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A New Anorectal Melanoma Cell Line Derived from a Primary Human Rectal Tumor

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Background: Anorectal melanoma is a rare disease with a poor prognosis. Symptoms are often nonspecific, which complicates preoperative diagnosis. Here, we describe the establishment of MELS, a new anorectal melanoma cell line derived from resection of a rectal tumor in a 40-year-old Japanese man.

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

Results: MELS cells are round or oval and have sharp thorn-like protrusions on some or all cell membranes. The cells form irregular attached colonies with numerous floating cells in two-dimensional culture. Transmission electron microscopy revealed that some MELS cells have cytoplasmic melanosomes. Immunocytochemically, MELS cells and surgical tissues had the same staining pattern. 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: No previous report provided detailed clinical information on an anorectal melanoma cell line. Thus, MELS cells should improve our understanding of the biological characteristics of anorectal melanoma and provide a novel platform for examining the effects of therapies for anorectal melanoma.
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Key words: 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 cutaneous, followed by ocular and mucous membrane sites[1]. Anorectal melanoma (<25% prevalence) is the second most common mucosal melanoma[1], although it accounts for fewer than 1% of all melanomas and approximately 0.5-4% of rectal and anal tumors[2–4]. However, the incidence of anorectal melanoma is increasing in some regions, such as the United States[5]. Anorectal melanoma has a 5-year survival rate of 10-20%[6,7], which is much lower than the 80% mean survival rate for cutaneous melanomas[1].

There is no specific pathological staging system for anorectal melanomas[8]. A recent large retrospective study of anorectal melanoma used the simplified three-tier sys-

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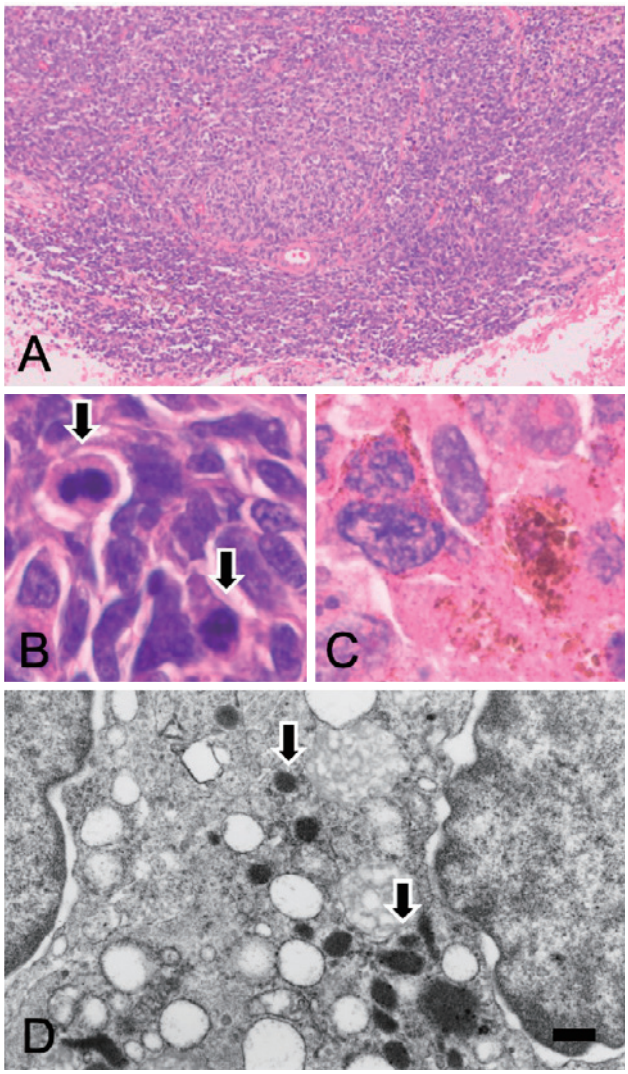

Fig. 1 Histological and transmission electron microscopic (TEM) features of surgically resected rectal tumor tissues
Hematoxylin and eosin staining showing that melanoma cells formed solid tumors with atypical mitoses (arrows in B) and brown melanin pigment in their cytoplasm (A-C). TEM revealed Stage IV melanosomes in the cytoplasm (D, arrows). Original magnification: A ×40; B, C ×600. D: Scale bar = 1 μm

tem for head and neck melanomas, which categorizes the disease as clinically localized (Stage I), regional lymph node involvement (Stage II), and distant metastasis (Stage III)[9]. These stages correlated with outcomes: the 5-year survival rates for stage I, II, and III anorectal melanoma were 26%, 9.8%, and 0%, respectively[10].

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 nonspecific[11–13]. Thus, 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 data, including sex and age, were not reported.

In this study, we established a new 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 and its response to colorectal cancer drugs. To our knowledge, this is the first report of a cell line established from primary rectum-derived melanoma cells.

Materials and Methods

Processing of the Original Tumor

MELS originated from a tumor resected from the rectum of a 40-year-old Japanese man who was 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 showed a filling defect in the rectum. Colonoscopy identified an ulcerated tumor, and a biopsy specimen of the lesion showed proliferation of epithelioid cells with pleomorphic features. Abdominoperineal resection revealed 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 features characteristic of melanoma. Stage III anorectal melanoma with distant metastasis to the liver was diagnosed[2]. Detailed clinical information was previously reported[14].

With the written informed consent of the patient and his family, a portion of the tumor was processed for primary culture 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 of the Nippon Medical School approved all procedures (registration no. 26-03, 2014).

Primary in Vitro Culture

After a rinse with sterile phosphate-buffered saline (PBS) supplemented with benzylpenicillin potassium (100 U/mL) and kanamycin sulfate, the tumor sample was cut into 1-mm fragments. The fragments were suspended in 20% fetal bovine serum (FBS; Nichirei Bioscience Inc., Tokyo, Japan) and seeded into 60-mm primary tissue culture dishes incubated in a humidified incubator at 37°C in 5% CO_2 for 4 h. Dishes were coated with 3-4 mL of Roswell Park Memorial Institute (RPMI)-

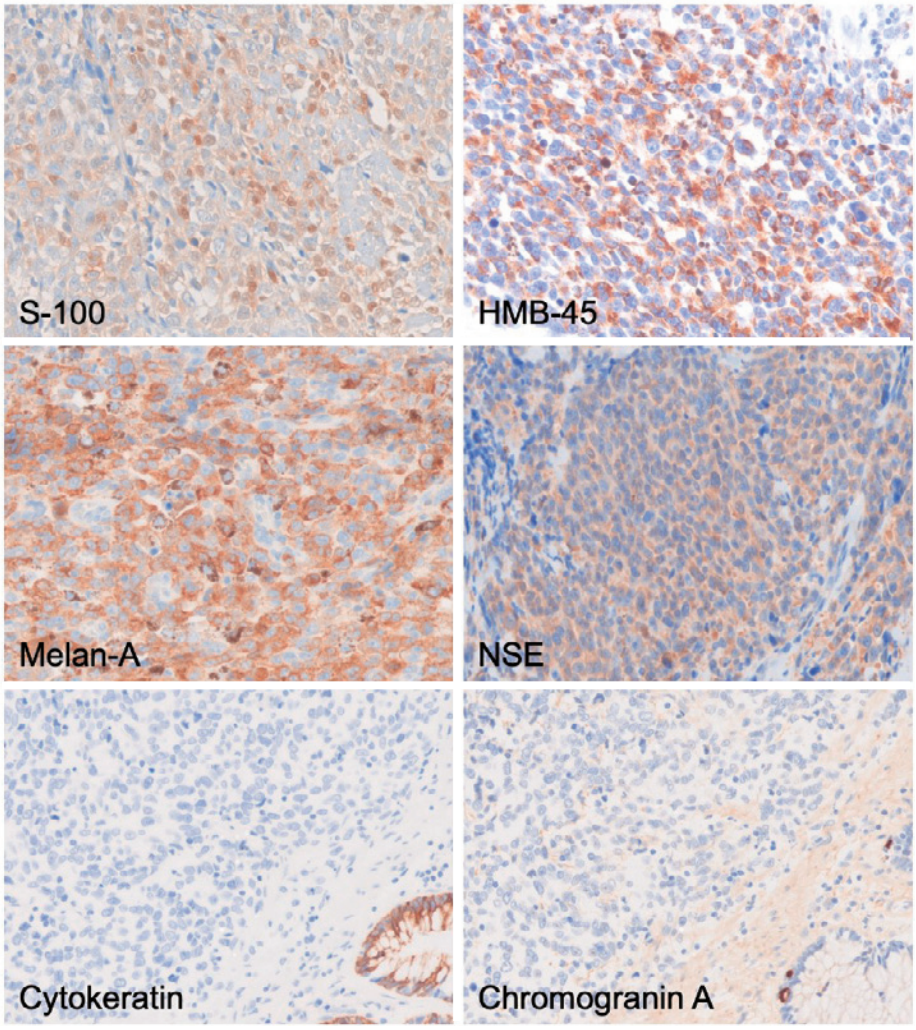

Fig. 2 Immunohistochemical analysis of surgically resected rectal tumor tissues
Rectal tumor cells were positive for S-100, HMB-45, Melan-A, and NSE, but negative for cytokeratin and chromogranin A. Original magnification: ×200

1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 15% FBS and antibiotics. This medium was replaced every 3 days. 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 1:1,000 rabbit anti-S-100 (Z0311; DAKO), 1:200 mouse anti-Pmel17/GP100 (HMB-45, M0634; DAKO), 1:200 mouse anti-Melan-A (M7196; DAKO), 1:1,000 mouse anti-NSE (M 0873; DAKO), 1:150 mouse anti-cytokeratin (M0821; DAKO), or 1:2,000 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 and 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% CO_2. The human melanoma cell line

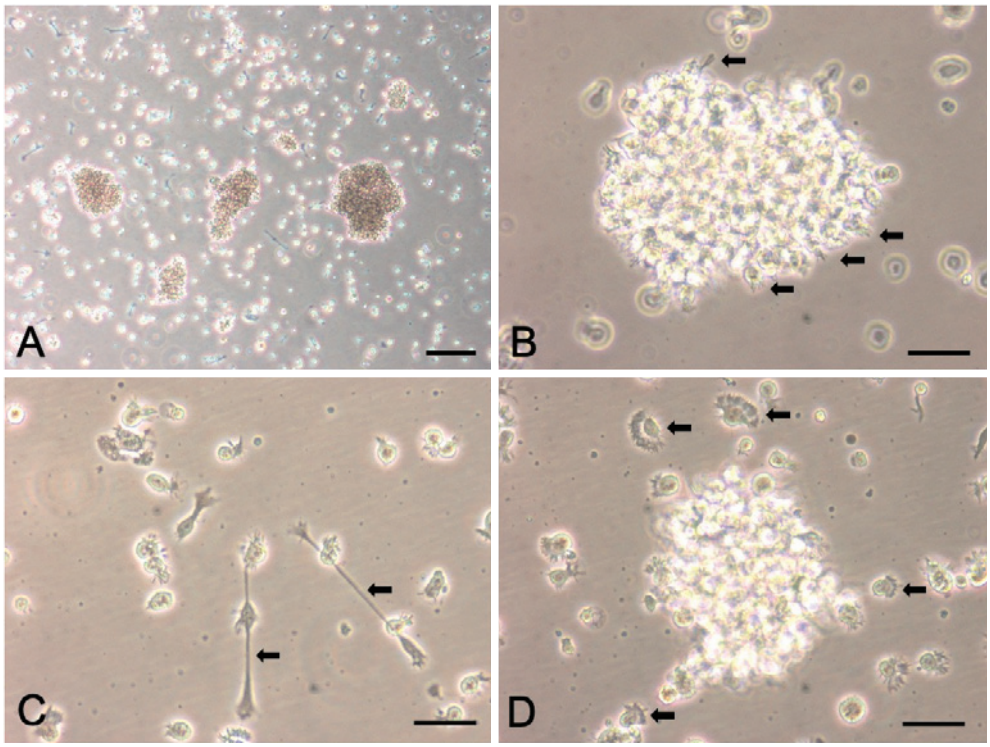

Fig. 3 Phase-contrast images of MELS cells
MELS cells were cultured in two-dimensional conditions. Cells formed irregular colonies with sharp protrusions (arrows in A and B). Morphology was varied, and cells had long protrusions or a semicircular appearance (arrows in C and D). A: Scale bar = 200 μm, B–D = 50 μm

A375 was obtained from American Type Culture Collection 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 by using an MSP-1S sputter coater (Shinku Device, Ibaraki, Japan). Cells were examined and photographed by 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% OsO_4 dissolved in distilled water. Samples were dehydrated using an ethanol gradient, embedded in Epon, and sliced into ultrathin sections with 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).

Anticancer Drug Resistance Assays

Cells (3.0×10^3 per well) were plated in 96-well culture dishes containing growth medium. Each anticancer 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, WI, USA). Cell viability was calculated as percentage of luminescence in drug-treated cells versus 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 with the Student 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

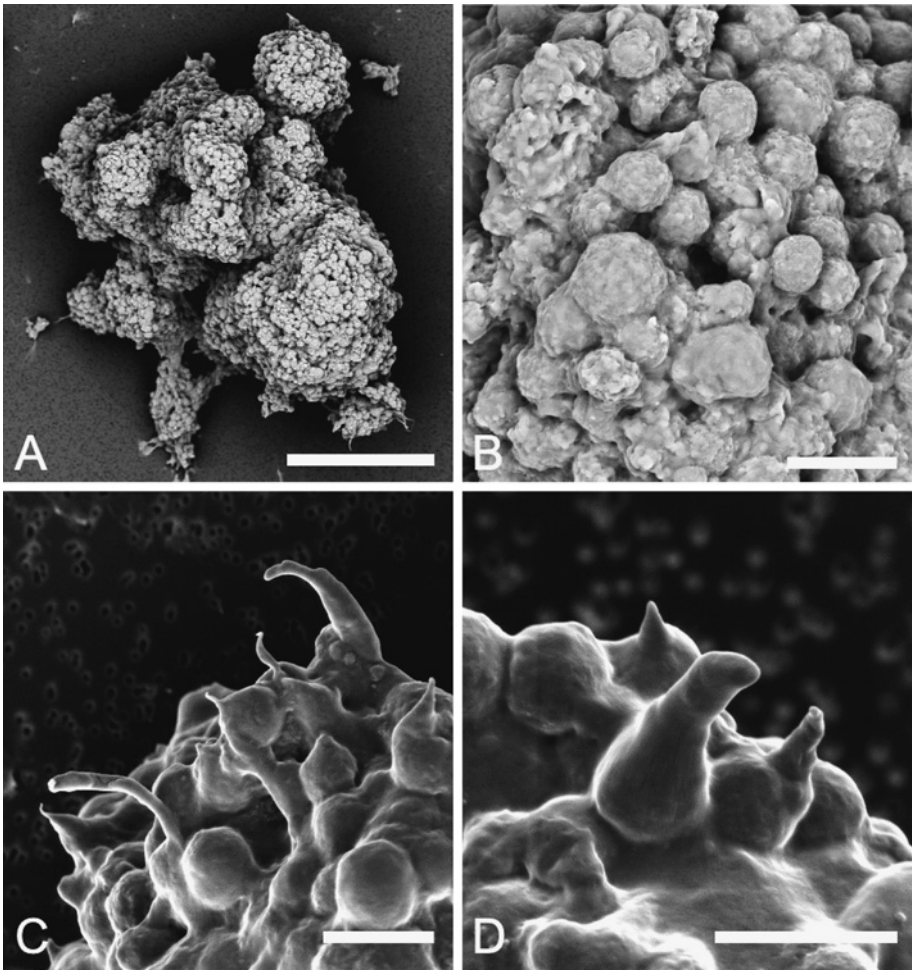

Fig. 4 Scanning electron microscopic (SEM) analysis of MELS cells
MELS cells formed grape-like colonies with short to long protrusions at the periphery (A-D).
Scale bar: A = 100 μm, B–D = 10 μm. A, B: reflective electrons, 10 kV; C, D: secondary electrons, 5 kV.

(**Fig. 1A**). Cells had atypical nuclei and numerous atypical mitoses (**Fig. 1B, arrows**); most exhibited epithelioid features and a partial-spindle appearance. Tissue sections contained less than 10% of all tumor cells which have small, dark brown pigment in their cytoplasm (**Fig. 1C**). TEM confirmed that these were stage IV melanosomes (**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. On the basis of these morphological characteristics, the tumor was diagnosed as a rectal melanoma.

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**). The morphology varied and included long protrusions (**Fig. 3C, arrows**) and a semicircular shape with short protrusions on the cell margin (**Fig. 3D, arrows**). SEM showed that colonies comprised round to oval-shaped MELS cells (**Fig. 4 A, 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 the 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 the properties of the original rectal melanoma tissues.

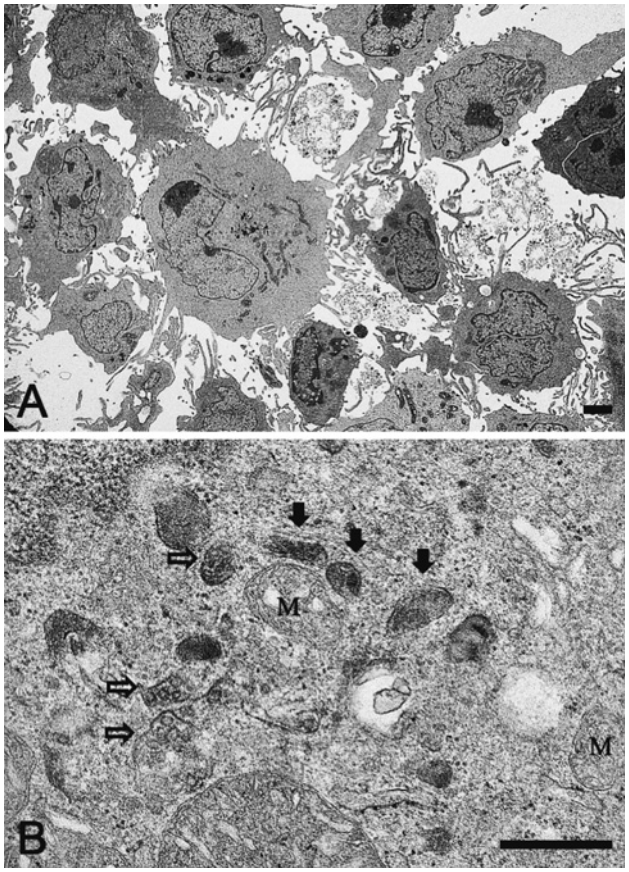

Fig. 5 TEM analysis of MELS cells
MELS cells had short to long protrusions (A) and premelanosomes (B, Stage I: white arrows, Stage II: black arrows) in the cytoplasm. M: mitochondria, Scale bar: A = 2 μm, B = 500 nm

Viability of MELS Cells after Addition of Anticancer Drugs

The ATP assays for comparing growth across the 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 anticolorectal cancer drugs (oxaliplatin, fluorouracil [5-FU], and irinotecan) on the three cell types. After adding 10 μM oxaliplatin, viability was lower for MELS cells than for the other two cell lines. Increasing the oxaliplatin concentration to 100 μM resulted in lower viability for MELS and A375 cells than for Caco-2 cells (**Fig. 7B**). Treatment with 100 μM 5-FU also resulted in lower viability in MELS and A375 cells than in Caco-2 cells (**Fig. 7B**). After treatment with 10 and 100 μM irinotecan, viability was lower for MELS and A375 cells than for Caco-2 cells (**Fig. 7B**).

Discussion

In this study, anorectal melanoma was diagnosed preoperatively by colorectal biopsy, among the most accurate ways to diagnosis this cancer[15]. The tumor was a poorly differentiated adenocarcinoma with solid proliferation. We did not test for melanin in the biopsy specimen or perform immunohistochemical or TEM analyses. We chose to establish the cultured cell line before confirming our melanoma diagnosis.

The surgically resected rectal tissues showed patterns typical of immunohistochemical staining for anorectal melanoma. In the established MELS cells, TEM revealed Stage I and II premelanosomes (classified according to morphology and melanin synthesis[16]). The proportion of melanin-positive cases is lower for anorectal melanoma than for cutaneous melanoma; melanin was found in fewer than 10% of tumor cells in our sample.

A previous SEM study reported that cutaneous melanoma cells had numerous extensions on their surface, including microblebs, microvilli, and some lamella[17]. Several cutaneous melanoma protrusions were extremely long, slender, and twisted between neighboring cells. MELS cells form aggregated colonies and exhibit a variety of morphologies, such as semicircular protrusions and very long, rod-like cytoplasm. In general, they were pleomorphic cell types with short-to-long protrusions on their surfaces. Previous SEM studies also noted that melanoma cell surfaces had numerous extensions, some with differentiations that suggested special functions, although cells with smooth surfaces were also present[17].

MELS cells were positive for melanoma-specific antigens, specifically anti-S-100, HMB-45, and Melan-A (also known as MART-1), and negative for epithelial and neuroendocrine markers. Anti-S-100 protein is the most common immunohistochemical stain used for diagnosing anorectal melanoma, as it is highly sensitive to melanocytic differentiation[18]. HMB-45 and Melan-A are melanocyte-specific markers used for diagnosis of melanoma[19]. These antibodies are present in most anorectal melanoma cases (94% and 93%, respectively)[18]. MART-1/Melan-A is reportedly required for maturation of Pme17, and HMB45 reacts with mature Pmel17[20]. The double positivity of MART-1/Melan-A and HMB-45 in MELS cells was unsurprising.

Anorectal melanoma is aggressive and outcomes are poor because of late diagnosis and rapid tumor growth in the rich vascular and lymphatic supply of the anorectal mucosa[21]. In this study, MELS cells had a far lower proliferation rate than conventional colorectal cancer and

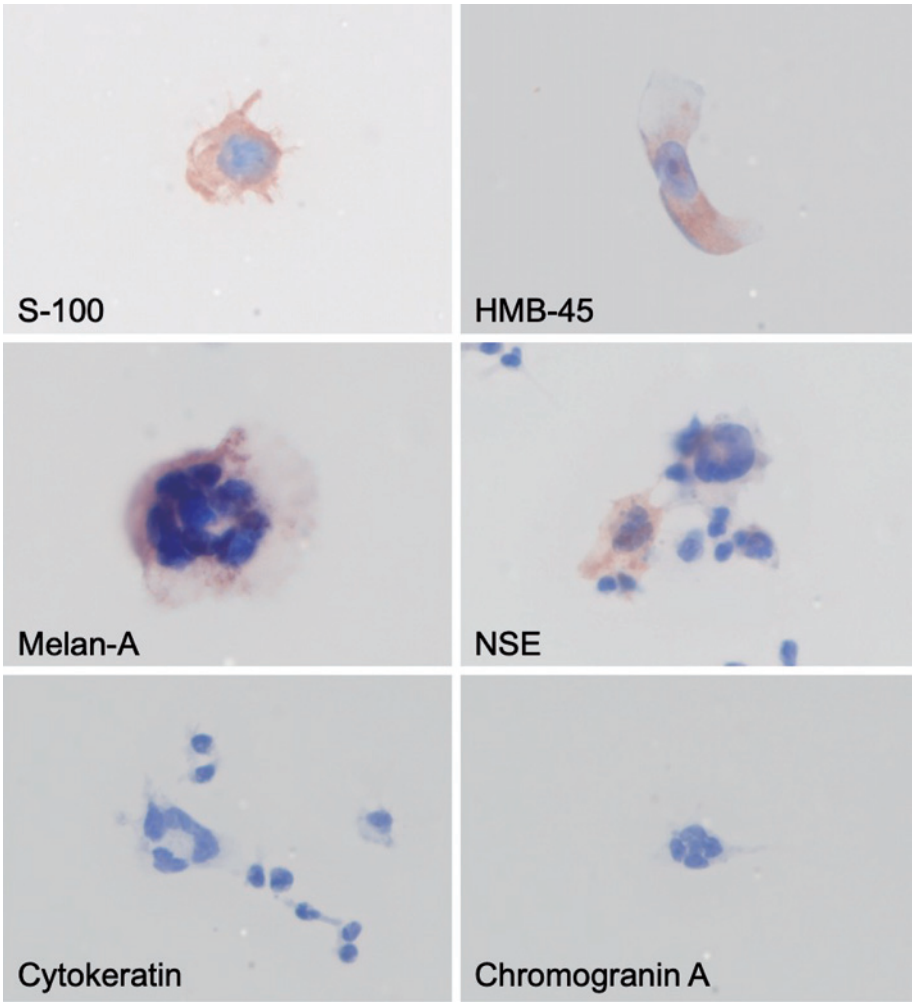

Fig. 6 Immunocytochemical analysis of MELS cells
MELS cells were positive for S-100, HMB-45, Melan-A, and NSE, but negative for cytokeratin and chromogranin A. Original magnification: ×600

cutaneous melanoma.

Treatment for anorectal melanoma includes surgery, radiotherapy, chemo-immunotherapy, and targeted therapy[22]. The JAK mutation is a major mechanism against anti-PD-L1 antibody in metastatic cutaneous melanoma[23]. Management of anorectal melanoma is hampered by the lack of randomized trials. Tumors tend to be resistant to radiotherapy and respond poorly to chemotherapy. Oxaliplatin and irinotecan are usually used to treat colorectal cancer, but melanomas derived from the colorectum are treated in the same manner as cutaneous melanomas. Our analysis of the effectiveness of therapeutic drugs for colorectal cancer in MELS cells showed that these anti-colorectal cancer drugs were effective for MELS. Future studies should attempt to clarify the underlying mechanisms and effectiveness of these drugs *in vivo*.

To our knowledge, SK-MEL-246 is the only other cell line derived from a human rectal melanoma cell line[24]. However, clinical details of that cell line, including donor sex and age, are unknown. The cell line was established from a metastatic rectal tumor site in a patient with melanoma; however, there appear to be no reports describing 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, will help us better understand anorectal melanomas and contribute to the development of anti-cancer drugs, immune therapy, and radiotherapy for this cancer.

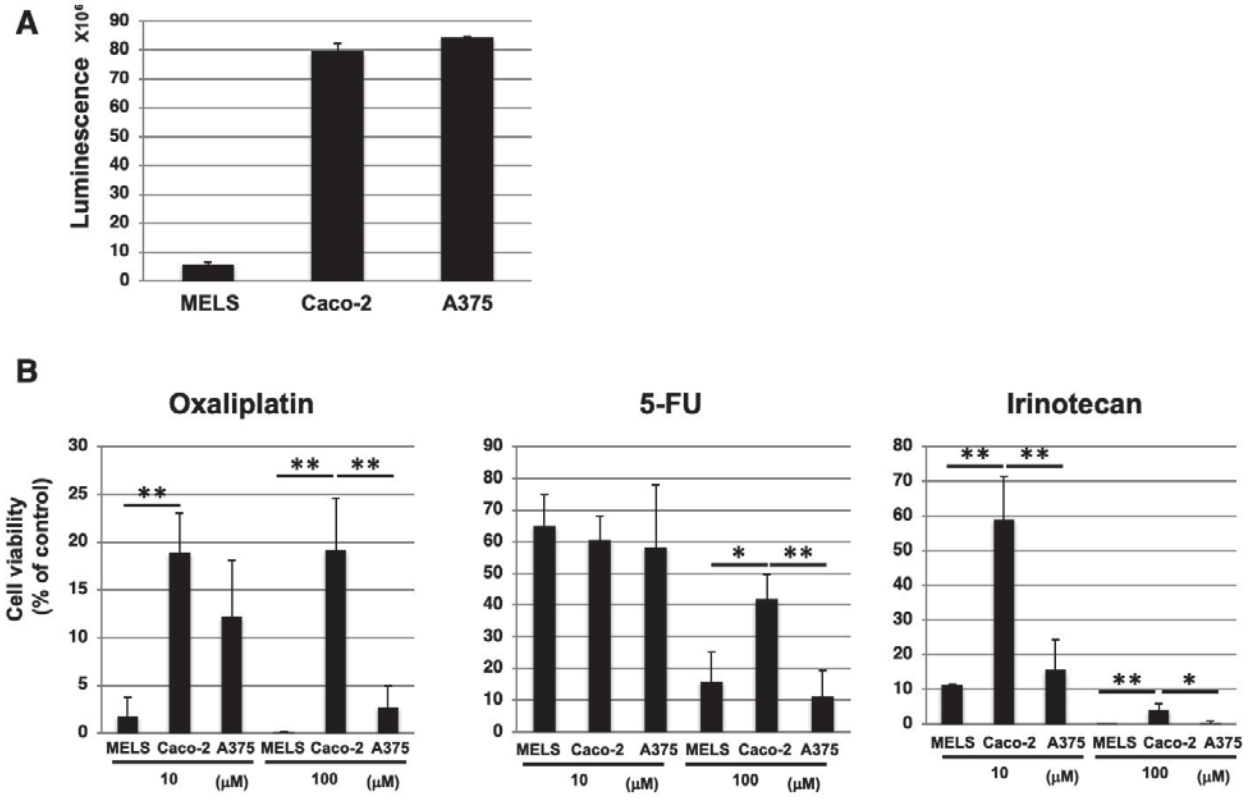

Fig. 7 Anti-cancer drug resistance assay of MELS, Caco-2, and A375 cells

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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Conflict of Interest: None declared.

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