The Development and Characterization of a Human Midgut Carcinoid Cell Line


Abstract

**Purpose:** Gastrointestinal neuroendocrine tumors (NET) are rare heterogeneous tumors that hypersecrete neuropeptides. The scarcity of good gastrointestinal NET models has limited the ability to study potential therapeutic agents. We describe and characterize the establishment of a human midgut carcinoid tumor cell line carcinomaum 2 (CNDT2).

**Experimental Design:** Tumor cells (CNDT2) were isolated from a liver metastasis from a patient with a primary ileal carcinoid. After 9 weeks in culture, the cells were plated in soft agar, and cells from a single colony were put back in culture (CNDT2.1). Those CNDT2.1 cells were injected s.c. into nude mice. Cells were isolated from a single resultant tumor (CNDT2.5), cultured, and characterized by electron microscopy, reverse transcription-PCR, serotonin enzyme immunoassay, Western blotting, and immunohistochemical analysis for NET markers and potential therapeutic targets.

**Results:** CNDT2 cells grew in monolayers in vitro, formed colonies in soft agar, and formed tumors in mice. Electron microscopy revealed round, pleomorphic, electron-dense neurosecretory granules characteristic of NETs. Tumor xenographs exhibited the appearance of NETs with small "salt-and-pepper" nuclei on H&E staining and chromogranin A, synaptophysin, and CD56 on immunohistochemical staining. CNDT2.5 cells produced serotonin and expressed insulin-like growth factor receptor-I, platelet-derived growth factor receptor-β, vascular endothelial growth factor receptor-1, cMET, epidermal growth factor receptor, neuropilin-1, and somatostatin receptors 1 to 5. Cytogenetic analysis revealed the presence of deletions at 2p and 6q and numerous translocations.

**Conclusion:** The establishment of this human midgut carcinoid tumor cell line may serve as a useful model system for studying cell biology and novel targeted agents in preclinical models.

Neuroendocrine tumors (NET) arise from cells derived from the embryonic neural crest, neuroectoderm, and endoderm. NETs occur at numerous anatomic sites, including the bronchial mucosa, thyroid, parathyroid, and adrenal glands; however, most occur within the gastroenteropancreatic axis (1). Gastroenteropancreatic NETs are classified as carcinoids (2) and originate from dispersed neuroectodermal cells, such as the enterochromaffin-like (Kultschitzky) cells of the intestine (3). They can produce a variety of peptides and hormones, including neuron-specific enolase, synaptophysin, chromogranin A, growth hormone, pancreatic polypeptide, calcitonin, growth hormone–releasing hormone, bombesin, adrenocorticotropin, hormone, glucagon, histamine, catecholamines, prostaglandins, gastrin, and insulin (3, 4). Tumors of the gastroenteropancreatic axis can be further subclassified according to their site of origin: foregut (carcinoid of the bronchus, lung, thymus, stomach, first portion of the duodenum, and pancreas), midgut (second portion of the duodenum, jejunum, ileum, appendix, and ascending colon), or hindgut (transverse colon, descending colon, and rectum; ref. 1).

The incidence of carcinoid tumors in the United States is approximately 2 per 100,000 individuals per year (3, 4). The overall 5-year survival rate for patients with any carcinoid tumor, regardless of its site, is ~67% (5). Carcinoids tend to be slow-growing, indolent tumors in comparison with adenocarcinomas. However, if unresectable, these tumors pose a tremendous challenge for treatment, as they are typically resistant to systemic therapy (2, 3). Clinically, carcinoids present with either local manifestations (mass, bleeding, and obstruction) or systemic symptoms (flushing, sweating, diarrhea, and bronchospasm). The classic carcinoid syndrome with flushing, diarrhea, abdominal pain, valvular heart disease, telangiectasia, wheezing, and pellagra is caused by an excess of biogenic amines, peptides, and other factors in the circulation (4, 6). However, their behavior and clinical presentation vary depending on their site of origin. Foregut carcinoids are typically argentaffin negative, have a higher incidence of MEN-1 gene mutations and deletions in chromosome 11, and tend to produce bioamines such as gastrin, glucagon, or insulin. Midgut carcinoids are typically argentaffin positive,
have a higher incidence of deletions in chromosome 18, and tend to produce bioamines such as serotonin and bradykinin (2, 3, 7-12).

Currently, very few models are available to study NETs, and still fewer are specific to carcinoid tumors. A few murine cell lines have been generated, but most are foregut carcinoids. Two of the most stable and widely used human NET cell lines, which may be a more realistic model for studying human disease, are also foregut tumor cell lines: BON, a human pancreatic carcinoid cell line, and H727, a human pulmonary carcinoid cell line (13, 14). However, the characteristics and behavior of NETs vary significantly according to their site of origin (2, 3, 7-12). Foregut NETs are both phenotypically and genotypically distinct from midgut carcinoid tumors. Attempts have been made to develop a stable carcinoid cell line that will grow in vitro and in vivo, but success has been rare (15-19).

The lack of adequate models for human NETs, particularly midgut and hindgut tumors, prompted us to seek new models that could be used to study signaling pathways and to develop targeted agents for the treatment of these tumors. We recently established a human carcinoid cell line isolated from an ileal carcinoid liver metastasis. In this article, we describe the development and characterization of this human midgut carcinoid cell line.

Materials and Methods

Tissue harvest. The source of the cells was resected malignant hepatic tissue from a 58-year-old woman with an ileal carcinoid and liver metastasis. Informed consent for use of residual tissue was obtained from the patient before surgery according to a protocol approved by the institutional review board of The University of Texas M. D. Anderson Cancer Center. The tumor tissue was harvested in a pathology suite, with care taken to maintain sterility, and placed in DMEM with 10% fetal bovine serum (FBS). The tissue was then minced with a scalpel, digested overnight with 660 ng/mL type II collagenase (Cell Isolation Optimizing System kit, Worthington Biochemical Corp.), and replated in fresh DMEM with 10% FBS the next morning. The supernatant with solid tissue remnants was removed 24 h later and replaced with fresh medium supplemented with nerve growth factor (10 ng/mL) and insulin-like growth factor (IGF)-1 (10 ng/mL), which was used for the first four passages. Cells were passaged at 50% to 75% confluence. Cells were passaged eight times and then plated in soft agar as a surrogate marker of tumorigenicity [tumorigenicity was subsequently confirmed in a murine model (see below)]. The resulting cell line was labeled carcinoid tumor 2 (CNDT2). These multiple in vitro passages and harvest after growth in soft agar were done to allow for loss of nontransformed cells.

When colonies formed in the soft agar, a single colony was harvested with a pipette tip and placed back into culture (CNDT2.1 cells). After being passaged 14 times, the CNDT2.1 cells were injected s.c. into nude mice to determine their tumorigenicity. The resulting s.c. tumors were reseeded under sterile conditions, and a portion was processed for immunohistochemical analysis and another portion was minced and digested as described above and placed back into tissue culture. Subsequent characterization of the cell line was done on cells derived from a single non-Matrigel tumor xenograft, which we named CNDT2.5 cells (Fig. 1).

Culture conditions. The human colorectal cancer cell line HT29 and human umbilical vascular endothelial cells (HUVEC) were obtained from the American Type Culture Collection. The human pancreatic NET cell line BON, derived from a lymph node metastasis, was provided by Dr. Kjell Oberg (Section for Endocrine Oncology, Department of Medical Sciences, University Hospital, Uppsala, Sweden) to J. Yao. CNDT2, CNDT2.1, and CNDT2.5 cell lines were developed as described above. With the exception of HUVECs, cell lines were cultured in DMEM supplemented with 10% FBS, penicillin-streptomycin, vitamins, sodium pyruvate, l-glutamine, nonessential amino acids (Life Technologies), and HEPES buffer (MP Biomedicals) at 37 °C in 5% CO2 and 95% air. The medium for the BON and CNDT2 cells was also supplemented with nerve growth factor (10 ng/mL). HUVECs were cultured in DMEM supplemented with 15% FBS and basic fibroblast growth factor (5 ng/mL). In vitro experiments were done when cells were at 50% to 70% confluence.

Cell counts. The growth rate of CNDT2.5 cells was evaluated with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as follows. Cells (1.5 × 104) were plated in 96-well plates in DMEM with 10% FBS overnight, and the medium was changed the next morning to DMEM with 1% FBS. All assays were done in quadruplicate. After 24, 48, or 72 h, 2 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich) in PBS was added (50 µL/well), and plates were incubated at 37 °C for 2 h. At that time, the medium and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were removed, DMSO was added for 10 min, and absorbance was measured at 570 nm. Doubling time was determined from these time points.

Colony formation in soft agar. To confirm the malignancy of the CNDT2 cells, we tested their survival and anchorage-independent growth in a colony formation assay as follows. Six-well plates were filled with semisolid medium containing MEM, 1% Bacto agar, 10% FBS, and penicillin-streptomycin, and cells in a semisolid medium (MEM with 0.5% Bacto agar, 10% FBS, and penicillin-streptomycin) were added at 400 per well. Additional medium (MEM with 10% FBS) was added to the wells after 2 days to keep the agar moist, and 100 mL of fresh medium was added to each well every 3 days thereafter. After incubation for 21 days at 37 °C, colonies larger than 50 μm in diameter were counted under a dissecting microscope at ×40 magnification. A single colony was then selected, harvested with a pipette tip, and placed back into culture, as described above, leading to the development of the cell line CNDT2.1.

Fig. 1. Algorithm showing the derivation of the CNDT2 cell lines.
The CNDT2 sublines were plated in the soft agar in a similar manner to test their anchorage-independent growth. Seven hundred cells of each cell line were plated in a six-well plate. The colonies that formed after 21 days were counted and averaged.

**Subcutaneous xenograft model.** Five 6-week-old male nude mice were purchased from the Animal Production Program of the National Cancer Institute’s Frederick Cancer Research and Development Center (Frederick, MD) and housed and maintained in accordance with the standards of the M. D. Anderson Cancer Center Institutional Animal Care and Use Committee.

CNDT2.1 cells were grown to 80% confluence and trypsinized, and >90% cell viability was confirmed by trypan blue exclusion. All five mice were injected s.c. with CNDT2.1 cells (2 × 10⁶) in 200 μL of HBSS in the right flank and with CNDT2.1 cells (2 × 10⁷) in 200 μL of Hank’s solution mixed with 200 μL of BD Growth Factor-Infused Matrigel (BD Biosciences) in the left flank. Tumors were measured with calipers every 3 days after first becoming visible or palpable, and tumor volumes (in cubic millimeters) were calculated as (length × 0.5) × (width)². At 30 days after injection, or earlier if tumors exceeded 1.5 cm in diameter, the mice were anesthetized by i.p. injection of pentobarbital sodium (Nembutal; 90 mg/kg) and killed by cervical dislocation. The s.c. tumors were excised and weighed, and a portion of each was placed in 10% formalin (for paraffin embedding), snap frozen, and placed in OCT solution (Miles Laboratories/Bayer AG) in preparation for subsequent immunohistochemical analyses. Another portion of each tumor was placed in medium with 10% FBS, minced, digested with collagenase overnight, and plated in DMEM with 10% FBS the next morning to ensure that the cells would be able to grow when put back into culture. The cell line derived from a single non-Matrigel tumor, labeled CNDT2.5, was used for all subsequent analyses.

**Immunohistochemical staining.** Immunohistochemical staining for chromogranin A, synaptophysin, CD56, and cytokeratin was done with formalin-fixed, paraffin-embedded tissue samples of the CNDT2.1 tumor xenografts prepared as described in the previous paragraph. Archived human pancreatic parenchymal tissue with islets was used as a positive control for NET-specific markers, and human squamous epithelium was used as a positive control for cytokeratins. In addition, paraffin-embedded sections from the patient’s primary tumor and the CNDT2.1 tumor xenografts were stained with H&E by standard methods, and tissue morphology was evaluated under high-power magnification by a gastrointestinal pathologist (A.R.). Other paraffin-embedded tissue sections (5 μm thick) on positively charged slides were deparaffinized in xylene, hydrated in graded alcohol, and pretreated for antigen retrieval in 10 mmol/L citrate buffer (pH 6.0) for 1 h in a steamer at 98°C for 45 min. Staining of individual slides was done with either mouse anti-chromogranin A, rabbit anti-synaptophysin, mouse anti-CD56, or an anticytokeratin cocktail as described in the Antibodies section below. Staining intensity in the CNDT2.1 tumors was compared with that of adjacent nonneoplastic tissue and with that of the positive controls. Overexpression was considered to be present if the staining intensity was at least twice the staining intensity of adjacent nonneoplastic tissue, as determined by an experienced gastrointestinal cancer pathologist (A.R.).

**Antibodies.** Antibodies used for immunohistochemical staining and Western blot analyses were as follows: goat anti-vascular endothelial growth factor receptor (VEGFR)-1 (Oncogene Research Products); mouse anti-VEGFR-2 (Zymed Laboratories); mouse anti-neurophilin (NRP)-1, mouse anti-NRP-2, rabbit anti-VEGFR-3, rabbit anti-NRP-2, and rabbit anti-IGF receptor-1 α and β (Santa Cruz Biotechnology); rabbit anti-platelet-derived growth factor receptor (Oncogene); rabbit anti-epidermal growth factor receptor (Cell Signaling Technology); rabbit anti-cMET and rabbit anti-somatostatin receptor (SSTR) 1 (Santa Cruz Biotechnology); rabbit anti-SSTR2, anti-SSTR3, anti-SSTR4, and anti-SSTR5 (Affinity BioReagents); mouse anti-chromogranin A (Chemicon); rabbit anti-synaptophysin (DAKO); mouse anti-neuron-specific enolase (Ventana); mouse anti-CD56 (Invitrogen); cytokeratin cocktail (anti-AE1/AE3 (DAKO), anti-CAM5.2 (Becton Dickinson, BD Biosciences), anti-MNF116 (DAKO), and UCD/PR-10.11 (recognizes human cytokeratin types 8 and 18); Zymed); and mouse anti-vinculin (Sigma-Aldrich).

**Electron microscopy.** HT29 (negative control), BON (positive control), CNDT2.1, and CNDT2.5 cells were grown to 50% to 75% confluence on 25 cm² tissue culture dishes. Electron microscopy was done as described elsewhere (20). Briefly, samples were fixed with 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 mol/L cacodylate buffer (pH 7.3) for 1 h. After fixation, samples were washed, treated with 0.1% filtered cacodylate-buffered tannic acid, postfixed with 1% buffered osmium tetroxide for 1 h, and stained en bloc with 1% filtered uranyl acetate. Samples were then dehydrated in increasing concentrations of ethanol, infiltrated, embedded in PolyBed 812 medium, and polymerized at 70°C for 2 days. Ultrathin sections were cut with an Ultracut microtome (Leica), stained with uranyl acetate and lead citrate.
in a Leica EM stainer, and examined in a JEM 1010 transmission electron microscope (JEOL USA) at an accelerating voltage of 80 kV. Digital images were obtained by an AMT Imaging System (Advanced Microscopy Techniques Corp.). The cell lines were imaged at \( \times 10,000 \) and \( \times 50,000 \) magnifications. Neuroendocrine secretory granules were assessed in randomly selected images at similar magnifications from each cell line.

**Serotonin enzyme immunoassay.** The serotonin enzyme immunoassay (BioSource International) was used for the quantitative determination of serotonin in cell lysate. Serotonin (5-hydroxytryptamine), a biogenic amine, is a neurotransmitter and can be found in high concentrations in the enterochromaffin-like cells of the intestinal mucosa. Serotonin is a hallmark of NET and, more specifically, midgut carcinoids (10). Serotonin enzyme immunoassay was done on CNDT2.5, BON (positive control), and HT29 cells (negative control). Whole-cell protein lysates were isolated from cells at 70% to 80% confluence as described previously (21). Cell lysate (500 µg per cell line) was resuspended in 100 µL of buffer and subjected to enzyme immunoassay as per the manufacturer’s instructions.

**Chromosome banding analysis.** CNDT2.5 cell cultures at 75% confluence were harvested for air drying according to standard techniques (22). Routine Giemsa (G-banding) staining was used for cytogenetic characterization. A minimum of five 15-banded metaphase spreads was analyzed from photographs to determine the frequency of altered chromosomes. Abnormal chromosomes observed in 85% or more of the metaphase spreads were listed as marker chromosomes. Additional metaphase spreads were counted to establish the mean, median, and mode numbers of chromosomes.

**Reverse transcription-PCR.** RNA was extracted from CNDT2.5, BON, HT29, and HUVECs that had been plated and grown to 70% to 80% confluence and assayed as described previously (23). Briefly, PCR amplification of chromogranin A, synaptophysin, neuron-specific enolase, VEGF-A, VEGF-B, VEGFR-2, and β-actin was done under the following conditions: 95°C for 5 min, 35 cycles of 30-s denaturing at 95°C, 1 min of annealing at 57°C, and 1 min of extension at 72°C. Products were analyzed by electrophoresis of 20 µL of each PCR mixture in a 2% agarose gel, and bands were visualized by ethidium bromide staining. β-Actin was used as a loading control. The following primers were used: chromogranin A, 5‘-CTCCCTGTGAACGCCCTATGAAT-3’ (sense) and 5‘-ACATCCTTGAGTGAGTGCTTCC-3’ (antisense); synaptophysin, 5‘-TGGCAAGAAGCCGAGTGA-3’ (sense) and 5‘-CCACATGAAGGGGAACGAC-3’ (antisense); neuron-specific enolase, 5‘-GAGCGGGCAGTGGAAAGG-3’ (sense) and 5‘-GTGGCAGAAAAGG-3’ (antisense); VEGF-A, 5‘-CCGCCGAAGAGAGTGCTTC-3’ (sense) and 5‘-CGGCCCTGAGAGGATGACTGC-3’ (antisense); VEGF-B, 5‘-CCTTCCTTGATGCTTACATG3-’ (sense) and 5‘-GTGCTGGCTCAGCAGTC-3’ (antisense); VEGFR-2, 5‘-CATCACATCCCTGATGAATG-3’ (sense) and 5‘-CCAAATCATATTGAGG-3’ (antisense); and

![Fig. 3.](image)

Characterization of a Midgut Carcinoid Cell Line

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-actin, 5'-GGAGTACTTGCGCTCAGGAGGAGC-3' (sense) and 5'-GCACTCTTCCAGCCTTCCTTCCTG-3' (antisense).

**Western blot analysis.** Whole-cell protein was isolated from CNDT2.5, BON, HT29, and HUVECs at 70% to 80% confluence by using radioimmunoprecipitation assay "B" protein lysis buffer as described previously (21). The isolated protein was quantified by a commercially available modified Bradford assay (Bio-Rad Laboratories). Protein samples for Western blotting were prepared by boiling the isolated protein with denaturing sample buffer. The protein was then separated by SDS-PAGE on an 8% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore Corp.). The membranes were then blocked with TBS containing 0.1% Tween 20 and either 5% nonfat dry milk or 3% to 5% bovine serum albumin for 1 h and probed with the appropriate primary antibody overnight at 4°C. The next morning, the membranes were washed, incubated with the appropriate horseradish peroxidase–conjugated secondary antibody (Amersham Biosciences) for 1 h at room temperature, and washed again, and protein bands were visualized by using a commercially available enhanced chemiluminescence kit (Amersham Biosciences). To verify the accuracy of whole-cell lysate protein loading, membranes were incubated in stripping solution for 30 min at 65°C, washed, and reprobed with a vinculin antibody as a loading control as described previously (24).

**Results**

**Derivation of cell lines.** Cells that were isolated from a human liver metastasis from an ileal carcinoid through collagenase digestion formed monolayers on plastic culture dishes. Cells of various morphology began to be lost with each successive passage over the first 4 to 6 weeks; after eight passages over a 9-week period, cells that were still present and viable in culture (called CNDT2 cells) were plated in soft agar. Cells that formed colonies in the soft agar, CNDT2.1, were then isolated from a single colony, passaged, and injected s.c. in nude mice. Cells isolated and cultured from a single tumor xenograft were called CNDT2.5 cells. All three of these cell lines grew in monolayers on plastic culture dishes, with an approximate doubling time of 20 h.

**Colony formation in soft agar.** CNDT2 cells, derived as described above, formed three colonies on a six-well plate at 18 days after having been plated in soft agar. All three colonies were larger than 50 μm in diameter.

CNDT2.1 and CNDT2.5 cells were plated in soft agar, at 700 per well, in a six-well plate. After 21 days, each plated cell line formed ~90 colonies (>50 μm in diameter) per well. There was
Morphologic characteristics of CNDT2.1 xenografts. Light microscopic analysis of H&E-stained sections of the patient's primary tumor and the CNDT2.1 tumor xenograft specimens revealed round, salt-and-pepper nuclei and acidophilic cytoplasm characteristic of NETs (Fig. 2; refs. 3, 9). The patient had a low-grade carcinoid tumor of the terminal ileum invasive into the serosa of the ileum and with metastasis to lymph nodes, omentum, and liver. The patient's primary tumor had an insular growth pattern, with focal tumor necrosis, 1 mitosis per 10 high-power fields, slight nuclear pleomorphism, and the characteristic clumped chromatin pattern (“salt-and-pepper nuclei”; refs. 2, 3, 8, 9). A similar pattern was seen in the CNDT2.1 tumors with an increase in nuclear pleomorphism and mitoses (five to six mitoses per 10 high-power fields; Fig. 2).

NETs are characterized by the presence of numerous neuroendocrine secretory granules on electron microscopy (2, 25–27). BON cells, from a pancreatic NET, are known to exhibit the round, pleomorphic, electron-dense neurosecretory granules and were used as a positive control (27). HT29 cells, which are of epithelial origin, lack the neurosecretory granules and were a negative control.

Electron microscopic evaluation of the ultrastructural features of the BON, CNDT2.1, CNDT2.5, and HT29 cells revealed neurosecretory granules in 90% of the BON cells, 30% of the CNDT2.1 and CNDT2.5 cells, and none in the HT29 cells (Fig. 3). The neurosecretory granules present in our CNDT2.1 and CNDT2.5 cells had a similar appearance to the granules in BON cells with a slightly less prominent halo, findings similar to that observed by others examining midgut carcinoid tumors (7–9).

NET markers. Several NET markers are used to characterize NETs. Neuron-specific enolase is a glycoprotein released from injured neural tissue that may also be secreted by neoplasms derived from neuronal or NETs. Synaptophysin is a synaptic vesicle glycoprotein that is present in endocrine cells, the brain, the spinal cord, and adrenal glands. Chromogranin A is a protein that is secreted in conjunction with a wide variety of peptide hormones and neurotransmitters and is a precursor for several peptides with a wide range of biological activity. The CD56 antigen is a glycoprotein of the IgG superfamily that recognizes the neural cell adhesion molecule. Cytokeratins are intermediate filament keratins generally found in epithelial tissue and some NETs (10, 28).

Reverse transcription-PCR (RT-PCR) analysis was used to determine whether NET markers were present on the CNDT2.5 cells, with BON human pancreatic carcinoid cells used as a positive control. We found that the CNDT2.5 cells expressed mRNA for neuron-specific enolase and synaptophysin but not for chromogranin A (Fig. 4). Immunohistochemical staining was also used to characterize the expression of various NET markers on the CNDT2.1 tumor xenografts in comparison with nonmalignant human pancreatic parenchymal tissue specimens with islets and squamous epithelium. Staining revealed that the CNDT2.1 tumor xenografts expressed chromogranin A, synaptophysin, and CD56 (Fig. 5) but not cytokeratin (data not shown).

Serotonin production. One of the hallmarks of midgut carcinoids is the production of serotonin. We did a serotonin enzyme immunoassay on our CNDT2.5, BON (positive control), and HT29 cells (negative control). We showed that our CNDT2.5 cells produced ~20 ng of serotonin per 500 µg of...
cell lysate. The BON cells produced ~4 ng, and the HT29 cells did not produce any detectable serotonin.

**Cytogenetics.** G-banding/karyotyping confirmed that the CNDT2.5 cells were of human origin. Cells had 60 to 94 chromosomes (mode, 66). Seven marker chromosomes were found (two deletions and five translocations) and characterized as follows: M1 = deletion (6q); M2 = translocation (7q to 8q); M3 = translocation (10q to 13q); M4 = deletion (2p); M5 = translocation (8q to 14q); M6 = translocation (10p to 13p); and M7 = translocation (18q to unknown).

**Ligand and receptor characterization.** We used RT-PCR and Western blotting to evaluate the expression of common therapeutic targets, such as VEGF ligands, VEGFRs, and other potential therapeutic targets in CNDT2.5 cells, HT29 cells (positive control for VEGF-A, VEGF-B, VEGFR-1, NRP-1, NRP-2, cMET, IGF receptor, platelet-derived growth factor receptor, epidermal growth factor receptor, and SSTR1-SSTR5), and HUVECs (positive control for VEGFR-2 and VEGFR-3). RT-PCR showed that the CNDT2.5 cell lines expressed mRNA for VEGF-A and VEGF-B ligands (Fig. 6A) but did not express mRNA for VEGFR-2 (Fig. 6C). Western blotting showed that CNDT2.5 cells expressed VEGFR-1, NRP-1, cMET, IGF receptor-1, platelet-derived growth factor receptor, epidermal growth factor receptor, and SSTR1 to SSTR5 (Figs. 6B and 7) but not VEGFR-3 or NRP-2 (Fig. 6B).

**Discussion**

The identification, development, and testing of therapeutic agents for gastrointestinal NETs have been hampered by the lack of available models. We present here details of the development and characterization of a midgut carcinoid cell line. This cell line, which we named CNDT2, could grow in culture, form colonies in soft agar, form solid tumors s.c. in mice, and express numerous markers of NETs. The ability of our cell line to consistently grow in tissue culture provides an ideal model to study receptor mechanisms, secretory mechanisms, genetics, and basic cell biology of human midgut NETs.

Midgut carcinoid models that rely solely on maintaining a transplantable tumor in vivo are limited by their inability to grow in the controlled setting of tissue culture (17). The ability of our cell line to form colonies in soft agar offers indirect (in vitro) proof of its tumorigenicity. More importantly, the soft agar assay also served as a good initial screening method to select clones with increased tumorigenicity.

CNDT2 cells also formed tumors in vivo. Of the five mice tested, two mice developed tumors from cells mixed with Matrigel and two other mice developed tumors from cells injected without Matrigel. The inconsistency of tumor formation probably reflects heterogeneity of the tumor cell line population. Our attempt to refine the cell line by selecting the most aggressive and tumorigenic clones led to the isolation of the CNDT2.5 cell line. The ability of these cells to reliably form tumors in vivo enables preclinical studies to test antineoplastic agents on tumor derived from midgut carcinoid cells.

After establishment of the initial CNDT2 cell line, it was important to characterize the NET markers to confirm that our cell line was of NET origin. Our findings showed some discrepancy between expression of NET markers in the in vitro (CNDT2.5) and in vivo (CNDT2.1 xenograft) conditions. Specifically, CNDT2.5 cells expressed mRNA for synaptophysin and neuron-specific enolase, but not chromogranin A, but the CNDT2.1 xenografts expressed the protein forms of synaptophysin, chromogranin A, and CD56. This discrepancy could arise from either the presence of stimuli in the tumor microenvironment in vivo that increase the production of the various NET markers or the heterogeneity of the tumor cell population leading to variable phenotypic expression. Although cytokeratins may be present on some NETs, they are not present on all midgut carcinoids and are not considered a specific marker of NETs. In addition to these NET markers, we showed that our CNDT2.5 cells produced high levels of serotonin in comparison with BON cells. The production of serotonin is a marker of NETs and more specifically midgut carcinoids (2, 3, 10).

Structural and ultrastructural features are also important components of characterizing our cell line as a NET. The clear presence of round, pleomorphic, electron-dense neurosecretory granules on electron microscopy is thought to be pathognomonic for NETs (2, 25–27) and further confirmed that our cell line was a NET. The slightly varied appearance of our cell line to form colonies in soft agar offers indirect (in vitro) proof of its tumorigenicity.
Midgut carcinoids, such as the source of our CNDT2 cells, originate from the enterochromaffin-like cells of the intestine, which may vary in their neurosecretory granule production (25). In addition to the presence of these ultrastructural features, on H&E staining, the patient’s primary tumor and the CNDT2.1 tumor xenografts showed morphologic characteristics of NETs, such as a round, condensed chromatin pattern (salt-and-pepper nuclei) and an acidic cytoplasm along with an insular growth pattern (8, 9). The increase in nuclear pleomorphism and mitosis in the CNDT2.1 xenograft compared with the patient’s primary tumor is likely due to clonal selection (3).

Genetic analysis of CNDT2.5 confirmed its human origin and ruled out the possibility that the cells were a contaminant from another source. Other groups have used genomic hybridization to analyze midgut carcinoids. In one such study, 18q22-qter was shown to be lost in 67% of 18 midgut carcinoids (12); another study of 14 midgut tumors showed gains in chromosomes 17q and 19p in 57% of cases, losses in chromosome 18 in 43% of cases, and loss of 9p in 21% of cases (29). In our study, we found deletions at 2p and 6q and translocations from 7q to 8q, 10q to 13q, 8q to 14q, 10p to 13p, and 18q to unknown locus. The translocation at 18q in the CNDT2.5 cell line does coincide with the defects found on chromosome 18 in other studies (11, 12).

Currently, systemic treatments have only minimal effect on the natural history of gastrointestinal NETs (6, 30, 31). Given the lack of efficacy of conventional agents, recent developments in molecular therapeutics make the cell surface receptor status of NETs an important issue. Characterization of tyrosine kinase receptor status in particular was important in our model to characterize its basic biology and facilitate its use to develop preclinical models for targeted molecular therapeutics in NETs.

VEGF ligands and associated receptors have become important therapeutic targets in treating gastrointestinal malignancies, such as metastatic colorectal cancer. Anti-VEGF therapy has become useful for treating metastatic colorectal cancer and various other neoplasms (32–34). For this reason, we chose to examine the status of VEGF ligands, VEGFRs, and VEGF-associated receptors (NRP-1 and NRP-2), a major interest of our laboratory. We detected VEGFR-1 and NRP-1 on our cells by Western blotting, but we could not detect VEGFR-2 or VEGFR-3 or NRP-2 by either Western blotting or RT-PCR. The presence of the VEGFR-1 receptor and NRP-1 on the CNDT2 cell line agrees with findings from other gastrointestinal malignancies and indicates that the VEGF ligand and receptors may have a role in the pathogenesis of NETs (35–39).

We also evaluated CNDT2.5 cells for the expression of other potential therapeutic targets and showed that CNDT2.5 cells expressed IGF receptor, platelet-derived growth factor receptor, epidermal growth factor receptor, and cMET. All of these tyrosine kinase receptors are possible valuable targets that warrant investigation. We were also able to show the presence of SSTR1 to SSTR5 as expected.

In conclusion, we have developed a midgut carcinoid cell line capable of being propagated in tissue culture and in nude mice. Our CNDT2 cell line expresses the morphologic and molecular markers of a NET as well as numerous potential therapeutic targets. This cell line will be a valuable tool for investigating the tumor biology of midgut carcinoids and for developing preclinical therapeutic models to treat NETs.

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