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Promoter methylation profiles of tumor suppressor genes in intrahepatic and extrahepatic cholangiocarcinoma

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Recent studies indicate that tumor suppressor genes can be epigenetically silenced through promoter hypermethylation. To further understand epigenetic alterations in cholangiocarcinoma, we have studied the methylation profiles of 12 candidate tumor suppressor genes (*APC*, *E-cadherin/CDH1*, *MGMT*, *RASSF1A*, *GSTP*, *RAR-β*, *p14^{ARF}*, *p15^{INK4b}*, *p16^{INK4a}*, *p73*, *hMLH1 and DAPK*) in 72 cases of cholangiocarcinoma, including equal number cases of intrahepatic cholangiocarcinoma and extrahepatic cholangiocarcinoma. A total of 10 cases of benign biliary epithelia were included as controls. The methylation status of tumor suppressor genes was analyzed using methylation-specific PCR. We found that 85% of all cholangiocarcinomas had methylation of at least one tumor suppressor gene. The frequency of tumor suppressor gene methylation in cholangiocarcinoma was: *RASSF1A* (65%), *p15^{INK4b}* (50%), *p16^{INK4a}* (50%), *APC* (46%), *E-cadherin/CDH1* (43%), *p14^{ARF}* (38%), *p73* (36%), *MGMT* (33%), *hMHL1* (25%), *GSTP* (14%), *RAR-β* (14%) and *DAPK* (3%). Although single tumor suppressor gene methylation can be seen in benign biliary epithelium, methylation of multiple tumor suppressor genes is only seen in cholangiocarcinoma. About 70% (50/72) of the cholangiocarcinomas had three or more tumor suppressor genes methylated and 52% (38/72) of cases had four or more tumor suppressor genes methylated. Concerted methylation of multiple tumor suppressor genes was closely associated with methylation of *RASSF1A*, p16 and/or *hMHL1*. Methylation of *RASSF1A* was more common in extrahepatic cholangiocarcinoma than intrahepatic cholangiocarcinoma (83 *vs* 47%, $P=0.003$) while *GSTP* was more frequently seen in intrahepatic compared to extrahepatic cholangiocarcinoma (31 *vs* 6%, $P=0.012$). Our study indicates that methylation of promoter CpG islands of tumor suppressor genes is a common epigenetic event in cholangiocarcinoma. Based on distinct methylation profiles, intrahepatic cholangiocarcinoma and extrahepatic cholangiocarcinoma are two closely related but biologically unique neoplastic processes. Taking advantage of the unique concurrent methylation profile of multiple genes in cholangiocarcinoma may facilitate the distinction of cholangiocarcinoma from benign biliary epithelium in clinical settings.
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Cholangiocarcinoma is an uncommon but highly aggressive malignancy, which arises from the biliary ductal epithelium, either intrahepatically or extrahepatically. There is evidence that the neoplastic transformation of biliary epithelial cells and malignant progression of cholangiocarcinoma is accompanied by a number of genetic and epigenetic alterations.[1,2] Genetic alterations such as point mutations of *K-ras* and *p53* have been found in a subset of cholangiocarcinoma.[3–6] Mutation or deletion of *p14^{ARF}* and *p16^{INK4a}* were not frequent events in cholangiocarcinoma.[7,8] Although overexpression of β-catenin was frequently encountered in cholangiocarcinoma, mutation of *β-catenin* has not been identified to date.[9] These studies indicate that genetic alterations frequently seen in other epithelial cancers are not commonly seen in cholangiocarcinoma.

Recent studies have shown that the function of a tumor suppressor gene can be silenced through promoter methylation. Epigenetic inactivation of a set of tumor suppressor genes through promoter methylation had been recently studied in intrahepatic cholangiocarcinoma in Asian populations.[2] However, to our knowledge, methylation profiles of extrahepatic cholangiocarcinoma has not been well characterized. To further understand the epigenetic

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changes in cholangiocarcinoma and to see the difference in epigenetic alterations between intrahepatic cholangiocarcinoma and extrahepatic cholangiocarcinoma, we have studied the promoter methylation profiles of 12 candidate tumor suppressor genes in 72 cases of cholangiocarcinoma, including 36 cases of intrahepatic cholangiocarcinoma and 36 cases of extrahepatic cholangiocarcinoma and compared to 10 cases of benign biliary epithelia. In all, 12 tumor suppressor genes, involved in several signal transductional pathways, are adenomatous polyposis coli (*APC*), *E-cadherin/CDH1*, O^6-methylguanine methyltransferase (*MGMT*), ras-associated secreted factor (*RASSF1A*), glutathione *S*-transferase (*GSTP*), retinoic acid receptor beta-2 (*RAR-β*), $p14^{ARF}$, $p15^{INK4B}$, $p16^{INK4A}$, *p73*, *hMLH1* and death-associated protein kinase (*DAPK*).

Materials and methods

Tumor Samples

In all, 72 cases of cholangiocarcinoma, including 36 cases of intrahepatic cholangiocarcinoma and 36 cases of extrahepatic cholangiocarcinoma, were collected from Johns Hopkins Pathology and Cytopathology Archives, with the permission of Johns Hopkins University Institutional Review Board. The specimens include 52 cases of paraffin-embedded surgical resection specimens and 20 cases of cytologic smears from fine-needle aspiration biopsies. All the cytologic cases had confirmed diagnosis from subsequent tumor biopsies or resections. In all, 10 cases of negative bile duct surgical margins were used as control. No initial chemotherapy or radiation therapy was instituted before tumor excision or fine-needle aspiration and biopsy procedure. The tissue was fixed either in buffered-formalin for surgical specimens or in ethanol-based fixative for fine-needle aspiration and biopsy specimens. Consecutive sections were cut at 4 μm from paraffin-embedded tissue blocks and mounted for histopathologic evaluation using conventional hematoxylin and eosin (H&E) staining. H&E-stained sections also served as a guide for DNA analysis. For DNA extraction, consecutive 10 μm sections from resected specimens were directly collected into sterile Eppendorff tubes. Cells from cytologic smears were scraped into Eppendorff tubes by sterile surgical blades. Genomic DNA was isolated by digestion with 100 μg/ml proteinase K and followed by conventional phenol/chloroform (1:1) extraction and ethanol precipitation.

Bisulfite Modification and Methylation-Specific PCR

Bisulfite modification and methylation-specific PCR were conducted based on the principle that bisulfite treatment of DNA converts unmethylated cytosine residues into uracil, whereas methylated cytosine residues remain unmodified. Thus, after bisulfite conversion, methylated and unmethylated DNA sequences can be distinguished by sequence-specific primers. DNA was treated with sodium bisulfite as previously described.[10] Briefly, 1 μg of genomic DNA was denatured by incubation with 0.2 M NaOH for 10 min at 37°C. Aliquots of 10 mM hydroquinone (30 μl; Sigma Chemical Co., St Louis, MO, USA) and 3 M sodium bisulfite (pH 5.0, 520 μl; Sigma Chemical Co.) were added and the solution was incubated at 50°C for 16 h. Treated DNA was purified by use of Wizard DNA purification System (Promega Corp., Madison, WI, USA), desulfonated with 0.3 M NaOH, precipitated with ethanol, and resuspended in water. Modified DNA was stored at −70°C until used. DNA sequences containing promoter regions of *APC*, *E-cadherin/CDH1*, *RASSF1A*, *GSTP*, *RAR-β*, $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$, *p73*, *MGMT*, *hMHL1* and *DAPK* genes were first amplified in a single PCR run with 30 cycles using flanking primer sets as previously described.[11,12] DNA methylation of CpG islands was then determined by PCR using specific primers for both methylated and unmethylated DNA.[11,12] Two sets of primers were used to amplify each region of interest: one pair recognized a sequence in which CpG sites are unmethylated (bisulfite modified to UpG), and the other recognized a sequence in which CpG sites are methylated (unmodified by bisulfite treatment). Negative control samples without DNA template (water only) and DNA were included for each set of PCR. PCR products were analyzed on 1% polyacrylamide gels. Methylation status of *hMHL-1* gene was analyzed only in 52 cases of surgical specimens; however not in cytologic specimens, due to the limitation of samples in the latter. Methylation-specific PCR of all the cases with negative methylation status and some of the methylation-positive cases were repeated to confirm the results.

Statistical Analysis

χ^2- or Fisher exact tests, depending on the absolute numbers included in the analysis, were used to analyze the frequency and association of concurrent tumor suppressor gene methylation in cholangiocarcinoma. χ^2- or Fisher's exact test were also applied to the correlation between tumor suppressor gene methylation profiles and clinicopathologic data. The strength of bivariate association between pairs of tumor suppressor genes was calculated using Pearson's correlation coefficients. A logistic regression analysis was used to analyze the correlation between tumor suppressor gene methylation profiles and the degree of tumor differentiation.

Results

Frequency of Tumor Suppressor Gene Promoter Methylation in Cholangiocarcinoma

Promoter methylation profiles of 12 tumor suppressor gene from 72 cases of cholangiocarcinoma and

10 cases of benign biliary epithelia is illustrated in Figure 1. The frequency of methylation of individual tumor suppressor gene is summarized in Table 1. Of 72 cases of cholangiocarcinoma, 61 cases (85%) had methylation of at least one tumor suppressor gene, while 11 cases (15%) had no detectable methylation of any 12 tumor suppressor genes studied. The frequency of tumor suppressor gene methylation in cholangiocarcinoma was: *RASSF1A* (65%), $p15^{INK4b}$ (50%), $p16^{INK4a}$ (50%), *APC* (46%), *E-cadherin/CDH1* (43%), $p14^{ARF}$ (38%), *p73* (36%), *MGMT* (33%), *hMHL-1* (25%), *GSTP* (14%), *RAR-β* (14%) and *DAPK* (3%). In benign biliary epithelial tissues, methylation of tumor suppressor genes were either nondetectable ($p14^{ARF}$, *p73*, *MGMT*, *hMLH1*, *E-cadherin/CDH1*, *GSTP*, *RAR-β* and *DAPK*) or with low frequency (20% for *RASSF1A* and 10% for $p15^{INK4b}$, $p16^{INK4a}$ and *APC*). There was a statistically significant difference between cholangiocarcinoma and benign biliary tissue in the frequency of methylation of *RASSF1A*, $p16^{INK4a}$, $p15^{INK4b}$, *APC*, $p14^{ARF}$ and *p73* (Fisher's exact test, $P=0.012$, 0.017, 0.020, 0.020, 0.026 and 0.026, respectively). There were marginal differences between cholangiocarcinoma and benign biliary epithelium in methylation of *MGMT* and *E-cadherin/CDH1* ($P=0.056$ and 0.078, respectively).

Concurrent Methylation of Multiple Tumor Suppressor Genes in Cholangiocarcinoma

Promoter methylation of multiple tumor suppressor genes was frequently seen in cholangiocarcinoma. Number of tumor suppressor genes methylated ranged from one to nine genes in cholangiocarcinoma. Although methylation of single tumor suppressor gene was seen in 50% (5/10) of cases of benign biliary epithelia, it was only encountered in two cases (3%) of cholangiocarcinoma. Of 72 cases of cholangiocarcinoma, 59 cases (82%) had methylation of two or more tumor suppressor genes (Table 2). In contrast, methylation of two or more tumor suppressor gene promoters was not seen in benign biliary tissue (Figure 1). Furthermore, 50 cases (70%) of cholangiocarcinoma had methylation of three or more tumor suppressor genes and 37 cases (52%) of cholangiocarcinoma had methylation of four or more tumor suppressor genes (Table 2). Methylation of multiple tumor suppressor genes tends to occur in those most frequently methylated genes, such as *RASSF1*, *APC*, $p15^{INK4b}$ and $p16^{INK4a}$. For instance, concurrent methylation of four or more tumor suppressor gene was seen in about 66% (31/47) cases of cholangiocarcinoma that harbored *RASSF1A* methylation; however, was found only in 32% (8/25) of cases without *RASSF1A* methylation. The difference between these two groups was statistically significant ($P=0.028$). Similarly, there was also a close correlation between methylation of *hMLH1* promoter and concurrent methylation of multiple tumor suppressor genes in cholangiocarcinoma. Of 52 cases of cholangiocarcinoma analyzed for *hMLH1*, methylation of the *hMLH1* promoter was found in 25% (13/52) cases. Among *hMLH1* methylators, methylation of five or more tumor suppressor genes was seen in 92% (12/13) cases. In contrast, only 39% (15/39) cases without *hMLH1* methylation had methylation of five or more tumor suppressor genes ($P=0.001$, Table 3).

Association between Methylation of Various Genes in Cholangiocarcinoma

To see whether epigenetic inactivation of a restricted number of pathways or of numerous signaling pathways involved in development of cholangiocarcinoma, we analyzed the strength of bivariate association between pairs of tumor suppressor genes methylated in cholangiocarcinoma using Pearson's correlation coefficients. Overall, methylation of all 12 tumor suppressor genes, except *DAPK*, showed some degree of association with each other. In all, 20 pairs of tumor suppressor genes were identified with significant correlation ($P<0.01$). Among these 20 pairs, 16 pairs associated with methylation of three core genes: *RASSF1A*, $p16^{INK4a}$ and *hMLH1* (Figure 2). Not only was methylation of these three tumor suppressor genes correlated each other, but also methylation of each of these three tumor suppressor genes (*RASSF1A*, $p16^{INK4a}$ and *hMLH1*) correlated significantly ($P<0.01$) with methylation of other tumor suppressor genes (Figure 2a). Specifically, methylation of *RASSF1A* promoter was closely associated with the methylation of *GSTP*, *hMLH1*, $p14^{ARF}$, $p16^{INK4a}$ and *p73*. Methylation of $p16^{INK4a}$ promoter was significantly associated with the methylation of *APC*, *E-cadherin/CDH1*, *hMLH1*, $p14^{ARF}$, *p15*, *p73*, *RAR-β* and *RASSF1A*. Whereas methylation of *hMLH1* promoter was strongly associated with the methylation of *MGMT*, $p14^{ARF}$, $p16^{INK4a}$, *RAR-β* and *RASSF1A* (Figure 2a).

Methylation Profiles between Intrahepatic Cholangiocarcinoma and Extrahepatic Cholangiocarcinoma

Promoter methylation profiles of tumor suppressor genes were compared between 36 cases of intrahepatic cholangiocarcinoma and 36 cases of extrahepatic cholangiocarcinoma. In terms of methylation

Figure 1 Promoter methylation profiles of 12 candidate tumor suppressor genes in intrahepatic cholangiocarcinoma, extrahepatic cholangiocarcinoma and benign biliary epithelia. Blank space represents no detectable promoter methylation of an individual tumor suppressor gene and black space represents detectable promoter methylation of an individual tumor suppressor gene. Space with cross represents the case that has not been analyzed for *hMHL1*.

Case #	RASSF1A	p15	p16	APC	CDH1	p14	p73	MGMT	hMLH1	GSTP	RAR-b	DAPK
BB1					■							
BB2	■											
BB3			■									
BB4												
BB5										■		
BB6	■											
BB7												
BB8				■								
BB9												
BB10												
IC1				■	■							
IC2				■	■			■				
IC3					■		■				■	
IC4												
IC5			■		■							
IC6	■	■			■		■	■		■		
IC7	■	■			■			■	■	■		
IC8												
IC9												
IC10			■	■	■					■	■	
IC11	■	■	■	■	■					■		
IC12	■	■	■	■				■	■	■	■	
IC13					■		■					
IC14	■	■	■		■		■			■	■	
IC15	■	■	■		■		■			■	■	
IC16												
IC17		■	■	■	■							
IC18	■	■	■	■	■	■		■	■		■	
IC19	■	■	■	■		■	■	■				
IC20		■	■	■		■		■		■		
IC21		■		■						■		
IC22		■		■		■						
IC23		■				■						
IC24	■	■	■			■						
IC25	■	■	■	■		■	■	■	■			
IC26	■	■	■	■		■		■	■			
IC27									x			
IC28						■			x			
IC29	■	■	■	■			■		x	■		
IC30			■		■	■			x			
IC31	■	■		■	■				x			
IC32	■	■	■	■	■		■		x			
IC33									x			
IC34	■			■			■	■	x	■		
IC35	■					■			x			
IC36	■	■			■				x			
EC1	■											
EC2												
EC3	■	■					■					
EC4	■		■					■				
EC5				■								
EC6	■						■					
EC7	■			■			■					
EC8	■		■		■							
EC9	■		■	■			■				■	
EC10	■	■	■	■		■	■		■		■	
EC11	■	■	■	■	■	■		■	■			
EC12	■	■	■	■			■					
EC13	■	■	■	■		■	■					
EC14	■	■	■	■		■			■		■	■
EC15	■	■	■	■	■	■					■	
EC16	■	■	■	■	■	■	■		■		■	
EC17	■	■	■	■	■	■	■	■	■			
EC18	■	■	■	■	■	■	■	■	■			
EC19												
EC20	■	■	■	■	■	■	■		■			
EC21	■				■	■				■		
EC22	■				■	■		■			■	
EC23	■					■		■				
EC24												
EC25	■	■	■		■	■	■					
EC26	■		■	■	■				■			
EC27	■	■	■	■	■	■		■	x			
EC28									x			
EC29	■		■						x		■	
EC30	■				■		■		x			
EC31	■	■	■	■		■	■	■	x			
EC32	■						■	■	x			
EC33	■							■	x			
EC34									x			
EC35	■	■	■		■	■		■	x	■		
EC36	■	■	■	■		■			x			■

Table 1 Frequency of methylation of tumor suppressor genes in cholangiocarcinoma

	RASSF-*1*	*p15*	*p16*	*APC*	*E-cad*	*p14*	*p73*	*MGMT*	hMLH1[a]	*GSTP*	*RAR-β*	*DAPK*
CC	65.3% (47)	51.4% (37)	50.0% (36)	45.8% (33)	43.1% (31)	37.5% (27)	36.1% (26)	33.0% (24)	23.6% (13)	18.1% (13)	18.1% (13)	2.7% (2)
ICC	48.6% (17)	54.1% (20)	48.6% (17)	47.2% (17)	48.6% (17)	30.0% (11)	27.0% (10)	27.0% (10)	18.5% (5)	31.4% (11)	16.0% (6)	0% (0)
ECC	83.3% (30)	48.6% (17)	54.3% (19)	44.4% (16)	40.0% (14)	46.0% (16)	43.0% (15)	40.0% (14)	32.0% (8)	5.7% (2)	20.0% (7)	5.7% (2)

CC—cholangiocarcinoma; ICC—intrahepatic cholangiocarcinoma; ECC—extrahepatic cholangiocarcinoma.
[a]Total 55 cases were used for *hMLH* MSP analysis.

Table 2 Concurrent methylation of tumor suppressor genes in cholangiocarcinoma

Number of TSG	*CC (%) (72)*	*ICC (%) (36)*	*ECC (%) (36)*
0 TSG	15.3 (11)	16.7 (6)	13.9 (5)
1 TSG	2.7 (2)	2.7 (1)	2.7 (1)
2 TSGs	11.2 (8)	13.9 (5)	8.3 (3)
3 TSGs	19.4 (14)	16.7 (6)	22.2 (8)
$\geq$4 TSGs	51.4 (37)	50.0 (18)	52.9 (19)

Absolute case numbers are included within parentheses. Abbreviations: TSG—tumor suppressor gene; CC—cholangiocarcinoma; ICC—intrahepatic cholangiocarcinoma; ECC—extrahepatic cholangiocarcinoma.

Table 3 Association of methylation status of *hMLH1* with concurrent methylation index of multiple tumor suppressor genes in cholangiocarcinoma

CMI	hMLH1 *methylator (%)* (n = *13*)	hMLH1 *nonmethylator (%)* (n = *39*)	P-*values*
0 TSGs	0 (0)	17.9 (7)	0.172
$\leq$5 TSGs	7.7 (1)	59.0 (17)	0.003
> 5 TSGs	92.3 (12)	23.1 (9)	0.001

CMI—concerted methylation index; TSG—tumor suppressor gene.

frequency, methylation of *RASSF1A* was seen more frequently in extrahepatic cholangiocarcinoma (30/36) than that in intrahepatic cholangiocarcinoma (17/36) (83 *vs* 47%, $P = 0.003$). Although methylation of $p14^{ARF}$, *p73*, *MGMT* and *hMLH1* was seen more often in extrahepatic cholangiocarcinoma than intrahepatic cholangiocarcinoma, the difference was not statistically significant ($P > 0.05$). On the other hand, methylation of *GSTP* promoter was more common in intrahepatic cholangiocarcinoma than in extrahepatic cholangiocarcinoma and this difference was statistically significant (31 *vs* 6%, $P = 0.012$). Although there was no significant difference in concurrent methylation of multiple tumor suppressor genes between intrahepatic and extrahepatic cholangiocarcinoma (Table 2), different bivariate association of pairs of tumor suppressor genes exists between intrahepatic cholangiocarcinoma and extrahepatic cholangiocarcinoma (Figure 2b and c). For example, methylation of *GSTP* or *MGMT* was not associated with methylation of other tumor suppressor genes in intrahepatic cholangiocarcinoma (Figure 2b), but methylation of *GSTP* was strongly ($P < 0.01$) associated with methylation of $p15^{INK4b}$, $p16^{INK4a}$, *p73* and *RAR-β* in extrahepatic cholangiocarcinoma. Similarly, methylation of *MGMT* was significantly ($P < 0.05$) associated with methylation of *APC*, *hMLH1* and *RASSF1A* in extrahepatic cholangiocarcinoma (Figure 2c).

Correlation of Tumor Suppressor Gene Methylation and Clinicopathologic Data

Methylation profiles have been correlated with clinicopathologic data, such as patient gender, tumor size and tumor differentiation. There was no significant difference in the frequency of tumor suppressor gene methylation between genders (50 males and 22 females, $P > 0.05$). The tumor size of cholangiocarcinoma ranged from 1.0 to 13 cm. Tumor differentiation of 52 cases of surgical specimens was graded histopathologically into well-, moderately- and poorly differentiated. For intrahepatic cholangiocarcinoma cases, 26 cases include eight cases of well differentiated, 12 cases of moderately differentiated and six cases of poorly differentiated. For extrahepatic cholangiocarcinoma, 26 cases consist of 13 cases of well differentiated, nine cases of moderately differentiated and four cases of poorly differentiated. All the cases of intrahepatic cholangiocarcinoma and extrahepatic cholangiocarcinoma are classified as adenocarcinoma, not otherwise specified. There was no statistical significance between the frequency of individual tumor suppressor gene methylation and the degree of tumor differentiation ($P > 0.05$, data not shown). In terms of concerted multiple tumor suppressor gene methylation, methylation of four and more tumor suppression gene promoters was seen relatively more frequently in poorly differentiated tumors (70%, 7/10) than moderately differentiated (47%, 10/21) and well-differentiated tumors (38%, 8/21). However, differences were not statistically significant between poorly- and moderately differentiated ($P = 0.28$) and between poorly- and well-differentiated ($P = 0.15$).

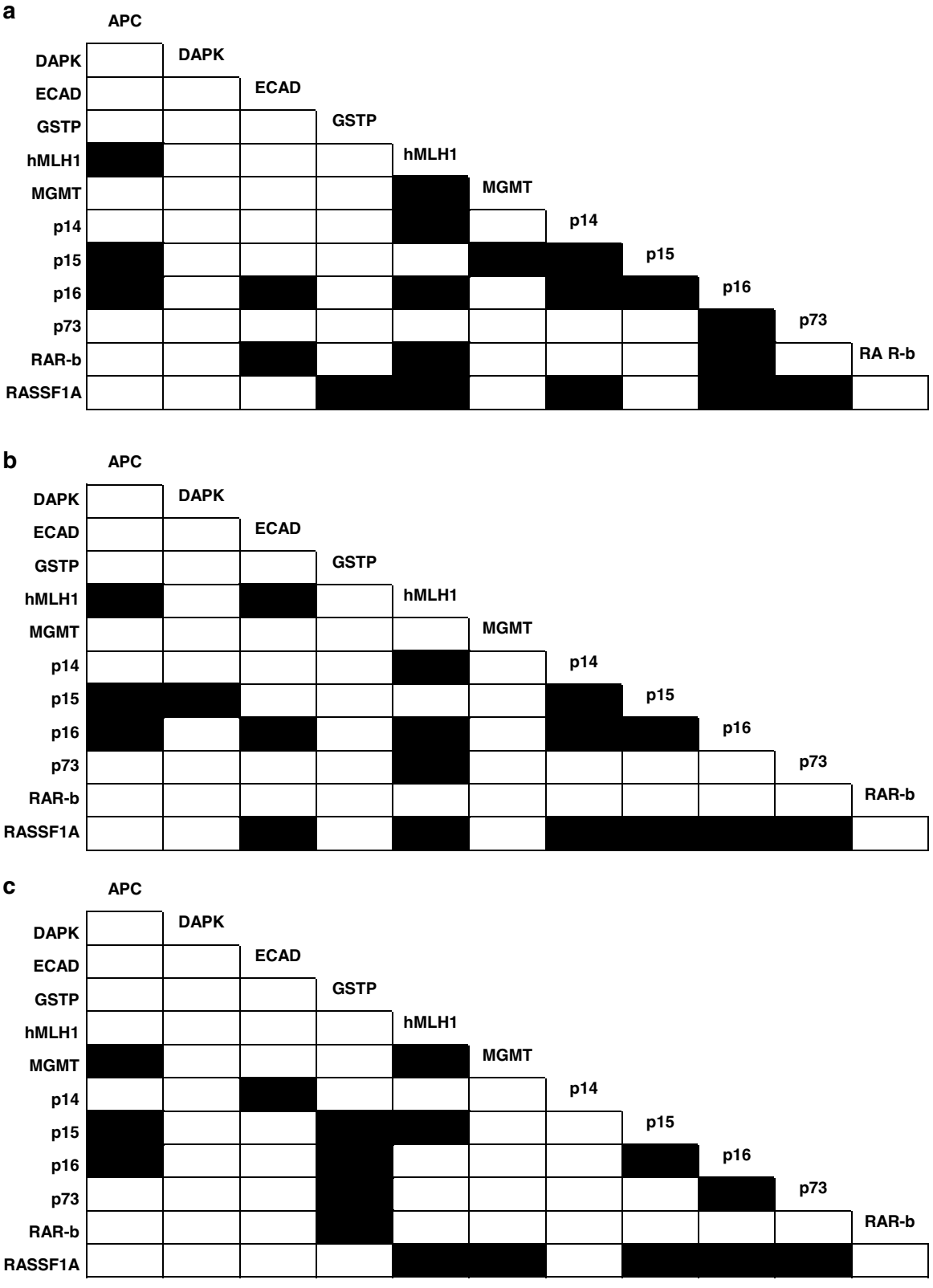

Figure 2 Many genes are coordinately methylated in cholangiocarcinoma. The strength of bivariate association between pairs of genes was calculated using Pearson's correlation coefficients. Black space represents methylation of pair of genes that demonstrates significant association ($P<0.01$). (**a**) Bivariate association of promoter methylation of 12 tumor suppressor genes in all 72 cases of cholangiocarcinoma. (**b**) Bivariate association of methylation of tumor suppressor genes in 36 cases of extrahepatic cholangiocarcinoma. (**c**) Bivariate association of methylation of tumor suppressor genes in 36 cases of intrahepatic cholangiocarcinoma.

Discussion

The molecular mechanisms of cholangiocarcinoma are 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.[13,14] Although epigenetic changes of intrahepatic cholangiocarcinoma had been recently studied in Asian populations,[2] methylation profiles of extrahepatic cholangiocarcinoma has not been reported to our knowledge. To further understand the epigenetic alterations in cholangiocarcinoma and to compare the methylation profiles between intrahepatic and extrahepatic cholangiocarcinoma, we have studied the methylation status of the promoter CpG islands of 12 tumor suppressor genes in 72 cases of cholangiocarcinoma, including 36 cases of intrahepatic cholangiocarcinoma and 36 cases of extrahepatic cholangiocarcinoma.

Our study demonstrates that epigenetic alteration of tumor suppressor genes, namely promoter hypermethylation, is a frequent event in cholangiocarcinoma. About 85% of cholangiocarcinoma had methylation of at least one tumor suppressor gene that we studied. Although methylation of each of 12 tumor suppressor gene promoters was seen in cholangiocarcinoma, the most frequently methylated tumor suppressor genes were *RASSF1A* (65%), $p15^{INK4b}$ (50%), $p16^{INK4a}$ (50%), *APC* (46%) and *E-cadherin/CDH1* (43%). One of the important findings from our study is that cholangiocarcinoma often had concurrent methylation of multiple tumor suppressor genes. Almost all methylated cases, except two, had methylation of two or more tumor suppressor genes. More than two-thirds of cholangiocarcinomas harbored methylation of three or more tumor suppressor gene promoters and more than half of the cases have methylation of four or more tumor suppressor gene promoters. It appears that development of cholangiocarcinoma may result from disruption of multiple signaling transduction pathways, such as the ras pathway (*RASSF1A*), Wnt pathway (*APC* and *E-cadherin/CDH1*), RB pathway ($p16^{INK4a}$), cell cycle control ($p15^{INK4b}$), *p53* pathway ($p14^{ARF}$ and *p73*) and microsatellite stability (*hMLH1*). Our data further indicate that accumulation of such epigenetic alterations through concerted methylation of multiple tumor suppressor genes is required during carcinogenesis of the biliary epithelium.

As 12 tumor suppressor genes examined here represents important elements of several signaling pathways, we are interested to see whether epigenetic inactivation of these tumor suppressor genes reflects disruption of either restricted or numerous signaling pathways involved in the development of cholangiocarcinoma and whether there are linkages between different pathways. Through the analysis of the strength of bivariate association between pairs of tumor suppressor genes methylated in cholangiocarcinoma, we found 20 pairs of tumor suppressor genes with significant correlation coefficients ($P<0.01$). Interestingly, 16 out of 20 pairs of such association are strongly correlated with the methylation of three core tumor suppressor genes: *RASS1A*, *p16* and *hMLH1*, which are involved in three separate signaling pathways (ras pathway, pRb pathway and DNA mismatch repair pathway). Our data suggest that these three signaling pathways are likely the main targets epigenetically altered during the development of cholangiocarcinoma.

The human *ras* association domain family *RASSF1A* gene was cloned from the lung tumor suppressor locus 3p21.3.[15] It has been shown that the expression of *RASSF1A* can be silenced through promoter methylation. Indeed, epigenetic inactivation is probably the main method that inactivates *RASSF1A* in cancer cells, since mutation of *RASSF1A* is extremely rare in cancer cells.[16] Methylation of *RASSF1A* has been found in lung, breast, ovarian, renal cell, bladder and gastric carcinomas.[17–22] We found that the vast majority cases of cholangiocarcinoma had methylation of *RASSF1A* promoter. Methylation of *RASSF1A* is often associated with concurrent methylation of multiple other tumor suppressor genes. The latter implies that methylation of *RASSF1A* may be one of the early events during biliary epithelial carcinogenesis. Although the biologic function of *RASSF1A* is still not entirely clear, recent studies indicate that *RASSF1A* is an important effector in mediating ras-induced apoptosis.[23–26] It has been long speculated that the ras pathway is one of the major targets altered during biliary epithelial carcinogenesis. Previous genetic studies have shown that activation of the *ras* oncogene through point mutations presented in a subset of cholangiocarcinoma, particularly in perihilar cholangiocarcinoma.[4,27] Our study further suggests that disruption of the ras pathway through the methylation of CpG islands in the *RASSF1A* promoter may also contribute to the development of cholangiocarcinoma. Recent studies indicate that methylation of *RASSF1A* and *ras* mutations are mutually exclusive in colorectal cancers.[28] Whether such phenomenon exists in cholangiocarcinoma needs further investigation.

Recent studies have indicated that the Wnt pathway is also involved in the pathogenesis of cholangiocarcinoma. Immunohistochemical study has shown that altered expression of β-catenin is found in up to 84% of cholangiocarcinoma.[9] However, mutation of *β-catenin* was not yet identified by sequencing 58 cases of cholangiocarcinoma.[9] Reduced expression of *E-cadherin/CDH1* was also reported in cholangiocarcinoma.[29] We found that about 69% of cases of cholangiocarcinoma had methylation of either *APC* or *E-cadherin/CDH1* or both, including 46% of cholangiocarcinoma with methylation of *APC* and 43% cases with methylation of *E-cadherin/CDH1*. Our data suggest that

promoter methylation of *APC* and/or *E-cadherin/CDH1* may contribute in certain extent to the disruption of Wnt pathway and perhaps the activation of β-catenin in cholangiocarcinoma. Interestingly, methylation of *APC* and *E-cadherin/CDH1* seems to be mutually exclusive in majority of cholangiocarcinoma. Approximately two-third of cases of cholangiocarcinoma had methylation of either *APC* or *E-cadherin/CDH1*; however, not both. A similar phenomenon was also observed in p53 pathway. We found that 68% of the cases had methylation of either $p14^{ARF}$ or *p73*. Our data suggest that disruption of one important element in the same signaling pathway is probably enough to diminish the function of the signaling pathway. According to Knudson's two-hit theory in cancer cells, it will be particularly interesting to see whether correlation between genetical loss of heterozygosity of one allele and epigenetic inactivation of remaining allele of such a frequently methylated tumor suppressor gene exists in cholangiocarcinoma.

Previous genetic studies have shown that there are different profiles between intrahepatic and extrahepatic cholangiocarcinoma. For instance, Kang *et al*[3] found more frequent *K-ras* mutation, in cholangiocarcinoma arising in major bile ducts such as hilar cholangiocarcinoma, but only very rare mutations seen in peripheral type or intrahepatic cholangiocarcinoma. A recent immunohistochemical study also suggests that intrahepatic cholangiocarcinoma and extrahepatic cholangiocarcinoma have different cytokeratin immunoprofiles.[30] Intrahepatic cholangiocarcinoma tends to be CK7+/CK20−, while extrahepatic cholangiocarcinoma is more frequently CK7+/CK20+.[30] To further understand the epigenetic changes in cholangiocarcinoma, we have compared the methylation profiles and patterns between intrahepatic- and extrahepatic cholangiocarcinoma. We found that different methylation profiles do exist between intrahepatic- and extrahepatic cholangiocarcinoma. Most of the tumor suppressor genes analyzed had similar methylation frequencies between intrahepatic- and extrahepatic cholangiocarcinoma, such as methylations of *APC*, *E-cadherin/CDH1*, $p15^{INK4b}$, $p16^{INK4a}$ and *MGMT*. However, methylation of *RASSF1A* and *GSTP* showed significant differences between intrahepatic- and extrahepatic cholangiocarcinoma. Methylation of *RASSF1A* was more frequently seen in extrahepatic cholangiocarcinoma than in intrahepatic cholangiocarcinoma (84 *vs* 47%), whereas methylation of *GSTP* was more commonly seen in intrahepatic cholangiocarcinoma (31 *vs* 6%). The high frequency of *RASSF1A* methylation closely resembles that seen in adenocarcinoma of the pancreas.[16] Whereas the higher frequency of *GSTP* methylation may be related to the close proximity between intrahepatic cholangiocarcinoma and hepatocellular carcinoma, which also harbors frequent methylation of *GSTP* promoter.[31] By dissecting the association between pairs of tumor suppressor gene methylation (Figure 2a and b), we found that although six pairs of such associations (mainly with *RASSF1A* and *APC*) were observed both in intrahepatic- and extrahepatic cholangiocarcinoma, majority of the bivariate associations were different between intrahepatic- and extrahepatic cholangiocarcinoma. Our findings support the notion that intrahepatic- and extrahepatic cholangiocarcinoma may be two closely related but different biologic entities.

Accurate diagnosis of cholangiocarcinoma in cytologic specimens, such as biliary brushing and fine-needle aspiration biopsy, is notoriously challenging for pathologists. One of the main reasons is that most cholangiocarcinomas have well-differentiated histopathologically and morphologically overlap with reactive biliary epithelia. As concurrent methylation of multiple tumor suppressor genes was not seen in benign biliary epithelium, methylation-specific PCR analysis of a set of tumor suppressor genes could be useful in facilitating accurate diagnosis of cholangiocarcinoma from benign/reactive biliary epithelium in preoperative biopsy or cytologic brushings. By analysis of a limited number of cytologic samples (biliary brushing and fine-needle aspiration and biopsy smears), we found that cytologic specimens (air-dried smears with Diff-Quik stain or alcohol-fixed smears with Papanicolaou stain) yield equal or better methylation-specific PCR results compared to recuts from paraffin-embedded tissues, probably due to less genomic fragmentation in cytologic specimens than formalin-fixed surgical specimens. Besides its fast and less complicated technical merits, one of the advantages of methylation-specific PCR technique is that it can differentially detect hypermethylated allele (often seen in malignant cells) from unmethylated allele (often seen in normal cells) by designing different size of the PCR products. This is particularly useful for applying it in clinical materials since it does not require the enrichment of cancer cells from background reactive inflammatory cells and stromal cells. It should be noted that our comparison tissues were not normal bile ductal epithelia, but rather benign biliary epithelia of negative surgical margins that are far (>2 cm) from cancerous lesions. In order to assess the utility of methylation profiling in diagnostic pathology, it is important to study the background benign tissue surrounding malignant lesions. Our data show that although single gene methylation can be seen in benign biliary epithelia, it is the concurrent multiple gene methylation profiling that may be clinically useful in distinguishing cholangiocarcinoma from benign biliary epithelium.

In summary, we found that promoter methylation of tumor suppressor genes was frequent in cholangiocarcinoma. Intrahepatic- and extrahepatic cholangiocarcinoma have overlapping but different methylation profiles. Concurrent methylation of

multiple tumor suppressor genes is a unique finding in cholangiocarcinoma since it was not found in benign biliary epithelia. Such concurrent methylation patterns in cholangiocarcinoma may be a useful adjunctive diagnostic test for cholangiocarcinoma in clinical samples, such as cytologic or small biopsy specimens that are not uncommonly frequent with difficulty using morphologic features alone.

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