Identification of Cancer Stem Cells in Human Gastrointestinal Carcinoid and Neuroendocrine Tumors

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BACKGROUND & AIMS: Metastatic gastrointestinal neuroendocrine tumors (NETs) frequently are refractory to chemotherapy. Chemoresistance in various malignancies has been attributed to cancer stem cells (CSCs). We sought to identify gastrointestinal neuroendocrine CSCs (N-CSCs) in surgical specimens and a NET cell line and to characterize novel N-CSC therapeutic targets. **METHODS:** Human gastrointestinal NETs were evaluated for CSCs using the Aldefluor (Stemcell Technologies, Vancouver, Canada) assay. An in vitro, sphere-forming assay was performed on primary NET cells. CNDT2.5, a human midgut carcinoid cell line, was used for in vitro (sphere-formation) and in vivo (tumorigenicity assays) CSC studies. N-CSC protein expression was characterized using Western blotting. In vivo, systemic short interfering RNA administration targeted Src. **RESULTS:** By using the Aldefluor assay, aldehyde dehydrogenase–positive (ALDH+) cells comprised 5.8% ± 1.4% (mean ± standard error of the mean) of cells from 19 patient samples. Although many primary cell lines failed to grow, CNDT96 ALDH+ cells formed spheres in anchorage-independent conditions, whereas ALDH- cells did not. CNDT2.5 ALDH+ cells formed spheres, whereas ALDH- cells did not. In vivo, ALDH+ CNDT2.5 cells generated more tumors, with shorter latency than ALDH- or sham-sorted cells. Compared with non-CSCs, ALDH+ cells demonstrated increased expression of activated Src, Erk, Akt, and mammalian target of rapamycin (mTOR). In vivo, anti-Src short interfering RNA treatment of ALDH+ tumors reduced tumor mass by 91%. **CONCLUSIONS: CSCs are present in NETs, as shown by in vitro sphere formation and in vivo tumorigenicity assays. Src was activated in N-CSCs and represents a potential therapeutic target in gastrointestinal NETs.**

Keywords: Carcinoid Tumors; Cancer Stem Cells; Src Family Kinase Inhibitor PP2; Neuroendocrine Tumors.

BASIC AND TRANSLATIONAL AT

Cancer stem cells (CSCs) typically comprise 1%–5% of the total tumor cell population, although some malignancies such as breast cancer (11%–35%) and glioblastoma (5%–30%)[1] possess a larger population. This cell population is considered primarily responsible for tumor initiation, growth, and metastasis.[2–5] According to the CSC theory, CSCs have properties similar to their non-malignant stem cell counterparts: self-renewal, anchorage-independent growth, and the potential for differentiation into heterogeneous tumors.[6] CSCs are thought to be chemoresistant and thus are spared by cytotoxic therapy; CSCs are thereby enriched in the residual tumor and subsequently mediate tumor recurrence.[5,7–11]

Gastrointestinal neuroendocrine tumors (NETs), including carcinoid tumors, are thought to originate from enterochromaffin cells[12] and are highly resistant to chemotherapy.[13] We hypothesized that NETs contain a subpopulation of cells termed *neuroendocrine cancer stem cells* (N-CSCs) that possess activated signaling pathways that could be exploited as therapeutic targets.

One major difficulty in investigating the mechanisms of NET growth is the limited number of gastrointestinal NET cell lines.[14,15] Our laboratory developed a midgut carcinoid cell line[15] and we used this cell line to identify and characterize a cell population enriched for N-CSCs using the Aldefluor assay. In addition, we used freshly isolated tumor cells from 14 midgut and 5 pancreatic NET surgical specimens to investigate a CSC population in NET tumors. Finally, we characterized mediators that can be targeted by available therapeutics and found the Src and mammalian target of rapamycin (mTOR) pathways to be activated. Because the mTOR inhibitor everolimus already has been validated in clinical trials[16–18] we chose to target Src because agents are available for the transition to clinical studies.

Materials and Methods

Human Tissue Specimens and Cell Lines

Patients undergoing NET resection at the University of Texas MD Anderson Cancer Center were identified. After informed consent was obtained under an Institutional Review

Abbreviations used in this paper: ALDH, aldehyde-dehydrogenase; CSC, cancer stem cell; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine; FACS, fluorescence-activated cell sorting; mTOR, mammalian target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5–diphenyltetrazolium bromide; N-CSCs, neuroendocrine cancer stem cells; NETs, neuroendocrine tumors; PBS, phosphate-buffered saline; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; siRNA, short interfering RNA.

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Board–approved protocol, a portion of resected tumor was harvested under sterile conditions in the pathology suite and placed on ice in Dulbecco's modified Eagle medium supplemented with F12 solution (DMEM/F12; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum, with care to avoid contaminating adjacent normal tissue. The specimen was transported to our laboratory, mechanically dissociated with sterile scalpels, and digested for 1 hour with 10 mg/mL type II collagenase (Cell Isolation Optimizing System; Worthington Biochemical Corp., Lakewood, NJ) in fresh DMEM/F12 media with 10% fetal bovine serum. The resulting single-cell suspension generated primary NET cultures.

The human midgut carcinoid cell line CNDT2.5 was developed in our laboratory.[15] Further examination of the CNDT2.5 cell line by short tandem repeat DNA analysis did not match the original patient tissue; however, because it strongly displays many NET features, it is considered to behave in a sufficiently NET-like manner for research purposes.[19] Cell lines were cultured in DMEM/F12 supplemented with 10% fetal bovine serum, penicillin-streptomycin, vitamins, sodium pyruvate, L-glutamine, and nonessential amino acids, at 37°C in 5% CO_2 and 95% air, as previously described.[20] All in vitro experiments were performed at 60%–80% confluence.

Identifying N-CSCs Using the Aldefluor Assay

An Aldefluor kit (Stemcell Technologies, Vancouver, CA) was used to isolate the population of cells with high aldehyde dehydrogenase (ALDH) enzymatic activity.[21,22] Cells were suspended in phosphate-buffered saline (PBS) after either trypsinization from in vitro culture or mechanical dissociation from surgical specimens and filtered through sterile 40-μm membranes to form single-cell suspensions. Red blood cells in the surgical samples were lysed using a red blood cell lysis buffer (eBioscience, Inc, San Diego, CA). All samples were subjected to the Aldefluor assay according to the manufacturer's protocol. The cells were resuspended in Aldefluor assay buffer containing ALDH substrate (BAAA, 1 μmol/L) and incubated at 37°C for 30 minutes. As a negative control, an aliquot from each sample was treated with 50 mmol/L diethyl-aminobenzaldehyde, a specific ALDH inhibitor. The fluorescently labeled product, BODIPY-aminoacetate, was produced by cells expressing the ALDH enzyme, and quantified using fluorescence-activated cell sorting (FACS) with the fluorescein isothiocyanate/FL1 channel and the diethyl-aminobenzaldehyde–treated cells as a negative control. Only the brightest 0.5%–1% of ALDH-positive (ALDH+) cells and most dim 0.5%–1% of ALDH-negative (ALDH-) viable cells were sorted for further in vitro and in vivo studies. Control cells (sham-sorted) were generated by subjecting parental cells to flow cytometry without any sorting markers. ALDH+, ALDH-, and sham-sorted cell viability was assessed by trypan blue staining and an automated cell counter.

Sphere-Forming Assay

ALDH+, ALDH-, and sham-sorted cells from fresh surgical specimens were cultured in 6-well, ultra-low-attachment plates (Corning Life Sciences, Lowell, MA) at a density of 10,000 cells per well because the overall low viability of primary cells precludes the use of single-cell culture techniques. Sorted CNDT2.5 cells were plated in 96-well, ultra-low-attachment plates (Corning Life Sciences) at a density of 1 viable cell per well. Cells were grown in cancer stem cell media consisting of DMEM (Invitrogen) supplemented with B27 Serum-Free Supplement (1:50; Invitrogen), 20 ng/mL epidermal growth factor, and 20 ng/mL basic fibroblast growth factor (both from R&D Systems, Minneapolis, MN) at 37°C and 5% CO_2. Fresh medium was added every 3–4 days and the formation of free-floating spheres was monitored. The experiment was terminated at 21 days and the development of any spheres (>50 μm) was quantified. The experiment was performed in triplicate.

Western Blotting

Whole-cell protein samples were isolated when cells reached 70%–80% confluence. Cells were solubilized in 20 mmol/L Tris-HCl (pH 8.0), 137 mmol/L NaCl, 1% Triton X-100, 1 mmol/L Na_3VO_4, 2 mmol/L ethylenediaminetetraacetic acid, and 1 complete Mini Protease Inhibitor Cocktail Tablet (Roche Diagnostics, Indianapolis, IN) per 10 mL of lysis buffer. Cell lysates were separated on sodium dodecyl sulfate–polyacrylamide gels at an 8%–15% concentration (determined by the target protein size) and transferred to polyvinylidene difluoride membranes (GE Healthcare, Piscataway, NJ). Membranes were probed with primary antibodies overnight at 4°C, washed 3 times for 10 minutes in Tris-buffered saline with 0.1% Tween-20, and probed with secondary antibody for 1 hour at room temperature. After incubation and 3 washes, immunostained proteins were detected using a chemiluminescence kit (Thermo Scientific, Waltham, MA). To confirm equal loading, membranes were re-probed with vinculin antibody.

Antibodies

The following primary antibodies were used for Western blotting: anti-pS2448–mTOR, anti-mTOR, anti-pS473–Akt, anti-Akt, anti-pY416–Src (all from Cell Signaling Technology, Danvers, MA), anti-Src (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Erk1/2 (Oncogene Science, Cambridge, MA), and antivinculin (Sigma-Aldrich, St. Louis, MO).

In Vivo Tumorigenicity Study

All animal studies were conducted under protocols approved by the institutional Animal Care and Use Committee. Male nude mice, 6–8 weeks old, were purchased from the National Cancer Institute at Frederick Center for Cancer Research (Frederick, MD) and maintained under specific pathogen-free conditions. All animal experiments met the requirements of the University of Texas MD Anderson Cancer Center Animal Care Facility and National Institutes of Health guidelines on animal care and use. We evaluated in vivo tumorigenicity of ALDH+ cells by subcutaneous injection of ALDH+, ALDH-, or sham-sorted CNDT2.5 cells, at various inoculums (100, 1000, 10,000, or 100,000 cells), suspended in 50 μL PBS mixed with 50 μL of basement membrane extract (Trevigen, Gaithersburg, MD) into right flanks of nude mice (5 per group). Tumors (firm masses larger than the original volume of injection, determined by a blinded observer) were monitored weekly for 12 weeks, at which point the mice were killed, tumors were harvested, and H&E staining was performed to confirm the characteristic CNDT2.5 morphology, using standard procedures.[23,24] This experiment was repeated 3 times.

In Vitro Src Inhibition

CNDT2.5 cells were plated in 10-cm plates (Corning Life Sciences) and allowed to adhere overnight. The next day, each subpopulation was treated with nothing, dimethyl sulfoxide (DMSO), or 10 μmol/L 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2) for 48 hours in 10% fetal bovine serum, DMEM/F12 media. After 48 hours, cells were harvested and subjected to the Aldefluor assay to evaluate the percentage of ALDH+ cells.