RESEARCH ARTICLE

Serum Adhesion Molecule-1 (ICAM-1) as a Potential Prognostic Marker for Cholangiocarcinoma Patients

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Abstract

   Cholangiocarcinoma (CCA) is a malignancy of bile ducts with a high incidence of invasion and metastasis. This disease is often detected in advanced stages because of the difficulties of early diagnosis, leading to a high mortality rate. However, biomarkers for early CCA detection are still lacking. In this study, to identify potential biomarker proteins, differential secretome analysis by the GeLC-MS/MS approach was applied with four CCA cell lines and a control immortalized cholangiocyte cell line. Among 78 up-regulated proteins, 53 including ICAM-1 were exclusively expressed in four CCA secretomes but not in MMNK1. Based on this result, we measured ICAM-1 levels in serum samples of CCA patients and healthy controls and found significantly higher values in CCA patients' sera. Receiver operating characteristic curve analysis suggested that serum ICAM-1 level could be a discriminatory diagnostic marker for CCA and healthy controls (area under curve=0.829) with a sensitivity of 77% and a specificity of 70% at a cut off value of 167 ng/ml. Moreover, the serum ICAM-1 showed positive correlations with alkaline phosphatase and carcinoembryonic antigen levels. Comparison of ICAM-1 levels of paired pre- and post-operative sera of 12 cases revealed significant decrease after tumor resection. However, serum ICAM-1 levels were not significantly different between CCA and benign biliary diseases with mainly inflammatory features.

Keywords: Cholangiocarcinoma - secretome - adhesion molecule-1 (ICAM-1)

Introduction

   Cholangiocarcinoma (CCA) is a malignancy of the biliary epithelium associated with a high incidence of invasion and metastasis, leading to a high mortality rate. The incidence of this cancer has been increasing worldwide (Patel, 2001; Khan et al., 2005; Blechacz and Gores, 2008; Khan et al., 2008; Mosconi et al., 2009). The highest incidence occurs in the northeastern region of Thailand, especially in Khon Kaen province (78.4 per 100,000 in male and 33.3 per 100,000 in female) (Sripa et al., 2010), where high prevalence of liver fluke (*Opisthorchis viverrini*) infection has been reported (Khuhaprema and Srivatanakul, 2007; Sripa and Pairojkul, 2008).

   CCA is notoriously difficult to diagnose and is usually fatal because of its late clinical presentation, resulting in poor prognosis. This disease remains an important public health problem due to lacking of biomarkers for early diagnosis (Khan et al., 2005). Clinical manifestation in the early stage of CCA is usually silent or associated with nonspecific symptoms. Several serum markers such as CA 19-9, CEA and ALP have been used for detecting CCA. However, these markers are not quite satisfactory for CCA detection because of their insufficient specificity and sensitivity for screening (Khan et al., 2005; Malhi and Gores, 2006; Aljiffry et al., 2009). Therefore, it is a pressing need to find novel biomarkers that can be utilized for detection of CCA in an early stage or in asymptomatic patients.

   The cancer secretome, the total of proteins that cancer cells secrete or shed into the extracellular environment, represents the main class of molecules involved in intercellular communication, cell adhesion, motility and invasion, and is a useful source for supplying potential cancer biomarkers (Wu et al., 2009; 2010). These proteins released by cancer cells *in vitro* possibly reflect the proteins released by cancer cells *in vivo*. The analysis of cancer cell culture media is an alternative approach to discover potential cancer biomarkers in body fluids, especially in blood samples (Wu et al., 2010). The present study the potential CCA biomarkers from a differential

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secretome analysis between four CCA and immortalized cholangiocyte cell, MMNK1. Aims of this study were to discover the potential biomarkers for CCA from secretome analysis using the proteomic-based approach via SDS-PAGE combined to LC-MS/MS and to demonstrate the practical valuable CCA related biomarkers for development of potential clinical application.

Accordingly, this secretome study facilitates establishment of potential biomarker, ICAM-1 for CCA patients. Although ICAM-1 protein was unsuitable molecule for CCA diagnosis due to the differentiate CCA from benign biliary diseases, this protein might be a useful prognostic biomarker for follow-up CCA patients after surgery or use as a monitoring molecule for assessing tumor additional advantage applications of ICAM-1 as well as a therapeutic drug target for CCA intervention are needed to be further investigated.

Materials and Methods

Cell lines and cell culture

Four CCA cell lines, KKU-OCA17, KKU-M213, KKU-M214 and KKU-100, were established from different histological types of a primary tumor mass of intrahepatic CCA (Sripa et al., 2005). They were kindly provided by the Liver Fluke and Cholangiocarcinoma Research Center (LFCRC), Faculty of Medicine, Khon Kaen University. Additionally, an immortalized cholangiocyte cell line, MMNK1 which was established by Maruyama et al. (Maruyama et al., 2004), was used as a control. All cell lines were cultured in Ham's F-12 culture medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin and 100 µg/ml of streptomycin. Cell lines were incubated at 37°C in 5% CO_2 air atmosphere. All cell lines were confirmed to be *Mycoplasma* free by specific PCR.

Serum samples

Pre-operative serum samples from 34 CCA patients (14 females and 20 males; 38-70 years of age with median 54 years) 12 CCA patients whose post-operative serum samples were available (3-9 months after surgery; median 5.5 months), and 20 serum samples from benign biliary diseases (9 females and 11 males; 31-78 years of age with median 62 years) were obtained from the Liver Fluke and Cholangiocarcinoma Research Center (LFCRC), Faculty of Medicine, Khon Kaen University. The normal control sera were the left-over of 20 healthy persons (13 females and 7 males; 26-58 years of age with median 48 years) who went to health check-up at the Office for Medical Technology and Physical Therapy Health Service, Faculty of Associated Medical Sciences, Khon Kaen University, with generally healthy appearance and had normal biochemical tests for blood glucose, liver function and renal function. The serum samples were kept at -70 °C until use. This project was approved by the Ethical Committee of Khon Kaen University,(HE521209), using samples collected with informed consent.

Preparation of the conditioned media and cell lysates

The condition for harvesting conditioned media

was modified from the optimal condition (Mbeunkui et al., 2006). The cells were grown in the compete media approximately to 70% confluence in 15 cm diameter dishes, then they were rinsed twice with 1x phosphate buffer saline (PBS) and twice with serum-free medium (SFM) to remove serum residues from the medium. After that, cells were incubated in 12 ml of SFM for 24 h. After complete incubation with SFM, the conditioned media (CM) containing secreted proteins were harvested and centrifuged at 600 x g for 10 min to remove the cell debris. The CM samples were concentrated and desalted by ultrafiltration with Amicon ultra-15 tubes (MW cut-off 3 kDa), centrifuged at 4,000 x g for 50 min at 4 °C, and the supernatants were stored as "secretome samples" at -20 °C until use. The remaining cells on the dishes were washed twice with 1x PBS and then lysed on ice for 10 min in lysis buffer containing protease and phosphatase inhibitors (mammalian protein extraction buffer). The lysates were centrifuged at 20,000 x g at 4 °C for 30 min and the proteins were precipitated by adding two volumes of acetone and kept at -20 °C overnight. Then, the precipitates were collected by centrifugation at 17,000 x g for 15 min and stored at -20 °C until use. The protein concentration of each samples were determined using the Lowry's method (Lowry et al., 1951).

In-gel tryptic digestion

Fifty micrograms of protein samples were dissolved in sample buffer and boiled for 5 min and were separated on 12.5% SDS-PAGE (ATTO AE-6530 system, Tokyo, Japan). The gel was run at a constant voltage of 70 V for 5 h at room temperature. The samples were loaded and run in parallel with standard molecular weight markers. After electrophoresis, the gel was soaked for 30 min with fixing solution (50% methanol, 12% acetic acid and 0.019% formaldehyde), washed twice in washing solution (35% ethanol) for 5 min each, and then sensitized by 0.02% $Na_2S_2O_3$ for 2 min. After washing twice for 5 min each with Milli Q® water, the gel was stained with silver nitrate solution (0.2% $AgNO_3$) for 20 min, washed twice in Milli Q® water for 1 min each prior to color with developing solution (6% Na_2CO_3, 0.019% formaldehyde, 0.0004% $Na_2S_2O_3$) for approximately 5 min (or until the desired protein bands attained). The staining was stopped by incubation in 1.5% Na_2 EDTA solution for 20 min. Finally, the stained gel was washed three times for 5 min each with Milli Q® water. The gel was scanned using a GS-710 scanner (Bio-Rad, Benicia, CA) before being stored in 0.1% acetic acid until in-gel tryptic digestion.

The gel lanes were divided into 5 wide ranges according to the standard protein markers and then sub-splitted into 15 ranges. Each gel range was chopped into many small pieces (1 mm³/piece), transferred to 96-well plate (5-10 pieces/well) and each gel was processed for in-gel digestion. Briefly, the gel pieces were dehydrated twice in 100% acetonitrile (ACN) for 5 min each with agitation and dried at room temperature for 15 min. Subsequently, cysteine bonds were reduced with 10 mM dithiothreitol (DTT)/10 mM ammonium bicarbonate (NH_4HCO_3) for 1 h at room temperature and alkylated with 100 mM iodoacetamide (IAA)/10 mM NH_4HCO_3

for 1 h at room temperature in the dark. The gel pieces were dehydrated twice in 100% ACN for 5 min each with agitation. After reduction and alkylation, 20 μl of 10 ng/μl trypsin in 50% ACN/10 mM NH_4HCO_3 was added and incubated for 20 min before adding 20 μl of 30% ACN and then incubated overnight at room temperature. The tryptic peptides were extracted from the gel three times with 30 μl of 50% ACN/0.1% formic acid. Finally, the tryptic peptide mixtures were dried and kept at -80 °C prior to LC-MS/MS analysis.

LC-MS/MS analysis

The extracted peptides sample was dissolved in 15 μl of 0.1% formic acid, centrifuged at 12,000 x g for 5 min, and then injected into LC-MS/MS system. The system composed of two main procedures including peptides separation by the liquid chromatography (LC system) and detection of eluted peptides mass. Briefly, the peptides were separated using a Dionex-Ultimate 3000 LC System. The mobile phase A (0.1% formic acid) and the mobile phase B (0.1% formic acid in 50% ACN) were. After injection, the peptides were separated on a Monolithic Nano Column (100 μm inner diameter, 5 cm length) with a flow rate of 1.0 μl/min by using multi-steps gradient of a linear concentration increase from 10% to 70% of mobile phase B for 13 min, 90% of mobile phase B at 13-15 min and decrease to 10% of mobile phase B at 15-20 min. The LC system was connected with an electrospray interface with ESI-Ion Trap MS (Bruker Daltonik GmbH, Bremen, Germany) for eluted peptide detection on a Monolithic Trap Column (200 μm inner diameter, 5 mm length) with a flow rate of 20 μl/min. The MS/MS data were simultaneously obtained.

Database searching and protein quantitation

The native MS/MS data were converted first into the common mzXML format using compassXport1.3.10 (Bruker Daltonik GmbH), and then the MS/MS data were processed using DeCyder MS Differential Analysis Software (version 2.0, GE Healthcare, Piscataway, NJ) which requires multi-steps performed by 3 modules. Firstly, import module was used to specify the optimal conditions in the Ion Trap mass analyzer, crop retention time from 4-12 min. Secondly, the peptide detection and background subtraction step were performed using the PepDetect module. Peptides were detected using charge state assignments from 1-4, mass resolution of 0.6 u, LC peak shape tolerance of 20.0, m/z shift tolerance of 0.1 u, m/z shape tolerance of 5% and using a s/n threshold of 1.0 and quantitation based on MS signal intensities of individual analyses. Finally, the matching peptides across different signal intensity were mapped using the PepMatch module resulting in a quantitative comparison with statistical significance at P≤0.05 (retention time and m/z tolerances of 5 min and 2 Da, respectively). All peptide data were exported into the mgf format and then searched against NCBInr human protein database by MASCOT MS/MS Ion search engine (Matrix Science, London, UK). The final data containing protein name, GI number, peptide sequence, P-value, ID score and signal intensity were exported into excel files.

Sandwich ELISA

The serum ICAM-1 levels of CCA patients and control groups were measured using the human ICAM-1 instant ELISA kit (cat no. BMS201INST, Bender MedSystems GmbH, Vienna, Austria), which is in principle a sandwitch ELISA using ELISA strips pre-coated with anti-human ICAM-1 monoclonal antibody and also contained lyophilized HRP-conjugated monoclonal anti-human ICAM-1 antibody. ELISA was performed according to the manufacturer's instruction. In brief, the 150 μl of distilled water was added to the each well of standard and blank and 140 μl of distilled water was added to each sample well. Then, 10 μl of each sample was added and incubated at room temperature for 2 h with shaking at 100 rpm. After 2 h incubation, the excess antibody was removed by washing three times with 400 μl of 1x washing buffer. Then, 100 μl of TMB substrate solution was added and incubated at room temperature for 10 min. The reaction was stopped by adding 100 μl of stop solution quickly into each well and read absorbance on a microplate reader (Tecan, Vienna, Austria) using 450 nm as the primary wave length and 620 nm as the reference wave length. A standard curve was constructed by MasterPlex® curve-fitting software (version 2.0.0.73, Hitachi Solutions America, South San Francisco, CA) into the five-parameter logistic (5PL) non-linear regression model which plotted absorbance values on y-axis against concentrations of seven ICAM-1 standards on x-axis in the log scale which represented ICAM-1 standard concentration of 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 ng/mL, respectively. The concentrations of unknown samples were determined by 5PL equation.

Bioinformatic analysis

The potential secretion through classical pathway of identifying proteins was predicted with SignalP 4.0 server (Petersen et al., 2011) that predicted the presence and location of signal peptide cleavage sites in amino acid sequences (FASTA format). Likewise, prediction of non-classical protein secretion was also performed on SecretomeP 2.0 server (Bendtsen et al., 2004). Cellular localization, protein classification and protein network analysis were analyzed using the Ingenuity Pathway Analysis software tool (IPA, Ingenuity Systems, Inc. Redwood City, CA). In addition, other information such as protein categorization, biological function was analyzed with PANTHER classification system (Mi et al., 2010).

Statistical analysis

The visualization and statistical analysis of identified proteins were performed by Multi Experiment Viewer (Mev) software (version 4.6.1, Dana-Farber Cancer Institute, Boston, MA). The different expression of identified proteins between CCAs and MMNK1 secretomes were estimated by t-test. Statistical analysis was conducted with SPSS software (version 17.0, SPSS Inc., Chicago, IL). The different values among two and more than two independent sample groups were estimated using the Mann-Whitney and Kruskal Wallis tests, respectively. The relationship between serum ICAM-1 levels and patient clinicopathological parameters were analyzed using Pearson's chi-squared and Fisher's extract

tests. The correlation between two variables was analyzed by Spearman's correlation test. In addition, comparison between pre-operative and post-operative serum levels was tested using Wilcoxon matched pairs test and data were plotted by GraphPad prism (version 5, GraphPad Software Inc., San Diego, CA). The statistical threshold was set at 0.05 (two-sided).

Results

Differential secretome analysis and candidate protein selection

Total comparative quantitation analysis using DeCyder MS software revealed that 129 proteins were aberrantly expressed in the conditioned media of CCA cell lines (P<0.01). Among them, 78 up-regulated and 51 down-regulated proteins represented the important molecular functions (data not shown). For CCA biomarker candidates, we considered proteins commonly expressed in four CCA cell lines secretomes but not in MMNK1 secretome. The 53 and called as "CCA-related proteins". The signal peptides of 53 CCA-related proteins were examined using SignalP 4.0 server. The protein contained a signal peptide was considered to be a classical secretory protein. In addition, SecretomeP 2.0 was used to seek for proteins secreted through non-classical secretory pathways. The results of showed that only 6 proteins contained the signal peptide sequence. In contrast, the SecretomeP predicted 21 proteins as non-classical secretory proteins. The residual 26 proteins were considered as non-secretory proteins. Subsequently, the secretory proteins were collected and non-secretory proteins were discarded. The 53 CCA-related proteins and their individual information are listed in Figure 1.

According to multi-steps of biomarker selection above, the results indicated that several proteins might be the potential biomarkers for CCA. Finally, among the considerable proteins, adhesion molecule-1 (ICAM-1) was selected for further validation steps because it met the criteria below; 1) In four CCA secretomes but was not found in MMNK1. 2) The predicted classical or non-classical secretory proteins that have a potential to enter into the circulation. 3) Availability of specific antibody and ELISA kit. 4) Association of molecular function with cancinogenesis and/or cancer progression.

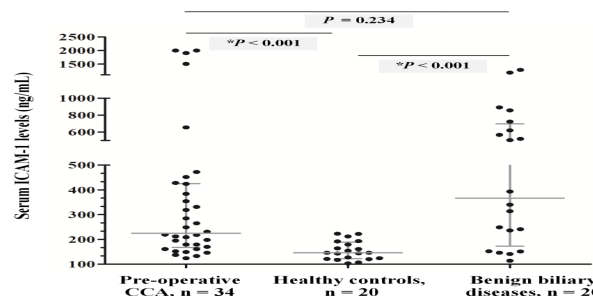### *ICAM-1 level of CCA, benign biliary patients and healthy controls*

According to the western blot analysis and mass spectrometry of the secretomes of CCA cell lines, ICAM-1 was distinctly upregulated. Therefore, we considered ICAM-1 protein might be a serum marker candidate for detecting CCA. Serum ICAM-1 levels of 34 pre-operative CCA patients and 20 healthy persons were evaluated by sandwich ELISA. A statistically significant increase of ICAM-1 was seen in CCA patients compared with healthy controls (defined as the median value and interquartile range) (225 (172-414) versus 146 (123-182) ng/ml; P<0.001). In addition, The 20 serum samples of patients with benign biliary diseases were analyzed. Significant increase of ICAM-1 was found also in this

group compared with healthy controls (367 (215-646) versus 146 (123-182) ng/ml; P<0.001). Serum ICAM-1 levels in benign biliary diseases patients were slightly higher than that in the pre-operative CCA group, although the difference was statistically not significant (P=0.234) (Figure 2). Thus, Serum ICAM-1 protein level may be unsuitable for differentiation of CCA from benign biliary diseases, but still this protein could be a potential serum biomarker for discriminating CCA from healthy persons.

To further evaluate the diagnostic significance of serum ICAM-1 for discriminating CCA from healthy persons, a receiver operating characteristic (ROC) curve was constructed by plotting sensitivity versus specificity or false positive rate (Figure 3). The area under the ROC curve (AUC): a commonly used as indicator for estimating the diagnostic efficiency of a potential biomarker, was subsequently calculated. The closer of AUC to 1 indicates the better diagnostic performance of the test. The ROC curve as shown in Figure 4 had an AUC of 0.829 which
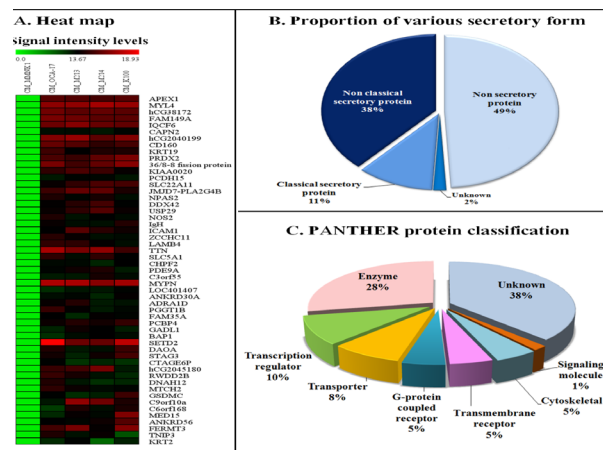
Figure 1. The Summary of 53 CCA-Related Proteins. (A) Heat map of the quantitative differential expression among four CCA secretomes and its control (MMNK1). Each row represents an individual protein and each column represents an individual sample (bar chart represents the signal intensity level as shown in corresponding color). (B) The proportion of protein form prediction was categorized into three groups including classical secretory proteins, non-classical secretory proteins and non-secretory proteins. (C) The pie chart represents molecular functional categorization which mostly involved in enzyme groups


Figure 2. Scatter Plots of Serum ICAM-1 Levels of Pre-operative CCA Patients, Healthy Controls and Benign Biliary Patients. Serum ICAM-1 levels of each group were defined as median value (long horizontal line) and interquartile range (upper and lower lines). The ICAM-1 level of pre-operative CCA and benign biliary diseases significantly increased healthy controls (P<0.001)

indicated the good diagnostic efficiency. When the cut off value of 167 ng/ml was set for ICAM-1, the sensitivity and specificity for discriminating CCA from healthy controls were 77% and 70%, respectively. Similarly, the positive predictive value, negative predictive value and the accuracy of the test were 81%, 64% and 74%, respectively.

Relationship between ICAM-1 level and clinicopathological status of patients

The relationships between serum ICAM-1 level and patients' clinical parameters are summarized in Table 1. The serum ICAM-1levels of 34 CCA patients ranged from 124 to 2,000 ng/ml with a median value of 225 ng/ml. In order to categorize the serum ICAM-1 level into low and high level value was used as the cut off. The high serum ICAM-1 level was significantly associated with the abnormal ALP (P<0.001) and CEA levels (P=0.025). However, serum ICAM-1 levels of CCA patients were not correlated with gender, age, histological type of tumor, lymph node metastatic behavior, survival time or CA19-9 level. The correlation between ICAM-1 and ALP or CEA levels were subsequently analyzed. The correlation was examined by the Spearman's correlation coefficient (R-value). The result between ICAM-1 and ALP levels showed remarkably positive correlation with R=0.703 (P<0.001) (Figure 4A). The ICAM-1 and CEA levels also positively correlated with R=0.563 (P=0.012) (Figure 4B). Since ALP and CEA levels have been commonly used to
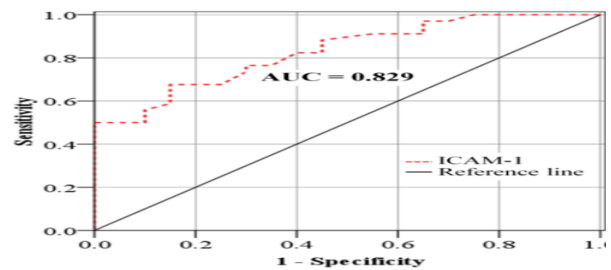
Figure 3. ROC Curve Analysis for the Diagnostic Efficiency of ICAM-1, Which was Constructed from Plotting Test Sensitivity as the y Coordinate Versus its False Positive Rate as the x Coordinate. ROC curve for discriminating CCA patients from healthy controls with AUC=0.829
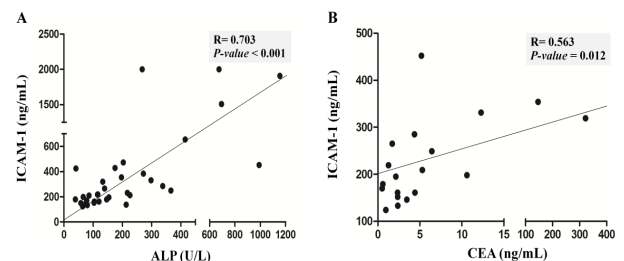
Figure 4. The Spearman's Correlation between ICAM-1 and ALP level (R=0.703, P<0.001) (A) and Correlation between ICAM-1 and CEA (R=0.563, P=0.012) (B).

Table 1. Relationship between Serum ICAM-1 Level and Patient Clinicopathological Characteristics

Characteristic parameters	Serum ICAM-1 level					
	No.	Mean±SD	p-value[a]	≤225ng/mL n=17 n(%)	>225ng/mL n=17 n(%)	p-value[b]
Gender			0.336			0.486
Male	20	504±582		9 (53)	11 (65)	
Female	14	367±481		8 (47)	6 (35)	
Age (years)			0.605			0.169
Median range				53(38-63)	56 (45-70)	
≤54	18	575±714		11 (65)	7 (41)	
>54	16	303±145		6 (35)	10 (59)	
Histological type			0.331			0.335
Papillary	14	415±554		9 (53)	5 (29)	
Non-papillary	16	504±602		7 (41)	9 (53)	
Mixed type	2	226±132		1 (6)	1 (6)	
Adenosquamous	2	440±17		0	2 (12)	
Survival (weeks)			0.428			0.732
≤67	17	346±417		9 (53)	8 (47)	
>67	17	548±636		8 (47)	9 (53)	
Lymph node metastasis			0.448			0.169
No	18	363±434		11 (65)	7 (41)	
Yes	16	542±640		6 (35)	10 (59)	
CA19-9 (n=21)			0.231			0.387
Normal (≤37U/mL)	10	228±109		7 (58)	3 (33)	
Abnormal (>37U/mL)	11	287±154		5 (42)	6 (67)	
CEA (n=19)			*0.025			0.057
Normal (≤2.5ng/mL)	9	178±44		8 (57)	1 (12)	
Abnormal (>2.5ng/mL)	10	270±96		6 (43)	7 (88)	
ALP (n=34)			*<0.001			*<0.001
Normal (42-121U/L)	14	189±74		13 (76)	4 (6)	
Abnormal (>121U/L)	20	628±646		4 (24)	16 (94)	

*Statistically significant correlation, [a]These variables were analyzed from the individual ICAM-1 level, [b]These variables were analyzed from two groups of ICAM-1 (low and high level groups)
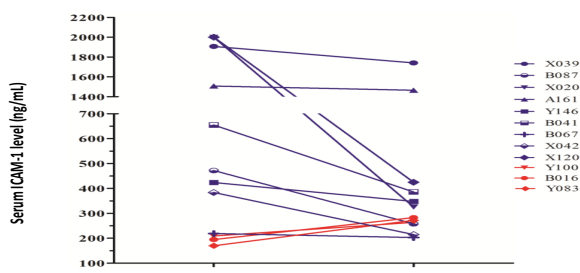
Figure 5. Comparison of Serum ICAM-1 Level between Pre-Operative and Post-Operative CCA of 12 matched Cases, Accounting 75% of Cases (9 of 12 cases) showed decrease of ICAM-1 Level after tumor Resection. (Wilcoxon matched pairs test, P=0.041). Blue color indicates the decreased ICAM-1 and red color indicates the increased ICAM-1 level after tumor

diagnose CCA patients, ICAM-1 might also be used as the additional biomarker for CCA patients.

Comparison of ICAM-1 levels between pre- and post-operative sera of CCA patients

The results shown above indicated that ICAM-1 protein can be a potential molecule for discriminating CCA from healthy persons. However, this protein was unsuitable to differentiate CCA from benign biliary diseases. To investigate an alternative merit of measuring serum ICAM-1 level for CCA patients, serum ICAM-1 levels were measured for the paired pre- and post-operative sera of 12 CCA patients (Figure 5). The decreased ICAM-1 level after surgery was defined as percent of decreased level when compared with pre-operative ones. Total nine cases showed that median value of ICAM-1 level was decreased for 41% after surgery. As the overall data, ICAM-1 levels in the pre-operative sera were significantly higher (P=0.041) than those in the post-operative sera. In the detail, 9 cases showed significant (P=0.008) decrease of ICAM-1 level after surgical resection of the tumor. In 3 cases, the serum ICAM-1 levels were slightly, although not significant (P=0.109), increased after surgery.

Discussion

Secretome profile analysis of four CCA cell lines in comparison with immortalized cholangiocyte cell line, MMNK1 revealed that ICAM-1 could be a potential candidate protein for an establishment of CCA biomarker. We selected this molecule based on the important characteristics of its involvement in cancer-associated biological processes, its potential secretory protein property and the availability of reliable commercial antibody and a ELISA kit. ICAM-1, also known as CD54, is a member of immunoglobulin superfamily that belongs to type I transmembrane glycoprotein possessing with an amino-terminus extracellular domain, a single transmembrane domain, and a carboxy-terminus cytoplasmic domain which exists in its native membrane-bound and soluble forms (Reilly et al., 1995; Wang and Springer, 1998). It is expressed in different types of cells including epithelial cells, endothelial cells and leukocytes (Yang et al., 2005; Vainer et al., 2006; Park et al., 2009). This protein is well known to modulate the essential

cell–cell and cell–matrix adhesion. Aberrant ICAM-1 expression has previously been reported regard to its role in inflammatory and malignant conditions (Okegawa et al., 2004; Pace et al., 2011). Especially, the soluble form of ICAM-1 has widely been described signaling molecule that plays important roles in tumor growth, angiogenesis, invasion and metastasis (Gho et al., 1999; 2001; Maruo et al., 2002; Kovacs, 2005; Gogali et al., 2010).

Enhanced ICAM-1 shedding in tumor culture media may occur due to the event of increased protease expression and activity, leading to proteolytic cleavage of membrane-bound ICAM-1 resulting in the generation of a soluble form ICAM-1 (Fiore et al., 2002; St-Pierre, 2005). Moreover, this mechanism was also supposed to play a role in tumor cell invasion of immune surveillance. In the present study, serum ICAM-1 levels in CCA patients were significantly higher that in the healthy group (P<0.001). The increased ICAM-1 shedding from CCA cells *in vitro* might be one possible explanation for the elevated serum ICAM-1 level that found in CCA patients.

In the present study, significant association of the high serum ICAM-1 level with abnormal of ALP and CEA observed. Especially, the correlation of ICAM-1 and ALP levels showed remarkably positive correlation with the high correlation coefficient value (R=0.703, P<0.001). Since ALP and CEA levels have been commonly used for diagnosis of CCA patients, ICAM-1 might be added as potential biomarker for CCA. Abnormal expression of serum ICAM-1 has been reported in various cancers such as breast, lung, colorectal and gastric cancer (Nakata et al., 2000; O'Hanlon et al., 2002; Mantur et al., 2009; Gu et al., 2011).

In this study, ROC curve plotting for estimating the diagnostic efficiency of serum ICAM-1 in CCA patients indicated indicator an effectively discriminate value for the CCA patients (AUC=0.829) with the sensitivity of 77% and specificity of 70% at an appropriate cut off value of 167 ng/mL. In addition, the combination of ICAM-1 and other often used markers including ALP, CA19-9 and CEA for CCA detection showed the notably better improve of the sensitivity than either one alone. Thus, the combination of these molecules for constructing a panel of biomarkers is considered to be a useful approach for CCA detection.

Regarding the limitation of ICAM-1 as a biomarker, it was unable to distinguish CCA from benign biliary diseases. However, as an alternative advantage of ICAM-1 protein in clinical application for CCA, it can be a potential indicator molecule for following-up of patients after curative surgery. ICAM-1 levels in the 12 paired pre- and post-operative sera showed the significant overall decrease by matched comparison (P=0.041). The decrease of ICAM-1 level after removal of tumor mass was observed in 9 of 12 cases (75%). These results suggested that ICAM-1 might be a useful prognostic marker for CCA.

Acknowledgements

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