

• BASIC RESEARCH •

Establishment and characterization of an opisthorchiasis-associated cholangiocarcinoma cell line (KKU-100)

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Abstract

AIM: To establish and characterize a new cholangiocarcinoma cell line from a patient living in the *Opisthorchis viverrini* (*O. viverrini*) endemic area of Northeast Thailand.

METHODS: Fresh liver biopsy and bile specimens were obtained from a 65-year-old Thai woman with cholangiocarcinoma of the *porta hepatis*. After digestion, the cells were cultured in Ham's F12 media. The established cell line was then characterized for growth kinetics, cell morphology, immunocytochemistry and cytogenetics. Tumorigenicity of the cell line was determined by heterotransplanting in nude mice.

RESULTS: The primary tumor was a poorly differentiated tubular adenocarcinoma. Examination of the bile revealed malignant cells with *O. viverrini* eggs. The cholangiocarcinoma cell line KKU-100 was established 4 mo after the primary culture-population doubling time was 72 h. KKU-100 possesses compact and polygonal-shaped epithelial cells. Immunocytochemically, this cell line exhibited cytokeratin, EMA, CEA, and CA125, but not α -fetoprotein (AFP), CA19-9, desmin, c-met, or p53. Such protein expressions parallel those of the primary tumor. Cytogenetic analysis identified aneuploidy karyotypes with a modal chromosome number of 78 and marked chromosomal structural changes. Inoculation of KKU-100 cells into nude mice produced a transplantable, poorly differentiated adenocarcinoma, similar to the original tumor.

CONCLUSION: KKU-100 is the first egg-proven, *Opisthorchis*

associated cholangiocarcinoma cell line, which should prove useful for further investigations of the tumor biology of this cancer.

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Key words: Cholangiocarcinoma; Cell line; Establishment; Characterization; Opisthorchiasis

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INTRODUCTION

Cholangiocarcinoma is rare but its prevalence in Northeast Thailand makes the region the area of highest incidence in the world^[1]. Coincidentally, Thailand has a high prevalence of infection of the liver fluke, *Opisthorchis viverrini*, and an estimated six million people are infected^[2]. Both experimental and epidemiological evidence implicate opisthorchiasis in the etiology of cholangiocarcinoma in this endemic area^[3,4]. Cumulative data suggest that the pathogenesis of this bile duct cancer in this region is different from that observed in Western countries with different etiologies^[4]. However, the detailed mechanisms of the cellular and molecular pathogenesis of *Opisthorchis*-associated cholangiocarcinoma and its biology are unclear.

Human solid tumor cell lines are important sources for studies of tumor biology including tumor cell growth, differentiation, metastasis, molecular pathogenesis, and susceptibility to drugs. A small number of reports, compared to other cancers, describing cholangiocarcinoma cell lines have been published, most from extrahepatic cholangiocarcinoma^[5-9] and a few from intrahepatic cholangiocarcinoma^[9,10-13]. Only one cholangiocarcinoma cell line was developed from a Thai^[14]. We therefore aimed to establish more cholangiocarcinoma cell lines from patients living in Northeast Thailand. Our report describes the establishment and characterization of an egg-proven, *Opisthorchis*-associated cholangiocarcinoma cell line, developed in our laboratory and designated as KKU-100.

MATERIALS AND METHODS

Patient

A 65-year old Thai woman was admitted to the Faculty of

Medicine, Srinagarind Hospital, Khon Kaen University, Thailand. She arrived with nausea, progressive jaundice and itching. She was previously diagnosed by ultrasonography at a provincial hospital as having cholangiocarcinoma. Significant laboratory investigations at admission showed elevated alkaline phosphatase (ALP) (266 U/mL, normal 42-120 U/mL), total bilirubin (23.1 mg/mL, normal 0.25-1.5 mg/mL), carcinoembryonic antigens (CEA) (44.6 ng/mL, normal <2.5 ng/mL), while the serum α -fetoprotein (AFP) (2.8 U/mL, normal <10 U/mL), HBsAg and HBsAg were negative. The stool examination revealed *O. viverrini* eggs. The serum counterpart was positive for the parasite-specific antibody using ELISA.

The diagnosis of cholangiocarcinoma was confirmed by ultrasonography, computer tomography, and endoscopic retrograde cholangiopancreatography (ERCP). Operative findings revealed skipped tumor lesions at the *porta hepatis* with bile duct obstruction and ascites (approximately 1 000 mL). The cul-de-sac, peritoneum and bowel wall were tumor free. A bilateral peripheral hepatojejunostomy (Roux-en-Y) with cystojejunostomy was the palliative treatment performed. Liver biopsy of the tumor mass was taken, the histopathology indicated a poorly differentiated tubular adenocarcinoma. Biliary cytology revealed clusters of tumor cells with *O. viverrini* eggs. The patient developed sepsis and died two follow-ups after surgery. Informed consent was obtained from the patient.

Establishment of tumor cell line

The liver biopsy was transported to the Department of Pathology immediately after surgery and used for cell culture. The tumor tissue was aseptically washed in Ham's F12 media (Seromed, Berlin, Germany) containing penicillin (200 U/mL) and streptomycin (200 μ g/mL), minced, then digested with 0.25% trypsin-EDTA (Gibco/BRL, Grand Island, MA) for 1 h at 37 °C. After dissociation, the tissue fragments were force-filtered through a 100- μ m pore mesh and the cell suspension was washed in the media 2-3 times to remove any residual blood. The washed cells were then cultured in Ham's F12 containing penicillin (100 U/mL) and streptomycin (100 μ g/mL) with 20% fetal bovine serum (Gibco/BRL) at 37 °C with 50 mL/L CO₂, the media were changed twice, weekly. Cultured tumor cells were observed daily under a phase-contrast microscope (Olympus, Tokyo, Japan). Contaminating fibroblasts were periodically removed with a cell scraper (Costar, Cambridge, MA) and by differential trypsinization.

Growth kinetics

After establishment, growth kinetics of the tumor cells was obtained by seeding the cells at 2×10^5 cells/well in duplicate in a 24-well plate (Nunc, Roskilde, Denmark). After seeding, the media were changed every two days. The cells were detached from the wells with trypsin-EDTA and the average number of viable cells was counted every 24 h in a hemacytometer in the presence of trypan-blue dye. Cells were counted for up to 12 d. The doubling time of the cell population was estimated during the logarithmic growth phase.

Morphology

Tissue culture flasks were routinely monitored for cell

morphology under a phase-contrast microscope.

Heterotransplantation

Four- to six-week-old athymic nude mice (BALB/cAnNCrj-nu/nu) were used for heterotransplantation. The mice were injected subcutaneously with KKU-100 cells (1×10^7 cells) suspended in culture medium and kept under specific pathogen-free conditions. The tumor was observed every week and when it reached a diameter of ≥ 1.5 cm, the mice were killed. The tumor was minced and transplanted again to other nude mice for serial transplantation. A portion of tumor was fixed in 10% buffered formalin, routinely processed for histopathology and immunocytochemistry.

Immunocytochemistry

To characterize the expression of cytokeratin, tumor markers and some gene products, the cells were grown as a confluent monolayer, washed in cold PBS, then scraped. After centrifugation, the cell pellet was fixed in buffered formalin and routinely processed in a cell block. Paraffin sections were cut and immunostained for cytokeratin, CEA, AFP, CA125, CA19-9, c-met and p53 using the standard avidin-biotin peroxidase method^[15]. Details of the antibodies are shown in Table 1. Mouse monoclonal antibody to pancytokeratins (Dako, Denmark, clone MNF 116) reacts with cytokeratins 5, 6, 8, 17, and probably 19 as shown in the specification sheet of the manufacturer. Primary and heterotransplanted tumors were similarly immunostained.

Cytogenetics

After 20 passages, the established tumor cells at the exponential phase were subjected to chromosomal analysis by treating them with 0.1 μ g/mL colcemid (Gibco/BRL). The cells were treated with a KCl/HEPES/EDTA hypotonic solution and harvested according to standard cytogenetic procedures. Slides of fixed cells were trypsin-Giemsa-banded to identify individual metaphase chromosomes. Representative chromosome sets were photographed and karyotyped. The modal chromosome number was determined from 100 cells.

Mycoplasma detection

Direct agar incubation of spent medium using a mycoplasma agar plate (Gibco/BRL) was routinely performed to detect any mycoplasma contamination.

RESULTS

Establishment of KKU-100 cell line

A few days after the primary culture, a few single colonies of epithelial-like round cells and fibroblasts attached to the 25 mL culture flask. The colonies were inactive and stable for several weeks while fibroblasts grew faster. Fibroblasts were periodically removed by scraping. Three months after the cell culture was initiated, the epithelial-like cells became active and propagated as monolayer cells. The colonies were then subcultured with trypsin-EDTA. Contaminating fibroblasts were removed by scraping and differential trypsinization. After four successive passages, a pure tumor cell line with continuous, stabilized multiplication was established and designated as KKU-100. The cell line KKU-100 was

maintained in our laboratory in >100 passages over the past 5 years.

Growth kinetics

KKU-100 cells followed a typical growth curve, including lag, logarithmic and stationary phases during the 12-d culture. By the tenth passage, the population doubling time in Ham's F12 with 10% fetal bovine serum, was approximately 72 h.

Morphology of KKU-100

Under a phase-contrast microscope, KKU-100 exhibited compact, monotonous polygonal to spindle cells. The cells were 'floating' or 'clumping' in a confluent monolayer that could be shaken free, mixed into the medium and transferred to another flask (Figure 1). Individual KKU-100 cells had a large nucleus containing 2-5 nucleoli and a clear cytoplasm.

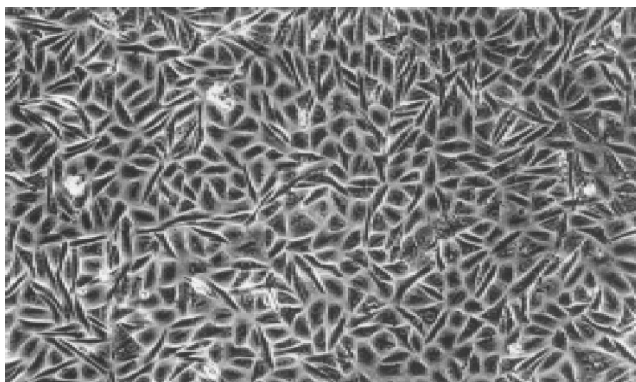


Figure 1 A confluent monolayer of KKU-100 cells shows compact polygonal to spindle cells. (Phase-contrast, original magnification, $\times 100$).

Heterotransplantation

Tumor nodules were developed in the nude mice three weeks after inoculation of KKU-100 cells and reached their greatest dimension of 1.6 cm in 10 wk. Serial transplantations into other nude mice had a shorter induction time for tumor nodules and grew larger. The second generation of transplanted tumors developed one week post inoculation and reached a maximum size of 4.4 cm in 10 wk. Histologically, the transplanted tumor was a poorly differentiated adenocarcinoma, similar to the primary tumor (Figures 2A and B).

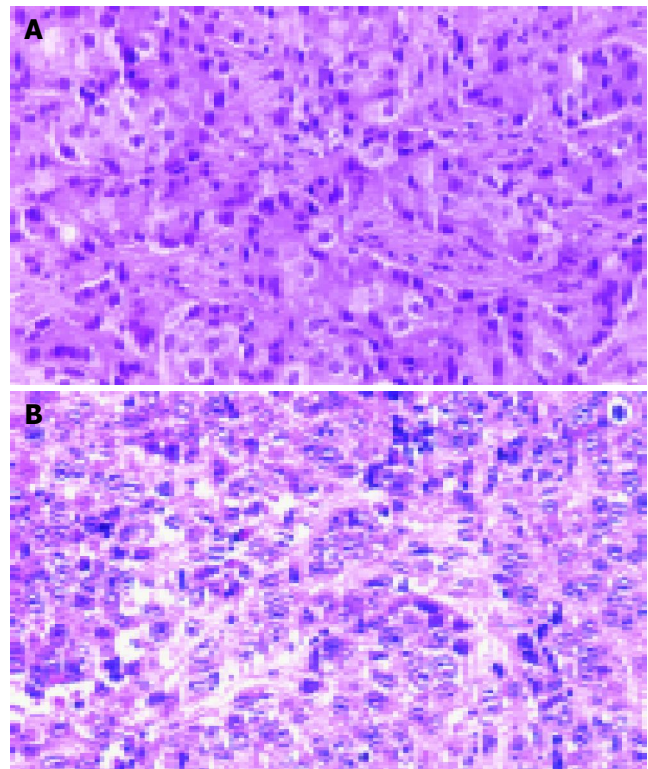


Figure 2 Histopathology of the primary tumor (A) and KKU-100 heterotransplanted tumors (B) in nude mice. (hematoxylin and eosin, original magnification $\times 200$).

Immunocytochemistry

Expression and grading of cellular antigens, tumor markers and cancer gene products of the original tumor and KKU-100 cells are shown in Table 1. KKU-100 cells expressed cytokeratin, EMA, CEA, and CA125 but not AFP, CA19-9, desmin, c-met and p53. These protein expressions were similar to those of the primary tumor. However, heterotransplanted tumors in nude mice retained only EMA protein (Figures 3A-D and Table 1).

Chromosomal analysis

A cytogenetics study revealed a number of chromosomes ranging between 56 and 92 with a modal chromosome number of 78. The G-banded analysis demonstrated aneuploidy karyotype with marked chromosomal structural abnormalities. Several marker chromosomes were noted. Example of the karyotype is shown in Figure 4.

Table 1 Details of antibodies used and their expression in primary tumor, KKU-100 cells and heterotransplanted tumors

Antibodies	Sources	Primary tumor ¹	KKU-100 cells ¹	Heterotrans-planted tumor ¹
Cytokeratin (clone MNF116)	Dako, Denmark	+++	++	-
EMA (clone E29)	Dako, Denmark	++	++	+++
Desmin (clone D33)	Dako, Denmark	-	-	-
CEA (polyclonal)	Dako, Denmark	+++	+	-
AFP (polyclonal)	Dako, Denmark	-	-	-
CA19-9 (clone 116-NS-19-9)	Dako, Denmark	-	-	-
CA125 (clone Ov185:1)	Novocastra, UK	+++	+	-
c-Met (polyclonal)	Santa Cruz, USA	-	-	-
p53 (clone DO-7)	Dako, Denmark	-	-	-

¹Grading: - = negative, + = <25% tumor cell stained, ++ = 25-50% tumor cell stained, +++ = >50% tumor cell stained.

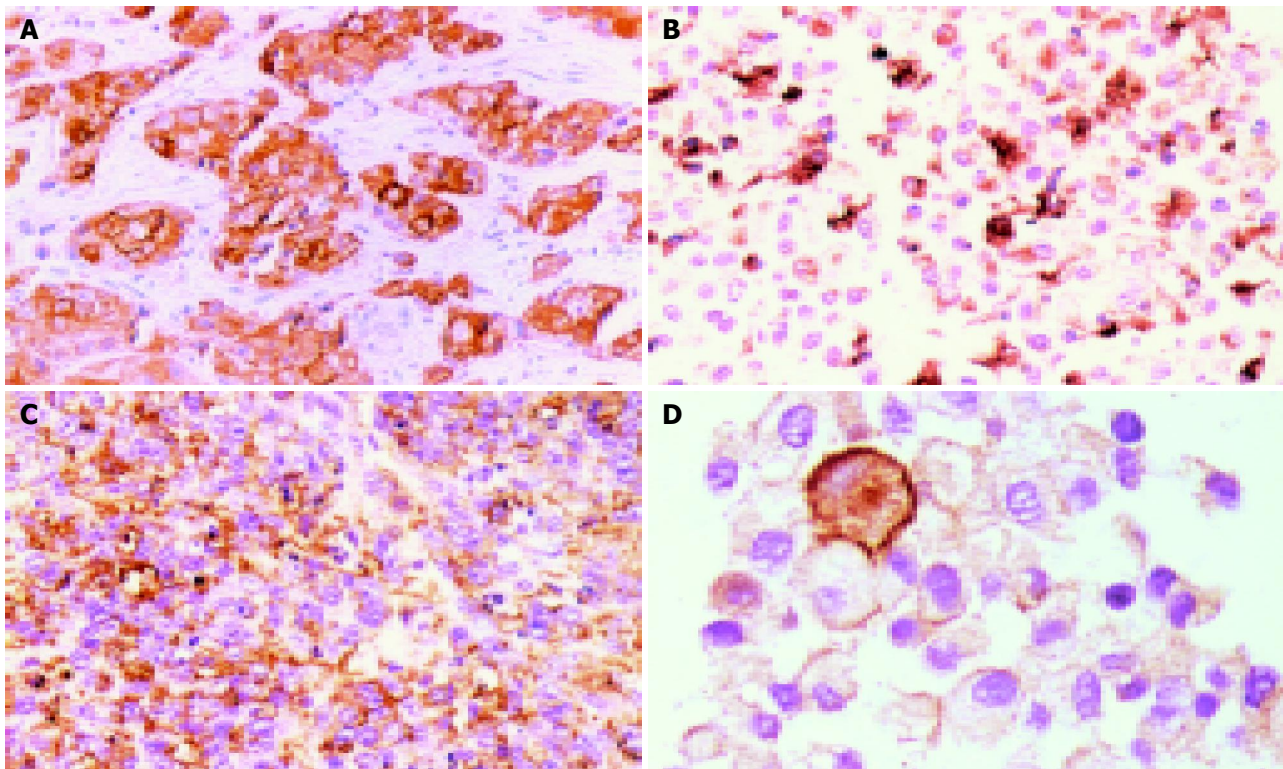


Figure 3 Immunocytochemistry of the primary tumor and KKU-100 cells. **A:** the primary tumor showing strong expression of cytokeratin; **B:** strong expression of

KKU-100 cell line; **C:** EMA expression in both heterotransplanted tumor cells; **D:** EMA expression in KKU-100 cells (immunoperoxidase, original magnification $\times 200$).

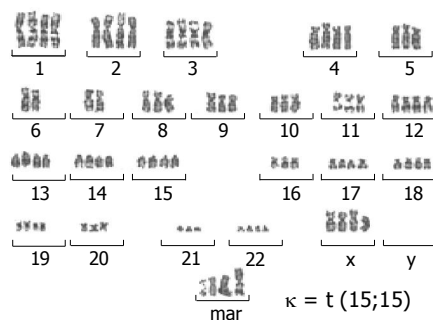


Figure 4 Representative G-banded karyotype of KKU-100 at passage 20 showing chromosomal abnormalities and marker chromosome (mar) : 84, +XX, +1, +2, +3, +4, +5, +8, +9, +10, +11, +12, +13, +14, +der(15)t(15;15), +16, +17, +18, +19, +20, +21, +22, +mar.

Mycoplasma detection

Mycoplasmas were not detected in the spent medium during cell culturing.

DISCUSSION

A small number of cholangiocarcinoma cell lines are available for cancer research and little is known about this bile duct cancer. To date, approximately eight cell lines have been developed from intrahepatic bile duct cancer, namely HChol-Y1^[5], SNU-1079^[9], HuCC-T1^[11], PCI:SG231^[12], CC-SW-1 and CC-LP-1^[13], HuCCA-1^[14], KMC-1^[16]. There is no cell line, to our knowledge, developed from porta hepatitis, though tumors at this site are lethal and account for about two-thirds of all cholangiocarcinomas^[17,18]. This is probably

due to the difficulty of tumor resection and obtaining specimens^[9]. KKU-100 may probably be the first porta hepatic-derived and egg-proven *Opisthorchis*-associated cholangiocarcinoma cell line. A previous cholangiocarcinoma cell line associated with opisthorchiasis, detected by ELISA, was isolated from a Thai patient^[14]. Our cell line is the second cell line developed in the area endemic for opisthorchiasis and cholangiocarcinoma in Thailand.

KKU-100 was derived from poorly differentiated tubular adenocarcinoma, the common histologic type of cholangiocarcinoma reported in Thailand^[20]. It possesses some characteristics of carcinoma in nature as evidenced by the expression of cytokeratin and EMA but not desmin. An electron microscopic study revealed microvilli and junctional complexes, the distinguishing ultrastructural features of adenocarcinoma (data not shown). Heterotransplantation of the KKU-100 cells into nude mice retained its histological features of the primary tumor. This is similar to several other cholangiocarcinoma cell lines that produce a similar histologic type in heterotransplanted animals^[8,10,12,13]. However, some immunocytochemical characteristics of the cell line were lost such as cytokeratin, CEA and CA125 upon transplantation. This may be due to the natural selection and adaptation of the cells to survive under culture condition or nude mouse microenvironment^[21,22].

Immunocytochemical analysis of tumor markers revealed that KKU-100 was positive for CEA, CA125 but not AFP and CA19-9-corresponding to the original tumor. The expression of CEA but not AFP in the primary tumor tissue and cells correlated with a marked elevation of serum CEA (44.6 ng/mL) and negative for AFP in this patient (data not

shown). This is common in pure cholangiocarcinoma, where elevated levels of serum CEA occur in $\leq 80\%$ of cases^[23,24], whereas AFP is almost negative. However, the expression of CEA and CA 125 in KKKU-100 cells was less than the primary tumor. This is similar to most established cholangiocarcinoma cell lines that show minimal or no CEA expression^[6,8,13]. The lowering or loss of some protein expression may be from the natural selection and adaptation of the cells to environment as previously mentioned. Only one cholangiocarcinoma cell line, HuCCA-1, exhibits CA 125^[14], our cell line is the second. In contrast, several cholangiocarcinoma cell lines can express or secrete CA19-9, such as HChol-Y1^[10], HuCC-T1^[11], KMBC^[8] and KMC-1^[16]. Other tumor markers secreted/expressed by KKKU-100 will be investigated for use in diagnostics and early detection in endemic areas.

Alterations of oncogenes and tumor suppressor genes are involved in the malignant transformation and progression of nearly all tumors. For cholangiocarcinoma, however, little is known about its molecular carcinogenesis. The p53 tumor suppressor gene, a common genetic alteration in various cancers, is over-expressed in up to 78.5% of cholangiocarcinomas^[25-27], while mutations of exon 5-8 range between 5% and 37.5%^[28-30]. Overexpression of c-met oncogene products has been described in about 60% of human cholangiocarcinomas^[31] and in furan-induced cholangiocarcinomas in rats^[32]. However, KKKU-100 cells show no expression of these two gene products. Besides the two genes studied, several other gene alterations may occur in KKKU-100 because there are marked chromosomal abnormalities in both number and structures. Detailed studies are in progress.

Cholangiocarcinoma is a major concern in Northeast Thailand because it is a fatal, malignant neoplasm with no curative treatment, neither chemotherapy nor radiation^[18]. At present, only surgical excision of the detectable tumor is associated with any improved survival^[18]. Since most patients have already advanced stage cancer when they arrive, palliative treatment is all that can be offered^[4]. Further study on the various aspects of cholangiocarcinoma, such as tumor biology, cellular and molecular carcinogenesis, biomarkers for early diagnosis and drug responses to new therapeutic agents, is needed. KKKU-100 cells should prove a valuable aid to such research and applicable management of cholangiocarcinoma.

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