RESEARCH ARTICLE

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 ≥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 person-years (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® 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™ 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® VILO™ 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 μM), 0.8 μl of $MgCl_2$ (25 mM), and 5.2 μl of water PCR grade, respectively. The 9 μl of PCR mix were pipetted into each pre-cooling Lightcycler capillary. The 1 μl 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®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- tiation	Metastasis	Survival Period (Mo.)
2[a]	37/M	Right lobe	5x3.5x4	Pap	Yes	15.8
3[a]	43/F	Left lobe	3x1x1.5	Tubular pap	Yes	17.0
4[a]	58/M	Left lobe	6x7x5	Moderate	Yes	6.6
5[a]	44/M	Right lobe	11x10x9	Mixed tubular+ mucinous	Yes	1.6
6[a]	65/M	Left lobe	5x5x5	Moderate	Yes	5.6
7[a]	73/M	Left lobe	4x5x1	Moderate	Yes	6.4
13[a]	65/M	Left lobe	6x4x3	Well	Yes	5.1
14[a]	62/F	Left lobe	4x3x1	Well	No	4.1
1[b]	70/M	Right lobe	4x2.5x3	Pap	No	3.4
8[b]	66/F	Right lobe	6x5x6	Pap	No	4.9
9[b]	67/F	Left lobe	6x4x5	Pap	Yes	9.5
10[b]	43/M	Right lobe	16x6x5	Pap+mucinous	Yes	27.7
11[b]	54/M	Left lobe	6x2x1	Pap	No	21.6
12[b]	76/M	Right lobe	3x3x1	Pap	No	3.0
15[b]	71/M	Right lobe	4.5x4x4	Pap	No	24.3

*pap – papillary; well - well-differentiated; moderate - moderately differentiated. M – male; F – female; [a]PI - periductal infiltrating type; [b]IG - intraductal growing type

Table 2. Primer Sequence used in qRT-PCR

Ref. Accession Number	Gene Symbol	Sense Primer (5'-->3')	Anti-sense Primer (5'-->3')
NM_021910	*FXYD3*	TTCTGCTGATCCTGAAATTGTA	TTCTTTTCCTTAGATGATGTGTTTT
NM_003979	*GPRC5A*	GCTCACTTGCTAAATAAGAATC*TAT*	ACCCTAACCATTGTCTCAGTA
NM_004363	*CEACAM5*	TACAAGTTTCTGATACCACTG	ATCCTCATTAGTTCATTTAGTC
NM_033049	*MUC13*	TCATCATACAGGTTGAGAATGTT	TCTGAGAGTC*TAT*CACATCAATG
NM_003979	*EPCAM*	TTCCTGTTGGCT*TAT*GTTAGTC	TTCTTCACGAGTTGAGGTTTAC
NM_002354	*TMC5*	AG*TAT*GAGAAGGCTGAGATAA	ATTTGTGTCCATTTGC*TAT*TTC
NM_012153	*EHF*	ACTTCAACCTCAACC*TAT*CTT	TCCTGCTACATTAC*TAT*GCTTA
NM_001122633	*CPS1*	C*TAT*ATCAGCAGATGGTAGACA	AACCTTACTTCCAAGT*TAT*TCC
NM_000353	*TAT*	TGAAAGTACCAGGTGAACAAAG	GGGCACAAATTCTCTCAATCTT
NM_002215	*ITIH1*	GGAGAAC*TAT*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 ≥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/β-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 channel-like 5), and *EHF* (ets homologous factor), and 3 down-regulated 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,

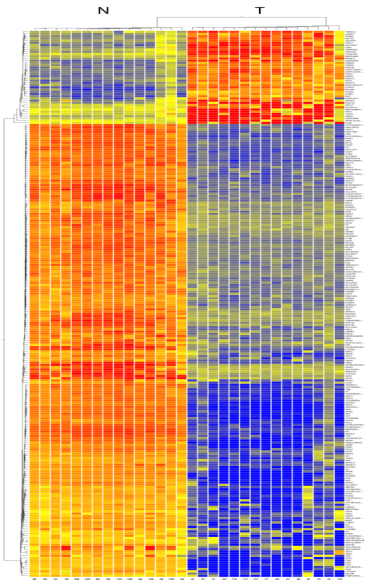

Figure 1. Hierarchical Clustering of ICC-associated Genes. Of the 246 genes indicated by Affymetrix Exon 1.0 ST array as being differentially expressed in ICC in comparison with matched normal tissues, 42 are up-regulated and 204 down-regulated (p<0.05 at magnitude of fold-change >20). Colorgram depicts 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)

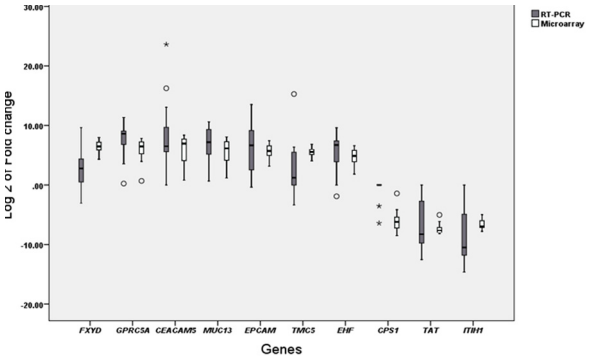

Figure 2. Box and Whisker Plot between Affymetrix Exon 1.0 ST Array (green) and qRT-PCR Data. Data include 7 genes randomly selected from the 2,821 up-regulated and 3 from 1,361 down-regulated genes. Box chart show a distribution of log2-transformed relative gene expression ratios of tumor to corresponding 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

Table 3. Top of 42 Up-regulated Genes in Intrahepatic Cholangiocarcinomas (p value >0.05, fold change >20)

Ref. seq	Gene symbol	Gene Description	Fold change
NM_000422	AGR2	anterior gradient homolog 2 (Xenopus laevis)	95.23
NM_014568	*FXYD3*	*FXYD* domain containing ion transport regulator 3	84.41
NM_003122	SLC44A4	solute carrier family 44, member 4	67.42
NM_005562|/NM_018891	*GPRC5A*	G protein-coupled receptor, family C, group 5, member A	61.94
NM_001170553|/NM_182607	CEACAM6	carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)	60.42
NM_005764	MUC1	mucin 1, cell surface associated	59.02
NM_007231	*CEACAM5*	carcinoembryonic antigen-related cell adhesion molecule 5	54.67
NM_002203	*MUC13*	mucin 13, cell surface associated	49.23
NM_001159352| NM_032044| NM_001159353| NM_001047980	*EPCAM*	epithelial cell adhesion molecule	46.98
NM_002639	CK*MT1A*|CKMT1B	creatine kinase, mitochondrial 1A | creatine kinase, mitochondrial 1B	46.84
NM_003225	*TMC5*	transmembrane channel-like 5	44.56
NM_033520	LIPH	lipase, member H	41.85
NM_182762	CK*MT1A*|CKMT1B	creatine kinase, mitochondrial 1A | creatine kinase, mitochondrial 1B	40.69
NM_000903| NM_001025433| NM_001025434| NM_138714	ITGB6	integrin, beta 6	40.27
NM_005727	AFAP1|LOC84740	actin filament associated protein 1 | hypothetical LOC84740	38.24
NM_021102|/NM_001166103	ESRP1	epithelial splicing regulatory protein 1	36.31
NM_012153	RAB25		35.46
NM_139053| NM_133181| NM_024526	KLK6	kallikrein-related peptidase 6	35.11
NM_000213| NM_001005731| NM_001005619	KRT19	keratin 19	33.95
NM_019894| NM_001083947| NM_001173551	*KLF5*	Kruppel-like factor 5 (intestinal)	33.93
NM_152386	POF1B	premature ovarian failure, 1B	31.17
NM_024921	SGPP2	sphingosine-1-phosphate phosphotase 2	29.43
NM_001730	TMPRSS4	transmembrane protease, serine 4	28.06
NM_002276	ITGB4	integrin, beta 4	28.05
NM_002774| NM_001012964| NM_001012965	EPS8L3	EPS8-like 3	27.06
NM_020387	*EHF*	ets homologous factor	26.42
NM_017697| NM_001034915| NM_001122826	SPINT2	serine peptidase inhibitor, Kunitz type, 2	26.37
NM_198595| NM_001134647| NR_026892	TSPAN1	tetraspanin 1	25.99
NM_000888	NQO1|NFAT5	NAD(P)H dehydrogenase, quinone 1 | nuclear factor of activated T-cells 5, tonicity-responsive	24.38
NM_001015001|/NM_020990	MACC1	metastasis associated in colon cancer 1	23.74
NM_139248	C19orf33	chromosome 19 open reading frame 33	23.64
NM_001105248| NM_024780| NM_001105249	TFF1	trefoil factor 1	23.12
NM_001015001|/NM_020990	SERPINB5	serpin peptidase inhibitor, clade B (ovalbumin), member 5	23.1
NM_002354	REG4|NBPF7	regenerating islet-derived family, member 4 | neuroblastoma breakpoint family, member 7	22.83
NM_033049	ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	22.7
NM_004363	*SLC6A14*	solute carrier family 6 (amino acid transporter), member 14	21.49
NM_002456| NM_001018016| NM_001018017| NM_001044390	PDZK1IP1	PDZK1 interacting protein 1	21.3
NM_002483	VSIG1	V-set and immunoglobulin domain containing 1	21
NM_003979	LAMC2	laminin, gamma 2	20.59
NM_025257| NM_001178044| NM_001178045	SPINK1	serine peptidase inhibitor, Kazal type 1	20.57
NM_021910|/NM_001136007	GALNT5	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 5 (GalNAc-T5)	20.56
NM_006408	KRT17	keratin 17	20.14

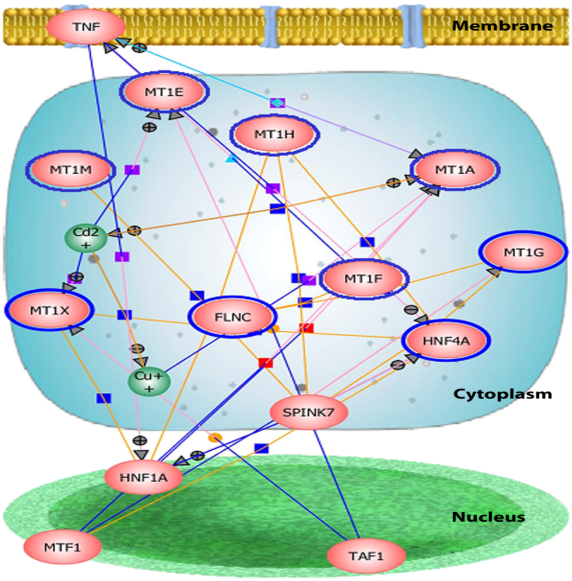

Figure 3. MT-heavy Metal Pathway. Differentially expressed genes (one up-regulated gene, *FLNC*, and 8 down-regulated genes, *MT1A*|*MT2A*, *MT1X*, *MT1H*|MT1P2, *MT1E*|MT1M|MT1JP, *MT1IP*|*MT1X*, *MT1H*|*MT1F*, *MT1G* and *HNF4A*) are highlighted in blue circle (bold circle indicating a significant role in the pathway)

namely, α6-β4-integrin (21/50 genes, 42%), androgen 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), *MT1IP* (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 down-regulated 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 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 (≥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*, *MT1IP*/*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

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 HNF4α 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.