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Chromosomal abnormalities and molecular landscape of metastasizing mucinous salivary adenocarcinoma

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ABSTRACT

Background: Mucinous adenocarcinoma of the salivary gland (MAC) is a lethal cancer with unknown molecular etiology and a high propensity to lymph node metastasis. Mostly due to its orphan status, MAC remains one of the least explored cancers that lacks cell lines and mouse models that could help translational and pre-clinical studies. Surgery with or without radiation remains the only treatment modality but poor overall survival (10-year, 44%) underscores the urgent need for mechanism-based therapies.

Methods: We developed the first patient-derived xenograft (PDX) model for pre-clinical MAC studies and a cell line that produces aggressively growing tumors after subcutaneous injection into nude mice. We performed cytogenetic, exome, and proteomic profiling of MAC to identify driving mutations, therapeutic targets, and pathways involved in aggressive cancers based on TCGA database mining and GEO analysis.

Results: We identified in MAC KRAS (G13D) and TP53 (R213X) mutations that have been previously reported as drivers in a variety of highly aggressive cancers. Somatic mutations were also found in KDM6A, KMT2D, and other genes frequently mutated in colorectal and other cancers: FAT1, NBEA, RELN, RLP1B, and ZFHX3. Proteomic analysis of MAC implied epigenetic up-regulation of a genetic program involved in proliferation and cancer stem cell maintenance.

Conclusion: Genomic and proteomic analyses provided the first insight into potential molecular drivers of MAC metastases pointing at common mechanisms of CSC propagation in aggressive cancers. The *in vitro*/*in vivo* models that we created should aid in the development and validation of new treatment strategies against MAC.

Introduction

While mucinous adenocarcinomas occur more frequently in the colon, appendix, breast, lung and ovary, mucinous adenocarcinoma of the salivary gland (MAC) represents only 0.1% of all salivary gland tumors. MAC is a rare cancer: only 73 MAC cases have been registered in the U.S. from 1998 to 2012 [1–3]. The majority of salivary MAC present with cervical nodal metastases (63%) and primary treatment is surgical excision, nodal dissection, with or without post-operative radiation therapy. Despite this aggressive treatment regimen, 10-year survival is only 44% [3]. Because of its orphan status, randomized clinical trials for MAC are not feasible and collection of tumor specimens for clinical research is rare, making salivary MAC one of the least studied cancers with yet

Abbreviations: MAC, mucinous adenocarcinoma of the salivary gland; CSC, cancer stem cells; PDX, patient-derived xenografts; GEO, gene expression omnibus; TCGA, the cancer genome atlas.

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unknown molecular drivers. Lack of MAC cell lines or patient-derived xenograft (PDX) models further complicates development of targeted therapies. To catalog molecular defects that may contribute to its MAC aggressive behavior and to identify potential therapeutic targets, we created the first MAC PDX model and cell line from a metastatic lymph node deposit. Cytogenetic analysis, exome sequencing, and shotgun proteomic studies revealed multiple chromosomal and molecular abnormalities that may contribute to aggressive behavior possibly via epigenetically regulated cancer stem cells (CSC).

Materials and methods

Clinical specimen

A high grade tumor with metastases to 3 out of 22 lymph nodes (largest node ~4.8 cm) was removed by parotidectomy and neck dissection in Vanderbilt-Ingram Cancer Center (WGY). A portion of the resected lymphatic metastases was collected after informed consent (IRB#030062, WGY), and diagnosed as a high-grade mucinous adenocarcinoma by a board-certified pathologist (AKE).

Mouse xenograft model

Athymic nude Foxn1 mice (Harlan Laboratories, Indianapolis, IN) were maintained in accordance with the Institutional Animal Care and Use Committee guidelines (Yale IACUC#12-11510). Male mice between 4 and 6 weeks of age were housed on irradiated corncob bedding (Harlan Laboratories) in individually ventilated cages on a 12-h light–dark cycle at 70–74 1F (21–23 1C) and 40–60% humidity. Mice were fed water *ad libitum* (reverse osmosis, 2 ppm Cl2) and an irradiated standard rodent diet (Teklad 2919) consisting of 18% protein, 5% fat and 4% fiber. To generate patient-derived xenografts (PDX), unprocessed tumor specimens were briefly washed with 70% ethanol, cut into ~30–50 mm^3 pieces and implanted subcutaneously in the shoulder area of anesthesized animals using sterile tools and staples for wound closure.

Primary cell culture

Resected tumor specimen was washed with PBS and digested for 30 min at 37 °C in serum-free KGM media (Life Technologies, Carlsbad, CA) that contained trypsin (0.05%) and 1 mg/ml collagenase from *Clostridium histolyticum* (Sigma-Aldrich, St. Louis, MO). FBS (Life technologies) was then added to the mixture (final conc. 10%) to quench trypsin and cells were pelleted by centrifugation (400*g*, 5 min) and washed twice with PBS. The cells were then resuspended in KGM with 10% FBS and 1X Antibiotic-Antimitotic (Invitrogen, Carlsbad, CA) and incubated overnight in collagen-coated plates. Attached cells were washed three times with PBS and incubated in antibiotic/antimitotic serum-free KGM media to produce primary culture. After 3 passages in serum-free media, fibroblast-free cells were transferred into KGM media supplemented with serum.

Microsatellite analysis

Using Promega GenePrint 10 STR analysis PCR kit with fluorescent tagging, PCR products were analyzed on Applied Biosystems 3730xL DNA Analyzer at the Yale Keck Facility and data processed using GeneMapper 3.7 (Applied Biosystems) software. The results were then compared to STR databases (http://www.cstl.nist.gov/strbase/) and (http://www.dsmz.de/services/services-human-and-animal-cell-lines/online-str-analysis.html).

Cytogenetics

G-banding and spectral karyotyping (SKY) analyses were performed as previously described [4]. Composite karyotype was generated based on all clonally occurring chromosome abnormalities in 10 analyzed metaphase spreads.

Molecular profiling

Whole exome sequencing was done at the New Generation Sequencing Core at Vanderbilt University Medical Center Core using Illumina Hi Seq 2000 with SureSelect All-Exon Target Enrichment System. Shotgun proteomic analysis was performed by first suspending the samples in LDS sample buffer (Life Technologies), resolving the proteins approximately 1 cm using a 10% Novex precast gel, and then performing in-gel tryptic digestion to recover peptides. These peptides were analyzed via MudPIT (Multidimensional Protein Identification Technology) essentially as previously described [5,6]. Briefly, digested peptides were loaded onto a biphasic pre-column consisting of 4 cm of reversed phase (RP) material followed by 4 cm of strong cation exchange RP material. Once loaded, this column was placed in line with a 20 cm RP analytical column packed into a nanospray emitter tip directly coupled to a linear ion trap mass spectrometer (LTQ). For a total of 11 salt steps, a subset of peptides was eluted from the SCX material onto the RP analytical via a pulse of volatile salt, those peptides separated by an RP gradient, and then ionized directly into the mass spectrometry where both the intact masses (MS) and fragmentation patters (MS/MS) of the peptides were collected. These peptide spectral data were searched against a protein database using Sequest and the resulting identifications collated and filtered using IDPicker and Scaffold (http://www.proteomesoftware.com). Relative protein abundances were evaluated via spectral counting techniques using the Quasitel program for P-value calculations.

Computational gene expression analysis

Expression array studies were performed using Multi-Experiment Matrix software [7,8] available at http://biit.cs.ut.ee/mem/index.cgi.

Results

Generation of a PDX model for metastasizing salivary mucinous adenocarcinoma (MAC)

Patient-derived xenograft models (PDX) produced via implantation of minimally processed tumor tissue into immunodeficient mice are emerging as a novel platform for clinical research that maintains tumor heterogeneity and faithfully recapitulates tumor response to treatment. PDX are also used for studying cancer stem cells (CSC) that have been linked to therapy resistance, metastases, and recurrence [9–11].

To produce a PDX model, fragments of tumor extracted from a patient's lymph node were minced and subcutaneously implanted into the flanks of 3 nude mice. Within two months, all animals grew tumors, which rapidly developed lobulated morphology and required frequent (monthly) passages into additional mice due to its aggressive growth. Comparative H&E staining demonstrated that PDX tissue recapitulated the histology of the original tumor (Fig. 1A).