CHAPTER III
RESEARCH METHODOLOGY

3.1 Materials

3.1.1 Chemicals

All chemicals used in the experiments were analytical grade. Names and sources of chemical were listed below.

Chemicals	Sources
Absolute ethanol	Merck, Darmstadt, Germany
Chloroform	Lab-Scan, Bankok, Thailand
Collagen sponge	Pfizer Japan Inc, Tokyo, Japan
Deoxynucleotide triphosphate (dNTP) Mix	Madison, WI, USA
Fetal bovine serum (FBS)	Gibco, Auckland, Newzealand
Hank's buffered salt solution (HBSS)	Sigma, St.Louis, MO, USA
Isopropanol	Lab-Scan, Bankok, Thailand
M-MLV RT 5X reaction buffer	Madison, WI, USA
Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT)	Madison, WI, USA
Penicillin-Streptomycin	Gibco, Rockville, MD, USA
Random primer	Madison, WI, USA
RNase inhibitor	Madison, WI, USA
RNA stabilization reagent	Qiagen, USA
RPMI 1640	Gibco, Rockville, MD, USA
Sodium-bicarbonate ($NaHCO_3$)	Merck AB, Stockholm, Sweden
Disodium succinate	Katayama, Osaka, Japan
TaqMan® Universal PCR Master Mix	Applied biosystems, Foster City, USA
TaqMan® MGB probes (FAM^{TM} dye labeled)	Applied biosystems, Foster City. USA
Trizol reagent	Invitrogen, Carlsbad, CA

Chemicals	Sources
5-fluorouracil	Abic, Netanya, Israel
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid(HEPES)	Gibco, Auckland, Newzealand
24-well microplate	Nunc, Roskilde, Denmark

3.1.2 Cholangiocarcinoma tissues

Cholangiocarcinoma tissues were obtained from patients who have been dignosed for intrahepatic cholangiocarcinoma and underwent surgical resection at the Department of Surgery, Srinagarind Hospital, Khon Kaen University. The number of tumor specimens were 35 specimens which obtained from 26 male patients and 9 female patients (range in age from 42 - 70 years).

3.1.3 Equipment

Instruments	Sources
CO_2 incubator	Binder, Tuttlingen, Germany
Larmina air flow	ESCO, Hatboro, USA
Microfuge Centrifuge	Beckman coulter, Fullerton, California, USA
Multiblock PCR system mechanic	Bio-active, California, USA
Real time PCR	Applied Biosystems, California, USA

3.2 Methods

3.2.1 Determination of 5-FU efficacy on CCA tissues

3. 2.1.1 Histoculture drug response assay (HDRA)

HDRA is a three-dimensional tissue culture method, in which intercellular connections are maintained (Furukawa, 1995). The HDRA was performed according to methods previously described (Hasegawa, 2007) with slight modifications. Tissues were washed 6 times in HBSS and aseptically cut into small pieces by using biopsy punch (3 mm diameter). Collagen sponge was cut into 1-cm squares, which were then placed into individual wells of a 24-well microtiterplate containing 1 ml RPMI 1640 medium containing 20% fetal bovine serum (FBS), 100 IU/ml of penicillin and 100 µg/ml streptomycin. 5-FU was dissolved in this media at various concentrations (i.e. 100, 200, 400 and 1000 µg/ml). A fragment of CCA tissue was then placed on the collagen sponge and cultured for 4 days at 37 °C in 5% CO_2 incubator. For the control group, CCA tissues were cultured for 4 days in the RPMI 1640 medium containing 20% FBS. Duplication of experiment were performed for each concentration of 5-FU.

After culture for 4 days, tumor tissue were examine by using formalin fixed paraffin embedded method and evaluated percentage of cancer cells survival by using microscopic examination as described below.

3.2.1.2 Microscopic examination

Four days after culture, a piece of each specimens incubated with complete medium alone or with various concentrations of 5-FU was processed for microscopic examination according to standard histological method. The tissue specimens were fixed in 10% buffered formalin for 24 h, before dehydrated in a graded ethanol series (70%, 95%, 95%, 95%, 100%, 100%, and 100; 1 h each), cleared in three changes of xylene for 1 h and two changed of paraffin each for 1.5 h at 60 °C, and embedded in melted paraffin. After embedding, the paraffin blocks were cooled down rapidly on a cooling plate before sectioning by a rotary microtome. The paraffin sections were placed on a water bath. After the sections had decompressed, coated slide was dipped under the sections at 45 ° angle, and the

sections were pulled out of the water. After draining off excess water, the slides, were dried in an oven at 37 °C for overnight. These sections can be stored at room temperature in slide boxes for a year. For hematoxylin and eosin staining, the sections were deparaffinized in xylene, rehydrated through graded alcohol series and rinsed for 5 minutes in distilled water. The slides were then stained with hematoxylin and eosin, dehydrated, cleared and mounted with Permount® before examining using an Olympus microscope by two investigators.

Histological findings were compared among those untreated tissues and 5-FU treated tissues after 4 days of culture. Critical histological examination was done in all liver tissue sections of a case. Total tumor cells in the tissue section were estimated as percentage. Total living tumor cells with hyperchromatic nuclei/cytoplasm were semi-quantitatively scored as percentage against total tumor cells (both viable and dead cells). Tumor cell death was justified by cells with eosinophillic cytoplasm with shrinkage (condensed) nuclei (pyknotic nuclei), nuclei fragmentation (karyorrhexis) and/or disappearing nuclei (karyolysis). Then percentage of inhibition index (I.I.%) was calculated using a following formular in duplicate samples.

I.I. % = (1- % living tumor cell of 5-FU treated tumor tissue/ % total tumor cell of control tissue)×100

3.2.1.3 Evaluation of optimal 5-FU concentration in HDRA

Evaluation of optimal 5-FU concentration used as a cut-off concentration to differentiate between poorly response and well response CCA tissues was performed. The dose response curve was constructed by using 5-FU concentrations and %I.I.

3.2.2 Determination level of mRNA expression by real-time PCR

3.2.2.1 RNA extraction

Total RNA was isolated from frozen tumor tissue using TRIzol reagent according to the manufacturer's protocol. Briefly, frozen tumor tissue (50-100 mg) was cut into small pieces then 1 ml of TRIzol reagent was added and immersed into liquid nitrogen for 1 min. After homogenized, the lysate was incubated for 5 min at 15-30 °C to permit the complete dissociation of nucleoprotein

complex. Then 0.2 ml of chloroform was added to the mixture and mixed vigorously for 15 sec, followed by incubated for 3 min at 15-30 °C. The mixture was centrifuged at 13000 rpm for 15 min at 2-8 °C. After centrifugation, 400 µl aqueous phase was transferred to new 1.5 ml microtube. RNA was precipitated by 0.5 ml isopropanol for 10 min at 15-30 °C and then centrifuged at 13000 rpm for 10 min at 2-8 °C. After centrifugation, supernatant was removed and RNA pellet was washed once by 1 ml of 70% ethanol, for 5 min at 15-30 °C and then centrifuged at 13000 rpm for 5 min at 2-8 °C. The RNA pellet was briefly air dried and dissolved by using 30µl RNase free water and stored at -70 °C until used.

3.2.2.2 Determination of RNA concentration

Amount of RNA was measure by using spectrophotometer. Total RNA yield and protein were determined absorbance (A) at the wavelength of 260 nm and 280 nm, respectively.

The RNA concentration (X) was then calculated using the following formular:

$$\text{RNA concentration} = \frac{\text{A 260 x dilution factor x 40}}{1{,}000} = \text{X } \mu g/\mu l$$

3.2.2.3 Reverse Transcription (RT) reaction

RT reaction was performed using method of Ishikawa *et al.* with some modifications (Ishikawa, 1999a). Briefly, 2.5 µg of total RNA from each samples was reverse-transcribed in a 20 µl reaction containing 500 µg of random hexamer, 20 units of RNase inhibitor (RNasin), 200 units of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV reverse transcriptase), 10 µM dNTPs Master Mix, and 4 µl of 5x RT buffer (1 mM Tris-HCl, 1.5 mM KCl, 60 µM $MgCl_2$ and 200µM Dithiothreitol (DTT)). Initially, RNA and random hexamer were mixed and heated for 10 min at 70 °C and immediately chilled on ice. Other reagents were then added and incubated for 10 min at 25 °C, 1 h at 37 °C, 5 min at 95 °C and subsequently for 5 min at 4 °C by using a Multiblock PCR system mechanic and the tube was chilled on ice.

3.2.2.4 Real-time Polymerase Chain Reaction (real-time PCR)

Gene involve in metabolic pathway including TS, DPD, TP, OPRT and GAPDH (glyceroldehyde-3-phosphate dehydrogenase) were determined the expression by using a TaqMan® gene expression assay. The assay numbers for the endogenous control (GAPDH) and target genes were as follow: Hs 02758991_g1(GAPDH), Hs 00426591_m1(TS), Hs 00157317_m1(TP), Hs 00559278_m1(DPD) and Hs 00923517_m1(OPRT). After RNA isolation, cDNA was derived from each sample and target cDNA sequences were amplified by semi-quantitative PCR using a fluorescence-based real-time detection method (ABI PRISM 7500 sequence detection system (Taqman); Applied Biosystems, Foster City, CA,USA). Each 20 µl PCR reaction mixture contained 10 µl of 2x Taqman Universal PCR mastermix with AmpErase UNG (Applied Biosystem), 1 µl of 20x Assay Mix (contains two target-specific primers and one TaqMan® MGB FAM™ dye-labeled probe) and 50 ng of cDNA sample. Each PCR was carried out in duplicate. Thermal cycling conditions comprised an initial step at 50 °C for 2 min was required for optimal AmpErase UNG activity followed by 95 °C for 10 min, each of 40 cycles for denature, annealing/extension at 95 °C for 15 sec and 60 °C for 1 min, respectively. All data were analyzed using the ABI PRISM 7500 Real-time PCR system sequence detection software v.1.4 (Applied Biosystem, Foster City, CA, USA). The data from the real-time PCR machine were exported into a Microsoft Excel spreadsheet to construct standard curves and to perform statistical analyses. The standard curve was created by plotting the threshold cycle (CT) against each input amount (containing 592.0, 118.4, 23.6, 11.8 and 4.7 µg) of control total RNA, prepared from EP5713 human CCA tissue. The coefficient of linear regression (r) for each standard curve was more than 0.990. The quantity of target cDNA or GAPDH PCR product was calculated using the corresponding standard curve and the amount of target cDNA in a given sample was normalized with that of GAPDH cDNA.

3.3 Statistical analysis

The student's t-test used to compare mean values between the drug response and non-response groups. Pearson's regression coefficient was calculated to evaluate correlation between TS, TP, DPD and OPRT gene expression.

STUDY DESIGN