Aberrant Promoter Hypermethylation of Multiple Genes in Gallbladder Carcinoma and Chronic Cholecystitis

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ABSTRACT

Purpose: **Aberrant methylation of 5′ gene promoter regions is an epigenetic phenomenon that is a major mechanism for silencing of tumor suppressor genes in many cancer types. There is limited information about the molecular changes involved in the pathogenesis of gallbladder carcinoma (GBC), including methylation status.**

Experimental Design: **We investigated the aberrant promoter methylation profile of 24 known or suspected tumor suppressor genes in 50 GBCs and compared those results with the findings in 25 chronic cholecystitis (CC) specimens without cancer. The methylation-specific polymerase chain reaction and combined restriction analysis methods were used to detect methylation, and the results were confirmed by sequencing of cloned polymerase chain reaction products.**

Results: **In GBC, gene methylation frequencies varied from 0% to 80%. Ten genes demonstrated relatively high frequencies of aberrant methylation: *SHP1* (80%), *3-OST-2* (72%), *CDH13* (44%), *P15^{INK4B}* (44%), *CDH1* (38%), *RUNX3* (32%), *APC* (30%), *RIZ1* (26%), *P16^{INK4A}* (24%), and *HPP1* (20%). Eight genes (*P73*, *RARβ2*, *SOCS-1*, *DAPK*, *DcR2*, *DcR1*, *HIN1*, and *CHFR*) showed low frequencies (2–14%) of methylation, and no methylation of the remaining six genes (*TIMP-3*, *P57*, *RASSF1A*, *CRBP1*, *SYK*, and *NORE1*) was detected. In CC, methylation was detected for seven genes: *SHP1* (88%), *P15^{INK4B}* (28%), *3-OST-2* (12%), *CDH1* (12%), *CDH13* (8%), *DcR2* (4%), and *P16^{INK4A}* (4%). Significantly higher frequencies of methylation in GBC compared with CC were detected for eight genes (*3-OST-2*, *CDH13*, *CDH1*, *RUNX3*, *APC*, *RIZ1*, *P16^{INK4A}*, and *HPP1*). Of those, four genes showed frequent methylation (>30%) in GBCs. The mean methylation index, an expression of the amount of methylated genes by case, was significantly higher in GBC (0.196 ± 0.013) compared with CC (0.065 ± 0.008; $P < 0.001$).**

Conclusions: **Our study constitutes the most comprehensive methylation profile report available in GBC and demonstrates that this neoplasm has a distinct pattern of abnormal gene methylation. Whereas gallbladders from healthy individual were not available, our finding of methylation in CC cases without cancer suggests that this phenomenon represents an early event in the pathogenesis of GBC.**

INTRODUCTION

Gallbladder carcinoma (GBC) is a relatively uncommon neoplasm that demonstrates considerable geographic and gender variation in incidence (1, 2). Mortality rates are highest among American Indian women from the southwestern United States and Chilean and Japanese women. In Chile, GBC is one of the most frequent cancers and represents the leading cause of cancer deaths in females (1). GBC is a highly malignant neoplasm with a poor prognosis, with most cases diagnosed at advanced stages. There is a clear worldwide association between GBC and cholelithiasis and chronic inflammation of the gallbladder [chronic cholecystitis (CC)], which are present in almost all GBC specimens (1).

There is limited information about the molecular changes involved in the pathogenesis of GBC. Most studies have focused on mutations of dominant oncogenes (*K-ras*) or tumor suppressor genes (TSGs) [*TP53*, *P16^{INK4A}*, and *FHIT* (3–5)]. Genome-wide and specific chromosome arm allelotyping analyses indicated that allelic losses at multiple sites of the genome are frequent in this neoplasm, suggesting the involvement of multiple TSGs in its pathogenesis (6, 7). Recently, Hansel *et al.* (8), using a RNA-based global gene expression microarray analysis of ~22,000 transcripts in a series of biliary tract tumors including 10 GBC specimens, detected a number of abnormally expressed genes, most of which had not been described previously in these tumor types. DNA methylation of the promoter regions is emerging as the major mechanism of inactivation of TSGs, and the abnormal methylation profile of human tumors has been studied in the recent years (9, 10). Individual tumor types frequently have characteristic patterns of acquired aberrant methylation (10, 11). In many cases, aberrant methylation of the CpG island genes has been correlated with a loss of gene expression, and it is proposed that DNA methylation provides an

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alternative pathway to gene deletion or mutation for the loss of TSG function (11, 12). The understanding of the methylation profile of tumors may impact on several clinical issues, including early detection, chemoprevention, prognosis, and cancer treatment (12).

In GBC, there is very limited information regarding abnormal methylation of cancer-related genes. There is only one report of methylation analysis of six candidate TSGs (*P16^{INK4A}*, *APC*, *MGMT*, *hMLH1*, *RAR*β*2*, and *p73*), which showed significant differences in gene methylation patterns between GBC cases from Chile and the United States (13). To further investigate the TSG methylation profile in GBC, we examined the methylation profile of 24 genes frequently silenced by aberrant methylation in a number of tumor types in 50 GBCs from Chile, and we compared the findings with those present in 25 CC cases. The 24 genes were chosen for study due to their presumed or known roles in various cellular functions related to cancer development, including cell cycle regulation, tissue invasion and metastasis, Janus-activated kinase (JAK)/signal transducers and activators of transcription (STAT) and transforming growth factor β signal pathways, key components of retinoid activity, signal transduction, apoptosis, angiogenesis, putative cytokine, mitotic stress checkpoint, methyltransferase superfamily, and *O*-sulfotransferase (refs. 14–35; Table 1).

MATERIALS AND METHODS

Clinical Samples. Fifty GBC samples and 25 CC samples were obtained from cases resected by cholecystectomy at the Catholic University Medical School Hospital (Santiago, Chile). The patients consisted of 39 women and 11 men ranging in age from 36 to 94 years (mean age, 65 years). The tumors consisted of 45 adenocarcinomas, 4 adenosquamous carcinomas, and 1 squamous cell carcinoma. Adenocarcinomas consisted of 30 tubular, 5 papillary, and 10 tubulo-papillary tumors. Most of the tumors were advanced GBCs with invasion of the gallbladder subserosa (16 cases, 32%) and serosa (29 cases, 58%), and the rest of the cases (5 cases, 10%) were early GBCs (mucosa or muscularis propia invasion). Twenty-five CC specimens obtained by cholecystectomy from 21 women and 4 men ranging in age from 34 to 80 years (mean age, 48 years) were also selected.

Methylation Assays. Genomic DNA was isolated from frozen tissue by digestion with 100 μg/mL proteinase K followed standard phenol-chloroform (1:1) extraction and ethanol precipitation. DNA was treated with sodium bisulfite as described previously (14). Briefly, 1 μg of genomic DNA was denatured by incubation with 0.2 mol/L NaOH for 10 minutes at 37°C. Aliquots of 10 mmol/L hydroquinone (30 μl; Sigma Chemical Co., St. Louis, MO) and 3 mol/L sodium bisulfite (pH 5.0; 520 μl; Sigma Chemical Co.) were added, and the solution was incubated at 50°C for 16 hours. Treated DNA was purified by use of the Wizard DNA Purification System (Promega, Madison, WI), desulfurated with 0.3 mol/L NaOH, precipitated with ethanol, and resuspended in water. Modified DNA was stored at −80°C until use. Gene name, gene location, function, and reference for methodology of all genes are summarized in Table 1. The methylation status of 23 genes was determined by methylation-specific polymerase chain reaction (MSP). We used a combined restriction analysis for DAPK (35). Negative control samples without DNA were included for each set of polymerase chain reaction (PCR). PCR products were analyzed on 2% agarose gels containing ethidium bromide.

Table 1 Summary data of genes tested for aberrant promoter hypermethylation in GBC ($n = 50$) and CC specimens ($n = 25$)

Gene abbreviation	Gene name	Gene location	Function	Reference for methodology
P16^{INK4A}	*Cyclin-dependent kinase inhibitor 2A*	9p21	Cell cycle regulation	14
P15^{INK4B}	*Cyclin-dependent kinase inhibitor 2B*	9p21	Cell cycle regulation	14
p57^{KIP2}	*Cyclin-dependent kinase inhibitor 1C*	11p15.5	Cell cycle regulation	15
CDH1	*E-cadherin*	16q22	Tissue invasion and metastasis	16
CDH13	*H-cadherin*	16q24	Tissue invasion and metastasis	17
TIMP-3	*Tissue inhibitor of metalloproteinase-3*	22q12–13	Tissue invasion and metastasis	18
SOCS-1	*Suppressor of cytokine signaling-1*	16p13	JAK-STAT pathway	19
SHP1	*Hematopoietic cell-specific protein-tyrosine phosphatase SHPTP1*	12p13	JAK-STAT pathway	20
SYK	*Spleen tyrosine kinase*	9q22	JAK-STAT pathway	21
CRBP1	*Cellular retinol-binding protein 1*	3q21–22	Key components in retinoid activity	22
RARb2	*Retinoic acid receptor β2 gene*	3p24	Key components in retinoid activity	23
RASSF1A	*RAS association domain family protein 1A*	3p21	Signal transduction	24
NORE1	*Novel potential RAS effector*	1q32.1	Signal transduction	25
APC	*Adenomatous polyposis coli gene*	5q21	Signal transduction	26
DcR1	*Decoy receptor 1*	8p22	Apoptosis	27
DcR2	*Decoy receptor 2*	8p22	Apoptosis	27
p73	*TP73*	1p36	Angiogenesis	28
RUNX3	*runt-related transcription factor 3*	1p36	TGF-β signal pathway	29
HPP1	*TMEFF2*	2q32.3	TGF-β signal pathway	30
HIN-1	*High-in normal-1*	5q35	Putative cytokine	31
CHFR	*Checkpoint with FHA and ring finger*	12q24.33	Mitotic stress checkpoint gene	32
RIZ1	*Rb-interacting zinc finger gene 1*	1p36	Methyltransferase superfamily	33
3-OST-2	*Heparan sulfate D-glucosaminyl 3-O-sulfotransferase-2*	16p12	*O*-Sulfotransferase	34
DAPK	*Death-associated protein kinase*	9q34	Proapoptotic serine/threonine kinase	35

Abbreviation: TGF, transforming growth factor.

Bisulfite Sequencing. The PCR products of MSP were cloned into plasmid vectors using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) as described previously (36). The inserted PCR fragments of four individual clones obtained from each sample were sequenced with M13 reverse primers using the Applied Biosystems PRISM dye terminator cycle sequencing method (Perkin-Elmer, Foster City, CA).

Data Analysis. Frequencies of methylation of two groups were compared using the χ^2 test or Fisher's exact test. The methylation index (MI), a reflection of the methylation status of all of the genes tested, is defined as the total number of genes methylated divided by the total number of genes analyzed. To compare the extent of methylation for the panel of genes examined, we calculated the MI for each case and then determined the mean for the different groups. Statistical analysis of MI between two variables was performed using the Mann-Whitney nonparametric *U* test. For all tests, $P < 0.05$ was regarded as statistically significant. For identification of a predictive model for GBC compared with CC based on gene methylation status, a multivariate penalized logistic regression model was used, and sensitivity and specificity were calculated.

RESULTS

Frequency of Gene Methylation in Gallbladder Carcinoma and Chronic Cholecystitis Specimens. We examined the methylation status of 24 genes (Table 1) in 50 GBC and 25 CC specimens (Fig. 1). In GBC, gene methylation frequencies varied from 0% to 80%. Ten genes demonstrated a relatively high frequency of aberrant methylation: *SHP1* (80%), *3-OST-2* (72%), *CDH13* (44%), *P15*^{INK4B} (44%), *CDH1* (38%), *RUNX3* (32%), *APC* (30%), *RIZ1* (26%), *P16*^{INK4A} (24%), and *HPP1* (20%). Eight genes (*P73*, *RAR*β*2*, *SOCS-1*, *DAPK*, *DcR2*, *DcR1*, *HIN1*, and *CHFR*) showed a low frequency (2–14%) of methylation, and no methylation was detected for the remaining six genes (*TIMP-3*, *P57*, *RASSF1A*, *CRBP1*, *SYK*, and *NORE1*; Fig. 2). In CC, methylation was detected for seven genes: *SHP1* (88%), *P15*^{INK4B} (28%), *3-OST-2* (12%), *CDH1* (12%), *CDH13* (8%), *DcR2* (4%) and *P16*^{INK4A} (4%; Fig. 2). The unmethylated form of *P16*^{INK4A}, run as a control for DNA integrity, was present in all tumor and inflammatory gallbladder samples examined. To confirm our MSP findings, bisulfite sequencing analysis of two representative methylated GBC or CC samples was performed in seven genes found to be frequently positive by the MSP technique (*3-OST-2*, *CDH13*, *CDH1*, *RUNX3*, *APC*, *SHP1*, and *p15*). All 14 samples in 56 individual clones analyzed demonstrated methylation of the CpG sites included in the amplicons, with methylation varying from 76% to 100%.

Comparative analysis of the methylation frequencies between GBC and CC classifies the 24 genes examined into three groups. The first group is that of genes showing significantly higher frequencies of methylation in GBC compared with CC ($n = 8$; *3-OST-2*, *CDH13*, *CDH1*, *RUNX3*, *APC*, *RIZ1*, *P16*^{INK4A}, and *HPP1*). Of these, four genes (*3-OST-2*, *CDH13*, *CDH1*, and *RUNX3*) showed methylation frequencies of >30%, and *3-OST-2* was the most frequently (72%) methylated gene in GBCs. Because our GBC and CC patients differed in age (patients ≥ 65 years, 50% GBC *versus* 12% CC; $P = 0.002$), we were unable to establish a predictive model for GBC based on methylation status of the above-mentioned eight genes. However, we performed a predictive model analysis using patients younger than 65 years ($n = 47$; GBC, 25 patients; CC, 22 patients) to minimize the age difference bias as much as possible. Our multivariate penalized logistic regression model analysis identified a five-gene (*3-OST-2*, *CDH13*, *RUNX3*, *P16*^{INK4A}, and *HPP1*) combination as a significant predictor of GBC compared with CC (92% sensitivity, 81% specificity). The second group was that of genes demonstrating frequent methylation in both GBC and CC specimens ($n = 2$; *SHP1* and

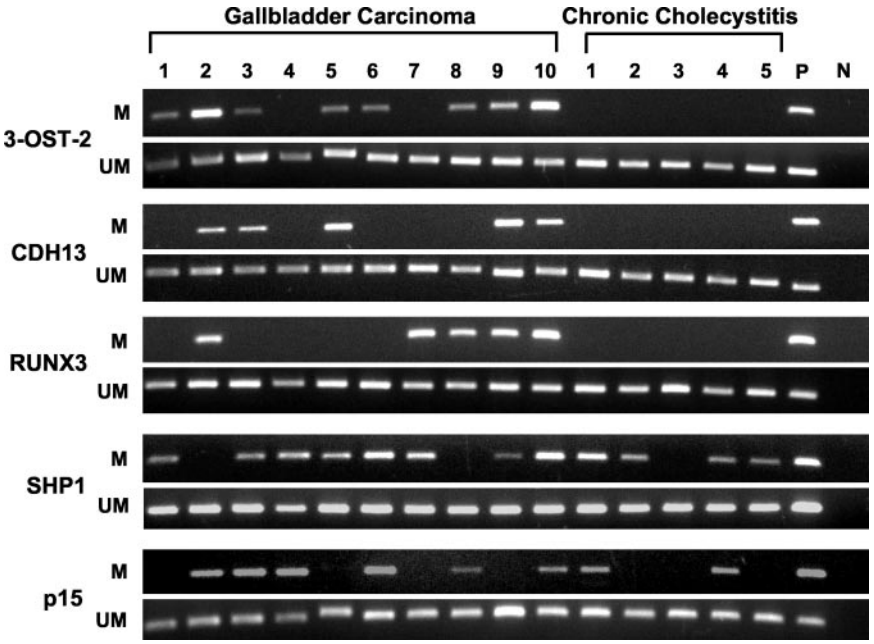

Fig. 1 Representative examples of MSP analyses of methylated form (*M*) or unmethylated form (*UM*) of five genes (*3-OST-2*, *CDH13*, *RUNX3*, *SHP1*, and *p15*) in GBC and CC samples. *P*, positive control; *N*, negative control.

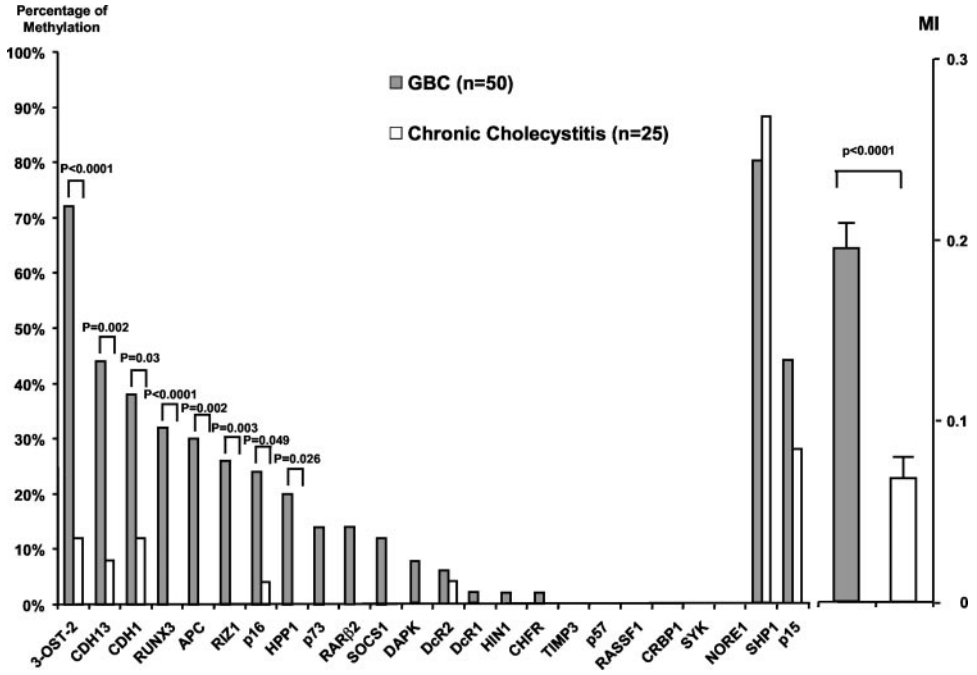

Fig. 2 Comparison of frequencies of aberrant methylation and mean MIs in GBC and CC samples. *P* values are shown when there was a significant difference between two groups.

P15^{INK4B}). The third group was that of genes showing low frequencies of methylation in both types of gallbladder specimens examined (the remaining 14 genes; Fig. 2).

Excluding *SHP1* and *P15*^{INK4B} genes, which were frequently methylated in malignant and nonmalignant gallbladder specimens, 96% of GBCs had methylation of one or more genes, whereas most of CCs (68%) had no gene methylated. In addition, a statistically significant ($P < 0.001$) higher mean MI was detected in GBC (0.196 ± 0.013) compared with CC (0.065 ± 0.008; Fig. 2).

Correlation between Methylation and Clinicopathological Features in Gallbladder Carcinoma. We compared the relationships between aberrant methylation of the eight genes (group 1) showing cancer specificity and clinicopathological features in GBCs. Interestingly, some statistically significant correlations between GBC features and gene methylation status of specific genes were established (Table 2): (*a*) *HPP1* was more frequently methylated in male patients (55%) than in female patients (10%; $P = 0.004$); (*b*) tumors displaying exclusively papillary or mixed papillary/tubular patterns demonstrated higher frequency of *APC* methylation than cancers exhibiting only tubular features (60% *versus* 13%, respectively; $P = 0.004$); (*c*) of interest, methylation of *RIZ1* correlated with tumor depth of invasion in the gallbladder wall and in adjacent liver tissue ($P = 0.0037$ and $P = 0.024$, respectively); and (*d*) *CDH1* methylation was associated with the presence of distant metastasis ($P = 0.024$). No significant association between methylation status and age was detected. In addition, no correlation between mean MI and clinicopathological features was noted.

DISCUSSION

Previous studies have reported the importance of DNA methylation in human cancers and focused on regions of the genome that may have functional significance resulting from suppression of gene activity. Whereas most individual cancers have several, perhaps hundreds, of methylated genes, the methylation profiles of individual tumor types are characteristic (10, 11). In GBC, there is very limited information regarding abnormal methylation of cancer-related genes (13). Thus, to further investigate the methylation profile of GBCs we tested the methylation status of a panel of 24 genes with presumed or known roles in various cellular functions related to cancer development that had been studied in many tumor types. Because of geography-related differences in the methylation profiles of GBCs, our studies were limited to tumors from Chile.

Analysis of the methylation frequencies in GBCs compared with the findings in CC specimens demonstrated a group of eight genes (*3-OST-2*, *CDH13*, *CDH1*, *RUNX3*, *APC*, *RIZ1*, *P16*^{INK4A}, and *HPP1*) that showed specificity for GBC, suggesting that they play an important role in GBC pathogenesis. A predictive model analysis based on these eight genes delineating GBC from CC failed because of substantial differences in age between both patient groups. However, if only patients < 65 years were considered, a five-gene (*3-OST-2*, *CDH13*, *RUNX3*, *P16*^{INK4A}, and *HPP1*) combination was a good predictor for GBC with 92% sensitivity and 81% specificity. Of these genes, the gene with the highest frequency (72%) of methylation in GBC was *3-OST-2*. This gene encodes an *O*-sulfotransferase that is involved in the final modification step of glycosaminoglycan chains of heparan sulfate proteoglycans (HSPGs), including glypicans and syndecans, whose altered expressions have been reported recently in human breast cancers (37). HSPGs are known to play major roles in cell growth, adhesion, and migration by interaction with a wide range of growth factors, morphogens, cytokines, and extracellular matrices (38). Silencing of *3-OST-2* suggests that altered modification of HSPGs is involved in cancer development in some tumor types.

Table 2 Relationship between aberrant promoter hypermethylation of eight genes (group 1) and clinicopathological features in GBCs

Clinicopathological characteristics	*3-OST-2*		*CDH13*		*CDH1*		*RUNX3*	
	Methylation (%)	*P*	Methylation (%)	*P*	Methylation (%)	*P*	Methylation (%)	*P*
Gender								
Male ($n = 11$)	8 (73)	>0.05	5 (45)	>0.05	4 (36)	>0.05	4 (36)	>0.05
Female ($n = 39$)	28 (72)		17 (44)		15 (38)		12 (31)	
Age (y)								
≤65 ($n = 25$)	19 (76)	>0.05	10 (40)	>0.05	10 (40)	>0.05	6 (24)	>0.05
>65 ($n = 25$)	17 (68)		12 (48)		9 (36)		10 (40)	
Histology								
Adenocarcinoma ($n = 45$)	34 (76)	>0.05	20 (44)	>0.05	17 (38)	>0.05	14 (31)	>0.05
Adenosquamous, squamous carcinoma ($n = 5$)	2 (40)		2 (40)		2 (40)		2 (40)	
Histology subtype								
Papillary, tubulo-papillary ($n = 15$)	11 (73)	>0.05	4 (27)	>0.05	6 (40)	>0.05	4 (27)	>0.05
Tubular ($n = 30$)	23 (77)		16 (53)		11 (37)		10 (33)	
Differentiation								
Well ($n = 7$)	5 (71)	>0.05	2 (28)	>0.05	2 (28)	>0.05	2 (28)	>0.05
Moderate ($n = 15$)	9 (60)		8 (53)		5 (33)		7 (47)	
Poor ($n = 28$)	22 (79)		12 (43)		12 (43)		7 (25)	
PT								
$T_{1a}/T_{1b}/T_2$ ($n = 21$)	14 (67)	>0.05	7 (33)	>0.05	11 (52)	>0.05	4 (19)	>0.05
T_3/T_4 ($n = 29$)	22 (76)		15 (52)		8 (29)		12 (41)	
PN								
N_0 ($n = 29$)	19 (66)	>0.05	13 (45)	>0.05	12 (41)	>0.05	12 (41)	>0.05
N_1 ($n = 21$)	17 (81)		9 (43)		7 (33)		4 (19)	
PM								
M_0 ($n = 44$)	33 (75)	>0.05	19 (43)	>0.05	14 (32)	0.024	15 (34)	>0.05
M_1 ($n = 6$)	3 (50)		3 (50)		5 (83)		1 (17)	
Liver invasion								
Positive ($n = 24$)	16 (67)	>0.05	10 (42)	>0.05	6 (25)	>0.05	10 (42)	>0.05
Negative ($n = 26$)	20 (77)		12 (46)		13 (50)		6 (23)	
Stage								
I/II ($n = 11$)	7 (64)	>0.05	2 (18)	>0.05	6 (55)	>0.05	2 (18)	>0.05
III/IVA/IVB ($n = 39$)	29 (74)		20 (51)		13 (33)		14 (36)	

Recently, silencing and aberrant promoter hypermethylation of *3-OST-2* has been demonstrated in breast cancer cell lines, as well as in several human tumors, including those of the breast (88%), colon (80%), lung (70%), and pancreas (100%; ref. 34). Our findings suggest that *3-OST-2* may play an important role in GBC development.

Two members of the cell adhesion cadherin family, *CDH13* and *CDH1*, which are related to tumor invasion and metastasis, also showed frequent methylation in GBC (44% and 38%, respectively). Inactivation by promoter methylation of both genes has been described in several tumor types including lung, breast, colon, and bladder cancers (17, 36, 39). Of interest, our data showed significant correlation between *CDH1* methylation and the presence of distant metastasis in our GBC cases, phenomena that have been reported previously in other tumor types (40).

RUNX3 has been suggested as a putative tumor suppressor involved in the development of gastric cancers (29). In this tumor type, hypermethylation combined with hemizygous deletion of the *RUNX3* correlates with a significant reduction in expression, and the tumorigenicity of cell lines in nude mice is inversely related to their level of *RUNX3* expression (29). A recent report showed that hypermethylation of the *RUNX3* gene promoter also occurs frequently (75%) in testicular yolk sac tumors of infants (41). Our result of frequent methylation of *RUNX3* (32%) in GBC expands the spectrum of tumor types in which this putative TSG may play an important role.

Selective methylation and silencing of the *APC1A* promoter and its specific products have been detected frequently in several human tumors (42). In GBC, we found methylation of this gene in 30% of the cases examined. Although the *APC* gene locus (5q21) allele losses were a rare event in GBCs (3), our methylation data are similar to other published findings (13). It is somewhat intriguing that we detected a substantially higher frequency of *APC* methylation in GBCs with papillary features.

The retinoblastoma protein-interacting zinc finger gene, *RIZ1*, a putative TSG, is a member of a nuclear histone/protein methyltransferase superfamily. *RIZ1* inactivation has been commonly found in many types of human cancers (43, 44). *RIZ1* mapped within a chromosomal region frequently deleted in many types of human tumors (1p36.1), including GBC (6). *RIZ1* was recently reported to be frequently methylated in several tumor types, such as breast (44%), liver (62%), and nasopharyngeal (60%) tumors (33, 45). In addition to the relatively (26%) high frequency of *RIZ1* methylation in our GBC cases, methylation of this gene was significantly associated with increasing depth of invasion and tumor-node-metastasis (TNM) stage of our GBCs.

$P16^{INK4A}$ gene abnormalities, including allelic losses at 9p21 (50%; ref. 3), gene mutations (20%; ref. 46), and methy-

Table 2 Continued.

APC		*RIZI*		*p16^{INK4A}*		*HPP1*	
Methylation (%)	*P*	Methylation (%)	*P*	Methylation (%)	*P*	Methylation (%)	*P*
1 (9)	>0.05	3 (27)	>0.05	1 (9)	>0.05	6 (55)	0.004
14 (36)		10 (26)		11 (28)		4 (10)	
9 (36)	>0.05	7 (28)	>0.05	7 (28)	>0.05	8 (32)	>0.05
6 (24)		6 (24)		5 (20)		2 (8)	
13 (29)	>0.05	11 (24)	>0.05	12 (27)	>0.05	8 (18)	>0.05
2 (40)		2 (40)		0 (0)		2 (40)	
9 (60)	0.0037	3 (20)	>0.05	7 (47)	>0.05	2 (13)	>0.05
4 (13)		8 (27)		5 (17)		6 (20)	
2 (28)	>0.05	1 (14)	>0.05	1 (14)	>0.05	1 (14)	>0.05
3 (20)		5 (33)		3 (20)		5 (33)	
10 (36)		7 (25)		8 (29)		4 (14)	
5 (24)	>0.05	1 (5)	0.0037	6 (29)	>0.05	4 (19)	>0.05
10 (34)		12 (41)		6 (21)		6 (21)	
9 (31)	>0.05	8 (28)	>0.05	9 (31)	>0.05	5 (17)	>0.05
6 (29)		5 (24)		3 (14)		5 (24)	
13 (30)	>0.05	12 (27)	>0.05	10 (23)	>0.05	9 (20)	>0.05
2 (33)		1 (17)		2 (33)		1 (17)	
9 (38)	>0.05	10 (42)	0.024	5 (21)	>0.05	5 (21)	>0.05
6 (23)		3 (12)		7 (27)		5 (19)	
3 (27)	>0.05	0 (0)	0.046	4 (36)	>0.05	2 (18)	>0.05
12 (31)		13 (33)		8 (21)		8 (21)	

lation (50%; ref. 13), as well as loss of protein immunohistochemistry expression (75%; ref. 47), have been detected frequently in GBC. Although our finding of 24% methylation of *P16^{INK4A}* in fresh frozen tumors is lower than the previously detected percentage of methylation (60%) in formalin-fixed, paraffin-embedded gallbladder tumors from Chilean patients, our data still support *P16^{INK4A}* as an important TSG in gallbladder tumorigenesis.

The *HPP1* gene was initially discovered because of its frequent hypermethylation in hyperplastic colon polyps, but it is also hypermethylated in colorectal adenomas and carcinomas (48) and gastric tumors (30). Our finding of 20% methylation in gallbladder tumors indicates that inactivation of *HPP1* may play an important role in the pathogenesis of a subset of GBCs.

Epidemiologic and clinical data support the current concept of chronic cholelithiasis and subsequent inflammation as established risk factors for GBC (1). Several molecular abnormalities, including allelic losses at TSG loci (7) and mitochondrial DNA mutations (49), have been demonstrated in a subset (up to 25%) of CC specimens, suggesting that they harbor clones of epithelial cells with malignant potential. Recently, House *et al.* (13) detected gene promoter methylation in three (*P16^{INK4A}*, 10%; *hMLH1*, 10%; *MGMT*, 15%) of six genes examined in formalin-fixed, paraffin-embedded CC specimens. Our finding that 7 of 24 known and candidate TSGs exhibited methylation in CC supports the concept that CC is a preneoplastic lesion for GBC. Unfortunately, it is virtually impossible to obtain normal gallbladder specimens (without gallstones and CC) because the organ is almost never surgically removed from disease-free individuals. Thus, our findings have to be interpreted in the context of not knowing the methylation status of normal gallbladder epithelium. Of those seven genes, four demonstrated substantially higher frequencies of methylation in GBCs compared with CCs. However, the two genes (*SHP1* and *P15^{INK4B}*) demonstrating the highest frequencies of methylation in CC specimens did not show any significant difference in methylation frequencies in GBC, suggesting that methylation of these genes is an early event. Gallbladder preneoplastic epithelium, such as dysplasia and carcinoma *in situ*, needs to be examined to investigate the stage of the sequential pathogenesis of GBC in which methylation of the above-mentioned genes occurs. *SHP1* is a negative regulator of the JAK/STAT signaling pathway (50). A high frequency of silencing of *SHP1* by promoter methylation was detected in most leukemias and lymphomas (20). *SHP1* may play a role in both GBC and CC. Similar to our findings, methylation of several TSGs has been detected in nonneoplastic epithelium from colon and stomach in patients without cancer (51, 52), indicating that early gene methylation could be a widespread phenomenon in human carcinoma pathogenesis.

The present study constitutes the most comprehensive methylation profile report available for GBC and demonstrates

that this neoplasm has a distinct pattern of abnormal gene methylation. Most of the genes showing high frequencies of abnormal methylation in GBC have not been studied previously in this neoplasm. Whereas gallbladders from healthy individual were not available, our finding of gene methylation in CC without cancer suggests that this phenomenon represents an early event in the pathogenesis of GBC.

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