RESEARCH ARTICLE

Identification of Pancreatic Cancer in Biliary Obstruction Patients by FRY Site-specific Methylation

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Abstract

Background: Methylation at cg 16941656 of FRY is exclusively found in normal pancreatic tissue and has been proven to be specific for pancreatic-in-origin among several adenocarcinomas. Here, we investigated methylated DNA in the bile as a biomarker to differentiate the cause of obstruction between pancreatic cancer and benign causes. Materials and Methods: Bile samples of 45 patients with obstructive jaundice who underwent ERCP were collected and classified into pancreatic cancer (group 1) and benign causes (group 2) in 24 and 21 patients, respectively. DNA was extracted from bile and bisulfite modification was performed. After, methylation in cg 16941656 of FRY was identified by real-time PCR, with beta-actin used as a positive control. Results: Methylated DNA was identified in 10/24 (41.67%) and 1/21 (4.8%) of cases in groups 1 and 2, respectively (P= 0.012). The sensitivity, specificity, positive predictive value and negative predictive value to differentiate pancreatic cancer from benign causes were 42%, 95%, 91%, and 59%, respectively. Conclusions: Detecting a methylation at cg 16941656 of FRY in bile has high specificity, with an acceptable positive likelihood rate, and may therefore be helpful in distinguish pancreatic cancer from benign strictures.

Keywords: Bile - biliary obstruction - pancreatic cancer - DNA methylation - FRY.

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Introduction

The differentiation between benign and malignant nature of biliary obstruction is essential. Conventionally, definite diagnosis of malignancy is based on histopathology, cytology or long-term clinical and image follow-up (Hadizadeh et al., 2014; Goral, 2015; Navaneethan et al., 2015). Detection of pancreatic cancer cell in bile is difficult. The current standard technique to obtain cells or tissue have limited diagnostic yield. The sensitivity of endoscopic retrograde cholangiopancreatography (ERCP)-based techniques, either brush cytology or intraductal biopsy, is less than 50% (Navaneethan et al., 2015). Although endoscopic ultrasonography with fine needle aspiration (EUS-FNA) has sensitivity of up to 80% (Sadeghi et al., 2016), the risk of tumor seeding at needle tract is still a concern (Katanuma et al., 2012). Moreover, serum carbonic antigen 19-9 which is a widely-used marker for pancreatic cancer and cholangiocarcinoma has low sensitivity (Patel et al., 2000). Hence, the pancreatic cancer DNA detection should be a better approach. Various molecular markers are proposed to be a biomarker for pancreatic cancer DNA detection (Majumder et al., 2015) but there are no perfect marker in bile specimen (Lourdusamy et al., 2015). CEAM6 and NGAL were promising in differentiating pancreatic cancer and cholangiocarcinoma from benign biliary strictures but they still needed further studies (Zabron et al., 2011; Budzynska et al., 2013; Farina et al., 2014).

From our previous study by bioinformatic approach, we reported the tissue-specific methylation database (Muangsub et al., 2014). Later using this information, we demonstrated that the methylation at cg16941656 of FRY is exclusively found in pancreatic tissue. Moreover, the methylation at this position is highly specific to pancreatic cancer and can be used to differentiate pancreatic cancer when from adenocarcinomas from other organs with high sensitivity and specificity (100% and 98.7%, respectively) (Srisuttee et al., 2016).

In this study, we aim to investigate the possibility of this marker in differentiating the cause of obstruction between pancreatic cancer and benign causes by real-time PCR approach.

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Materials and Methods

Ethics statement and subject population

The study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB number 360/57). All patient volunteers with obstructive jaundice who underwent ERCP at the Excellence Center for Gastrointestinal Endoscopy, King Chulalongkorn Memorial Hospital during 2012-2015 were screened. The inclusion criteria were 1) age of 18 years or more, 2) indicated for therapeutic ERCP, 3) diagnosis of pancreatic cancer or benign biliary obstruction, and 4) obtained informed consent. Criteria for diagnosis of pancreatic cancer were 1) positive histopathology or 2) positive cytology or 3) dynamic cross-sectional imaging demonstrated hypovascular mass at pancreas which progressed after follow up. The criteria for benign causes were 1) demonstration of stone during ERCP and final cholangiogram revealed no residual stricture or filling defect or 2) negative cytology or biopsy for malignancy, no progression of stricture and no development of mass and patient survival after 6-month follow up. Exclusion criteria were 1) contraindicated for ERCP, 2) recent pancreatitis within 1 month, or 3) contrast media was injected into bile duct before bile aspiration.

The staging of pancreatic cancer is based on the American Joint Committee on Cancer (AJCC) 7th edition (2010). In brief, stage 0 includes carcinoma in-situ without nodal or distant metastasis. Stage I includes tumor limited to the pancreas without nodal or distant metastasis. Stage II includes tumor extends beyond the pancreas without involvement of celiac axis, superior mesenteric artery, and no nodal or distant metastasis, or tumor of any size with regional lymph node metastasis, but no distant metastasis. Stage III includes tumor invades celiac axis or superior mesenteric artery without distant metastasis. And stage IV includes tumor with distant metastasis. Sample size calculation was based on previous studies which reported the methylation level at cg16941656 of FRY of pancreatic tissue and liver of 66.77% (Bibikova et al., 2009) and 2.21% (Bibikova et al., 2009; Pai et al., 2011; Shen et al., 2012), respectively. The sample size of 20 samples per group is required to detect the difference with type-I error of 0.05 and power of 80%.

Specimen Recruit

During ERCP, biliary cannulation was done by catheter preloaded with guide-wire. After successful biliary cannulation, fluoroscopic images were used to guide the guide-wire to negotiate the stricture without contrast injection. When the guide-wire was passed through the stricture, the catheter followed the guide-wire proximity to the stricture where bile aspiration with double-syringe technique was done. The bile aspirated by the first syringe was discarded to prevent possible contamination and 3-5 ml of bile sample collected by the second syringe was immediately stored in a sterile cryotube freezer at -80°C without preservation solution.

DNA extraction and sodium bisulfite modification

One milliliter of bile sample was centrifuged for 10 minutes at 15,000 g and then the precipitated pellet was lysed by proteinase K and lysis buffer overnight at 50°C before transferring for DNA extraction by a standard phenol-chloroform extraction protocol. After precipitation, the isolated genomic DNA was eluted using distilled water and, after elution, used for bisulfite treatment. Bisulfite modification of DNA was performed by using the EZ DNA methylation kit (Zymo Research, ZYMO RESEARCH CORP, Orange, CA) according to the manufacturer's instructions.

Quantitative Real time PCR of FRY methylation

The quantitative real time (qRT) PCR of FRY methylation was previously described (5). In brief, 2 μl of bisulfite DNA was performed with duplex quantitative real time of methylation product of cg16941656 of FRY PCR by adding to 18 μl of master mix (containing TaqMan Fast Universal master mix (2x), the forward and reverse primer of FRY and Beta-actin and their probes) on a 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The sequence for FRY amplification are forward primer : 5'-GTTGCGTATTTGGTTGGAGTC-3', FRY reverse primer : 5'-CGAAACTAT CATCACCAAAAACG-3' and FRY TaqMan probe : 5' Fam-TGGTGGGCGGTCGGTA-MGB 3' whereas Beta-actin PCR was used as positive control using forward primer :5'-GTGTATTTGATTTTTGAGGAGA-3', reverse primer : 5'-CCTTAATACCAACCTACCCAA-3' and probe 5'Cy5-AAGGTGAAYGTGGATGAAGTTGGTGGTGAGG-BHQ 3'. Real-Time PCR System using thermal cycling conditions: firstly, uracil-N-glycosylase was activated at 50°C 2 min afterwards followed by the activation of Taq polymerase enzyme at 95ºC 10 min. Next, PCR steps were done by 60 cycles of 95ºC 15 sec and 60ºC 1 min.

Sensitivity test and methylated FRY loci calculation

Methylated human genomic DNA (EpiTect®PCR control kit, Qaigen, USA) was diluted in serial dilution from 20 ng/μl to 0.02 ng/μl and 0.01 ng/μl for sensitivity investigation. These samples were performed with real-time PCR using a specific probe to methylated FRY loci and then set as a standard curve. To calculate FRY methylated level, the Ct values derived from standard curve were calculated with results to the formula y=7E+08e-0.597x. Consequently, the absolute quantification of each sample was calculated.

Statistical Analysis

Chi-squared test was used to determine the difference between two groups. All differences were considered significant when $P<0.05$. Graphs and formulas of methylated FRY levels were created with Microsoft Excel Program.

Results

Forty-five patients with obstructive jaundice underwent ERCP were included in this study. The causes of biliary obstruction were pancreatic cancer and benign causes in 24 and 21 patients, respectively. The mean ages were 60.3 and 56.7 years. The proportion of male gender was 33.3% in

Table 1. Demographic Data and Percentage of FRY Methylation

	Pancreatic cancer (n=24)	Benign causes (n=21)	p-value
Age	60.3 (11.5)	56.7 (18.6)	0.445
Gender	Male 8 (33.3%) Female 16 (66.7%)	Male 7 (33.3%) Female 14 (66.7%)	1
Diagnosis	Pancreatic cancer by Pathology 14 Cytology 9 Imaging 1	Common bile duct stone 18 Chronic pancreatitis 1 Papillary stenosis 1 Autoimmune pancreatitis 1	NA
FRY methylation	10 (41.7%)	1 (4.8%)	0.005
Methylated FRY concentration [mean(SD)] (ng/µl)	0.573 (1.8325)	0.0005 (0.0022)	0.14

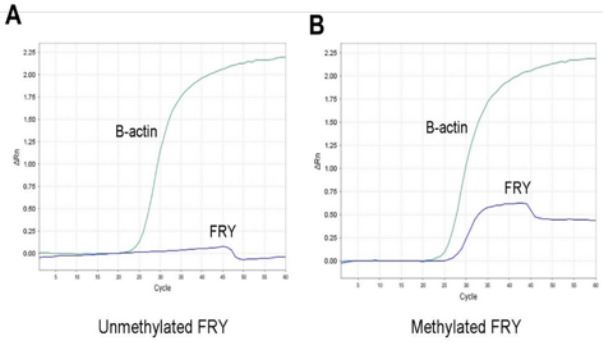

Figure 1. qRT-FRY Methylation Results, A) for Unmethylated FRY and B) for Methylated FRY

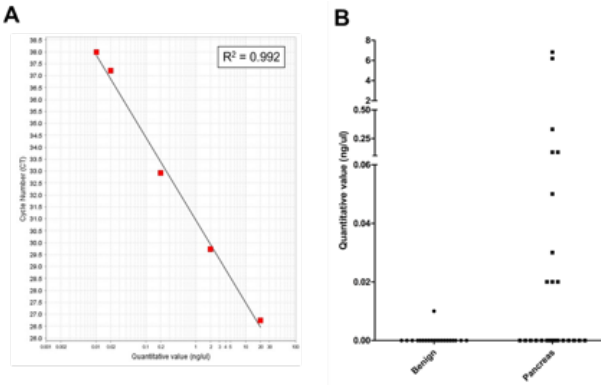

Figure 2. Measurement of FRY Methylation Levels. A: Sensitivity test of qRT-FRY methylation from five DNA concentrations including 20, 2, 0.2, 0.02 and 0.01 ng/µl. B: Comparing methylation levels of FRY between benign causes patients and pancreatic cancer patients

pancreatic cancer and 33.3% in benign causes. Pancreatic cancer patients were diagnosed by histopathology, cytology and clinical and image follow-up in 14, 9 and 1 patients, respectively. Cancer staging was I, II, III, and IV in 2, 7, 2, and 13 patients, respectively. The causes of biliary obstruction in benign causes were biliary stone, chronic pancreatitis, papillary stenosis and autoimmune pancreatitis in 18, 1, 1 and 1 patients, respectively.

The qRT methylated and unmethylated results are depicted in Figure 1. Beta-actin can be detected in all bile specimens. The reliability of this method was confirmed (R2=0.992), and the qRT-PCR was capable to detect methylated FRY at the concentration of 0.01 ng/µl (Figure 2). After calculation of methylated loci, the methylated FRY ranged from 0.02-6.83 ng/µl in bile from pancreatic cancer and 0.01 ng/µl in benign causes. Methylated FRY were detected in 41.7% and 4.8% in pancreatic cancer and benign causes, respectively (OR=8.26 and P=0.004). In pancreatic cancer group, the methylated FRY levels were detected in 50%, 28.6%, 100% and 38.5% in stage I, II, III and IV, respectively (P=0.333). The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were 41.7%, 95.2%, 91% and 59%, respectively. Moreover, the positive likelihood ratio (LR+), (95% confidence interval) and negative likelihood ratio (LR-), (95% confidence interval) of this marker were 8.75 (1.22,63) and 0.61 (0.43,0.87), respectively (Table 1).

Discussion

Bile aspiration is a simple process routinely involved as a part of ERCP which is considered not to increase post-ERCP complication or tumor seeding. Here, we performed the qRT-PCR to detect the methylation at cg16941656 of FRY which is present in pancreatic cancer DNA from bile specimens of obstructive jaundice patients. Pancreatic cancer cases had significantly higher FRY methylation when compared with benign causes (41.7% vs 4.8%, respectively). This showed a satisfactory marker with high specificity (95%), PPV (91%), and LR+ (8.75) for differentiation of pancreatic cancer from benign obstruction.

We found a false positive FRY methylation in 1 patient of benign obstruction (autoimmune pancreatitis) considering that the distal common bile duct joins with the main pancreatic duct to form the common channel in 68-86% of normal human (Allescher, 1989). We did not elucidate this marker in pancreatic juice from normal subjects because pancreatic juice aspiration might increase risk of post-ERCP pancreatitis (Salehimarzijarani et al., 2012). Prior studies demonstrated that some DNA methylations can be observed in pancreatic juice of normal patients (Kisiel et al., 2015). Then, we hypothesized that the detection of FRY methylation in benign group may be caused by contaminated pancreatic juice in bile sample which in turn, lowers the specificity. Therefore, the utility of this marker in bile is only in case of bile duct invasion from pancreatic cancer.

A prior study (Srisuttee et al., 2016), extracting DNA from tissue specimens were demonstrated the sensitivity of 100% of this marker in differentiation between pancreatic adenocarcinoma and other adenocarcinomas (stomach, lung, endometrium, ovary, prostate gland, bile duct, colon and breast). In present study bile samples, the sensitivity is only 41.7% and is not associated with cancer staging. This means the low efficiency may depend on the number

of cancer DNA contaminated in samples. Even though the qRT-PCR has ability to detect DNA concentration at minimum of 0.01 ng/μl, a false negative could come from bile duct obstruction without invasion from pancreatic cancer or sporadic mutation at cg16941656 which limited the sensitivity.

The high percentage of specificity and PPV showed that this methylation is a promising marker for pancreatic cancer and benign obstructive jaundice as it causes differentiation in biliary stricture patients. In further study, we will examine this promising marker by 1) extending sample size for unequivocal results, 2) develop more sensitive techniques to detect pancreatic cancer DNA, such as digital PCR and 3) verify the possibility of this marker to detect pancreatic cancer circulating DNA in patient blood.

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