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Identification of Specific Biomarker Genes for Separating Intrahepatic Cholangiocarcinoma Type

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Abstract **Intrahepatic cholangiocarcinoma (ICC) is one of the most lethal liver cancers. ICC is classified into 3 types: mass-forming (MF), periductal infiltrating (PI), and intraductal growth (IG). Among these, the PI and IG types are located in the bile duct, so that histopathologic diagnosis may be quite difficult. This study aims to identify the specific biomarkers for distinguish between PI and IG types. The top five up-regulated genes from PI (C19ORF33, UPK1B, CTHRC1, GREM1, KLK11) and IG type (CFTR, SLC5A1, MUC17, BDKRB2, CCL18) were examined in ICC samples (11 PI and 11 IG samples) using SYBR green-based real-time reverse transcription-PCR (qRT-PCR). Among these genes, the results showed that the CTHRC1 gene of the PI type identified with the highest up-regulation in PI (72.7%) and 27.3% in IG samples (*P*=0.034), whereas the CFTR gene of the IG type showed the highest up-regulation in 72.7% of IG and 9.1% of PI samples (*P*=0.001). Hence, the up-regulation of CTHRC1 and CFTR may serve as biomarkers for separating PI from IG types, respectively, and is also a potential diagnostic in ICC.** *(Thai Cancer J 2020;40:76-85)*
Keywords: intrahepatic cholangiocarcinoma, biomarker, genes

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การศึกษาตัวบ่งชี้ทางชีวภาพในการจำแนกชนิดของมะเร็งท่อน้ำดีภายในตับ

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บทคัดย่อ มะเร็งท่อน้ำดีภายในตับเป็นมะเร็งชนิดที่ร้ายแรงที่สุดชนิดหนึ่ง แบ่งได้เป็น 3 ลักษณะตามพยาธิสภาพ ได้แก่ massforming (MF), periductal infiltrating (PI) และ intraductal growth (IG) และเนื่องจากมะเร็งท่อน้ำดีภายในตับชนิด PI และ IG เกิดบริเวณภายในท่อน้ำดีจึงอาจทำให้ตรวจหาด้วยวิธีทางจุลพยาธิวิทยาได้ค่อนข้างยาก ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์เพื่อหาตัวบ่งชี้ทางชีวภาพในการจำแนกมะเร็งท่อน้ำดีภายในตับ โดยคัดเลือกยีนที่มีการแสดงออกสูงสุด 5 ลำดับแรกจากแต่ละชนิด ได้แก่ กลุ่ม PI: C19ORF33, UPK1B, CTHRC1, GREM1, KLK11 กลุ่ม IG: CFTR, SLC5A1, MUC17, BDKRB2, CCL18 มาทดสอบกับตัวอย่างมะเร็งท่อน้ำดีภายในตับ (ชนิด PI 11 ตัวอย่างและชนิด IG 11 ตัวอย่าง) ด้วยเทคนิคเรียลไทม์อาร์ทีพีซีอาร์ (SYBR green-based real-time reverse transcription-PCR) ผลการศึกษาพบว่ายีน CTHRC1 จากกลุ่ม PI มีการแสดงออกสูง (up-regulated) มากที่สุด (ร้อยละ 72.7) ในตัวอย่างชนิด PI และพบร้อยละ 27.3 ในตัวอย่างชนิด IG (P=0.034) ในขณะที่ยีน CFTR จากกลุ่ม IG มีการแสดงออกสูงมากที่สุด (ร้อยละ 72.7) ในตัวอย่างชนิด IG ส่วนในตัวอย่างชนิด PI พบเพียงร้อยละ 9.1 (P=0.001) ดังนั้นยีน CTHRC1 จากกลุ่ม PI และยีน CFTR จากกลุ่ม IG สามารถนำมาใช้เป็นตัวแทนของตัวบ่งชี้ทางชีวภาพในการจำแนกความแตกต่างระหว่างมะเร็งท่อน้ำดีภายในตับชนิด PI และ IG ซึ่งผลที่ได้นี้สามารถนำมาใช้ต่อยอดในการวินิจฉัยมะเร็งท่อน้ำดีภายในตับได้อย่างมีศักยภาพต่อไปในอนาคต *(วารสารโรคมะเร็ง 2563;40:76-85)*

คำสำคัญ: มะเร็งท่อน้ำดีภายในตับ ตัวบ่งชี้ทางชีวภาพ ยีน

Introduction

ICC is the second most common primary hepatobiliary cancer globally and is a major cause of cancer-related deaths that shows no indication of a decrease in mortality rate[1-3]. In developing countries especially in Thailand has the highest incidence of ICC in the world, which may be related to *Opisthorchis viverrini* infection[4-6]. The prevalence of liver fluke infection in northeast Thailand is about 317.6 per 100,000 person-years[6].

In detection, it should be emphasized that imaging is not always reliable for the diagnosis of some ICC. For instance, ICC <2 cm in size mimic HCC because of the absence of the progressive enhancement pattern[7]. Moreover, it may quite difficult to use only histopathologic diagnostic for differential three types of ICC (MF, PI and IG) which are located in bile duct. From the above problems, there are several studies searching for novel biomarkers that are likely to be helpful in diagnosis,

characterization, therapy selection including separating type. A deep understanding of the relative relevance of each biomarker will be a key to successfully diagnose diseases, therapy and monitoring[8-11].

To date, 2821 up-regulated genes in ICC for Thai patients have been detected. Moreover, 531 and 1535 genes were specific up-regulated in PI and IG types, respectively[12,13]. The present study aims to validate these selected specific genes especially in PI and IG types, which may be used as a molecular biomarker to identify type of ICC.

Materials and Methods

Patients and clinical features

ICC fresh tumor samples (11 PI and 11 IG samples) including with corresponding normal samples were leftover specimen collection at Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, Thailand which transferred from Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand. The study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Thailand (MUTM 2008-004-02) and (MUTM 2014-057-03).

Sample processing

Total RNA was isolated by TRIzol® reagent (Invitrogen, USA) according to the manufacturer's instructions. The total amount of RNA isolated was quantified using a Nanodrop 2000 c spectrophotometer (Thermo Fisher Scientific, USA) OD_{260} measurements, and its quality evaluated by visualizing specific bands (18S and 28S rRNA) using 1.5% gel electrophoresis. First strand cDNA was performed using Superscript® VILO™ cDNA Synthesis kits (Invitrogen, USA) and purified by DNAclear™cDNA Purification Kit (Applied Biosystems, USA). The quantity of purified cDNA was measured by Nanodrop2000 c spectrophotometer and qualified using conventional PCR with GAPDH primer.

Validation of candidate gene expression by SYBR green-based real-time reverse transcription-PCR (qRT-PCR)

Primers were designed by Primer-BLAST program (NCBI) using nucleotide sequences from the NCBI database. The nucleotide sequences of all primers are shown in Table 1. The LightCycler® FastStart DNA Master SYBR Green I (Roach, Germany) was used to perform SYBR green qPCR. Each 10 µl of reaction mixture contained 5 µl of 2X MasterMix SYBR Green, 0.3 µM of forward primer, 0.3 µM of reverse primer, 3.4 µl of sterile distilled water and 1 µl of cDNA. The reactions were carried out using a Roche Lightcycler® 480 Real-Time PCR System (Roche, Germany), with the following cycling conditions: pre-incubation at 95°C for 5 minutes, followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing temperature (specific to the primer) for 20 seconds, and extension at 72°C for 20 second. The $2^{-\Delta\Delta CT}$ method was used to calculate relative quantification in gene expression. Over-expression of mRNA was specified as N-fold change ≥1.5, normal mRNA expression was specified as N-fold change (range 0.5001-1.4999), and under-expression was specified as N-fold change ≤0.5. All samples were performed at least twice[20,21].

Table1 The oligonucleotide sequences of PI and IG types

Type	Gene	Sequence (5′ to 3′)
PI	C19ORF33-F	AGCATTCCTCCAACGGGCA
	C19ORF33-R	GTCGAACTCCATGGCGGTAA
	UPK1B-F	AGCCTCTACCCACTGCTTGA
	UPK1B-R	GGAAGAGGTTGGGTGTGAAA
	CTHRC1-F	TTGTTCAGTGGCTCACTTCG
	CTHRC1-R	TTCAATGGGAAGAGGTCCTG
	GREM1-F	GCTCTGGCATTCAGAGAACC
	GREM1-R	AAATTCGCCTAGCGTGAGAA
	KLK11-F	ATCACCATCATTGAGCACCA
	KLK11-R	CCCAGGAGATAATGCCTTGA
IG	CFTR-F	ACAGGATGGTTCCCTTGATG
	CFTR-R	GGGCTGTCCTGTGACAATTT
	SLC5A1-F	CAACATCGCCTATCCAACCT
	SLC5A1-R	TAAACAACCTTCCGGCAATC
	MUC17-F	CGCTTTCTGCAACCAGTACA
	MUC17-R	ACTGGAGTGAGCAGGAGGAA
	BDKRB2-F	GGTTGTGCTGCTGCTATTCA
	BDKRB2-R	GCACACTCCCTGGTACACCT
	CCL18-F	AGAAGGAGGCCAGGAGTTGT
	CCL18-R	GTGGAATCTGCCAGGAGGTA

F: forward primer, R: reverse primer

Statistical analysis

The association between gene expression of PI and IG types were performed with SPSS statistical software. Differences in expression levels in these types were analyzed using the student t-test, and $P<0.05$ was considered statistically significant.

Results

Among top five of up-regulated genes selected from each PI and IG types, CTHRC1 gene was found as the most up-regulated gene in PI sample (72.7%) followed by C19ORF33 (63.6%), GREM1 (54.5%), KLK11 (45.5%), MUC17 (45.5%), UPK1B (36.4%), BDKRB2 (27.3%), CCL18 (27.3%), SLC5A1 (18.2%), and CFTR (9.1%), as shown in Figure 1.

Table 2 Gene expression patterns of CTHRCl and CFTR in PI and IG samples

sample	No.	Gene expression patterns	
		CTHRCl	CFTR
PI	1	+	-
	2	+	-
	3	-	-
	4	+	+
	5	-	-
	6	+	-
	7	+	-
	8	-	-
	9	+	-
	10	+	-
	11	+	-
IG	1	-	-
	2	-	-
	3	-	+
	4	-	+
	5	+	+
	6	-	-
	7	+	+
	8	-	+
	9	-	+
	10	-	+
	11	+	+

+ = up-regulated, - = not up-regulated

Meanwhile, the result of up regulated gene in IG sample show that CFTR gene is the most predominant gene which was found in 8 from 11 samples (72.7%) followed by MUC17 (63.6%), BDKRB2 (63.6%), GREM1 (63.6%), C19ORF33 (54.5%), UPK1B (54.5%), CCL18 (36.4%), KLK11 (36.4%), SLC5A1 (27.3%), and CTHRC1 (27.3%), as shown in Figure 2.

Of 22 ICC samples (11 PI and 11 IG samples), the up-regulated CTHRC1 gene was found in 8/11 (72.7%) of PI samples but was detected at relatively low frequency in 3/11 (27.3%) of IG samples(P=0.034), whereas up- regulate of CFTR gene was presented only in 1 (9.1%) of PI sample and were identified with high frequency in 8/11 (72.7%) of IG samples (P=0.001), the data are shown in Table 2 and Figure 3.

Discussion and Conclusion

ICC is the cancer of the intrahepatic bile ducts and together with hepatocellular carcinoma (HCC), which is the majority of primary liver cancers[1-3]. Previous studies have raised some concern about the incidence and mortality related to ICC, whereas the incidence of extrahepatic cholangiocarcinoma (ECC) has remained relatively stable over time[14-16]. Patients with early stage of ICC are usually asymptomatic. Moreover, the clinical presentation is non-specific and difficult to establish diagnosis[17]. Therefore, the identification of ICC novel biomarkers are the keys of successful early diagnosis and it is also useful for effective treatment of patients with CCA.

In the present study, of top five of up-regulated genes from either PI or IG type of ICC determined by a previous report[12] were validated by SYBR green qRT-PCR showed that CTHRC1 and CFTR genes could be the representative of biomarkers for identification PI and IG types, respectively. CTHRC1 was the predominant gene from PI type, which showed significantly different of up-regulated gene between PI and IG samples. Previous studies revealed that collagen triple helix repeat containing 1 is a protein that in humans is encoded by the CTHRC1 gene and it has been

identified as a cancer-related gene[18]. Numerous reports showed the association between expression of CTHRC1 and cancer proliferation, invasion, metastasis, clinical stages, tumor size, including nodal metastasis. Moreover, up-regulation of CTHRC1 is a positive indicator of poor prognosis in malignant tumor patients including lung cancer, epithelial ovarian cancer, colorectal cancer, hepatocellular carcinoma and breast cancer[19-23] .

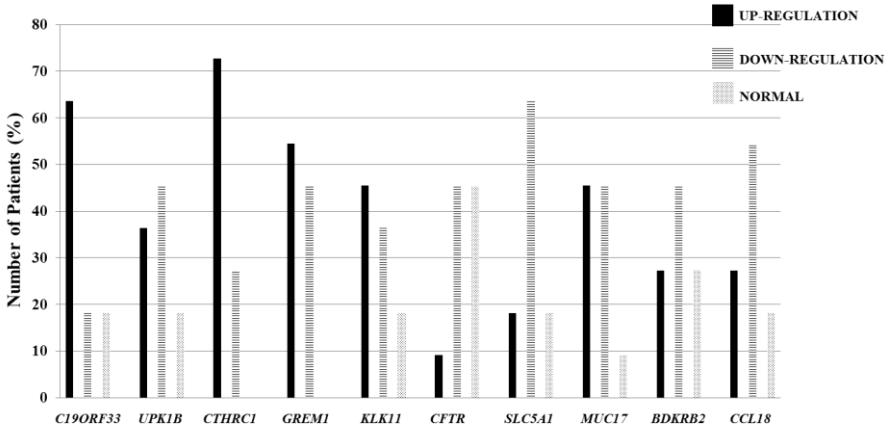

Figure 1 Differential gene expression in PI samples by SYBR Green qRT-PCR

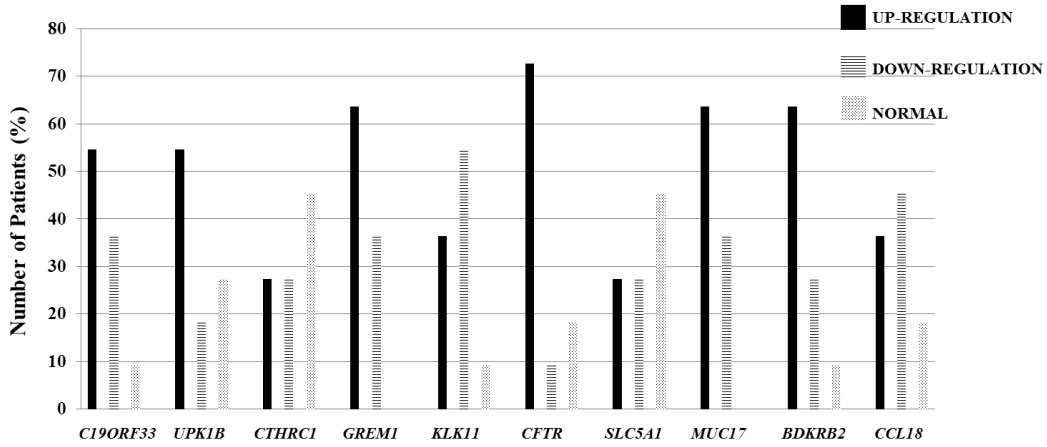

Figure 2 Differential gene expression in IG samples by SYBR Green qRT-PCR

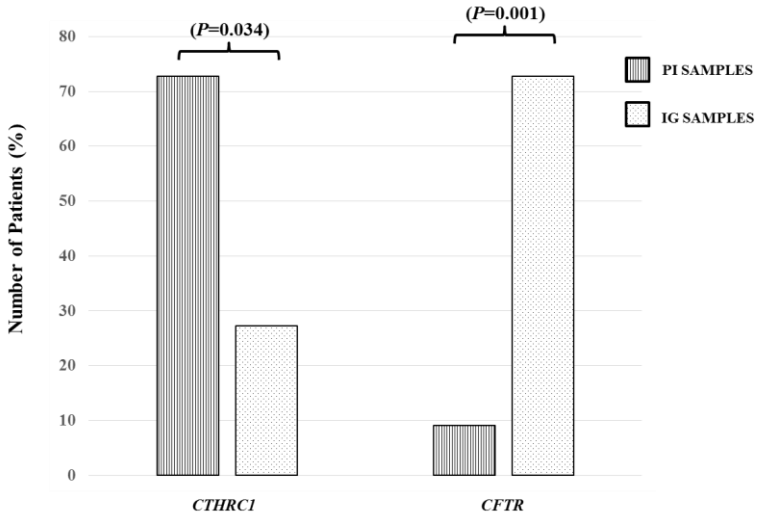

Figure 3 Comparison between up-regulation of CTHRC1 and CFTR genes in PI and IG samples by SYBR

Green qRT-PCR

A series of clinical studies have demonstrated that CTHRC1 protein and mRNA expression were detected ubiquitously in many human solid tumors[24], and the overexpression of CTHRC1 in tumor cells increased the characteristics of malignant aggressiveness including increased tumor growth, invasion and metastasis. Furthermore, our results provided experimental evidence that up-regulated of CTHRC1 gene could be found in ICC. Therefore, CTHRC1 gene may be developed as a potential crucial biomarker for detection of PI type in the ICC cases in Thai patients.

The CFTR gene was the most dominant gene from IG type and the up-regulation of CFTR gene was significantly different between IG and PI samples. Previous studies have suggested that cystic fibrosis transmembrane conductance regulator (CFTR) is a membrane protein and chloride channel in vertebrates that is encoded by the CFTR gene, which it may be associated with several cancers[25,26]. It has been shown the evidence of CFTR gene expression could serve as a biomarker for lung cancer metastasis and prognosis, a prognostic predictor in breast cancer and cervical cancer[27-29].

Previous research suggested that CFTR may have a more generic function in development of cancer, both in vitro and in vivo studies[28]. Additionally, based on previous observation showed the CFTR gene provides instructions for making a protein called the CFTR protein, which is a membrane of glycoprotein expressed in certain epithelia, liver and predominantly found in the intrahepatic bile duct cells, where it plays a role in ductular bile formation[25,30]. This information is consistent to other study which reported that CFTR is expressed in the epithelia of the intrahepatic and extrahepatic bile ducts and the gallbladder and the CFTR protein is localized to the apical domain of these cells[25]. In particular, the report[31] revealed that CFTR expression was enhanced in patients with hepatolithiasis may support the supposition that CFTR is causally related to intrahepatic stones formation[32]. Interestingly, it is widely known that longstanding intrahepatic stones lead to ICC[33]. Moreover, this study indicated that the CFTR gene could be the potential biomarker for identification IG type of ICC.

From our knowledge, several studies have been searching for a novel biomarker for detection or separating type of cancer using immuno histochemical assay (IHC), fluorescence in situ hybridization (FISH) technique, including qRT-PCR. Interestingly, the previous studies showed that qRT-PCR has the potential to become standard in terms of its performance, accuracy, sensitivity, broad dynamic range, and high throughput capacity[34]. Moreover, qRT-PCR is more convenient, easier, and rapid compared to IHC and FISH[35,36].

In summary, our observations indicated that the up-regulation of CTHRC1 and CFTR genes were mainly exhibited in PI and IG types of ICC and these genes may be developed as valuable prognosis biomarkers for PI and IG types, respectively. However, a greater number of cases should be included to make the prospective studies valid in the future. Interestingly, to the best of our knowledge, our data have provided clinical findings for the first time that CTHRC1 and CFTR genes expression are well correlated with ICC.

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