RESEARCH ARTICLE

Plasma Peptidome as a Source of Biomarkers for Diagnosis of Cholangiocarcinoma

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Abstract

Cholangiocarcinoma (CCA) is the bile duct cancer which constitutes one of the important public health problems in Thailand with high mortality rate, especially in the *Opisthorchis viverrini* (a parasite risk factor for CCA) endemic area of the northeastern region of the country. This study aimed to identify potential biomarkers from the plasma peptidome by CCA patients. Peptides were isolated using 10 kDa cut-off filter column and the flow-through was then used as a peptidome for LC-MS/MS analysis. A total of 209 peptides were obtained. Among these, 15 peptides were concerned with signaling pathways and 12 related to metabolic, regulatory, and biosynthesis of secondary metabolite pathways. Five exclusive peptides were identified as potential biomarkers, i.e. ETS domain-containing transcription factor ERF (P50548), KIAA0220 (Q92617), phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit beta isoform isoform 1 (P42338), LP2209 (Q6XYC0), and casein kinase II subunit alpha (P19784). Three of these biomarkers are signaling related molecules. A combination of these biomarkers for CCA diagnosis is proposed.

Keywords: Cholangiocarcinoma - biomarker - peptidome - LC-MS/MS

Asian Pac J Cancer Prev, **17 (3)**, 1163-1168

Introduction

Cholangiocarcinoma (CCA) is the bile duct cancer which constitutes one of the important public health problem in Thailand with high mortality rate, especially in *Opisthorchis viverrini* (the risk factor of CCA) endemic area of the northeastern region of the country (Sripa and Pairojkul, 2008; Pinlaor et al., 2013). Most patients are present to the hospitals or health facilities at the later stage of disease progression due to asymptomatic and unavailability of diagnostic tool for early detection of the tumor. These factors together with limited effective therapeutic treatment options, have led to unsatisfactory control of this type of cancer. Identification of potential biomarkers for early diagnosis of CCA is required (Jia et al., 2014; Xu et al., 2014). Several attempts have been successfully identified the potential peptidome biomarkers for other types of cancer as well as infectious diseases (Terracciano et al., 2011; Xiao et al., 2011; Fan et al., 2012; Yang et al., 2012). The peptidome includes small proteins (10 kDa or smaller), cleaved proteins, and protease digested proteins. Both the pattern and the level of these expressed peptides in disease conditions can be exploited as biomarkers for disease diagnosis (Fukuoka et al., 2013; Maldaner et al., 2013; Kadoglou et al., 2014; Yin et al., 2014). The aim of the study was to investigate the plasma peptidome as potential biomarkers for the diagnosis of CCA using LC-MS/MS.

Materials and Methods

Samples

Plasma samples obtained from (i) 20 CCA patients (intrahepatic cholangiocarcinoma, 13 males and 7 females, aged 36-72 years), (ii) 12 patients with *Opisthorchis viverrini* (OV, 9 males and 3 females, aged 32-71 years) infection, and and 8 healthy subjects (7 males and 1 female, aged 40-63 years) were kindly provided by Dr. Petcharin Srivatanakul, the National Cancer Institute of Thailand. Ethical approval of the study protocol was obtained from the Ethics Committee of the Ministry of Public Health of Thailand. Written informed consent for study participation was obtained from all subjects prior to the study. The diagnosis of CCA was based on clinical and histopathological examination. The levels of liver enzymes (AFP, AST, ALT, and ALP) and CA19-9 in CCA patients were significantly higher than non-CCA subjects in group (ii) and (iii) [median (range): AFP 3.17 (1.47-

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291.7) *vs*. 3.47 (1.59-9.2) *vs*. 2.77 (1.23-4.39) U/ml; AST 68 (18-253) *vs*. 34 (15-79) *vs*. 24.5 (20-33) U/ml; ALT 54 (12-201) *vs*. 39.5 (16-67) *vs*. 22.5 (8-76) U/ml; and ALP 391 (83-1,912) *vs*. 93.5 (55-143) *vs*. 73.5 (44-98) U/ml for group (i) *vs*. (ii) *vs*. (iii), respectively, Mann-Whitney U test, $p < 0.05$]. Plasma peptide concentrations were determined using Lowry's method (Lowry et al., 1951) with bovine serum albumin (SIGMA-Aldrich, MO, USA) as a reference standard.

Preparation of plasma peptides and LC-MS/MS analysis

Plasma peptides (≤ 10 kDa in size) from CCA patients and non-CCA subjects were isolated using a 10 kDa cut-off column (Pall corporation, NY, USA). Plasma sample (200 μl) was loaded into and separated from the column through centrifugation at 7,000 ×g for 30 minutes. The separated plasma peptides were concentrated using vacuum concentrator and treated with buffer I (10 mM each of dithiothreitol and ammonium bicarbonate) and buffer II (100 mM iodoacetamide and 10 mM ammonium bicarbonate) for carbamidomethyl reaction at room temperature. The reaction was terminated by the addition of buffer I. Finally, plasma peptides were digested with trypsin (10 ng trypsin in 50% acetonitrile and 10 mM ammonium bicarbonate) through an overnight incubation at room temperature and samples were injected into LC-MS/MS.

Analysis of LC-MS/MS data

The LC-MS/MS data were analyzed using DeCyder MSTM (Amersham Bioscience AB, Uppsala, Sweden) and MASCOT (http://www.matrixscience.com) programs. Gene ontology and signaling pathways were analyzed using STRAP (Vivek Bhatia, Boston University School of Medicine) and PANTHER (http://www.pantherdb.org) (Mi et al., 2013), respectively. The metabolic, regulatory, and biosynthesis of secondary metabolites pathways were analyzed using iPath (http://pathways.embl.de/iPath2.cgi) (Yamada et al., 2011). The presence of specific peptides in either the CCA patients or non-CCA subjects were investigated to identify potential biomarkers for early diagnosis of CCA. The protein-protein interactions of potential biomarkers were investigated using STRING (http://string-db.org/) to elucidate the involvement of potential biomarkers in carcinogenesis.

Results

Analysis of CCA plasma peptidome

A total of 209 peptides were obtained from all plasma samples (CCA patients and non-CCA subjects) and identified from LC-MS/MS data by DeCyder MSTM and MASCOT programs. Out of these, 148 and 115 peptides were retrieved from UniProt ID and KEGG, respectively. The UniProt ID retrieved peptides were further analyzed by STRAP for identification of their functions. Results suggest that most of these peptides were involved in cellular process in biological function category, nucleus in cellular component category, and binding in molecular function category (Figure 1A, 1B, and 1C, respectively).

Table 1. The analysis of cell signaling pathways by PANTHER

Protein	UniProt ID	Cell Signaling Pathway
DNAH6 protein	Q9C0G6	· Huntington disease (Dynein complex)
Kainate receptor subunit KA2a	Q16478	· Huntington disease (Kainate receptor) · Ionotropic glutamate receptor pathway (Kainate Receptor) · Metabotropic glutamate receptor group III pathway (AMPA/Kainate Receptor) · Ionotropic glutamate receptor pathway (Glutamate receptor, ionotropickainate 5) · Metabotropic glutamate receptor group I pathway (Kainate Receptor) · Ionotropic glutamate receptor pathway (AMPA/Kainate Receptor)
Insulin-like growth factor 1 receptor precursor	P08069	· Insulin/IGF pathway-protein kinase B signaling cascade (Insulin receptor/Insulin like growth factor receptor/insulin related receptor) · Gonadotropin releasing hormone receptor pathway (IGF-1R) · Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade (Insulin/Insulin like growth factor) · Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade (Insulin receptor/Insulin like growth factor receptor/insulin related receptor)
Collagen alpha-3(IX) chain precursor	Q14050	· Integrin signalling pathway (Collagen)
ITGAX protein	P20702	· Integrin signalling pathway (Integrin alpha)
IRS-1	P35568	· Interleukin signaling pathway (Insulin receptor substrate family) · Gonadotropin releasing hormone receptor pathway (IRS) · Gonadotropin releasing hormone receptor pathway (IRS-1) · Insulin/IGF pathway-protein kinase B signaling cascade (Insulin receptor substrate family) · PI3 kinase pathway (Insulin Receptor Substrate) · Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade (Insulin receptor substrate family)

Table 1. The analysis of cell signaling pathways by PANTHER (continued)

Protein	UniProt ID	Cell Signaling Pathway
SCK	P98077	· VEGF signaling pathway (Shc-Like Protein) · EGF receptor signaling pathway (Src homology 2 domain-containing transforming protein 1) · Angiogenesis (Shc-like protein) · PDGF signaling pathway (SH2 homology domain containing transforming protein)
KIAA1568 protein	Q9HCK4	· Axon guidance mediated by Slit/Robo (Roundabout)
Myosin light chain kinase	Q15746	· Cytoskeletal regulation by Rho GTPase (Myosin light chain kinase) · Inflammation mediated by chemokine and cytokine signaling pathway (Myosin light chain kinase)
Rho kinase	O75116	· Cytoskeletal regulation by Rho GTPase (Rho-associated coiled-coil-containing protein kinase)
Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit beta isoform isoform 1	P42338	· FGF signaling pathway (Phosphatidylinositol 3-kinase) · PI3 kinase pathway (Activated p110) · Ras Pathway (Phosphatidylinositol 3-kinase) · Angiogenesis (Phosphatidylinositol 3-kinase) · EGF receptor signaling pathway (Phosphatidylinositol 3-kinase) · Insulin/IGF pathway-protein kinase B signaling cascade (Phosphatidylinositol 3-kinase) · Integrin signalling pathway (Phosphatidylinositol 3-kinase) · p53 pathway (Phosphatidylinositol 3-kinase) · VEGF signaling pathway (Phosphatidylinositol 3-kinase) · Interleukin signaling pathway (Phosphatidylinositol 3-kinase) · Inflammation mediated by chemokine and cytokine signaling pathway (Phosphatidylinositol 3-kinase) · T cell activation (Phosphatidylinositol 3-kinase) · Axon guidance mediated by netrin (Phosphatidylinositol 3-kinase) · p53 pathway feedback loops 2 (Phosphatidylinositol 3-kinase) · Hypoxia response via HIF activation (Phosphatidylinositol 3-kinase) · B cell activation (Phosphatidylinositol 3-kinase) · Apoptosis signaling pathway (Phosphatidylinositol 3-kinase) · Endothelin signaling pathway (Phosphatidylinositol 3-kinase) · PI3 kinase pathway (p110) · PDGF signaling pathway (Phosphatidylinositol 3-kinase)
ETS domain-containing transcription factor ERF	P50548	· PDGF signaling pathway (Ets)
Protocadherin alpha-C1	Q9H158	· Wnt signaling pathway (Cadherin) · Cadherin signaling pathway (Cadherin)
Casein kinase II subunit alpha	P19784	· Wnt signaling pathway (Casein Kinase 2) · Parkinson disease (Casein kinase II) · Cadherin signaling pathway (Casein kinase II)
Ankyrin repeat domain 6	Q9Y2G4	· Wnt signaling pathway (Diversin)

Analysis of signaling pathway related peptides by PANTHER showed 15 peptides having a role in cell signaling such as growth factor receptor-, hormone receptor-, cadherin-, and integrin- signaling pathways (Table 1). iPath analysis suggested the involvement of 12 peptides in metabolic pathway, e.g., cytosolic purine 5'-nucleotidase of purine metabolism, chain A, structure of human placental S-adenosylhomocysteine hydrolase (P23526) of cysteine and methionine metabolism, and peroxisomal sarcosine oxidase (Q9P0Z9) of amino acid metabolism. In addition, some are also involved in regulation and biosynthesis of secondary metabolite pathways (Table 2).

Identification of potential biomarkers for CCA from plasma peptidome

Out of the 209 peptides, three were specifically detected in plasma samples of the CCA patients but not in non-CCA subjects. These included ETS domain-containing transcription factor ERF (UniProt ID No. P50548) (30%, 6 from 20), KIAA0220 (UniProt ID No.Q92617) (30%, 6 from 20), and phosphatidylinositol 4, 5-bisphosphate 3-kinase catalytic subunit beta isoform isoform 1 or PIK3CB (UniProt ID No.P42338) (50%, 10 from 20). The two peptides namely LP2209 (UniProt ID No.Q6XYC0) and casein kinase II subunit alpha (UniProt ID No.P19784) were detected in plasma samples of the

Table 2. Analysis of the Metabolic Pathways, Regulatory Pathways, and Biosynthesis Pathways of Secondary Metabolites of the Identified Proteins by iPath

Protein	KEGG	Metabolic pathway	Regulatory pathway	Biosynthesis pathway of secondary metabolites
PGS1 protein, partial (Q32NB8)	hsa:9489	Glycerolphospholipid metabolism	-	-
N-glycosylase/DNA lyase isoform 1a (O15527)	hsa:4968	-	Base excision repair	-
MRP5 (O15440)	hsa:10057	-	ABC transporters	-
Chain A, Crystal Structure Of The Ubc Domain Of BaculoviralIap Repeat-Containing Protein 6 (Q9NR09)	hsa:57448	-	Ubiquitin mediated proteolysis	-
Cytosolic purine 5'-nucleotidase (P49902)	hsa:22978	Purine metabolism Pyrimidine metabolism Nicotinate and nicotinamide metabolism	-	Purine metabolism Pyrimidine metabolism Nicotinate and nicotinamide metabolism
DNA repair and recombination protein RAD54B isoform 1 (Q9Y620)	hsa:25788	-	Homologous recombination	-
Threonine--tRNA ligase, mitochondrial isoform a (Q9BW92)	hsa:80222	-	Aminoacyl-tRNA biosynthesis	-
Glutamate decarboxylase 1 isoform GAD67 (Q8IVA8)	hsa:2571	Alanine, aspartate and glutamate metabolism Beta-alanine metabolism Taurine and hypotaurine metabolism Butanoate metabolism Valine, leucine and isoleucine biosynthesis Type I diabetes mellitus	-	Alanine, aspartate and glutamate metabolism Beta-alanine metabolism Taurine and hypotaurine metabolism Butanoate metabolism Pantothenate and CoA biosynthesis Type I diabetes mellitus
MutL protein homolog 1 variant (Q59EG3)	hsa:4292	-	Mismatch repair	-
Peroxisomalsarcosine oxidase (Q9P0Z9)	hsa:51268	Glycine, serine and threonine metabolism Lysine degradation Peroxisome	-	-
Chain A, Structure Of Human Placental S-adenosylhomocysteine Hydrolase: Determination Of A 30 Selenium Atom Substructure From Data At A Single (P23526)	hsa:191	Cysteine and methionine metabolism	-	-
Alpha-mannosidase 2x (P49641)	hsa:4122	N-glycan biosynthesis High-mannose type N-glycan biosynthesis	-	-

OV and CCA patients, but not in healthy subjects (Table 3). The string result showed interaction of PI3KCB with v-akt murine thymoma viral oncogene homolog 1 (AKT1, UniProt ID No. P31749), phosphatase and tensin homolog (PTEN, UniProt ID No.P60484), and phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1, UniProtID

Table 3. Identified Proteins that were Absent in Plasma of Healthy Subjects (H) and Patients with OV Infection (OV)

Protein name	Uniprot	KEGG	Not found
Casein kinase II subunit alpha	P19784	hsa:1459	H
ETS domain-containing transcription factor ERF	P50548	hsa:2077	OV+ H
KIAA0220	Q92617	-	OV + H
LP2209	Q6XYC0	-	H
Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit beta isoform isoform 1	P42338	hsa:5291	OV+ H

Table 4. Battery Test for Diagnosis of CCA Based on 5 Potential Proteins

CCA screening		Diagnosis
Screening Part I*	Screening Part II**	
Positive for> 1 proteins	Negative for all of the three proteins	CCA + OV
Positive for> 1 proteins	Positive for> 1 proteins	CCA
Negative for both proteins	Negative for all of the three proteins	H
Negative for both proteins	Positive for> 1 proteins	CCA

* LP2209, Casein kinase II subunit alpha; ** ETS domain-containing transcription factor ERF, PIK3CB, KIAA0220; The abbreviation list: CCA + OV, the patients with both cholangiocarcinoma and *Ophisthorchis viverrini* infection; CCA, patients with; * LP2209, Casein kinase II subunit alpha ** ETS domain-containing transcription factor ERF, PIK3CB, KIAA0220; The abbreviation list: CCA + OV, the patients with both cholangiocarcinoma and *Ophisthorchis viverrini* infection; CCA, patients with cholangiocarcinoma

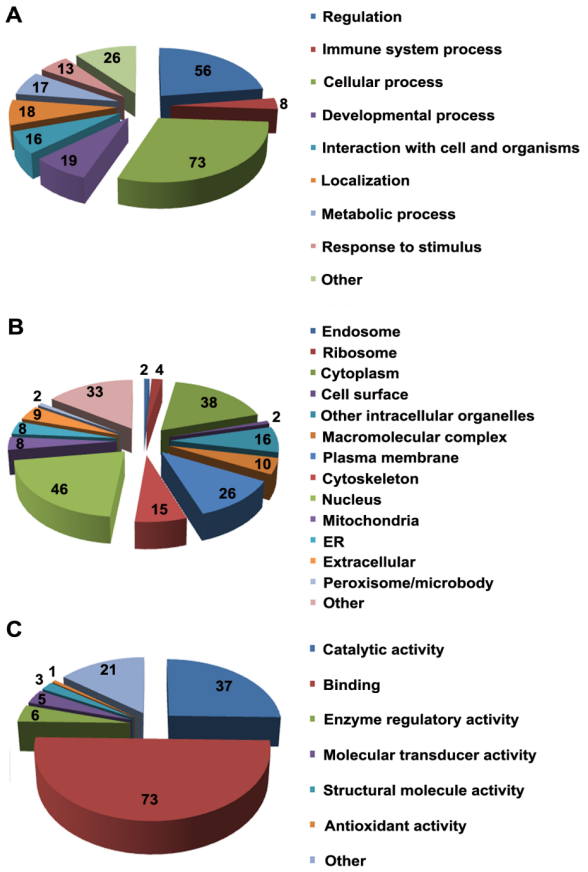

Figure 1. STRAP analysis of the functions of identified proteins based on (A) biological process, (B) cellular component, and (C) molecular function

No.P27986) (Figure 2).

Discussion

Analysis of plasma peptidome of CCA patients identified only 5 out of 209 peptides as potential biomarkers

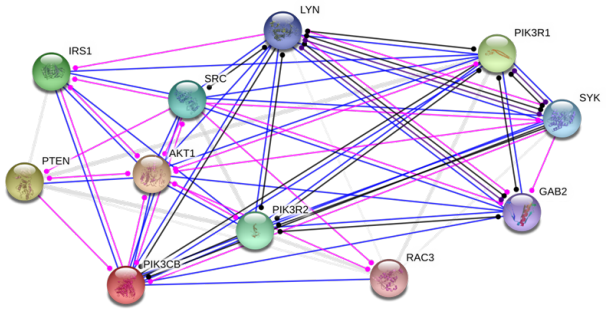

Figure 2. Analysis of Phosphatidylinositol 4, 5-bisphosphate 3-kinase Catalytic Subunit Beta Isoform Isoform 1 by STRING

for CCA, i.e., ETS domain-containing transcription factor ERF, PIK3CB, casein kinase II subunit alpha, KIAA0220, and LP2209. The first three have been reported to play major roles in signaling pathways that are associated with cell growth and proliferation (Zaldumbide et al., 2002; Jia et al., 2008). ETS domain-containing transcription factor ERF is a transcription repressor of ETS2 promoter which is regulated by MAPK1/ERK2 phosphorylation (Sgouras et al., 1995). PIK3CB plays a role in phosphatidylinositol (PI) signaling pathway (Wee et al., 2008; Dbouk et al., 2010) through interacting with AKT1, PTEN, and PIK3R1 (Figure 2). Up-regulation of this protein may affect the downstream signaling cascade of PI signaling pathway (Wee et al., 2008), which may promote cancer cell growth and differentiation. Casein kinase (CK) II subunit alpha is involved in cell proliferation. Inhibition of CK II kinase was demonstrated to activate cell apoptosis in cancer cell (Hamacher et al., 2007). In addition, the protein was shown to be activated following Wnt signaling pathway activation via frizzled receptor (Song et al., 2000; Seldin et al., 2005). The functions of KIAA0220 and LP2209 remain unclear. KIAA0220 cDNA has been characterized as nuclear pore complex-interacting protein, while LP2209 has been shown to promote mice NIH/3T3 cells' growth.

The combination of these identified biomarkers could be applied as a battery of test for CCA diagnosis for diagnosis of CCA alone or CCA with OV infection (Table 4). The efficiency of the test for CCA diagnosis should be confirmed in a larger number of patients.

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