Generation and Sequencing of Pulmonary Carcinoid Tumor Cell Lines

Michael K. Asiedu, PhD, Charles F. Thomas, Jr., MD,† Sandra C. Tomaszek, MD,* Tobias Peikert, MD,† Bharati Sanyal, PhD,†Shari L. Sutor, MS,* Marie-Christine Aubry, MD,‡ Peter Li, PhD,§ and Dennis A. Wigle**

Introduction: Pulmonary carcinoid tumors account for approximately 5% of all lung malignancies in adults, and comprise 30% of all carcinoid tumors. There are limited reagents available to study these rare tumors, and consequently no major advances have been made for patient treatment. We report the generation and characterization of human pulmonary carcinoid tumor cell lines to study underlying biology, and to provide models for testing novel chemotherapeutic agents.

Methods: Tissue was harvested from three patients with primary pulmonary typical carcinoid tumors undergoing surgical resection. The tumor was dissociated and plated onto dishes in culture media. The established cell lines were characterized by immunohistochemistry, Western blotting, and cell proliferation assays. Tumorigenicity was confirmed by soft agar growth and the ability to form tumors in a mouse xenograft model. Exome and RNA sequencing of patient tumor samples and cell lines was performed using standard protocols.

Results: Three typical carcinoid tumor lines grew as adherent monolayers in vitro, expressed neuroendocrine markers consistent with the primary tumor, and formed colonies in soft agar. A single cell line produced lung tumors in nude mice after intravenous injection. Exome and RNA sequencing of this cell line showed lineage relationship with the primary tumor, and demonstrated mutations in a number of genes related to neuronal differentiation.

Conclusion: Three human pulmonary typical carcinoid tumor cell lines have been generated and characterized as a tool for studying the biology and novel treatment approaches for these rare tumors.

Key Words: Typical carcinoid, Neuroendocrine cells, Lung cancer; Atypical carcinoid, Exome sequencing, Cell line generation.

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*Division of General Thoracic Surgery, Department of Surgery, Mayo Clinic College of Medicine, Rochester, MN; †Thoracic Diseases Research Unit, Division of Pulmonary, Critical Care, and Internal Medicine, Department of Medicine; ‡Department of Laboratory Medicine and Pathology; and §Department of Health Sciences Research, Mayo Clinic College of Medicine, Rochester, MN.

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Address for correspondence: Dennis A. Wigle, MD, PhD, Division of General Thoracic Surgery, Department of Surgery, Department of Physiology and Biomedical Engineering, Mayo Clinic, 200 First Street SW, Rochester, MN 55905. E-mail: wigle.dennis@mayo.edu

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Pulmonary carcinoid tumors represent a subset of up to 5% of all primary bronchopulmonary neoplasms.[1,2] Their incidence has rapidly increased within the past 30 years, and was 1.57 per 100,000 in 2003[3]. Overall, carcinoid tumors may be found elsewhere in the body, most commonly in the appendix, followed by rectum, ileum, lungs, bronchi, and stomach.[2] Pulmonary carcinoid tumors are thought to arise from the neuroendocrine cells of the bronchopulmonary epithelium.[4]

The 2004 World Health Organization classification of lung tumors groups pulmonary neuroendocrine tumors into four main tumor types: low-grade typical carcinoid tumors (TC), intermediate-grade atypical carcinoid tumors (AC), high-grade large cell neuroendocrine carcinomas, and small-cell lung cancers.[5,6] Immunohistochemical and morphologic features are currently utilized to establish a diagnosis and to distinguish between typical and atypical carcinoid tumors.[7] Early stage typical carcinoid tumors are associated with a good prognosis after surgical resection, with a 5-year survival of ~88% versus ~50% for atypical carcinoid tumors.[1]

Complete surgical resection of the tumor with regional and mediastinal lymph node dissection currently remains the treatment of choice for cure in patients able to tolerate surgery. In those patients with tumors not amenable to surgical resection or with systemic metastatic disease, chemotherapeutic options have failed to succeed with low response rates of ~20% or less.[8–11] This suggest that an acceleration of preclinical research to better understand the biology of these tumors is required to facilitate identification of novel targeted agents and improve chemotherapeutic options for advanced tumors.

Carcinoid cell line resources to study the biology of pulmonary carcinoid tumors are currently very limited. Since the original description of four carcinoid cell lines by the NCI-Navy Medical Oncology Branch cell line bank, NCI-H292 has been reclassified as a mucoepidermoid pulmonary carcinoma.[12] NCI-H727, classified as a typical carcinoid tumor, is clinically inconsistent with the biology of a typical carcinoid tumor. It was derived from a stage 3A right upper lobe lung mass from a 65-year old white female, 60 pack-years smoker on April 24, 1984. She had chemotherapy and radiation therapy and died 4 months later (August 19, 1984) from brain metastases.[13] NCI-H727 grows as an adherent monolayer and harbors the G12V K-ras mutation. Given the highly unusual clinical outcome of this patient from which this cell line was derived and the presence of K-ras mutation, we believe that

NCI-H727 is likely a non–small-cell lung cancer (NSCLC) with neuroendocrine features instead of a typical carcinoid tumor. Up to 30% of NSCLC in patients who smoke harbor K-ras mutations, while we have found 0/31 K-ras mutations in 24 TC and 7 AC tumors tested to date.[14] NCI-H720 is derived from a lung tumor (stage not reported) from a male (age and race not reported) and is a presumed atypical carcinoid tumor.[13] NCI-H720 grows as a cell suspension in HITES medium supplemented with 5% fetal bovine serum. NCI-H835 is a presumed typical carcinoid tumor from a 48-year old black nonsmoking female and was established as floating cells in ACL-4 medium.[13] Patient treatment and follow-up was not reported for either of NCI-H720 or NCI-H835. The currently available cell lines unfortunately lack detailed clinical and histologic information. This may explain why a small number of in vitro studies have failed so far in translating to clinical application.[15] For these reasons, this study aimed to create cell lines as necessary research resources for advancing understanding of the biology and treatment response of pulmonary carcinoid tumors. These resources will provide the foundation for functionally dissecting cancer pathways and testing novel targeted agents for improved therapies.

MATERIALS AND METHODS

Establishment of Cell Lines

The Mayo Clinic College of Medicine Institutional Review Board approved this study. Three patients scheduled for resection of suspected primary bronchopulmonary carcinoid tumors were consented for cell line generation. Tumors were reviewed and confirmed based on the 2004 WHO classification by a lung pathologist experienced in the assessment of neuroendocrine lung cancers. Tissue from the resected typical carcinoid tumors harvested at the time of surgery was dissected from surrounding tissue and mechanically homogenized into a cell suspension. Cells were filtered through a 100 micron filter to disperse individual tumor cells and centrifuged twice at 1000 rpm for 10 minutes to remove debris. Cells were plated in RPMI 1640 + 10% FBS or DMEM + 10% FBS medium and incubated at 37°C and 5% CO_2 with daily examination of the plates. Medium was changed every 2–7 days based on observation of the cells and progression of the culture. Cells were passaged using trypsin dispersion when the plates became 75% or more confluent.

Immunohistochemistry

Carcinoid cell lines were examined for expression of neuroendocrine markers synaptophysin (SYP) and chromogranin A (CGA). After cytospin of 75,000 cells in 100 µl of medium onto a charged slide for 6 minutes at 600 rpm, the slides were air-dried for 4 hours at room temperature and acetone-fixed for 20 min at -20°C before blocking for 2 hours at 4°C with 10% horse serum. Thereafter the slides were incubated for 2 hours at 4°C with anti-SYP 1:1000 (rabbit) and anti-CGA 1:100 (mouse) in PBS + 0.2% Triton x-100. After washing the slides they were incubated at room temperature for 30 minutes in the dark with 1:200 dilution of anti-mouse rhodamine. Incubation followed for 30 minutes in the dark with 1:1000 dilution of anti-rabbit FITC after washing the slides again. After removal of the salts, the air-dried slides were stained with DAPI and covered.

The resected tumor specimens were stained with hematoxylin and eosin (H&E), SYP, and CGA. The stained sections were then photographed.

Western Blotting

Carcinoid cells were harvested with lysis buffer containing 1% Nonidet 40, 150 mM NaCl, 50 mM HEPES, 2m MEDTA, 3 mM EGTA, and 50 mM β-glycerophosphate with protease inhibitors pepstatin, leupeptin, aprotinin (10 µg/ml), PMSF (2 mM), and NaF (50 mM). Protein concentration was measured using the BCA protein assay reagent (Pierce Biotechnology, Rockford, IL). 100 µg of each protein lysate was separated on a long 10% SDS-PAGE gel for 16 hours at 120 volts and transferred to PDVF membrane. Nonspecific sites were blocked with TTBS containing 5% milk before addition of the primary antibodies. Primary antibody for CGA (Chemicon, Billerica, MA) was used at 1:500 dilution, followed by application of secondary antibody antimouse HRP conjugate (1:1000) dilution. Bound antibodies were detected with enhanced chemiluminescence (Amersham Bioscience, Piscataway, NJ).

Cell Proliferation

The CyQUANT NF assay is based on measurement of cellular DNA content via fluorescent dye binding. Because cellular DNA content is highly regulated, it is closely proportional to the cell number. The CyQuant NF protocol requires aspiration of growth medium (for adherent cells), replacement with dye binding solution, incubation for 30–60 minutes, and then measurement of fluorescence in a microplate reader. Cells were plated in quadruplicate in a 96-well microplate at a density of 5000 cells per well. At various time points the growth medium was removed and washed with 1× PBS. 100 µl of 1× dye binding solution prepared according to protocol (Invitrogen Grand Island, NY) was aliquoted into each microplate well. The covered microplate was incubated at 37°C for 60 minutes. This incubation period is required for equilibration of dye–DNA binding, resulting in a stable fluorescent endpoint. The fluorescence intensity was measured using a Fluoroskan Ascent fluorescence microplate reader (Thermo Scientific, Waltham, MA) with excitation at 485 nm and emission detection at 538 nm.

Soft Agar Assays for Colony Formation

Six-well plates were layered with media containing 0.8% agar to create a bottom layer. The fluorescence intensity was measured using a Fluoroskan Ascent fluorescence microplate reader (Thermo Scientific) with excitation at 485 nm and emission detection at 538 nm. Visible colonies greater than 50 microns were counted by light microscopy. All conditions were performed in triplicate.

Mouse Xenograft Model

Study approval was obtained from the Mayo Clinic Institutional Animal Care and Use Committee (IACUC).