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Establishment and Characterization of New Cell Lines Derived from Human Pancreatic Cancers

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SUMMARY

Two tumorigenic human cell lines, HPC-3 and HPC-4, were established from the metastatic ascitic fluid of two male patients with pancreatic adenocarcinoma of ductal origin. Both of these lines showed epithelioid configurations on the confluent monolayer culture. The doubling time during the exponential cell growth was approximately 21 hr for HPC-3 and 24 hr for HPC-4, respectively. These lines can grow in an anchorage independent manner in 0.3% soft agar, and they also develop tumors in nude mice. However, the tumorigenicity of HPC-4 was relatively weaker than that of HPC-3. These tumors showed a histological architecture compatible with an anaplastic carcinoma. Electronmicroscopically these tumors indicated intercellular junctions as well as a few rough endoplasmic reticulum. Furthermore, they expressed pancreatic cancer associated antigen that was defined by HC-2 monoclonal antibody. The data suggested that these lines could be useful for the study of immunobiological characterization of human pancreatic cancers.

Key words: HPC-3, HPC-4, Human pancreatic cancer line, Tumorigenesis

INTRODUCTION

The incidence of adenocarcinoma of the exocrine pancreas is increasing in Japan as well as in other countries. For the immunobiological studies of this cancer, it is practical to use established pancreatic cancer cells. However, there are only a few such lines. The paucity of pancreatic cancer lines available for the experiment may be due to the difficulty in successful establishment of the primary

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culture for this particular tumor because of a strong protease activity usually present in the tumor tissue itself. We demonstrated previously a human pancreatic line HPC-1 that was derived from an ascitic fluid in a pancreatic cancer patient(1). This opportunity of a line establishment showed us that the tumor cells in metastatic ascitic fluid rather than the primary pancreatic tumor mass may provide a more successful chance of cell adaptation for *in vitro* culture. Therefore, we attempted to establish other pancratic cancer lines starting with the metastatic ascitic materials from patients.

In this report, we have demonstrated the establishment of two human new cancer lines. These lines showed not only the epithelioid nature of cells, but expressed the pancreatic tumor associated antigen that was defined by monoclonal antibody. Furthermore, they are tumorigenic *in vitro* and *in vivo,* and were available for the investigations of immunobiological aspects of human pancreatic cancers.

MATERIALS AND METHODS

1. *Cell culture*

Tumor cells were obtained from metastatic ascitic effusions of a 67-year-old and a 54-year-old male patient with primary adenocarcinomas of pancreatic ductal origin. 500–700 m*l* of the ascitic effusion obtained by paracenthesis was centrifuged at 250×g for 10 min. Cell pellets were resuspended in 20 m*l* of RPMI-1640 medium containing 10% fetal calf serum (FCS), and cell suspensions were carefully layered on a 20 m*l* of Ficoll-Conray density gradient. After spinning down at 1,000×g for 25 min at room temperature, an interface in which tumor cells, mesothelial cells, fibroblasts, macrophages and lymphocytes were found was separated. This was washed three times with RPMI-1640 medium. Then the cells were immediately seeded in a 25 cm^2 culture flask (Falcon #3013, Oxnard, CA.), and were cultured under 37℃ in 5% CO_2 in humidified air. The supernatant of each flask was daily reseeded into other flasks for the first week, and in this manner the newly seeded cells were enriched with respect to slowly attaching tumor cells. In contrast, other more rapidly attaching cells, particularly macrophages, mesothelial cells and fibroblasts were selectively reduced. After one month from the initiation of culturing, attaching cells such as non-malignant epitheloid cells were almost reduce, and floating cancer cells began to adhere onto the underface of culture flasks. These were serially transferred by treating with 0.05% trypsin (Sigma Co., St. Louis, MO.) plus 0.02% EDTA in a phosphate-buffered saline solution, pH7.4 (PBS).

2. *Morphological examination*

A conventional Giemsa staining of HPC-3 cells at 24th passage generation *in*

vitro and HPC-4 cells at 18th was performed, and the light-microscopical morphology of cells was observed. HPC-3 and HPC-4 tumors that were developed in nude mice were also examined histologically with H-E stain.

3. Electronmicroscopical examination

Tumor tissues growing in nude mice were fixed with 0.1M cacodylate buffer overnight. They were treated with 1% OsO_4 in phosphate buffer and were washed with 7.5% sucrose for 5 min. The fixed cells were dehydrated in a graded series of ethanol from 50% to 100% and propylene oxide, and were embedded in epon-propylene oxide. The thin sections were stained with 5% uranyl acetate and were observed under a JEM-100B-TR electron microscope.

4. Doubling time of cells

5×10^4 HPC-3 cells at 16th passage generation *in vitro* and the same number of HPC-4 cells at 12th were seeded into 25 cm^2 culture flasks (Falcon #3013). The cells were cultured and counted at 12 hr intervals for five days after trypsinizing cell monolayers, and the doubling time of these cells was determined.

5. Culture in soft agar

HPC-3 and HPC-4 were brought into the cultivation in soft agar as described previously by several investigators(2, 3). 10^3 HPC-3 or HPC-4 were plated in dishes (Falcon #3002) containing 0.3% bacto-agar (Difco, Detroit, NJ.), and incubated at 37℃ under 5% CO_2 in moistured air. For the control of positive anchorage-independent growth of cells, a highly tumorigenic murine colon line C-C 36 was employed(4, 5). NIH 3T3 and BALB 3T3 were both employed for negative controls in the cultures. The number of colony formations in soft agar with % plating efficiency after 2 weeks and 3 weeks of cultivation as follows: (No. of clusters—No. of original cell aggregates) $\times$ 100/No. of viable nucleated cells plated.

6. Transplantation study of HPC-3 and HPC-4 in nude mice

HPC-3 cells and HPC-4 cells grown *in vitro* were harvested by trypsinization, and 10^6 cells/mouse were inoculated subcutaneously in the back of five BALB/c nude mice obtained from CLEA Japan Co., Shizuoka, Japan. The mice were observed weekly for tumor development up to 20th week after cell inoculations.

7. Assays for tumor-associated antigens of HPC-3 and HPC-4 using monoclonal antibodies.

10^5 HPC-3 and HPC-4 cells were seeded into the tissue culture flask and were cultured for 72 hr. To determine whether HPC-3 and HPC-4 express the pancreatic cancer associated antigens, we employed monoclonal antibodies HC-1 and HC-2 (6). These monoclonal antibodies were developed in our laboratory and were shown to be associated with the human pancreatic cancer lines. These tests were performed by an indirect immunofluorescence. Briefly, 10^4 of HPC-3 and HPC-4 cells were reacted with a saturated amount of HC-1 and HC-2 monoclonal

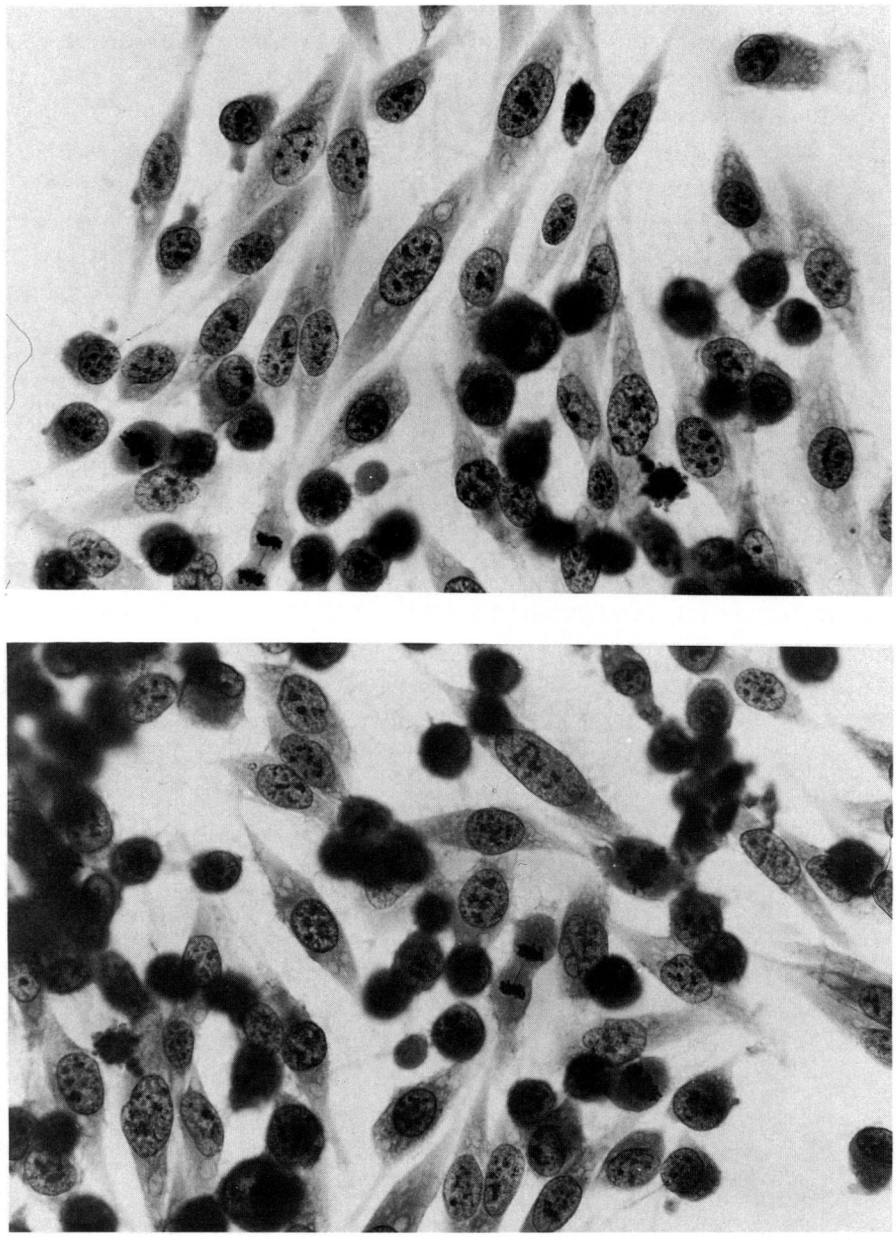
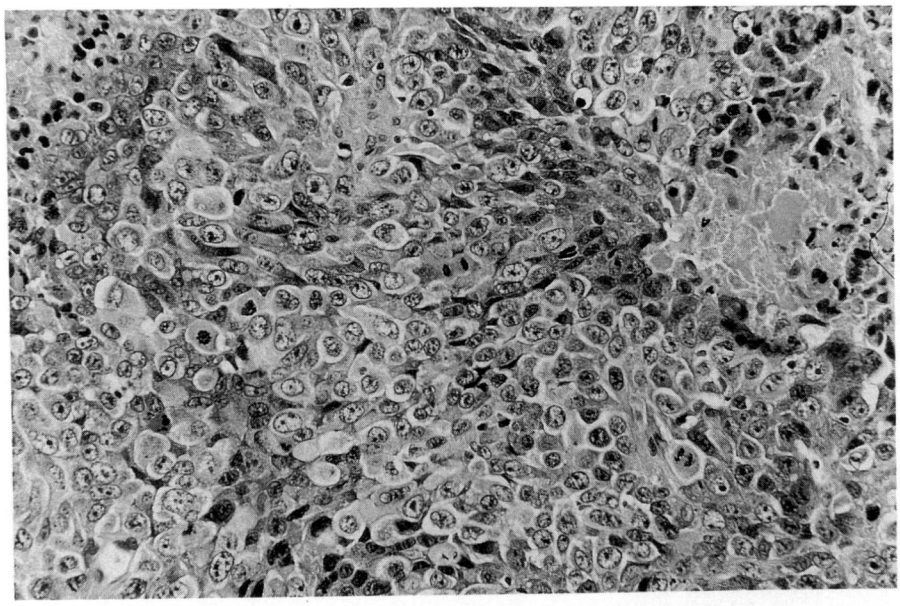
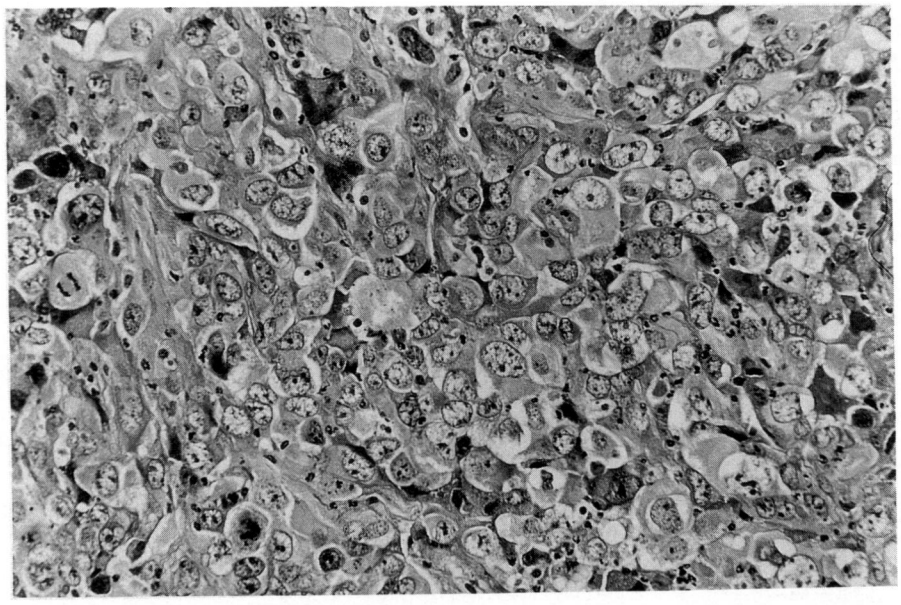


Fig. 1 Microscopic views of continuous cultured tumor cell lines. HPC-3 (A); HPC-4 (B). These lines showed a spindle or round form, and multiplied in adherent fashion on the plastic surface. Giemsa stain ×400.



A



B

Fig. 2 Histology of HPC-3 (A) and HPC-4 (B) tumors developed in nude mice, both showing a histological architecture consistent with an undifferentiated or anaplastic carcinoma. H. E. stain, ×200.

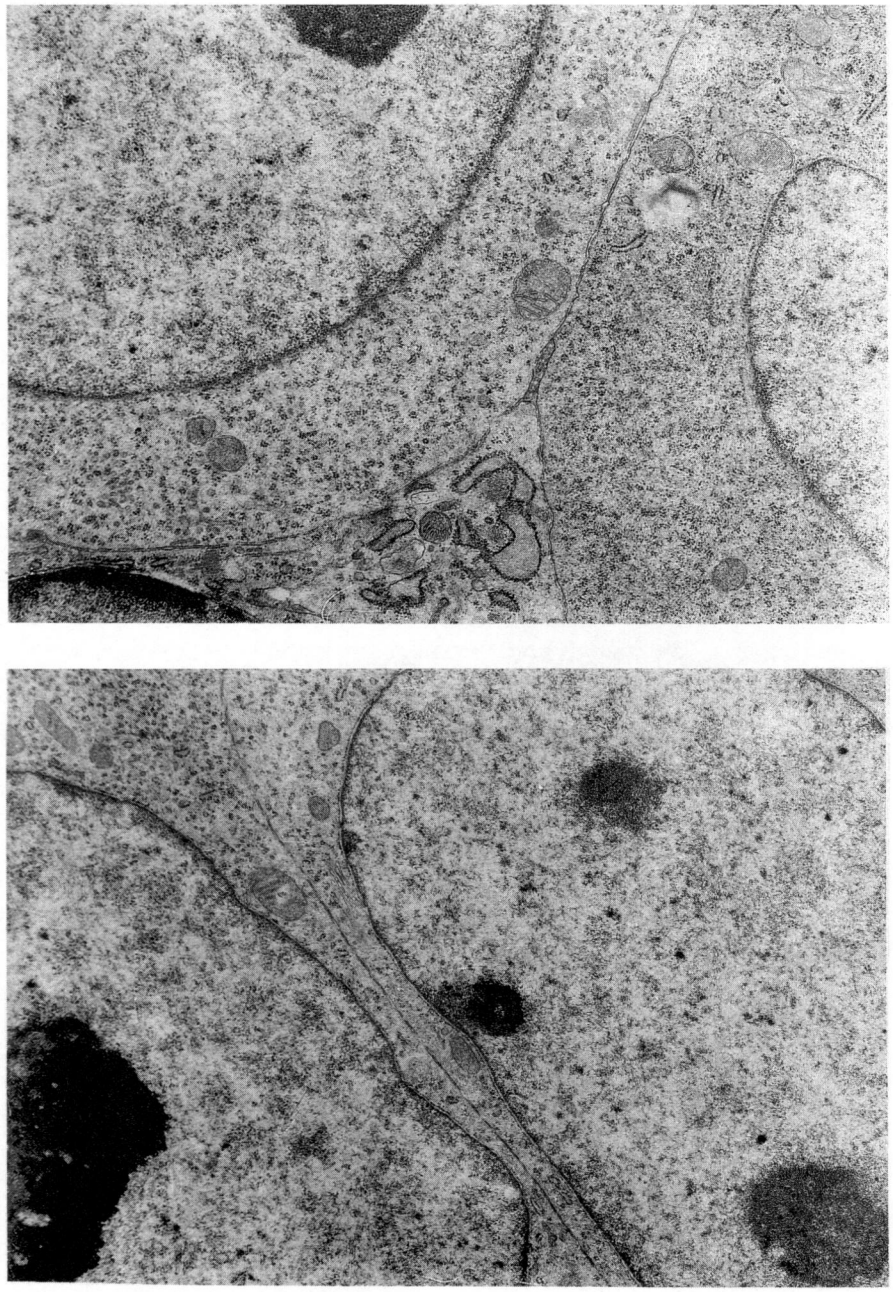


Fig. 3 Electron microscopic view of tumors developed in nude mice. A, HPC-3, ×6000; B, HPC-4, ×5000. Both tumors show intercellular junctions and a few rough endoplasmic reticulum.

antibodies at room temperature for 30 min, and were washed twice with PBS. The cells were admixed with 40 x diluted goat anti-mouse Ig conjugated with FITC. The cells were also washed twice with PBS, and were observed under a Nikon Fluorophoto microscope equipped with a vertical UV illuminator.

RESULTS

1. *Morphological characteristics of cells*

Tumor cells in ascitic fluid indicated a round shape in various sizes with a single or multiple nuclei, and mitoses were frequently obseved. In the primary culture, tumor cells were adapted to grow *in vitro* in RPMI-1640 medium supplemented with 10% FCS in a floating fashion rather than an adherent fashion. After culturing for five weeks, HPC-3 cells propagated on the bottom of plastic culture flasks, and established a confluent monolayer of cells (Fig. 1-A). Thereafter, cells were continuously multiplying in an adherent fashion on the tissue culture flasks. HPC-4 showed a similar course of growth adaptation *in vitro* as HPC-3. HPC-4 propagated on the bottom of plastic culture flasks after culturing for 8 weeks (Fig. 1-B). Both of these lines showed round or spindle shape of cell morphology on the monolayer. It was also indicated that both of lines was free of mycoplasma contamination as determined by immunofluorescence using a mycoplasma stain kit (Flow Laboratories Inc., Va.).

2. *Doubling time*

The doubling time of HPC-3 and HPC-4 cells was determined during an exponential phase of cell growth. It was calculated that HPC-3 and HPC-4 cells

Table 1 *Tumorigenicity of HPC-3 and HPC-4 in soft agar and nude mice.*

cell lines	% plating efficiency[a] in 0.3% agar at		palpable tumor development[b] in nude mice at	
	2nd week	3rd week	12th week	20th week
HPC-3	20.3±2.5[c]	22.8±3.7	3/5	3/5
HPC-4	8.2±1.0	12.2±1.2	0/5	1/5
C-C36	33.8±7.1	55.6±8.0		
NIH3T3	0.	0.		
BALB3T3	0.	0.		

[a] 10^3 per 6 cm dish were inoculated into a seeder layer containing 0.3% agar. % plating efficiency is expressed as follows: (the number of clusters—the number of original cell aggregates) × 100/the noumber of viable cell plated.

[b] 10^6 HPC-3 or HPC-4 were injected subcutaneously in the back of five nude mice for each line. Mice were observed weekly for palpable tumor development. The data indecates palpable tumors/the number of mice.

[c] Mean±S. E.

had an approximately 21 hr and 24 hr doubling time, respectively.

3. Anchorage-independent growth of cells

The growth capability of two cell lines in an anchorage-independent manner was assessed using 0.3% soft agar. It was shown in Table 1 that at the 2nd week of cultivation HPC-3 and HPC-4 formed colonies with a plating efficiency of 20.3% and 8.2%, respectively. A highly tumorigenic murine colon cancer line C-C 36 showed higher plating efficiency than these lines in this experimental situation. At the 3rd week of agar culture, each of plating efficiency increased up to 22.8% for HPC-3 and 12.2% for HPC-4. On the other hand, there were no colony formations observed for NIH 3T3 and BALA 3T3 cells. These data indicate clearly that both HPC-3 and HPC-4 have tumorigenic characteristics *in vitro*.

4. Tumorigenicity of cells in nude mouse

For the examination of the tumorigenicity *in vivo* in HPC-3 and HPC-4 cells, 10^6 cells/mouse of these cells were inoculated subcutaneously into five nude mice for each line. The mice were observed weekly for tumor development up to 20th week after cell inoculations. As shown in Table 1, three out of five mice injected with HPC-3 showed a palpable tumor development by the 12th week after injection. The most early appearance of a tumor was seen at 5th week after injection. In contrast, one out of five mice injected with HPC-4 showed palpable tumor development at 20th week after injection.

5. Tumor-associated antigens expressed on cells

The reactivity of HPC-3 and HPC-4 cells with monoclonal antibodies HC-1 and HC-2 which were recently developed in our laboratory(6) was examined. It was

Table 2 *The reactivity of lines with HC-1 and HC-2 monoclonal antibodies.*

cell lines	HC−1	HC−2	cell lines	HC−1	HC−2
Pancreatic cancers			Bladder cancers		
HGC-25	+	+	KU-1	+	−
PANC-1	+	+	NAT	+	−
HPC-1	+	+	Colon cancers		
HPC-3	+	+	M-7609	+	−
HPC-4	+	+	Lung cancers		
Gastric cancers			PC-1	−	−
KATO-III	+	−	PC-6	−	−
MKN-28	+	−	PC-10	+	−
MKN-45	−	−	PC-15	−	−
OKAJIMA	+	−	OG-90	+	−

The reactivity of indirect immunofluorescence on the cell surface was represented by positive (+) or negative (−) fluorescence.

shown that HC-1 reacted with the cell surface antigens which were expressed on the pancreatic cancer cell lines as well as in other epitheloid tumorigenic cell lines. In contrast, HC-2 reacted with the surface antigens expressed specifically on the pancreatic cancer-derived cells, but not on other epithelial cancer-derived cells. Therefore, the reactivity of HPC-3 and HPC-4 with HC-2 monoclonal antibody was considered to be important for the implication of tissue origin of these lines. As shown in Table 2, HC-2 reacted with HPC-3 and HPC-4. It was also indicated that HC-2 reacted specifically with the pancreatic cancer-derived lines such as HGC-25, PANC-1 and HPC-1, but not with the cells derived from the gastric, gall bladder, colon and pulmonary cancers. HC-1 also reacted with HPC-3 and HPC-4. However, the antigen defined by HC-1 monoclonal antibody was expressed broadly among epithelial tumor cell lines.

DISCUSSION

It is still difficult to adapt all tumor cells into the *in vitro* culture environment. The successful adaptation of the neoplastic cells to *in vitro* cultivation usually seems to depend upon an incidental chance of the cell culture attempts. Hence there are only a limited number of reports(7—13) regarding the human pancreatic cancer lines as well.

We have previously reported(1) a successful establishment of a human pancreatic cancer line derived from a metastatic fluid of pancreatic carcinoma. Our experience in endeavoring to adapt the pancreatic tumor cells into *in vitro* cultivation suggested that tumor cells in the metastatic ascitic materials were more likely to grow *in vitro* than those in the primary solid tumor materials. This may imply that the neoplastic cells in the ascitic fluid are those that have already adapted form of cells in which the growing capability was conferred in a different milieu rather than the primary organ of cells. Therefore, we attempted to establish pancreatic tumor lines using metastatic ascitic fluid with the same culturing protocol described previously(1). We have definitely showed here that successful cultures of two human pancreatic tumor lines HPC-3 and HPC-4 have been established.

Both of these lines clearly demonstrated that several morphological characteristics which are consistent with the epithelioid nature of cells exist. Intercellular junctions were observed electronmicroscopically in these tumors which developed in nude mice. It was confirmed in chromosomal analysis that both lines have human chromosomes with a 75 modal number (data not shown). The malignant potential of these lines was clearly revealed by an anchorage-independent growth *in vitro* and a heterotransplantation experiment using nude mice. Recent studies have shown that the cell growth potential in an anchorage-independent manner was a very reliable parameter to indicate the neoplastic nature of cells(14). In fact, mouse

3T3 cells transformed by various oncogenes can grow anchorage-independently(15). HPC-3 and HPC-4 grew in 0.3% soft agar, although there was a difference in the tumorigenic potential between these cells. HPC-3 had a high anchorage-independent growth potential and tumorigenic capability in nude mice as compared with those of HPC-4. However, it is at present not known why this difference of the tumorigenicity was detected.

It has been reported that some lines derived from the pancreatic carcinomas maintained their biological functions and the expression of antigens(11, 12). Our preliminary study suggested that there was no overt production of the tumor associated antigens such as CA19-9, carcinoembryonic antigens (CEA) and tissue polypeptide antigens (TPA) in the culture supernatants of HPC-3 and HPC-4 (data not shown). As to the investigations of the immunological properties of tumor cells, monoclonal antibodies are available for detecting the tumor associated antigens as well as for searching the tumor specific antigens. Usui *et al.* (6) recently developed two mouse monoclonal antibodies, HC-1 and HC-2, which react with the human pancreatic cancers. HC-1 demonstrated the reactivity against the pancreatic tumor lines and freshly isolated pancreatic tumor tissues from the patients. This monoclonal antibody also reacted with some epithelial tumor lines including the gastric and pulmonary carcinomas. However, HC-2 reacted specifically only with the pancreatic tumors. The antigens defined by HC-1 and HC-2 were identified as the glycoproteins with 130 and 95 k, and 360 k daltons of the molecular weights, respectively. These molecular features suggested that these antigens are different from CA19-9 antigens, CEA and TPA in terms of the reactivity patterns as well as the molecular characteristics. We studied the reactivity of these monoclonal antibodies against HKC-3 and HPC-4. It was demonstrated that both of HC-1 and HC-2 reacted strongly with both of these lines. This indicates that HPC-3 and HPC-4 still expressed the epithelial tumor associated antigens detected by HC-1. Furthermore, the putative tumor antigen specific for pancreatic neoplasms that was defined by HC-2 monoclonal antibody was expressed on the cell surface of these lines. Therefore, these lines could be very useful for the studies of analysing immunobiological features of the pancreatic carcinomas.

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