Original Article

Establishment and characterization of a novel anorectal melanoma cell line derived from primary human rectal tumor

Running title: A novel anorectal cancer cell line

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Abstract

Background: Anorectal melanoma is a rare disease with poor prognosis and nonspecific symptoms, leading to difficulties in preoperative diagnosis. Here, we describe the establishment of MELS, a novel anorectal melanoma cell line derived from tumor resection of the rectum in a 40-year-old Japanese man.

Methods: Histological, electron microscopic, and immunohistochemical features of S-100, HMB-45, Melan-A, and NSE positivity in the patient's tumor were confirmed as typical of surgically resected anorectal melanoma.

Results: MELS cells are round or oval cells with sharp thorn-like protrusions on some or all of the cell membrane. They form irregular attached colonies with numerous floating cells in two-dimensional culture. Transmission electron microscopy revealed that some MELS cells possess cytoplasmic melanosomes. Immunocytochemically, MELS cells showed the same staining patterns as surgical tissues. MELS cells had lower growth rates than Caco-2 (a colon adenocarcinoma cell line) and A375 (a cutaneous melanoma cell line) cells. Oxaliplatin and irinotecan were more effective in MELS cells than in Caco-2 and A375 cells.

Conclusions: Prior to our study, no reports had provided detailed clinical information on anorectal melanoma cell lines. Thus, MELS cells should improve current knowledge on the biological behaviors of anorectal melanoma, while providing a novel platform for examining the effects of anorectal therapies.

Keywords; anorectal melanoma, novel cell line, colorectal cancer, electron microscopy, HMB-45

Introduction

Melanoma can arise at any site where melanocytes exist, although the most common site of melanoma is the cutaneous, followed by the ocular and mucous membrane site¹. Of the mucosal melanomas, anorectal melanoma (<25% prevalence) is the second most common¹, though it accounts for less than 1% of all melanomas and approximately 0.5–4% of rectal and anal tumors^{2–4}. However, incidence of this cancer is increasing in certain regions, such as the United States⁵. Five year survival rate of anorectal melanoma has a 5-year survival rate of only 10–20%^{6, 7}, very low compared with the 80% mean survival rate of cutaneous melanomas¹.

Currently, anorectal melanomas have no specific pathologic staging system⁸. A recent large retrospective study on this cancer therefore used the simplified three-tiered system for head and neck melanomas, which categorizes disease into clinically localized (Stage I), regional lymph node involvement (Stage II), and distant metastasis (Stage III)⁹. Researchers found that these stages correlated with outcome; the 5-year survival rates of anorectal melanoma at stages I, II, and III were 26%, 9.8%, and 0%, respectively¹⁰.

Diagnosis of anorectal melanoma is difficult because up to 80% of lesions lack obvious pigmentation, and up to 20% are histologically amelanotic; moreover, symptoms are usually nonspecific¹¹⁻¹³. Owing to this difficulty, extremely few anorectal melanoma cell lines have been reported. We are aware of SK-MEL-246 (Memorial Sloan Kettering Cancer Center) from a metastatic rectal tumor site in a patient with melanoma, but clinical information, including sex and age, is lacking.

In this study, we established a novel human anorectal melanoma cell line (MELS) derived from a 40-year-old Japanese man. In addition, we examined the immunohistochemical and electron microscopic features of this cell line, along with how it is affected by colorectal cancer drugs. To the best of our knowledge, this report is the first cell line established from primary rectal-derived melanoma cells.

Materials and Methods

Original tumor processing

The origin of MELS was a tumor resected from the rectum of a 40-year-old Japanese man, referred to Nippon Medical School Chiba Hokusoh Hospital in 2005 with

anal pain and bleeding. Digital examination revealed a hard mass, and a barium enema test demonstrated a filling defect in the rectum. Colonoscopy identified an ulcerated tumor, and biopsy of the lesion showed the proliferation of epithelioid cells with pleomorphic features. Abdominoperineal resection was performed to reveal a brown to black tumor (45 × 50 mm) with ulceration at the top. Tumor tissues exhibited vascular and lymph vascular invasion, as well as regional lymph node metastases. Immunohistochemical staining and transmission-electron-microscopic analysis of the resected tumor revealed characteristic features of melanoma. The patient was diagnosed with stage III anorectal melanoma with distant metastasis to the liver². Detailed clinical information was previously reported¹⁴.

With the written informed consent of the patient and his family, a portion of the tumor was processed for primary culturing to establish a rectal melanoma cell line. This study was conducted in accordance with the Declaration of Helsinki, 2013. The Ethics Committee and Institutional Review Board at the Nippon Medical School approved all procedures (registration no. 26-03, 2014).

Primary in vitro culture

After a rinse using sterile phosphate-buffered saline (PBS) supplemented with benzylpenicillin potassium (100 U/mL) and kanamycin sulfate, the tumor sample was cut into 1 mm fragments. Fragments were suspended in 20% fetal bovine serum (FBS; Nichirei Bioscience Inc., Tokyo, Japan) and seeded into 60 mm primary tissue culture dishes that were incubated in a humidified incubator at 37°C in 5% CO₂ for 4 h. Dishes were coated with 3–4 mL of Roswell Park Memorial Institute (RPMI)-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 15% FBS and antibiotics. This medium was replaced every 3 d. When fibroblast cell growth was observed, an essentially pure tumor cell population was extracted via differential trypsinization, followed by five to six passages. The new cell line was cultured for more than 60 passages in RPMI-1640 medium containing 10% FBS.

Immunohistochemical and immunocytochemical analyses

Paraffin-embedded tissue sections (3.5 µm) were immunostained using Histofine Simple Stain Max PO (R) or (M) kits (Nichirei Bioscience Inc., Tokyo, Japan). Sections were incubated with 0.3% hydrogen peroxide in methanol for 30 min, to block endogenous peroxidase activity. Slides were then incubated for 20 h at 4°C in 1:1000

rabbit anti-S-100 (Z0311; DAKO), 1:200 mouse anti-Pmel17/GP100 (HMB-45, M0634; DAKO), 1:200 mouse anti-Melan A (M7196; DAKO), 1:1000 mouse anti-NSE (M0873; DAKO), 1:150 mouse anti-cytokeratin (M0821; DAKO), or 1:2000 anti-chromogranin A (A0430; DAKO) antibodies. Bound antibodies were detected using Simple Stain Max PO (R) or (M) with diaminobenzidine tetrahydrochloride (DAB) as the substrate and counterstained with Mayer's hematoxylin for visualization. Negative controls were not stained with primary antibodies.

For immunocytochemical analysis, MELS cells were fixed using 4% paraformaldehyde/PBS for 10 min and treated with 0.2% TritonX-100/PBS for 5 min. Cells were incubated with primary and secondary antibodies, then stained as described for the tissue sections.

Human colon and melanoma cell lines

Caco-2, a conventional human colon cancer cell line, was obtained from Riken BRC Cell Bank (Ibaraki, Japan), and cultured in 10% FBS-supplemented RPMI-1640 media at 37°C in 5% CO₂. Human melanoma cell line A375 was obtained from American

Type Culture Collection (ATCC) and cultured under the same conditions in 10% FBS-supplemented DMEM.

Scanning electron microscopy (SEM)

MELS were fixed overnight with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C. Glutaraldehyde was then removed, and cells were washed with PBS. Cells were post-fixed with osmium tetroxide for 30 min to prevent sphere samples from collapsing during sample preparation. After complete dehydration via a graded ethanol series, spheres were suspended in 100% ethanol, air-dried, and covered with a platinum layer using an MSP-1S sputter coater (Shinku Device, Ibaraki, Japan). Cells were examined and photographed using a Phenom Pro desktop scanning electron microscope with reflective or secondary electrons (Thermo Fisher Scientific).

Transmission electron microscopy (TEM)

Rectal tumor tissues and MELS cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), then post-fixed for 1 h with 2% OsO₄ dissolved in distilled water. Samples were dehydrated using an ethanol gradient, embedded in Epon, and sliced into ultrathin sections using an ultramicrotome. Sections were then stained with uranyl

acetate and lead citrate for examination under a transmission electron microscope (H-7500; Hitachi High-Technologies, Tokyo, Japan).

Anti-drug resistance assays

Cells (3.0×10^3 per well) were plated in 96-well culture dishes containing growth medium. Each anti-cancer drug was administered at the indicated concentrations after 1 d. Four days later, cell growth rates were measured with ATP assays, performed using CellTiter-Glo[®] 2.0 (Promega, Madison, USA). Cell viability was calculated as percentage of luminescence in drug-treated cells versus in untreated control cells.

Statistical analyses

Data were analyzed in Easy R (EZR; Saitama Medical Center, Jichi Medical University, Saitama Japan), a GUI for R (The R Foundation for Statistical Computing, Vienna, Austria) that adds functions frequently used in biostatistics. Continuous variables were analyzed using the student's t-test. Significance was set at $P < 0.05$.

Results

Pathological features of surgically resected rectal tissues

Tumor cells in the rectum formed a solid structure (Fig. 1A). Cells possessed atypical nuclei and numerous atypical mitoses (Fig. 1B, arrows); most exhibited epithelioid features and partial-spindle appearance. Tissue sections had less than 10% of tumor cells, which contained small dark brown pigment localized to the cytoplasm (Fig. 1C). These were revealed to be Stage IV melanosomes under TEM (Fig. 1D, arrows).

Immunohistochemical analysis revealed that tumor cells were positive for S-100, HMB-45, Melan-A, and NSE, but negative for cytokeratin and chromogranin A (Fig. 2). Cytokeratin was localized in normal epithelial cells, while chromogranin A was localized in neuroendocrine cells of normal ducts. Based on these morphological characteristics, the tumor was diagnosed as a melanoma in the rectum.

Establishment of MELS

MELS was weakly attached to culture plates and formed irregularly shaped colonies (Fig. 3A), with sharp thorns at the periphery (Fig. 3B, arrows). Morphology was variable, including long protrusions (Fig. 3C, arrows) and a semi-circular shape with short protrusions on the cell margin (Fig. 3D, arrows). Observations under SEM showed that colonies comprised round to oval-shaped MELS cells (Fig. 4A, B), with peripheral cells

possessing protrusions of various lengths (Fig. 4C, D). Furthermore, TEM analysis showed that MELS cells had numerous short-to-long protrusions (Fig. 5A), and stage I and II premelanosomes were present in their cytoplasm, (Fig. 5B white and black arrows respectively). Immunocytochemical analysis indicated that MELS cells cultured in chamber slides were positive for S-100, HMB-45, Melan-A, and NSE, but negative for cytokeratin and chromogranin A (Fig. 6). Thus, established MELS cells retained properties of the original rectal melanoma tissues.

Viability of MELS cells after the addition of anti-cancer drugs

The ATP assays for comparing growth across MELS, Caco-2 (human colon adenocarcinoma line), and A375 (human cutaneous melanoma line) cells showed that growth rates were markedly lower in MELS cells than in Caco-2 and A375 cells (Fig. 7A). Next, we used viability assays to compare the effects of common anti-colorectal cancer drugs (oxaliplatin, fluorouracil [5-FU], and irinotecan) on the three cells types. After adding 10 μ M oxaliplatin, MELS cells had lower viability than in the other two cells. Increasing oxaliplatin concentration to 100 μ M resulted in lower viability for MELS and A375 cells compared with Caco-2 cells (Fig. 7B). Treatment with 100 μ M 5-FU also

caused lower viability in MELS and A375 cells than in Caco-2 cells (Fig. 7B). Finally, under both 10 and 100 μ M irinotecan treatment, MELS and A375 cells exhibited lower viability than Caco-2 cells (Fig. 7B).

Discussion

In this study, we performed preoperative diagnosis of anorectal melanoma using colorectal biopsy, among the most accurate ways to diagnosis this cancer¹⁵. We determined that the observed tumor was a poorly differentiated adenocarcinoma with solid proliferation. Additionally, we did not observe melanin in the biopsy sections, nor did we perform immunohistochemical and TEM analyses. We chose to prepare for establishing a cultured cell line before confirming our melanoma diagnosis.

The surgically resected rectal tissues showed typical immunohistochemical staining patterns for anorectal melanoma. In the established MELS cells, we observed Stage I and II premelanosomes using TEM (classifications are according to morphology and melanin synthesis¹⁶). Anorectal melanoma has fewer melanin-positive cases than cutaneous melanoma; accordingly, melanin was found in less than 10% of tumor cells in our sample.

A previous report using SEM showed that cutaneous melanoma cells possess numerous extensions on their surface, including microblebs, microvilli, and some lamella¹⁷. Several cutaneous melanoma protrusions were extremely long, slender, and twisted between neighboring cells. We observed that MELS cells form aggregated colonies, with cells exhibiting a variety of morphologies, such as semi-circular protrusions and very long, rod-like cytoplasm. In general, they were pleomorphic cell types with short to long protrusions on their surfaces. Corroborating our findings, previously published SEM data indicated that melanoma cell surfaces had numerous extensions, some with differentiations that suggested special function, although smooth-surfaced cells were also present¹⁷.

MELS cells were positive for melanoma-specific antigens, specifically anti-S-100, HMB-45, and Melan-A (also known as MART-1), while being negative for epithelial and neuroendocrine markers. Anti-S-100 protein is the most common immunohistochemical stain used for diagnosing anorectal melanoma, being highly sensitive to melanocytic differentiation¹⁸. HMB-45 and Melan-A are melanocyte-specific markers used for the diagnosis of melanoma¹⁹. In particular, the latter two antibodies are present in most

anorectal melanoma cases (94% and 93%, respectively)¹⁸. MART-1/Melan-A is reportedly required for the maturation of Pmel17 and HMB45 reacts with the matured Pmel17²⁰. The double positivity of MART-1/Melan-A and HMB-45 in MELS cells is reasonable.

Anorectal melanoma is characterized by aggressive behavior, poor outcome due to late diagnosis, and fast tumor growth in the rich vascular and lymphatic supply of the anorectal mucosa²¹. In this study, we found that MELS cells had a far lower proliferation rate than conventional colorectal cancer and cutaneous melanoma.

For patients with anorectal melanoma, treatments include surgery, radiotherapy, chemo-immunotherapy, and targeted therapy²². It is known that JAK mutation has been a major mechanism against anti-PD-L1 antibody in metastatic melanoma of the cutaneous²³. Management strategies for anorectal melanoma have been hampered by a lack of randomized trials. Tumors tend to be considerably resistant to radiotherapy and show a poor response to chemotherapy. Oxaliplatin and irinotecan are usually used for colorectal cancer treatments, but melanomas derived from colorectum are treated in the same manner as cutaneous melanomas. We evaluate the effectiveness of therapeutic drugs for

colorectal cancer in MELS cells and found that these anti-colorectal cancer drugs were effective for MELS. Further studies are needed to clarify the underlying mechanisms and the effectiveness of these drugs *in vivo*.

To the best of our knowledge, SK-MEL-246 is the only other cell line that has been derived from a human rectal melanoma cell line²⁴. However, clinical details of this cell line, including donor gender and age, are unknown. Moreover, the cell line was established from a metastatic rectal tumor site in a patient with melanoma. As far as we are aware, no reports are available concerning the immunohistochemical, electron microscopic, and genetic characteristics of SK-MEL-246 cells. Our study thus fills an important gap in anorectal melanoma research.

In conclusion, we established MELS, a new anorectal melanoma cell line derived from a 40-year-old Japanese man. This cell line expresses typical melanoma-specific antigens and possesses melanosomes in the cytoplasm. Further studies of MELS, including identification of genetic mutations, should help us better characterize anorectal melanomas and contribute to the development of anticancer drugs, immune therapy, and radiotherapy for treating this cancer.

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Disclosure Statement

The authors have no conflicts to declare.

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Figure legends

Figure 1. Histological and transmission electron microscopic (TEM) features of surgically resected rectal tumor tissues

Hematoxylin and eosin staining showing that melanoma cells formed solid tumors with atypical mitoses (arrows in B) and brown melanin pigment in their cytoplasm (A-C).

TEM revealed Stage IV melanosomes in the cytoplasm (D, arrows). Original magnification: A $\times 40$; B, C $\times 600$. D: Scale bar = 1 μm

Figure 2. Immunohistochemical analysis of surgically resected rectal tumor tissues

Rectal tumor cells were positive for S-100, HMB-45, Melan-A, and NSE, but negative for cytokeratin and chromogranin A. Original magnification: $\times 200$

Figure 3. Phase-contrast images of MELS cells

MELS cells were cultured in two-dimensional conditions. Cells formed irregular colonies with sharp protrusions (arrows in A and B). Morphology was varied, with long protrusions or a semi-circular appearance (arrows in C and D). A: Scale bar = 200 μm , B–D = 50 μm

Figure 4. Scanning electron microscopic (SEM) analysis of MELS cells

MELS cells formed grape-like colonies with short to long protrusions at the periphery (A-D).

Scale bar: A = 100 μm , B–D = 10 μm . A, B: reflective electrons, 10 kV; C, D: secondary electrons, 5 kV.

Figure 5. TEM analysis of MELS cells

MELS cells had short to long protrusions (A) and premelanosomes (B, Stage I: white arrows, Stage II: black arrows) in the cytoplasm. M: mitochondria, Scale bar: A = 2 μm , B = 500 nm

Figure 6. Immunocytochemical analysis of MELS cells

MELS cells were positive for S-100, HMB-45, Melan-A, and NSE, but negative for cytokeratin and chromogranin A. Original magnification: $\times 600$

Figure 7. Anti-cancer drug resistance assay in MELS, Caco-2, and A375 cells

ATP assays compared growth across MELS, Caco-2 (human colon adenocarcinoma cell line), and A375 cells (human cutaneous melanoma cell line) (A). Dose response (10 or 100 μM) of MELS, Caco-2, and A375 cells to oxaliplatin, 5-FU, and irinotecan was determined using ATP assays (B). $*p < 0.05$, $**p < 0.01$.

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