Establishment and Characterization of a New Human Pancreatic Cancer Cell Line, YPK-1

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Abstract A new human pancreatic cancer cell line, designated YPK-1, was established from ascites collected from a 62-year-old Japanese man. This cell line has been maintained in vitro for 96 months with stable growth through 260 passages. We characterized the YPK-1 cell line morphology, doubling time, levels of tumor markers secreted into culture medium, chromosomal content, and tumorigenicity in nude mice. YPK-1 cells have a clear polygonal-shaped cytoplasm, round nuclei, and prominent nucleoli. The cells grew as an adherent monolayer and lacked contact inhibition. The doubling time at the tenth passage was 49.3 hours. YPK-1 cells secreted detectable amounts of carcinoembryonic antigen, carbohydrate antigen 19-9, carbohydrate antigen 125, carbohydrate antigen 50, DU-PAN-2, and SPan-1. Chromosome analysis revealed that the diploid chromosome number ranged from 57 to 62 with a mode of 61. Tumorigenicity was demonstrated by development of tumors after subcutaneous injection of YPK-1 cells into nude mice. We believe that this cell line will contribute to studies of the biological and molecular properties of pancreatic carcinoma and to the development of therapeutic strategies for this tumor.

Introduction

Pancreatic carcinoma is a disease with a poor prognosis and is considered by many to be one of the deadliest malignancies\(^1\). Although many therapeutic strategies have been developed, most have not appreciably improved the prognosis of patients with pancreatic carcinoma, which has an overall 5-year survival rate of less than 5%\(^2\). Therefore, it is important to establish human pancreatic cancer cell lines as in vitro models for studying the biological and molecular properties of this tumor. During the past 20 years, establishment of many human pancreatic cancer cell lines has been reported \(^2\text{--}^25\). These cell lines have provided valuable information about various aspects of pancreatic cancer, including carcinogenesis, histogenesis, diagnosis, and therapy. Because pancreatic carcinoma remains a disease with a generally poor prognosis\(^1\), more cell lines are needed to investigate this neoplasm in greater depth.

In our laboratory, six esophageal cancer cell lines have been established \(^26,27\), and these cell lines have provided some valuable information about tumor markers \(^28\)
and the biological and molecular properties of esophageal cancer. Since 1994, we have attempted to establish additional pancreatic cancer cell lines based on our experience with cell culture techniques. This paper reports the establishment and characterization of a new human pancreatic cancer cell line that we have designated YPK-1.

Materials and methods

Patient

A 62-year-old Japanese man was admitted to our hospital on August 23, 1994, because of epigastralgia and a weight loss of 15 kg over 6 months. He was diagnosed as having pancreatic cancer with multiple liver metastases. On admission, his serum levels of tumor markers were 27.1 ng/ml in carcinoembryonic antigen (CEA), 54963 IU/ml in carbohydrate antigen (CA) 19-9, 156 IU/ml in CA125, 13000 IU/ml in DU-PAN-2, and 5600 IU/ml in SPan-1. He received irradiation therapy. Ascites appeared, and cytologic examination with Papanicolaou, Giemsa, alcian blue, and periodic acid Schiff (PAS) staining was performed. Adenocarcinoma cells were seen frequently (Fig. 1) and were positive for PAS and alcian blue. The patient died 2 months after admission.

Culture medium

Dulbecco’s modified Eagle’s medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 100 U/ml of penicillin, 100 

 µg/ml of streptomycin (GIBCO BRL, Rockville, MD), 12 mM sodium bicarbonate, and 20% fetal calf serum (FCS) (M.A. Bioproducts, Walkersville, MD) was used as the primary culture medium (PCM). DMEM containing 10% FCS was used as the maintenance culture medium.

Culture procedure

Collected serous ascites was placed in tissue culture flasks with a plug seal screw cap (25 cm² style; Becton Dickinson, San Jose, CA) at 37°C in an atmosphere of 5% CO₂ in air. After tumor cells adhered to the bottom of the flasks and formed nests, the cells were washed in Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS) (Nissui) and cultured in PCM at 37°C in an atmosphere of 5% CO₂ in air. The medium was changed three times each week. For subculture, cells were detached by treatment with trypsin-EDTA (0.05% trypsin, 0.53 mM ethylenediamine tetraacetic acid Na₂; GIBCO BRL). The cells were observed

Fig. 1. Cytologic appearance of the original tumor cells in ascites (Papanicolaou, original magnification, x100).
daily with a phase-contrast microscope (Nikon, Tokyo, Japan).

**Growth curve**

Growth curve was obtained by seeding the cells at 1x10^5 cells per tissue culture dish (60 x 15 mm style; Becton Dickinson) in 5 ml of DMEM containing 10% FCS in duplicate. The medium was changed every 2 days after seeding. The cells were detached with trypsin-EDTA, and the average number of viable cells from two dishes was determined by counting cells that excluded trypan blue with a Bürker-Türk hemocytometer. The doubling time of the cell population was estimated from the logarithmic growth phase.

**Tumor markers**

Secretion of tumor markers was assessed in cells grown for 48 hours in 5 ml of DMEM containing 10% FCS. Supernatants were obtained by centrifugation at 3000 rpm for 10 minutes at 4°C, and assayed for CEA, CA 19-9, CA 125, CA 50, DU-PAN-2, and SPan-1 with the use of commercially available assay kits (CEA, CEA RIABEAD, Dainabot, Tokyo, Japan; CA 19-9, Centocor CA19-9 RIA Kit, TFB, Tokyo, Japan; CA125, Centocor CA125II IRMA Kit, TFB; CA50, TR-FIA Kit, Pharmacia, Tokyo, Japan; DU-PAN-2, Determiner DU-PAN-2, Kyowa, Tokyo, Japan; SPan-1, SPan-1 RIABEAD, Dainabot).

**Chromosome study**

Three days after seeding the cells were placed into 25 cm² style tissue culture flasks, colcemid (0.04 μg/ml) was added to the flasks, and cells were incubated at the 37°C for 2 to 4 hours. Cells were detached with 0.25% trypsin and treated with hypotonic 0.075 M KCl solution for 10 minutes. They were then centrifuged at 3000 rpm for 10 minutes at 4°C, and fixed with a mixture of methanol and acetic acid (3:1) twice. Cells suspended in fixative were dropped onto slides, dried over a flame, and stained with Giemsa. G-banding was carried out by staining the cells with Giemsa after 5 minutes of treatment with 0.0125% trypsin

**Heterologous transplantation**

Five- to 6-week-old male athymic nude mice (Balb/c nu/nu mice; Japan SCL, Hamamatsu, Japan) were used to examine the tumorigenicity of YPK-1. Cells (1x10^6) were suspended in 100 μl of PBS and injected subcutaneously into the abdomens of mice. The mice were killed 4 weeks after inoculation, and the subcutaneous tumors were processed for light microscopic examination, immunohistochemical staining, and electron microscopic examination.

**Light microscopic examination**

For light microscopic observation, formalin-fixed paraffin blocks of specimens were sectioned at 3 μm. Sections were dewaxed, rehydrated, and stained with hematoxylin-eosin. For immunohistochemical staining, sections were dewaxed, rehydrated, and stained with antibody to CEA (Kyowa Medicus Co., Tokyo, Japan), CA 19-9 (TFB), and CA 125 (TFB) and an avidin-biotin complex immunoperoxidase technique (DAKO LSAB kit; DAKO Co., Carpinteria, CA) as described below. Briefly, nonspecific binding was blocked by incubation of sections with non-immune goat serum diluted in 0.05 M Tris/HCl buffer, pH 7.6, containing 6% carrier protein and 15 mM sodium azide as preservative. Samples were incubated with primary antibody (diluted 1:100 in PBS) for 30 minutes, incubated in secondary antibody (diluted 1:100 in PBS) for 15 minutes, and then incubated in 1:400 streptavidin for 15 minutes at room temperature. Peroxidase activity was detected with 3,3'-diaminobenzidine as a chromogen with H₂O₂ as a substrate. Samples were counterstained with hematoxylin and mounted. As negative controls, samples were stained as above but with PBS substituted for the primary antibody.

**Electron microscopic examination**

For electron microscopic observation, specimens were fixed in Karnovsky’s fixing fluid for 1 hour at room temperature. After rinsing three times with 0.1 M sodium cacodylate buffer, samples were postfixed in 2% osmium tetroxide with Millonig’s buffer (1:1), dehydrated in a graded series
of ethanols, and embedded in Epon 812. Specimens were sectioned on an ultra-microtome, stained with uranyl acetate and lead citrate, and examined under a Hitachi H-500 electron microscope.

**Mycoplasma detection**

After the cells were passed twice through antibiotic-free medium, the medium to be tested was exposed to the cells for 3 days. Mycoplasma contamination was examined by Gen-Probe Mycoplasma T.C. Rapid Detection Kit (Gen-Probe Co., Inc. San Diego, CA).

**Results**

*Establishment and morphology of the YPK-1 cell line*

Serous ascites was collected and placed in 25 cm² style tissue culture flasks. A few days after primary culture, small colonies of epithelial-like cells were observed in several flasks. The ascites was then removed, and PCM was added. These colonies increased in size, and piling up of the cells was observed in the center of the colonies. When the epithelial-like cells reached semi-confluence at 14 days after primary culture, cells were subcultured into two 25 cm² style tissue culture flasks and maintained in DMEM containing 10% FCS. Thereafter, subcultures were performed periodically. Growth of fibroblast-like cells, which were easily distinguished from epithelial-like cells under phase con-

![Fig. 2. Phase contrast microscopic appearance of YPK-1 cells (original magnification, (a) x100 (b) x400).](image)

![Fig. 3. Growth curve of YPK-1 cells at the tenth passage.](image)
trast microscopy, was also observed around the colonies. The growth of the epithelial-like cells appeared to be inhibited or limited by the overgrowth of the fibroblast-like cells. The fibroblast-like cells were removed by differential trypsinization and serial passages. The resulting cell line grew continuously and was passaged for 260 generations over 96 months. It has been designated YPK-1.

YPK-1 cells were found to proliferate in a pavement-like arrangement. After reaching confluence, the cells had a tendency to pile up and showed lack of contact inhibition. These cells were shown to have pleomorphic features, being primarily polygonal-to-spindle shaped with eosinophilic cytoplasm and oval-to-round nuclei (Fig. 2). Occasionally, multinucleated giant cells were observed.

*Growth curve*

The growth curve was obtained by seeding YPK-1 cells at the tenth passage. After a lag phase of 48 hours, cells entered a rapid logarithmic growth phase. The doubling time of the cell population in the logarithmic phase was 49.3 hours (Fig. 3).

*Tumor markers*

Tumor markers CEA, CA 19-9, CA 125, CA 50, DU-PAN-2, and SPan-1 were detected in culture media. The secretion of tumor markers paralleled the growth curve in all phases (Table 1). CEA, CA 19-9, CA 125, CA 50, DU-PAN-2, and

<table>
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<th>Days after seeding</th>
<th>CEA (ng/ml)</th>
<th>CA19-9 (IU/ml)</th>
<th>CA125 (IU/ml)</th>
<th>CA50 (IU/ml)</th>
<th>DU-PAN-2 (IU/ml)</th>
<th>SPan-1 (IU/ml)</th>
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<td>250</td>
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Fig. 4. Distribution of chromosomal number at the twelfth passage.
Fig. 5. A G-band karyotype of YPK-1 cells at the twelfth passage. Note that there are 61 chromosomes.

*Chromosome study*

YPK-1 cells at the twelfth passage were used for chromosome analyses. The diploid number of chromosomes ranged from 57 to 62, with a mode of 61 (Fig. 4). G-band analysis of cells containing 61 chromosomes revealed a karyotype of XX, +2, +4, +5, +7, +8x2, +9, +11, +12, +14, +15, +16, add(16)(qter)x2, +mar (Fig. 5).

*Heterologous transplantation and morphology of tumor cells transplanted into nude mice*

YPK-1 cells at the ninth passage were suspended in PBS and injected subcutaneously into the abdomens of nude mice. Visible subcutaneous tumors were detected approximately 18 days after inoculation and gradually increased in size. The subcutaneous tumors reached their greatest dimension (10 mm) by 28 days after inoculation, but metastatic foci in the internal organs were not observed.

Under light microscopic examination, tumors showed the histological features of moderately differentiated tubular adenocarcinoma (Fig. 6). The tumor cells had eosinophilic, columnar or polygonal cytoplasm, and round-to-oval nuclei (Fig. 6a). Thin bundles of collagen fibers were present in the stroma (Fig. 6b). Expression of CEA, CA 19-9, and CA 125 in the...
cytoplasm of subcutaneous tumors was
detected by immunohistochemical staining
(data not shown).
Under electron microscopic examination,
the tumor cells had microvilli at the api-
ces of the cells (Fig. 7a). In the cytoplasm,
organelles were poorly developed, and no
zymogen or endocrine granules were ob-
served (Fig. 7b).

Mycoplasma detection
Mycoplasma contamination was not pre-
ent.

Discussion
Since the first report of transplantation
of a human colon cancer into nude mice
by Rygaard and Povlsen in 1969 32, trans-
plantation of human malignant tumors into
nude mice has been reported by many authors
26. Because transplanted tumors retain
the morphological and functional char-
acteristics of the original tumor, transplan-
tation into nude mice is recognized as a useful in vivo tumor model. More-
over, the chances of obtaining fresh cancer
cells are increased if the transplanted tumors
develop. Therefore we subcutaneously
transplanted human esophageal cancer cells
into nude mice to establish tumor cell lines.
The overall success rate for transplanta-
tion of esophageal cancers is 83% in our
laboratory 26. Six human esophageal can-
cer cell lines have been established from
subcutaneously transplanted cells 26, 27. How-
ever, this method is complicated and ex-
pendive. In the present study, we attempted
to establish cell lines by use of an easy
method in which ascites was placed in
tissue culture flasks. Using ascites as a
culture material has several advantages,
including no or less contamination by nor-
mal epithelial cells and fibroblasts, ease
of obtaining many free cancer cells,
 repeatability of cultivation 6, and lack of
damage because tumor cells are obtained
without the use of enzymes. We established
the YPK-1 cell line with this method.

YPK-1 cells were characterized as human
pancreatic cancer cells on the basis of the
following observations. Morphologically,
YPK-1 cells exhibit an epithelial cell
growth pattern with a pavement-like arrange-
ment and show a lack of contact inhibi-
tion. YPK-1 cells had stable growth for
more than 96 months (260 passages), and
tumorigenicity was shown by the develop-
ment of tumors after subcutaneous injection
of YPK-1 cells into nude mice. Light and
electron microscopic examination of tumors
transplanted into nude mice showed that
these cells had the features of adenocar-
cinoma cells. We confirmed that these
cells were of human origin by chromosome
analyses, and we concluded that the cell
line established was derived from a human
pancreatic cancer.

Recently, many tumor markers have
been used for diagnosis and management
of malignant tumors. Tumor markers have
five potential uses: screening, diagnosis,
establishing prognosis, monitoring treat-
ment, and detecting relapse 33. For
pancreatic cancer, CA19-9 and CEA are useful tumor markers\textsuperscript{34,35}. YPK-1 cells secrete detectable amounts of not only CEA and CA 19-9 but also CA125, CA50, DU-PAN-2, and SPan-1. Serum levels of these tumor markers in our patient were elevated. High serum levels of these tumor markers are sometimes found in patients with pancreatic cancer, but the mechanism of secretion remains unclear. It may be revealed by further investigation of YPK-1 cells.

With respect to morphology, doubling time, levels of tumor markers secreted into culture medium, and karyotype, there are some differences in YPK-1 cells compared with other previously established pancreatic cancer cell lines. In general, clinical courses of pancreatic cancers vary. This means that additional cell lines that have different characteristics are necessary.

In conclusion, the YPK-1 cell line is a new human pancreatic cancer cell line that should contribute to studies of the biological and molecular properties of pancreatic carcinoma and to the development of therapeutic strategies for this pancreatic cancer.

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