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Whole-Exome Sequencing of Salivary Gland Mucoepidermoid Carcinoma

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Abstract

Purpose—Mucoepidermoid carcinoma (MEC) is the most common salivary gland malignancy. To explore the genetic origins of MEC, we performed systematic genomic analyses of these tumors.

Experimental Design—Whole-exome sequencing and gene copy number analyses were performed for 18 primary cancers with matched normal tissue. Fluorescence in situ hybridization (FISH) was used to determine the presence or absence of the *MECT1-MAML2* translocation in 17 tumors.

Results—*TP53* was the most commonly mutated gene in MEC (28%), and mutations were found only in intermediate- and high-grade tumors. Tumors with *TP53* mutations had more mutations overall than tumors without *TP53* mutations (p=0.006). *POU6F2* was the second most frequently mutated gene, found in three low-grade MECs with the same in-frame deletion. Somatic alterations in *IRAK1, MAP3K9, ITGAL, ERBB4, OTOGL, KMT2C, and OBSCN* were identified in at least two of the 18 tumors sequenced. FISH analysis confirmed the presence of the *MECT1-MAML2* translocation in 15 of 17 tumors (88%).

Conclusions—Through these integrated genomic analyses, *MECT1-MAML2* translocation and somatic *TP53* and *POU6F2* mutations appear to be the main drivers of mucoepidermoid carcinoma.

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Keywords

Whole-exome sequencing; Mucoepidermoid carcinoma; Salivary gland cancer; Translocation

Introduction

Mucoepidermoid carcinoma (MEC) is the most common malignant neoplasm arising from the salivary gland(1). MEC is characterized by its cellular heterogeneity and consists of mucin-producing, epidermoid, and intermediate cells. These tumors are also known for their variable biological and clinical behavior(2). Several histologic grading systems have been proposed, which classify MEC tumors into low, intermediate, and high grades and have been shown to correlate with clinical outcome. However, issues with consistency and reproducibility in histologic grading still exist(3, 4). Furthermore, systemic treatment options remain limited, necessitating a deeper understanding of the molecular underpinnings of this cancer.

One important genetic aberration in MEC is the translocation of chromosomes 11q and 19p, which has been proposed as an early event in the pathogenesis of the disease(5, 6). This translocation, reported in over 50% of MEC tumors(7), results in fusion of the *MECT1* and *MAML2* genes, forming a fusion protein that causes disruption of cell cycle regulation and differentiation(8). Low-grade tumors have a higher incidence of the fusion compared to high-grade tumors(9), and patients with fusion-positive cancer tend to have improved survival, with significantly lower risk of local recurrence, metastases, or cancer-related mortality(10).

Other genetic alterations have been identified in MEC. For example, copy number variations (CNVs) have been found to occur more frequently in fusion-negative cancers, with potential loss of tumor suppressor genes, such as *DCC, SMAD4, GALR1*, and *CDKN2A/B*, and gain of oncogenes, such as *MAFA, LYN, MOS*, and *PLAG1*(9).

Comprehensive analysis of genetic alterations underlying MEC has not been reported. In order to shed light on the genomic landscape of MEC, we performed whole-exome sequencing and copy number analysis of tumors from 18 patients.

Materials and Methods

Sample preparation

A retrospective review of patients treated for MEC was performed after obtaining approval from the Institutional Review Board of the Johns Hopkins Medical Institutions. Clinical and demographic data including age, gender, tobacco use, primary site, and lymph node involvement were extracted from electronic medical records. Tumors were scored using the Armed Forces Institute of Pathology grading scheme(11).

Formalin-fixed, paraffin-embedded (FFPE) tissues were dissected to achieve a neoplastic cellularity of >60%. DNA was purified from these tumors, as well as matched non-neoplastic tissue adjacent to tumor, using the AllPrep DNA/RNA purification kit (Qiagen,

catalog # 80204) according to the manufacturer's instructions. Extracted DNA was then used to generate libraries suitable for massively parallel sequencing.

DNA sequencing

Sample library construction, next generation sequencing (NGS), and bioinformatic analyses of tumor and normal samples were performed at Personal Genome Diagnostics (Baltimore, MD). In brief, genomic DNA from tumor and normal samples were fragmented and used for Illumina TruSeq library construction (Illumina, San Diego, CA) and captured using the Agilent V4 exome panel per the manufacturers's instructions. Paired-end sequencing, resulting in 100 bases from each end of the fragments, was performed using a HiSeq 2000 Genome Analyzer (Illumina, San Diego, CA).

Somatic mutations were identified using VariantDx(12) custom software for identification of mutations in matched tumor and normal samples. Prior to mutation calling, primary processing of sequence data for both tumor and normal samples was performed using Illumina CASAVA software (v1.8), including masking of adapter sequences. Sequence reads were aligned against the human reference genome (version hg18) using ELAND. Candidate somatic mutations, consisting of point mutations and small (<50bp) insertions and deletions were then identified using VariantDx across the coding exomic regions. VariantDx examines sequence alignments of tumor samples against a matched normal while applying filters to exclude alignment and sequencing artifacts. In brief, an alignment filter was applied to exclude quality failed reads, unpaired reads, and poorly mapped reads in the tumor. A base quality filter was applied to limit inclusion of bases with reported Phred quality scores >30 for the tumor and >20 for the normal (http://www.phrap.com/phred/). A mutation in the tumor was identified as a candidate somatic mutation only when (i) distinct paired reads contained the mutation in the tumor; (ii) the number of distinct paired reads containing a particular mutation in the tumor was at least 10% of read pairs for exome; (iii) the mismatched base was not present in >1% of the reads in the matched normal sample as well as not present in a custom database of common germline variants derived from dbSNP; and (iv) the position was covered in both the tumor and normal. Mutations arising from misplaced genome alignments, including paralogous sequences, were identified and excluded by searching the reference genome. Candidate somatic mutations were further filtered based on gene annotation to identify those occurring in protein coding regions. Functional consequences were predicted using snpEff and a custom database of CCDS, RefSeq and Ensembl annotations using the latest transcript versions available on hg18 from UCSC (https://genome.ucsc.edu/). Predictions were ordered to prefer transcripts with canonical start and stop codons and CCDS or Refseq transcripts over Ensembl when available. Finally, mutations were filtered to exclude intronic and silent changes, while retaining mutations resulting in missense mutations, nonsense mutations, frameshifts, or splice site alterations. A manual visual inspection step was used to further remove artifactual changes. We have optimized our sequencing and bioinformatics approaches in the past so that specificity of mutations is extremely high. This has been extensively validated not only by Sanger sequencing but also by NGS at high depth. A minimum of 95% of the mutations identified using these approaches are bona fide(13, 14). Copy number alterations were identified by comparing normalized average per-base coverage for a particular gene in a

tumor sample to the normalized average per-base coverage in a matched normal sample for that patient(15). Focal amplifications (≥3-fold or six copies) and homozygous deletions were reported.

MECT1-MAML2 translocation

MECT1-MAML2 translocation was determined by fluorescence in situ hybridization (FISH) performed on FFPE sections using a commercially available MAML2 dual-color, break-apart probe (Z-2014-200, Zytovision, Germany). Prior to hybridization the slides were deparaffinized using a VP 2000 processor (Abbott Molecular, Des Plains, IL) in which pretreatment with protease I was used. Following deparaffinization the slides and the MAML2 probe were co-denatured at 80 °C for 7 minutes and allowed to anneal over night at 37 °C in humidified atmosphere. At the end of the incubation the slides were washed in 2 × SSC/0.3% NP-40 for 2 min at 72 °C and for 2 min at room temperature, with agitation. Traces of detergent were removed with a wash in 2 × SSC at room temperature. The slides were counterstained with DAPI and a cover slip was applied using Vectashield mounting medium (H-1000, Vector Laboratories, Inc.).

A fluorescence microscope was used to evaluate the probe pattern. Cells with two fusion signals of one orange and one green fluorochrome were scored as normal. Cells with rearrangements for MAML2 gene had one normal fusion signal and one orange and one green signal at a distance from each other.

Statistics

All statistical analyses were carried out with two sided tests with statistical significance level set at p-value of 0.05. STATA 10 software (Stata Corp, College Station, TX) was used for the analyses.

Results

Whole-exome sequencing was performed for 18 tumors with matched normal tissue as described in the Methods section. The average high quality per base coverage of the normal samples was 101-fold and 174-fold for the tumors, with 90% and 92% of targeted bases represented by at least 10 reads, respectively (Supplementary Table S1).

Somatic mutations

Using stringent criteria for analyses of these data(16), we identified 774 candidate somatic mutations in 705 genes among the 18 tumors (Supplementary Table S2). The range of mutations per tumor was 3 to 242, with a mean and standard deviation of 43 ± 63.5 mutations per tumor (Table 1).

There were differences in the genetic landscapes of tumors based on histologic grade. Intermediate- and high-grade tumors tended to harbor *TP53* mutations (5 out of 9) while low-grade tumors did not have any *TP53* mutations (0 out of 9; p=0.03, Fisher's exact test) (Supplementary Table S3). More C:G>G:C substitutions were seen in intermediate- and

high-grade tumors compared to low-grade tumors (p=0.03, Fisher's exact test) (Supplementary Table S4).

Somatic mutations were identified in at least two tumors in eight different genes (Table 1 and Supplementary Table S2). *TP53* was the most frequently mutated gene, with mutations found in five tumors. Among the five *TP53* mutations, two were frameshift alterations and three were missense mutations (Q331H, G245S, R280T). Tumors harboring *TP53* mutations had significantly more mutations overall than tumors without *TP53* mutations (104.4 ± 99.6 versus 17.8±14.2, p=0.006, Student's t-test). The only two high-grade tumors without known *MECT1-MAML2* had *TP53* mutations. One of these patients, who presented with locally advanced disease and later developed distant metastasis, was found to have a truncating *TP53* mutation (insertion frameshift).

Three recurrent in-frame deletions were observed in *POU6F2;* mutations in this gene were found exclusively in low-grade MEC tumors, but the difference was not statistically significant, potentially due to the limited sample size (p=0.21, Fisher's exact test). Other genes with mutations in more than one tumor included *IRAK1, MAP3K9, ITGAL, ERBB4, OTOGL, KMT2C*, and *OBSCN*. Of these, *MAP3K9* and *OTOGL* mutations were seen only in intermediate- or high-grade tumors.

Several known oncogenes and tumor suppressor genes were noted to harbor mutations in a single tumor each. Mutations were observed in the oncogenes *ARID1A, CBL, ABL1, AR, EPHA5, FH, INSR, PRKDC, RET*, and *HRAS* and the tumor suppressor genes *PBRM1, SMAD2, SMAD3, FBXW7*, and *HNF1A*.

Copy number variation

Copy number analysis revealed 34 copy number variations (CNVs) in nine tumors, of which all but one were focal amplifications. One recurrent CNV occurred in 2 intermediate-grade tumors (tumor samples 17 and 20) with amplification of 17q21.2. One high-grade tumor (tumor sample 29) had amplification of 19q13.2-q13.3. *KLK2* amplification (tumor sample 29) was the only potentially biologically significant gene.

MECT1-MAML2 translocation

Fluorescence in situ hybridization (FISH) was employed to determine the presence or absence of the *MECT1-MAML2* translocation (Supplementary Fig. S1). The translocation was present in 15 of 17 tumors, including all of the low- and intermediate-grade tumors and three out of five high-grade tumors; translocation status could not be determined for one patient with intermediate-grade mucoepidermoid carcinoma (Table 1).

Discussion

Whole-exome sequencing of 18 MEC samples revealed that *TP53* mutation burden differed based on histologic grade of the tumors. The most frequently mutated gene identified through these analyses was *TP53*, though *TP53* mutations were isolated to intermediate- and high-grade tumors. We also identified mutations in *POU6F2, IRAK1, MAK3K9, ITGAL, ERBB4, OTOGL, KMT2C*, and *OBSCN* in more than one tumor. Copy number analyses

demonstrated that most observed CNVs were amplifications. We also confirmed *MECT1-MAML2* translocation in 88% of tumors.

Our analyses found that the most commonly mutated gene in MEC is *TP53*, the most frequently altered gene in human cancer(17). *TP53* mutations have been previously reported in 25–33% of MEC, although only small numbers of MEC cases were included in those studies, which were not performed in an unbiased fashion(18–20). Of the five tumors with *TP53* mutations in our study, three were missense mutations and two were frameshift alterations. *TP53* mutations were identified in only intermediate- or high-grade tumors, consistent with one study that suggested detection of aberrant p53 expression using immunohistochemistry was associated with higher histologic grade. This study also found that aberrant p53 expression was associated with local recurrence(21). Furthermore, we found that the presence of *TP53* mutation was significantly associated with higher mutation frequency overall. However, the clinical implications of these findings are hard to assess given the heterogeneity of the patients. Further research is therefore warranted to investigate possible role of the mutation as a prognostic biomarker.

POU6F2 was the second most frequently mutated gene, with mutations found in three low-grade MECs. All three *POU6F2* mutations were in-frame deletions at the same location (187Q>-) and were found in low-grade MEC tumors without *TP53* mutation. In COSMIC, the same in-frame deletion was seen in twelve specimens from various human cancers, including lung adenocarcinoma, endometroid carcinoma, melanoma, hemangioblastoma, esophageal squamous cell carcinoma, intestinal adenocarcinoma, and Wilms tumor. Among those, *TP53* mutations were seen in only three of twelve specimens. *POU6F2* encodes a member of the POU protein family, characterized by the presence of two DNA binding subdomains (a POU-specific domain and a homeodomain) separated by a variable polylinker. The POU family members are transcriptional regulators, many of which are known to control cell type-specific differentiation pathways(22). *POU6F2* is involved in the development of the pituitary(23) and kidney(24). Loss of heterozygosity in regions containing *POU6F2* has been reported in Wilms tumor(25). Further studies are necessary to elucidate the role of *POU6F2* in human cancers, including MEC.

The other recurrently mutated genes included *IRAK1, MAP3K9, ITGAL, ERBB4, OTOGL, KMT2C*, and *OBSCN*. *MAP3K9*(26) and *ERBB4*(27) encode for protein kinases that have been frequently implicated in human cancers and may be potential therapeutic targets. *IRAK1* encodes interleukin-1 (IL-1) receptor-associated kinase 1, which is a serine/threonine protein kinase that associates with the IL-1 receptor upon stimulation. IRAK1 can mediate stimulation of the NF-κB and MAPK pathway, thereby promoting cell proliferation(28). *ITGAL* encodes the integrin alpha L chain, which combines with the beta 2 chain to form LFA-1, which plays a critical role in cell-cell interactions, cellular adhesion, and cytotoxic T-cell mediated killing(29, 30). *KMT2C* encodes for Mixed Lineage Leukemia 3 (MLL3), which acts as a histone H3 lysine 4 (H3K4)-specific methyltransferase(31). Although mutations in *KMT2C* have been reported in various solid tumors, its role as a true driver of oncogenesis is debated(32, 33). As MLL3 is a coactivator of p53, its inactivation may result in reduced p53 function and subsequent accumulation of genetic damage(34). The role of *OTOGL* and *OBSCN* in MEC tumorigenesis is unclear.

Alterations in genes with potential clinical utility included recurrent mutations in *ERBB4* and isolated mutations in *ARID1A, ABL1, INSR, RET*, and *HRAS*.

Using FISH, we verified the presence of the *MECT1-MAML2* translocation in 88% of tumors, slightly higher than the previously reported range of 38% to 81%(35). Due to limited availability of fresh frozen tumors, we were unable to perform whole-genome sequencing to detect *MECT1-MAML2* or other chromosomal rearrangements. Consistent with the literature, all low- or intermediate-grade MEC tumors in this study were positive for the translocation, while 60% of high-grade tumors were positive(36). *MECT1-MAML2* translocation produces a fusion protein that disrupts Notch signaling through the effects of Maml2, which normally acts as a co-activator of Notch receptors and transactivates Notch target genes(8, 37). By forming a novel Mect1-Maml2 fusion product, Maml2 replaces its Notch ligand binding domain with the *CRTC1* promoter and CREB binding domain, which allows the fusion protein to act as a co-activator of the cAMP/CREB signaling pathway(38, 39). It has been shown that ectopic expression of Mect1-Maml2 may be tumorigenic in rat epithelial cells, and inhibition of the fusion protein using RNA interference can suppress growth in fusion-positive MEC cell lines(40). A recent report suggests that the fusion protein may also interact with Myc and activate Myc transcription targets, including genes involved in cell growth, metabolism, survival, and tumorigenesis(41). These findings, along with lack of significant driver oncogene mutations in MEC, strongly suggest that *MECT1-MAML2* translocation may be the main oncogenic driver in this tumor type. In our analysis of 18 patients, two patients who did not harbor the *MECT1-MAML2* translocation had *TP53* mutations, including a frameshift insertion and a missense mutation in the DNA binding domain (R280T), which provides an alternative mechanism of tumorigenesis in the absence of the translocation.

In recent years, comprehensive genomic analyses have been performed in a number of salivary gland neoplasms(42). Chromosomal translocations resulting in gene fusions and mutations in known cancer pathways appear to be of particular importance in several tumors of salivary origin. In adenoid cystic carcinoma, for example, the t(6;9) translocation resulting in the MYB-NFIB fusion product is well-described, and two recent next-generation sequencing studies found alterations in chromatin regulation and Notch signaling pathways(43, 44). The significance of the *MECT1-MAML2* translocation and of *TP53* mutation demonstrated here in MEC is therefore consistent with findings in other salivary tumors.

The results of this study provide evidence that MEC tumors have few common genetic aberrations other than the *MECT1-MAML2* translocation and *TP53 and POU6F2* mutations. The *MECT1-MAML2* translocation was seen in all low-grade tumors and most intermediate- and high-grade tumors; in two high-grade cases without *MECT1-MAML2* translocation, we identified *TP53* mutations. The presence of *TP53* mutations was significantly associated with higher histologic grade and a higher number of mutations overall. *POU6F2* may also represent a driver mutation as three mutations were found in low-grade MEC. Future investigation to elucidate the functional roles of *MECT1-MAML2* translocation and the somatic mutations in MEC tumorigenesis would be highly desired.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Translational Relevance

Mucoepidermoid carcinoma (MEC) is the most common malignancy of the salivary gland. However, its molecular underpinnings remain unclear, and few systemic therapeutic options are available for this tumor type. We therefore performed whole-exome sequencing and copy number analyses of 18 primary cancers to identify possible drivers of tumorigenesis in MEC. Our data demonstrate that the *MECT1/MAML2* translocation may be the main oncogenic driver in these tumors. In tumors without the translocation, *TP53* mutation may act as an alternate mechanism of tumorigenesis. In addition, *POU6F2* mutations may act as drivers of oncogenesis in low-grade tumors. We also identified somatic mutations in a number of other genes, not previously implicated in MEC, which may serve as therapeutic targets. These findings should be further investigated for their therapeutic potential.

Table 1

Summary of clinical and sequencing data of 18 MEC cases

Tumor Sample	**1**	**3**	**5**	**7**	**8**	**9**	**10**	**11**	**13**	**17**	**18**	**19**	**20**	**21**	**29**	**31**	**32**	**33**
Sex	F	M	F	F	F	F	F	F	F	F	F	F	M	F	F	F	M	M
Age	28	46	66	76	48	17	54	37	42	52	61	57	49	65	86	44	50	36
Site	Par	Par	FOM	OP	Pal	Par	Pal	Par	Par	Par	Par	Max	Par	SMG	Par	Par	Par	SMG
Grade	Low	Low	Low	Low	Low	Low	Low	Low	Low	Int	Int	Int	Int	High	High	High	High	High
MECT1/MAML2 translocation	+	+	+	+	+	+	+	+	+	Ind	+	+	+	+	-	-	+	+
Distant met	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Lymph node involvement	N	N	N	Y	N	N	N	N	N	N	N	Y	N	Y	N	Y	N	Y
Tobacco exposure	N	N	Y	Unk	N	N	N	N	N	N	N	N	N	Y	Y	N	Y	Y
TP53										+	+		+		+	+		
POU6F2	+				+		+											
IRAK1		+															+	
MAP3K9													+				+	
ITGAL					+													+
ERBB4					+					+								
OTOGL										+			+					
KMT2C				+									+					
OBSCN				+														+
# of mutations *	50	17	31	38	35	3	8	5	3	177	43	14	242	10	48	12	11	27
CNVs	0	0	0	0	3	14	7	0	0	1	1	5	1	0	1	0	0	1

Abbreviations: F, female; M, male; OP, oropharynx; Par, parotid; FOM, floor of mouth; Pal, palate; Max, maxilla; SMG, submandibular gland; Int, intermediate; Ind; Indeterminate.

* Identified by whole-exome sequencing.