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Establishment and characterization of a human cholangiocarcinoma cell line

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Abstract. Cholangiocarcinoma (CC) is a rare malignant tumor arising from the biliary tract. The disease is notoriously difficult to diagnose and is usually fatal due to its late clinical presentation and the lack of effective non-surgical therapeutic strategies. To date, little is known about the cancer biology of the disease and the establishment and characterization of only a few CC cell lines have been reported. We report here the establishment of a new human cancer cell line, HKGZ-CC, from a moderate to poorly differentiated intrahepatic bile duct carcinoma from a Chinese patient. Morphological characteristics, growth kinetics, ability to grow on anchorage-independent soft agar, tumorigenicity in nude mice and cytogenetic features of the cell line were investigated. Chromosome banding karyotype and comparative genomic hybridization analyses revealed chromosomal changes in 1pter-p31, 1q31-qter, 3q, 8q21-qter, 9pter-9q34, 10, 13q21-qter and X. This newly established cell line should serve as a useful model for studying the molecular pathogenesis of CC.

Introduction

Cholangiocarcinoma (CC), tumor of the biliary epithelium, accounts for 3% of all gastrointestinal cancers and is the second most common primary hepatic tumor (1). The incidence of and mortality rate for the disease varies considerably in different geographic regions, with the incidence highest in Thailand, P.R. China and other parts of Southeast Asia. Yet for reasons that are not clear, rising incidence rates, paralled by mortality rates, have also been documented globally (1-3). The prognosis of patients with CC is dismal as the disease is notoriously difficult to diagnose and is usually fatal due to its late clinical presentation and the lack of effective non-surgical therapeutic modalities. Most patients have unresectable disease at presentation and die within 12 months following diagnosis. Overall survival rate is poor, with less than 5% of patients surviving to 5 years (1). Established risk factors, including conditions associated with chronic biliary tract inflammation account for a small proportion of cases; and additional risk factors such as cirrhosis, infection with hepatitis B virus and hepatitis C virus are now also being recognized to contribute to the development of the disease (1,2). Yet despite our growing understanding of the disease, the precise molecular and genetic steps for the oncogenesis of CC remain largely unknown. This underlines the need for a better understanding of the molecular pathogenesis of the disease. For this purpose, a permanently growing, well-characterized cell line can be the most indispensable tool. Although a number of biliary tract cancer cell lines have been reported in the literature to date, most of these cell lines either lack detailed characterization or are mostly established from the more common extrahepatic CC (4-15). Of the cell lines established from intrahepatic CC (16-22), all are either of Japanese, Korean or Thai origin (Table I). Despite the prevalence of CC in P.R. China, no cell lines have been established from a Chinese patient to date. We report here the establishment and characterization of a new human intrahepatic CC cell line, named HKGZ-CC from a Chinese patient. This newly established cell line would provide a useful *in vitro* model for the study of the pathogenesis of CC.

Materials and methods

Specimen collection. The specimen was obtained from a 76-year-old Chinese man who underwent surgical resection at the First Affiliated Hospital of Sun Yat-sen University (Guangzhou, P.R. China) for intrahepatic CC in the right lobe of the liver. The size of the specimen was 6x5x3 cm. No portal vein, lymph node or distant metastasis was detected at the time of surgical resection. Laboratory investigations at admission

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Table I. Documented human cholangiocarcinoma cell lines.

Cell line	Age (years)	Sex	Site	Race	Differentiation	Ref.
KKU-100	65	F	Porta hepatis	Thai	Poor	4
TGBC-47	-	-	Extrahepatic	Japanese	Poor	12
TGBC-51	-	-	Papilla of vater	Japanese		
TBCN6	-	-	Extrahepatic	Japanese	Poor	
SNU-245	-	-	Extrahepatic	Korean	Well	5
SNU-308	-	-	Gall bladder	Korean	Moderate	
SNU-478	-	-	Ampulla of vater	Korean	Poor	
SNU-869	-	-	Ampulla of vater	Korean	Well	
SNU-1079	-	-	Intrahepatic	Korean	Moderate	
SNU-1196	-	-	Extrahepatic	Korean	Moderate	
SCK	57	M	Intrahepatic	Korean	Moderate	16
JCK	59	M	Intrahepatic	Korean	Moderate	
Cho-CK	72	M	Intrahepatic	Korean	Moderate	
Choi-CK	57	M	Intrahepatic	Korean	Poor	
HBDC	75	F	Extrahepatic	Japanese	Poor	7
ICBD-1	71	M	Extrahepatic	Japanese	Poor	13
OCUCh-LM1	61	M	Extrahepatic	Japanese	Well	8
TFK-1	63	M	Extrahepatic	Japanese	-	9
KMBC	73	M	Extrahepatic	Japanese	Moderate to poor	14
KMC-1	62	M	Intrahepatic	Japanese	-	22
CC-SW-1	-	-	Intrahepatic	-	Moderate	21
CC-LP-1	-	-	Intrahepatic	-	Moderate	
HuCCA-1	-	-	Intrahepatic	Thai	-	17
MEC	-	-	Extrahepatic	Japanese	Moderate	10
PCI:SG231	-	-	Intrahepatic	-	-	20
HuCC-T1	56	-	Intrahepatic	Japanese	Moderate	19
CHGS	-	-	-	Japanese	-	6
HuH-28	-	-	Extrahepatic	Japanese	-	11
HChol-Y1	-	-	Intrahepatic	Japanese	-	18
Mz-ChA-1	-	-	Gall bladder	-	-	15
Mz-ChA-2	-	-	Gall bladder	-	-	
SK-ChA-1	-	-	Extrahepatic	-	-	

Bold, intrahepatic bile duct carcinoma.

showed normal levels of α-fetoprotein (AFP) (4.63 μg/l, normal 0-20 μg/l) but elevated levels of CA125 (85.03 U/l, normal 0-35 U/l), CA19-9 (4402.07 U/l, normal 0-35 U/l) and carcinoembryonic antigen (CEA) (34.7 μg/l, normal 0-5 μg/l). Serologic hepatitis B virus (HBV) examination of this patient was negative. The tumor was histopathologically classified as moderate to poorly differentiated intrahepatic bile duct carcinoma.

Primary culture and establishment of cell line. Tumor specimens were washed in complete DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). After rinsing in the same medium twice, tumor tissues were minced into 1-mm^3 pieces and incubated with 1X Accumax (1200-2000 U/ml proteolytic activity containing collagenase and DNase; Innovative Cell Technologies, Inc., San Diego, CA, USA) at 10 ml/g tissue in DPBS (Invitrogen,

 CA, USA) for 20 min at 37˚C under constant rotating s. Single cell suspension was obtained by filtering the supernatant through a 100-μm and 40-μm cell strainer (BD Biosciences, San Jose, CA). The resulting filtrate was topped up with an equal volume of complete DMEM and centrifuged at 1000 rpm at 4˚C for 5 min. The pellet was resuspended in complete DMEM and seeded onto plastic flasks. Cultures were incubated at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. Medium was changed twice a week. Cells were subcultured when they reached 70-80% confluence.

During the subsequent period of continuous propagation by subculture, the cells were sampled at intervals, put in freezing medium containing 40% DMEM, 50% FBS and 10% DMSO, and stored in liquid nitrogen. After thawing, stored cells could be propagated in culture without noticeable change in growth and morphology. The cell line was designated HKGZ-CC (Hong Kong Guangzhou - Cholangiocarcinoma).

Morphologic examination and growth kinetics. Unstained cultured cells were routinely monitored and photographed with a phase-contrast microscope.

Cells of passage 15 were studied to estimate the population-doubling time. A suspension of $2x10^5$ cells was plated in 25-cm^2 culture flasks. Cells were detached from the flasks with trypsin and the average number of viable cells from two culture flasks was counted every 24 h in a hemacytometer chamber in the presence of trypan-blue dye. Cells were counted for up to 17 days. The growth curve was plotted and the doubling time of the cell population was estimated during the logarithmic growth phase.

Anchorage-independent growth assay in soft agar. Anchorage-independent growth was assessed by colony-formation ability in soft agar. Cells were suspended in soft agar and growth medium in 6-well plates at a density of 5000 cells per well. After 2-3 weeks, colonies (≥10 cells) were counted under the microscope in ten fields per well and photographed. Data represent results from at least two independent experiments performed in triplicate.

Tumorigenicity in nude mice. The study protocol was approved by and performed in accordance with the Committee of the Use of Live Animals in Teaching and Research at the University of Hong Kong. Cells of passage 8 were prepared to determine their tumorigenicity in nude mice. Cultured cells ($2x10^6$) were harvested, washed, resuspended in 0.1 ml of complete DMEM, and injected subcutaneously into the right flanks of two 4- to 5-week-old male nude mice. Animals were examined every week for the development of tumors. Tumor-bearing mice were sacrificed. Tumor tissue was excised, fixed in 10% formalin, and processed for routine histopathologic examination.

Cytogenetic analysis. Chromosome harvest was performed after short-term culture. Metaphase chromosomes were stained by standard trypsin-G banding method. Twenty-five metaphases were characterized. The description of karyotypic abnormalities followed the recommendations of the International System for Human Cytogenetic Nomenclature (23). Structural and numeric abnormalities were identified as clonal changes when found in ≥10 cells.

Comparative genomic hybridization (CGH). Genomic DNA was extracted from the tumor sample with proteinase K/sodium dodecyl sulfate digestion and phenol/chloroform/isoamyl alcohol extraction. Normal reference DNA was prepared from peripheral blood lymphocytes of healthy donors. Metaphases were prepared following standard procedures from peripheral blood lymphocytes of a healthy male donor. CGH was performed as described previously (24). Briefly, 1 μg of genomic DNA from the tumor sample and sex-matched normal reference was labeled directly with SpectrumGreen-dUTP and SpectrumRed-dUTP (Vysis, Downers Grove, IL, USA) by nick translation, respectively. Labeled tumor DNA (500 ng) and normal DNA probes were denatured and hybridized to normal metaphase chromosomes at 37˚C in a moist chamber for 48 h. The slide was then washed in 0.4X SSC/0.3% NP-40 at 75˚C for 2 min and then in 2X SSC/0.1% NP-40 at room temperature for 2 min. Slides were briefly counterstained with 1 μg/ml DAPI in an antifade solution.

Digital image analysis. Hybridized metaphase chromosomes were analyzed using a digital image analysis system containing a Zeiss Axiophot microscope equipped with a Metachrome II cooled-charged device camera (Zeiss, Oberkochen, Germany). Three images of each metaphase were captured using filter wheel-mounted, single band excitation Rhodamine, FITC, and DAPI filters. The image analyses were carried out using Quips CGH program (Vysis). Ten metaphases were analyzed to generate fluorescent ratio profiles in each case. Interpretation of the profiles was performed according to the program guidelines. The threshold used for the interpretation of gains and losses of a DNA sequence copy number was defined as a tumor/reference ratio >1.2 or <0.8, respectively, with both the standard and the reverse hybridization methods. High-copy-number amplification was defined as a tumor/reference ratio >1.50.

Results

Morphology and culture characteristics. The HKGZ-CC epithelial cells grew gradually from explants of a CC specimen and formed sparse colonies within 26 days of primary culture. The first successful subculture was performed at approximately one and a half months and the second one 7 days later. Once cell growth started, it tended to proceed progressively without periods of acute cell death. The contaminating fibroblasts gradually decreased and finally disappeared. The cells began to grow quickly at the 6th passage and were passaged for more than 40 generations thereafter.

The cultured HKGZ-CC cells grew as an adherent monolayer with characteristic epithelial morphologic features (Fig. 1A and B). The cells maintained consistent morphology from the primary culture to the following passages. After thawing, the cryopreserved cells could be propagated in culture without noticeable change in growth and morphology. The growth curve of HKGZ-CC cells is shown in Fig. 1C. The population doubling time of HKGZ-CC was approximately 48 h. HKGZ-CC cells were able to produce recognizable colonies in soft agar (data not shown).

Tumorigenicity in nude mice. The HKGZ-CC cells were highly tumorigenic in athymic nude mice. Within 21 days after

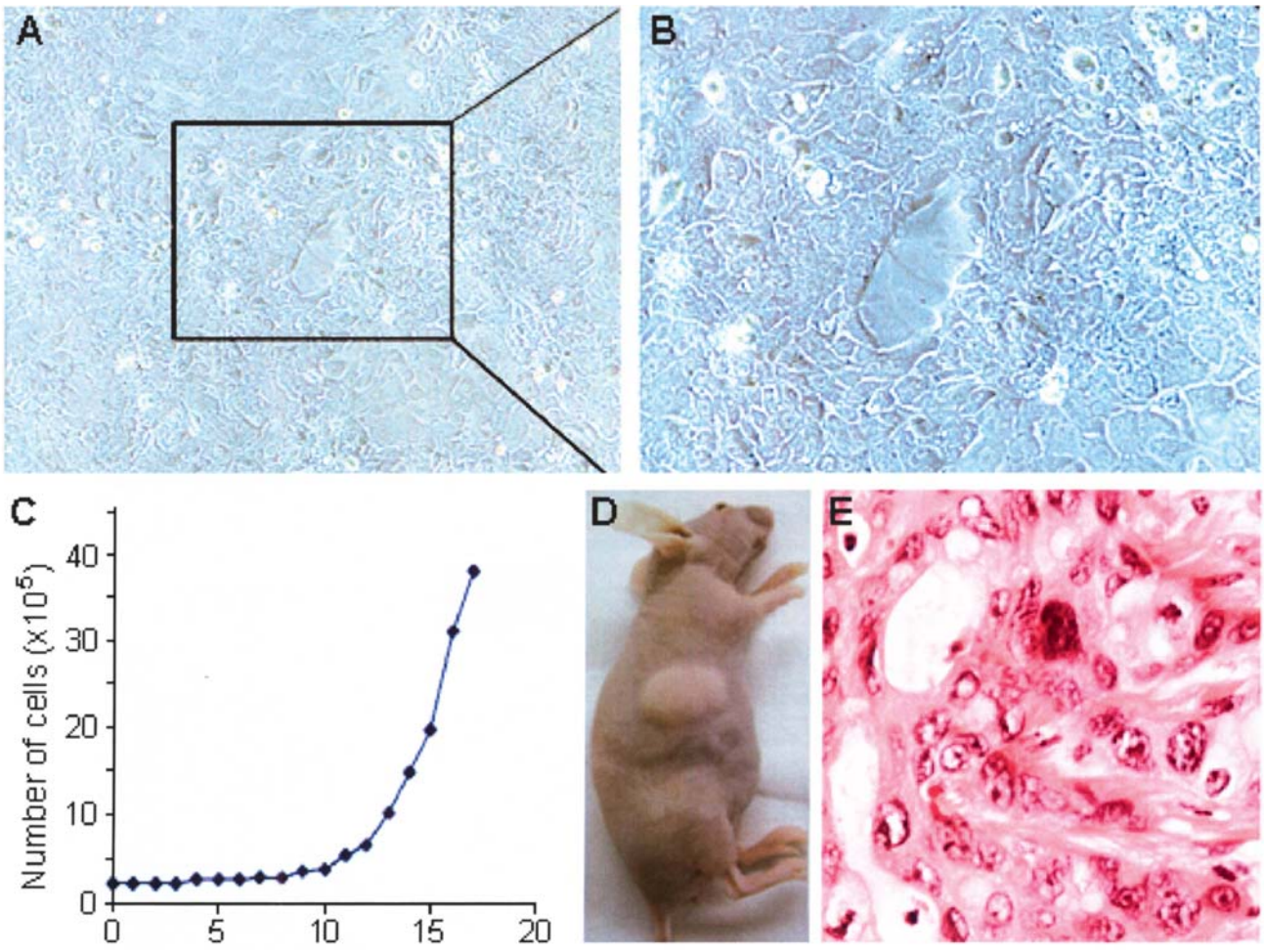

Figure 1. (A and B) HKGZ-CC cells in tissue culture. The cells grew in monolayer sheets and exhibited typical malignant epithelia in morphology. Micrographs of cultured HKGZ-CC cells at phase contrast x100 (A) and x200 (B). (C) Growth curve of HKGZ-CC cells. (D) Tumorigenicity test of HKGZ-CC cells in nude mice showing a tumor mass 3 weeks following subcutaneous injection of $2x10^6$ HKGZ-CC cells. (E) Histology of xenografted tumor of HKGZ-CC cells shows a moderate to poorly differentiated CC (H&E x400 magnification).

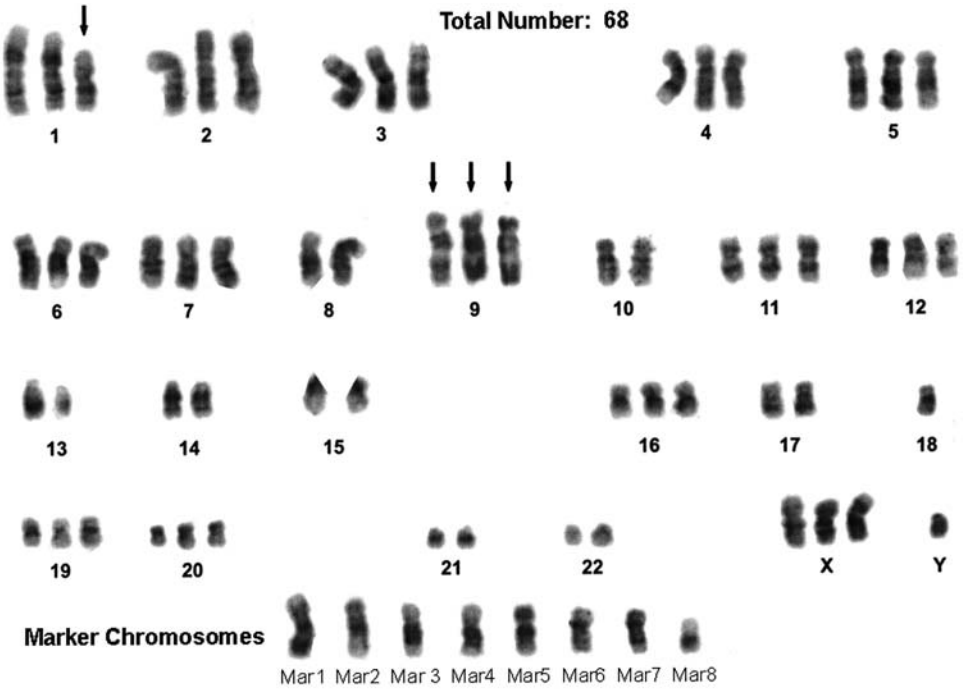

Figure 2. Representative G-banded karyotype of HKGZ-CC cells.

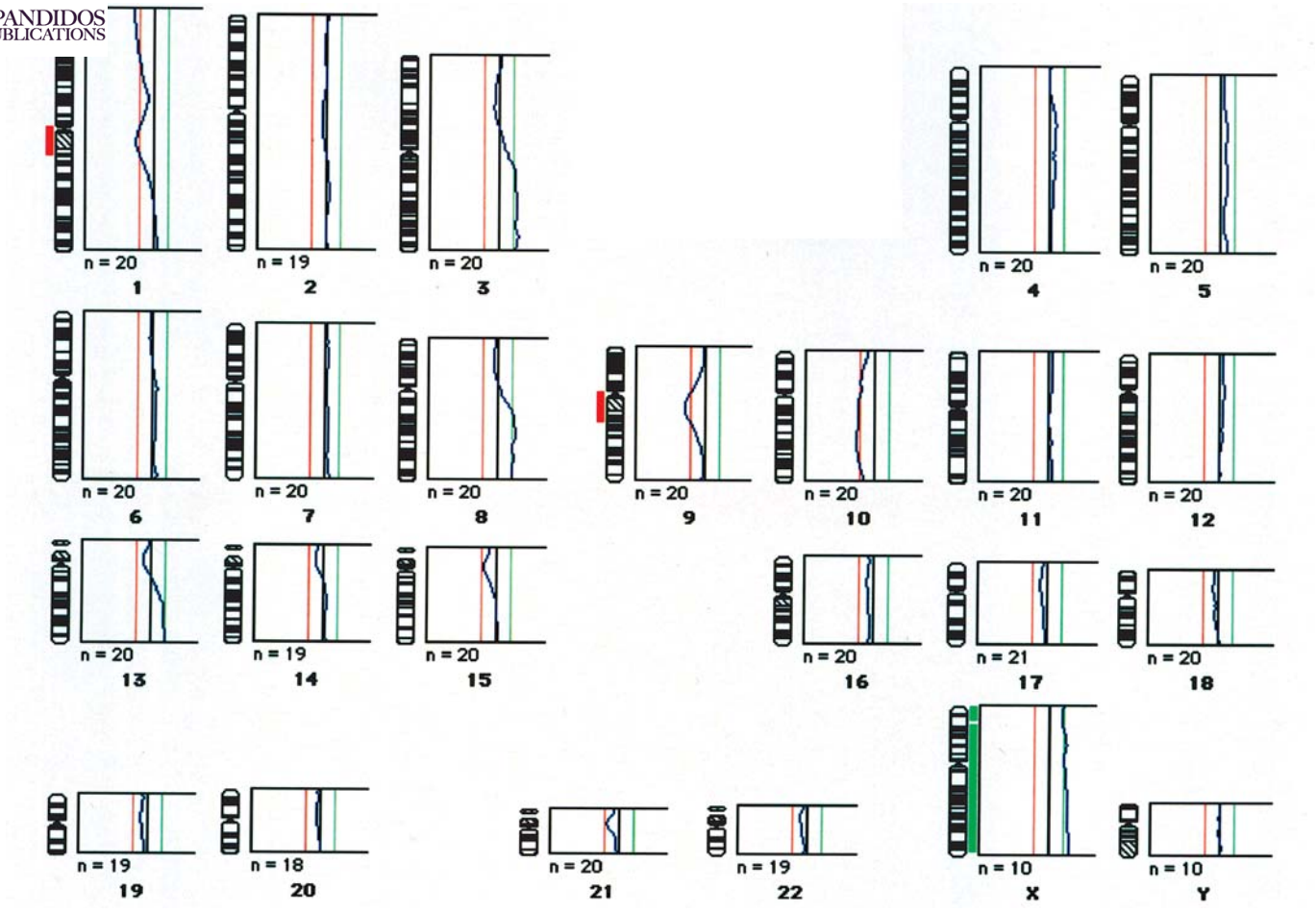

Figure 3. Comparative genomic hybridization analysis of chromosomal alterations in HKGZ-CC cells. Ratio profiles are shown to the right of chromosome ideograms. The baseline (middle) ratio is 1.0. The left-sided shift indicates a ratio of 0.8 and the right-sided shift indicates a ratio of 1.2. The ratio profile was calculated as a mean of ten chromosomes. The solid bars to the left and right of chromosome ideograms indicate loss and gain of DNA sequence, respectively.

subcutaneous injection of cells, visible tumors developed in all of the 2 nude mice at the site of inoculation (Fig. 1D). Histological examination of the xenotransplanted nodules showed moderate to poorly differentiated CC, similar to the description of the original resected tumor of the bile duct from which the HKGZ-CC cell line was derived (Fig. 1E).

Cytogenetic analysis. GTG banding analysis found that the HKGZ-CC cell line is a triploid. A representative karyotype is shown in Fig. 2 and the composite karyotype is summarized as follows: 64~74,XXXY,-1,-1,-8,-10,-13,-14, -15, -17, -18, -18, -21, -22, +der(?;1)(?::1q31_1qter),+der(9;?)(9pter_9q34::?), +der(9;?)(9pter_9q34::?),+der(9;?)(9pter_9q34::?),+3~9mar.

CGH analysis. Changes in the genomic copy number in HKGZ-CC cells were identified by CGH analysis. The profile of chromosome copy number change detected by CGH is shown in Fig. 3. Gains of 3q, 8q21-qter, 13q21-qter and X; and losses of 1pter-p31 and 10 were detected.

Discussion

Advances in cell culture methods have made it possible to establish a variety of human carcinoma cell lines from surgical and autopsy tissues. Since pure cells in cultures can be used for a vast array of studies that cannot normally be carried out using tissue specimens, the study of permanent cell lines established from human cancers has played a pivotal role in our study and understanding of cancer cell biology.

Only a few long-term intrahepatic CC cell lines have been reported to date. Although the incidence of the disease is prevalent in P.R. China, no cell lines have been established from a Chinese patient thus far.

In this study, we report the establishment and characterization of a new CC cell line, HKGZ-CC, derived from a moderate to poorly differentiated intrahepatic bile duct carcinoma from a Chinese patient. The HKGZ-CC cells grew as an adherent monolayer with characteristic epithelial morphology, with a population doubling time of approximately 48 h. Cultured cells maintained consistent morphology from the primary culture to the following subculture passages. The HKGZ-CC cells had been grown continuously for approximately 5 months, undergoing >40 passages; and growth continued even after recovery from cryopreservation. The HKGZ-CC cells also exhibited an ability to form colonies in soft agar. Further, heterotransplantation of the HKGZ-CC cells into nude mice resulted in tumor growth and histological features of the tumor resembled those of the original primary tumor that the cell line was established from. Cytogenetic analysis confirmed that HKGZ-CC cells were of human origin.

Chromosome aberrations are involved in the malignant transformation and progression of nearly all tumors. Chromosome banding karyotype and CGH analyses allow for the characterization of and global overview of chromosomal alterations involved in the entire genome of a tumor. To date, only a limited number of studies have investigated the cytogenetic changes in CC and our understanding of the molecular carcinogenesis of the disease remains limited. Previous investigations of CC-derived cell lines showed chromosomal gains in 2q, 5p, 7p, 8q, 13q, 17q, 18q, 19q and X and chromosomal losses in 1p, 4q, 5q 8p, 9p, 17p, 18q, 19p, X and Y (16,18,25-27). These results are relatively well matched with our findings where gains in chromosomes 8q, 13q, X and losses in 1p were also detected. Yet it is also interesting to note that the newly established cell line only displayed few chromosomal alterations, when compared to other cell lines that have been characterized previously. Our findings suggest that the chromosomal abnormalities detected in this newly established cell line might play a crucial role in the early steps of carcinogenesis of CC.

In this study, we report the establishment and characterization of a new human cholangiocarcinoma cell line derived from a Chinese patient, termed HKGZ-CC. Further study on the various aspects including tumor biology, cellular and molecular carcinogenesis, biomarkers for early diagnosis and drug responses to new therapeutic agents, is needed for a better understanding of CC. The new cell line should provide us with a new experimental model for the research of this disease in the future.

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