Global and gene-specific DNA methylation pattern discriminates cholecystitis from gallbladder cancer patients in Chile

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Ethical conduct of research
The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Financial & competing interests disclosure
This research was supported by National Cancer Institute grants U01CA84986 and K01CA164092. This work was supported by NCI’s Clinical Proteomic Tumor Analysis Consortium initiative (U24CA160036). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.
Abstract

Aim—The aim of the study was to evaluate the use of global and gene-specific DNA methylation changes as potential biomarkers for gallbladder cancer (GBC) in a cohort from Chile.

Material & methods—DNA methylation was analyzed through an ELISA-based technique and quantitative methylation-specific PCR.

Results—Global DNA Methylation Index (p = 0.02) and promoter methylation of SSBP2 (p = 0.01) and ESR1 (p = 0.05) were significantly different in GBC when compared with cholecystitis. Receiver curve operator analysis revealed promoter methylation of APC, CDKN2A, ESR1, PGP9.5 and SSBP2, together with the Global DNA Methylation Index, had 71% sensitivity, 95% specificity, a 0.97 area under the curve and a positive predictive value of 90%.

Conclusion—Global and gene-specific DNA methylation may be useful biomarkers for GBC clinical assessment.

Keywords
gallbladder cancer; global and gene-specific DNA methylation; molecular biomarkers panel; tumor suppressor genes

Gallbladder cancer (GBC) is the fifth most common cancer of the GI tract [1] and the most prevalent malignancy of the biliary tract [2,3]. GBC has marked regional and ethnic variation worldwide, with the highest incidence rates being reported in Chile, Mexico, Bolivia, India, Central European countries and among Native Americans in North America [3–6]. While in Chile GBC is the leading cause of cancer deaths among women, with a mortality rate of 16.2/100,000 [7], in the USA it is a rare neoplasm, where 10,650 new cases are expected in 2014 representing 0.7% of all cancer cases [8].

GBC is a highly aggressive malignancy and is usually detected at an advanced unresectable stage [9], when the disease has already metastasized. Only 20% of the patients have disease confined to the gallbladder at the time of diagnosis [9]. This late detection is associated with poor prognosis; the 5-year survival rate is only 10% [9]. Although little is known about genetic predisposing factors associated with GBC, a few studies have reported mutations in KRAS, TP53, p16/CDKN2A, microsatellite instability, overexpression of COX2, VEGF, hTERT and ERBB2 in GBC [10]. Besides the occurrence of genetic alterations, other risk factors have been associated with GBC: gallbladder disease (cholecystitis, chronic inflammation of the gallbladder, congenital biliary abnormalities and polyps) age, obesity or environmental factors such as exposure to carcinogens. However, the etiology of GBC is not well understood and the influence of these risk factors for GBC tumorigenesis is not clearly established [5,11–12].

There are two carcinogenic pathways known for GBC, the metaplasia–dysplasia–carcinoma and the adenoma–carcinoma, arising from two different types of epithelial lesions. The most frequent type, the metaplasia–dysplasia–carcinoma seen in most epithelial tumors, is
secondary to chronic irritation or inflammation, as a result of gallstones and biliary tract infections. The evolution of chronic gallbladder inflammation, or cholecystitis, into dysplasia, carcinoma in situ and progression to invasive carcinoma, tracks at the molecular level, with tumor suppressor gene silencing by DNA methylation, together with global and gene-specific loss of methylation [7,10]. However, there are only limited studies [13–16] related to GBC and its pre-neoplastic lesions’ methylome and methylation alterations.

We conducted a study akin to a Phase I Biomarker Development Trial [17], to identify a panel of epigenetic biomarkers that can distinguish cholecystitis from GBC patients. We quantified the GBC global methylome with an ELISA-based technique and promoter DNA methylation of eight genes that regulate multiple oncogenic pathways with quantitative methylation-specific PCR (qMSP) in patients from Chile: 19 GBC cases and seven chronic cholecystitis cases, which were used as non-cancer controls for this study. We examined gene-specific promoter methylation in a panel of eight tumor suppressor genes (TSG) reported to be frequently methylated in various tumor types (APC, CDKN2A, ESR1, MCAM, MGMT, PGP9.5, RARβ and SSBP2) [13,18–21]. We hypothesized that a subset of these eight genes, together with the global DNA methylation index (GMI) would significantly discriminate between cholecystitis and gallbladder cancer patients.

Materials & methods

Sample collection

Tissue samples from 19 GBC cases and seven chronic cholecystitis cases were collected from 2004 to 2008, at the clinic of Doctor Hernán Henríquez Aravena (HHA) tertiary care regional hospital, in Temuco, Chile. The diagnosis was confirmed by histological examination (biopsy) performed by a team of three pathologists from HHA. A random set of pathology slides from the study samples was sent for diagnostic confirmatory review to a pathologist at Johns Hopkins School of Medicine. The Institutional Review Boards of the HHA and the Johns Hopkins School of Medicine approved the protocol for this study.

DNA extraction

DNA was extracted from 5 mg of frozen tissue from each sample by digestion with 1% SDS and 20 μg/ml proteinase K (Roche, Manhein, Germany) at 48°C for 24 h, followed by phenol/chloroform extraction and ethanol precipitation of DNA as previously described [22].

Bisulfite treatment

Genomic DNA extracted from the tissues was subjected to bisulfite modification, which converts unmethylated cytosine residues to uracil, using EpiTect Bisulfite Kit (QIAGEN, CA, USA) according to the manufacturer’s protocol as previously described [23].

Global DNA methylation index

Global DNA methylation levels on tissue and cell line DNA were obtained with an ELISA-based commercial kit (MDQ1, Imprint® Methylated DNA Quantification Kit; Sigma Aldrich, MO, USA). The MDQ1 kit is a high-throughput, molecular biology kit, which uses
a 96-well plate format to provide accurate differential global DNA methylation absorbance readings with as little as 50 ng of genomic DNA. In total, 2 μl of DNA at a concentration of 100 ng/μl were diluted with 28 μl of lysis and binding buffers and incubated at 60°C. The samples were incubated with capture and detection antibodies and absorbance was read at 450 nanometers. Quantification of global DNA methylation was obtained from calculating the amount of methylated cytosines in the sample (5 mC) relative to global cytidine (5 mC + dC) in a positive control that had been previously methylated. All samples were analyzed in duplicate as previously described [24].

Quantitative methylation-specific PCR

We used previously developed qMSP primers and probes, which amplify the promoters of eight genes that play an important role in oncogenesis: cell adhesion, cellular proliferation, cell cycle control, cellular differentiation, cell migration, apoptosis, DNA repair, cell growth and protein degradation – MCAM [25,26], SSBP2 [21,27], ESR1 [28,29], APC [30,31], CDKN2A [32,33], MGMT [33,34], RARβ [35] and PGP9.5 [36,37] and the promoter of the internal control ACTB (β-actin gene). The primer and probe sequences, which we designed for our previous methylation studies based on bisulfite sequencing data, along with the annealing temperatures are provided in Supplementary Table 1 (see www.futuremedicine.com/doi/suppl/10.2217/fon.14.165).

Fluorogenic PCR reactions were performed in duplicates in a reaction volume of 20 μl that contained 3 μl of bisulfite-modified DNA; 600 nM of each primer; 200 nM probe; 0.75 U of platinum Taq polymerase (Invitrogen, MD, USA); 200 μM of each dATP, dCTP, dGTP and dTTP; 200 nM ROX dye reference; 1X buffer (16.6 mM ammonium chloride; 67 mM Trizma [Sigma]; 6.7 mM of magnesium chloride; 10 mM of mercaptoethanol and 0.1% dimethyl-sulfoxide). Amplifications were performed using the reaction profile: 95°C for 3 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min in a 7900 HT sequence detector (Applied Biosystems, CA, USA) and were analyzed by a sequence detector system (SDS 2.4; Applied Biosystems). Each plate included patient DNA samples, positive controls (leukocytes from a healthy individual were methylated in vitro using SssI methyltransferase; New England Biolabs, MA, USA) and multiple water blanks as non-template controls. Serial dilutions (90–0.0009 ng) of in vitro methylated DNA were used to construct a standard curve for each plate. The relative level of methylated DNA for each gene in each sample was determined as a ratio of the amplified gene quantity to the quantity of β-actin multiplied by 1000.

Quantitative real-time reverse transcription PCR

RNA samples from three GBC cell lines (SNU308, GBD1 and G415) and from four GBC samples (GB82, GB95, GB126 and GB127) were assessed for ESR1, PI6, PGP9.5, APC, SSBP2 and GAPDH expression levels using quantitative real-time reverse transcription (qRT-PCR). Reverse transcription was performed with random hexamer primers and Superscript II Reverse Transcriptase (Invitrogen) according to manufacturer’s instructions. qRT-PCR was then carried out on the Applied Biosystems 7900HT Sequence Detection Instrument using TaqMan expression assays (Applied Biosystems). The 2^−ΔΔCt method was used to quantify relative gene expression [38].

Future Oncol. Author manuscript; available in PMC 2015 February 18.
Statistical analysis for qMSP data

qMSP values were adjusted for DNA input by expressing results as ratios between 2 absolute measurements. The relative level of methylated DNA for each gene in each sample was determined as a ratio of qMSP for the amplified gene to ACTB and then multiplied by 100 for easier tabulation ([(average DNA quantity of methylated gene of interest/average DNA quantity for internal reference gene β-actin) × 100]) [28]. The samples were categorized as unmethylated or methylated based on detection of methylation above a threshold set for each gene. For quality control, all amplification curves were visualized and scored without knowledge of the clinical data. Receiver operator characteristic (ROC) curves were used to identify a cutoff ratio above the highest control ratio observed for each gene to set specificity at the percentage that maximizes the number of samples correctly classified. Promoter methylation ratios for each gene were compared between cancer GBC and cholecystitis samples. The Fisher’s exact and χ² tests (significance level = 0.05; CI: 95) were used to compare GMI and qMSP methylation levels. Results with a p ≤ 0.05 were considered significant. Once the best individually discriminating genes were found, a stepwise bootstrapping approach was used to identify the gene panel with the highest sensitivity, specificity, area under the curve (AUC), and positive and negative predictive value. All analyses were performed using STATA version 12.

Results

Patient characteristics

A sequential cohort of patients were selected for this study in Temuco, Chile. The available demographic and clinical patient data is listed in Table 1. The age among GBC patients varied between 41 to 84 years (median: 62 years; average: 63.5 years) and between 33 to 88 years (median: 68 years; average: 63.5 years) for cholecystitis patients. The mean age for both groups showed no significant statistical difference. The majority of patients (78.6%) selected for the present study were women and 21.4% were of Mapuche descent, the indigenous inhabitants of south-central Chile.

Global DNA methylation

GMI was evaluated in 26 patients: 19 cancer and seven cholecystitis tissue samples. We observed a significant global loss of DNA methylation in GBC (p = 0.02). Most GBC patients (74%) had a GMI below the cutoff value of 60. The median GMI in two of three GBC cell lines, GBD1 and SNU308, was also below the cutoff value of 60. Figure 1. shows a boxplot of the GMI of the two patients groups and the three cell lines.

Quantitative methylation-specific PCR

Promoter methylation of APC, CDKN2A, ESR1, MCAM, MGMT, PGP9.5, RARβ and SSBP2 was evaluated in DNA samples from 19 primary tumors and in seven cholecystitis tissue samples (Figure 2). We found that SSBP2 (p = 0.01) and ESR1 (p = 0.05) significantly discriminated GBC from cholecystitis patients (Table 2). Promoter methylation scatter plots of the eight genes are shown in Figure 3 & 4. APC was methylated in six out of 19 (32%) of GBC samples and in one in seven (14%) of chronic cholecystitis samples. CDKN2A was
methylated in five in 19 (26%) of GBC samples and in one of seven (14%) of chronic cholecystitis samples. \textit{ESR1} was methylated in eight of 19 (42%) GBC samples and in zero out of seven (0%) of chronic cholecystitis samples. \textit{MCAM} was methylated in seven of 19 (37%) of GBC samples and in one out of seven (14%) of chronic cholecystitis samples. \textit{MGMT} was methylated in two out of seven (29%) of GBC samples and in one out of seven (14%) chronic cholecystitis samples. \textit{PGP9.5} was methylated in four of 19 (21%) GBC samples and in one of seven (14%) chronic cholecystitis samples. \textit{RAR}\textsubscript{β} was methylated in seven out of 19 (37%) GBC samples and in two out of seven (29%) chronic cholecystitis samples. \textit{SBPP2} was methylated in ten of 19 (53%) of GBC samples and in zero out of seven (0%) of chronic cholecystitis samples. The association of aberrant DNA methylation and demographic features was also examined. Detectable methylation levels were not associated with age or gender in this group of patients except for \textit{SBPP2}, which is associated to gender (Supplementary Table 2). However, the small sample size makes it difficult to identify strong associations.

ROC curve analysis revealed that \textit{SBPP2} had 68% sensitivity, 86% specificity and a 0.83 AUC; \textit{ESR1} had 42% sensitivity, 100% specificity and a 0.56 AUC; \textit{CDKN2A} had 47% sensitivity, 86% specificity and a 0.69 AUC; \textit{APC} had 32% sensitivity, 86% specificity and a 0.47 AUC (Figure 5). A gene panel combining the GMI with promoter DNA methylation results for these five genes had 71% sensitivity, 95% specificity, a 0.97 AUC, a positive predictive value (PPV) of 90% and a negative predictive value (NPV) of 83% (Figure 5).

\section*{Quantitative real-time reverse transcription PCR}

To examine if the promoter methylation data we have generated relates to gene silencing or not, we quantified expression levels of the five genes included in the molecular panel in three GBC cell lines (SNU308, GBD1 and G415) and four GBC tissues. Differential transcript levels for \textit{APC, CDKN2A, ESR1, SSBP2} and \textit{PGP9.5} were confirmed by quantitative RT-PCR in some of the RNA samples used for qMSP analysis and in GBC cell lines RNA (Supplementary Table 1). The relative expression levels showed consistency with the qMSP results obtained for \textit{ESR1} and \textit{SSBP2} in GBC tissue and cell lines (Supplementary Figures 1A–1D).

\section*{Discussion}

Our main objective in this study was to determine the methylation profile of a panel of genes in GBC using a quantitative method (qMSP) as well as assess global methylation in GBC. We report here for the first time a global loss of DNA methylation and \textit{ESR1} and \textit{SSBP2} promoter methylation in GBC. Using ROC curve analysis we identified a panel of five TSG, which combined with the GMI have the potential of serving as biomarker(s) for GBC clinical management.

Global loss of methylation and gene-specific DNA promoter methylation occur frequently during carcinogenesis and have been considered as potential molecular markers for cancer initiation and progression [39]. DNA methylation in mammals is mostly seen at position 5′ of the cytosine ring in CpG through the covalent bond of a methyl group. NonCpG
sequences can also become methylated but with less frequency [40–42]. In normal tissue, methylation of CpG islands usually increases with age, although the total genomic content of the methylcytosines decreases [43]. During carcinogenesis a global loss of DNA methylation, together with tumor suppressor gene silencing by promoter DNA methylation, has been observed in most tumor types [44]. Promoter methylation in CpG islands of tumor suppressor genes has been demonstrated as a hallmark in cancer [39]. Earlier studies have profiled gene-specific promoter methylation in head and neck squamous cell carcinoma [45,46], bladder cancer [47,48], lung cancer [49] and liver cancer [50], among others.

Epigenetic studies conducted to determine the promoter methylation profile of GBC have found promoter methylation of multiple genes associated with poor survival, disease progression and histology subtype – 3-OST-2, CDH1, CDH13, RUNX3, APC, RIZI, CDKN2A, HPP1, MGMT, hMLH1, DAPK and many others – were shown to be methylated in their promoter regions by conventional PCR. Presence of aberrant promoter DNA methylation in some genes was associated with poor survival, disease progression and histology subtype [14,16,51–52]. So far, only PGP9.5 has been described to lose promoter methylation in GBC [53].

Previous studies evaluated promoter methylation status in GBC by MSP. One of the drawbacks of this approach is that it is a subjective technique, as it scores for methylation based on visualization of bands in an electrophoresis gel [54]. Real-time qMSP allows a rapid detection and quantitation of promoter methylation and is better suited than conventional MSP for high-throughput studies, due to its high sensitivity and ease of automation [55]. qMSP has been put forward as a platform to develop biomarkers for early detection, diagnosis and clinical management of cancer [56]. We designed the qMSP primers and probes tested in this study using the pipeline first published by our laboratory in 2001 [57]. In this pipeline bisulfite sequencing is performed at the onset of primer development stage, not only to assess the methylation status of the CpGs contained in that area but also to verify that we are amplifying the genomic region of interest. The fact that qMSP is an easily reproducible and quantitative method that can be used to query large number of samples, makes this method a feasible approach to be used in the clinics to test tissue and biofluid samples from GBC patients for the presence of methylation (Supplementary Figure 2A & 2B).

In this study we identified a molecular panel of DNA methylation events that can distinguish GBC from chronic cholecystitis. The panel consists of the qMSP values for five TSGs (APC, SSBP2, CDKN2A, PGP9.5 and ESR1) together with the GMI. While ESR1 and SSBP2 could significantly distinguish between GBC and cholecystitis by themselves, it was not until they were combined with APC, CDKN2A, PGP9.5 and the GMI that the panel’s sensitivity (71%) and specificity (95%) attained clinically relevant levels. Our study was designed to identify a panel of epigenetic biomarkers that could discriminate between GBC and chronic cholecystitis, akin to a Phase I biomarker development trial. Phase I biomarker development trials are designed to identify biomarkers that can discriminate between cancer and noncancer tissue. The small sample size did not permit us to carry out a properly powered trial. This design does not lend its self to classify biomarkers as drivers or passengers of the oncogenic process, nor to examine their functional characteristics in GBC.
cell lines. We did however test the expression status of this gene panel in three GBC cell lines, SNU308, GBD1, G415 and four GBC tumor samples. The relative expression levels showed consistency with the qMSP results obtained for \textit{ESR1} and \textit{SSBP2} in GBC tissue and cell lines. Given these results we plan to examine the functional characteristics of \textit{ESR1} and \textit{SSBP2} in future studies (Supplementary Figure 2C).

\textit{SSBP2} is a tumor-suppressor gene that belongs to a very conserved family of DNA binding proteins. The SSBP family components stabilize single strand DNA (ssDNA) regions avoiding degradation and incorrect processing until the regular cellular processes can be performed. Because of this crucial function \textit{SSBP2} plays a role in DNA replication and repair [58]. \textit{SSBP2} promoter methylation is associated with gene silencing in prostate cancer and esophageal carcinomas. In prostate cancer, aberrant methylation was not detected in normal tissue, whereas 61.4\% of the neoplastic samples showed the epigenetic alteration [25,59]. Methylation of this gene was reported to be present in 86\% of esophageal cancer samples [25,59]. \textit{SSBP2} seems to have an effective role in cell proliferation and cell cycle control. Induction of its expression in prostate and esophageal cancer cell lines resulted in reduced cell proliferation and cell cycle arrest [25,59]. We observed a significant difference on \textit{SSBP2} DNA methylation frequency when comparing GBC and noncancer samples. \textit{SSBP2} aberrant methylation in GBC, as well in other tumor types, might result in gene silencing which will impact in DNA replication and repair and genome stability. We found promoter methylation of \textit{SSBP2} and minimal \textit{SSBP2} expression in one of the three GBC cell lines we tested: GBD1. On the contrary, \textit{SSBP2} expression does not seem to be regulated by promoter methylation in SNU308 and G415 GBC cell lines (Supplementary Figures 1A & B).

\textit{MCAM}, also known as \textit{CD146}, is a calcium-independent transmembrane glycoprotein adhesion molecule. It is expressed in endothelial cells and plays a role in cohesion of the endothelial monolayer. It was initially identified as a marker for progression and metastasis in melanoma [60]. Later studies showed decreased expression of \textit{MCAM} and its role in tumor progression and metastasis in multiple cancers including GBC [61] and breast [62]. Promoter methylation of \textit{MCAM} has been associated with advanced tumor stage in prostate cancer [25]. However, the authors detected high frequency of promoter methylation in the gene promoter but with positive protein expression by immunohistochemistry suggesting that, at least in prostate cancer, methylation is not the major factor controlling MCAM expression. Positive expression of MCAM has been described in GBC, while the protein levels were reduced in noncancer gall-bladder tissue. Higher levels of MCAM have been associated with increased angiogenesis and lymphangiogenesis and this gain of expression showed important impact on disease progression, metastasis and survival in gallbladder adenocarcinomas [61]. Here, we investigated the methylation status of \textit{MCAM} in an attempt to understand its regulation in GBC. However, no difference was observed between the cancer and noncancer gallbladder tissue.

The high incidence rates of GBC among women may be partly attributed to hormonal factors [63]. \textit{ESR1} encodes for a ligand-activated transcription factor involved in regulation of gene expression that affects cellular proliferation and differentiation in specific tissues [64]. Genetic alterations in \textit{ESR1} gene seem to have different impacts in the risk for GBC. A
susceptibility risk polymorphism on ESR1, rs1801132, is associated with higher risk to bile duct and ampulla of Vater cancers but not GBC [63]. However, presence of the homozygous variant genotype for the marker rs2234693 (ESR1-397TT) is associated with significant higher risk to GBC (odds ratio: 1.8) [65]. Promoter methylation of ESR1 in association with gene silencing has been observed in other tumor types such as breast cancer [66], rectal cancer [67] and cervical cancer [28]. Our study is the first to evaluate the methylation status of ESR1 in GBC. Here, we described a methylation frequency of 42% among cancer patients, while no methylation was detected in noncancer samples. ESR1 hypermethylation followed by transcriptional blockade may result in downregulation of cell cycle and growth regulators, once this gene is known to have a role in transcription regulation by the recruitment of proteins of the transcriptional protein complex [68]. Our cell line data supports the mechanistic link between promoter metylation and down-regulation of ESR1 in GBC. Two of the three cell lines we tested, GBD1 and G415, have evidence of ESR1 promoter methylation and expression downregulation. Conversely, ESR1 downregulation in SNU308 does not seem to be under methylation control (Supplementary Figures 1C & D).

APC, a tumor suppressor gene, encodes a protein involved in cell migration, cell adhesion, transcriptional activation and apoptosis. Promoter methylation of APC and its role in tumorigenesis has been reported in rectal [67], esophageal [69], breast [29] and cervical [28] cancers. In GBC, promoter methylation has been observed to be present in 30–40% of cases [13–15]. APC regulates the level of free β-catenin, which is involved in Wnt signaling. Besides APC gene mutation, inactivation of APC by aberrant promoter methylation results in increased β-catenin levels, which subsequently triggers tumor formation, through Wnt signaling [70,71]. The relevance of APC hypermethylation in the development of GBC might be associated with the dysregulation of the Wnt pathway, showing the necessity of follow-up investigations on this important pathway.

Silencing of CDKN2A by promoter methylation is frequently observed in solid tumors [72–74]. This gene encodes a protein (p16) that negatively regulates cell cycle progression. p16 protein binds to CDK4/6 inhibiting their ligation to cyclin D [75]. In GBC, CDKN2A methylation has been described in multiple studies with varied frequencies, (14.5–80%) but there is no reported association with prognosis or clinicopathologic variables [1,14–16,52,76–77]. Besides the fact that CDKN2A methylation showed no association with GBC, this gene is an important cell cycle regulator and further analysis on the epigenetic and genetic alterations in these tumors is critical for the comprehension of their biology.

MGMT gene is a DNA methyltransferase. The aberrant methylation of this gene is known to be associated with response to alkylating chemotherapeutic agents in glioblastomas [78,79]. House et al. reported MGMT methylation in 13% of GBC cases evaluated [13]. Here, maybe due to the fact that we used a more sensitive method for methylation detection, we found that 30% of GBC samples present methylation in this gene promoter, while the frequency in noncancer patients was 14%. MGMT DNA methylation, as well as in glioblastoma, seems to be associated to therapeutic response with alkylating agents. Gallbladder cell lines treated with the bifunctional alkylating agent 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea showed different proliferation rates according to MGMT protein expression status, GBC cell lines harboring MGMT downregulation were more sensitive to
1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea [80]. Aberrant methylation of MGMT might be a useful tool to determine the better treatment modality to be applied in each GBC patient.

RARβ encodes a receptor that when activated participates in cell growth and differentiation. RARβ is a tumor suppressor gene and loss of this gene plays an important role in tumorigenesis [81]. RARβ promoter methylation has been reported in rectal [67] and cervical [28] cancers. RARβ methylation in gall bladder cancer is associated with disease progression from cholecystitis to GBC. Progressive increase in DNA methylation level of this gene was observed following the evolution of chronic inflammation to invasive cancer, suggesting that this event plays a role in gall bladder tumorigenesis [13,16]. Methylation of RARβ promoter region was observed in a significant proportion of cancer-free high-fat consumers [82]. Gallstones disease, a known GBC risk factor, shows higher incidence among overweight and obese patients [5]. This evidence indicates that presence of RARβ promoter methylation may be associated with obesity in GBC patients. The information about obesity was not collected on our cohort, however, in future studies it might be a relevant fact to be taken into account when evaluating the association between GBC and RARβ methylation.

PGP9.5 is the only gene selected for our study with a reported loss of promoter methylation in GBC. Also known as UCHL1, PGP9.5 prevents protein degradation by the proteosome-dependant pathway by removing ubiquitin from ubiquitinated proteins [83]. In a previous study, PGP9.5 methylation was detected in 84.6% of normal gall bladder [35] epithelia and in only 27.2% of the GBC samples. Methylation status was inversely correlated with protein expression levels. These data suggest that PGP9.5 in GBC might have a role as an oncogene [53]. We report a similar frequency of PGP9.5 promoter methylation in GBC patients (21%). In addition, none of our three cell lines show evidence of methylation and the difference in PGP9.5 promoter methylation frequency between cholecystitis (14%) and GBC patients is not significant (p = 0.69). Our data suggest that the normal GB epithelium undergoes a loss of PGP9.5 promoter methylation early in the inflammation-associated metaplasia–dysplasia–carcinoma pathway for GBC.

To our knowledge this is the first study to report a global loss of DNA methylation in GBC. A global loss of DNA methylation is a hallmark of human cancer, first reported more than 30 years ago [84]. This global loss, commonly referred to as global DNA hypomethylation, is mostly seen in the repetitive elements, interspersed repeats and tandem repeats that comprise approximately half of the human genome [85]. The global loss of DNA methylation in repetitive elements can lead to neighboring gene disruption via transcriptional interference and activation of transposable elements, which can lead to chromosomal and microsatellite instability throughout the genome [43,86]. Global DNA hypomethylation has been studied as a marker of cancer risk in peripheral blood leukocytes [87–89] and as a biomarker for breast [90], prostate [91], ovarian [92], lung [93], liver [94], bladder [95], oral [94] and laryngeal cancer [96]. Our data in GBC tissues and cell lines is consistent with published data for other tumor types. We observed a statistically significant difference in the GMI when comparing cholecystitis with GBC patients. The GMI index in cell lines provides further evidence to the hypothesis that there is a progressive global loss of DNA methylation in GBC.
In the present study we observed that 52.6% (ten out of 19) of the GBC cases presented two or more methylated genes. In these cases, more than one TSG may have their functions disrupted by promoter methylation, suggesting a probable imbalance in the activity of the signaling pathways those genes help to regulate. Subsequent concomitant deregulation of multiple pathways might be important for the initiation and progression of this tumor type. However, further investigation to determine the importance of each of the regulatory pathways implicated by this study in GBC, as well as the identification of the driving and passenger molecular alterations associated with them, are crucial for a better understanding of GBC biology.

The majority of GBC are not resectable when diagnosed as a result of lack of specific symptoms in early stages of the disease. This late diagnosis results in a 5-year survival rate of only 10%. The highest occurrence rates among men and women are registered in Chile with this last group being more frequently affected. It is well established that chronic cholecystitis and gallstones are risk factors for the development of GBC, however the etiology of these tumors is poorly understood [5,97]. Identification of the molecular mechanisms involved in GBC tumorigenesis is of extreme importance for better understanding of their biological behaviour as well as prognosis determination. A variety of genetic alterations (mutations, microsatellite instability and aberrant expression) are present in GBC and are associated with bad prognosis for patients harboring these changes [10]. Aberrant DNA methylation including global loss of methylation and gene-specific gain of methylation in the promoter are also common features of this cancer type (Supplementary Figure 3). DNA promoter methylation is a cumulative event during cancer progression and is implicated in gene silencing of the majority of its target genes, resulting in downregulation of important tumor suppressors [7].

Hence, a panel that incorporates global and gene-specific changes can serve as a useful early detection molecular tool. A panel of genetic and epigenetic changes would be even more relevant if proven to be detected in bodily fluids (bile, serum, plasma or urine) from GBC patients. Evaluation of cell-free DNA is an approach that has to be explored in this tumor type, one that can lead to a noninvasive screening method for GBC.

**Conclusion**

Using a quantitative method we show for the first time that GBC undergoes a significant loss of global DNA methylation and two genes, SSBP2 and ESR1 that are differentially methylated in GBC. We also show that the GMI, together with promoter methylation in a panel of five genes (APC, CDKN2A, ESR1, SSBP2 and PGP9.5), may act as a potential early detection biomarker panel for GBC. Further studies including larger groups of GBC and cholecystitis patients are important to validate our findings and examine their association with somatic mutations and known GBC prognostic factors using tissue and body fluids, including bile.
Future perspective

GBC is a gastrointestinal neoplasia with specific geographic and ethnic variation with the highest incidence rates described in Chilean women. It is an aggressive disease associated with low survival rates. Late diagnosis, due to lack of symptoms in early stages, is the main factor associated with poor prognosis. Gallbladder stones and inflammatory conditions are risk factors involved in the ethiology of GBC. The signaling pathways that are disturbed and culminate in gallbladder tumorigenesis are not well understood.

The results of this study suggest that a panel of five methylated TSGs combined with the GMI can be potential biomarkers for early detection of GBC. Further molecular studies involving larger cohorts that examine combined somatic mutations and epigenomic alterations in crucial genes in the GBC tumorigenesis process are essential to enable the clinical application of these markers in liquid biopsies, as they become part of routine clinical follow-up in the future. Liquid biopsies in bile, blood or urine, testing for panels of combined genetic and epigenetic alterations associated with GBC early detection, response to treatment and survival, will inform the differential diagnosis of gastrointestinal oncologists in the not-so-distant future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

Papers of special note have been highlighted as:

• of interest;
•• of considerable interest


Future Oncol. Author manuscript; available in PMC 2015 February 18.


EXECUTIVE SUMMARY

Background

- Gall bladder cancer (GBC) is a rare disease associated with poor survival rates due to late diagnosis.
- In the present study, global and DNA methylation was evaluated in GBC samples and cholecystitis tissue to identify biomarkers for prognosis, early detection and treatment.

Material & methods

- Frozen tissue samples were obtained from patients with cholecystitis (non-neoplastic tissue) and with GBC.
- GBC tissue and cell line DNA was used for global DNA methylation index determination and for promoter methylation profile analysis of APC, CDKN2A, ESR1, MCAM, MGMT, PGP9.5, RARβ and SSBP2.

Results

- A significant difference was observed in global methylation index between the cholecystitis and GBC samples.
- DNA methylation analysis showed that promoter methylation of SSBP2 and ESR1 are significantly more frequent in GBC patients.
- A panel that includes the GMI and promoter methylation of five genes – SSBP2, ESR1, CDKN2A, APC and PGP9.5 – discriminates GBC from cholecystitis samples with 71% sensitivity, 95% specificity, a 0.97 area under the curve, and a positive predictive value of 90% and a negative predictive value of 83%.

Conclusion

- Global and gene-specific differential promoter DNA methylation panels can be used as biomarkers for GBC early detection.
- A biomarker development trial examining the global DNA methylation index (GMI), and the promoter methylation status of SSBP2, ESR1, CDKN2A, APC and PGP9.5 in a larger and well-characterized group of samples is warranted to verify the results obtained in this study.
- A sensitive and specific panel of global and promoter DNA methylation biomarkers will enable a new generation of early detection, diagnostic and prognostic devices, and reduce gall bladder cancer mortality rates worldwide.
Figure 1. Global DNA methylation index in three gall bladder cancer cell lines (G-415, GBD1 and SNU308), cholecystitis patients (n = 7) and gall bladder cancer (n = 19) patients.

Global DNA methylation was determined by the ratio between the amount of methylated cytosines in the sample and a fully methylated positive control. The red line represents the cutoff value for the global DNA methylation index in gall bladder cancer (60).

GBC: Gall bladder cancer.
Figure 2. Promoter methylation frequency for APC, CDKN2A, ESRI, MCAM, MGMT, PGP9.5, RARβ and SSBP2 in cholecystitis (n = 7) and gallbladder cancer (GBC) patients (n = 19)

Samples are ordered by frequency of methylation, first in the cholecystitis patients and then in the GBC patients. The frequency of promoter methylation for cholecystitis patients ranges from 0 to 38%. The frequency of promoter methylation for patients ranges from 0 to 100%.

GBC: Gallbladder cancer; NA: Not applicable.
Figure 3. Quantitative methylation-specific PCR results for APC, CDKN2A, ESR1, MCAM, MGMT, PGP9.5, RARβ, and SSBP2

Graphical expression of the logistic regression, Pr (GBC = 1) = \logit^{-1} (b_0 + b_1 \times \text{methylation}) in tissue from 26 participants with data overlain. The predictor methylation is the qMSP value for each case (1) and each control (0). Cutoff methylation values for APC, CDKN2A, ESR1, MCAM, MGMT, PGP9.5, RARβ, and SSBP2 are shown by the vertical dotted line. Probability of GBC is shown in red.

GBC: Gallbladder cancer.
Figure 4. Quantitative methylation-specific PCR analysis of candidate gene promoters in the validation screen cohort, which consisted of 17 gallbladder cancer tumor tissue samples and seven cholecystitis tissue samples.

The relative level of methylated DNA for each gene in each sample was determined as a ratio of methylation-specific PCR for the amplified gene to ACTB and then multiplied by 1000 ([average value of duplicates of gene of interest/average value of duplicates of ACTB] \times 1000) for APC, CDKN2A, ESR1, MCAM, MGMT, PGP9.5, RARβ and SSBP2. Red line denotes cutoff value.
Figure 5. Receiver operator characteristic curve analysis for molecular panel: GMI, APC, CDKN2A, ESR1, PGP9.5 and SSBP2

(A) Sensitivity, specificity and area under the curve results for quantitative methylation-specific PCR analysis, and (B) receiver operator characteristics curve for the GMI and promoter methylation of APC, CDKN2A, ESR1, PGP9.5 and SSBP2. Demonstrates that for this molecular panel the global DNA methylation index and qMSP results have 71% sensitivity, 95% specificity, a 0.97 AUC, a PPV of 90% and a NPV of 83%.

†SSBP2, CDKN2A, ESR1, APC and PGP9.5.

AUC: Area under the curve; NPV: Negative predictive value; PPV: Positive predictive value.
Table 1

Demographic and clinicopathological characteristics.

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Table 2

Comparison of promoter and global DNA methylation frequencies in cholecystitis and cancer patients.

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†DNA available to test only seven tumor samples with MGMT quantitative methylation-specific PCR primers and probe.
‡Statistically significant χ².
GMI: Global Methylation Index.