# **Gene Expression Profiling of Intrahepatic Cholangiocarcinoma**

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# Abstract

Intrahepatic cholangiocarcinoma (ICC) is ranked as one of the top five causes of cancer-related deaths. ICC in Thai patients is associated with infection with the liver fluke, *Opisthorchis viverrini*, but the molecular basis for development remains unclear. The present study employed a microarray approach to compare gene expression profiles of ICCs and normal liver tissues from the same patients residing in Northeast Thailand, a region with a high prevalence of liver fluke infection. In ICC samples, 2,821 and 1,361 genes were found to be significantly up- and down-regulated respectively (unpaired t-test, p<0.05; fold-change  $\geq$ 2.0). For validation of the microarray results, 7 up-regulated genes (*FXYD3, GPRC5A, CEACAM5, MUC13, EPCAM, TMC5,* and *EHF*) and 3 down-regulated genes (*CPS1, TAT,* and *ITIH1*) were selected for confirmation using quantitative RT-PCR, resulting in 100% agreement. The metallothionine heavy metal pathway contains the highest percentage of genes with statistically significant changes in expression. This study provides exon-level expression profiles in ICC that should be fruitful in identifying novel genetic markers for classifying and possibly early diagnosis of this highly fatal type of cholangiocarcinoma.

Keywords: Intrahepatic cholangiocarcinoma - gene expression profile - metallothione heavy metal pathway

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# Introduction

Intrahepatic cholangiocarcinoma (ICC), the second most common primary hepatobiliary cancer globally, is a major cause of cancer-related deaths and shows no indication of a decrease in mortality rate (Khan et al., 2005; Ustundag and Bayraktar, 2008; Mosconi et al., 2009). Although the incidence of ICC had been primarily associated with developing countries, ICC is now increasing in developed countries, especially in the United Kingdom and Japan (Kato et al., 1990; Taylor-Robinson et al., 1997; McLean and Patel, 2006; West et al., 2006). Thailand has the highest incidence of ICC in the world, perhaps related to a tradition of eating raw fish, which may be contaminated with the liver fluke parasite, Opisthorchis viverrini, a cause of cholangiocarcinoma (Kurathong et al., 1985; Vatanasapt et al., 1990; Parkin et al., 1991; Thamavit et al., 1993; 1994; Sripa and Pairojkul, 2008). The prevalence of liver fluke infection in northeast Thailand is about 317.6 per 100,000 personyears (Sriamporn et al., 2004). O. viverrini induces a chronic inflammatory mechanism that may result in DNA damage, leading to a neoplastic transformation of biliary epithelial cells (Haswell-Elkins et al., 1994; Satarug et al., 1996). However, the molecular mechanisms underlying this process remain unclear. Gene expression profiles

using cDNA microarray have been generated, showing that *O. viverrini* associated ICC in Thai patients exhibited up-regulated of genes involve in xenobiotic metabolism whereas that in non *O. viverrini* associated ICC in Japanese patients presented enhanced gene expression in growth factor signaling pathway (Jinawath et al., 2006).

The introduction of oligonucleotide microarrays has enabled simultaneous detection of many thousands of expressed genes, making it highly useful for identifying genetic mechanisms and for providing biomarkers. Although various genetic markers in different types cancers and their sub-classification have been widely exploited using this technique (Chee et al., 1996; Golub et al., 1999; Kim et al., 2004), there are only two publications using in-house cDNA microarrays to produce a comprehensive analysis of gene expression profiles in ICC mass forming subtype (Obama et al., 2005; Jinawath ,et al., 2006) and another for gene expression profile in billiary tract cancer (gallbladder carcinoma, ICC and distal bile duct carcinoma) using Affymetrix GeneChip U133A expression array (Hansel et al., 2003).

In this study, gene expression profiles using Affymetrix microarray expression platform, Exon 1.0 ST of 15 Thai patients with two different types of ICC, namely, intraductal growth type and periductal infiltrating type, were generated and compared with the corresponding

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normal tissues, in order to establish a common gene expression profile of ICC. These data may allowing for better understanding of the development of ICC.

### **Materials and Methods**

### Patients and tissue samples

ICC tumor (8 periductal infiltrating tissues and 7 intraductal growth tissues) and corresponding normal liver tissue samples were obtained from Biobank of Liver Fluke and Cholangiocarcinoma Research Center, Srinagarind Hospital, Khon Kaen University, Thailand. The average age of patients was 59.6 years (ranging from 37-76 years), with an average survival period of 10.4 months (ranging from 1.6-27.7 months). Hematoxylin and eosin-stained sections of formalin-embedded tissues were examined under a light microscope in order to classify tumor types. All tissues were diagnosed clinically and pathologically as ICC according to WHO classification (Hamilton et al., 2000). The study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Thailand (MUTM 2008-004-02) and Faculty of Medicine, Khon Kaen University (HE471214) and informed consents of the patients were obtained prior to undergoing hepatectomy.

#### Microarray profiling

A portion of each frozen tissue biopsy (approximately 3x3x3 mm) was homogenized using a freeze-thaw protocol of TRIzol<sup>®</sup> RNA Isolation kit (Invitrogen, CA, USA) and RNA was isolated according to the manufacturer's protocol. Quantity and quality of RNA samples were assayed using a NanoDrop<sup>TM</sup> 1000 (Thermo Fisher Scientific, MA, USA) spectrophotometer. Total RNA was adjusted to a final concentration of 1 µg/µl. Integrity of the extracted RNA (RIN) was measured using Agilent RNA 6000 Nano Kit and BioAnalyzer 2100 (Agilent Technologies, CA, USA), resulting in an acceptable score >5.5.

# Whole-transcript expression array and microarray image processing

cRNA was prepared from 1 µg of total RNA using GeneChip® WT Terminal Labeling kit (Affymetrix, Inc., CA, USA). First-strand cDNA synthesis was primed using a T7-(dT24) oligonucleotide primer with and RNA polymerase. After second-strand synthesis, in vitro transcription was performed to produce biotin-labeled cRNA. After fragmentation of the cRNA products (20 µg at 94°C for 35 min.) to lengths of 35-200 bp, the samples were added to a hybridization solution to a final cRNA concentration of 0.05 mg/ml. Hybridization was performed by incubation overnight (17 hours.) of 200 ul of the sample to an Affymetrix Exon 1.0ST in the GeneChip® Hybridization (Affymetrix, Inc., CA, USA). Washed and stained the arrays in GeneChip® Fluidics station 450 (Affymetrix, Inc., CA, USA) with the Wash, and Stain Kit (Affymetrix, Inc., CA, USA). The arrays were scanned using a GeneChip® Scanner 3000 7G (Affymetrix, Inc., CA, USA).

Validation of microarray data by quantitative reverse transcriptase PCR (qRT-PCR)

Total RNA was reverse transcribed into cDNA using SuperScript® VILOTM cDNA Synthesis Kit (Invitrogen, CA, USA) according to the manufacturer's instructions. Expression of gene of interest was quantified by qPCR using a SYBRGreen I PCR kit (Roche Diagnostics, Germany) and gene specific primers (Table 2). The Lightcycler solution mixture (Fast Start DNA master SYBR Green I) was containing of 1 µl (0.5 µM) of primer mix (5  $\mu$ M), 0.8  $\mu$ l of MgCl<sub>2</sub> (25 mM), and 5.2  $\mu$ l of water PCR grade, respectively. The 9 µl of PCR mix were pipetted into each pre-cooling Lightcycler capillary. The 1  $\mu$ l of cDNA template was added. Each capillary was sealed with the stopper and centrifuged at 700 g for 5 sec. Thermal cycling and fluorescent monitoring were performed in LightCycler<sup>®</sup>2.0 instrument (Roche Diagnostics, Germany). The qRT-PCR was performed by all 30 samples (15 tumor tissues and 15 their corresponding normal liver tissues). The GAPDH gene in tumor and their corresponding normal live tissue was also quantified as the control gene copy number. The point at which the PCR product was first detected above the fixed threshold and terms the cycle threshold (Cp) was determined for each sample by LightCycler® Software version 4.1 (Roche Diagnostics, Germany). The relative gene amplification in cDNA samples was determined by comparative Cp method, as previously described (Livak and Schmittgen, 2001).

#### Statistical analysis

Pre- and post-data analyses were conducted using GeneSpring GX 11.5 (Agilent Technologies, CA, USA). Data were normalized using Iterative PLIER default protocol for background correction. Expression profiles of tumor samples and their corresponding normal liver tissues were determined according to the following parameters: corrected p-value cut-off of <0.05; unpaired t-test; asymptotic and multiple testing corrections; Benjamini-Hochberg. A hierarchical cluster analysis was also performed to assess correlations among the samples

 Table 1. Clinico-pathological Features of the 15 ICC

 Samples Studied

Patient No.	Age/ Sex	Tumor Location*	Size (cm.)	Differen- Me tiation	tastas	is Survival Period (Me	o.)
2ª	37/M	Right lobe	5x3.5x4	Рар	Yes	15.8	
3 <sup>a</sup>	43/F	Left lobe	3x1x1.5	Tubular pap	Yes	17.0	
4 <sup>a</sup>	58/M	Left lobe	6x7x5	Moderate	Yes	6.6	
5ª	44/M	Right lobe	11x10x9	Mixed tubula	r+		
				mucinous	Yes	1.6	
6ª	65/M	Left lobe	5x5x5	Moderate	Yes	5.6	
7 <sup>a</sup>	73/M	Left lobe	4x5x1	Moderate	Yes	6.4	
13ª	65/M	Left lobe	6x4x3	Well	Yes	5.1	100.
14 <sup>a</sup>	62/F	Left lobe	4x3x1	Well	No	4.1	
1 <sup>b</sup>	70/M	Right lobe	4x2.5x3	Рар	No	3.4	
8 <sup>b</sup>	66/F	Right lobe	6x5x6	Pap	No	4.9	
9 <sup>b</sup>	67/F	Left lobe	6x4x5	Pap	Yes	9.5	75 (
10 <sup>b</sup>	43/M	Right lobe	16x6x5	Pap+mucinou	is Yes	27.7	75.
11 <sup>b</sup>	54/M	Left lobe	6x2x1	Pap	No	21.6	
12 <sup>b</sup>	76/M	Right lobe	3x3x1	Pap	No	3.0	
15 <sup>b</sup>	71/M	Right lobe	4.5x4x4	Pap	No	24.3	_50 (

\*pap – papillary; well - well-differentiated; moderate - moderately differentiated. M – male; F – female; PI - periductal infiltrating type; <sup>b</sup>IG - intraductal growing type

25.0

Λ

6.3

56.3

Ref. Accession Number	Gene Symbol	Sense Primer (5'>3')	Anti-sense Primer (5'>3')
NM_021910	FXYD3	TTCTGCTGATCCTGAAATTGTA	TTCTTTTCCTTAGATGATGTGTTTT
NM_003979	GPRC5A	GCTCACTTGCTAAATAAGAATCTAT	ACCCTAACCATTGTCTCAGTA
NM_004363	CEACAM5	TACAAGTTTCTGATACCACTG	ATCCTCATTAGTTCATTTAGTC
NM_033049	MUC13	TCATCATACAGGTTGAGAATGTT	TCTGAGAGTCTATCACATCAATG
NM_003979	EPCAM	TTCCTGTTGGCT <i>TAT</i> GTTAGTC	TTCTTCACGAGTTGAGGTTTAC
NM_002354	TMC5	AGTATGAGAAGGCTGAGATAA	ATTTGTGTCCATTTGCTATTTC
NM_012153	EHF	ACTTCAACCTCAACCTATCTT	TCCTGCTACATTACTATGCTTA
NM_001122633	CPS1	CTATATCAGCAGATGGTAGACA	AACCTTACTTCCAAGT <i>TAT</i> TCC
NM_000353	TAT	TGAAAGTACCAGGTGAACAAAG	GGGCACAAATTCTCTCAATCTT
NM_002215	ITIH1	GGAGAAC <i>TAT</i> GGAGCAATTCAC	GGCTTGACTTTGATGACAATTTC

and genes of interest using Euclidean distance and average linkage statistical methods.

# Results

#### Identification of expressed genes related to ICC

Using the criteria of statistical significance of p-value<0.05 and fold-change  $\geq$ 2 compared with matched normal tissues, a total of 2,821 genes were identified as being up-regulated and 1,361 genes as down-regulated. Using unsupervised hierarchical clustering analysis, with p-value <0.05 fold change cut-off >20, 42 genes were up-regulated in tumor tissues (Figure 1, Table 3) and 204 down-regulated compared with normal control samples. The 42 up-regulated genes included GPRC5A (molecular transducer), FXYD3 (ion transporter), SLC6A14 (amino acid transporter), EHF and KLF5 (transcription factors), GALNT5, SGPP2, LIPH, and TMPRSS4 (catalytic activity), and SPINK1, SERPINB5 and SPINT2 (protease inhibitors). Among the 204 down-regulated genes, the 5 most lowest were SERPINC1 (serpin peptidase inhibitor, clade C/antithrombin member 1), APOH (apolipoprotein  $H/\beta$ -2-glycoprotein I), *HRG* (histidine-rich glycoprotein), KNG1 (kininogen 1) and HPX (hemopexin).

#### Validation of microarray technology by qRT-PCR

In order to verify the reliability of the microarray data, 7 up-regulated [*FXYD* (domain containing ion transport regulator 3), *GPRC5A* (G protein-coupled receptor family C group 5 member A), *CEACAM5* (carcinoembryonic antigen-related cell adhesion molecule 5), *MUC13* (mucin-13 cell surface associated), *EPCAM* (epithelial cell adhesion molecule), *TMC5* (transmembrane channellike 5), and *EHF* (ets homologous factor), and 3 downregulated genes (*CPS1* (carbamoyl-phosphate synthetase 1/mitochondrial carbamoyl-phosphate synthetase 1), *TAT* (tyrosine amino transferase), and *ITIH1* (inter-alpha (globulin) inhibitor H1]. were selected for validation by measuring their expression levels using qRT-PCR. The results of qRT-PCR and microarray data were in very good agreement (Figure 2).

### Metabolic pathway analysis

Metabolic pathway analysis using Genespring GX version 11.5 of the significantly differentially expressed genes in ICC, compared with matched normal tissues, indicated that these genes are present in 8 pathways,



**Figure 1. Hierarchical Clustering of ICC-associated Genes.** Of the 246 genes indicated by Affymetrix Exon 1.0 ST array as being differentially expressed in ICC in comparison with matched normal tissues, 42 are up-regulated and 204 downregulated (p<0.05 at magnitude of fold-change >20). Colorgram depicts relative levels of gene expression (high in red and low in blue). The horizontal bar (left) indicates genes that are expressed in ICC cases, and vertical bar (top) indicates the expression profile of 246 genes in each sample (N=normal, T=tumor)



Figure 2. Box and Whisker Plot between Affymetrix Exon 1.0 ST Array (green) and qRT-PCR Data. Data include 7 genes randomly selected from the 2,821 up-regulated and 3 from 1,361 down-regulated genes. Box chart show a distribution of log2-transformed relative gene expression ratios of tumor to corresponding normal liver tissues (log2 T/N) from microarray and qRT-PCR assays using the same RNA samples. Expression of *GAPDH* was used for normalization of qRT-PCR

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Table 3. Top of 42 Ur	o-regulated Genes in Intrahe	patic Cholangiocarcinomas (	p value >0.05, fold change >20)

NU_00422         AGR2         anterior gradient bornlog 2 (Xempore line's)         95.23           NU_00428         FXU23         FXU20	Ref. seq	Gene symbol	Gene Description	Fold change	
NM, 043498       FXD2	NM_000422	AGR2	anterior gradient homolog 2 (Xenopus laevis)	95.23	
NM_01212       SI_CLALA       volute currier family 44, member 4       67.42         NM_010170330NM_118200       CFACAM6       61.94         NM_010170330NM_118200       CFACAM6       60.42         NM_010170330NM_118200       CFACAM6       60.42         NM_0010170330       MUC10       consintemproprior antigen related all adhesion molecule. 5       54.67         NM_001017030       MUC13       consintemproprior antigen related all adhesion molecule. 5       44.55         NM_001017030       CKM771AFCKNTTB       contine Kingg priorbinal 1.01 contine Kingg and provide antigen related all adhesion molecule. 5       44.55         NM_0010170300       CKM771AFCKNTTB       contine Kingg and provide all all contine Kingg and provide all adhesion molecule. 5       44.55         NM_00102531       THPH       contine Kingg and provide all adhesion molecule. 5       44.55         NM_00102531       THPH       contine Kingg and provide all adhesion molecule. 5       44.55         NM_00102531       THPH       contine Kingg and provide all adhesion molecule. 5       43.53         NM_00102531       RAB25       karini 19       0       32.41         NM_00102531       KAB25       karini 19       0       33.93       33.93         NM_00102531       KAB25       karini 19       0       33.93	NM_014568	FXYD3	FXYD domain containing ion transport regulator 3	84.41	
NM_003520/NM_01890         GPRC5A         G provins-organization molecule         61.94           NM_003734         NRC1         MCR2A         61.04           NM_003734         NRC1         MCR2A         61.04           NM_003734         CRACAM6         61.04         92.02           NR_003731         CRACAM6         61.04         92.02           NR_0013733         MCR3         92.02         92.02           NR_0013733         CRACAM6         61.04         92.02           NR_0013733         RACCA         65.41         92.02           NR_00147890         CRATTACKNTTB         65.41         10.0         20.3           NR_0012331         TRG6         resting kinese, minochand gg 3.1         65.41         90.02           NR_001231         TRG6         scitn filterent associated         90.62         31.3         30.0           NR_001231         TRG6         resting kinese, minochand gg 3.1         65.41         90.02         30.0           NR_001231         RND         51.0         31.3         31.3         31.3         31.3           NR_001231         RND         resting kinese, minochand gg 3.1         65.0         31.3         31.3         31.3         31.3         3	NM_003122	SLC44A4	solute carrier family 44, member 4	67.42	
NM_0011055310M_18207       CEACAM6       cancincentryourie artigen related call adhesion molecule       60.42         NM_005764       MUCLI       marcine antigent call of adhesion molecule       50.47         NM_002130       CEMUT1       marcine antigent call of adhesion molecule       50.47         NM_002130       CEMUT1       marcine adhesion molecule       50.47         NM_002130       CEMUT1ACKNT1B       creatine kinase, raincher biggats       46.58         NM_002235       TMC5       lipse, marcher H       45.5         NM_0023430       CEMUTAKCKNT1B       creatine kinase, raincher Biggats       46.54       43.66         NM_00254331       TROB       50.0       56.2       31.3       30.0         NM_0021253       KR17       KR17       karls       56.2       31.3       35.11       30.0         NM_0021234       TROB       S50.0       sin filament associated preprint adjace preprint in the set of t	NM_005562l/NM_018891	GPRC5A	G protein-coupled receptor, family C, group 5, member A	61.94	
ML005761         Grout-specific cross rescting antigen         60-42           ML005761         CECM4         50-42           ML00170320         CECM4         service associated         50-42           ML00170320         EPCAM         epithelial <b>200</b> -0 eson molecule         46-38           ML00170320         EPCAM         epithelial <b>200</b> -0 eson molecule         46-38           ML00173800         CKMT7LACKMT1B         creatine kinege patiecheoticial 1A1 creatine kinege matechandraft associated         46.44           ML00173800         TMC3         TMC3         45.64         41.65           ML00173800         TMC3         TMC3         45.64         41.64           ML00173800         TMC3         TMC3         45.64         41.64           ML001024141         Create kinege patiecheotifial 1A1 creatine kinege matechootifial 1A1         46.84         43.6           ML001024141         AFAP1ILOC84740         actin filament associated metalent logotificate iL DC84740         49.23         49.23           ML00102311         KLFS         kalikrein-schaot peptida 31.3         31.31         33.56         30.00           ML00102311         KLFS         kalikrein-schaot poptida 20.00         49.23         33.95         49.23           ML00107301         KLFS <td>NM_001170553I/NM_182607</td> <td>CEACAM6</td> <td>carcinoembryonic antigen-related cell adhesion molecule</td> <td></td> <td></td>	NM_001170553I/NM_182607	CEACAM6	carcinoembryonic antigen-related cell adhesion molecule		
NML 09754       MUC1       much 1. cell surface associated       \$9.02         NML 09721       CL2LAMS       much 1. cell surface associated       \$9.03         NML 0010303321       DPCAM       epithelial g000-g000 molecule       \$9.03         NML 0010303321       DPCAM       epithelial g000-g000 molecule       \$6.33         NML 0010303321       DPCAM       epithelial g000-g000 molecule       \$6.34         NML 001033332       CKM77ACKMTTB       creatine kinace_mine file 5       \$1.01       20.3         NML 00103330       TRGS       lipse.minethial g000-g000 molecule       \$4.55       \$4.55         NML 00103331       TRGS       lipse.minethial g000-g000 molecule       \$4.55       \$5.42       \$3.33       \$3.00       \$3.00       \$3.00       \$3.00       \$3.00       \$3.00       \$3.00       \$3.00       \$3.00       \$3.00       \$3.00       \$3.00 <td< td=""><td></td><td></td><td>6 (non-specific cross reacting antigen)</td><td>60.42</td><td></td></td<>			6 (non-specific cross reacting antigen)	60.42	
NU_01/21       C/AL/M0       circincemprone antigen reliable cell altebolm molecule S       3-3-67         NU_01/213       B/C/J1       circincemprone antigen reliable cell altebolm molecule S       3-3-67         NU_01/213       B/C/J1       circincemprone antigen reliable cell altebolm molecule S       3-3-67         NU_001/213       B/C/J1       circince intrage apticeholytical 1-late ceatine kinase minitechol 2003       40.51         NU_001/2333       NU_001/2333       ceatine kinase apticeholytical 1-late ceatine kinase minitechol 2003       44.55         NU_001/23431       TACS       revalues minitechol 2003       54.2       31.3         NU_001/23431       RAD2       ceatine kinase, minitechol 2003       54.2       31.3         NU_001/23431       KLPS       kultecin reliated perioda 31.3       38.0       23.7       31.3       35.61         NU_001/23431       KLPS       kultecin reliated perioda 31.3       38.0       23.7       31.3       35.61       30.00         NU_001/23431       KLPS       kultecin reliated perioda 31.3       38.0       23.7       31.3       35.61       30.00         NU_001/23431       KLPS       kultecin reliated perioda 31.3       38.0       23.7       31.3       35.61       30.00         NU_001/23541       KRTP	NM_005764	MUC1	mucin 1, cell surface associated	59.02	
AM_0012013       BM_0L2D       Inten 1.5 cell utilize alsociated       40.23         AM_00120344       BPCAM       cell utilize alsociated       40.33         AM_00120341       BPCAM       cell utilize alsociated       40.33         AM_001203431       CKMT/ACKMTIB       creatine kinase, mitochondreil 1.1 creatine kinase, mitochondreil 1.8       44.85         AM_00123431       TGB6       integrin, bes 6       54.2       31.3       30.0         AM_00123431       TGB6       integrin, bes 6       54.2       31.3       30.0         AM_00123431       TGB6       integrin, bes 6       50.0       54.2       31.3       30.0         AM_00123431       KRT19       actin filance factor 5 (integrin) by the factor 5 (integrin) by th	NM_007231	CEACAMS	carcinoembryonic antigen-related cell adhesion molecule 5	54.67	
Micro Constraint         Deckal         Enclain         Enclain <thenclain< th="">         Enclain         <thenclain< th=""></thenclain<></thenclain<>	NM_002203	MUC13 EDCAM	mucin 13, cell surface associated	49.23	
0010137373       001047930         NM_001047930       6.3         NM_00127372       106.1         NM_0012313       1101         NM_00103131       1100         NM_00103131       1100         NM_00103131       1100         NM_00103131       1100         NM_00103131       1100         NM_00103131       1100         NM_0010311       1100         NM_00103101       11000	NM_022044	EFCAM		40.96	
Number of the second	NM_001159353		6.3 10.1 20.2		
NML 003639       CKMT/ICKNTIB manamethed is large_gritechare final 1/h (creatine kinase minochondra) 1/20 MA (03320       46.84 H3.5       40.01         NML 03252       LIPH MA (11)       Impact, member H       Impact, member H       41.85         NML 03252       CKMT/ICKNTIB (TGB6       revalue kinase, minochondral 1/16       41.85         NML 0010254141       ITGB6       actin filment associated meting. Hystotherial L0 C84730       38.24         NML 0101254141       actin filment associated meting. Hystotherial L0 C84730       38.24         NML 0101254141       KLK6       actin filment associated meting. Hystotherial L0 C84730       38.24         NML 0101254141       KLK6       actin filment associated meting. Hystotherial L0 C84730       38.24         NML 01012541       KLK6       actin filment associated meting. Hystotherial L0 C84730       38.24         NML 01013711       KLK6       keratin 19       0       33.95       32.13         NML 01005619       Fermature ovarian finiter. High       ga g	NM_001047980		20.3	7	
NAT_00225         TMC.5         TMC.5         Transmer/AC humac-like 5         Transmer/AC humac-like 5         Table 5         4:5         4:5         40.0           NM_003250         LIPH         impart, member H         errasine, hate 6.5         40.0	NM 002639	CKMT1AICKMT1B	creatine kinase, mitochondrial 1A   creatine kinase mitochondrial 1B	46.84	
NM_032520       LIPH       Upset, microber PI       incertaine kinese, microber PI       inceraine kinese, microber PI       incer	NM 003225	TMC5	transmembrane channel-like 5	44.56	30.0
NM_1272c2       CKM71/ACKMT1B       creatine kinase, micchoneige, 301 cre_465 Shase, micchoner 11B       40.69         NM_000025433       ITGIA       integrin, height, and the social of the state integrin, base integrindinter, integrin, and intered integrin, and i	NM_033520	LIPH	lipase, member H	41.85	
NM_000033       ITGB6       integrin, beta 6       50.0       54.2       31.3       40.27         NM_001025434       AFAPILOC84740       ESRP1       actin filament associated metein       Ithoutestat       33.3       33.4       33.9       33.9       30.0       33.9       30.0       33.9       30.0       33.9       30.0       33.9       30.0       33.9       30.0       33.9       30.0       33.9       30.0	NM_182762	CKMT1AICKMT1B	creatine kinase, mitochond <b>56.3</b> A   creatine & mitochondrial 1B	40.69	
NM_00125433       50.0       54.2       31.3       30.0         NM_00125434       actin filament associated meters (hypothetisal LOC8474)       38.24       33.3       33.41       30.0         NM_00125431       skilixrein-related peptida 33.43       38.0       23.7       31.3       33.41       30.0         NM_012153       KIK6       kaltixrein-related peptida 33.43       38.0       23.7       31.3       35.11       30.0         NM_012153       KR19       keratin 19       0       33.95       32.9       39.9	NM_0009031	ITGB6	integrin, beta 6	40.27	
NM_00125134       AFAP1ILOC84740       actin filament associated poptial.       1000       38.24       36.31         NM_0211221NN_001166103       RAB25       retin filament associated poptial.       38.0       23.7       31.3       35.46       30.0         NM_021213       RAB25       RAB25       RAB25       RAB25       38.0       23.7       31.3       35.46       30.0         NM_02132       KRT19       keratin 19       0       33.95       32.3       33.95       3	NM_001025433I		50.0 31.3		20.0
NM_33714 NM_005727       AFAP[ILOCS4740 ESRP]       actin filament associated motion [hopothetical LOC84740 cpithelial sQ500 regulatory protein ]       38.24 35.46         NM_012153 NM_1300531       KLK6       kalikrein-related poptida 34.3       38.0       23.7       31.3       35.46         NM_021213 NM_000105731       KRT19       keratin 19       0       33.95       39.0       33.93	NM_001025434l				50.0
NM_007272       AFAPILOC84740       Sati filtiment associated moteing 1 hypothetical 1.0C84740       Sati 5.41         NM_0121021NM_00166103       RAB25       kallikrein related peptidas 3.4.3       Sat. 3.5.46       Sat. 3.5.46         NM_01230531       KLK6       kallikrein related peptidas 3.4.3       Sat. 3.5.46       Sat. 3.5.46         NM_01030531       KKT19       keratin 19       0       Sat. 3.5.46       Sat. 3.5.46         NM_001037311       KKT19       keratin 19       0       Sat. 3.5.46       Sat. 3.5.46         NM_001037311       KKT9       remature ovarian failure. 105       Sat. 3.5.46       Sat. 3.5.46       Sat. 3.5.46         NM_001037311       KKT9       remature ovarian failure. 105       Sat. 3.5.46       Sat. 3.5.46 <td>NM_138714</td> <td></td> <td></td> <td></td> <td></td>	NM_138714				
NM_021123       ESRP1       cpthclais fg25q2 regulatery protein       36.0       23.7       31.3       35.40         NM_130031       KLK6       kalifikrein-related peptida       34.3       35.40       35.41       35.40         NM_012133       KR19       keratin 19       0       33.95       35.90       33.95       35.90       33.95       35.90       33.95       35.90       33.95       35.90       33.95       35.90       33.95       35.90       33.95       35.90       33.95       35.90       33.95       35.90       33.95       35.90       33.95       35.90       33.95       35.90       33.95       35.90 </td <td>NM_005727</td> <td>AFAP1ILOC84740</td> <td>actin filament associated protein 1   hypothetical LOC84740</td> <td>38.24</td> <td></td>	NM_005727	AFAP1ILOC84740	actin filament associated protein 1   hypothetical LOC84740	38.24	
NML 1303       KLK6       kalikrein-related peptina       33.43       33.00       35.11       30.00         NML 13033       KLK6       kalikrein-related peptina       34.3       35.11       30.00         NML 232526       KRT19       kernatin 19       0       33.95       9         NML 001057311       NML 001057311       The period p	NM_021102l/NM_001166103	ESRPI	epithelial splig regulatory protein 1	36.31	
NL 1331811       XLK0       Kulliklein (ealed pejulialeeven       23.7       Eale       3.11       Automatical (Constraints)         NM 202326       KRT19       keratin 19       0       33.95       90         NM 0010057311       NM 0010057311       KEF5       Kruppel-like factor 5 (intesting)       10 <t< td=""><td>NM_012153</td><td>KAB25</td><td>Itallitatia related partido 31.3</td><td>35.40</td><td>30.0</td></t<>	NM_012153	KAB25	Itallitatia related partido 31.3	35.40	30.0
Null 2013/201       KRT 19       keratin 19       33.95       39       39       39       39       39       39       30       30       50       30       50       30       50       30       50       30       50       30       50       30       50       30       50       30       50       30       50       30       50       30       50       30       50       30       50       30       50       30       50       30       50       30       50       30       50       30	NM 133181	KLK0	kallikrein-related peptidase of 23.7	55.11	50.0
Number 2013         KRT19         keratin 19         June 10	NM 024526				
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	NM_0002131	KRT19	keratin 19 0	33.95	
CM_001005610         End of the second s	NM_001005731	inter 19		55.55	ne
$\begin{tabular}{ c c c c c c c c c c c c c c c c c c c$	NM 001005619		issi nei nei		No
NM_0010339471       N.T. I.	NM_019894I	KLF5	Kruppel-like factor 5 (intesting)	33.93	
NM_001173551       FFIB       premature ovarian failure, IU       premature ovarian failure, IU <td>NM_001083947I</td> <td></td> <td>R re tre</td> <td></td> <td></td>	NM_001083947I		R re tre		
NM_01232366       POF1B       premature ovarian failure. 115       50       31.17         NM_012421       SGPP2       splingosine-1-phosphate phosphotose 2       29.43         NM_001730       TMPRSS4       transmembrane protease, series 4       50       50       28.06         NM_002764       EPSL3       EPSL3ke 3       27.06       28.05         NM_001012965       EVSL3       EPSL3ke 3       27.06       27.06         NM_001012965       EHF       ets homologous factor       50       50       27.06         NM_00102965       EHF       ets homologous factor       50       26.42       27.06         NM_00112965       EHF       ets homologous factor       50       26.43       27.06         NM_0010349151       EHF       serine peptidase inhibitor, Kunitz type, 2       26.37       26.43         NM_1085951       TSPAN1       tetraspanin 1       25.99       25.99         NM_001050011/NM_020990       MACC1       metastasis associated in colon cancer 1       23.74         NM_00105248       TFF1       trefoid factor 1       23.12         NM_00105249       Serpin peptidase inhibitor, clade B (ovalburnin), member 5       23.1         NM_00105249       Serpin peptidase inhibitor, facel B (avalburnin), member	NM_001173551		vith out		
NM_020210       SGRP2       sphingosine-1-phosphate phosphotase 2       op	NM_152386	POF1B	premature ovarian failure, 1 🖉 🗧 🖉	31.17	
NM_001730       TMPRSS4       transmembrane protease, sering 4       Original Constraints       28.05         NM_0027761       FIGB4       integrin, beta 4       Original Constraints       27.06         NM_0010129051       EPS8-like 3       Original Constraints       26.42         NM_01020565       EHF       ets homologous factor       Original Constraints       26.42         NM_01020565       EHF       ets homologous factor       Original Constraints       26.42         NM_001128266       FER       Serine peptidase inhibitor, Kunitz type, 2       26.37         NM_00182826       TSPAN1       tetraspanin 1       25.99         NM_00101346471       Activated T-cells 5, tonicity-responsive       24.38         NM_001015001/VM_020990       MACC1       metastasi associated in colon cancer 1       23.74         NM_001105011/VM_020990       MACC1       metastasi associated in colon cancer 1       23.12         NM_001105011/VM_020990       MACC1       metastasi associated in colon cancer 1       23.12         NM_001015011/VM_020990       SERPINB5       serpin peptidase inhibitor, clade B (ovalburnin), member 5       23.1         NM_00101501/VM_020990       SERPINB5       serpin peptidase inhibitor, clade B (ovalburnin), member 5       23.1         NM_00101501/VM_020990       SERP	NM_024921	SGPP2	sphingosine-1-phosphate phosphotase 2 🖉	29.43	
NM_002276         ITGB4         integrin. beta 4         Operation         Top         Description         Description <thdescription< th="">         Description         <thdescript< td=""><td>NM_001730</td><td>TMPRSS4</td><td>transmembrane protease, serie 4</td><td>28.06</td><td></td></thdescript<></thdescription<>	NM_001730	TMPRSS4	transmembrane protease, serie 4	28.06	
NM_001012964l27.06NM_001012965lImage: Second State	NM_002276	TTGB4	integrin, beta 4 O E A	28.05	
NM_001012964i         Second Status         Second S	NM_002/74I	EPS8L3		27.06	
NM_020387         EHF         ets homologous factor         Z         26.42           NM_01013903         SPINT2         serine peptidase inhibitor, Kunitz type, 2         26.37           NM_00112826         NM_00112826             NM_0011346471         SPINT2         Serine peptidase inhibitor, Kunitz type, 2         25.99           NM_0011346471         NR_026892           24.38           NM_0010150011/NM_020990         MACC1         metastasis associated in colon cancer 1         23.74           NM_0010152481         TFF1         trefoil factor 1         23.12           NM_001015249         SERPINB5         serpin peptidase inhibitor, clade B (ovalbumin), member 5         23.1           NM_001015249         SLC6A14         solute carrier family, member 7         22.83           NM_00103043         SLC6A14         solute carrier family 6 (amino acid transporter), member 14         21.49           NM_001018017         NM_001018017         21.3         NM_001018017           NM_001018017         SPINK1         serine peptidase inhibitor, Kazal type 1         20.59           NM_00108017         NM_00108017         SLC6A14         solute carrier family 6 (amino acid transporter), member 14         21.49           NM_001080171         NM_0001044390<	NM_001012965		ew c		
Inf_DotSid         Inf	NM 020387	FHF	ets homologous factor $\Psi$	26.42	
NL_0010349151Entric Construction product matched rules (product matched rules) (product matched rules	NM 017697	SPINT2	serine peptidase inhibitor Kunitz type 2	26.37	
NM_001122826         TSPAN1         tetraspanin 1         25.99           NM_0011346471         NR_026892         TSPAN1         tetraspanin 1         25.99           NM_000888         NQO1INFAT5         NAD(P)H dehydrogenase, quinone 1   nuclear factor of activated T-cells 5, tonicity-responsive         24.38           NM_0010150011/NM_020990         MACC1         metastasis associated in colon cancer 1         23.74           NM_010152481         TFF1         trefoil factor 1         23.12           NM_001105248         TFF1         trefoil factor 1         23.12           NM_00105249         Serpin peptidase inhibitor, clade B (ovalbumin), member 5         23.1           NM_00105011/NM_020990         SERPINB5         serpin peptidase inhibitor, clade B (ovalbumin), member 5         23.1           NM_00105249         regenerating islet-derived family, member 7         22.83           NM_002354         REG4INBPF7         regenerating islet derived family, member 14 neuroblastoma breakpoint family, member 7         22.83           NM_00108016         NM_00108016         11.49         21.49           NM_00108016         PDZK1 Interacting protein 1         21.3           NM_00108016         NM_00144390         21.3           NM_00178043         VSIG1         V-set and immunoglobulin domain containing 1         21.2	NM 001034915	011112	Serine peptidase initiation, riantiz (jpe, 2	20107	
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NM_001018016l       NM_001018017l         NM_001044390       NM_002483       VSIG1       V-set and immunoglobulin domain containing 1       21         NM_003979       LAMC2       laminin, gamma 2       20.59         NM_025257l       SPINK1       serine peptidase inhibitor, Kazal type 1       20.57         NM_001178044l       VSIG1       UDP-N-acetyl-alpha-D-galactosamine:polypeptide       20.57         NM_021910l/NM_001136007       GALNT5       UDP-N-acetyl-alpha-D-galactosamine:polypeptide       20.56         NM_006408       KRT17       keratin 17       20.14	NM_002456l	PDZK1IP1	PDZK1 interacting protein 1	21.3	
NM_001018017!         NM_001044390         NM_002483       VSIG1       V-set and immunoglobulin domain containing 1       21         NM_003979       LAMC2       laminin, gamma 2       20.59         NM_025257!       SPINK1       serine peptidase inhibitor, Kazal type 1       20.57         NM_001178044!       VSIG1       UDP-N-acetyl-alpha-D-galactosamine:polypeptide       20.57         NM_021910!/NM_001136007       GALNT5       UDP-N-acetyl-alpha-D-galactosamine:polypeptide       20.56         NM_006408       KRT17       keratin 17       20.14	NM_001018016				
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NM_002483VSIG1V-set and immunoglobulin domain containing 121NM_003979LAMC2laminin, gamma 220.59NM_0252571SPINK1serine peptidase inhibitor, Kazal type 120.57NM_0011780441V20.57NM_001178045UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 5 (GalNAc-T5)20.56NM_006408KRT17keratin 1720.14	NM_001044390	10004			
NM_003979     LAMC2     Iaminin, gamma 2     20.59       NM_0252571     SPINK1     serine peptidase inhibitor, Kazal type 1     20.57       NM_0011780441     NM_001178045     UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 5 (GalNAc-T5)     20.56       NM_006408     KRT17     keratin 17     20.14	NM_002483	VSIG1	V-set and immunoglobulin domain containing 1	21	
NM_0232371     SPINK1     serine peptidase innibitor, Kazal type 1     20.57       NM_001178044l     NM_001178045     UDP-N-acetyl-alpha-D-galactosamine:polypeptide     20.56       NM_006408     KRT17     keratin 17     20.14	INIM_003979	LAMC2	iaminin, gamma 2	20.59	
NM_001178045         NM_021910I/NM_001136007       GALNT5         UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 5 (GalNAc-T5)       20.56         NM_006408       KRT17       keratin 17       20.14	NIVI_0232371 NM_0011780441	STINKI	senne pepudase minonor, Kazai type 1	20.37	
NM_001136007     GALNT5     UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 5 (GalNAc-T5)     20.56       NM_006408     KRT17     keratin 17     20.14	NM 001178045				
N-acetylgalactosaminyltransferase 5 (GalNAc-T5)20.56NM_006408KRT17keratin 1720.14	NM 021910I/NM 001136007	GALNT5	UDP-N-acetyl-alpha-D-galactosamine:polypentide		
NM_006408 KRT17 keratin 17 20.14			N-acetylgalactosaminyltransferase 5 (GalNAc-T5)	20.56	
	NM_006408	KRT17	keratin 17	20.14	

12.8

51.1

33.1

Chemotherapy



**Figure 3. MT-heavy Metal Pathway.** Differentially expressed genes (one up-regulated gene, *FLNC*, and 8 down-regulated genes, *MT1A*|*MT2A*, *MT1X*, *MT1H*|MT1P2, *MT1E*|MT1M|MT1JP, *MT11P*|*MT1X*, *MT1H*|*MT1F*, *MT1G* and *HNF4A*) are highlighted in blue circle (bold circle indicating a significant role in the pathway)

namely,  $\alpha 6$ - $\beta 4$ -integrin (21/50 genes, 42%), and rogen receptors (28/94 genes, 30%), epidermal growth factor receptor 1 (EGFR1) (48 /177 genes, 27%), interleukin 3 (IL3) (21/71 genes, 29%), metallothionine (MT) heavy metal pathway (9/14 genes, 64%), transforming growth factor, beta receptor (TGFBR) (40/136 genes, 29%), TNF-alpha/NF-kB (54/191 genes, 28%), and Wnt signaling pathway (30/104 genes, 29%). Among these pathways, genes in the MT heavy metal pathway (one up-regulated gene FLNC (filamin C, gamma), and 8 down-regulated genes, MT1A (metallothionein 1A), MT1E (metallothionein 1E), MT1F (metallothionein 1F), MT1G (metallothionein 1G), MT1H (metallothionein 1H), MT11P (metallothionein 1I (pseudogene)), MT1X (metallothionein 1X), and HNF4A (hepatocyte nuclear factor 4, alpha), exhibit significant changes in expression in cholangiocarcinoma patients (Figure 3).

# Discussion

Global gene expression profiling of billiary tract cancer has been applied in 7 cases of gallbladder carcinoma, 2 ICC, 2 distal bile duct carcinoma and 9 biliary cancer cell lines, revealing 282 up-regulated and 513 downregulated genes (Hansel et al., 2003). Gene expression profile of 25 Japanese ICC patients (10 mass-forming, 2 periductal infiltrating, 11 mixed subtypes (mass-forming and periductal infiltrating) and 2 unknown subtype), using an in-house cDNA microarray demonstrated 52 up-regulated and 421 down-regulated genes (Obama et al., 2005). A comparison of global gene expression profiles of 20 Opisthochis viverrini-associated Thai ICC patients [10 mass-forming type and 10 mixed subtype (mass-forming and intraductal growth type)], with 20 Japanese ICC patients [8 mass-forming, 2 intraductal growth type and 10 mixed subtypes (mass-forming

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and intraductal growth type)], using an in-house cDNA microarray, indicated 77 up-regulated genes and 325 down-regulated genes in common (Jinawath et al., 2006). The current study of 15 Thai ICC patients (8 periductal infiltrating and 7 intraductal growing tissues) using Affymetrix GeneChip®Human Exon 1.0ST identified 2,821 up-regulated and 1,361 down-regulated genes at the same fold change cut off ( $\geq 2$ ). Using unsupervised hierarchical clustering analysis, with fold change cut-off >20, 42 genes were up-regulated in tumor tissues and 204 down-regulated with only 3 up-regulated genes (BUB1B, HOXB7 and TOP2A) in agreement with the previous report (Hansel et al., 2003; Obama et al., 2005; Jinawath et al., 2006). BUB1B [budding uninhibited by benzimidazones 1 homolog beta (yeast)] encodes a kinase involved in spindle checkpoint function and plays a role in the inhibition of anaphase-promoting complex/cyclosome, delaying the onset of anaphase and ensuring proper chromosome segregation (Davenport et al., 1999), over expression of BUB1B is significantly correlation with a less advanced pathologic stage in oral squamous cell carcinoma (Rizzardi et al., 2011). HOXB7 (homeobox protein Hox-B7) is a member of the Antp homeobox family, which functions as a sequence-specific transcription factor involved in cell proliferation and differentiation (McAlpine and Shows, 1990), over expression of HOXB7 is significantly correlate with advance stage and poor prognosis of colorectal cancer (Liao et al., 2011) and oral squamous cell carcinoma (Bitu et al., 2012), TOP2A (DNA topoisomerase II-alpha) encodes an enzyme that controls and alters the topologic state of DNA during transcription (Watt and Hickson, 1994), over expression of TOP2A is associated with better overall survival and disease-free survival in early breast cancer treated with anthracyclines (Arriola et al., 2007). The association of these genes expression with the clinical features of cholangiocarcinoma are worthy further studied due to these information have not been reported elsewhere.

Several studies have provided evidence for an association of expressed gene member in the MT heavy metal pathway with human breast, colon, kidney, liver, lung, nasopharyngeal, ovarian, prostate, salivary gland, testicular, thyroid and urinary bladder cancers (Schmidt et al., 1985; West et al., 1990; Stennard et al., 1994; Cherian et al., 2003). Expression of MT isoform genes depends on differentiation status and proliferative index of the tumor (Cherian et al., 2003). In human liver tissues, MTs are expressed at high levels, whereas there is no MT expression in hepatocellular carcinomas. Down-regulation of MT genes can be caused by hypermethylation of MT promoter and by mutations in other genes, such as p53 tumor suppressor gene (Jacob et al., 2002). In this study, the Exon 1.0 ST microarray set was designed to capture the 14 major genes in MT pathway, and among these 14 genes, 9 were identified having significant changes in expression in ICC samples, namely, one up-regulated gene (FLNC) and 7 down-regulated genes of MT1 isoforms (MT1/ MT2A, MT1X, MT1H/MT1P2, MT1E/MT1M/MT1JP, MT11P/MT1X, MT1H/MT1F, MT1G and HNF4A).

*FLNC* is a member of the filamin family, which organizes actin polymerization in response to various signals (Stossel et al., 2001), and a defect in a member

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of the filamin family is most commonly linked with neuromuscular disorders (Dalkilic et al., 2006). This is the first report, as far as we know, of an increase in *FLNC* expression in cancer.

The expression patterns of MT isoforms have been reported in various human tumors, such as the increase in mRNA levels of MT1A, MT1E, MT1F, MT1G, MT1H, and MT1X (but not MT1B), in breast cancer tissues (Bay et al., 2001). MT2A mRNA transcript has been reported to positively correlate with cell proliferation and histological grading of breast cancer (Jin et al., 2002). In urological malignancies, up-regulation of MT2A and down-regulation of MT1A and MT1G mRNA levels have been detected in renal cancer tissues (Nguyen et al., 2000). HNF4A encodes a transcription factor regulating the expression of several hepatic genes. Increase of HNF4a mRNA was observed in ampullary cancer and HNF4A protein expression was an independent predictor of good prognosis in carcinoma of the papilla of Vater (Ehehalt et al., 2011). ICC can now be added to the list of cancers affected by aberrant expression of HNF4A.

In summary, using Affymetrix GeneChip®Human Exon 1.0ST microarray system we have produced a gene expression profile of liver fluke-associated ICC in comparison with matched normal tissues. The changes in gene expression levels demonstrated by microarray analysis were confirmed using qRT-PCR of 10 randomly selected genes. Data from this study have provided a data set of candidate genes involved in ICC, which should lead to a better understanding of the molecular mechanisms underlying ICC and may serve as the beginning of the establishment a database for the discovery of novel diagnostic markers and perhaps even novel drug targets in ICC.

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# References

- Arriola E, Rodriguez-Pinilla SM, Lambros MB, et al (2007). Topoisomerase II alpha amplification may predict benefit from adjuvant anthracyclines in HER2 positive early breast cancer. *Breast Cancer Res Treat*, **106**, 181-9.
- Bay BH, Jin R, Jayasurya A (2001). Analysis of metallothionein expression in human cancers. Acta Histochem Cytochem, 34, 171-6.
- Bitu CC, Carrera M, Lopes MA, (2012). HOXB7 expression is a prognostic factor for oral squamous cell carcinoma. *Histopathology*, **60**, 662-5.
- Chee M, Yang R, Hubbell E, et al (1996). Accessing genetic information with high-density DNA arrays. *Science*, **274**, 610-4.

- Cherian MG, Jayasurya A, Bay BH (2003). Metallothioneins in human tumors and potential roles in carcinogenesis. *Mutat Res*, **533**, 201-9.
- Dalkilic I, Schienda J, Thompson TG, Kunkel LM (2006). Loss of FilaminC (*FLNC*) results in severe defects in myogenesis and myotube structure. *Mol Cell Biol*, **26**, 6522-34.
- Davenport JW, Fernandes ER, Harris LD, Neale GA, Goorha R (1999). The mouse mitotic checkpoint gene *BUB1B*, a novel bub1 family member, is expressed in a cell cycle-dependent manner. *Genomics*, **55**, 113-7.
- Ehehalt F, Rummele P, Kersting S, et al (2011). Hepatocyte nuclear factor (HNF) 4alpha expression distinguishes ampullary cancer subtypes and prognosis after resection. *Ann Surg*, **254**, 302-10.
- Golub TR, Slonim DK, Tamayo P, et al (1999). Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science*, **286**, 531-7.
- Hamilton SR, Aaltonen LA, International Agency for Research on Cancer., World Health Organization. 2000. Pathology and genetics of tumours of the digestive system. Lyon: IARC Press. 314 p. pp.
- Hansel DE, Rahman A, Hidalgo M, et al (2003). Identification of novel cellular targets in biliary tract cancers using global gene expression technology. *Am J Pathol*, **163**, 217-29.
- Haswell-Elkins MR, Satarug S, Tsuda M, et al (1994). Liver fluke infection and cholangiocarcinoma: model of endogenous nitric oxide and extragastric nitrosation in human carcinogenesis. *Mutat Res*, **305**, 241-52.
- Jacob ST, Majumder S, Ghoshal K (2002). Suppression of metallothionein-I/II expression and its probable molecular mechanisms. *Environ Health Perspect*, **110**, 827-30.
- Jin R, Chow VT, Tan PH, et al (2002). Metallothionein 2A expression is associated with cell proliferation in breast cancer. *Carcinogenesis*, **23**, 81-6.
- Jinawath N, Chamgramol Y, Furukawa Y, et al (2006). Comparison of gene expression profiles between *Opisthorchis* viverrini and non-*Opisthorchis viverrini* associated human intrahepatic cholangiocarcinoma. *Hepatology*, 44, 1025-38.
- Kato I, Kuroishi T, Tominaga S (1990). Descriptive epidemiology of subsites of cancers of the liver, biliary tract and pancreas in Japan. Jpn J Clin Oncol, 20, 232-7.
- Khan SA, Thomas HC, Davidson BR, Taylor-Robinson SD (2005). Cholangiocarcinoma. Lancet, 366, 1303-14.
- Kim IJ, Kang HC, Park JG (2004). Microarray applications in cancer research. *Cancer Res Treat*, **36**, 207-13.
- Kurathong S, Lerdverasirikul P, Wongpaitoon V, et al (1985). Opisthorchis viverrini infection and cholangiocarcinoma. A prospective, case-controlled study. Gastroenterology, 89, 151-6
- Liao WT, Jiang D, Yuan J, et al (2011). HOXB7 as a prognostic factor and mediator of colorectal cancer progression. Clin Cancer Res, 17, 3569-78.
- Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, **25**, 402-8.
- McAlpine PJ, Shows TB (1990). Nomenclature for human homeobox genes. *Genomics*, **7**, 460.
- McLean L, Patel T (2006). Racial and ethnic variations in the epidemiology of intrahepatic cholangiocarcinoma in the United States. *Liver Int*, 26, 1047-53.
- Mosconi S, Beretta GD, Labianca R, et al (2009). Cholangiocarcinoma. Crit Rev Oncol Hematol, 69, 259-70.
- Nguyen A, Jing Z, Mahoney PS, et al (2000). *In vivo* gene expression profile analysis of metallothionein in renal cell carcinoma. *Cancer Lett*, **160**, 133-40.
- Obama K, Ura K, Li M, et al (2005). Genome-wide analysis of gene expression in human intrahepatic cholangiocarcinoma.

Hepatology, 41, 1339-48.

- Parkin DM, Srivatanakul P, Khlat M, et al (1991). Liver cancer in Thailand. I. A case-control study of cholangiocarcinoma. *Int J Cancer*, **48**, 323-8.
- Rizzardi C, Torelli L, Barresi E, et al (2011). BUBR1 expression in oral squamous cell carcinoma and its relationship to tumor stage and survival. *Head Neck*, **33**, 727-33.
- Satarug S, Haswell-Elkins MR, Tsuda M, et al (1996). Thiocyanate-independent nitrosation in humans with carcinogenic parasite infection. *Carcinogenesis*, **17**, 1075-81.
- Schmidt CJ, Jubier MF, Hamer DH (1985). Structure and expression of two human metallothionein-I isoform genes and a related pseudogene. *J Biol Chem*, **260**, 7731-7.
- Sriamporn S, Pisani P, Pipitgool V, et al (2004). Prevalence of *Opisthorchis viverrini* infection and incidence of cholangiocarcinoma in Khon Kaen, Northeast Thailand. *Trop Med Int Health*, 9, 588-94.
- Sripa B, Pairojkul C (2008). Cholangiocarcinoma: lessons from Thailand. Curr Opin Gastroenterol, 24, 349-56.
- Stennard FA, Holloway AF, Hamilton J, West AK (1994). Characterisation of six additional human metallothionein genes. *Biochim Biophys Acta*, **1218**, 357-65.
- Stossel TP, Condeelis J, Cooley L, et al (2001). Filamins as integrators of cell mechanics and signalling. *Nat Rev Mol Cell Biol*, 2, 138-45.
- Taylor-Robinson SD, Foster GR, Arora S, Hargreaves S, Thomas HC (1997). Increase in primary liver cancer in the UK, 1979-94. Lancet, 350, 1142-3.
- Thamavit W, Pairojkul C, Tiwawech D, et al (1993). Promotion of cholangiocarcinogenesis in the hamster liver by bile duct ligation after dimethylnitrosamine initiation. *Carcinogenesis*, 14, 2415-7.
- Thamavit W, Pairojkul C, Tiwawech D, Shirai T, Ito N (1994). Strong promoting effect of *Opisthorchis viverrini* infection on dimethylnitrosamine-initiated hamster liver. *Cancer Lett*, 78, 121-5.
- Ustundag Y, Bayraktar Y (2008). Cholangiocarcinoma: a compact review of the literature. *World J Gastroenterol*, **14**, 6458-66.
- Vatanasapt V, Uttaravichien T, Mairiang EO, et al (1990). Cholangiocarcinoma in north-east Thailand. *Lancet*, 335, 116-7.
- Watt PM, Hickson ID (1994). Structure and function of type II DNA topoisomerases. *Biochem J*, **303**, 681-95.
- West AK, Stallings R, Hildebrand CE, et al (1990). Human metallothionein genes: structure of the functional locus at 16q13. *Genomics*, **8**, 513-8.
- West J, Wood H, Logan RF, Quinn M, Aithal GP (2006). Trends in the incidence of primary liver and biliary tract cancers in England and Wales 1971-2001. *Br J Cancer*, **94**, 1751-8.