การตรวจหา markers ที่จำเพาะในมะเร็งท่อน้ำดี: ผลการศึกษาเบื้องต้น

Identification of Specific Markers for Cholangiocarcinoma: A Preliminary Study

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บทคัดย่อ

การใช้เทคนิค real-time RT-PCR แทนการดูเซลล์ด้วยกล้องจุลทรรศน์ ซึ่งเป็นวิธีดั้งเดิม น่าจะสามารถเพิ่มความไวในการตรวจหาเซลล์มะเร็งท่อน้ำดีในน้ำล้างช่องท้องของผู้ป่วยมะเร็งท่อน้ำดีได้ แต่เนื่องจากในปัจจุบันยังไม่มียีนที่จำเพาะต่อมะเร็งท่อน้ำดี โดยการศึกษาก่อนหน้าในระดับโปรตีนด้วยเทคนิค immune-histochemistry พบว่า HNF1β และ CK7 มีความจำเพาะต่อเซลล์เยื่อบุท่อน้ำดี และเซลล์มะเร็งท่อน้ำดี การศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อตรวจหายีนที่จำเพาะต่อเซลล์มะเร็งท่อน้ำดี ในชิ้นเนื้อที่เก็บแบบ formalin-fixed and paraffin-embedded (FFPE) โดยใช้เทคนิค real-time RT-PCR ถึงแม้ว่า RNA ที่ได้จากชิ้นเนื้อ FFPE จะมีคุณภาพไม่ดีเท่าชิ้นเนื้อที่เก็บโดยวิธี fresh frozen แต่ยังสามารถนำมาใช้ในการวิเคราะห์การแสดงออกของยีน *HNF1β* และ *CK7* ได้ จากผลการทดลองพบว่ามีการแสดงออกของยีน *HNF1β* และ *CK7* ในชิ้นเนื้อมะเร็งท่อน้ำดี 80% และ 36.67% ตามลำดับ แต่ถ้าใช้ยีนทั้งสองร่วมกันพบว่าการแสดงออกเพิ่มขึ้นถึง 90%

ABSTRACT

The using of real-time RT-PCR technique instead of cytology technique might increase sensitivity of the detection of cholangiocarcinoma (CCA) cell in peritoneal lavage from CCA patients. However, nowadays, the promising genes for CCA cell are still limited. *HNF1β* and *CK7* have been previously shown to be specific to biliary epithelial cell and CCA cell by immunohistochemistry. Hence, this work aims to identify the expression of *HNF1β* and *CK7* genes in CCA cell compared to hepatocyte in FFPE tissue using real-time RT-PCR. Although the quality of RNA retrieved from FFPE tissue is not as good as from fresh frozen one but still, it can be analysed for gene expression. The result shows that the expression of *HNF1β and CK7* in cholangiocarcinoma FFPE tissues can be detected 80% and 36.67%, respectively. Interestingly, when combine the expression of these two genes together, the detection rate rises to 90%.

คำสำคัญ: เอฟเอฟพีอี, เรียล-ไทม์ พีซีอาร์, ตัวบ่งชี้, มะเร็งท่อน้ำดี

Key words: FFPE, real-time PCR, marker, cholangiocarcinoma

INTRODUCTION

Cholangiocarcinoma is an adenocarcinoma that derives from bile duct epithelium along the biliary tree. It can be classified into 2 types due to the anatomical sites: intrahepatic cholangiocarcinoma and extrahepatic cholangiocarcinoma. The epidemiology and risk factors is regional diverse and nearly all early CCA patients are underdiagnosed due to the lack of specific symptoms. Therefore, most of the CCA patients are diagnosed at advance stage (Patel T, 2011). At present, surgical resection is the most effective method for curing. The survival rate is better comparing to the past three decades (Friman S, 2011). Laparoscopy is performed in order to investigate the CCA staging. The cytology of the peritoneal lavage collected by laparoscopy is very beneficial to exclude the metastatic case. Nevertheless, this technique has very low sensitivity (Wong J, 2012). Therefore, we plan to use real-time RT-PCR technique to increase the sensitivity. Our major problem is that there are no promising genes for the detection of CCA cells from peritoneal lavage. Interestingly, HNF1β and CK7 has previously reported to be strongly expressed to biliary epithelial cell and CCA cells but weakly expressed in hepatocyte by immunohistochemistry technique (Limaye PB, 2010), (Bateman AC, 2010). To date, the molecular study for these 2 genes is still limit. This work aims to identify the expression of *HNF1*β and *CK7* in CCA cell in FFPE tissues compared to hepatocyte using real-time RT-PCR. Due to RNA is unstable and easy degradable and the formalin is the cause of fragmented and cross-linked of RNA, RNA retrieved from FFPE gives not very promising results (Deben C, 2013). However, they can be used to analyse for gene expression and a lot of FFPE are available in the hospital. We expect these markers to be able to differentiate CCA cells from hepatocytes. The findings would have a significant clinical impact; in particular, the obtained genes that might be further developed for detecting biliary epithelial cells and CCA cells in peritoneal lavage from cholangiocarcinoma patients.

MATERIALS AND METHODS

50 FFPE tissues from CCA patients who underwent surgery at Siriraj Hospital from 2009 to 2012 were obtained at the department of pathology. This study was approved by Siriraj Institutional Review Board at faculty of Medicine Siriraj Hospital, Mahidol University. RNA was isolated from sections with approximately 1.5 cm^2 surface area. The concentration, A260/A280, and A260/230 ratio were measured by NanophotometerTM (Implen, Germany).

After the RNA quality was assessed, the RNA template was used for cDNA synthesis. The real-time PCR was performed on the Strategene Mx3005P real-time PCR machine (Strategene, USA) against a reference gene. The sequences of each primer were shown in Table1. The PCR reaction were incubated at 94°C for 10 min, following by 40 cycles of 94°C for 35 sec, 58°C for 20 sec, and 72°C for 20 sec according to previous study.

PCR primers were designed to amplify 89 and 110 bp fragments of *HNF1*β and *CK7*, respectively. Their performance were checked in normal white blood cells, pancreatic cell lines, CCA fresh frozen sample, CCA FFPE samples, non-template control, and non RT control. The reaction mixtures and the conditions were mentioned earlier as *ACTB*.

Table 1 Primers used in this study

Gene	Primer	Product size (bp)
ACTB	F-5'-CAACCGCGAGAAGATGCC-3'	94
	R-5'-AGAGGCGTACAGGGTTAGCA-3'	
HNF1β	F-5'-GGGCGGAGGTGGACCGGAT-3'	89
	R-5'-TCTGGGATGTTGTGTTGCT-3'	
CK7	F-5'-ATTAGACCACCGCACAG-3'	110
	R-5'-ATTCAGGGCATCCACCTT-3'	

To study the expression of *HNF1β* and *CK7*, real-time PCR amplification was performed on the Strategene Mx3005P real-time PCR machine (Strategene, USA). The optimum condition started from initial denaturation at 94°C for 10 min followed by 45 cycles of 94°C for 35 sec, 58°C for 20 sec, and 72°C for 20 sec. Each sample was run in triplicates. For the melting curves analysis, the fluorescence signals were measured continuously. The PCR products were denatured into single strand during reducing temperature. The different melting temperature represented the different lengths and sequences of PCR product.

RESULTS AND DISCUSSION

The total RNA concentrations were in range of 24 – 1,268 ng/μL. The estimated mean concentration was 246 ng/μL. The mean of A260/A280 and A260/A230 ratio were 1.969 and 1.753 with the values ranging from 0.922 to 2.333 and 0.321 to 2.476, respectively. Since the very poor quality of RNA was extracted from FFPE sources, as expecting the value showed greatly variation in both concentration, the ratio of A260/A280, and A260/A230. As the initial concentration 1 μg, we excluded 5 cases with too little amount of RNA concentration. The other 45 cases were amplified using the Brilliant II SYBR Green Master Mix in a Strategene Mx3005P real-time PCR machine against *ACTB*. Its PCR product size is 92 base pair.

The results showed that only 30 cases can be amplified over the threshold within 40 cycles by using the pancreatic cell line as an inter-run calibrator. Figure 1 showed that even the initial RNA concentration is nearly the same; the quality of each sample is very diverse. The later quantification cycle (Cq) the more distinct between triplicate reactions reflected the lower RNA performance.

To validate the in-house primers performance, they were tested with many types of sample. First, they were proved with normal white blood cells (WBC) since the peritoneal lavage normally contains them. The results represented that there was no expression of both genes in normal WBC. Furthermore, both *HNF1β* and *CK7* expressed in CCA fresh frozen tissue and CCA FFPE. On the other hand, they did not express in pancreatic cell lines, non-template control, and non-reverse transcription control. This represented that the PCR product amplify from mRNA not gDNA.

From the preliminary study, 30 CCA FFPE samples were amplified against *HNF1β* and *CK7*. Expression of *HNF1β* was detected in 24 of 30 cases or 80% while expression of *CK7* was detected in 11 of 30 cases or 36.67%. Interestingly, when combine the expression of these two genes together, the detection rate increased to 90% (27/30). Therefore, these 2 candidate genes might be interesting for further study not only in CCA cells but also in normal biliary duct and normal gall bladder.

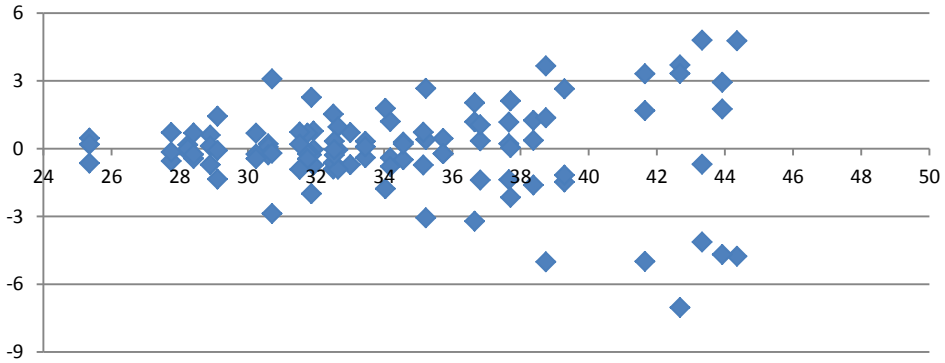

Figure 1 Plot of the % difference in Ct values (in triplicate) vs. mean Ct of ACTB

CONCLUSION

This preliminary showed that the expression of *HNF1β* was detected in 80% of FFPE samples. Although, the expression of *CK7* was not very impressive, the combination of *HNF1β* and *CK7* expression as molecular multimarkers could increase the detection rate up to 90% of cases. However, work is on the way to study the expression of these two candidate genes in CCA cell, normal hepatocyte, normal cholangiocyte and gall bladder epithelial cell to confirm that they are specific to CCA cell.

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