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Salivary gland cancer patient-derived xenografts enable characterization of cancer stem cells and new gene events associated with tumor progression

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Abstract

Purpose—Salivary gland cancers (SGC) frequently present with distant metastases many years after diagnosis, suggesting a cancer stem cell (CSC) subpopulation that initiates late recurrences; however current models are limited both in their availability and suitability to characterize these rare cells.

Experimental Design—Patient-derived xenografts (PDX) were generated by engrafting patient tissue onto nude mice from one acinic cell carcinoma (AciCC), four adenoid cystic carcinoma (ACC), and three mucoepidermoid carcinoma (MEC) cases, which were derived from successive relapses from the same MEC patient. Patient and PDX samples were analyzed by RNA-seq and Exome-seq. Sphere formation potential and *in vivo* tumorigenicity was assessed by sorting for Aldefluor (ALDH) activity and CD44 expressing subpopulations.

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Conflict of Interest

The authors declare no conflict of interest.

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Data and materials availability: Materials will be shared per the University of Colorado's Office for Technology Transfer policies and Institutional Review Board.

Results—For successive MEC relapses we found a time-dependent increase in CSCs (ALDH⁺CD44^{high}), increasing from 0.2% to 4.5% ($P=0.033$), but more importantly we observed an increase in individual CSC sphere formation and tumorigenic potential. A 50% increase in mutational burden was documented in subsequent MEC tumors, and this was associated with increased expression of tumor promoting genes (*MTIE*, *LGR5*, *LEF1*), decreased expression of tumor suppressor genes (*CDKN2B*, *SIK1*, *TP53*), and higher expression of CSC-related proteins such as SOX2, MYC, and ALDH1A1. Finally, genomic analyses identified a novel *NFIB-MTFR2* fusion in an ACC tumor and confirmed previously reported fusions (*NTRK3-ETV6* and *MYB-NFIB*).

Conclusions—Sequential MEC PDX models preserved key patient features and enabled the identification of genetic events putatively contributing to increases in both CSC proportion and intrinsic tumorigenicity, which mirrored the patient's clinical course.

Introduction

Salivary gland cancer (SGC) afflicts ~4,000 adults in the US annually and includes several distinct histotypes, of which mucoepidermoid carcinoma (MEC) and adenoid cystic carcinoma (ACC) are the most prevalent (1, 2). Preclinical models of SGC are scarce due to the difficulty in establishing models (3). These already limited models are further diluted by disease histotype (4) and cell line misidentification (5). Research using patient-derived xenografts (PDXs) from more common malignancies has led to enhanced preclinical and translational research (6). However, to date, only ACC PDX models have been reported (7, 8), with no reports characterizing PDX models from the most frequent subtype, MEC.

Late recurrences (longer than 5 years after diagnosis) manifesting primarily as distant metastases occur in upwards of 26% and 17% of ACC and MEC patients respectively (9). This is particularly striking in ACC patients with a promising 5-year survival rate of 75–80%, which plummets to 35% at 10 years and to 10% by 20 years (10, 11). Recurrences after a decade or more suggest the persistence of a tumor cell population that is senescent, can self-renew, and upon migration to distant sites can replicate the morphology and heterogeneity of the originating tumor; these characteristics are consistent with the accepted definition of a cancer stem cell (CSC) subpopulation (12).

Earlier studies inferred the presence of SGC CSCs and tested surface markers (CD44, CD133, ABCG2) and CSC-related factors (SOX2, OCT4, NANOG) (13, 14), however these did not prospectively define CSCs by sphere formation or tumor initiation (15). Aldefluor was the sole marker used to determine that the ALDH⁺ population was tumorigenic in ACC PDX models (16), and ALDH⁺CD44^{high} cells from MEC cells lines formed spheres and initiated tumors (10, 17). Using ACC PDX models and primary cultures, Panaccione *et al.* determined that CD133⁺ CSCs were maintained through SOX10 and Notch signaling and high expression of CD44 (18, 19).

Here we report the successful engraftment of the first three known PDX models of a high grade MEC (derived from the same patient after successive relapses), as well as one acinic cell carcinoma (AciCC), and four ACC cases. We have characterized the somatic mutational landscape, confirmed the presence of previously reported gene fusion events, and identified

a novel gene fusion. We determined that the ALDH⁺CD44^{high} phenotype defines the CSC fraction across ACC and MEC tumors using sphere formation and *in vivo* tumor initiation. The detailed analysis of MEC models generated from successive relapses identified an increase in mutation load, associated with increased expression of tumor promoting genes, and decreased transcription of tumor suppressor genes. Finally, in a landmark finding in solid tumor CSC biology, we found a time and relapse-dependent increase in both CSC frequency, as well as individual CSC sphere forming potential *in vitro* and tumorigenicity *in vivo*. The accumulation of CSCs over time contributing to disease progression or cancer relapse is considered a key principle in CSC biology, but this concept has been elusive and difficult to demonstrate in solid tumors.

Materials and Methods

Generation of patient-derived xenografts (PDX) of salivary gland cancers (SGC)

The protocol for studies involving human subjects was approved by the Colorado Multiple Institutional Review Board (COMIRB #08-0552) in accordance with the Belmont Report and U.S. Common Rule. Informed written consent was obtained from all patients whose tissues were used for this study. The University of Colorado Institutional Animal Care and Use Committee (IACUC) approved all experiments involving mice. PDX generation and characterization was previously reported (20).

Fluorescence activated cell sorting (FACS) and CSC implantation in vivo

Processing of tumor tissue for sorting, analysis, and *in vivo* implantation of CSCs was previously reported (21).

Immunohistochemistry (IHC)

IHC analyses were performed as previously described (3). Primary antibodies and dilutions; 1:750 ALDH1A1 (#61195 BD Biosciences), 1:50 CK5 (#CM353C BioCare), 1:200 Cleaved caspase-3 (#9664 Cell Signaling), 1:100 EGFR (#4267 Cell Signaling), 1:100 phospho-EGFR (#3777 Cell Signaling), 1:100 Ki67 (#RM-9106-S1 Thermo), and 1:2000 phospho-SMAD2 (#ab188334 Abcam).

Protein isolation and western blotting

Protein analyses were performed as previously described (21). Primary antibodies and dilutions; 1:2000 Actin (pan) (Cell Signaling), 1:500 ALDH1A1 (Sigma Aldrich, St. Louis, MO), 1:750 p4EBP1 (Cell Signaling), 1:1000 SMAD2/3 (Cell Signaling), 1:1000 pSMAD2 (Cell Signaling), 1:1000 pSMAD3 (Cell Signaling), 1:500 SOX2 (Cell Signaling).

Sample preparation, whole exome sequencing (WES), and RNA-seq

DNA was isolated from blood and tumor with a DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA), while total RNA was isolated from tumor tissue using an RNeasy kit (Qiagen, Valencia, CA, USA). Isolated DNA and RNA were sent to the University of Colorado Cancer Center Genomics and Microarray Core, which performed library preparation, sequencing, and FASTQ generation. Single-end sequencing of mRNA (Poly A)

was performed on an Illumina HiSEQ instrument with read lengths of 100bp (Illumina, San Diego, CA). DNA underwent paired-end sequencing using Agilent Technologies SureSelect Human All Exon Versions 5 and 6 with read lengths of 150bp (Agilent, Santa Clara, CA, USA).

Sequencing confirmation of gene fusions

DNA sequencing was conducted as previously described (22), and new primer sets were developed for the fusions listed below.

NTRK3-Forward:	5'-CACTGCATCGAGTTTGTGGTGCG
ETV6-Reverse:	5'-CTTATGGTTTCCCCACAGTCGAGC
Product:	393 bp
NTRK3:ETV6 sequencing:	5'-CCAACGCTGCACTGGCTG
NFIB-Forward:	5'-GGAACCAAGTCCTACAGGAGACTTTTACC
MTFR2-Reverse:	5'-GCAAGTGTAGGCAAAATGTGTCAAGAAGAG
Product:	372 bp
NFIB:MTFR2 sequencing:	5'-ATCCTTGAGTTTAGAAGGCTTGTGTTGC
MYB-Forward:	5'-CCTTGTAGCAGTACCTGGGAACCTGC
NFIB-Reverse:	5'-TTGGACATTGGCCGTAAGATGG
Product:	186 bp
MYB:NFIB sequencing:	5'-CCTGTGGAAAGATGGAGGAGCAG

Fluorescent in situ hybridization (FISH) analysis of gene fusions

FISH analysis of gene fusions was previously described (6) and is further described in detail in the supplementary materials.

Statistics

Experiments were compared with a two-group t-test. Calculations were done using GraphPad Prism version 7.0 and SPSS version 11. Data are represented graphically as mean $\pm$ SEM.

Results

The success of SGC PDX generation correlates with disease stage

After securing patient informed consent, excess tumor tissue samples from twelve SGC surgeries were implanted subcutaneously on the flanks of athymic nude mice, including 5 ACC, 4 MEC, 1 salivary duct carcinoma (SDC), 1 AciCC, and 1 mammary analogue secretory carcinoma (MASC). Engraftment rates varied between histotypes resulting in PDX models of 3 MEC (75%), 3 ACC (60%), and 1 AciCC (100%) (Table S1 and Table S2). The engraftment rate of relapsed tumors was higher than that of primary cases and were 63% and 20% respectively (Table S1), while time to initial engraftment and subsequent passaging for MEC (3), ACC (3) and a single acinic cell carcinoma required on average 170 ± 46 , 401 ± 7 and 343 days to generate tumors ($>1,500\text{mm}^3$) respectively (Fig. S1A). Morphology (H&E staining) and cytokeratin 5 (CK5) staining were conserved in PDX tumors when compared to the originating patient tissue (Fig. 1A).

The most unique PDX were three models (CUSG006, –007, –012) generated from the same patient with a high grade MEC from three successive surgeries of relapses occurring over three years (Fig. 1B). The time between surgeries was similar (~9–10 months), but by the third operation the tumor had migrated outside of the primary parotid bed site, invading into the deep neck space and around the carotid artery. The growth rate of CUSG012 was nearly identical to CUSG006 and CUSG007 once stably passaged in mice (Fig. S1B).

SGCs are hypo-mutated tumors with limited oncogene overlap

Whole exome sequencing (WES) of nine tumors from seven patients with paired normal samples determined the mutation burden for each case (Fig. 1C). Possibly damaging somatic mutations present in more than one sample are displayed in a co-mutation (coMut) plot (Fig. 1D), while all mutations are reported in Table S3. We observed substantial overlap in the three MEC cases and a ~50% increased mutation burden in the two later samples, CUSG007 (62 mutations) and CUSG012 (58 mutations), compared to CUSG006 (41 mutations). We found that CUSG012 had acquired an inactivating mutation in the *SHPRH* gene. This gene has been shown to suppress genomic instability (23), which may allow for rapid accumulation of mutations and tumor progression. When filtered by known somatic cancer-related mutations (COSMIC) we observed few oncogenic mutations per tumor (0 mutations in CUSG002, CUSG003, CUSG006, CUSG007; 1 mutation in CUSG004, CUSG005, CUSG012; 2 mutations in CUSG013 [*ATM*, *KDM6A*]), except for the SDC case (CUSG008; 5 COSMIC mutations), which included missense mutations in *ERBB2*, *CDH1*, and *BAP1* (Fig. S1B). We also identified inactivating mutations in genes with putative roles as tumor suppressors and promoting genomic stability including missense mutations of *RB1CC1* in multiple histotypes (MEC, SDC, MASC), while *SHPRH* mutations were found in two cases (MEC, SDC). We found that two ACC tumors (CUSG004, –005) had frameshift mutations in *SPEN* and *KMT2D*.

Gene fusions are a dominant source of genomic damage in some cases of SGC

The analysis of SGC tumor RNA-seq data using the STAR-fusion pipeline identified multiple high-confidence gene fusion events in patient tumors. Since single-end sequencing was performed, junction read counts, or the number of reads that split alignment between two genes, were used as fusion evidence (Table S4). We identified a novel fusion event, *NFIB-MTFR2*, in the hypo-mutated ACC model CUSG004. This molecular fusion event was confirmed by Sanger sequencing (Fig. 1E) and subsequently visualized by fluorescence *in situ* hybridization (FISH) using green 5' *NFIB* and the red 3' *MTFR2* DNA probes in the CUSG004 patient tumor, but not in a matched normal tissue sample (Fig. 1F) or other controls (Fig. S2 and Table S5). We identified several previously reported fusions, including *NTRK3-ETV6* (CUSG002, MASC) and *MYB-NFIB* (CUSG005, ACC) (Table S4). Coinciding with its high mutation burden (Fig. 1C), CUSG008 (SDC) had numerous genomic rearrangements, including *ADNP2-KCNG2*, *YTHDF1-NKAIN4*, and *LAMA5-FTCD* (Table S4). Finally, the *NFIB-MTFR2* (CUSG004) and *MYB-NFIB* (CUSG005) fusions observed in patient tumor was conserved following engraftment on mice.

SGC gene expression analysis overlaps in histologic subtypes

We first assessed unsupervised gene clustering and the drift in gene expression following PDX engraftment on mice and subsequent passaging by analyzing patient tissue and multiple PDX passages for three cases by RNA-seq. We found that the two ACC cases, CUSG004, -005, clustered more closely than the MEC case, CUSG006 (Fig. S3A). We also found that 85% of genes that had significant changes in expression following engraftment on nude mice overlapped across all three cases (Fig. S3B to E) and were related to immune response (Fig. S4A to D). Numerous differentially expressed genes were identified when comparing ACC or MEC samples vs. all others (Table S6). Gene set enrichment analysis (GSEA) identified significant upregulation of the Hallmark Pathways, “MYC Targets”, “Mitotic Spindle”, and “E2F Targets” in ACC tumors (Fig. 2A).

GSEA comparing the PDX tumors engrafted from subsequent MEC relapses identified the upregulation of Hallmark pathways over time, including “E2F Targets”, “Myc Targets”, “DNA Repair”, and “TGF-beta” and downregulation of “Epithelial to Mesenchymal Transition” and “Inflammatory Response” (Fig. 2B). When comparing tissue collected from the first two surgeries (CUSG006, CUSG007) we observed progressively increased expression of growth promoting genes (*CR1* [97-fold], *MAGEC2* [21-fold], *MMP1* [3.1-fold], and *HEY1* [2.1-fold]). We next compared tissue from the second and third surgeries and found expression of genes related to migration (*MTIE* [1,445-fold]), survival (*EN1* [6.9-fold]) and CSCs (*LGR5* [28-fold], *LEF1* [19-fold]) to be dramatically enriched in the relapsed third tumor (Fig. 2C). Just as striking, expression of key tumor suppressors (*CDKN2B* [-1,628-fold], *TP53* [-2.3-fold], *SIK1* [-1,709-fold]) was also inhibited in this same case.

Next, we assessed gene expression changes throughout disease progression by examining protein levels in patient tissue by IHC. Tumor cell proliferation, Ki67 expression, did not change between cases CUSG006, -007, and -012 (Fig. 2D). However, we did observe incremental increases in pSMAD2 staining and ALDH1A1-high cells by IHC (Fig. 2D). Changes in gene expression and IHC were confirmed by western blot for key pathways, which were replicated three times with different PDX tumors. Levels of pSMAD2, pSMAD3, NOTCH1, HES1, SOX2, ALDH1A1, and MYC increased over disease progression in the three PDX cases, while EGFR signaling (EGFR, pEGFR, pMAPK) decreased (Fig. 2E).

SGC subpopulations with sphere forming potential share a common phenotype

We determined Aldefluor activity and CD44 expression in two ACC and three MEC PDX models and found the ALDH⁺CD44^{high} population consistently fell between 0.15% and 4.5%, except for the ACC PDX model CUSG005 that had a dual positive population of 17% (Fig. 3A and Table S7). Cells from PDX tumors were sorted by Aldefluor activity and CD44 expression and seeded as spheres (5×10^3 cells/well ALDH⁺CD44^{high}, ALDH⁺CD44^{low}, ALDH⁻CD44^{high} or 5×10^4 cells/well ALDH⁻CD44^{low}) in 96-well low-bind plates. The ALDH⁺CD44^{high} population generated the most tumor spheres for both ACC (CUSG004 $P=0.032$, CUSG005 $P<0.001$) and MEC (CUSG007 $P<0.001$, CUSG012 $P=0.018$) when sorted from PDX. The initial MEC tumor of three successive surgeries (CUSG006) was the

sole exception, where no sorted subpopulation generated more than 3 spheres per well (Fig. 3B, and Fig. S5). Out of all subpopulations sorted across cases, the CUSG012 ALDH⁺CD44^{high} subpopulation consistently formed the largest spheres across all cases and histotypes (Fig. 3B).

The ALDH⁺CD44^{high} subpopulation from the three MEC relapses increased from 0.2% (CUSG006) to 0.3% (CUSG007) and then to 4.5% (CUSG012) (Fig. 3C and Table S7). As noted above, subpopulations sorted from CUSG006 tumors had difficulty generating spheres, while CUSG007 ALDH⁺CD44^{high} cells readily formed spheres (Fig. 3C). Of note, all subpopulations sorted from CUSG012 tumors were able to generate spheres (Fig. 3C and D), however the ALDH⁺CD44^{high} cell subpopulation had the greatest sphere generating potential, forming the most and the largest spheres across all three cases (Fig. 3C). Comparing sorted ALDH⁺CD44^{high} and bulk tumor cells we found that HES1, SOX2, and pSMAD2 were enriched in sphere-forming ALDH⁺CD44^{high} cells (Fig. 3D).

Tumorigenic populations induce in vivo tumors in cell dilution studies

Tumor formation in mice using cell dilution assays remains the gold standard to identify CSCs (24, 25). All four tumor subpopulations (10^2 and 10^3 cells for ALDH⁺CD44^{high}, ALDH⁺CD44^{low}, ALDH⁻CD44^{high}; 10^5 cells for ALDH⁻CD44^{low}) and bulk tumor cells (10^4 and 10^5 cells) from one ACC (CUSG004) and three MEC (CUSG006, -007, -012) PDX were implanted into the flanks of nude mice and monitored for up to 500 days for tumor formation. The ALDH⁺CD44^{high} subpopulation was the most tumorigenic when 10^3 cells were injected (Table S8), but tumor initiation/growth took over a year when tenfold fewer (10^2) ALDH⁺CD44^{high} cells were implanted (Fig. 4A), suggesting CSCs can remain senescent for long periods before actively proliferating. Importantly, 10^3 ALDH⁺CD44^{high} cells were as tumorigenic as 10^5 bulk tumor cells supporting that it is the ~1% CSC fraction within bulk cells that bears tumorigenicity; indeed, 10^5 ALDH⁻CD44^{low} cells rarely formed tumors (Table S8). Tumors resulting from CSC implantation harbored CSC fractions similar to the originating PDX (Table S7), and subsequent passaging of tumor subpopulations sorted from CSC-generated tumors (CUSG004, CUSG007) gave similar results (Table S8 and Fig. 4B).

MEC CSCs increase their fraction and individual tumorigenicity upon successive relapses

No subpopulations sorted from CUSG006 tumors generated tumors while ALDH⁺CD44^{high}, and to a lesser extent ALDH⁺CD44^{low} cells, from CUSG007 and CUSG012 cells readily formed tumors in cell dilution studies with inoculates as low as 10^2 cells (Table S8). It is important to note again that the ALDH⁺CD44^{high} fraction increased from 0.3% to 4.5% during the progression of CUSG007 to -012, possibly accounting for the subtly lower single marker (ALDH⁻CD44^{high} and ALDH⁺CD44^{low}) subpopulation tumorigenicity in CUSG012.

Discussion

Orphan diseases like SGC are often understudied due to a scarcity of laboratory models such as cell lines or PDX for functional, mechanistic, and/or therapeutic experiments. Currently,

research into the molecular drivers responsible for SGC initiation and progression is limited, and this is perhaps reflected in the lack of targeted therapies for this disease. There is also incomplete evidence that CSCs promote proliferation and relapse of SGC tumors across histotypes (16, 17). Therefore, establishing PDX models will greatly aid in the identification of key genomic and expression alterations in engrafted cases and in the definition of the CSC subpopulations across SGC histotypes. This will provide additional and valuable tools in determining the processes that lead to late recurrences and metastasis (9) and developing hypothesis-based novel therapeutics (26). In this report we show the first successful establishment of high grade MEC PDX models reported to date, the fact that these originated from the same individual at different disease times, and the observation of mutation accumulation and enhanced growth promoting gene dysregulation over time. Additionally, a novel *NFIB-MTFR2* gene fusion was identified in an ACC case. Importantly we demonstrate that both CSC frequency and individual CSC “aggressiveness” (sphere forming potential, tumorigenicity) increase in subsequent relapses.

The characterization of the genomic and expression changes of successive relapses of an increasingly aggressive tumor over time revealed that during the ~10 months between the first (CUSG006) and second (CUSG007) relapses there were dramatic increases in *CR1* and *MAGEC2* expression, which regulate the CSC compartment of colorectal tumors (27) and can promote metastases through STAT3 (28), respectively. In comparing CUSG007 and CUSG012 we found that CUSG012 had acquired an inactivating mutation in *SHPRH*, a gene that suppresses genomic instability (23), which could account for the accompanying genomic and gene expression changes. Along with downregulation of tumor suppressors (*CDKN2B*, *SIK1*, *TP53*), we noted increased expression of genes associated with survival (*ENI*) (29), Wnt signaling (*LEF1*), and colon stem cells and colorectal CSCs (*LGR5*). These gene expression changes were accompanied by increased TGF- β signaling and expression of known protein effectors of the CSC phenotype (SOX2, MYC, ALDH1A1), suggesting a transition to a CSC-enriched state.

To formally characterize CSCs, we used both sphere formation and tumor initiation in mice using cell dilution assays, the *in vitro* and *in vivo* “gold standards” when prospectively defining CSCs (15). Similar to previous CSC studies in solid tumors (15) the ALDH⁺CD44^{high} fraction ranged between ~0.1 and 3% in four out of five cases. However, the ALDH⁺CD44^{high} population in the CUSG005 tumor was >15%, which may be due to the homogeneity of this MYB-NFIB fusion-driven tumor (30). The ALDH⁺CD44^{high} population consistently generated the most, and the largest, spheres compared to other subpopulations across ACC and MEC subtypes. As few as 10² ALDH⁺CD44^{high} cells initiated tumors *in vivo*, and resulting tumors recapitulated the morphology, tumor cell populations, and CSC fraction as that of the originating tumor. Possibly the most striking data arose from the inherently controlled assessment of CSC profiles and properties from PDX models arising from three subsequent relapses from the same subject. Increased mutation load and ensuing gene dysregulation ultimately led to both increased CSC fraction and individual CSC aggressiveness (sphere formation, tumorigenicity), in what we propose is a relevant observation highlighting the translational value of PDX models. This finding supports a core tenet in CSC biology. Similarly, it has been reported that CSC directly extracted from pre- and post-treatment samples of breast cancers showed increases in breast CSC numbers and

sphere formation potential in response to cytotoxic therapy (31), emphasizing that originating patient samples are critical in order to study delicate biological processes such as the characterization of the CSC fraction.

The timing of CSC initiated tumor formation closely followed what is observed in the clinic, as 100 highly tumorigenic CSCs took nearly 250 days to give rise to measurable (~100 mm³) tumors on mice, a size still several fold smaller than what is clinically or radiologically detectable in patients. The ability of a few cells to survive undetected for many months in a hostile environment before proliferating are characteristics consistent with residual disease leading to late recurrence and metastases in SGC patients (9). The observation that PDX-derived CSCs can model the clinical behavior of SGC is critical to support the utilization of these models to study the process of metastasis and to identify relevant therapies.

We successfully engrafted PDX models for three MEC, three ACC, and one AciCC cases that generally preserved the originator tumor's morphology. The time required for initial SGC PDX engraftment following patient tumor implantation exceeded a year in some cases, which was much longer than what we have previously observed in our extensive experience with head and neck squamous cell carcinomas (HNSCC). HNSCC commonly grow within 3–4 months (22); notably this follows the usual timing of HNSCCs relapses that commonly occur in the first year after diagnosis (32). The growth dynamic of SGC PDX is also consistent with the protracted natural history where SGC distant relapses decades after diagnosis are common (9, 10) and previous SGC PDX reports (7, 8). To our knowledge we have generated the first functional PDX models of MEC, which retain the morphology of their respective histotypes. An interesting observation is that the majority of successfully engrafted cases were relapses, consistent with pancreatic and breast cancers (33, 34).

SGCs are hypo-mutated compared to other neoplasms (35). Through WES analysis we determined that ACC tumors (CUSG004, CUSG005, CUSG013) had between 19–25 somatic mutations, which is consistent with a previous study that reported 2–35 mutations per ACC case (36). We did not observe high levels of *TP53* mutations or alterations in PI3K signaling that were reported in studies using targeted NextGen Sequencing of 182–315 cancer genes (37–39). This may be due in part to the lack of sequenced paired normal tissue to remove germline SNPs/mutations in these other reports. However, we did observe mutations in cancer associated genes that have been reported using multiple platforms. We found that two of three ACC tumors harbored frameshift mutations in *SPEN*, a negative regulator of Notch signaling, which is mutated in up to 20% of ACC cases (36, 38). The third ACC case had mutations in both *KDM6A* and *ATM* that are mutated in ~15% and ~5% of ACCs respectively (38, 40). The BRCA1-Associated Protein 1 (*BAP1*) was mutated in the single SDC case (CUSG008) analyzed, which is a gene consistently mutated in 5–10% of SGCs across disease histotypes (37, 38, 40). We also observed inactivating mutations across histotypes in putative tumor suppressors *RB1CC1*, *RARRES1* and *TP53BP1* (41–43), as well as in *SHPRH*, which suppresses genomic instability (23) by promoting error free replication (44). Loss of *SHPRH* function may account for rapid changes in gene expression and progression of the CUSG012 tumor. Also, two ACC cases with low mutation burdens shared damaging rearrangements in the tumor suppressor *KMT2D* (45). Finally, we have

confirmed the presence of common SGC genomic alterations and novel mutations that may allow for tumorigenesis and disease progression.

Given this low frequency of oncogenic mutations we tested gene fusions known to occur in several SGC histotypes, including *ETV6-NTRK3* fusions in MASCs and *MYB-NFIB* fusions in ACCs (46, 47), and these genetic events were identified in cases CUSG002 (MASC) and –005 (ACC), respectively. We identified a novel gene fusion between the *NFIB* and *MTFR2* genes in an ACC PDX model (CUSG004). This is likely a driver event since the MYB domain of the *MYB-NFIB* fusion promotes carcinogenesis through its role as a transcription factor (30). Notably, *NFIB* has been identified as an oncogene in small cell lung cancer where it appears to regulate cell proliferation and viability through transcriptional regulation (48). Furthermore, the *NFIB-MTFR2* fusion results in the loss of the transcriptional regulatory domain of *NFIB* and leads to a truncated NFIB protein. Further studies examining the consequences of this truncation are merited as NFIB is thought to be important in the regulation of the TGF-beta and Sonic Hedgehog pathways (49).

In summary, here we report detailed genomic and CSC characterization from a comprehensive panel of SGC PDX, which includes three sequential models from the same MEC patient that have enabled us to dissect the molecular evolution and functional changes in CSCs over time. This is the first report of successful MEC PDX generation. We observed a doubling in the mutation burden in subsequent relapses and found an enrichment of pro-survival gene expression and “stemness.” The size of the CSC subpopulation increased over time, and in addition the CSCs had a significant rise in sphere initiation capacity and *in vivo* tumorigenicity. Overall these findings suggest that CSCs may be associated with tumor progression in MEC, as well as highlighting the importance of SGC PDX as a valid tool in expanding our understanding of the molecular progression of cancer and the impact of disease progression on tumorigenic cell subpopulations, such as CSCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Salivary gland cancers are an orphan disease, and we have established the first patient-derived xenograft models of mucoepidermoid carcinoma (MEC) from tissue collected during subsequent surgeries for the same individual. CSCs were defined by Aldefluor activity- and CD44-positive subpopulations, as well as sphere formation and *in vivo* tumor initiation. The novel findings in solid tumor CSC biology were increases in the CSC fraction (from 0.2% to 4.5%), as well as increased individual CSC sphere formation and *in vivo* tumorigenicity in the three successive MEC relapses. A 50% increase in mutational burden occurred in subsequent MEC relapses, associated with tumorigenic changes in tumor promoting and suppressing genes; expression of the CSC-related proteins SOX2, MYC, and ALDH1A1 increased with relapses. A novel *NFIB-MTFR2* fusion in an ACC tumor was identified. The frequency of cancer stem cells as well as their associated sphere forming potential and tumorigenicity increased with disease progression and accumulating mutational burden.

Fig. 1. Exome-seq and RNA-seq analyses identifying somatic mutations and gene fusions in SGC patient samples and PDX models

(A) CUSG PDX models recapitulated the morphology (H&E) and CK5 staining of the originating patient tumor. (B) Three PDX models engrafted from successive surgeries by the same patient. The time between tissue collection of CUSG006 and CUSG007, as well as CUSG007 and CUSG012, was consistent at ~10 months. (C) Mutation burden (somatic mutations scored as possibly damaging) for each SGC case analyzed by Exome-seq. (D) CoMut plot of somatic mutations scored as possibly damaging occurring in two or more cases. Grey boxes= missense mutations, blue boxes= nonsense mutations, yellow boxes= frameshift mutations. **Bold** cases are from tissue collected from successive surgeries from the same patient. (E) Identified *NFIB-MTFR2* gene fusion product. From top to bottom: Chromosomal locations, exon numbers, Sanger sequencing, and protein product. (F) CUSG004 patient normal tissue, patient tumor tissue (F0), and PDX tumor tissue (F1, F5) hybridized with the *NFIB(SG)/MTFR2(SR)* FISH fusion probe set. Patient tumor and PDX tissue showed a positive pattern. Tumor adjacent normal tissue collected at the time of surgery was negative for the fusion product. Arrows indicate fused signals.

Fig. 2. Gene expression analysis of SGC patient tissue and PDX models

(A) Hallmark gene sets enriched for both upregulation and downregulation in ACC vs. all other SGC cases. (B) Hallmark gene sets enriched for both upregulation and downregulation when comparing PDX models arising from successive surgeries. CUSG006= 1st surgery, CUSG007= 2nd surgery, CUSG012= 3rd surgery. (C) Waterfall plots denoting changes in gene expression of >Log2 fold change ($P<0.05$) between three PDX cases engrafted from successive surgeries. (D) IHC staining of patient tumor tissue collected from the same patient over consecutive surgeries showed pSMAD2 staining increased over time along with the number of highly positive ALDH1A1 cells (arrows). (E) Western blot analysis of PDX tumor tissue highlights increased expression of CSC-related, Notch signaling, and TGF-β pathway proteins during tumor progression.

Fig. 3. Sphere formation by SGC PDX tumor subpopulations

(A) Representative histograms of sorted ALDH⁺CD44^{high} populations. (B) Generation of spheres by tumor subpopulations sorted from two ACC (CUSG004, –005) and three MEC (CUSG006, –007, –012) PDX models measured by **Top** spheres per well (>50μm) and **Bottom** sphere size. Gray shaded graphs are for PDX tumors generated from the same tumor over successive surgeries. (C) ALDH⁺CD44^{high} percentage, spheres per well, and sphere size generated by the ALDH⁺CD44^{high} subpopulation sorted from PDX models that were engrafted from tissue collected from successive surgeries in the same patient. (D) CUSG012 ALDH⁺CD44^{high} cells have increased SOX2, Hes1, and pSmad2 levels than bulk tumor cells. * *P*<0.05.

Fig. 4. Tumor initiation by SGC PDX tumor subpopulations

(A) 10^3 ALDH⁺CD44^{high} cells initiate tumors that proliferate as rapidly as 10^5 bulk tumor cells. When injected into the flanks of nude mice, as few as 10^2 CUSG004 ALDH⁺CD44^{high} cells initiated tumors after not proliferating (forming detectable tumors) for nearly 1 year.

(B) Tumors initiated by SGC CSCs (ALDH⁺CD44^{high}) recapitulate the morphology (H&E) and CK5 staining for the originating patient and PDX tumors.