Epigenetic marker for diagnosis of cholangiocarcinoma in clinical endoscopic biliary specimens of patients with bile duct stricture

Jiraporn Kanchanapermpoon[1,2],Chanitra Thuwajit[2], WarayuPrachayakul[3] and Peti Thuwajit[2]*

[1]Graduate Program in Immunology, [2]Department of Immunology, [3]Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700

**Corresponding author: petthu@msn.com*

ABSTRACT

Cholangiocarcinoma can cause obstructive jaundice which should be discriminated from other benign diseases. Recently, cholangioscope and cytology study are used for diagnosis with high specificity; however, the sensitivity of these examinations is quite low. The use of DNA marker such as methylation may help with the diagnosis. *HOXA1* and *RASSF1A* genes are interesting targets since they are tumor suppressor genes, which usually have hypermethylated promoter during carcinogenesis. Analysis of these genes indicated that DNA methylation status of *HOXA1*promoter but not *RASSF1A* may be useful for diagnosis of cholangiocarcinoma in biliary obstruction patients.

Keywords: DNA methylation, cholangiocarcinoma, biliary obstruction

INTRODUCTION

Cholangiocarcinoma (CCA) is a malignant tumor arising from the neoplastic transformation of cholangiocytes, the epithelial cells lining of bile ducts (Welzel *et al*., 2006). CCA has been classified into two groups consisting of intrahepatic CCA (IHC) arising within the liver and extra hepatic CCA (EHC) originating in the bile duct along the hepatoduodenalligament (Aljiffry *et al*., 2009). Patients usually show non-specific symptoms such as right upper quadrant abdominal pain, flatulence and fatigue (Pungpak *et al*., 1983). The clinical presentation of extra hepatic CCA is usually jaundiced from bile duct obstruction, which is difficult to distinguish from benign etiology. There are three modalities available for diagnosis of CCA in the cases of biliary obstruction including gendoscopic retrograde cholangiopancreatography (ERCP), endoscopic ultrasound with fine needle aspiration and cholangioscopy (Nguyen *et al*., 2008). Endoscopic procedure plays a role in the diagnosis as cells from the stricture lesion are examined by cytology study. However, the results are usually inconclusive. To improve the efficacy of endoscope-based technique, the molecular markers can be used to study the specimens obtained from the endoscope. While the molecular mechanisms of CCA have been largely unknown, aberrant methylation of

promoter CpG islands associated with human tumor suppressor genes has been studied as an alternative mechanism of gene inactivation that contributes to the biology of several human neoplasms (Baylin and Herman, 2000). Promoter region hypermethylation typically results in down-regulation or silencing of gene transcription; consequently, the expression of a tumor suppressor gene can be silenced through aberrant hypermethylation in cancer (Baylin and Herman, 2000). Since alterations of the cellular epigenome usually precede morphologic changes and genetic alterations, identification of related aberrant DNA methylation profiles may serve as a diagnostic marker and an intervention target for CCA (Huang L*et al*., 2010). RAS association domain family 1A (*RASSF1A*) gene is a tumor suppressor gene, which was previously reported as having hypermethylation in its promoter in CCA, suggesting that *RASSF1A* might play an important role in the pathogenesis of CCA. In addition, a previous study has investigated the DNA methylation of Homeobox protein Hox-A1 (*HOXA1*), showing that *HOXA1* was hypermethylated more than 95% in the EHC. These findings indicated that *RASSF1A* and *HOXA1* methylation might be used as biomarkers for the detection of bile duct stricture in patients.

MATERIALS AND METHODS

Cell lines

CCA cell lines including KKU-M055, KKU-M213, KKU-M214 (adenocarcinoma) and KKU-M139 (squamous cell carcinoma) were kindly donated from Associate Professor Dr. Banchob Sripa, Department of Pathology, Faculty of Medicine, KhonKaen University. The non-malignant MMNK1 cells which express bile duct epithelial marker were kindly donated from Professor Dr. Sopit Wongkham, Department of Biochemistry, Faculty of Medicine, KhonKaen University. Hepatocellular carcinoma (HCC) cell line (HepG2) was kindly donated from Associate Professor Dr. Thawornchai Limjindaporn, Department of Anatomy, Faculty of Medicine Siriraj hospital, Mahidol University

Human endoscopic brushed cells

EDTA blood and endoscopic brushed cells from biliary stricture patients in Siriraj Hospital were taken by Dr. Warayu Prachayakul, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University. Informed consent was obtained from each patient. The *Siriraj Institutional Review Board,* Faculty of Medicine Siriraj Hospital, Mahidol University, approved the research protocols (Si521/2010). White blood cells were removed from endoscopic brushed biliary cells using CD45 Microbeads human kit (Miltenyi, Auburn, CA, USA).

Measurement of DNA methylation of *RASSF1A* and *HOXA1* by EpiTect® Methyl DNA Methylation PCR System

Genomic DNA extraction

To measure the methylation of *RASSF1A* and *HOXA1* genes, genomic DNA extraction was performed using QIAamp® DNA Mini Kit (Qiagen, Germany) following

instructtion manual. The amount of the total DNA was measured by Nanodrop®. DNA samples are kept at -20°C for future use.

Determination of DNA methylation

There were 2 types of restriction endonuclease (RE) used in this assay, methylation sensitive RE (Ms), which digests unmethylated DNA and methylation dependent RE (Md), which digest methylated DNA. Restriction digestion was performed with the EpiTect® Methyl DNA Restriction Kit (Cat. no. 335451) (SA Biosciences, Qiagen). Reaction cocktail without enzymes was prepared as a control. For each gene (*RASSF1A* and *HOXA1*), individual PCR cocktails were prepared using the primers from SA Biosciences. The methylation analysis was performed using real time PCR and percentage of methylation was calculated to categorize intermediate methylated and unmethylated promoters as recommended by the manufacturer's instructions. Promoter hypermethylation would be considered when percent hypermethylation is higher than 80%.

RESULTS AND DISCUSSION

DNA methylation of *HOXA1* and *RASSF1A*

DNA methylation PCR system of *HOXA1* and *RASSF1A* was performed in DNA sample of KKU-M055, KKU-M139, KKU-M213, KKU-M214, HepG2**,** healthy control blood, patients' endoscopic blushed biliary cell and blood cells. Hypermethylation of *HOXA1* was shown in KKU-M055, KKU-M213, KKU-M214 adeno-CCA cell lines but not in KKU-M139 squamous CCA cell line, HepG2 HCC cells and MMNK1 non-malignant biliary epithelial cell line (Figure 1A).Healthy control blood cells showed fully unmethylated *HOXA1* promoter (Figure 1A). First patient's specimens did not exhibit hypermethylation of *HOXA1* whereas the second patient's showed full hypermethylation. White blood cells isolated from brushed specimens showed percent DNA methylation between brushed biliary cells and blood cells (Figure 1A). For *RASSF1A* promoter, only MMNK1 and HepG2 cells showed

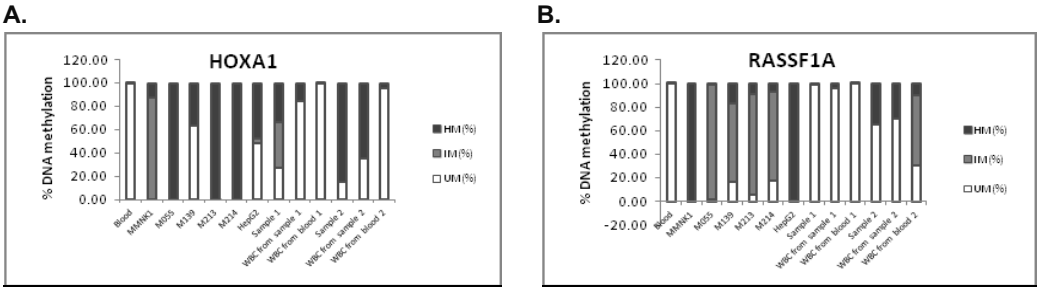

Figure 1 DNA methylation of *HOXA1* and *RASSF1A*.Blood sample in the in first column of both figures was healthy control blood. A., DNA methylation of *HOXA1*; B., DNA methylation of *RASSF1A*; HM, hypermethylation; IM, intermediate methylation; UM, unmethylation.

hypermethylation whereas all 4 CCA cell lines did not (Figure 1B). Healthy control blood showed no methylation as expected (Figure 1B). First patient's specimens also showed no methylation, which as similar to blood samples, and second patient's showed low level of methylation (Figure 1B).

The results indicated that brushed biliary cells from CCA patients might exhibit hypermethylation of *HOXA1* promoter. The difference in methylation level might result from the purity of malignant cell in the specimens with few normal epithelial and blood cells. The gene *RASSF1A* could not be used as marker for diagnosis of CCA. The results indicated that *HOXA1* promoter methylation status can be used for diagnosis of CCA in biliary stricture patients but with adequate malignant cells.

CONCLUSION

We demonstrated hypermethylation in cancer cell lines when compared with healthy control blood. Hypermethylation of *HOXA1* promoter has been observed in brushed biliary samples from CCA patients but not *RASSF1A*. *HOXA1* hypermethylation may be considered as an alternative diagnostic choice for CCA in patients with bile duct stricture.

ACKNOWLEDGEMENTS

This work was supported by Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University

REFERENCES

Aljiffry M, Walsh MJ and Molinari M (2009) Advances in diagnosis, treatment and palliation of cholangiocarcinoma: 1990-2009. World J Gastroenterol 15: 4240–4262.

Baylin SB and Herman JG (2000) DNA hypermethylation in tumorigenesis: epi genetics joins genetics. Trends Genet 16: 168–174.

Huang L, Frampton G, Liang LJ and Demorrow S (2010) Aberrant DNA methylation profile in cholangiocarcinoma. World J Gastrointest Pathophysiol 1: 23–29.

Nguyen K and Sing JT Jr (2008) Review of endoscopic techniques in the diagnosis and management of cholangiocarcinoma. World J Gastroenterol 14: 2995–2999.

Pungpak S, Bunnag D and Harinasuta T (1983) Clinical and laboratory evaluation of praziquantel in opisthorchiasis. Southeast Asian J Trop Med Public Health 14: 363–366.

Welzel TM, McGlynn KA, Hsing AW, O'Brien TR and Pfeiffer RM (2006) Impact of classification of hilarcholangiocarcinomas (Klatskin tumors) on the incidence of intra- and extrahepaticcholangiocarcinoma in the United States. J Natl Cancer Inst 98: 873–875.