

SHORT COMMUNICATION

Establishment and Characterization of a Cholangiocarcinoma Cell Line from a Thai Patient with Intrahepatic Bile Duct Cancer

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Liver cancer is still a leading cause of morbidity and mortality of the population in many parts of the world including Thailand.¹ Of the two primary liver cancers, cholangiocarcinoma (CCA), a malignant tumor of the epithelial lining of the biliary system, is more common in the north-eastern part of Thailand where the prevalence of liver fluke infection is the highest. The association between *Opisthorchis viverrini* (*Ov*) infestation and CCA has been established from epidemiological, clinical and pathological studies,²⁻⁵ and more recently confirmed in a case control study by Parkin and associates.⁶ Altogether, different lines of evidence clearly demonstrate that the development of CCA in Thailand depends on the interaction between *Ov* infection and nitrosamines, either consumed as contaminants in foods or endogenously produced.⁴⁻⁸

A specific diagnosis for CCA in humans is not yet available.⁹ Ultrasonography is a useful tool but it often detects the disease when it is too late to be successfully managed. A specific tumor marker(s) is not yet available although such non-specific markers as carbohydrate associated antigens (i.e., CA 19-9,

SUMMARY A new human cholangiocarcinoma cell line (HuCCA-1) was established from cholangiocarcinoma (CCA) tissue fragments surgically removed from a Thai patient with intrahepatic bile duct cancer. The growth medium used for the primary cell culture was Ham's F12 supplemented with 10% fetal bovine serum (FBS) and 10 ng/ml epithelial growth factor (EGF). Approximately one month later, the cells were subcultured in Ham's F12 supplemented with only 10% FBS. The population doubling time was approximately 55 hr. Staining of the cells for cytokeratin and mucin confirmed that the cells were mucin-secreting tumor of epithelial cell origin. The supernatant fluid secreted a number of non-specific tumor markers including CA 125 and traces of MCA and AFP. The ability of the HuCCA-1 cell line to synthesize specific marker that may have potential in the diagnosis of cholangiocarcinoma is now being investigated.

CA 125 and CA 50) have been reported to be elevated in the serum from a high proportion of cholangiocarcinoma patients, particularly in those with metastasis.⁷⁻¹¹ However, a number of other benign diseases of the biliary system are known to be associated with the elevation of these markers.¹⁰ Recently it was demonstrated that cholangiocarcinoma chemically induced in rats contained large amounts of a biliary cell membrane glycoprotein that could be readily detected in the serum of tumor-bearing animals.¹² If this is the case and can be subsequently confirmed, it may lead to the discovery of a diagnostically useful tumor marker in humans.

Up to now, there have been a few reports on human cholangiocarcinoma cell lines¹³⁻¹⁵ and a variety of common tumor markers have been detected in the supernatant culture fluid.^{13,14} Whether or not

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these cell lines also produce other tumor markers, particularly those specifically associated with CCA, remains to be determined. In the present study, we attempted to establish and characterize a human CCA cell line with the objective to simplify our task of identifying specific tumor marker(s). Our ultimate goal is to eventually identify and isolate potential candidates for the very early detection of cholangiocarcinoma in humans.

MATERIALS AND METHODS

Clinical specimens

Liver tissue and serum specimens were taken from a 54-yr old male patient admitted to Siriraj Hospital with an enlarged left lobe of the liver. Clinical signs and symptoms gave an initial impression of a primary liver cancer. Analysis of serum for tumor markers showed an elevated level of carcinoembryonic antigen (CEA) but a normal level of alpha-fetoprotein (AFP). Additionally, the serum was ELISA positive for antibody to *Ov* antigen, suggesting a current or previous infection with the liver fluke. Histological examination of liver tissue sections revealed cells with the characteristics of adenocarcinoma. Together, these laboratory, pathological and clinical findings were consistent with a diagnosis of peripheral cholangiocarcinoma with metastasis to the stomach and diaphragm.

Fragments of the intrahepatic tissue were surgically removed from the patient, suspended in Ham's F12 culture medium (GIBCO) at 4°C and immediately transferred to the laboratory for processing. All steps were carried out under sterile conditions in the presence of penicillin (100 U/ml), streptomycin (100 µg/ml) and gentamycin (100 µg/ml).

Cell cultures

Techniques for the establishment of our human cholangiocarcinoma cell line (HuCCA-1) were slight-

ly modified from those previously described.^{13,14,16} Briefly, the tumor tissue was quickly washed in phosphate buffered saline (PBS), pH 7.2, and minced with scissors, after which a single cell suspension was prepared by using a stainless steel mesh. The cell suspension was washed twice in Ham's F12 culture medium prior to being cultured. Cells were placed into 25 cm² plastic tissue culture flasks (Nunc) with 3-4 ml of the growth medium containing Ham's F12, 10% fetal bovine serum (FBS, Flow Laboratories), 10 ng/ml epithelium growth factor (EGF, GIBCO), 100 U/ml penicillin and 100 µg/ml streptomycin. The cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere.

Cultures were observed daily for 3 days following the initial seeding. During this interval, a majority of the cells attached to the flasks. Floating cells in the supernatant fluid were transferred to new flasks and allowed to adhere. These flasks were subsequently treated in the same manner as the primary seeding flasks. After attachment, fresh culture growth medium was changed every 2-5 days. One month after initial cultivation, monolayers of polygonal-shaped cells began to appear with minimum fibroblast overgrowth. Cells in the primary cultures were then removed for subculturing ($1-2 \times 10^6$ cells per flask) using 0.25% trypsin and 0.1% EDTA. New growth medium (4-5 ml) consisting of Ham's F12 supplemented with 10% FBS but without EGF was added every 3-4 days. The cultured were passaged every 7-10 days.

Growth curves

The method used to study cell growth was essentially the same as described elsewhere.^{13,14} Specifically, 2×10^5 cells cultured in Ham's F12 supplemented with 10% FBS (Passage 28) were added to a series of 60-mm plastic Petri dishes (Nunc) containing 5 ml of the same growth medium which was subsequently replaced every 2 days. At time inter-

vals, the cells were checked for viability and counted. The population doubling time was calculated from the exponential phase of growth.

To determine the colony-forming efficiencies, 250, 500 and 1000 cells were incubated for 14 days in 100-mm plastic dish (Nunc) containing 10 ml of growth medium. The number of colonies (≥ 10 cells) were counted.

Indirect immunofluorescence

Cell monolayers at Passage 5 were subcultured and allowed to grow on sterile microscope slides. After confluent growth was obtained, the slides were washed with PBS, air-dried and fixed in cold acetone for 15 min. The slides were then overlaid with mouse monoclonal antibodies to human cytokeratin for 30 min and thoroughly washed with PBS. Biotinylated rabbit antibody to mouse immunoglobulins was subsequently applied for 30 min and washed. The slides were finally flooded with fluoresceinated streptavidin and examined under a fluorescent microscope.

Two different mouse monoclonal antibodies were used. The DAKO-CKI clone LP34 is known to react with human epithelial tissues and is useful in confirming the epithelial origin of tumor cells. According to the manufacturer, it reacts with keratin intermediate filaments with molecular weights of 45 and 56 kDa. Another monoclonal, AE1/AE3 (Boehringer monoclonal antibody, (AE1/AE3 (Boehringer Mannheim), is claimed by the manufacturer to be more specific and is therefore useful in distinguishing carcinomas from non-carcinomas. It recognizes human cytokeratin antigens with molecular weights of 50, 56.5, 58 and 65-67 kDa.

Other techniques

Chromosome analysis of the cell monolayer at Passage 52 was carried out as previously described.¹⁷ Mucin production by cells at Passage 42 was determined by the Mayer's

mucicarmine reaction.¹⁸ Tumor markers CA 19-9, CA 125 and mucin-like carcinoma associated antigen (MCA) were quantitated by enzyme immunoassay (EIA) using commercial kits (Roche) and AFP was quantitated by a sandwich ELISA.¹⁹

RESULTS AND DISCUSSION

Under the cultivation conditions described herein, a majority of the dispersed cells prepared from the human CCA tissue fragments adhered to the bottom of the flask. Within a few weeks of growth, monolayers began to appear (Fig. 1) and polygonal-shaped epithelial cells could be readily distinguished from spindle-shaped cells which most likely represented fibroblasts. The latter gradually decreased in number with each passage and finally disappeared after about one month of primary culture. Although the maintenance of the HuCCA-1 cell line was routinely carried out in the presence of 10% serum supplement, the latter was gradually reduced and once a confluent growth was obtained, it was omitted without adverse effect on cell integrity. Our results were essentially identical to those reported by other investigators. Yamaguchi and associates were able to establish a cholangiocarcinoma cell line, HChol-Y1, from an autopsy of a patient with a primary liver tumor. They used Ham's F12 medium containing 0.1% FBS for a primary culture and subsequently used serum-free, chemically defined medium for culture maintenance.¹³ Another cholangiocarcinoma cell line, HuCC-T1, was similarly established with ascites fluid cells from a patient with a primary liver tumor, using RPMI 1640 medium originally supplemented with 10% serum that was subsequently replaced with 0.2% lactalbumin hydrolysate.¹⁴

With the hematoxylin and eosin (H&E) stain (Fig. 2), the monolayer cells showed prominent round-to-oval nuclei, occasionally with multiple nucleoli, and the cytoplasm

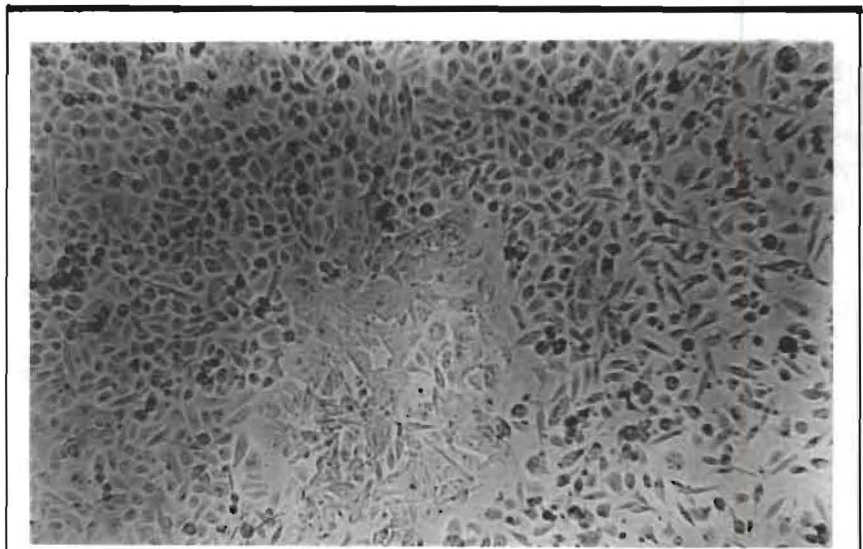


Fig. 1 Appearance of unstained primary cell culture from human CCA tissue fragments. The photograph was taken 20 days after the initial seeding ($\times 100$).

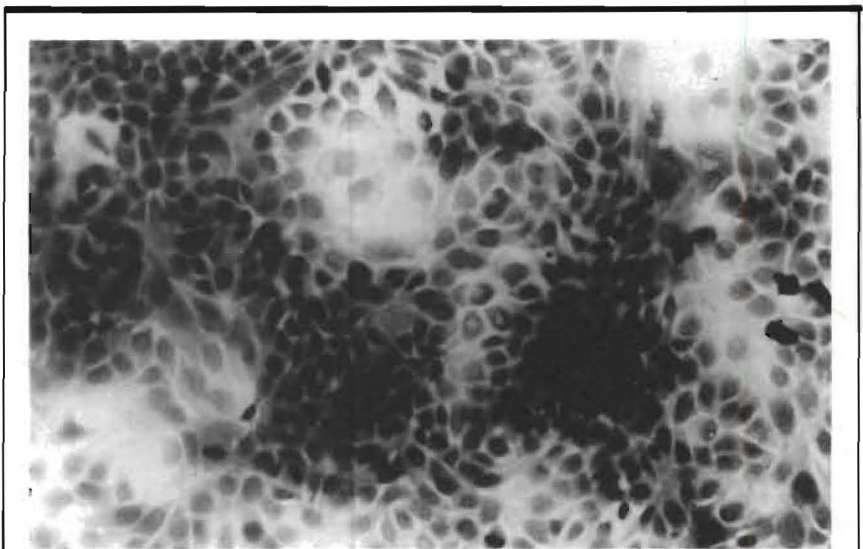


Fig. 2 Morphology of the HuCCA-1 cell line at Passage 23. Piling up of cells and occasional gland-like appearance are seen among the confluent monolayers. The cells were stained with H & E ($\times 200$).

was filled with granules. This was particularly evident with the phase contrast microscope (Fig. 3). On occasion, gland-like structures could be noticed. When the cell line was cultured a Passage 28 in the presence of 10% serum supplement and the growth rate was determined, the

results were very similar to those reported previously for the other two human cholangiocarcinoma cell lines described above.^{13,14} The growth curve of our cell line is shown in Fig. 4. The population doubling time was about 55 hr. The mean colony-forming efficiency of the cell line

was 25.9%. The limited data from chromosome analysis done at Passage 52 indicated an abnormal chromosome number. More detailed cytogenetic analysis of other cholangiocarcinoma cell lines has shown numerous other chromosomal aberrations including many breakpoints.¹⁵

To ascertain whether our HuCCA-1 cell line was a mucin secreting tumor of epithelial cell origin, the cells were cultured as monolayers on microscope slides and stained with specific anticytokeratin for epithelial cell type or with Mucicarmine reagent for mucin production. Isolated cells exhibited positive stainings for these components. These observations are consistent with those obtained from histopathological studies of tissue sections by other investigators.

Because one of the main objectives in establishing human cholangiocarcinoma cell lines was to use them for the study of specific tumor marker(s), we evaluated this cell line for its capacity to synthesize commonly known tumor markers, i.e., CA 19-9, CA 125, MCA and AFP. In this experiment, confluent monolayers grown in the presence of 10% FBS were evaluated for their capacity to synthesize these markers in the presence or absence of serum supplement. Results presented in Table 1 shows the capacity to synthesize some of these common tumor markers and indicates that the synthesis was slightly enhanced by 10% FBS. The other 2 cholangiocarcinoma cell lines, i.e., HChol-Y1 and HuCC-T1, also synthesized these markers in a manner that was affected by the growth conditions.^{13,14} It is hoped that cell line HuCCA-1 also synthesizes a number of other tumor associated markers and that some of these may be cholangiocarcinoma specific. We have found a 200 kDa glycoprotein in CCA extracts by SDS-PAGE and immunoblotting techniques (Unpublished observations) and feel that this protein is a potential candidate for the diagnosis of CCA in

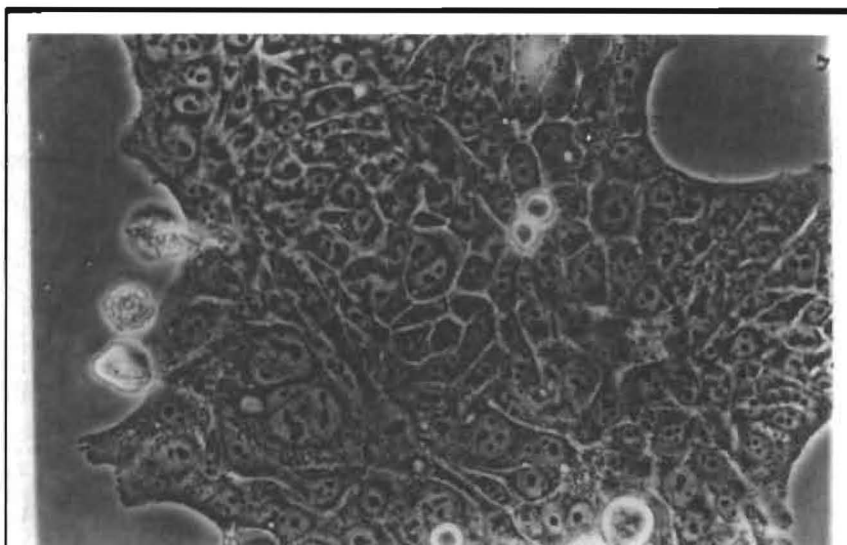


Fig. 3 Morphology of the HuCCA-1 cell line at Passage 53 seen under the phase contrast microscope. A large number of polygonal shaped cells possessing nuclei with multiple nucleoli can be readily observed ($\times 200$).

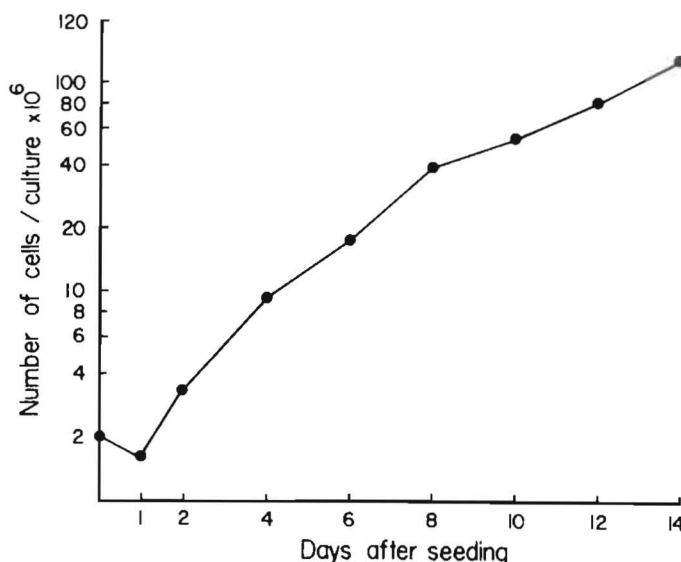


Fig. 4 Growth curve of the HuCCA-1 cells cultured in 10% FBS. 2×10^5 cells at Passage 28 were plated in 60-mm plastic Petri dishes and triplicate cell counts were made every other days for 2 weeks. Population doubling time was 55 hr.

humans. Studies are now underway to determine whether HuCCA-1 synthesizes and secretes this component into the medium. Chemically induced cholangiocarcinoma in rats is known to secrete membrane asso-

Table 1. Tumor markers secreted in the spent media by HuCCA-1 cell line at Passage 44.

Medium	CA 125 ^a	CA 19-9 ^a	MCA ^a	AFP ^a
Serum-free	222	0	4.8	11.0
10% FBS	438	0	38.0	3.6

^a Tumor markers, measured by enzyme immunoassay, were produced by 10⁶ cells under confluent growth and expressed as U/ml/10⁶ cells/48 hr.

ciated protein that can be readily detected in the blood circulation of tumor-bearing animal.¹²

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