ment and maintenance of tissue-specific gene expression patterns in mammals. Disruption of epigenetic regulation can lead to altered gene function and malignant cellular transformation[47]. Recent cancer epigenetic studies have revealed various alterations in the epigenetic machinery in GC, including DNA methylation, histone modifications, nucleosome positioning, noncoding RNAs and miRNAs[48-52]. Aberrant DNA methylation in the promoter CpG islands of genes results in inactivation of tumor suppressor and other tumor-related genes in cancer cells and is the most well-defined epigenetic hallmark in GC. Methylation of a large number of genes with different biological functions has been found to be correlated with the clinicopathological characteristics and prognosis in GC[48-52]. DNA methylation with its advantages as a biomarker for the detection of cancer in biopsy specimens and body fluids that can be obtained non-invasively, such as serum and gastric washes, may have a clinical application in GC. Detection of aberrant DNA methylation of genes, such as *CDH1*, *DAPK*, *GSTP1*, *p15*, *p16*, *RARβ*, *RASSF1A*, *RUNX3* and *TFPI2*, in the serum may be a useful biomarker for the detection of GC[50]. Studies of DNA methylation and histone modification using NGS technologies, such as whole-genome bisulfite sequencing and targeted bisulfite sequencing, will lead to new discoveries and improve our knowledge of the epigenomics of GC[11].

Association of the aberrant methylation of RASGRF1 with an epigenetic field defect and an increased risk of GC

Aberrant DNA methylation is implicated in the epigenetic field defect seen in GC. Thus, it is important to identify predictive biomarkers by screening for DNA methylation in the noncancerous background gastric mucosa of patients with GC. Using methylated-CpG island amplification coupled with CpG island microarray (MCAM) analysis, Takamaru *et al*[53] found 224 genes that were methylated in the noncancerous gastric mucosa of patients with GC. Among them, RASGRF1 methylation was significantly elevated in the gastric mucosa from patients with either intestinal- or diffuse-type GC, compared with the mucosa from healthy individuals. RASGRF1 methylation was independent of mucosal atrophy and could be used to distinguish both serum pepsinogen test-positive and -negative patients with GC from healthy individuals. Ectopic expression of RASGRF1 suppressed colony formation and Matrigel invasion by GC cells. RASGRF1 methylation appears to be significantly involved in the epigenetic field defect of the stomach and to be a useful biomarker to identify individuals at high risk for GC.

Association of aberrant methylation of miR-34b/c with an epigenetic field defect and an increased risk of GC

The silencing of miRNAs is often associated with CpG island hypermethylation. Thus, to identify epigenetically silenced miRNAs in GC, Suzuki *et al*[54] screened for miRNAs that were induced by treatment of GC cells with 5-aza-2'-deoxycytidine and 4-phenylbutyrate. Hypermethylation of the neighboring CpG island epigenetically silenced miR-34b and miR-34c. Methylation of the miR-34b/c CpG island was frequently observed in GC cell lines (13/13, 100%) but not in normal gastric mucosa from healthy *H. pylori*-negative individuals. Transfection of the precursors of miR-34b and miR-34c into GC cells suppressed growth and changed the gene expression profile. Methylation of miR-34b/c was found in a majority of primary GCs (83/118, 70%). Notably, analysis of the non-cancerous gastric mucosae from GC patients (n = 109) and healthy individuals (n = 85) revealed that methylation levels were higher in the gastric mucosae of patients with multiple GC lesions than in the mucosae from those patients with single GC and the mucosae from healthy *H. pylori*-positive individuals. These results suggest that miR-34b and miR-34c are novel tumor suppressors frequently silenced by DNA methylation in GC. Methylation of miR-34b/c appears to be significantly involved in an epigenetic field defect in the stomach and to be a useful biomarker to identify individuals at high risk for multiple GC.

Methylation of miR-34b/c in the mucosa of the noncancerous gastric body may be a useful biomarker for predicting the risk of metachronous GC

Metachronous GC can develop after endoscopic resection of GC and is not predictable based on the clinical characteristics alone. Aberrant DNA methylation in noncancerous gastric mucosa has been implicated in gastric carcinogenesis and may be a useful biomarker of GC risk. Suzuki *et al*[55] evaluated the clinical utility of DNA methylation as a biomarker of metachronous GC risk. Scheduled follow-up endoscopy was performed in 129 patients after curative endoscopic resection of early GC. Biopsy specimens were collected from noncancerous mucosa in the gastric antrum and body. A quantitative methylation analysis of miR-34b/c, SFRP1, SFRP2, SFRP5, DKK2 and DKK3 using bisulfite pyrosequencing was performed on the collected biopsy specimens. The utility of the methylation status for predicting the risk of developing metachronous GC was analyzed using Kaplan-Meier and Cox proportional hazards models. During the follow-up period, 17 patients (13%) developed metachronous GCs. The cumulative incidence of metachronous GC was significantly higher among patients with elevated miR-34b/c, SFRP2 and DKK2 methylation in the gastric body. Elevated methylation of miR-34b/c showed the most significant association with the risk of metachronous GC; the cumulative incidence of metachronous GC was much higher in the high miR-34b/c-methylation group than in the low methylation group. Multivariate analysis adjusted for age, sex, *H. pylori* status and pathological findings showed that miR-34b/c methylation in the gastric body was an independent predictor of metachronous GC risk. Methylation of miR-34b/c in the mucosa of the noncancerous gastric

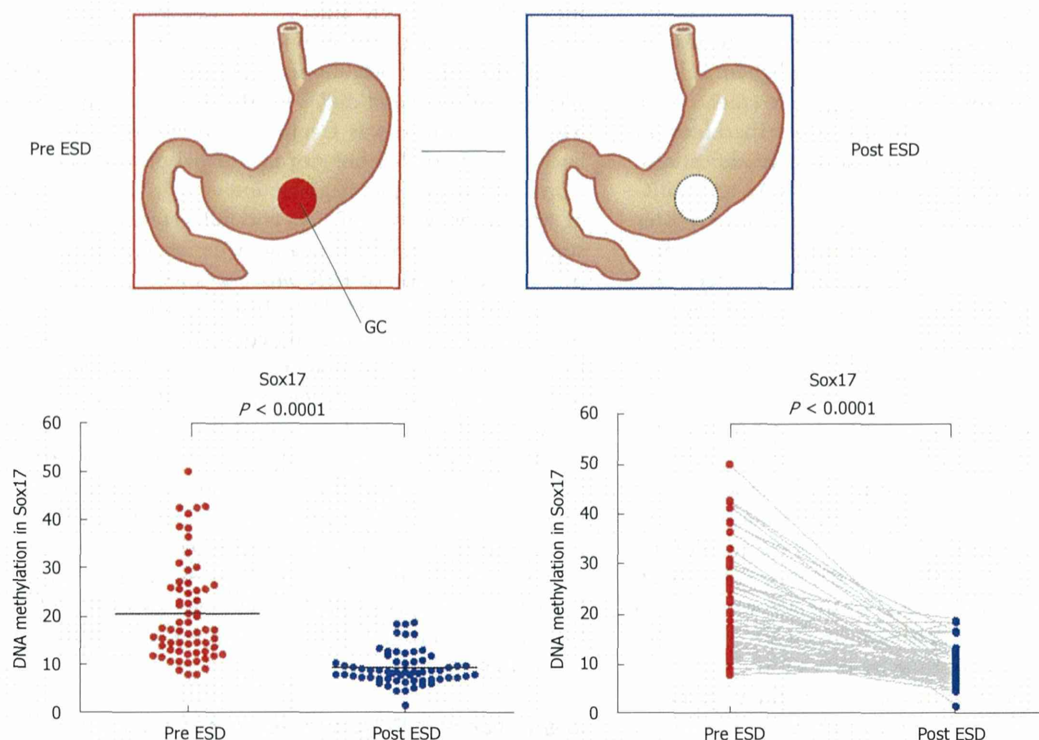

Figure 3 Methylation levels of Sox17 before and after endoscopic submucosal dissection. Methylation levels of Sox17 were analyzed by pyrosequencing using the DNA recovered from gastric washes before and after endoscopic submucosal dissection[57].

body may be a useful biomarker for predicting the risk of metachronous GC. Finally, NGS technologies may characterize an epigenetic field defect more clearly and highlight more useful biomarkers.

Sensitive and specific detection of early GC by DNA methylation analysis of gastric washes

Because many mucosal cells can be found in the gastric juice, the detection of molecular markers in the gastric juice was a possible noninvasive approach to detect GC. However, the use of gastric juice as a molecular diagnostic or predictive tool has been previously reported to be impractical because the DNA is easily degraded by gastric acidity. In this regard, Watanabe *et al*[56] have developed a new method for GC detection by DNA methylation in gastric washes but not in gastric juice. These authors analyzed 51 candidate genes in 7 GC cell lines and 24 GC samples (training set). They then selected 6 genes (*MINT25*, *RORA*, *GDNF*, *ADAM23*, *PRDM5* and *MLF1*) for further analyses. The methylation status of these genes was analyzed in a test set consisting of 131 GCs at various stages. The 6 candidate genes were validated in a different population of 40 primary GC samples and 113 noncancerous gastric mucosa samples. The 6 genes showed differential methylation in GC and normal mucosa in the training, test and validation sets. GDNF and MINT25 were the most sensitive molecular markers of early-stage GC, whereas PRDM5 and MLF1 were markers of a field defect. A close correlation between methylation levels in tumor biopsy samples and gastric washes was noted. MINT25 methylation showed the best sensitivity (90%) and specificity (96%), and it had the greatest area under the receiver operating characteristic curve (0.961) in terms of tumor detection in gastric washes. MINT25 methylation in gastric washes may be a sensitive and specific marker for the screening of GC.

Detection of early GC by DNA methylation analysis of Sox17 in gastric washes

Although minimally invasive treatment is widely accepted for early-stage GC, appropriate risk markers to detect residual cancer after endoscopic resection and the potential for recurrence are not available. To find candidate genes that might be markers for the detection of early GC, Oishi *et al*[57] performed methylated CpG island amplification microarray analysis on 12 gastric washes (from the pre- and post-endoscopic treatment of six patients). Among the candidate genes, the *Sox17* gene was selected for further analysis. The DNA methylation status of Sox17 was examined in a validation set consisting of 128 gastric wash samples (64 pre-treatment and 64 post-treatment) from cases of early GC. Sox17 showed significant differential methylation in the pre- and post-treatment gastric washes of early GC patients (Figure 3). Moreover, the treatment of GC cells that lacked Sox17 expression with the methyltransferase inhibitor 5-aza-2'-deoxycytidine restored the gene's expression. Additionally, the introduction of exogenous Sox17 into silenced

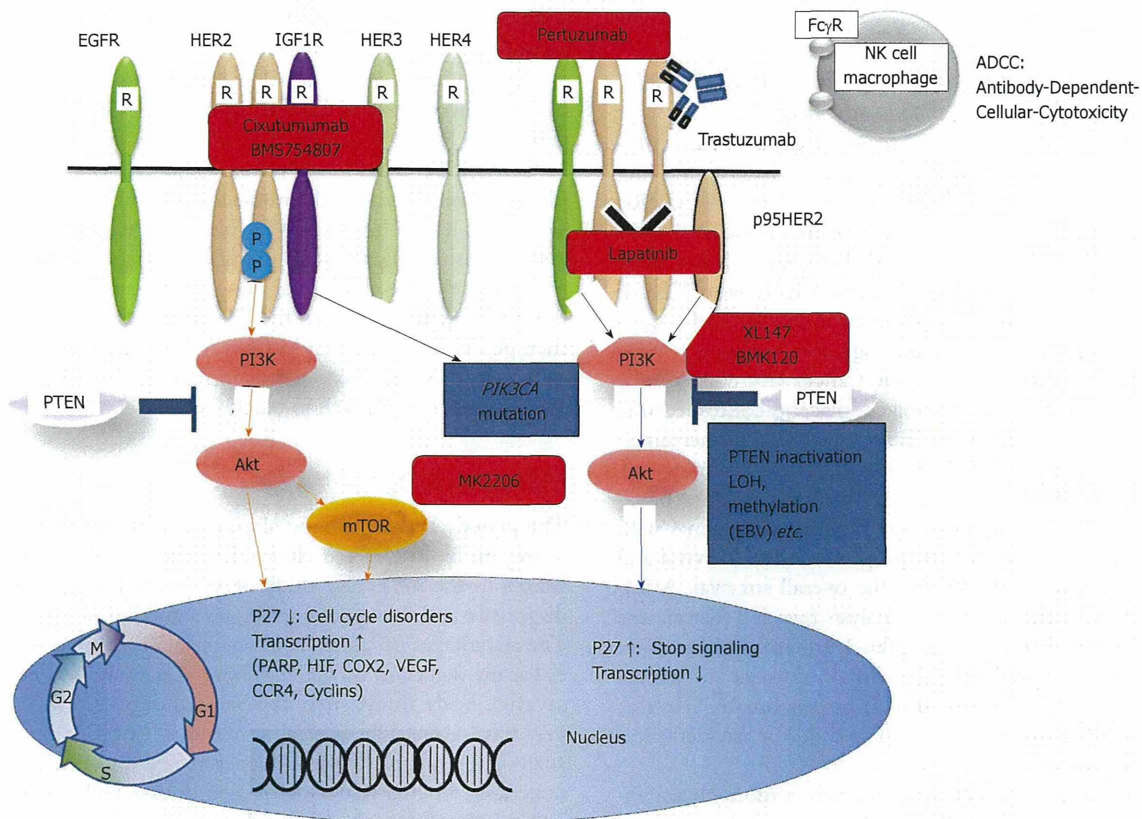

Figure 4 Human epidermal growth receptor family members, the PI3K/Akt pathway, and targeted drugs. HER: Human epidermal growth receptor; NK: Natural killer; IGF1R: α-insulin-like growth factor 1-receptor; EGFR: Epidermal growth factor receptor; PI3K: Phosphatidylinositol-3-kinase; PTEN: Phosphatase and tensin homologue.

GC cells suppressed colony formation. The data suggest that the silencing of Sox17 occurs frequently in early GC and plays a key role in the disease. Gastric wash-based DNA methylation analysis could be useful for the early detection of recurrence following endoscopic resection in early GC patients. Interestingly, the usefulness of gastric wash-based molecular testing for antibiotic resistance in *H. pylori* has also been reported[58]. It will be interesting to analyze gastric washes using NGS.

Anti-HER2 antibody trastuzumab has led to an era of personalized therapy in GC

Trastuzumab is an antibody that targets the HER2 extracellular domain and induces antibody-dependent cellular cytotoxicity and inhibition of the HER2 downstream signals (Figure 4). In the ToGA study, standard chemotherapy regimens (capecitabine plus cisplatin or fluorouracil plus cisplatin) combined with trastuzumab resulted in a longer survival time than standard regimens without trastuzumab in patients with HER2-positive GC[59]. Thus, HER2 expression has become a major concern in GC[60]. HER2 overexpression is observed in 7%-34% of GC cases. Mechanisms of resistance to trastuzumab have been reported in breast cancer. There are various mechanisms underlying trastuzumab resistance, such as alterations of the HER2 structure or surroundings, dysregulation of HER2 downstream signal effectors and interaction of HER2 with other membrane receptors (Figure 4). The PI3K-Akt pathway is one of the main downstream signaling pathways of HER2. It is well known that PIK3CA mutations and PTEN inactivation cause over-activation of a downstream signal without activation of an upstream signal. The frequencies of PIK3CA mutations and PTEN inactivation in GC have been reported to be 4%-25% and 16%-77%, respectively. However, little is known about the association between HER2 expression and PI3K-Akt pathway alterations in GC. Sukawa *et al*[29] have found that HER2 overexpression was significantly correlated with pAkt expression in GC tissues. Furthermore, pAkt expression was correlated with poor prognosis. These results suggest that the PI3K-Akt pathway plays an important role in HER2-positive GC. Moreover, PIK3CA mutations and PTEN inactivation could affect the effectiveness of HER2-targeting therapy. Thus, it is necessary to clarify not only HER2 alterations but also PI3K-Akt pathway alterations to optimize HER2-targeting therapy in patients with GC. In this regard, NGS will be useful for the identification of complicated mechanisms of trastuzumab resistance in GC. The only approved targeted therapy for patients with advanced GC is trastuzumab. It is hoped that NGS will reveal a driver gene alteration that will make other targeted

therapies possible[13,61].

Monoclonal antibodies targeting VEGF (AVAGAST trial) and VEGFR-2 (REGARD trial) in advanced GC

Several vascular endothelial growth factor (VEGF)-targeted agents have been developed, including neutralizing monoclonal antibodies (MoAbs) to VEGF/VEGFRs, soluble VEGF receptors and tyrosine kinase inhibitors (TKIs). The anti-VEGF MoAb bevacizumab has been approved for colorectal cancers. VEGF and VEGF receptor-2 (VEGFR-2)-mediated signaling and angiogenesis contribute to the pathogenesis and progression of GC. The Avastin in Gastric Cancer (AVAGAST) trial was a multinational, randomized, placebo-controlled trial designed to evaluate the efficacy of adding bevacizumab to capecitabine-cisplatin in the first-line treatment of advanced GC[62]. The study showed that adding bevacizumab to the chemotherapy regimen in patients with advanced GC improved the progression-free survival and tumor response rate but not the overall survival. A following biomarker evaluation analysis revealed that plasma VEGF-A and tumor neuropilin-1 are strong biomarker candidates for predicting the clinical outcome in patients with advanced GC treated with bevacizumab[63]. In this regard, NGS will be a powerful method for the identification of predictive biomarkers.

To analyze whether ramucirumab, a monoclonal antibody targeting VEGFR-2, prolongs survival in patients with advanced GC, an international, randomized, double-blind, placebo-controlled, phase 3 trial was conducted in 29 countries[64]. In total, 355 patients with advanced gastric or gastro-esophageal junction adenocarcinoma and disease progression after first-line chemotherapy were randomly assigned (2:1) to receive best supportive care plus either ramucirumab 8 mg/kg (n = 238) or placebo (n = 117), intravenously once every 2 wk. The primary endpoint was overall survival. The median overall survival was 5.2 mo in the ramucirumab group and 3.8 mo in the placebo group (HR = 0.776, 95%CI: 0.603-0.998, P = 0.047). The survival benefit with ramucirumab remained unchanged after multivariate adjustment for other prognostic factors (multivariate HR = 0.774, 95%CI: 0.605-0.991, P = 0.042). Thus, ramucirumab is the first biological treatment given as a single drug that showed survival benefits in patients with advanced gastric or gastro-esophageal junction adenocarcinoma who progressed after first-line chemotherapy. The findings also validate VEGFR-2 signaling as an important therapeutic target in advanced GC.

Potential targeted drugs for GC

Using NGS to target a subset of druggable genes becomes a more effective way to discover therapeutic targets[13,14,61]. There are several potential targeted drugs, either MoAb or small-molecule TKIs, that are being investigated either in synergy with, or in place of, established treatments. These drugs include inhibitors of growth factors and their receptors [*i.e.*, VEGF, epidermal growth factor receptor, HER2, insulin-like growth factor 1 (IGF1) receptor, c-MET], MEK inhibitors and drugs targeting the Hedgehog pathway[65].

Dysregulation of the IGF1 and IGF2/IGF1R system has been implicated in the pathogenesis of GC[66-69]. The expression levels of both IGFs and IGF1R are increased in GC. IGF1R is also involved in angiogenesis and lymphangiogenesis through the modulation of VEGF expression in a GC cell line[70]. IGF1R blockade reduced tumor angiogenesis and enhanced the effects of bevacizumab in a GC cell line. Thus, targeting IGF1R in combination with agents that block the VEGF pathway may have therapeutic utility in GC. Moreover, targeting the novel miR-7/IGF1R/Snail axis has been reported to be useful as a therapeutic approach to block GC metastasis[71].

CONCLUSION

The genetic and epigenetic alterations in GCs continue to inspire biological and clinical implications. Recent advances in the molecular study of GC have brought new diagnostic and therapeutic strategies into clinical settings. The advantages of using DNA methylation as a biomarker for the detection of GC in biopsy specimens and non-invasive body fluids such as serum and gastric washes may have a possible clinical application in GC. Further analysis is required to gain a deeper insight into GC carcinogenesis, a better understanding of disease pathogenesis and the development of new diagnostic and therapeutic approaches targeting essential pathogenic alterations. In this regard, the rapid advances in NGS technologies will hopefully continue to reveal driver alterations of GC, further our understanding of gastric carcinogenesis and improve the therapy for each individual tumor. The characterization of genes that were discovered by NGS rather than by laboratory and clinical research is also necessary.

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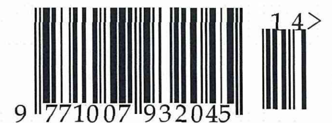

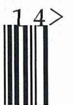

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ORIGINAL ARTICLE

Cancer detection by ubiquitin carboxyl-terminal esterase L1 methylation in pancreatobiliary fluids

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Abstract

AIM: To evaluate the utility of measuring epigenetic alterations in pancreatic and biliary fluids in determining molecular markers for pancreatobiliary cancers.

METHODS: DNA was extracted from undiluted pancreatic and biliary fluids. As a surrogate for a genome-wide hypomethylation assay, levels of long interspersed nuclear element-1 (LINE-1) methylation were analyzed using bisulfite pyrosequencing. CpG island hypermethylation of 10 tumor-associated genes, aryl-hydrocarbon receptor repressor, adenomatous polyposis coli, calcium channel, voltage dependent, T type α1G subunit, insulin-like growth factor 2, *O*-6-methyl-guanine-DNA methyltransferase, neurogenin 1, CDKN2A, runt-related transcription factor 3 (RUNX3), secreted frizzled-related protein 1, and ubiquitin carboxyl-terminal esterase L1 (UCHL1), was analyzed using MethyLight. To examine the role of CpG methylation and histone deacetylation in the silencing of UCHL1, human gallbladder carcinoma cell lines and pancreatic carcinoma cell lines were treated with 2 or 5 μmol/L 5-AZA-dC for 72 h or 100 nmol/L Trichostatin A for 24 h. After the treatment, UCHL1 expression was analyzed by real-time reverse transcription-polymerase chain reaction.

RESULTS: Pancreatobiliary cancers exhibited significantly lower LINE-1 methylation levels in pancreatic and biliary fluids than did noncancerous pancreatobiliary disease (58.7% ± 4.3% *vs* 61.7% ± 2.2%, P = 0.027; 53.8% ± 6.6% *vs* 57.5% ± 1.7%, P = 0.007); however, LINE-1 hypomethylation was more evident in pancreatic cancer tissues than in pancreatic fluids (45.4% ± 5.5% *vs* 58.7% ± 4.3%, P < 0.001). CpG island hypermethylation of tumor-associated genes was detected at various frequencies, but it was not correlated with LINE-1 hypomethylation. Hypermethylation of the *UCHL1* gene was cancer-specific and most frequently detected in pancreatic (67%) or biliary (70%) fluids from patients with pancreatobiliary cancer. As a single marker, hypermethylation of the *UCHL1* gene in pancreatic and biliary fluids was most useful for the detection of pancreatic and pancreatobiliary cancers, respectively (100% specificity). Hypermethylation of the *UCHL1* and *RUNX3* genes in pancreatic and biliary fluids was the most useful combined marker for pancreatic (87% sensitivity and 100% specificity) and pancreatobiliary (97% sensitivity and 100% specificity) cancers. Treatment with a demethylating agent, 5-AZA-

2'-deoxycytidine, restored UCHL1 expression in pancreatobiliary cancer cell lines.

CONCLUSION: Our results suggest that hypermethylation of UCHL1 and RUNX3 in pancreatobiliary fluid might be useful for the diagnosis of pancreatobiliary cancers.

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Key words: Pancreatobiliary cancers; DNA methylation; Pancreatobiliary fluids; Ubiquitin carboxyl-terminal esterase L1; Runt-related transcription factor 3

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INTRODUCTION

Despite recent advances in diagnosis and treatment, the prognosis of patients with pancreatobiliary cancer is still poor. Surgical resection is possible in only a small proportion of patients[1,2]. Consequently, elucidation of the biological characteristics of pancreatobiliary carcinomas is necessary to improve the prognosis of patients and to devise better treatment strategies. Various genetic and epigenetic alterations play a role in pancreatobiliary cancer[3-6].

Two contradicting epigenetic alterations often coexist in cancer: global or genome-wide hypomethylation, which is mainly observed in repetitive sequences within the genome, and regional hypermethylation, which is frequently associated with CpG islands within gene promoters[7]. Hypermethylation of CpG islands is a common feature of cancer that is associated with gene silencing[7,8]. A number of genes are aberrantly methylated and silenced in pancreatobiliary cancer that are rarely methylated in non-neoplastic counterparts[4-6,8], and this methylation is detectable in pancreatic and/or biliary fluids[9-12]. The detection and/or quantification of these alterations in pancreatic and/or biliary fluids has promise for facilitating the differentiation of benign and malignant pancreatic and/or biliary strictures.

In contrast to CpG islands, repetitive DNA elements are normally heavily methylated in somatic tissues. Approximately 45% of the human genome is composed of repetitive sequences, including long interspersed nuclear elements (LINEs) and short interspersed nuclear elements[13]. Liquid chromatography-mass spectrometry analysis has shown that levels of LINE-1 methylation strongly correlate with methyl cytosine content. This strong correlation enables LINE-1 methylation to be used as a proxy for genome-wide methylation[14]. Moreover, LINE-1 hypomethylation is known to occur during the development and progression of various human malignancies[15,16]. Additionally, we recently reported that LINE-1 hypomethylation correlates significantly with the aggressiveness of gastrointestinal stromal tumors and that LINE-1 methylation could be a useful marker for risk assessment[17]. Array comparative genomic hybridization analysis revealed a significant correlation between LINE-1 hypomethylation and chromosomal aberrations[17]. Chromosomal gains and losses are also common in pancreatic and biliary cancers[18,19]; their detection by fluorescence *in situ* hybridization modestly improves the prediction of cancer using biliary brushings[20,21]. Gene hypomethylation has been reported to be a frequent epigenetic event in pancreatic cancer and is commonly associated with the overexpression of affected genes[22]. A previous study showed that hypomethylation is more common in carcinoid tumors than in pancreatic endocrine tumors and is associated with clinicopathologic features, including lymph node metastasis, as well as genetic and epigenetic alterations in these tumors[23].

To date, however, only a few groups have reported the methylation of LINE-1 and/or other repetitive sequences in pancreatobiliary cancer[23,24], and there are no published studies analyzing LINE-1 methylation in pancreatic and/or biliary fluids. We found correlations between the level of LINE-1 methylation and the methylation of other repetitive sequences[17]. In this study, we analyzed LINE-1 methylation and its relationship with hypermethylation of CpG islands in pancreatic and biliary fluids, and we investigated whether the detection and/or quantification of these epigenetic alterations can be used as markers for pancreatobiliary cancer.

MATERIALS AND METHODS

Clinical samples and cell lines

Pancreatic and biliary fluids were obtained at the time of endoscopic retrograde cholangiopancreatography (ERCP) and ERCP/percutaneous transhepatic cholangiography and drainage, respectively[9-12]. Pancreatic and biliary fluids were collected from 30 and 48 patients, respectively. Informed consent was obtained from each subject. Tumors were classified according to the tumor-node-metastasis classification system of the International Union Against Cancer. The absence of cancer was based on clinical evaluation and follow-up of one or more years. Human gallbladder carcinoma cell lines TGBC1TKB and TGBC2TKB and pancreatic carcinoma cell lines PANC-1, PK-1, PK-45P and PK59 were purchased from Riken Cell Bank (Tsukuba, Japan). Cells were cultured in RPMI1640 or DMEM supplemented with 10% fetal bovine serum.

Extraction and bisulfite treatment of DNA

DNA was extracted from undiluted pancreatic and bili-