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APPENDICES

APPENDIX A

Reagents for laboratory experiments

1 Reagent for Direct Immunofluorescent staining

1X FACS lysis buffer

BD FACS^{TM} Lysing solution (10X) (BD Biosciences)	10	ml
Sterile DW	90	ml

1X Permeabilizing Solution

BD FACS Permeabilizing Solution (10X) (BD Biosciences)	10	ml
Sterile Deionized water (DI)	90	ml

(Do not dilute in phosphate buffer saline or other buffer)

0.1% Saponin in FACS medium for permeabilization

Saponin	0.1	g
FACS medium	100	ml

10X Phosphate buffer saline

NaCl	40	g
KCl	1	g
Na_2HPO_4	5.75	g
KH_2PO_4	1	g

Adjust pH to 7.4 then adjust final volume to 500 ml by DW. The solution was sterilized by autoclave at 121 °C for 20 min, and store at room temperature.

FACS medium

1XPBS	100	ml
0.1 % Sodium azide (NaN_3)	0.1	g
3% Fetal bovine serum	3	ml

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1% Paraformadehyde

Paraformadehyde powder	1	g
1X PBS	100	ml
0.1 % NaN_3	0.1	g

Heat preparation and stir need for solubility of paraformadehyde powder and also need light protection

PI staining buffer

FACS medium	100	ml
1 μg/ml Propidium iodide (PI)		

2 Reagents for PBMC and monocyte, lymphocyte and neutrophil separations

Red Blood Cell Lysis Buffer

NH_4Cl	8.26	g
$KHCO_3$	1	g
EDTA	0.037	g

Adjust pH to 7.4 then adjust final volume to 1,000 ml by DW. The solution was sterilized by autoclave at 121 °C for 20 min, and store at room temperature.

RPMI-1640 medium with 10% FCS, and 1% antibiotic

	Final conc.	**Volume**	
RPMI-1640 medium with L-Glutamine		900	ml
with 2 gram of sodium bicarbonate ($NaHCO_3$)			
Fetal bovine serum	10%	100	ml
Penicilin-Streptomycin stock solution	1%	10	ml

(All chemical reagents from GIBCO, Invitrogen)

Mix the solution at the sterile area (laminar flow) with sterilized technique, and store at 4-8 °C.

63% Percoll Solution

Percoll™(Density 1.130 g/ml)(GE Healthcare)	63	ml
10X sterile PBS	7	ml
1X sterile PBS	30	ml

72% Percoll Solution

Percoll™(Density 1.130 g/ml)(GE Healthcare)	72	ml
10X sterile PBS	8	ml
1X sterile PBS	20	ml

3 Reagents for RNA extraction by Trizol®reagent (Invitrogen)

70% Ethanol

	Final conc.	**Volume**	
Absolute ethanol	70%	35	ml
DW		15	ml

Mix the solution and store at room temperature.

Diethylpyrocarbonate (DEPC) treated water

	Final conc.	**Volume**	
DEPC (MW 162.141)	0.1%	1	ml
DW		999	ml

Mix well for at least 1 hr, let the solution stand at room temperature for an overnight then subject to autoclave, and stored at room temperature or aliquots store at -20 °C for longer time.

3 Reagents for reverse transcription (RT) reaction (first standed cDNA synthesis)

First strand cDNA synthesis kit for RT-PCR (Roche)

	Final amount	**Volume**
Total RNA	0.25 μg	8.2 μl
10X Reaction Buffer	1X	2 μl
25 mM $MgCl_2$	5 mM	4 μl
dNTP mix	1 mM	2 μl
Random hexamer (0.5 μg/μl)	1 μg	2 μl
RNase inhibitor	50 Units	0.5 μl
AMV reverse transcriptase	≥ 20 Units	0.8 μl
	Total volume	20 μl

Ready To – Go You Prime First strand Bead (GE HealthCare)

	Final amount	**Volume**
Total RNA	0.25 μg	25 μl
Random hexamer (0.5 μg/μl)	0.5 μg	1 μl
DEPC water		7 μl
	Total volume	33 μl

4 Reagents for SYBR green real-time PCR

LightCycler® FastStart DNA Mater SYBR Green I (Roche)

	Volume	
LightCycler® FastStart DNA Mater SYBR Green I	2	µl
25 mM $MgCl_2$	1.6	µl
10 µM Primer set (Forward and Reverse primer)	1	µl
DEPC water	10.4	µl
cDNA	5	µl
Total volume	20	µl

SYBR® Green PCR Mater Mix (Applied Biosystem)

	Volume	
SYBR® Green PCR Mater Mix (2X)	10	µl
10 µM Primer set (Forward and Reverse primer)	1	µl
DEPC water	4	µl
cDNA	5	µl
Total volume	20	µl

8.2 Reagents for immunohistochemistry and double Immunofluorescent staining in paraffin sections.

0.05 M Tris HCl buffer pH = 7.6

Tris (MW 121.14)	6.1	g

Dissolve the component in 800 ml DW and adjust pH to 7.6* by using HCl . Then adjust volume up to 1000 ml. Store at room temperature.

3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate

DAB	1	g
DW	40	ml

Mix and transfer to aliquot in 1.5 ml tube and keep at -20 °C

Dissolve in beaker that cover with foil (light sensitive)

20X Citrate buffer stock pH = 6

Citric acid	420	g
NaOH	210	g
DW	8000	ml

Adjust pH to 6 by using 1 M NaOH. Then adjust volume up to 10 liters

Autoclave and store at room temperature.

APPENDIX B

Supplementary Data

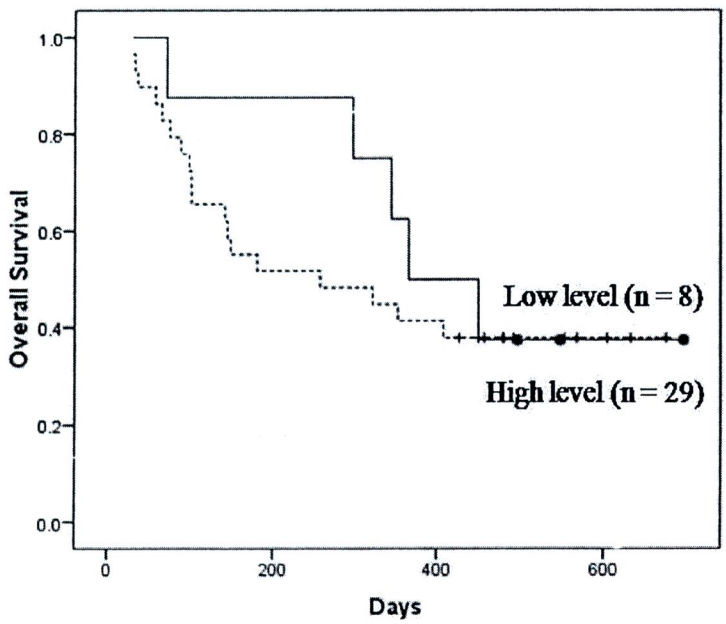

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Figure B1 Kaplan-Meier survival curves of CCA patients with high or low level of $CD14^{+}CD16^{+}$ monocytes. The median survivals of patients with low or high levels of CD14+CD16+ monocytes are 257 days and 365 days respectively. There was no different survival time between both groups of the patients.

Table B1 Characteristics of the CCA and BBD patients in microarray study

No.	Age (yr)	Sex	Diagnosis	Histopathology	WBC counts (cells/ml)
Cholangiocarcinoma (CCA) patients					
1	33	F	Extrahepatic CCA	WD tubular adenocarcinoma	8.3×10^3
2	68	M	Extrahepatic CCA	PD adenocarcinoma	7.22×10^3
3	59	M	Intrahepatic CCA	MD tubular adenocarcinoma	9.55×10^3
4	67	M	Extrahepatic CCA	MD tubular adenocarcinoma	21.8×10^3
5	70	M	Intrahepatic CCA	Papilotubular adenocarcinoma	18.11×10^3
6	52	M	Extrahepatic CCA	WD tubular adenocarcinoma	6.24×10^3
7	51	F	Intrahepatic CCA	WD tubular adenocarcinoma	14.1×10^3
8	67	M	Intrahepatic CCA	Invasive papillary adenocarcinoma	8.4×10^3
9	52	M	Extrahepatic CCA	Invasive papillary adenocarcinoma	16.5×10^3
Benign biliary tract disease (BBD) patients					
1	53	M	BBD	Chronic inflammation at intrahepatic bile duct	16.9×10^3
2	55	M	BBD	Chronic inflammation at intrahepatic bile duct	13.6×10^3
3	47	M	BBD	Cholangitis	16.3×10^3
4	64	M	BBD	Heamangioma	5.9×10^3
5	47	M	BBD	Granulomatous of parasitic infection	9.9×10^3

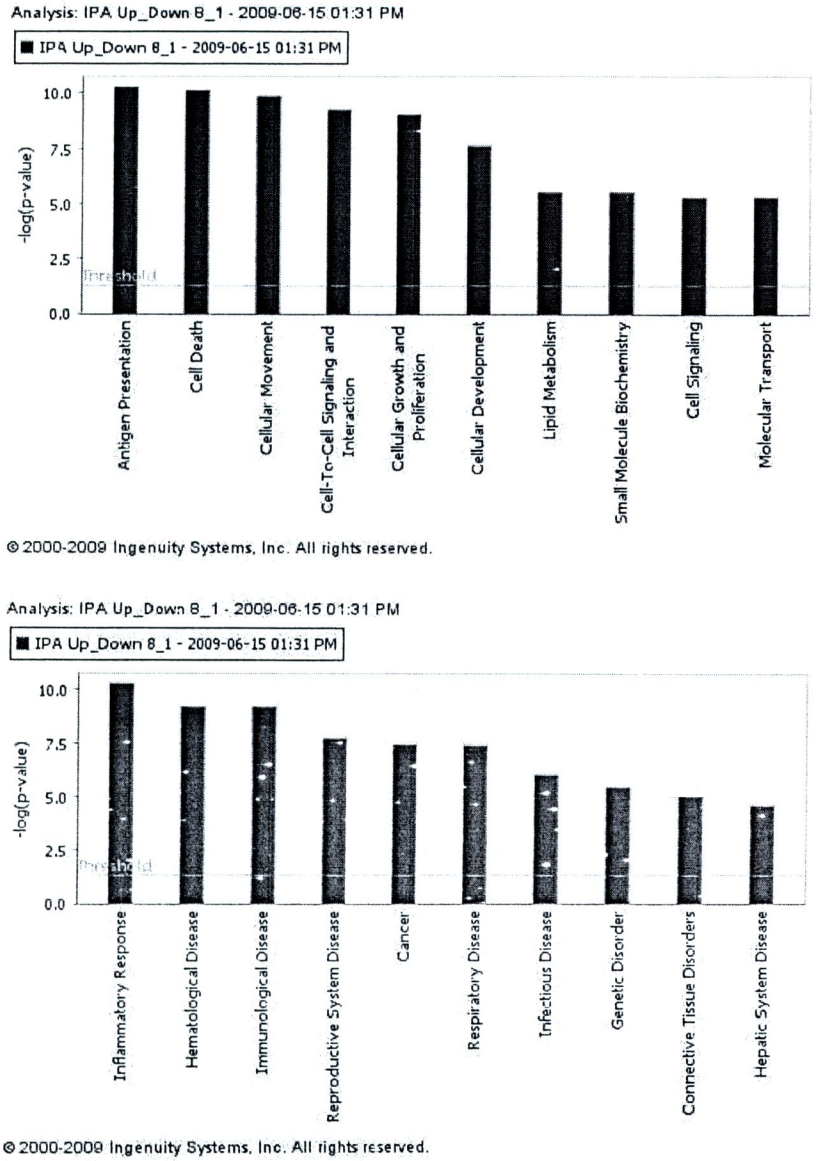


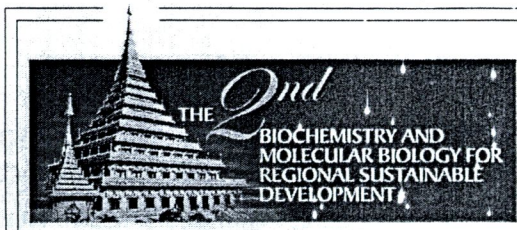
Figure B2 Gene Ontology of differentially expressed genes in CCA peripheral leukocytes was grouped based on molecular and cellular functions (Upper panel), and disease and disorder (Lower panel) from ingenuity pathway analysis (IPA).

Table B2 Unique set of differentially expressed genes in CCA patients.

Probeset ID	Gene Symbol	Gene Title	Fold Change
Up-regulated genes			
224776_at	AGPAT6	1-acylglycerol-3-phosphate O-acyltransferase 6 (lysophosphatidic acid acyltransf	1.71013
202381_at	ADAM9	ADAM metallopeptidase domain 9 (meltrin gamma)	1.96734
207992_s_at	AMPD3	adenosine monophosphate deaminase (isoform E)	2.19578
209122_at	ADFP	adipose differentiation-related protein	2.55761
202888_s_at	ANPEP	alanyl (membrane) aminopeptidase (aminopeptidase N, aminopeptidase M, microsomal	2.26976
225524_at	ANTXR2	anthrax toxin receptor 2	1.72658
203388_at	ARRB2	arrestin, beta 2	1.52865
202874_s_at	ATP6V1C1	ATPase, H+ transporting, lysosomal 42kDa, V1 subunit C1	1.55422
202391_at	BASP1	brain abundant, membrane attached signal protein 1	1.72384
202087_s_at	CTSL	cathepsin L	2.64564
200663_at	CD63	CD63 molecule	1.67048
202878_s_at	CD93	CD93 molecule	1.96563
223454_at	CXCL16	chemokine (C-X-C motif) ligand 16	1.56218
209774_x_at	CXCL2	chemokine (C-X-C motif) ligand 2	2.88502
207850_at	CXCL3	chemokine (C-X-C motif) ligand 3	4.90128
221676_s_at	CORO1C	coronin, actin binding protein, 1C	1.78989
201201_at	CSTB	cystatin B (stefin B)	1.5805
200998_s_at	CKAP4	cytoskeleton-associated protein 4	1.6506
226064_s_at	DGAT2	diacylglycerol O-acyltransferase homolog 2 (mouse)	1.69009
202887_s_at	DDIT4	DNA-damage-inducible transcript 4	1.8846
200664_s_at	DNAJB1	DnaJ (Hsp40) homolog, subfamily B, member 1	1.85094
209037_s_at	EHD1	EH-domain containing 1	2.03626
205767_at	EREG	epiregulin	4.01995
221267_s_at	FAM108A1	family with sequence similarity 108, member A1 /// family with sequence similari	1.68284
213746_s_at	FLNA	filamin A, alpha (actin binding protein 280)	2.06052
218880_at	FOSL2	FOS-like antigen 2	2.57995
1553723_at	GPR97	G protein-coupled receptor 97	1.99421
213524_s_at	G0S2	G0/G1switch 2	1.70691
208308_s_at	GPI	glucose phosphate isomerase	1.69501
205349_at	GNA15	guanine nucleotide binding protein (G protein), alpha 15 (Gq class)	1.70535
243819_at	GNG2	Guanine nucleotide binding protein (G protein), gamma 2	1.75065
205133_s_at	HSPE1	heat shock 10kDa protein 1 (chaperonin 10)	1.7213
200800_s_at	HSPA1A /// HSPA1B	heat shock 70kDa protein 1A /// heat shock 70kDa protein 1B	2.12322
229521_at	FLJ36031	hypothetical protein FLJ36031	1.82943
224826_at	RP5-1022P6.2	hypothetical protein KIAA1434	1.57548
213300_at	KIAA0404	hypothetical protein LOC23130	1.56666
203006_at	INPP5A	inositol polyphosphate-5-phosphatase, 40kDa	1.67145
209185_s_at	IRS2	insulin receptor substrate 2	1.98094

APPENDIX C

Certificate of Poster Presentation Award



Organized by
Biochemistry and Molecular Biology Section of The Science Society of Thailand
Department of Biochemistry, Faculty of Science, Khon Kaen University
Department of Biochemistry, Faculty of Medicine, Khon Kaen University

This is to certify that

Chutima Subimerb

was awarded **HONORABLE** mention
For the Poster Presentation
At The Second Biochemistry and Molecular Biology Conference,
Khon Kaen University, May 7- 8, 2009,
Khon Kaen, Thailand

Assoc. Prof. Dr. Rudee Surarit
Chairperson, Biochemistry and Molecular Biology Section
of The Science Society of Thailand

Assoc. Prof. Dr. Puangrat Yongvanit
Chairperson of the Organizing Committee

Assist. Prof. Dr. Nipa Milintawisamai
Deputy Chairperson of the Organizing Committee

RESEARCH PUBLICATIONS AND PRESENTATIONS

Research publications

- Chutima Subimerb, Viraphong Lulitanond, Narong Khuntikeo, Chanvit Leelayuwat, Seiji Okada, Michael S McGrath, Sopit Wongkham. "Enhanced frequencies of circulating CD16 monocytes in cholangiocarcinoma" ***(Will be submitted to Clinical Exp Immunology)***
- Chutima Subimerb, Somchai Pinlaor, Vatjarabhongsa Bhudisawasdi, Chanvit Leelayuwat, Michael S McGrath, Sopit Wongkham. "Transcription profiles of peripheral blood leukocytes and prognostic of clinical outcome in patients with cholangiocarcinoma" ***(Will be submitted to Cancer Research)***
- Chutima Subimerb, Somchai Pinlaor, Narong Khuntikeo, Alanna Morris, Michael S McGrath, Sopit Wongkham, "Tissue invasion macrophage density correlates with prognosis in cholangiocarcinoma" ***(Submitted to World Journal of Gastroenterology)***

Research presentation

- **Poster presentations**
    1. Chutima Subimerb, Somchai Pinlaor, Chanvit Leelayuwat, Michael S McGrath, Sopit Wongkham "Anti-inflammatory effect on pathogenesis of Opisthorchiasis in hamsters" RGJ-Ph.D. congress VII, Chonburi, Thailand. April 20-22, 2006.
    2. Chutima Subimerb, Viraphong Lulitanond, Chanvit Leelayuwat, Somchai Pinlaor, Sopit Wongkham "Increased level of $CD14^{+}CD16^{+}$ monocyte in peripheral blood related to tumor type of cholangiocarcinoma" The 3 rd APOCP Conference, Bangkok, Thailand. November 3-5, 2006.
    3. Chutima Subimerb, Viraphong Lulitanond, Chanvit Leelayuwat, Somchai Pinlaor, Michael S McGrath, Sopit Wongkham "Increased levels of $CD14^{+}CD16^{+}$ monocytes in blood and infiltrating

macrophages in tissue related to tumor type and prognosis of cholangiocarcinoma" Society of leukocyte biology, Boston, United state of America. October 11-13, 2007.

4. Chutima Subimerb, Viraphong Lulitanond, Chanvit Leelayuwat, Somchai Pinlaor, Kenneth G headlock, Michael S McGrath, Sopit Wongkham "The expression profile of peripheral blood cells in cholangiocarcinoma patients" Princess Congress VI, Bangkok, Thailand. November 25-26, 2007.
5. Chutima Subimerb, Chanvit Leelayuwat, Somchai Pinlaor, Chawalit Pairojkul, Michael S McGrath, Sopit Wongkham "High level of infiltrating macrophages related to poor prognosis of cholangiocarcinoma" The Asia-Africa International Network Symposium of JSPS, Asia and Africa Science Platform Program and, The Fourth LiverCare center Symposium Infection-Immunity and Cancer; " Infection-Immunity and Cancer" , Khon Kaen , Thailand. February 19-20, 2008.
6. Chutima Subimerb, Chanvit Leelayuwat, Somchai Pinlaor, Alanna Morris, Michael S McGrath, Sopit Wongkham "The presence of recent blood derived macrophage migrants in cholangiocarcinoma tissues is associated with elevation of activated blood $CD14^{+}CD16^{+}$ cells and poor survival". 10th World Congress on Gastro Intestinal Cancer, Barcelona, Spain. Presented by Professor Michael S McGrath (MD.Ph.D). June 25-28. 2008
7. Chaisiri Wongkham, Chutima Subimerb, Chanvit Leelayuwat, Somchai Pinlaor, Michael S McGrath, Sopit Wongkham "Cholangiocarcinoma associated blood transcriptome" 20^{th} Meeting of European Society for Cancer Research (EACR), Lyon, France. Presented by Associated Professor Chaisiri WongKham. July 5-8, 2008.
8. Chutima Subimerb, Chaisiri Wongkham, Viraphong Lulitanond, Chanvit Leelayuwat, Somchai Pinlaor, Narong Khuntikeo, Vatcharaphong Bhudhisawasdi, Michael S McGrath, Sopit

Wongkham "Differential expression profile of peripheral blood leukocytes related to invasive activity in cholangiocarcinoma".
American Association for Cancer Research; "100th Annual Meeting 2009", Denver, Colorado, United State of America. April 18-22, 2009.

- **Oral Presentation**

  Chutima Subimerb, Chaisiri Wongkham, Viraphong Lulitanond, Chanvit Leelayuwat, Somchai Pinlaor, Narong Khuntikeo, Vatcharaphong Bhudhisawasdi, Michael S McGrath, Sopit Wongkham "Differential Expression profile of peripheral blood leukocytes related to poor survival of cholangiocarcinoma patients" RGJ-Ph.D. congress X, Chonburi, Thailand. April 3-5, 2009.

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Present	Ph.D. candidate	Medical Biochemistry	Khon Kaen University	Thailand

Scholarships

- **2004-2009 (for 5 years)**

  The Royal Golden Jubilee Ph.D. Program, Thailand

  (Under supervision of Associated Professor Dr. Sopit Wongkham)

- **May 2007- Oct 2007 (for 6 moonths)**

  University of California, San Francisco, USA

  (Training: gene expression profile by cDNA microarray, under supervision of Professor Michael S McGrath MD. Ph.D.)

- **October 2008 - January 2009 (for 4 months)**

  Japan Student Services Organization-JASSO, Japan

  (Training: Flow cytometer analysis, under supervision of Professor Seiji OKADA MD. Ph.D.)