Establishment of a New Extrahepatic Bile Duct Carcinoma Cell Line, TFK-1

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The First Department of Surgery, Tohoku University School of Medicine, Sendai 980–77, *Cancer Cell Repository, and †Department of Cancer Chemotherapy, Institute of Development, Aging and Cancer, Tohoku University, Sendai 980–77

Sajiyo, S., Kudo, T., Suzuki, M., Katayose, Y., Shinoda, M., Muto, T., FukuharA, K., Suzuki, T. and Matsuno, S. Establishment of a New Extrahepatic Bile Duct Carcinoma Cell Line, TFK-1. Tohoku J. Exp. Med., 1995, 177 (1), 61–71 — A new human extrahepatic bile duct carcinoma cell line (TFK-1) was established from a surgically resected tumor specimen, which was histologically diagnosed as partly papillary adenocarcinoma and partly differentiated tubular adenocarcinoma. The tumor cells cultured in RPMI-1640 medium supplemented with 10% FBS grew as monolayers showing epithelial-like morphology with a population doubling time of 37 hr during exponential growth at passage 40. Chromosome number was distributed in the range of 72 to 76, with a modal number of 73. Tumor markers (CEA, CA19-9, ST-439, DUPAN-2) were negative in culture supernatant and plasma of SCID mice grafted with TFK-1 cells. Though no point mutation at 12 codon of K-ras was detected, expression of c-erbB-2 product and MUC1 antigen was positive. TFK-1 is the third cell line established from extrahepatic bile duct carcinomas in the world literature, and should provide useful information on various aspects of this type of neoplasm.

In cases of bile duct carcinoma (BDC), massive infiltration of tumor cells along the bile duct tree and a multicentric origin of tumors are very frequently observed (Suzuki et al. 1989). Therefore curative resection of BDC is usually difficult, resulting in a poor prognosis of BDC patients. The limits of surgery for BDC indicate the need for adjuvant therapy to control residual BDC tissues. To develop an effective therapy for BDC, it is necessary to establish cell lines for
better understanding of its biology. However, only a limited number of BDC cell lines have so far been established, and this is especially true for the extrahepatic BDC case.

In this paper, we report the establishment of a new extrahepatic BDC cell line, TFK-1, cultured directly from surgically resected tumor tissue.

**MATERIALS AND METHODS**

*Patient*

A 63-year-old male, complaining of jaundice and general malaise, was hospitalized in our clinic. He received partial resection of the bile duct, cholecystectomy and right lobectomy of the liver. The main tumor was located in the middle of the common bile duct, where lumen was occupied by a soft papillary mass. Histologically, the tumor mainly consisted of papillary elements, but in parts demonstrated features of a moderately differentiated tubular adenocarcinoma (Fig. 1). Serum tumor marker levels on admission were as follows: CEA, 4 ng/ml; CA19-9, 11 U/ml; AFP, 4 ng/ml. These values were within the normal ranges.

*Cell culture*

A specimen, obtained at surgical operation from the main tumor, was cut into pieces, digested with 1,000 U/ml Dispase (Collaborative Research, Bedford, MA, USA) for 30 min at 37°C and the large tissue fragments allowed to settle. Supernatant fluid containing dissociated cells was collected, centrifuged at 100×g and the cell pellets were resuspended in RPMI-1640 containing 10% fetal bovine

![Fig. 1. Light micrograph of the primary bile duct tumor obtained at surgery. It shows the features of both papillary adenocarcinoma and partially tubular adenocarcinoma. (H & E, ×50)](image-url)
serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Culture was in 12-well plates (Costar, Cambridge, MA, USA) in a 5% CO₂ incubator at 37°C. Initially, contaminating fibroblast cells were found in the cultures, but after serial passage for about 6 months, remaining fibroblasts could be removed by mechanical scraping and differential attachment selection with trypsin. Thereby, a BDC cell line, designated TFK-1, forming typical epithelial monolayers was obtained. The TFK-1 cells have been serially cultured for 44 months (75 passages) since the primary culture in our laboratory.

**Heterologous transplantation**

Growing culture cells were harvested with trypsin (0.25%)/EDTA (0.02%), washed and suspended in PBS. The cells (1 × 10⁷) were then injected s.c. into the backs of CB17/Icr-SCID, Jcl mice (female, 7-week-old). Under anesthesia, the mice bearing tumors were autopsied and the tumor tissues were examined histologically.

**Karyotype analysis**

Chromosomal analysis was performed on metaphase cells prepared by treatment with 0.05 mg/ml colcemid for 12 hr. The cells were subjected to hypotonic conditions for 15 min, then fixed in methanol: acetic acid (3 : 1) solution. The treated cells were smeared on slide-glasses and air-dried slides were G-banded for analysis.

**Cell growth analysis**

Growth rate of the TFK-1 cell line at passage 40 was examined by seeding 35-mm cell culture dishes with 2 × 10⁵ cells/dish in 2 ml of RPMI-1640 supplemented with 10% FBS. The cells were counted in triplicate every day with a hemocytometer. Doubling time was calculated using a semilogarithmic graph.

**Tumor markers secreted by TFK-1 cells**

To examine for secretion of tumor markers into the culture medium, confluently growing TFK-1 cells were cultured for 2 days. Then, collected supernatant was assayed for tumor markers including CEA, CA19-9, ST-439 and DUPAN-2 by radioimmunoassay. Plasma from TFK-1 xenografted SCID mice, which were sacrificed when subcutaneous tumor size was about 1.5 × 1.5 cm, was also assayed for the tumor markers by the same method.

**Mutation analysis of the K-ras gene**

Initially, DNA was isolated from TFK-1 cells (1 × 10⁷) by the phenol-chloroform extraction method. Oligonucleotide primers used for PCR amplification of K-ras were as follows; K-ras-HindIII (5'-GGGA/AGCTTGTGGTAGTTGGAGC-3') K-ras-EcoRI (5'-CCCG/AATTCCTTG)
CACCAGTAATATGCA-3'). Genomic DNA was amplified by PCR using the above two primers, and the PCR products were digested with \textit{HindIII} and \textit{EcoRI}. Then, they were ligated into plasmid vector pUC18. The vector was transfected to \textit{E. coli} (DH5\textalpha{} strain), and six clones were obtained. To identify \textit{K-ras} mutations at codon 12, we subsequently sequenced \textit{K-ras} exon containing amplified fragments by the dideoxy chain termination method.

**Immunohistological study of the c-erbB-2 product**

Xenograft TFK-1 tumors were fixed in neutral formalin and embedded in paraffin. Tissue sections (4 \(\mu\)m thick), after deparaffinization, were treated with 0.3\% \(\text{H}_2\text{O}_2\) in methanol and incubated with rabbit polyclonal anti-c-erbB-2 product (31004; Nichirei, Tokyo). Then, they were incubated with biotinylated anti-rabbit IgG, followed by incubation with ABC complex (Vector Lab. Inc., Burlingame, CA, USA). The sections were developed with substrate solution which contained 3, 3'-diaminobenzidine and \(\text{H}_2\text{O}_2\).

**FACS analysis of MUC1 expression**

MUC1 expression on the surfaces of TFK-1 cells was examined by indirect membrane immunofluorescence tests using Muse-11 monoclonal antibody (kindly supplied by Dr. Imai, Sapporo Medical College), and FITC conjugated anti-mouse IgG (EY Lab., San Mateo, CA, USA) as the second antibody. The stained cells were analyzed by flow cytometry (FACSsort, Beckton Dickinson, Lincoln Park, NJ, USA).

**Morphologic study**

Tissue sections of the BDC tumor obtained at surgery and xenografted tumors were stained with hematoxylin and eosin (H & E). Monolayer cells after fixation with 2\% glutaraldehyde were processed by routine methods for transmission electron microscopy (TEM).

**RESULTS**

**Morphological characteristics of cultured TFK-1 cells**

Fig. 2 shows the typical morphological features of TFK-1 cells on a plastic dish, with formation of polygonal epithelial monolayers showing a pavement stone-like arrangement.

**Electron microscopic findings**

Ultrastructural observation by TEM revealed the cells to contain irregular orthochromatic nuclei and to be relatively rich in mitochondria and ribosomes, with moderate amounts of rough endoplasmic reticulum and microfilaments (tonofilaments). On the surfaces of the cells, pseudopods and numerous microvilli were evident (Fig. 3).
Growth kinetics

Cell doubling times around passage 40 were 37 hr, as shown in Fig. 4.

Chromosome analysis

Chromosome analyses at passage 40 revealed a modal number of 73 with a
range of 72–76 (Fig. 5). A representative G-banded karyotype is shown in Fig. 6, exhibiting some common structural changes. Abnormalities of i (1q), dup (1) (p22 p32), t (1; 9) (p13 : p21), del (3) (p13 p21), t (6; 9) (q11 ; q11), t (11; 15) (p11, p11) were found in 100% of the cells, of i (15q), i (17q), i (21q) in 90%, and of del (11) (p11) in 60% (Fig. 6).

**Heterotransplantation**

With an inoculum of $1 \times 10^7$ cells, progressively growing tumors were regular-
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Fig. 6. A representative G-band karyotype for a TFK-1 cell at passage 40 showing
73, XY, +i(1q), +der(1)t(1; 15)(p11; p11), dup(1)(p22 p32), +2, +2,
+del(3)(p13 p21), +del(3)(p13 p21), +del(3), +4, +der (6)t(6; 15)(q11;
q11), +8, +10, +der(11)t(11; 15)(p11; p11), +der(11)(p11), +12, −13,
+der(14)t(14; 18)(p11; q11), +der(15)t(11; 15)t(p11; p11), +der(15)t(14;
15)(p11; q11), +16, +16, +i(17q), +der(19)t(1; 19) (p21; p13) +20,
+i(21q), +20, +i(21q), +22, +mar, +mar.

Fig. 7. Light micrograph of a xenografted tumor in a SCID mouse.
ly observed in SCID mice. Light microscopy of the xenografted tumor specimens, obtained one month after inoculation, demonstrated the same morphology as the surgically resected BDC tumor, that is, the main component was invasive papillary adenocarcinoma accompanied by the areas of moderately differentiated solid structures (Fig. 7). Expression of c-erbB-2 product was weakly demonstrated by immunohistological investigation of xenograft tumor tissue (Fig. 8).

![Image](image-url)

**Fig. 8.** Moderate c-erbB-2 expression in a xenograft TFK-1 tumor (SCID mouse tumor). Expression of c-erbB-2 protein was detected immunohistochemically, using a rabbit anti-c-erbB-2 polyclonal antibody specific for the intracellular domain of the protein (Nichirei Co., Inc., Tokyo).

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<tr>
<th>Table 1. Tumor marker levels in plasma and culture supernatant</th>
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<td>Patient's plasma</td>
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<td>(on admission)</td>
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<td>Plasma of tumor bearing SCID mice (n=2)*</td>
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<td>Culture supernatant*</td>
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Tumor marker levels measured by radioimmunoassay.
*Aliquots of \(1 \times 10^7\) TFK-1 cells in 0.2 ml PBS were injected subcutaneously into the backs of SCID mice. Sacrifice was 1 month after tumor injection, when the tumor size was about \(1.5 \times 1.5\) cm.
*Confluent TFK-1 cells were cultured for 48 hr at 37°C, then culture supernatant was obtained for tumor maker determination.
*Not detected.
Secretion of tumor markers

In Table 1, tumor marker levels are shown. Two tumor markers (CEA and CA19-9) in the patient's plasma taken at admission were within normal range. In the plasma of tumor-bearing SCID mice, no increase in CEA or CA19-9 was found. Similarly, four tumor markers (CEA, CA19-9, ST-439, and DUPAN-2) did not show positive values in the TFK-1 culture supernatant.

MUC1 expression on TFK-1 cells

As shown in Fig. 9, FACS analysis revealed positive expression of MUC1 antigens on the TFK-1 cells (74.5%).

K-ras gene analysis

No mutations of K-ras at codon 12 were found on sequencing of amplified DNA from TFK-1 cells.

DISCUSSION

In the present study, we succeeded in establishing a new human extrahepatic BDC cell line from a surgically resected specimen, without xenotransplantation. The cell line shows the typical morphology of polygonal epithelial monolayers with a pavement stone-like cell arrangement. The electron microscopic demonstration of numerous microvilli on the luminar cell surfaces, with bundles of intracytoplasmic tonofilaments, further confirms a typical epithelial cell phenotype. Although secretion of tumor markers (CEA, CA19-9, ST-439, DUPAN-2) from TFK-1 cells was not detected, expression of c-erbB-2 product and MUC1 antigen (Yamashita et al. 1993) was positive. In addition the histology of xenotransplanted lesion closely resembled that of the original surgically resected tumor. These results indicate that the TFK-1 cell line is indeed derived from
BDC tissue.

In the literature, establishment of SK-ChA-1 (Knuth et al. 1985), KMBC (Yano et al. 1992), HuCC-T1 (Miyagiwa et al. 1989), HChol-YI (Yamaguchi et al. 1985) and KMC-1 (Iemura et al. 1992) BDC cell lines has been reported. Among them, HuCC-T1, HChol-YI and KMC-1 cells were derived from intrahepatic BDC, leaving only two lines (SK-ChA-1, and KMBC) as extrahepatic BDC cell lines. Therefore TFK-1 is the third established cell line originating from a human extrahepatic BDC. Since considerable biological differences between intrahepatic and extrahepatic BDC have been observed, this TFK-1 as an in vivo/in vitro tool should prove very valuable for the study of BDC biology.

The reason why we could succeed in establishing a BDC cell line directly from the surgically resected tumor tissue is not clear, although the fact that the obtained tissue showed good viability with rapid proliferation of the cells in the culture medium, may have been important. SK-ChA-1 was established from malignant ascites with primary adenocarcinoma of the extrahepatic biliary tree, and KMBC was established from a serially transplanted tumor in nude mouse. Most culture cell lines established from xenografted tumor tissues are known to be infected with murine C-type viruses, while TFK-1 cell line has been established from surgically resected tumor specimen. The method applied for establishment of the cell line should give more value to TFK-1 cell line.

KMBC cells, originated from well-differentiated adenocarcinoma, possess various functional characteristics, such as CA19-9, tissue peptide antigen, CEA, ferritin, $\beta_2$-microglobulin, fibronectin and $\alpha_2$-macroglobulin. Contrary, TFK-1 cells secrete neither CA19-9 nor CEA. The difference between the two cell lines might be related to differentiation stage of the two BDC tissues.

According to a recent paper (Imai et al. 1994), K-ras codon 12 mutations are detectable in a high proportion of bile duct tumors (13 of 23, 56.5%) when tumor samples are obtained by microdissection from paraffin-embedded tissue sections. However, K-ras mutations do not appear to be correlated with tumor size, histologic type or metastatic potential (Imai et al 1994). In the present study, TFK-1 was not found to show K-ras codon 12 mutation, but the fact that it does express c-erbB2 product, and MUC1 antigen, is of interest in terms of studies of BDC carcinogenesis.

Acknowledgment

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Note Added in Proof
In this cell line, mutation of p53 was demonstrated by functional analysis of separated 