Pathol. Oncol. Res. (2012) 18:227–237
DOI 10.1007/s12253-011-9432-5

RESEARCH

# Down-Regulated Expression of HSP70 in Correlation with Clinicopathology of Cholangiocarcinoma

**Sirintip Boonjaraspinyo · Thidarut Boonmars · Sasithorn Kaewkes · Porntip Laummaunwai · Somchai Pinlaor · Watchalin Loilome · Puangrat Yongvanit · Zhiliang Wu · Anucha Puapairoj · Vajarabhongsa Bhudhisawasdi**

Received: 25 November 2010 / Accepted: 24 June 2011 / Published online: 13 July 2011


**Abstract** Cholangiocarcinoma is a crucial health problem in northeast Thailand. Although rare, it is a highly fatal disease and the prognosis of CCA patients is very poor. To determine if expression of specific genes is useful for diagnosis and prognosis for CCA. We examined the expression of HSP70, HSP90, RB1, cyclin D1, and HDAC6 in 50 resections of human CCA tissues by quantitative real-time PCR. The expression of HSP70, RB1, and HDAC6 was "dominant down-regulation," while the expression of cyclin D1 and HSP90 was "dominant up-regulation." There were no correlations between RB1, cyclin D1, HSP90, and clinicopathological parameters such as status, histology type, histological grading, stage of CCA, and metastasis. A significant association was found between HDAC6 and CCA staging ($p$=0.000), CCA gross type and HSP70 ($p$=0.046) as well as RB1 expression ($p$=0.046). Patients with down-regulation of HSP70 had significantly poorer prognosis than those in the up-regulation group ($p$=0.002). Expression of HSP70 may be useful as a new prognostic marker for CCA.

**Keywords** Cholangiocarcinoma · Clinicopathology · Retinoblastoma 1 · Cyclin D1 · Heat shock protein 70 · Heat shock protein 90 · Histone deacetylase 6

**Abbreviations**

| | |
|---|---|
| CCA | cholangiocarcinoma |
| RB1 | retinoblastoma 1 |
| HSP70 | heat shock protein 70 |
| HSP90 | heat shock protein 90 |
| HDAC6 | histone deacetylase 6 |

S. Boonjaraspinyo · T. Boonmars (✉) · S. Kaewkes · P. Laummaunwai · S. Pinlaor
Department of Parasitology, Faculty of Medicine,
Khon Kaen University,
Khon Kaen 40002, Thailand
e-mail: bthida@kku.ac.th

S. Boonjaraspinyo · T. Boonmars · S. Pinlaor · W. Loilome · P. Yongvanit · V. Bhudhisawasdi
Liver Fluke and Cholangiocarcinoma Research Center,
Khon Kaen University,
Khon Kaen 40002, Thailand

W. Loilome · P. Yongvanit
Department of Biochemistry, Faculty of Medicine,
Khon Kaen University,
Khon Kaen 40002, Thailand

Z. Wu
Department of Parasitology, Gifu University Medicine,
Graduate School,
Gifu 5011194, Japan

A. Puapairoj
Department of Pathology, Faculty of Medicine,
Khon Kaen 40002, Thailand

V. Bhudhisawasdi
Department of Surgery, Faculty of Medicine,
Khon Kaen University,
Khon Kaen 40002, Thailand

## Introduction

Cholangiocarcinoma (CCA) arises from the epithelium within the intrahepatic and extrahepatic bile ducts. The highest worldwide prevalence of CCA is found in Thailand, especially in the northeast which is an endemic area of *O. viverrini* infection [1, 2]. CCA is a crucial health problem to people in this region. Opisthorchiasis leads to many pathological changes of the intra- and extrahepatic bile ducts and the gallbladder. Chronic infection, accompanied by chronic

inflammation caused by mechanical damage, parasite excretion/secretion and release of inflammatory cytokines, leads to development of CCA [3]. A combination of factors (*O. viverrini* infection, nitrosamine exposure, host immune response, etc.) contribute to mutagenesis and multistep processes involving several groups of genes. Among the relevant genes, HSP90 regulates the signaling pathways needed for growth, survival, and the unlimited replicating potential of the tumor [4]. HSP70 plays a role both in facilitating the neoplastic transformation of cells and in inhibiting apoptosis. HSP70 blocks the assembly of the apoptosome complex [5]. Retinoblastoma 1 (RB1) is a tumor-suppressor gene [6] which prevents cells from replicating damaged DNA by preventing its progression along the cell cycle through G1 (first gap phase) into S (synthesis phase) [7]. Cyclin D1 interacts with tumor suppressor protein RB1; the expression of this gene promotes cell cycle progression by phosphorylating and inactivating the retinoblastoma proteins [8]. Histone deacetylases (HDACs) create a non-permissive chromatin conformation that prevents the transcription of genes that encode proteins involved in tumorigenesis [9]. HDAC6 is a specific deacetylase of several proteins including α-tubulin, cortactin, peroxiredoxins, chaperone protein and HSP90. Inhibition of HDAC6 function makes cells more sensitive to misfolded protein stress induced by protease inhibition and, as a consequence, to cell death [10].

CCA is an incurable and rapidly lethal disease if the tumors cannot be removed completely. In surgical patients, the cure rate depends on the stage of tumorigenesis, tumor location, and complete or partial tumor resection, as demonstrated in several previous studies. The five-year survival rates after curative resection (R0) for intrahepatic, perihilar and distal CCA tumors were 63%, 30% and 27%, respectively [11]. The median survival of patients with non-resection was 6–12 months [12]. Because CCA is asymptomatic in the early stages [13] many patients present to the hospital with advanced-stage CCA, and with consequently short survival times [14].

In the present study we investigated the correlation between the expression of tumor-relevant genes and clinicopathological parameters in patients with intrahepatic CCA using real-time RT-PCR (reverse transcription–polymerase chain reaction). The overexpression or underexpression of target genes may play a critical role in the carcinogenesis and tumor progression of CCA. We found that down-regulation of HSP70 may be used as a new marker in the diagnosis and prognosis of CCA.

## Materials and Methods

### Collection of Human CCA and Adjacent Tissues

Fifty frozen liver tissue samples were provided from the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand. Selected CCAs and adjacent tissues were histological confirmed by a medical pathologist. Two cases of CCA with a history of opisthorchiasis were included. Total RNA from CCA and adjacent tissues from each case was extracted, and complementary DNA (cDNA) was prepared as described below [15]. The utilization of the specimens in the present study was approved by the Human Ethics Committee of Khon Kaen University Khon Kaen Thailand (Ethical Clearance No. HEKKU501153).

The clinicopathological features of CCA patients were represented by clinical status, gross type, histological type, histological grading, staging and metastasis. Intrahepatic CCA is classified as mass-forming, periductal-infiltrating, or intraductal-growing, based on its growth characteristics as ascertained by the Liver Cancer Study Group of Japan [16]. The histological grading classifications are: well differentiated, moderately differentiated, and poorly differentiated [17]. The most common histologic type of intrahepatic CCA is adenocarcinoma with tubular and/or papillary structures. The stage of CCA was classified using the International Union Against Cancer (UICC)/American Joint Committee on Cancer (AJCC) TNM (tumor-node-metastasis) system (Table 1) [18].

### Primers for Real-Time PCR

The primers for amplification of human CCA samples were designed based on the published sequences in GenBank: HSP70 (accession no. NM_ 005345, forward tcaccatcaccaac gacaag and reverse agcccctcatcctccacg); HSP90 (accession no. NM_ 007355, forward ccaaaaagcacctggagatca and reverse tgtcggcctcagccttct); RB1 (accession no. NM_000321, forward aggtctgccaacaccaacaa and reverse tccttcagcacttctttt-gagc); cyclin D1 (accession no. NM_053056, forward ctcacacgcttcctctccag and reverse acctcctcctcctcctcttcc); HDAC6 (accession no. NM_006044, forward ccctccagttc taagttggtca and reverse tcttttctcgtgtggtcatcc); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; accession no. NM_002046, forward gaacatcatccctgcctctact and reverse cctgcttcaccaccttcttg).

### Total RNA Isolation and Complementary DNA Synthesis

Total RNA was extracted from liver tissues (200 mg) using TRIzol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The isolated RNA was treated with DNase I (RNase-Free DNase; Fermentas, Burlington, ON, Canada) in the presence of ribonuclease inhibitor (Fermentas). The treated RNA was extracted with phenol/chloroform, precipitated with ethanol, and dissolved in RNase-free water. Reverse transcription was performed with M-MLV reverse transcriptase (Fermentas) according to the manufacturer's

**Table 1** TNM classification and AJCC/UICC staging system for intrahepatic CCA [18]

| TNM stage | Tumor spread |
|---|---|
| T1 | Solitary tumor without vascular invasion[a] |
| T2a | Solitary tumor with vascular invasion[a] |
| T2b | Multiple tumors, with or without vascular invasion[a] |
| T3 | Tumor perforating the visceral peritoneum or involving local extrahepatic structures by direct invasion |
| T4 | Tumor with periductal invasion[b] |
| N0 | No regional lymph node metastases |
| N1 | Regional lymph node metastases[c] |
| M0 | No distant metastases |
| M1 | Distant metastases |

| Stage | Tumor | Node | Metastases |
|---|---|---|---|
| I | T1 | N0 | M0 |
| II | T2 | N0 | M0 |
| III | T3 | N0 | M0 |
| IVA | T4 | N0 | M0 |
| | Any T | N1 | M0 |
| IVB | Any T | Any N | M1 |

[a] Includes major vascular (portal or hepatic vein) and microvascular invasion.

[b] Includes tumors with periductal-infiltrating or mixed mass–forming and periductal-infiltrating growth pattern.

[c] Nodal involvement of the celiac, periaortic, or caval lymph nodes is considered to be distant metastasis (M1).

instructions. Three μg total RNA of each sample was prepared in a new tube and 1 μl of oligo(dT) 18 primer and 11 μl DEPC-treated water added. The samples were gently mixed, spun down, and incubated at 70°C for 5 min. They were then chilled on ice, and the following components added: 5x reaction buffer, 4 μl; ribonuclease inhibitor, 1 μl; and 10 mM dNTP mix, 2 μl. This was mixed gently and incubated at 37°C for 5 min. After that, 1 μl of M-MLV reverse transcriptase was added to the mixture. The reaction was incubated at 42°C for 60 min and then inactivated by heating at 70°C for 10 min [15].

### Absolute Quantification by Real-Time PCR

Real-time RT-PCR using the SYBR® Green method was performed to analyze the absolute quantification of mRNA expression. Five μl cDNA template was added into a PCR plate. The PCR reaction mixture consisted of 2 μl of 10x HotStar Taq buffer, 1 μl of each 5 mM dNTP, 2.4 μl of 25 mM $MgCl_2$, 1 μl of 5 μM primer pairs, 0.2 μl of HotStar Taq DNA polymerase (Fermentas) and 7.4 μl of distilled water to give a final volume of 20 μl. The reaction mixture was added with cDNA into a 96-well real-time PCR plate and briefly spun down. PCR analysis was performed using an Applied Biosystems 7500 Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). All values were normalized with a standard curve, and reported as a copy number change over the background of the housekeeping gene (G3PDH) level.

### Statistical Analysis

Statistical correlation between expression of HSP70, HSP90, RB1, cyclin D1, and HDAC6 and various clinicopathological variables (status, gross type, histological type, histology grading, staging and metastasis) was performed by the chi-square and Student's *t*-tests using SPSS version 16.0. Survival curves were constructed by the Kaplan-Meier method and compared with the log-rank test using SigmaPlot version 11.0. The results were considered statistically significant when the *P*-value was less than 0.05.

## Results

### Expression of HSP70, HSP90, RB1, Cyclin D1 and HDAC6 in Human CCA Tissues

Figure 1a-e show expression of HSP70, HSP90, RB1, cyclin D1 and HDAC6 in human CCA tissues. The expression of HSP70 was down-regulated in 33 cases (66%; 0.0- to 0.84-fold). The expression of HSP90 was up-regulated in 38 cases (76%; 1.01- to 335-fold). The expression of RB1 was down-regulated in 32 cases (64%; 0.0- to 0.98-fold). The expression of cyclin D1 was up-regulated in 15 cases (30%; 1.66- to 666-fold). The expression of HDAC6 was down-regulated in 49 cases (98%; 0.01- to 0.58-fold).

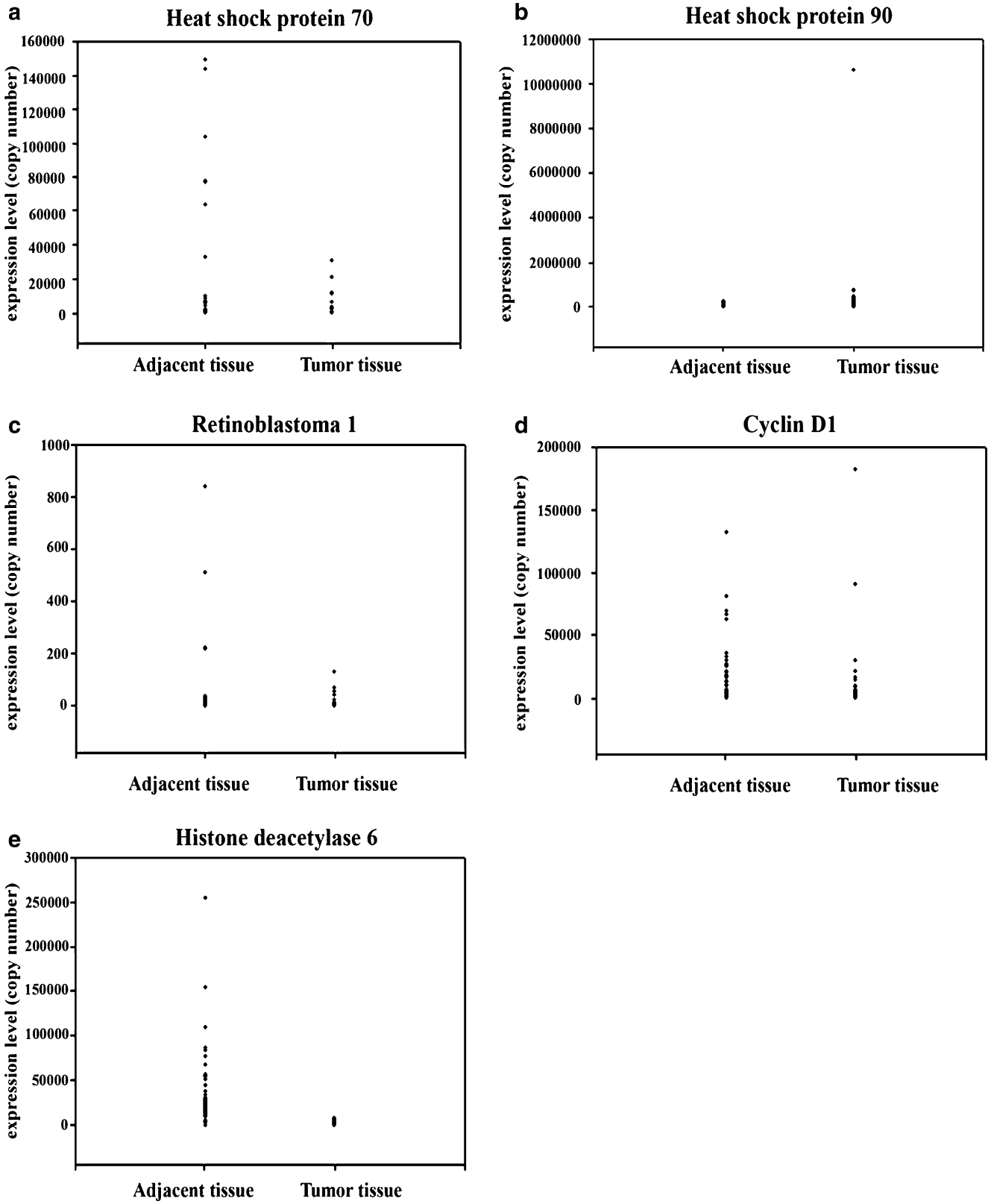



**Fig. 1** Real-time RT-PCR results of the expression levels of: **a**, HSP70; **b**, HSP90; **b**, RB1; **d**, cyclin D1; **e**, HDAC6 in liver tissue of human CCA

### Correlation Between Gene Expression and Clinicopathological Features of CCA Patients

The clinicopathological characteristics of the 50 CCA patients are summarized in Table 2. Thirty-two patients (64%) presented up-regulation of RB1 but did not show a statistically significant association with clinicopathological parameters such as status, histology type, histological grading, stage of CCA, and metastasis. A significant association was found between CCA gross type and RB1 expression ($p$=0.046) (Table 4). There were no correlations between HDAC6 and various clinicopathological parameters (status, histology type, histological grading and metastasis) excepted stage of CCA($p$=0.000) (Table 3). Moreover, there were no correlations between HSP70 and various clinicopathological parameters (status, histology type, histological grading, stage of CCA, and metastasis). A significant association was found between CCA gross type and HSP70 expression ($p$=0.046) (Table 3). Moreover, no relationship between cyclin D1 and the various clinicopathological parameters was observed, as well as these HSP90 expressions (Tables 3 and 4).

**Table 2** Clinicopathological characteristics of 50 CCA patients

| Clinicopathological factors | Number | |
|---|---|---|
| Age ($n$=50) | 56.12±9.63 | |
| Gender ($n$=50) | | |
| Male | 33 | (66.00%) |
| Female | 17 | (34.00%) |
| Survival (Year) ($n$=50) | 1.8±1.24 | |
| Status ($n$=50) | | |
| Dead | 35 | (70.00%) |
| Alive | 15 | (30.00%) |
| Liver lobe of CCA ($n$=50) | | |
| Right | 37 | (74.00%) |
| Left | 13 | (26.00%) |
| CCA gross type ($n$=30) | | |
| Mass forming | 19 | (63.33%) |
| Intraductal growing | 6 | (20.00%) |
| Periductal infiltrating | 2 | (6.67%) |
| Mixed type | 3 | (10.00%) |
| Histology type ($n$=40) | | |
| Tubular type | 20 | (50.00%) |
| Papillary type | 15 | (37.50%) |
| Mixed type | 5 | (12.50%) |
| Histology grading ($n$=23) | | |
| Well differentiation | 13 | (54.17%) |
| Moderate differentiation | 8 | (33.33%) |
| Poor differentiation | 2 | (8.33%) |
| Staging of CCA ($n$=50) | | |
| I, II | 21 | (42.00%) |
| III | 10 | (20.00%) |
| IVA | 17 | (34.00%) |
| IVB | 2 | (4.00%) |
| Metastasis ($n$=50) | | |
| No metastasis | 30 | (60.00%) |
| Metastasis | 20 | (40.00%) |

### Overall Survival Rate of Patients with CCA

The calculated expression levels of HSP70 and related genes (HSP90, RB1and HDAC6) was correlated with patient survival as shown in Fig. 2. There were 35 deaths (70%) and the mean survival time was 1.19 years after resection. The overall survival rates for the HSP70 down-regulation group at 1, 2 and 3 years were 30.1%, 7.51% and 3.76%, and for the up-regulation group 69.1%, 62.2% and 51.8%, respectively (Fig. 2c). The patients in the down-regulation group had a significantly poorer prognosis than those in the up-regulation group ($p$=0.002). In contrast, the 3-year survival rate of patients with HSP90 up-regulation was 12.9%, while that of patients with HSP90 down regulation was 36.1% (Fig. 2d). Even though the survival rate of patients with HSP90 up-regulation was shorter, the difference was not statistically significant ($p$=0.219). Additionally, the survival rate of patients with RB1 down-regulation was similar to that of RB1 up-regulation at a 2-year survival rate ($p$=0.891); after 2 years the survival rate of RB1 down-regulation was longer (Fig. 2a). The survival rates for the cyclin D1 up-regulation group at 1, 2 and 3 years were 59.3%, 51.9% and 34.6%, and for the down-regulation group 37.0%, 9.71% and 9.71%, respectively (Fig. 2b). The patients in the cyclin D1 up-regulation group had a significantly better prognosis than those in the down-regulation group ($p$=0.044). The HDAC6 survival rate was not statistically significant between up-regulation and down-regulation ($p$=0.407) because the up-regulation group had only 1 case, and that patient died before 1 year. The survival rate of the down-regulation group was longer than that of the up-regulation group (Fig. 2e).

## Discussion

CCA is a cancer of the bile ducts, and is a rapidly lethal disease. Many factors can cause CCA development, such as *O. viverrini* infection, nitrosamine exposure, etc. A combination of these factors leads to mutagenesis and multistep processes involving several groups of genes. Many kinds of genes are involved in the cell cycle, such as RB1 and related genes. In the present study, the expressions of HSP70, HSP90, RB1, cyclin D1 and HDAC6 in 50 cases of

**Table 3** Gene expression related to clinicopathological parameters of patients with CCA using chi-square analysis

| Clinicopathological parameters | | HSP 90 | | HSP 70 | | HDAC 6 | |
|---|---|---|---|---|---|---|---|
| | | Up-regulation 38 (76%) | Down-regulation 12 (24%) | Up-regulation 17 (33%) | Down-regulation 33 (66%) | Up-regulation 1 (2%) | Down-regulation 49 (98%) |
| Status ($n$=50) | | | | | | | |
| Dead | ($n$=35) | 28 (80.0%) | 7 (20.0%) | 7 (20.0%) | 28 (80.0%) | 1 (2.9%) | 34 (97.1%) |
| Alive | ($n$=15) | 9 (60.0%) | 6 (40.0%) | 10 (66.7%) | 5 (33.3%) | 0 (0.0%) | 15 (100.0%) |
| | | $X^2$=47.619 | $P$=0.447 | $X^2$=43.651 | $P$=0.150 | $X^2$=24.048 | $P$=0.345 |
| CCA gross type ($n$=30) | | | | | | | |
| Mass forming | ($n$=19) | 15 (78.9%) | 4 (21.1%) | 6 (31.6%) | 13 (68.4%) | 1 (5.3%) | 18 (94.7%) |
| Intraductal growing | ($n$=6) | 4 (66.7%) | 2 (33.3%) | 1 (16.7%) | 5 (83.3%) | 0 (0.0%) | 6 (100.0%) |
| Periductal infiltrating | ($n$=2) | 2 (100.0%) | 0 (0.0%) | 2 (100.0%) | 0 (0.0%) | 0 (0.0%) | 2 (100.0%) |
| Mixed type | ($n$=3) | 2 (66.7%) | 1 (33.3%) | 2 (66.7%) | 1 (33.3%) | 0 (0.0%) | 3 (100.0%) |
| | | $X^2$=90.000 | $P$=0.307 | $X^2$=90.000 | $P$=0.046* | $X^2$=49.167 | $P$=0.547 |
| Histology type ($n$=40) | | | | | | | |
| Papillary type | ($n$=20) | 14 (70.0%) | 6 (30.0%) | 9 (45.0%) | 11 (55.0%) | 0 (0.0%) | 20 (100.0%) |
| Tubular type | ($n$=15) | 13 (86.7%) | 2 (13.3%) | 4 (26.7%) | 11 (73.3%) | 1 (6.7%) | 14 (93.3%) |
| Mixed type | ($n$=5) | 4 (80.0%) | 1 (20.0%) | 2 (40.0%) | 3 (60.0%) | 0 (0.0%) | 5 (100.0%) |
| | | $X^2$=72.333 | $P$=0.533 | $X^2$=68.933 | $P$=0.314 | $X^2$=40.722 | $P$=0.438 |
| Histology grading ($n$=24) | | | | | | | |
| Well differentiation | ($n$=13) | 10 (76.9%) | 3 (23.1%) | 2 (15.4%) | 11 (84.6%) | 0 (0.0%) | 13 (100.0%) |
| Moderate differentiation | ($n$=8) | 7 (87.5%) | 1 (12.5%) | 3 (37.5%) | 5 (62.5%) | 1 (12.5%) | 7 (87.5%) |
| Poor differentiation | ($n$=2) | 2 (100.0%) | 0 ( 0.0%) | 1 (50.0%) | 1 (50.0%) | 0 (0.0%) | 2 (100.0%) |
| | | $X^2$=46.000 | $P$=0.389 | $X^2$=28.890 | $P$=0.523 | $X^2$=31.736 | $P$=0.285 |
| Staging of CCA ($n$=50) | | | | | | | |
| I, II | ($n$=21) | 13 (61.9%) | 8 (38.1%) | 6 (28.6%) | 15 (71.4%) | 0 (0.0%) | 21 (100.0%) |
| III | ($n$=10) | 8 (80.0%) | 2 (20.0%) | 4 (40.0%) | 6 (60.0%) | 0 (0.0%) | 10 (100.0%) |
| IVA | (n=17) | 14 (82.4%) | 3 (17.6%) | 7 (41.2%) | 10 (58.8%) | 0 (0.0%) | 17 (100.0%) |
| IVB | ($n$=2) | 2 (100.0%) | 0 (0.0%) | 0 (0.0%) | 2 (100.0%) | 1 (50.0%) | 1 (50.0%) |
| | | $X^2$=3.103 | $P$=0.376 | $X^2$=1.857 | $P$=0.603 | $X^2$=24.490 | $P$=0.000* |
| Metastasis ($n$=50) | | | | | | | |
| No metastasis | ($n$=30) | 21 (70.0%) | 9 (30.0%) | 11 (36.7%) | 19 (63.3%) | 0 (0.0%) | 30 (100%) |
| Metastasis | ($n$=20) | 16 (80.0%) | 4 (20.0%) | 6 (30.0%) | 14 (70.0%) | 1 (5.0%) | 19 (95.0%) |
| | | $X^2$=45.833 | $P$=0.521 | $X^2$=37.500 | $P$=0.355 | $X^2$=26.389 | $P$=0.235 |

* $P$-value is significant

**Table 4** Gene expression related to clinicopathological parameters of patients with CCA using chi-square analysis

| Clinicopathological parameters | | RB1 | | Cyclin D1 | |
|---|---|---|---|---|---|
| | | Up- regulation 18 (36%) | Down-regulation 32 (64%) | Up-regulation 15 (30%) | Down-regulation 35 (70%) |
| Status ($n$=50) | | | | | |
| Dead | ($n$=35) | 12 (34.3%) | 23 (65.7%) | 9 (25.7%) | 26 (74.3%) |
| Alive | ($n$=15) | 6 (40.0%) | 9 (60.0%) | 6 (40.0%) | 9 (60.0%) |
| | | $X^2$=43.651 | $P$=0.150 | $X^2$=30.499 | $P$=0.592 |
| CCA gross type ($n$=30) | | | | | |
| Mass forming | ($n$=19) | 7 (36.8%) | 12 (63.2%) | 3 (15.8%) | 16 (84.0%) |
| Intraductal growing | ($n$=6) | 2 (33.3%) | 4 (66.7%) | 1 (16.7%) | 5 (83.3%) |
| Periductal infiltrating | (n=2) | 1 (50.0%) | 1 (50.0%) | 2 (100.0%) | 0 (0.0%) |
| Mixed type | ($n$=3) | 2 (66.7%) | 1 (33.3%) | 2 (66.7%) | 1 (33.3%) |
| | | $X^2$=90.000 | $P$=0.046* | $X^2$=71.93 | $P$=0.381 |
| Histology type ($n$=40) | | | | | |
| Papillary type | ($n$=20) | 6 (30.0%) | 14 (70.0%) | 5 (25.0%) | 15 (75.0%) |
| Tubular type | ($n$=15) | 6 (40.0%) | 9 (60.0%) | 4 (26.7%) | 11 (73.3%) |
| Mixed type | ($n$=5) | 1 (20.0%) | 4 (80.0%) | 5 (100.0%) | 0 (0.0%) |
| | | $X^2$=68.933 | $P$=0.314 | $X^2$=62.889 | $P$=0.191 |
| Histology grading ($n$=24) | | | | | |
| Well differentiation | ($n$=13) | 3 (23.1%) | 10 (76.0%) | 3 (23.1%) | 10 (76.9%) |
| Moderate differentiation | ($n$=8) | 4 (50.0%) | 4 (50.0%) | 3 (37.5%) | 5 (62.5%) |
| Poor differentiation | ($n$=2) | 2 (100.0%) | 0 (0.0%) | 0 (0.0%) | 2 (100.0%) |
| | | $X^2$=28.890 | $P$=0.523 | $X^2$=30.59 | $P$=0.723 |
| Staging of CCA ($n$=50) | | | | | |
| I, II | ($n$=21) | 6 (28.6%) | 15 (71.4%) | 6 (28.6%) | 15 (71.4%) |
| III | ($n$=10) | 4 (40.0%) | 6 (60.0%) | 3 (30.0%) | 7 (70.0%) |
| IVA | ($n$=17) | 8 (47.1%) | 9 (52.9%) | 5 (29.4%) | 12 (70.6%) |
| IVB | ($n$=2) | 0 (0.0%) | 2 (100.0%) | 1 (50.0%) | 1 (50.0%) |
| | | $X^2$=2.600 | $P$=0.458 | $X^2$=0.404 | $P$=0.939 |
| Metastasis ($n$=50) | | | | | |
| No metastasis | ($n$=30) | 11 (36.7%) | 19 (63.3%) | 8 (16.0%) | 22 (84.0%) |
| Metastasis | ($n$=20) | 7 (35.0%) | 13 (65.0%) | 7 (14.0%) | 13 (86.0%) |
| | | $X^2$=37.500 | $P$=0.355 | $X^2$=38.442 | $P$=0.237 |

* $P$-value is significant

resected human CCA were investigated. The results indicated that the expressions of RB1, HSP70 and HDAC 6 were down-regulated and the expressions of cyclin D1 and HSP90 were up-regulated in CCA tissues. The correlations between RB1, cyclin D1, HSP90, HDAC6 and various clinicopathological parameters such as status, histological type, histological grading, stage, and metastasis were not significant. However, a significant association was found between CCA gross types and HSP70 ($p$=0.046) as well as RB1expression ($p$=0.046). The patients in the down-regulation group of HSP70 had a significantly poorer prognosis than those in the up-regulation group ($p$=0.002).

HSP70 facilitates neoplastic transformation of cells by inhibiting apoptosis. It functions as a unique chaperone to the malignant transformation state by maintaining several oncoproteins in a functionally active conformation. In addition, HSP70 blocks the assembly of apoptosome, a multiprotein complex involved in apoptosis [5]. The present study showed the expression of HSP70 was downregulated in 66% of cases and was associated with CCA more advance gross lesions. The survival rates for patients with HSP70 down-regulation were significantly lower than for those in the up-regulation group ($p$=0.002). In agreement with these results, it has been shown that over-expression of HSP70 was correlated with good prognosis in esophageal cancer, renal cancer, and melanoma [19]. In contrast, HSP70 downregulation is associated with carcinogenesis of the oral epithelium, and is a marker of early hepatocel-

lular carcinoma [20]. HSP70 downregulation also correlates with poor prognosis in breast cancer [21], endometrial cancer [22], uterine cervical cancer [23], and pancreatic cancer [24].

HSP90 regulates signaling pathways needed for growth, survival and unlimited replicating potential of the tumor [4]. HSP90 establishes a tight association with client oncoproteins, supporting their aberrant state and function, which is essential for malignant transformation. HSP90 and its co-chaperones also modulate tumor apoptosis, mainly through their effects on AKT kinase, TNF-$\alpha$ receptors, and the NF-kB transcriptional factor [25–27]. The present study showed that the expression of HSP90 was up-regulated in 76% of cases. As reported previously, there was over-expression of HSP90 in breast cancer [28, 29], and gastric cancer [30], and well as in cancer of the pancreas [31], and bladder [32]. The three-year survival rate of patients with HSP90 up-regulation was lower than that of the down-regulation group, but the difference was not significant ($p$= 0.219). Our results are consistent with other reports, showing that HSP90 has been correlated with poor prognosis in breast cancer [28, 29]. In contrast, HSP90 expression is associated with good prognosis in endometrial cancer [22].

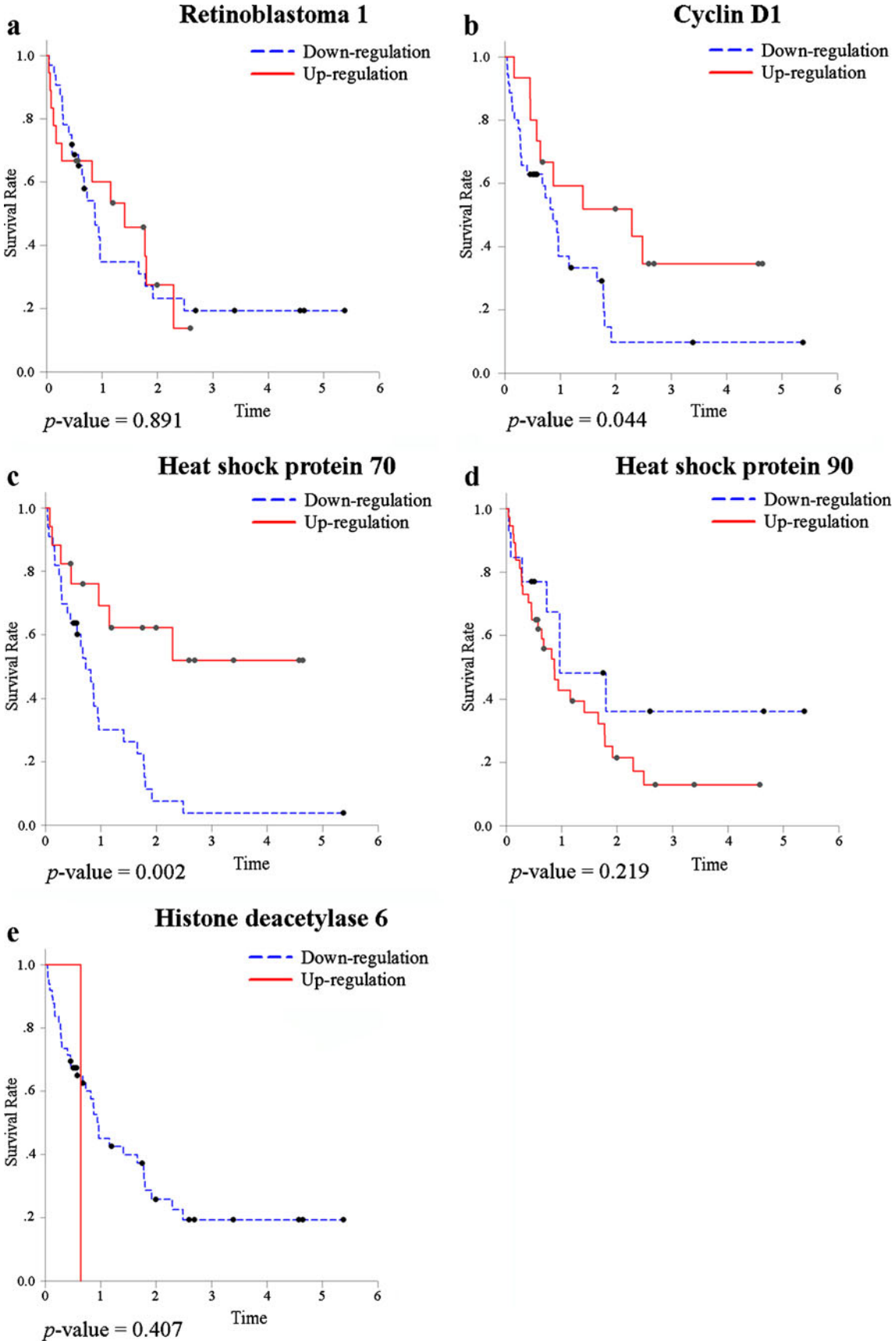
Retinoblastoma (RB) is a tumor suppressor gene that is dysfunctional in many types of cancer. RB1 prevents the cell from replicating damaged DNA by preventing its progression along the cell cycle through G1 (first gap phase) into S (synthesis phase) [7]. RB1 inactivation may result in the genesis of malignant or benign tumors such as cholangiocarcinoma [15], breast cancer [33], esophageal cancer [34] and lymphoma [35]. The present study showed that the expression of RB1 was down-regulated in 64%, similar to the results described by Chatterjee et al. [36] and Sgambato et al. [37] in bladder cancer patients, and by Deeb et al. [38] for prostate cancer. This down-regulation frequency was higher than in previous reports by Boonmars et al. [15] and Kang et al. [39] on CCA. Moreover, our present study is the first paper that found a correlation between RB1 expression and CCA gross type in CCA (Tables 3 and 4). However, the overall survival rate of patients with RB1 down-regulation was similar to those with RB1 up-regulation. RB1 gene alterations such as mutation or phosphorylation are not common in CCA. The statistical significance of RB1 expression in this study was difficult to determine because it was unclear whether RB1 expression is phosphorylate protein or not a mutation. Further study is necessary to investigate the function (or non-function) of RB1.

The cyclin D1 proto-oncogene is an important regulator of G1 to S phase progression in many different cell types. Cyclin D1 forms active complexes that promote cell cycle progression by phosphorylating and inactivating the retinoblastoma protein (RB) [8]. Cyclin-dependent kinase phosphorylate RB protein plays a critical role in neuronal cell cycle control and apoptosis [40], mutations, amplification and overexpression of this gene, which alters cell cycle progression, were frequently observed in a variety of tumors and may contribute to tumorigenesis, e.g. in cases of lymphoid, parathyroid and breast cancer [41, 42]. The present study showed that the expression of cyclin D1 was up-regulated in 30% of cases, which supported previous reports that cyclin D1 was up-regulated 30–70% in breast cancer [33, 43] and 20–80% in bladder cancer [37, 44]. However its down-regulation was lower than in previous reports by Boonmars et al. [15] and Kang et al. [39] in CCA, Deeb et al. [24] in lung cancer, and Garcea et al. [45] in pancreatic cancer. However, we did not find any correlation between cyclin D1 expression and clinicopathological parameters in CCA.

Histone deacetylases (HDACs) regulate the expression and activity of proteins involved in both cancer initiation and cancer progression, and create a non-permissive chromatin conformation that prevents the transcription of genes that encode proteins involved in tumorigenesis [9]. HDAC6 is a component of the aggresome, a cellular structure that constitutes a major site of degradation for misfolded protein aggregates, both non-ubiquitinated and ubiquitinated misfolded proteins. Inhibition of HDAC6 function makes cells more sensitive to misfolded protein stress induced by protease inhibitor and, as a consequence, can lead to cell death [10]. HDAC6 is a specific deacetylase of several proteins including a-tubulin, cortactin, peroxiredoxins, chaperone protein and HSP90. The present study showed that the expression of HDAC6 was down regulated in 98% of cases and no relationship was found between HDAC6 mRNA expression and survival rate, even though it correlated with the CCA staging. Recently, high levels of expression of HDAC6 mRNA and protein expression have been reported to correlate with improvement of disease and overall survival rates in patients with hormone-sensitive cancer [46].

In conclusion, our results suggest that HSP70 may be involved in CCA progression; thus, they may be markers of poor prognoses of CCA patients. However, the importance of RB1, HSPs and HDAC6 expression appears to be more complicated, in that there were significant differences in expression between tumor cell types. Therefore, further studies of time profiles for each gene using animal models or in vitro cell line studies may be needed to clarify the mechanisms of gene-CCA involvement.

**Fig. 2** Overall survival curves for groups of CCA patients: **a**, RB1; **b**, ▶ cyclin D1; **c**, HSP70; **d**, HSP90; **e**, HDAC6. Down-regulation (dotted line) and up-regulation (solid line)


a
Retinoblastoma 1
Down-regulation
Up-regulation
Survival Rate
Time
p-value = 0.891
b
Cyclin D1
Down-regulation
Up-regulation
Survival Rate
Time
p-value = 0.044
c
Heat shock protein 70
Down-regulation
Up-regulation
Survival Rate
Time
p-value = 0.002
d
Heat shock protein 90
Down-regulation
Up-regulation
Survival Rate
Time
p-value = 0.219
e
Histone deacetylase 6
Down-regulation
Up-regulation
Survival Rate
Time
p-value = 0.407

**Acknowledgments** This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission and the Thailand Research Fund through the Royal Golden Jubilee PhD program (Grant No. PHD/0139/2551) to Miss Sirintip Boonjaraspinyo and Associate Professor Thidarut Boonmars. The authors also gratefully acknowledge partial financial support from the Grant-in-Aid for Scientific Research (21590463) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We also wish to thank the Department of Parasitology, Liver Fluke and Cholangiocarcinoma Research Center, Animal Experimental Unit, Faculty of Medicine, Khon Kaen University, for their assistance. We also thank Stewart Sell, MD, Ordway Research Institute for his review of the manuscript.

## References

1. International Agency for Research on Cancer (IARC) (1994) Infection with liver flukes (*Opisthorchis viverrini, Opisthorchis felineus* and *Clonorchis sinensis*). IARC Monogr Eval Carcinog Risks Hum 61:121–175
2. Sithithaworn P, Haswell-Elkins MR (2003) Epidemiology of *Opisthorchis viverrini*. Acta Trop 88:187–194
3. Sripa B, Pairojkul C (2008) Cholangiocarcinoma: lessons from Thailand. Curr Opin Gastroenterol 24:349–156
4. Freeman BC, Yamamoto KR (2002) Disassembly of transcriptional regulatory complexes by molecular chaperones. Science 296:2232–2235
5. Beere HM, Wolf BB, Cain K et al (2000) Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. Nat Cell Biol 2:469–475
6. Madhavan J, Ganesh A, Kumaramanickavel G (2008) Retinoblastoma: from disease to discovery. Ophthalmic Res 40:221–226
7. Das SK, Hashimoto T, Shimizu K et al (2005) Fucoxanthin induces cell cycle arrest at G0/G1 phase in human colon carcinoma cells through up-regulation of p21WAF1/Cip1 2. Biochim Biophys Acta 1726:328–335
8. Lundberg AS, Weinberg RA (1998) Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. Mol Cell Biol 18:753–761
9. Glozak MA, Seto E (2007) Histone deacetylases and cancer. Oncogene 26:5420–5432
10. Hideshima T, Bradner JE, Wong J et al (2005) Small-molecule inhibition of proteasome and aggresome function induces synergistic antitumor activity in multiple myeloma. Proc Natl Acad Sci U S A 102:8567–8572
11. DeOliveira ML, Cunningham SC, Cameron JL et al (2007) Cholangiocarcinoma: thirty-one-year experience with 564 patients at a single institution. Ann Surg 245:755–762
12. Kondo S, Takada T, Miyazaki M et al (2008) Guidelines for the management of biliary tract and ampullary carcinomas: surgical treatment. J Hepatobiliary Pancreat Surg 15:41–54
13. Uttaravichien T, Bhudhisawasdi V, Pairojkul C et al (1999) Intrahepatic cholangiocarcinoma in Thailand. J Hepatobiliary Pancreat Surg 62:128–135
14. Green A, Uttaravichien T, Bhudhisawasdi V et al (1991) Cholangiocarcinoma in north east Thailand: a hospital-based study. Trop Geogr Med 43:193–198
15. Boonmars T, Wu Z, Boonjaruspinyo S et al (2009) Alterations of gene expression of RB pathway in *Opisthorchis viverrini* infection-induced cholangiocarcinoma. Parasitol Res 105:1273–1281
16. Liver Cancer Study Group of Japan (2003) The general rules for the clinical and pathological study of primary liver cancer, 2th ed. Tokyo: Kanehara 15
17. Nakanuma Y, Sripa B, Batanasapt V et al (2000) Intrahepatic cholangiocarcinoma. In: Hamilton SR, Aaltonen LA (eds) World Health Organization classification of tumours: pathology and genetics—tumours of the digestive system. IARC Press, Lyon, pp 173–180
18. Edge SB, Byrd DR, Compton CC et al (2009) AJCC cancer staging manual, 7th edn. Springer-Verlag Press, New York
19. Ciocca DR, Calderwood SK (2005) Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. Cell Stress Chaperones 10:86–103
20. Chuma M, Sakamoto M, Yamazaki K et al (2003) Expression profiling in multistage hepatocarcinogenesis: identification of HSP70 as a molecular marker of early hepatocellular carcinoma. Hepatology 37:198–207
21. Lazaris AC, Chatzigianni EB, Panoussopoulos D et al (1997) Proliferating cell nuclear antigen and heat shock protein 70 immunolocalization in invasive ductal breast cancer not otherwise specified. Breast Cancer Res Treat 43:43–51
22. Nanbu K, Konishi I, Komatsu T et al (1996) Expression of heat shock proteins HSP70 and HSP90 in endometrial carcinomas. Correlation with clinicopathology, sex steroid receptor status, and p53 protein expression. Cancer 77:330–338
23. Abd el All H, Rey A, Duvillard P (1998) Expression of heat shock protein 70 and c-myc in cervical carcinoma. Anticancer Res 18:1533–1536
24. Aghdassi A, Phillips P, Dudeja V et al (2007) Heat shock protein 70 increases tumorigenicity and inhibits apoptosis in pancreatic adenocarcinoma. Cancer Res 67:616–625
25. Basso AD, Solit DB, Chiosis G et al (2002) Akt forms an intracellular complex with heat shock protein 90 (Hsp90) and Cdc37 and is destabilized by inhibitors of Hsp90 function. J Biol Chem 277:39858–39866
26. Vanden Berghe T, Kalai M, van Loo G et al (2003) Disruption of HSP90 function reverts tumor necrosis factor-induced necrosis to apoptosis. J Biol Chem 278:5622–5629
27. Chen G, Cao P, Goeddel DV (2002) TNF-induced recruitment and activation of the IKK complex require Cdc37 and Hsp90. Mol Cell 9:401–410
28. Jameel A, Skilton RA, Campbell TA et al (1992) Clinical and biological significance of HSP89 alpha in human breast cancer. Int J Cancer 50:409–415
29. Yano M, Naito Z, Yokoyama M et al (1999) Expression of Hsp90 and cyclin D1 in human breast cancer. Cancer Lett 137:45–51
30. Liu X, Ye L, Wang J et al (1999) Expression of heat shock protein 90 beta in human gastric cancer tissue and SGD7901/VCR of MDR-type gastric cancer cell line. Chin Med J (Engl) 112:1133–1137
31. Ogata M, Naito Z, Tanaka S et al (2000) Overexpression and localization of heat shock proteins mRNA in pancreatic carcinoma. J Nippon Med Sch 67:177–185
32. Cardillo MR, Sale P, Di Silverio F (2000) Heat shock protein-90, IL-6 and IL-10 in bladder cancer. Anticancer Res 20:4579–4583
33. Bosco EE, Wang Y, Xu H et al (2007) The retinoblastoma tumor suppressor modifies the therapeutic response of breast cancer. J Clin Invest 117:218–228
34. Contu SS, Contu PC, Damin DC et al (2007) pRB expression in esophageal mucosa of individuals at high risk for squamous cell carcinoma of the esophagus. World J Gastroenterol 13:1728–1731
35. Chim CS, Wong KY, Loong F et al (2007) Frequent epigenetic inactivation of Rb1 in addition to p15 and p16 in mantle cell and follicular lymphoma. Hum Pathol 38:1849–1857
36. Chatterjee SJ, Datar R, Youssefzadeh D et al (2004) Combined effects of p53, p21, and pRb expression in the progression of bladder transitional cell carcinoma. J Clin Oncol 22:1007–1103
37. Sgambato A, Migaldi M, Faraglia B et al (2002) Cyclin D1 expression in papillary superficial bladder cancer: its association with other cell cycle-associated proteins, cell proliferation and clinical outcome. Int J Cancer 97:671–678

38. Deeb KK, Michalowska AM, Yoon CY et al (2007) Identification of an integrated SV40 T/t-antigen cancer signature in aggressive human breast, prostate, and lung carcinomas with poor prognosis. Cancer Res 67:8065–8080
39. Kang YK, Kim WH, Jang JJ (2002) Expression of G1-S modulators (p53, p16, p27, cyclin D1, Rb) and Smad4/Dpc4 in intrahepatic cholangiocarcinoma. Hum Pathol 33:877–888
40. Sumrejkanchanakij P, Tamamori-Adachi M, Matsunaga Y et al (2003) Role of cyclin D1 cytoplasmic sequestration in the survival of postmitotic neurons. Oncogene 22:8723–8730
41. Kella VK, Constantine R, Parikh NS et al (2009) Mantle cell lymphoma of the gastrointestinal tract presenting with multiple intussusceptions – case report and review of literature. World J Surg Oncol 7:60
42. Gillett C, Smith P, Gregory W et al (1996) Cyclin D1 and prognosis in human breast cancer. Int J Cancer 69:92–99
43. Azechi H, Nishida N, Fukuda Y et al (2001) Disruption of the p16/cyclin D1/retinoblastoma protein pathway in the majority of human hepatocellular carcinomas. Oncology 60:346–354
44. Mitra AP, Datar RH, Cote RJ (2006) Molecular pathways in invasive bladder cancer: new insights into mechanisms, progression, and target identification. J Clin Oncol 24:5552–5564
45. Garcea G, Neal CP, Pattenden CJ et al (2005) Molecular prognostic markers in pancreatic cancer: a systematic review. Eur J Cancer 41:2213–2236
46. Zhang Z, Yamashita H, Toyama T et al (2004) HDAC6 expression is correlated with better survival in breast cancer. Clin Cancer Res 10:6962–6968