RESEARCH ARTICLE

Serial Analysis of Gene Expression Reveals Promising Therapeutic Targets for Liver Fluke-associated Cholangiocarcinoma

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Abstract

Cholangiocarcinoma (CCA) continues to be a serious health problem and is the most common fatal cancer in northeastern Thailand. Comprehensive gene expression analysis was here used to identify possible therapeutic targets for CCA treatment. We assessed liver fluke-associated CCA tissue using serial analysis of gene expression (SAGE) and compared the data to normal liver tissue as a part of the Cancer Genome Anatomy Project (CGAP). The analysis identified 509 differentially expressed genes. Of 142 up-regulated examples, we selected candidates including TMSB10, GAL3, VDR, CYPA and CD147 for further validation in CCA tissues by immunohistochemistry. VDR, CYPA and CD147 were confirmed to be consistently overexpressed in the samples tested. The therapeutic and diagnostic potential of these genes warrants further investigation.

Keywords: Cholangiocarcinoma - bile duct cancer - serial analysis of gene expression - SAGE

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Introduction

Cholangiocarcinoma (CCA) is the most common liver cancer in the northeastern Thailand where the incidence rate is the highest in the world (Shin et al., 2010). Liver fluke (*Opisthorchis viverrini*) infection has been proven to be the major risk factor of CCA in this endemic area (Sripa et al., 2007). Most CCA patients present at the invasion/metastasis stage and this leads to a high mortality rate (Patel, 2002). Discovery of new promising therapeutic targets may be the alternative approach for CCA treatment. Progression of tumor cells toward a high malignancy phenotype and metastasis is a multi-event cascade involving alterations in the expression of various genes. Thus, global gene expression profiling is the appropriate tool to study this complex disease. Serial analysis of gene expression (SAGE) (Velculescu et al., 1995) has been used to identify differentially expressed genes in many types of human cancer. This quantitative method for high throughput gene expression analysis can be compared with experiments from different laboratories done at different times (Boon et al., 2002). The Cancer Genome Anatomy Project (CGAP) provides a SAGE database of various normal and cancer tissues and also several online tools on this user-friendly website http://cgap.nci.nih.gov/SAGE. To identify the potential therapeutic targets, we performed a large scale gene expression profiling of liver fluke-associated CCA tissues using SAGE and then compared these profiles with the gene profile of normal liver provided from the SAGE public database. Among the differentially expressed genes, we focused on the up-regulated genes whose altered expression may lead directly or indirectly to an increased malignancy phenotype. We finally selected 5 candidate genes (TMSB10, GAL3, VDR, CYPA and its receptor CD147) for further validation of SAGE analysis in different sets of CCA tissues by immunohistochemistry.

Materials and Methods

CCA patient samples for SAGE

The paired CCA tissues were selected to establish two SAGE libraries: an intrahepatic metastatic nodule (SAGE_Liver_Cholangiocarcinoma_B_K1) and a primary CCA tumor tissue (SAGE_Liver_Cholangiocarcinoma_B_K2D). The tissues were obtained from a Thai male, 55 years old with an intrahepatic mass forming CCA. The histology proved to be a poorly differentiated adenocarcinoma (Figure 1). Tumor invaded the intrahepatic vein, and invaded beyond Glisson's capsule involving diaphragm, right adrenal gland and peritoneum. The cut sections for establishing SAGE contained more than a 60% CCA cell population as analyzed by histology.

SAGE library construction and analysis

Construction of the SAGE library was performed following the instructions of the Micro-SAGE protocol

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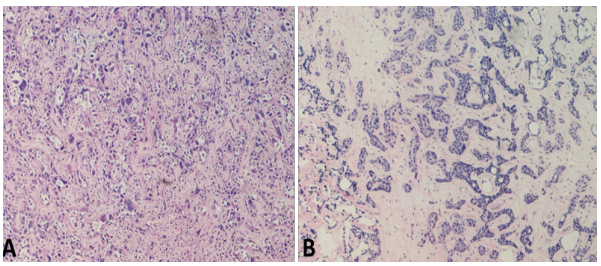

Figure 1. Histopathological Examination of the Samples Used for SAGE Library Construction. Hematoxylin and eosin staining for (A) an intrahepatic metastatic nodule and (B) a primary CCA

(Datson et al., 1999). RNA was isolated using RNeasy (Qiagen) according to manufacturer's instructions. Five µg of total RNA was used as starting material. The SAGE library clones containing inserts were purified and sequenced by Agencourt through the SAGE sequencing service (CGAP collaboration, GR). Sequence data from SAGE libraries were posted on the CGAP website http://cgap.nci.nih.gov/SAGE.

The SAGE Differential Gene Expression Display (DGED) tool was used to compare SAGE data between the pools of two CCA samples with a normal human liver which was provided on the CGAP website. DGED results were reported only when odds ratio (fold change) was significantly greater than 2 or significantly less than 0.5. The odds ratio was calculated by the following formula [(Tag count sum/ total tag count of CCA libraries)/ (Tag count/ total tag count of normal liver library)].

Immunohistochemistry

Surgical specimens from CCA patients who underwent operations in Srinagarind hospital, Faculty of Medicine, Khon Kaen University, Thailand were collected. Informed consent was obtained from all patients before surgery, and the research protocol (#HE471214) was approved by the Human Research Ethics Committee at Khon Kaen University. All specimens were histologically proved and subjected to the standard protocol. Briefly, deparaffinized 4 µm sections of formalin-fixed specimens were submitted to heat-induced antigen retrieval, incubated with the specific antibodies, and processed using the 3,3'-diaminobenzidine (DAB) as the chromatogen. The sections were then counterstained with hematoxylin and mounted with coverslips. The optimized dilutions used were previously reported (Seubwai et al., 2007; Junking et al., 2008; Obchoei et al., 2011)

Results

SAGE profiles of CCA

SAGE data of two CCA tissues generated by us were pooled and compared with SAGE data of normal liver tissues (library name: SAGE_Liver_normal_B_1) provided on the CGAP website by the SAGE DGED online tool. The numbers of total tags and unique tags of each library are shown in Table 1. At least 46,000 total tags were reported in CCA libraries. Using the criteria mentioned in the method, we identified 620 SAGE tags as differentially expressed. Of these, 111 tags were undefined or mapped to incomplete cDNA clones on the SAGE database while 509 tags (142 up-regulated and 367 down-regulated) were assigned to genes. These genes were analyzed by Ingenuity pathway analysis (IPA; Ingenuity® Systems, http://www.ingenuity.com) in order to determine enriched functional categories. As seen in Figure 2, the three most abundant categories found in this analysis were lipid metabolism, energy production and molecular transport.

Selection of therapeutic targets for CCA

To identify the therapeutic targets for CCA, we selected 4 up-regulated genes, TMSB10, LGALS3 (GAL3), VDR and PPIA (CYPA) as shown in Table 2 based on these criteria: (i) gene function involved in carcinogenesis/ metastasis; (ii) had specific drugs or inhibitors which could be used for targeted therapy; and (iii) the availability of antibodies that label formalin-fixed paraffin-embedded archival material. We further previewed the expression levels of these selected candidate genes in normal and tumor tissues of various gastrointestinal tract organs such as stomach, pancreas and colon by the SAGE Anatomic viewer tool as shown in Figure 3.

Validation of SAGE data by immunohistochemistry (IHC) of selected candidates

Selected up-regulated genes in CCA including TMSB10, GAL3, VDR and CYPA were investigated in an independent set of CCA samples by the IHC method in order to validate the SAGE data using a different technique and to localize the origin of cell types that expressed the selected genes. The data demonstrated that TMSB10 and GAL3 were detected in normal and malignant biliary cells. High intensity of positive staining of these two proteins was demonstrated in most of CCA tissues. Moreover, VDR and CYPA were significantly overexpressed at the protein

Table 1. SAGE Library Information

Library	CCA_K1	CCA_K2D	Normal liver
Total clones	2,592	2,688	2,688
Total tags	60,319	46,853	66,308
Unique tags	40,476	20,722	15,496
No. of differentially expressed tag		509	
Up-regulated		142	
Down-regulated		367	

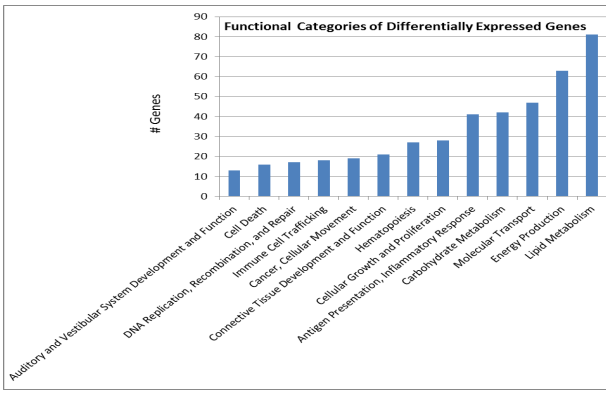

Figure 2. Functional Categories Assigned to Differentially Expressed Genes by IPA. There are 433 genes included in the gene ontology classification

Table 2. Information of 4 Selected Candidates

SAGE tag	CCA (K1+K2D)	Normal liver	Odds ratio	Unigene cluster	Gene symbol	Gene name
GGGGAAATCG	48	0	NaN	Hs.446574	TMSB10	Thymosin beta 10
TTCACTGTGA	100	1	61.93	Hs.531081	LGALS3, GAL3	Lectin, galactoside-binding, soluble, 3
GAGAAACCCT	25	2	7.74	Hs.524368	VDR	Vitamin D (1,25- dihydroxyvitamin D_3) receptor
CCTAGCTGGA	232	52	2.76	Hs.356331	PPIA, CYPA	Peptidylprolyl isomerase A (cyclophilin A)

*NaN stands for not a number

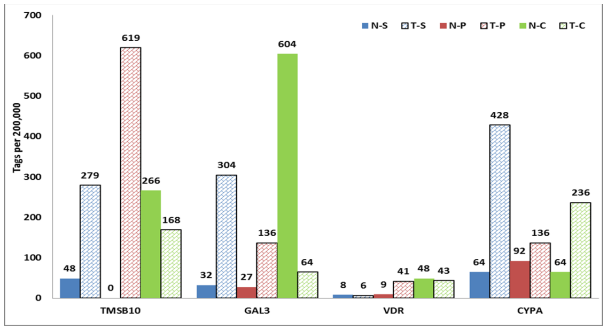

Figure 3. Expression Levels of 4 Selected Up-Regulated Genes in Other Gastrointestinal (GI) Tract Organs. Up-regulation of these genes is also demonstrated in other GI tract cancers, indicating cancer associated function. Data obtained from SAGE anatomic viewer tool. N, normal; T, tumor; Stomach (S): SAGE_Stomach_normal_ epithelium_B_body1 and SAGE_Stomach_carcinoma_B_G189. Pancreas (P): SAGE_Pancreas_normal_B_1 and SAGE_Pancreas_adenocarcinoma_B_91-16113. Colon (C): SAGE_Colon_normal_B_NC1and SAGE_Colon_adenocarcinoma_B_Tu102

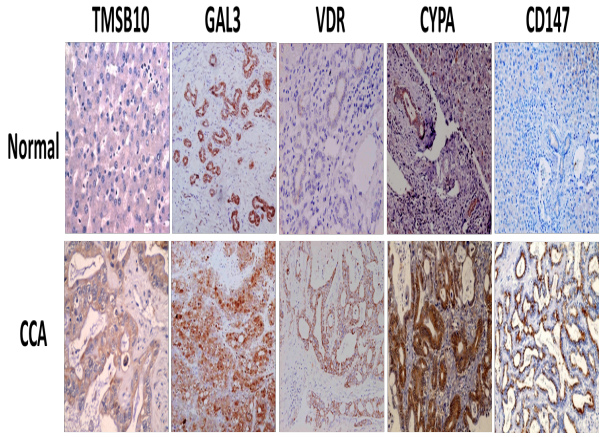

Figure 4. Immunohistochemical (IHC) Analysis of Candidate Genes. Representative IHC Staining of normal bile duct or liver and CCA tissues for the indicated proteins (TMSB10, GAL3, VDR, CYPA and CD147) are shown. The positive staining of TMSB10 and GAL3 was found in normal bile duct or liver and CCA tissues. Overexpression of VDR, CYPA and CD147 was obviously detected in CCA tissues. DAB staining; original magnification 20X

level in CCA tissues compared to normal bile ducts. We next investigated the cell surface signaling receptor of CD147 or extracellular matrix metalloproteinase inducer (EMMPRIN). CD147 expression was also highly elevated in CCA tissues and correlated with CYPA expression (Figure 4).

Discussion

The identification of genes differentially expressed in CCA tissues relative to normal tissues provides a basis for the development of novel strategies to detect and treat this highly lethal cancer. Because SAGE data can be compared between studies and laboratories, we constructed SAGE libraries of CCA and then compared to the publicly available SAGE library of normal liver tissue by using the SAGE DGED online tool on the CGAP website. We identified 509 genes that are differentially expressed in CCA. Of these 142 were up-regulated and 367 down-regulated relative normal liver tissues. These genes were functionally categorized based on their gene ontology classification. The most frequent tags correspond to genes involved in lipid metabolism, energy production and molecular transport. These findings were as expected since liver is a major organ in energy metabolism. Consistently, alteration of several lipid-related pathways including apolipoproteins (APOA1, APOA2) and fatty acid metabolism (fatty acid binding protein 1, liver; FABP1) were documented in intrahepatic cholangiocarcinoma (ICC) (Nishino et al., 2008).

Among 142 up-regulated genes, we validated 4 selected genes from SAGE comparison including TMSB10, GAL3, VDR and CYPA using immunohistochemistry. Their expression levels of normal and tumor tissues in a variety of gastrointestinal tract organs were analyzed by the SAGE Anatomic viewer (http://cgap.nci.nih.gov/SAGE/AnatomicViewer). The results indicated that most of the differentially expressed genes in CCA were also differentially expressed in other cancers. Noteworthy, the pattern of 4 selected genes of CCA were similar to those of stomach and pancreatic cancers.

IHC analysis demonstrated that TMSB10 and GAL3 were constitutively expressed in normal bile duct epithelia even their SAGE tag counts were very low. Although a count of zero tags indicates absence of detection in SAGE analysis, there may still be expression below the level of detection, which in this case is approximately 1 transcript per 50,000. The expression of these 2 genes was not drastically changed among the normal bile duct epithelia, but markedly enhanced in CCA cells. However, we additionally investigated TMSB10 protein expression in match pairs of primary and metastatic CCA. Preliminary results showed that under expression of TMSB10 has been found in metastatic tumors (unpublished manuscript). TMSB10 is the abbreviated gene symbol for thymosin β 10 which is widely distributed in many tissues with proven biological activities as an actin sequestering protein involved in cell motility. It has been reported to correlate with tumor biology such as cell proliferation, apoptosis, angiogenesis and metastasis behavior of several types of human cancers (Chen et al., 2005; Sribenja et al., 2009). The roles of TMSB10 and its signaling pathway in CCA cell migration and metastasis is now under investigation

in our laboratory.

GAL3, a β-galactoside-binding lectin, is a multifunctional protein implicated in a variety of biological functions, including tumor cell adhesion, proliferation, differentiation, cancer progression and metastasis (Castronovo et al., 1996). Our previous published study suggested that low GAL3 expression was significantly associated with lymphatic invasion. Suppression of GAL3 expression in two human CCA cell lines using siRNA substantially increased cell migration and invasion of CCA cells without alterations in cell proliferation (Junking et al., 2008). Similar to what we observed, Shimonishi and colleagues reported that GAL3 tends to disappear at later stages of ICC (Shimonishi et al., 2001). Taken together, regulation of GAL3 expression may therefore be an alternative therapeutic approach to control metastasis of CCA.

The vitamin D (1,25- dihydroxyvitamin D_3) receptor or VDR belongs to the steroid/thyroid hormone nuclear receptor superfamily. VDR up-regulation has been shown in primary tumor tissues of breast, colon and pancreatic cancers (Friedrich et al., 1998; Cross et al., 2001; Albrechtsson et al., 2003).

Validation of the VDR expression pattern corresponded to SAGE analysis. VDR was rarely expressed in normal bile duct epithelia but highly expressed in 74% of CCA tissues. The survival rate of CCA patients with positive VDR expression in tumor tissue was significantly better than that of patients with negative expression of VDR. In addition, treatment with $1,25(OH)_2D_3$, an active metabolite of vitamin D_3, in the CCA cell lines with high expression of VDR significantly reduced cell proliferation in a dose-dependent manner. The effect was not shown in lower VDR expressing CCA cell lines (Seubwai et al., 2007). These results are consistent with those seen in carcinomas of gastric, breast and colon (Albrechtsson et al., 2003; Banerjee and Chatterjee, 2003; Pelczynska et al., 2005). These findings suggest that supplementation of $1,25(OH)_2D_3$ or its analogs may be a potential strategy for long-term control of tumor development and progression in CCA patients. To prove this hypothesis, Seubwai et al. recently reported that supplementation of 22-oxa-D_3 to CCA-inoculated mice effectively suppressed tumor growth and induced cellular apoptosis in tissue samples from patients with CCA analyzed by using a histodrug response assay (Seubwai et al., 2010). These data encourage further investigation of $1,25(OH)_2D_3$ or its analogues as therapeutic agents in the treatment of CCA patients.

Cyclophilin A (CYPA) is an 18 kDa cytosolic protein that is thought to be the major intracellular target of the immunosuppressive drug cyclosporin A (CsA) (Handschumacher et al., 1984). Various forms of evidence exhibited elevation of CYPA in several types of cancers including non-small cell lung cancer, pancreatic adenocarcinoma, hepatocellular carcinoma, oral cancer, buccal squamous cell carcinoma and CCA (Campa et al., 2003; Howard et al., 2004; Obama et al., 2005; Yang et al., 2005; Li et al., 2006; Wang et al., 2006). Consistent with the literature, strongly positive staining of CYPA in CCA tissues has been revealed. We have recently reported that increasing of CYPA expression could accelerate CCA cell proliferation and tumor growth. Moreover, treatment of CsA, an inhibitor of CYPA inhibited CCA cell proliferation in a dose-dependent fashion (Obchoei et al., 2011). Application of CsA or CYPA deletion may be used as the molecular target for CCA therapy.

Interplay of CYPA and CD147/EMMPRIN (extracellular matrix metalloproteinase inducer) has been demonstrated to affect cell proliferation, migration and differentiation (Sherry et al., 1992; Kim et al., 2004; Yang et al., 2005). These citations led us to determine the expression pattern of CD147 in CCA tissues. Highly elevated CD147 expression in the studied CCA tissues has been detected. The results were consistent with neoplasias such as lung, breast, oral squamous cell carcinoma, melanoma and follicular thyroid carcinoma (Polette et al., 1997; Bordador et al., 2000; Kanekura et al., 2002; Omi et al., 2012). CD147, is a transmembrane glycoprotein that is categorized as a member of the immunoglobulin superfamily and can stimulate both tumor cells and nearby fibroblasts and endothelial cells to produce MMPs, facilitating cancer cell invasion (Nabeshima et al., 2006). In 2006, Chen et al. developed iodine (^{131}I) metuximab injection (Licartin), a novel CD147-specific monoclonal antibody which was safe and active for hepatocellular carcinoma (HCC) patients in phase I/II trials (Chen et al., 2006). This information suggest that CD147 may be the potential target for cancer treatment.

In conclusion, we constructed SAGE libraries of liver fluke- associated CCA. These gene expression profiles provided candidate genes including TMSB10, GAL3, VDR, CYPA and CD147 which are potentially involved in carcinogenesis, invasion/ metastasis of CCA. Furthermore, these selected candidates may represent some promising therapeutic targets for CCA.

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