR*, *FGFR*) signaling characteristic of cutaneous SCCs of other sites[10, 18].

Our tumor analysis also revealed a high frequency of telomerase reverse transcriptase (*TERT*) amplifications and alterations to DNA methyltransferase 1 (*DNMT1*). *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[21]. *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[22]. *TERT* amplification and *DNMT1* inactivation are quite rare

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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[9-10]. 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[23-24].

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[25]. 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[26].

At present, there is no universally accepted staging system for SCCs of the EAC and temporal bone. The Pittsburgh classification[11] and the Stell and McCormick[27] 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 (e.g., *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 (i.e., cetuximab), PI3K inhibitors and emerging therapeutic approaches for NOTCH-disrupted tumors[25]. 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

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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.

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

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 signalizing, *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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Table and Figure Legends

TABLE 1. Clinical, demographic, and survival characteristics of our EAC SCC patient cohort.

† According to Pittsburgh Staging System

‡ LTBR: lateral temporal bone resection; SP: superficial parotidectomy, SND: selective neck dissection

FIGURE 1. Frequency distribution of high-confidence somatic mutation and copy number alteration calls (A), and frequently mutated genes (B) in our seven EAC SCC tumors.

FIGURE 2. Genes with recurrent copy number alterations across all seven EAC SCC tumors.

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Supporting Information

Supplemental Table 1.

List of the 227 genes used in our targeted sequencing panel representing genes mutated at >1% frequency in the original HNSCC TCGA project[9]

Supplemental Table 2. Total number of reads per tumor, % mapped, and % uniquely mapped to the human genome. (T = tumor; N= normal)

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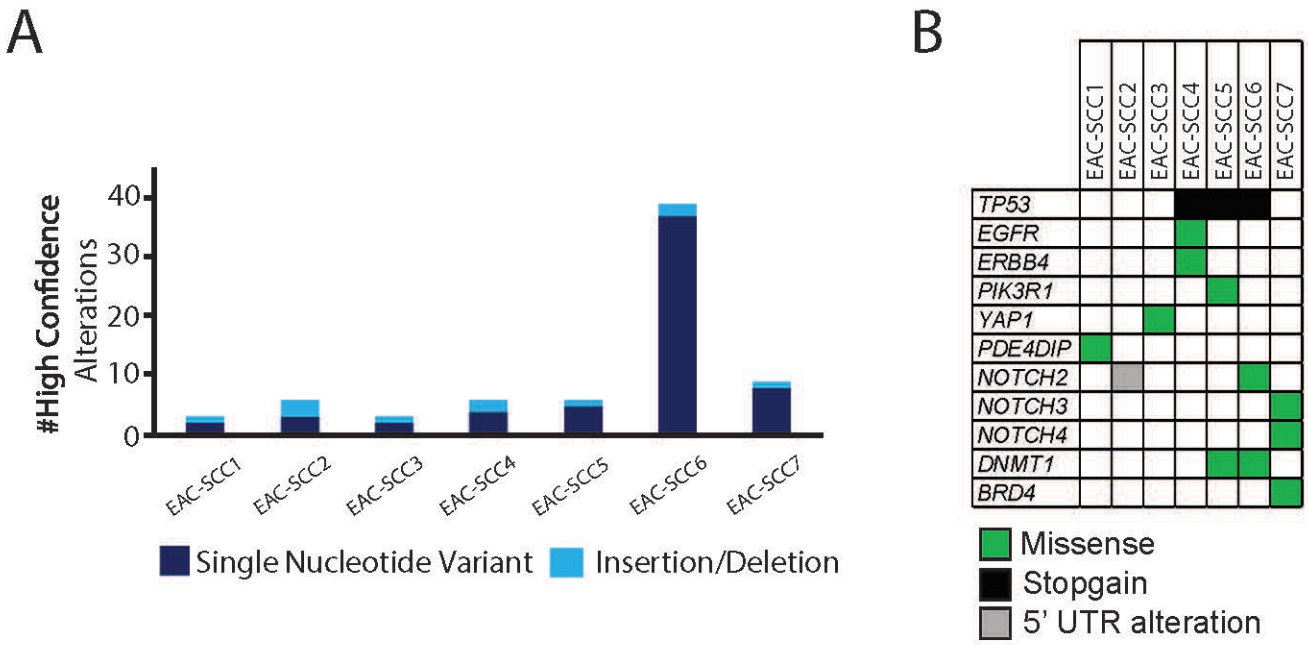

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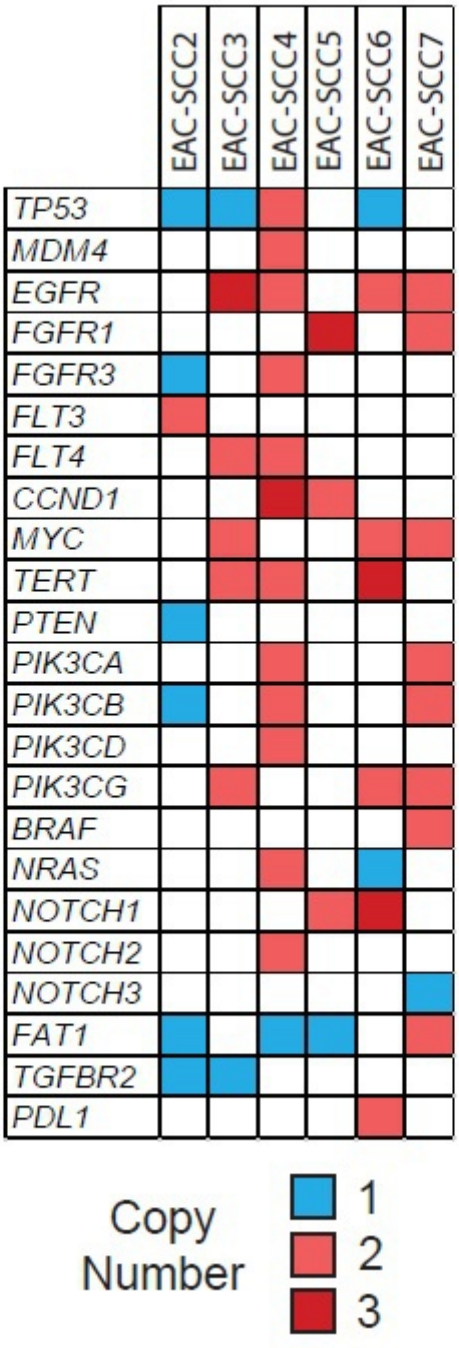

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	EAC-SCC1	EAC-SCC2	EAC-SCC3	EAC-SCC4	EAC-SCC5	EAC-SCC6	EAC-SCC7
Age - Yr	71.5	54.6	86.1	58.7	79.2	84.9	73.0
Sex	Male	Female	Male	Female	Male	Male	Male
ACE-27 Index	1	0	1	1	1	1	3
Immunosuppression	No	No	No	No	No	No	No
Previous Non-Melanoma Skin Cancer	No	Yes	No	No	Yes	Yes	Yes
Ear Affected	Left	Left	Left	Left	Left	Left	Left
T/N Stage†	3/0	2/0	2/1	3/0	2/0	4/0	2/1
Overall Stage†	III	II	IV	III	II	IV	IV
Operation Performed‡	LTBR, SP, SND	LTBR, SP, SND	LTBR, SP, SND	LTBR, SP, SND	LTBR	LTBR	LTBR, SP, SND
Adjuvant Treatment	RT	None	RT	CRT	RT	None	RT
Surgical Margins	Negative	Negative	Positive	Negative	Negative	Positive	Positive
Differentiation	Well	Well	Moderate	Moderate	Poor	Poor	Well
Perineural Invasion	Negative	Negative	Negative	Negative	Negative	Negative	Positive
Bone Invasion	Negative	Negative	Negative	Positive	Positive	Negative	Negative
Angiolymphatic Invasion	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Duration of Follow-Up (months)	56.6	54.9	57.4	19.6	37.1	9.8	4.3
Recurrent Disease	No	No	No	Yes - Local	No	Yes - Persistent Unresectable Disease	Yes - Local & Distant
Died of Disease	No	No	No	Yes	No	Yes	Yes