Establishment and Characterization of a New Human Gallbladder Carcinoma Cell Line

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Abstract. *Background: Prognosis for patients with gallbladder carcinoma (GBC) is poor and the standard treatment for GBC has not yet been established. Materials and Methods: We established the human GBC cell line TYGBK-1, from a patient with papillary, tubular adenocarcinoma. Results: The doubling time was 48 hours. This cell line has a missense mutation of p53 and no mutation of the K-RAS gene. This cell line was transplantable to nude mice. We characterized the sensitivity of TYGBK-1 to gemcitabine. We also examined the association of two gemcitabine-related genes (deoxycytidine kinase, dCK, and Hu antigen R, HuR). Among four GBC cell lines (TYGBK-1, NOZ, G-415, TGBC2TKB), TYGBK-1 and NOZ exhibited sensitivity to gemcitabine. Furthermore, these cells expressed both dCK and HuR mRNA, rather than gemcitabine-resistant cells. Conclusion: The newly established GBC cell line TYGBK-1, may represent an effective tool for development of chemotherapeutic treatment for GBC.*

Gallbladder carcinoma (GBC) is a common biliary malignancy with high mortality, generated from the gallbladder or cystic duct. Most patients with GBC are treated at an advanced stage, and the prognosis remains poor despite the development of modern diagnostic modalities. Jaundice and abdominal pain of ten indicate an advanced cancer that is already unresectable (1, 2). GBC resection rate was 69.7% and the rate of curative resection was 37.7%, from 1998 to 2004 (1). Prognosis for patients with GBC is poor compared to that for other types of gastrointestinal cancers. Surgical resection is the only available option for a cure. However, this is possible only for selected patients. The treatment for GBC has not yet been established (3, 4). Although many therapeutic strategies have been developed, the majority have not improved the prognosis of patients with GBC appreciably. Therefore, it is important to establish a human GBC cell line as an *in vitro* model for studying tumor biology, including susceptibility to drugs. In this study, we report the establishment and characterization of a new human GBC cell line, TYGBK-1. We described the cell phenotypes and checked for genetic alterations of *K-RAS* and *p53* genes.

For the treatment of biliary tract carcinoma, gemcitabine (2', 2'-difluorodeoxycytidine) is a key drug. The response rate of biliary tract cancer to gemcitabine monotherapy is 22-36%. The median survival time (MST) has been reported to range from 7 to 14 months (5-10). Regarding transport into the cell, gemcitabine is phosphorylated to its mononucleotide moiety by deoxycytidine kinase (dCK), a rate-limiting enzyme in the salvage of deoxyribonucleosides that provides deoxynucleotid triphosphates for replicative and repair DNA synthesis. In pancreatic carcinoma cells, *HuR* associates with *dCK* mRNA. Exposure to gemcitabine of pancreatic carcinoma cells enhances the association between *HuR* and *dCK* mRNA and increases cytoplasmic HuR levels (11). HuR is a useful prognostic biomarker for patients with pancreatic carcinoma (12). On the other hand, even though gemcitabine has been used in patients with GBC, no such studies have been performed. Using this new resource, we assessed GBC's sensitivity to gemcitabine. We also examined the association between the expression levels of dCK, HuR and sensitivity to gemcitabine.

Materials and Methods

Patients' history. The primary gallbladder adenocarcinoma cells were obtained from a lymph node of a 67-year-old female patient who underwent cholecystectomy in 2008. Macroscopic examination revealed that the gallbladder was occupied by a tumor which was histologically characterized as a papillary, tubular type. The tumor had invaded to the serosa. Defined direct invasion around the

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gallbladder bed and invasion of portal veins had been recorded. There was lymph node metastasis (T4, N1, M0 according to the UICC standardization) (Figure 1A).

Primary culture. The resected tumor was surgically removed from the patient and was mechanically minced with sharp scissors. Cells were initially cultured in Dulbecco's Modified Eagles' medium + HAM's F12 medium (Wako, Osaka, Japan), supplemented with 5% fetal bovine serum containing antibiotics (GIBCO, Grand Island, NY, USA) at 4˚C. The cell cultures were maintained in humidified incubators at 37˚C in an atmosphere of 5% CO_2 and 95% air. Eight weeks after the start of primary culture, the first passage was performed. Then a homogeneous layer of epithelial tumor cells with a sustained growth pattern was established.

Heterotransplantation. Four-week-old athymic female nude mice (BALB/CAN.Cg-*Foxn1*^{nu}/CrlCrlj; Charles River Laboratories, Yokohama, Japan) were used for heterotransplantation. TYGBK-1 cells (1×10^7 cells) suspended in culture medium and matrigel were injected. The tumor was observed for three weeks and was again subcutaneously transplanted to other nude mice for serial transplantation. A portion of the tumor was fixed in 10% buffered formalin, and routinely processed for histopathology and immunocytochemistry.

Growth characteristics. The cells were plated on 6-well culture plates and cultured for 7 days in an incubator with CO_2. Cells were counted daily in three wells. The doubling time of the cell population was determined from the exponential phase of the growth curve.

Chromosome analysis. The established tumor cells from the 50th passage were subjected to chromosomal analysis. The cytogenetic study was performed on G-banded metaphase cells obtained from a 5-day-old culture. A total of 50 cells were analyzed.

Assay for tumor markers in the conditioned medium. The concentration of carcinoemboryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) in the conditioned medium (used for 3 days' culture) of TYGBK-1 cells and in a control growth medium (without cells, also incubated for 3 days), were measured by radioimmunoassay (RIA).

Mutation screening. For the analysis of *p53*, polymerase chain reaction (PCR) was performed for 40 cycles. After purification of the product by PCR, mutation screening of exons 5, 6, 7 and 8 of *p53* was performed by DNA direct sequencing analysis. Mutations of codons 12 and 13 in the *K-RAS* gene were also screened by direct sequencing analysis.

Susceptibility to anticancer drugs. Gemcitabine (Sigma-Aldrich, St Louis, MO, USA) susceptibility tests were carried out on four gallbladder cell lines (TYGBK-1, G-415, NOZ and TGBC2TKB). G-415 and TGBC2TKB were obtained from Riken Bioresource Center (Tsukuba, Japan). NOZ was purchased from the Japanese Collection of Research Bioresources (Osaka, Japan) (13, 14). Sensitivity to gemcitabine was analyzed by methylthiazoletrazolium (MTT) assay using 96-well plates and the cell proliferation reagent WST-1 (Roche Chemicals, Osaka, Japan). Cells were added to the plate at a density of 1×10^4 cells/well in a volume of 100 μl/well and were incubated for 24 h to ensure sufficient cell growth. After a 24-hour culture, the cells were exposed for 48 h to gemcitabine at a concentration ranging from 1 nM to 1 mM. The proliferation curves were then constructed by calculating the mean value of the optical density measurements at 450 nm using a 96-well plate reader (NJ-2100; Japan Intermed, Osaka, Japan).

Analysis of cell cycle distribution and apoptosis. The cell preparation was the same as described in the section of growth characteristics. After plating and a 24-hour recovery period, TYGBK-1 cells were treated with 50% of the IC_{50} concentration of gemcitabine for 24 h. After a 48 h culture, both adherent and detached cells were collected. In brief, cells were washed twice with phosphatebuffered saline (PBS)and counted. Then 1×10^6 cells were collected and examined. The percentage of cells in each growth phase was determined using the Cell Cycle Phase Determination Kit (Cayman Chemical Company, Ann Arbor, MI, USA). Analysis of propidium iodide (PI) fluorescence was performed using FACSCcant II (BD Biosciences, San Jose, CA, USA).

Quantitative real-time PCR. Total RNA extractions were performed using Trizol reagent (Life Technologies, Tokyo, Japan). The RNA was subsequently treated with RNase-free DNase I. Total RNA was reverse-transcribed (RT) to first-stand cDNA using a PrimeScript® II Firststrand cDNASynthesis Kit (TaKaRa Bio, Shiga, Japan), according to the manufacturer's instructions. For validation of RT-PCR, quantitative real-time PCR was used in order to examine the expression levels of mRNA. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the internal control to quantify initial cellular transcripts. The sequences of the primers were as follows: dCK (326 bp): TCTCTGAATGGCAAGCTCAA (sense), CTATGCAGGAGCCAGCTTTC (antisense); HuR (189 bp): ATGAAGACCACATGGCCGAAGACT (sense), AGTTCACAAAGC CATAGCCCAAGC (antisense); *GAPDH* (105bp); AGCCTCAAGAT CATCAGCAATGCC (sense), TGTGGTCATGAGTCCTTCCACGAT (antisense). The PCR primers were obtained from Life Technologies (Tokyo, Japan). PCR reactions were carried out in 20 μl, containing cDNA, each forward and reverse primer, and 2 × SyBer® Primix Ex Taq™ II (TaKaRa Bio). Real-time PCR was performed using Mx3000P QPCR System(Agilent Technologies; Santa Clara, CA, USA). PCR was carried out as follows: 10 s at 95˚C, followed by 40 cycles of 10 s at 95˚C, then 40 s at 55˚C.

Immunohistochemistry. Immunohistochemical analysis of dCK and HuR expression was carried out using paraffin-embedded sections of the TYGBK-1 cells cultured block. We used a rabbit anti-dCK polyclonal antibody (LS-B1825; Lifespan Biosciences, Seattle, WA, USA) at a dilution of 1:200, and anti-HuR polyclonal antibody (sc-56709; Santa Cruz, CA, USA), at a dilution of 1:200.

Results

Morphology and culture characteristics. Histologically, the TYGBK-1 cell line is similar to the original tumor in both hematoxylin and eosin (HE) and human epithelial membrane antigen (EMA) staining (Figure1A-D). TYGBK-1 epithelial cells grew as an adherent monolayer with characteristic epithelial morphological features (Figure 1E). The cells maintained consistent morphology from the primary culture to the subsequent passages. The growth curve of TYGBK-1

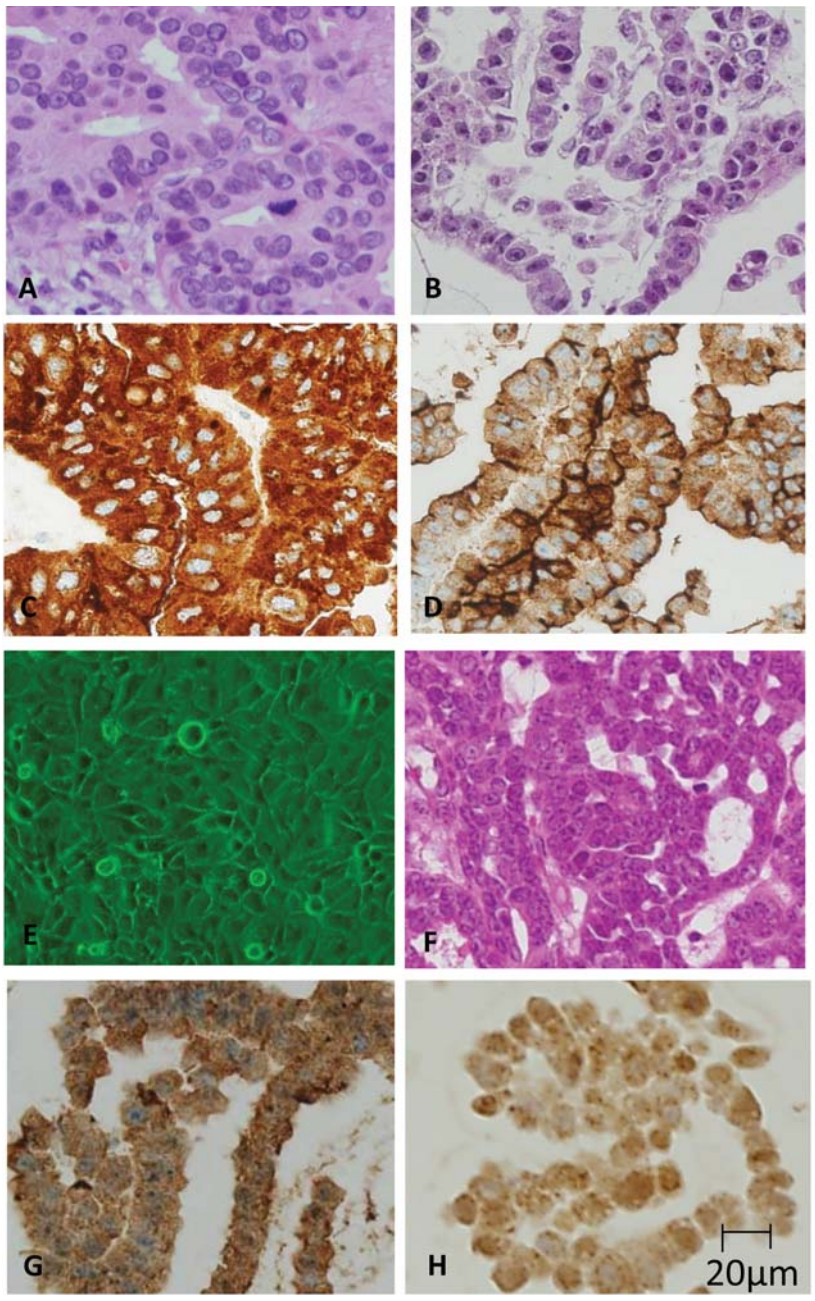
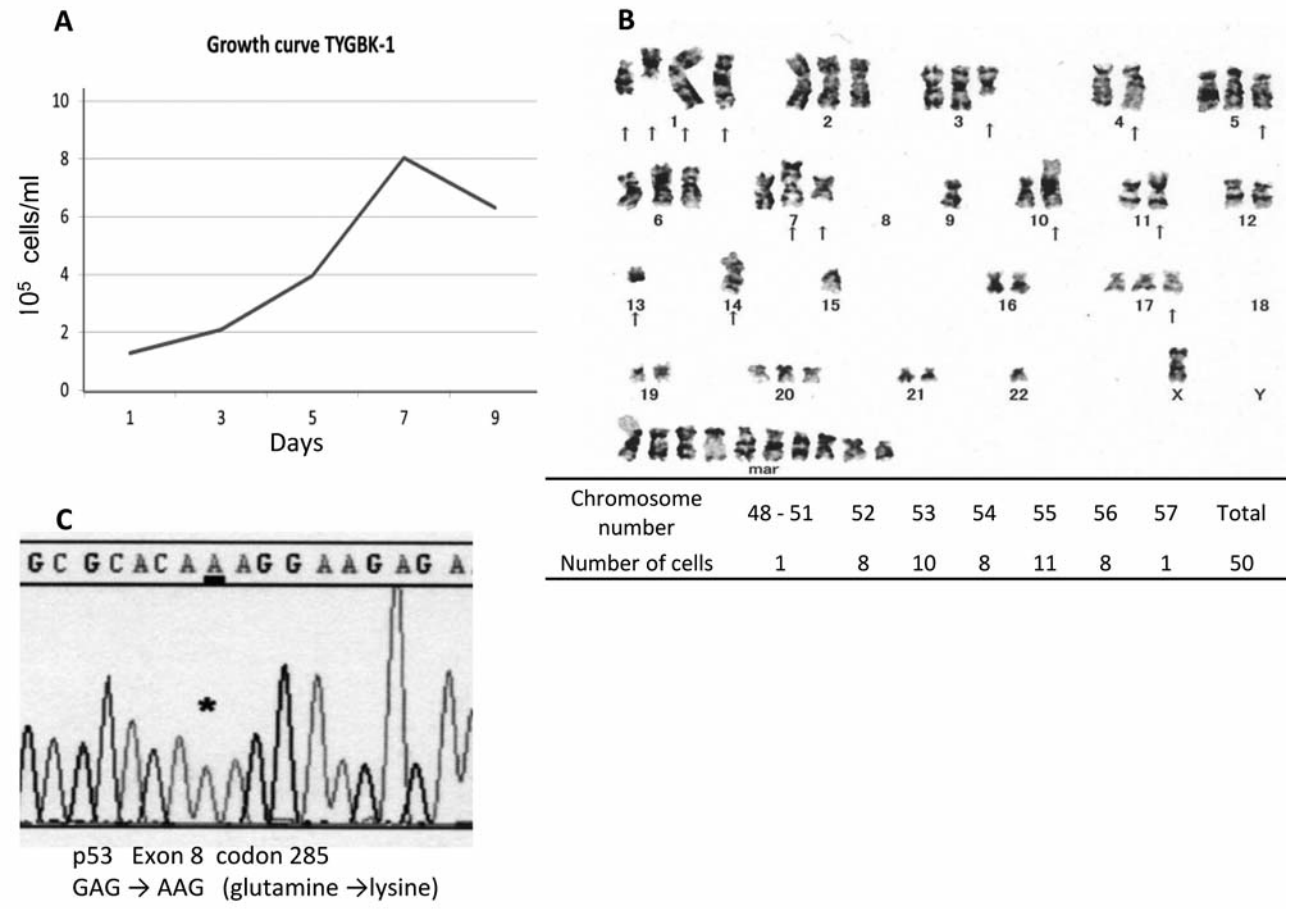


Figure 1. *Histopathological features of the original tumor and TYGBK-1 cell line. Hematoxylin and eosin (HE) staininig of the original tumor (A) and of the TYGBK-1 cell line (B). Staining with Human epitherial membrane antigen (EMA) of the original tumor (C) and of TYGBK-1 cell line (D). The TYGBK-1 epithelial cells grew as an adherent monolayer with characteristic epithelial morphological features (E). Heterotransplantation of TYGBK-1 cell to nude mice. Histologically, the transplanted tumor was an adenocarcinoma, similar to the primary tumor (F). Immunohistochemical staining of dCK (G) and HuR (H) in the TYGBK-1 cell line.*

cells is shown in Figure 2A. The population doubling-time of TYGBK-1 cells was approximately 48 h, determined from the slope of the growth curve. In total, the cells were passaged more than 120 times.

Heterotransplantation. Tumor nodules developed in the nude mice three weeks after their initial inoculation with TYGBK-1 cells. Histologically, the transplanted tumor was adenocarcinoma, similar to the primary tumor (Figure 1F).



Chromosome number	48 - 51	52	53	54	55	56	57	Total
Number of cells	1	8	10	8	11	8	1	50

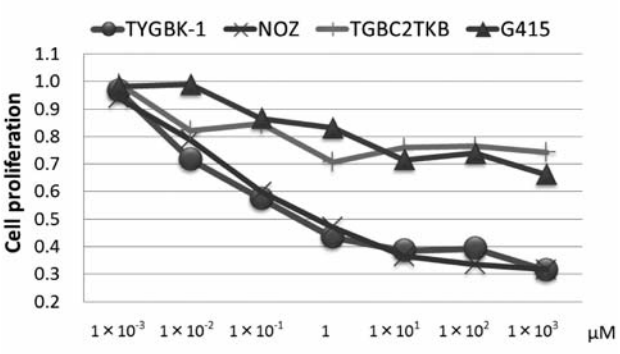
Figure 2. *Characteristics of the TYGBK-1 cell line. A: Growth curve. The population doubling time of TYGBK-1 determined from the slope of the growth curve was approximately 48 h. B: Chromosome analysis. The G-banding analysis for the TYGBK-1 cell line, showing the number of chromosomes ranging between 52 and 56, with a modal chromosome number of 55. C: Mutations of the p53 gene. For the p53 gene, TYGBK-1 had a missense mutation from glutamine (GAG) to lysine (AAG), at codon 285 of exon 8.*

Chromosome analysis. The modal chromosome number was 55, ranging from 52 to 56. The G-band karyotype is shown in Figure 2B. Distribution of the chromosome numbers is shown in (Table I). The composite karyotype is summarized as follows: 52–56,X,-X[10],+add(1) (p11),add(1)(q11)[10], +add(1)(q21)[6],+der(1)add(1)(p36.1)add(1)[2],der(1;14) (q10;q10)[10],+2[8],add(3)(p25)[3], +add(3)(q11.2)[10], add (4)(q21)[6],+5[9],add(5) (p11.2)[3],add(5)(p15)[5],+6[10], add(7)(p15)[2],+i(7)(p10)[10],-8[10],-8[9],-9[10],-10 [4],add(10)(p11.2)[2],add(11)(p11.2)[2],add(11)(p15)[10],add(12)(q24.1) [2],-13[10], del(13)(q?)[10],der(14;15)(q10;q10) [10],+add(17)(p11.2)[10],-18[10],-19[5],+20[2], -21[7],-22[8], +10~13mar.

Assay for tumor markers in the conditioned medium. The tumor markers in the conditioned medium of the TYGBK-1 were as follows: CEA, 0.5 ng/ml; and CA19-9, 117 U/ml.

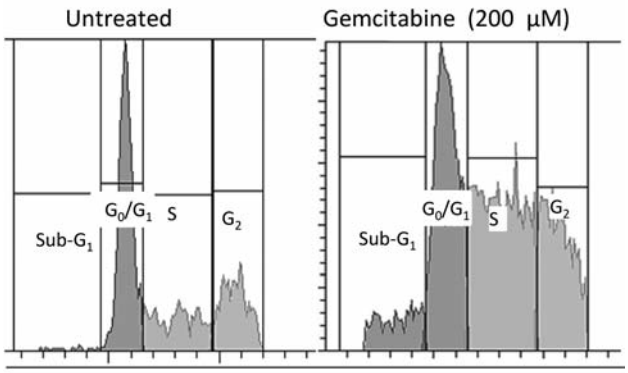
Mutations in the p53 and K-RAS genes. For the *p53* gene, TYGBK-1 had a missense mutation from glutamine (GAG) to lysine (AAG) at codon 285 of exon 8 (Figure2C). *K-RAS* mutation was not detected.

Sensitivity to gemcitabine. Sensitivity to gemcitabine in the TYGBK-1, NOZ, G-415 and TGBC2TKB cell lines was assessed by the MTT assay. The cells were treated with different concentrations of gemcitabine for 48 h. The proliferation curves and IC_{50} of gemcitabine in the four cell lines are shown in Figure 3. TYGBK-1 was the most sensitive to gemcitabine. We were able to classify these cell lines into two groups: a gemcitabine-sensitive group that included TYGBK-1 and NOZ, and a gemcitabine-resistant group that included G-415 and TGBC2TKB. The IC_{50} values of the resistant group were beyond the range of our measurement (>100 μM).



Cell line	IC_{50}(μM)
TYGBK-1	0.36
NOZ	0.66
G-415	>100
TGBC2TKB	>100

Figure 3. *Sensitivity to gemcitabine in four gallbladder carcinoma cell lines. The ratio of cell proliferation compared to the control at each concentration of gemcitabine is shown. According to the proliferation curves and to gemcitabine IC_{50} values, TYGBK-1 and NOZ were sensitive to gemcitabine.*



Cells (%)	Sub-G_1	G_0/G_1	S phase	G_2/M
Untreated	1.6±0.8	54.0±8.4	18.5±2.3	23.4±7.2
Gemcitabine (200 nM)	6.9±0.2	30.7±0.9	32.4±4.9	24.0±4.3

Figure 4. *Effect of gemcitabine on the cell cycle distribution of TYGBK-1 cell line. The percentage of cells in S phase increased, and that of the sub-G_1 population also increased on treatment with gemcitabine. Values are mean percentages±SD of three experiments.*

Analysis of cell cycle and apoptosis. Flow cytometry showed that gemcitabine was able to affect the cell cycle distribution of TYGBK-1 cell. As a result, the percentage of cells in the S phase increased, and the sub-G_1 population also increased (Figure 4).

DCK and HuR expression among the GBC cell lines. Figure 5 shows the *dCK* and *HuR* mRNA expression in the four GBC cell lines. The ratio of each level to that of the control represented the relative quantity of mRNA expression in the cells. The TYGBK-1 and NOZ cell lines highly expressed *dCK* and *HuR* mRNA. On the other hand, low *dCK* expression was found in the G-415 and TGBC-2TKB cells. Expression levels of *dCK* and *HuR* were associated with gemcitabine sensitivity of GBC cell lines to gemcitabine. Immunohistochemical staining for dCK and HuR in the original tumor and TYGBK-1 cells revealed that, TYGBK-1 cells exhibited strong expression (Figure 1G and 1H).

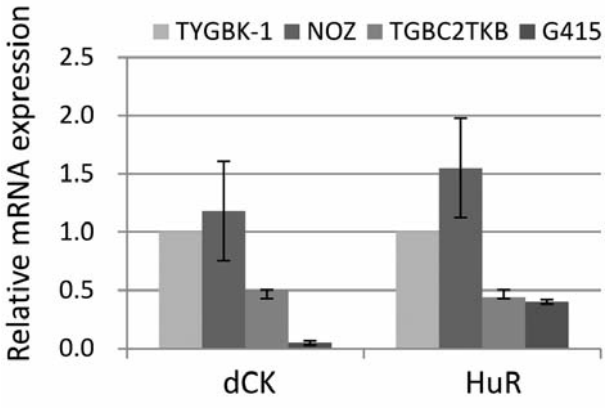


Figure 5. *Deoxycytidine kinase (dCK) and Hu antigen R (HuR) expression among the gallbladder carcinoma cell lines. The TYGBK-1 and NOZ cells, highly expressed dCK and HuR mRNA. On the other hand, low dCK expression was found in the G-415 and TGBC-2TKB cells.*

Discussion

The study of permanent cell lines, established from human cancer has played a major role in our understanding over the biology of cancer. However, there are few studies about GBC cell lines compared to other types of cancer (15). The following gallbladder cancer cell lines have been reported in the world literature: G-415 (13), NOZ (14), OCUG-1 (16), KMG-C (17), GBK-1 (18), GB-d1 (19), TGBC series (20), FU-GBC-2 (21), SNU-308 (22) among others (13, 14, 16-22).

In this article, we present a newly established gallbladder cancer cell line. It possesses some characteristics of carcinoma in nature, as evidenced by the expression of EMA, of CA19-9 production and the fact that its being transplantable to nude mice. CA19-9 is widely used as a tumor marker for the initial diagnosis of gallbladder carcinoma, cholangiocarcinoma, and pancreatic carcinoma. However, it is more useful in measuring

the effectiveness of cancer treatment by studying the patient's CA19-9 levels over time. Serum CA19-9 has been evaluated as a predictive indicator of treatment efficacy and outcome in various clinical settings (23). Out of the gallbladder carcinoma cell lines mentioned above, TGBC-44 and FU-GBC-2 have been reported to have high CA19-9 values in the conditioned medium of the cell line. To evaluate the sensitivity to chemotherapy *in vitro*, the CA19-9-producing ability may be useful.

It is still unclear whether loss of heterozygosity (LOH) plays any significant role in gallbladder carcinogenesis, but recent studies have found a high incidence of LOH at several chromosomes in GBC. LOH on chromosomes 1p, 3p, 5p, 8p, 9p, 9q, 13q, 16q, and 17p has been frequently found in GBC (24, 25). We detected 1q21, 1p36, 3p25, 5p11, 5p15, 13q, 17p11 in the TYGBK-1 cell line. In GBC, LOH at 1p34-36 (53%) may be related to progression of the disease (24, 26). Tumor suppressor gene p73 is a protein related to the p53 tumor protein. It has been identified in this region. LOH on chromosome 13q has been observed frequently in many types of cancer (27, 28). The retinoblastoma *(RB)* gene is located at 13q14 and is the prototype tumor suppressor gene, encoding a nuclear protein that acts as a cell cycle control checkpoint at the G_1 phase (29). Limited information is available regarding the genetic changes occurring during gallbladder carcinogenesis. Mutations in *K-RAS* have been reported in 2.7-39.1% of patients with GBC (30-33). Of the GBC cell lines examined, the *K-RAS* mutation has been identified only in NOZ (34). In the TGBC series, chromosomal gain of 12p12 (*K-RAS* locus) was observed in 4 out of 5 TGBC cell lines (20). However we could not detecte *K-RAS* mutations in codon 12 and 13.

In biliary tract carcinoma, the role of *p53* is still controversial. Abnormalities of the *p53* tumor suppressor gene are seen in 27-70% of GBC cases (34-39). On the other hand, discrepancies in the *p53* status was detected for 23% of cell lines. The TYGBK-1 cell line had a missense mutation from G to A at E285K. This mutation is the same as that in the multiple myeloma cell line, RPMI-8226 (40, 41). This is a temperature-sensitive mutation. Glu^{285} is the human 'hot-spot' *p53* mutant (42-45). Thus, TYGBK-1 might be sensitive to thermal therapy.

In our study, NOZ and TYGBK-1 cell lines exhibited sensitivity to gemcitabine. Our results also showed that not only NOZ but TYGBK-1 cells as well, were positive for dCK and HuR expression. dCK arrests tumor growth, and results in apoptosis (45-47).The stress response protein HuR, an RNA-binding protein, modulates *dCK* mRNA expression and was associated with a patient who received a gemcitabine-based adjuvant therapy (48). TYGBK-1 is a good research model for sensitivity to gemcitabine.

In summary, we have established and characterized a new human gallbladder adenocarcinoma cell line. This cell line has a missense mutation of *p53,* production of CA19-9, is gemcitabine-sensitive, and has high dCK and HuR expression. Using this cell line, we hope to contribute to the development of individualization of cancer treatment.

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