Journal of Cell Science & Therapy

Walawalkar, et al., J Cell Sci Ther 2015, 6:1
DOI: 10.4172/2157-7013.1000196

Review Article Open Access

Significance of Microsatellite Instability and Gene Methylation as Prognostic Biomarkers during Gallbladder Cancer Progression: A Review

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Rec date: Jan 25, 2015; **Acc date:** Feb 18, 2015; **Pub date:** Feb 20, 2015

Abstract

Gallbladder cancer is a common malignancy of the biliary tract with increasing incidences seen in Chile and Northern India. The disease is aggressive with poor prognosis and a median survival rate of less than 6 months following diagnosis. The aetiology of the tumour is complex with early lymph node metastasis and direct invasion into the liver and peritoneal cavity. Diagnosis is usually incidental during pathological review of cholecystectomy due to non-specific symptoms. Chemotherapy has no significant impact on gallbladder carcinoma as seen in other solid gastrointestinal malignancies. Various pre-disposing factors underlie the progression towards gallbladder cancer, but a strong correlation exists with chronic cholelithtiasis and inflammation.

A number of molecular alterations have been reported during gallbladder disease progression which may be associated with prognosis and certain risk factors. But the mechanisms contributing to gallbladder cancer are poorly understood. Various studies report the importance of DNA methylation and microsatellite instability in pathogenesis of gallbladder carcinogenesis. Their involvement in cell cycle pathways and DNA repair mechanisms respectively could make them potential candidates for biomarkers in early detection, diagnosis and therapeutics. Further elucidation of molecular and pathological events during gallbladder disease progression would help to identify novel targets for diagnosis and disease management. This review summarizes significant data related to microsatellite instability and specific gene methylation patterns, and concludes their importance as possible molecular markers of gallbladder cancer.

Keywords: Microsatellite instability; Gene methylation; Gallbladder cancer; Chronic cholelithiasis

Introduction

Gallbladder disease and progression

Gallbladder carcinoma is the cancer of gallbladder epithelium with low incidence rates compared to other cancer types. Among populations at highest risk, about 1% deaths occur because of gallbladder cancer due to poor survival rates. During 2008, the cases of gallbladder cancer incidence at the global level were 145, 662 with an Age-Standardized Rate (ASR) of 2.0 per 105 person years. Incidence varies geographically with higher rates in certain areas of Latin America (Colombia, Peru, and Ecuador), Japan, and Eastern Europe (Poland, the Czech Republic, Slovakia, Hungary, and the former East Germany). High rates of gallbladder cancer have been noted in Hispanic and American Indian populations in North America. The incidence among women is approximately double than that of men in high-risk populations [1,2].

In India, during 2001, the estimated number of gallbladder cancer was 14,986 and is likely to increase to 23,750 by 2016 as a result of aging and increase in size of the population. The GBC incidence rates have been reported to be highest in women from India (21.5 out of 100,000), Chile (18.1 out of 100,000), Pakistan (13.8 out of 100,000) and Ecuador (12.9 out of 100,000) [3,4]. Thus with ever-increasing incidence and poor prognosis, early diagnosis and treatment of gallbladder carcinoma is essential. Various markers according to the stages of gallbladder cancer are yet to be experimentally documented and thus still require extensive research.

The advancement of cancer up to the stage of gallbladder carcinoma is a lengthy process. There are many risk factors currently proposed to be involved in progression of gallbladder cancer. One of the main causes is chronic cholecystisis (CC) which may lead to various molecular changes (like continuous release of inflammatory factors), therefore resulting in the progression from inflammation to malignancy. The staging towards gallbladder cancer is usually characterized by chronic cholelithiasis with inflammation, metaplasia, dysplasia, carcinoma in situ and then finally invasive carcinoma [1,2]. Based on these observations researchers propose the period for progression from dysplasia to advanced gallbladder carcinoma to be approximately 15 years. Studies examining various tumor-related genes and gene products have shown great promise as possible prognostic and diagnostic markers, which are yet to be explored and well-understood in gallbladder cancers [5].

Genetic Alterations in Gallbladder Cancer

There are a number of reasons for GBC which include a) mutation b) loss of heterozygosity c) microsatellite instability (MSI) and d) promoter methylation. GBC could be the result of any of these phenomena or due to the cumulative effect of these phenomena [6]. Identifying these alterations and mapping their regulation is of utmost importance for improved prognosis and diagnosis of gallbladder carcinoma. In this study we focus on two such parameters (i.e. MSI

and gene methylation) which have shown some promising results in GBC.

Microsatellite instability as a molecular marker of gallbladder disease progression

Microsatellites are the tandemly repeated short sequence motifs that range from 1-6 base pairs. These DNA regions are simple sequences or Short Tandem Repeats (STRs) and classified as mono-, di-, tri-, tetra-, penta or hexanucleotide repeats on the basis of the number of repeating units. A typical microsatellite can be repeated up to 100 times and consists of a single repeat type only or they may consist of more than a single repeat type known as compound microsatellite. A single microsatellite locus can be amplified using specific Polymerase Chain Reaction (PCR) which is possible due to the fact that microsatellites are embedded in single copy DNA. Microsatellite stretches have often been reported to be disrupted by base substitutions which are called imperfect microsatellites or by insertions called interrupted microsatellite [7-9].

Microsatellite Instability (MSI) was initially reported in solid tumors in patients with colon cancer and Hereditary Non-Polyposis Colorectal Cancer (HNPCC) syndrome. MSI is probably caused by an aberrant system of gene repair during DNA replication. When the repair mechanism is altered, it results in mutations in the DNA sequences, further giving rise to either accumulated single nucleotide mutations, or altered length of repetitive microsatellite sequences which ultimately leads to MSI [9,10]

Failure to repair errors in repetitive sequences may be due to mutations in DNA mismatch repair genes (such as MLH1, MSH2 or MSH6) that leads to Microsatellite Instability (MSI) of the tumours. MSI can occur in tumours of many organs, but it has been largely documented in colorectal cancer. Studies have shown that among consecutive colorectal cancers, MSI is usually due to somatic MLH1 gene methylation; constitutional mutations of MLH1, MSH2 or MSH6 are the initial stage of development of MSI in the majority of HNPCC associated cancers [8,11].

Microsatellites form a huge reservoir for polymorphic genetic markers, this is because there are several hundred microsatellites present in eukaryotic genomes, and each of the loci can be subjected to DNA replication slippage. Microsatellite alleles also differ in their number of repeats. Therefore, an efficient and cost-effective method of genotyping can be the PCR amplification of a microsatellite locus which is followed by sizing of the. Moreover, a high sample throughput analysis is nowadays possible due to the availability of capillary sequencers and mass spectroscopy [8,9].

Studies have reported Microsatellite Instability (MSI) in colon cancer, gastric cancer, endometrium cancer, ovarian cancer, hepatobiliary tract cancer, urinary tract cancer, brain cancer and skin cancers [7,8]. Thus MSI cannot be neglected as a potential biomarker and needs to be well understood in every cancer type.

Genetic basis for MSI: Alterations in the genome are normally repaired by the DNA mismatch repair (MMR) system. These proteins correspond to several MutS and MutL homologues from the prokaryotic MutHL DNA repair system, hMLH1 (human MutL Homologue 1), and hMSH2, hMSH3, and hMSH6 (human MutS homologues 2, 3, and 6). They bind the mismatched DNA, excise the desired region and repair the sequence. Due to mutation or promoter methylation of critical MMR genes, normal mismatch repair fails to function. Alterations in the repeat sequences remain unrepaired due to which alleles of different sizes will be formed at the next replication. These different sized alleles form microsatellite instability [7,8,11].

Subsets of sporadic pancreatic, endometrial, prostate, and gastric carcinomas are affected by MSI. The inactivation of genes containing repeat sequences alters the tumor biology. Transforming growth factor beta receptor type II, BAX, and E2F4 are some examples of such kind of vulnerable genes [11].

A possible correlation between aberrant expressions of certain tumor suppressor genes and co-existing MSI during gallbladder cancer, has been reported by certain researchers. Studies were conducted to check co-relation between the p53, K-ras and MSI presence in a population with gallbladder carcinoma in Japan and Hungary. It appears that the p53 mutations and MSI differ in patients with gallbladder carcinoma between two distinct high-incidence areas. But, the results were not as significant as that in case of colorectal cancer where there is a prominent inverse relation between the presence of p53 and MSI [12].

Another study screened the genomic DNA from 21 gallbladder carcinomas using PCR-SSCP followed by sequencing to check for alterations in exon 15 of the B-raf gene where. The results obtained showed abnormal bands. Other features also examined were the association of the mutational status with the presence of K-ras or p53 mutations, MSI and the clinicopathological features (namely age, gender, histological type and stage of the tumor). Sporadic MSI-H colorectal Cancers (CRCs) harbouring hMLH1 methylation but not Lynch syndrome-related CRCs has earlier been shown to be associated with the BRAF V600E mutation. This study on gallbladder carcinoma showed B-raf mutations in 7 of 21 (33%) of the gallbladder carcinomas and were all located at the hot spot codon 599 of exon 15. K-ras mutations at codon 12 had been previously found in 4 of the 23 specimens (25%) and p53 mutations in 5 of 21 cases (24%). BAT26 used as a marker for indicating the presence of MSI. Alterations in BAT26 were not observed in these specimens. Both B-raf and p53 mutations were determined whereas no K-ras and B-raf mutations were identified simultaneously in the same samples [13].

Mismatch repair genes are the responsible genes for hereditary non polyposis colon cancer, and mutation of these genes causes replication error (RER). In several RER-positive colon cancer cell lines, mutations of repetitive sequences of transforming growth factor beta (TGF-β) type H receptor (RII) gene have been reported. Since TGF- β inhibits cell proliferation, loss of response to TGF- β is an important tumor progression step [14]. Two separate studies conducted by Saetta A et. al., evaluated MSI in gallbladder cancer with p53, BAX and TGF- β (RII). In this study with 20 gallbladder carcinomas from Greek patients, alterations in length of the BAT-26 mononucleotide marker (as an indicator of microsatellite instability) were correlated with the presence of p53 and ras mutations, alterations of the bax and TGF-beta RII genes and tumors' clinicopathological features. In another study by the same researchers none of the specimens showed microsatellite instability at the BAT-26 marker. BAT-26 is an indicator of high-level microsatellite instability. Thus, while determining the microsatellite instability status of gallbladder carcinoma, it may not be that sufficient, when used alone. A possible explanation for this may be that gallbladder carcinomas are characterized by low-level instability. They also stated that Bax and TGF-beta RII genes may not also be targets of instability in this type of tumors. They concluded from their study that as far as flat type of cancer is concerned Ras and p53 genes do not appear to cooperate during gallbladder cancer. Further they

stated that p53 alterations might have a role in the de novo pathway of gallbladder carcinogenesis [15].

MSI as biomarker for gallbladder cancer: Gallbladder cancer is a highly aggressive disease with a poor prognosis, and has a 5 year survival rate of less than 5%. Therefore, it would be very beneficial to identify the molecular mechanisms responsible for this condition. Even though a lot of studies have been done on the presence of mutated genes for example K-ras and p53 but still more detailed genetic information relating to gallbladder cancer is yet to be done.

One of the ways to investigate the molecular mechanism is to examine the microsatellite instability (MSI). With the increase in the presence of MSI in tumors, the National Cancer Institute Workshop on Microsatellite Instability recommended that tumors be divided into three groups, depending on the frequency of MSI. These three groups are: high frequency MSI (MSI-H), characterized by more than two of the five microsatellite markers showing instability; low-frequency MSI (MSI-L), characterized by only one of the five markers showing instability; and microsatellite stable (MSS), where no genetic instability is seen [9]. There are recommendations currently provided by the NCI (National Cancer Institute) panel for evaluation of MSI-H and MSI-L. These consisted of different MSI markers studied under colon cancer eg. BAT25, BAT26, D2S123, D5S346 and D17S250 etc. These guidelines are thus also useful for checking MSI in gallbladder cancers [16]. In prior studies of gallbladder cancer, there were only few reports with small number of cases and comparison with other tumor types was difficult. Using varied numbers and types of microsatellite markers is important. Using the NCI criteria, Carlos Roa et. al., analyzed a large series of early and advanced gallbladder cancers for MSI. They also examined pre-malignant glands for the same alterations as those seen in the adjacent tumors, and correlated their results with the immunohistochemical (IHC) expression of the MMR system and the clinicopathological findings. The presence of MSI in gallbladder cancers from a high-risk population were demonstrated by these studies. They also detected MSI in intestinal metaplasia and dysplasias adjacent to tumors with MSI-H and, occasionally, adjacent to tumors with MSI-L. These results indicate that in a subset of patients MSI might be participating in the early stages of gallbladder carcinogenesis [11]. In a study by Saetta et al., a group of 37 gallbladder carcinomas was analyzed for alterations in a proposed panel of mononucleotide and dinucleotide markers of MSI. Somatic frameshift mutations at repeated sequences in the coding regions of TGF bRII, Bax, hMSH3, hMSH6 were also examined. The results thus obtained were correlated with the presence of K-ras and p53 alterations, and tumors' clinicopathological features. In 9 gallbladder carcinomas microsatellite instability and/or LOH were observed. Cases showing microsatellite instability displayed alterations only in dinucleotide markers and were classified as MSI-L carcinomas. Based on the analysis of the above mentioned panel of markers a subset of gallbladder carcinomas is characterized by low-level instability. The study concluded that the pathway of microsatellite instability plays a minor role in the pathogenesis of gallbladder cancer [17]. Using an automated fluorescent DNA sequencer employing four microsatellite markers (p53, APC, DCC, NM23-H1) Yoshida et. al., examined loss of heterozygosity (LOH) in the p53, APC, DCC, RB, and NM23-H1generegions by polymerase chain reaction (PCR)-LOH assay. They also used five additional microsatellite markers for the determination of MSI. An inverse correlation between MSI and LOH in gallbladder carcinoma was observed. They also concluded that there is a lower incidence of lymph node metastasis in gallbladder carcinoma patients with MSI [18]. Microsatellite instability and fragile histidine triad (FHIT) loss have been seen to be involved in gallbladder carcinogenesis. Niraj Kumari et. al., studied the frequency of expression loss of MMR proteins and loss of FHIT expression increased from dysplasia to carcinoma. This suggested that both these aberrancy have a role in pathogenesis of gallbladder cancer. The study also showed the anomaly to be occurring at an early stage in carcinogenesis of gallbladder. Moreover, 53% of gallbladder cancer which express loss of MMR proteins also showed loss of FHIT expression, which was more frequently observed in advanced stage disease. All the results implied that reduced FHIT expression may be correlated with expression loss of MMR proteins on immunohistochemistry [19].

All these studies reveal the biomarker capabilities of MSI in order to detect gallbladder carcinomas. Also the importance of this marker has been evaluated in early stages of gallbladder disease. A study by Yanagisawa et al revealed MSI in 30% of severe chronic cholelithiasis cases suggesting it may play a key role in early stages of gallbladder carcinogenesis [20].

The correlation studies with known tumor suppressor genes as well as oncogenes report some significant data with a requirement for extensive analysis. Figure 1 depicts the overall relation between various molecular changes and MSI that occur during gallbladder cancer.

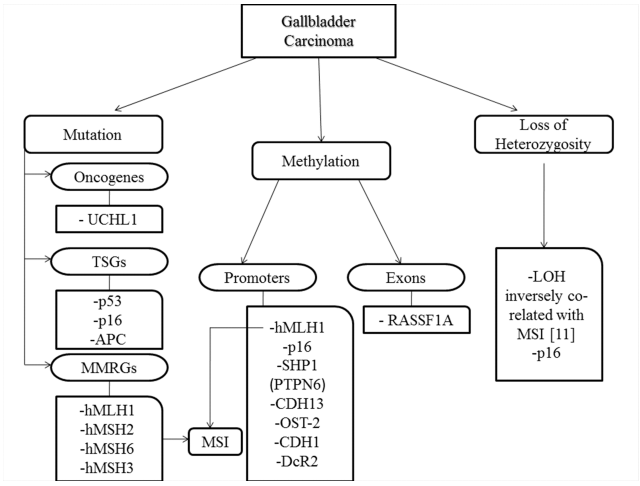

Figure 1: Molecular mechanisms contributing to Microsatellite instability during gallbladder carcinogenesis

DNA methylation in gallbladder cancer

DNA methylation causes 'turning off' of the genes and occurs at the CpG islets of the DNA, residing in the promoters, first exons and 5' untranslated regions. Tumor Suppressor Genes (TSGs) are said to the 'Gatekeeper' that regulate cell proliferation by providing check points in the cell cycle. Aberrant promoter methylation silences TSGs in many cancers. This methylation process of CpG islets is known as hypermethylation [21]. When loss of methylation occurs in case of oncogenes, they get activated. This process is known as hypomethylation. Methylation of TSGs is one of the main causes for GBC. This epigenetic modification is also observed in chronic cholecystisis (CC) during gallbladder disease [21-23]. Figure 2 highlights some important genes showing increasing frequency in methylation status during progression of gallbladder disease.

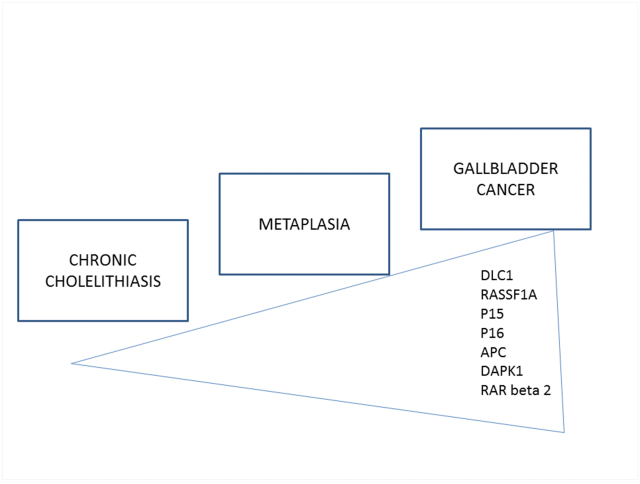

Figure 2: Increasing methylation patterns seen in candidate genes during progression of gallbladder carcinoma

There are many genes involved in GBC which get methylated at variable frequencies. They are: SHP1, 3-OST-2, CDH13, P15INK4B, CDH1, RUNX3, APC, RIZ1, P16INK4A, HPP1, P73, RAR_2, SOCS-1, DAPK, DcR2, DcR1, HIN1, CHFR, TIMP-3, P57, RASSF1A, CRBP1, SYK, NORE1, DLC1, RARb2, MGMT, FHIT, RASSF1, hMLH1, GSTP1, SEMA3B, BLU, DUTT1. Some of these genes are also methylated during chronic cholelithiasis and thus may be of prognostic significance in gallbladder disease progression. For instance, in one study by Takahsi et al., it was observed that ten genes showed a relatively high frequency of abnormal methylation: SHP1 (80%), 3-OST-2 (72%), CDH13 (44%), P15INK4B (44%), CDH1 (38%), RUNX3 (32%), APC (30%), RIZ1 (26%), P16INK4A (24%), and HPP1 (20%) [22,24]. In the same study, eight genes (P73, RAR_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. Similarly, methylation in CC was observed in seven genes: SHP1 (88%), P15INK4B (28%), 3-OST-2 (12%), CDH1 (12%), CDH13 (8%), DcR2 (4%) and P16INK4A (4%) [22,24]. Table 1 summarizes some of the important genes methylated in gallbladder cancers with variable frequencies.

Gene	Function	Methylation State in GBC	Remarks
SHP1	Cell growth, differentiation and mitotic cycle.	Hypermethylated	No significant difference in methylation between CC and GBC [2]
p15	Cell cycle regulator	Hypermethylated	Poor survival rates [8]
3OST-2	Encodes an O-sulfotransferase	Hypermethylated	Altered expression in breast cancers as well [21]
CDH13	Promotes cell-cell adhesion	Hypermethylated	Causes cancer infiltration to serosa [8]
RUNX3	Role in TGF-beta signal pathway	Hypermethylated	Helicobacter pylori can accelerate its methylation status [1]
APC	Negative regulatorcontrollingbeta-catenin and thus involved in adhesion and apoptosis	Hypermethylated	Poor survival rates [8]
p16	Cyclin dependent kinase	Hypermethylated	Significant prognostic factor; better survival rate without its methylation [1]
HPP1	Involved in TGF- beta signaliing pathway	Methylation status not checked in normal gall bladder cells	
MGMT	Methyltransferase	Hypermethylated	Significant correlation between survival rate and methylation status [8]; favourable prognosis with alkylating agents [8]
RIZ1	Retinoblastoma protein-interacting zinc finger gene, a putative TSG	Hypermethylated	Associated with increasing depth of invasion and tumor-node-metastasis (TNM) [2]
CDH1	Cellular adhesion;loss leads to metastasis and invasion	Hypermethylated	No significant difference between methylated and non-methylated cases [1]
DLC1	GTPase activating protein	Hypermethylated	Poor survival rates [8]; higher methylation through cancer progression [8]
Reprimo	Cell cycle regulator	Hypermethylated	
SEMA3B	Induces apoptosis	Hypermethylated	

FHIT	Role in apoptosis and DNA replication	Hypermethylated	No correlation between survival rate and methylation status [7]
p73	Involved in apoptosis and cell cycle regulation	Hypermethylated	Low methylation frequency [2]
SOCS 1	Involved in JAK STAT pathway; alsotakes part in a negative feedback loop to attenuate cytokine signaling	Hypermethylated	Low methylation frequency [2]
RAR 2	Role in cell signalling	Hypermethylated	Higher methylation through cancer progression [8]
DAPK	Serine throenine Kinase; positive mediator of gamma-interferon induced programmed cell death	Hypermethylated	Higher methylation through cancer progression [8]
DcR2	TNF-receptor superfamily	Hypermethylated	4% methylation in CC [2]
RASSF1A	Inhibits the expression of the RAS oncogene, acting as a tumor suppressor gene	Hypermethylated	Methylation in exon 1 of this gene was 36.4% in carcinoma samples, 25.0% in adenoma and 8.0% in normal epithelium [5,24]
UCHL1	Belongs to peptidase C12 family	Hypomethylated	Methylation frequency decreases from normal epithelium to adenoma to carcinoma [1]
TIMP3	Encodes inhibitors of thematrix metalloproteinases, a group of peptidases involved in degradation of theextracellular matrix	Hypermethylated	Higher methylation through cancer progression [8]
p14	Regulates cell cycle	Hypermethylated	High methylation frequency [4]
DUTT 1	Role in metastasis and migration	Hypermethylated	
BLU	Role in cell cycle	Hypermethylated	
MLH1	Mismatch repair		GBC due to MSI [1]
GSTP1	Involved in detoxification by catalyzing theconjugation of hydrophobic and electrophilic compounds		
DrC 1	Key component of the nexin-dynein regulatory complex (N-DRC), essential for N-DRC integrity		Low methylation frequency [2]
HIN1	Inhibits cell growth(mainly secreted in Breast epithelium)	Hypermethylated	Low methylation frequency [2]
CHFR	Cell cycle regulator; provides a checkpoint that delays entry into metaphase		Low methylation frequency [2]

Table 1: Methylation pattern of some of the significant genes in gallbladder cancer

Important genes involved in methylation and could serve as biomarkers

SHP1: is one of the most frequently methylated genes in both GBC and CC. In one study, forty out of fifty cases (i.e. 80% frequency) of GBC, this gene was methylated. Its frequency was found to be 88% for CC [25]. However, the two genes (SHP1 and P15INK4B) demonstrating the highest frequencies of methylation in CC specimens did not show any significant difference in methylation frequencies in GBC, which suggests that methylation of these genes could be an early event [25]. This gene is responsible for the regulation of cell growth, differentiation and mitotic cycle.

P15INK4B: is a cell cycle regulator. Its methylation frequency in GBC was 44% in one study [25] and in another one it was only 22% [26]. The survival chances of patients with this gene methylation were quite poor (along with APC and CDH13) [26]. In another study, the frequency of P15 methylation was determined from CC without metaplasia to advanced stage cancer. In CC without metaplasia the frequency was 13.3%, in CC with metaplasia it was 25%, in early carcinoma it was 21.1%, 36% and 21.7% in subserous layer and serous layer (both represent advanced stage of cancer) respectively [26]. The frequency was not seen to be increasing with the progression towards GBC. But this cannot be concluded since the sample size was small.

3-OST-2: had a frequency of 72% in GBC and 12% in CC [25]. Their multivariate penalized logistic regression model analysis identified a five-gene (3-OST-2, CDH13, RUNX3, P16INK4A and HPP1) combination as a significant predictor of GBC compared with CC (92% sensitivity, 81% specificity) [25]. The second group of genes demonstrating frequent methylation in both GBC and CC specimens includes SHP1 and P15INK4B [25]. 3-OST-2 encodes an O-

sulfotransferase that is involved in the final modification step of glycosaminoglycan chains of heparan sulfate proteoglycans. Its altered expressions have been reported recently in human breast cancers [27].

CDH13: CDH13 is a cadherin and it promotes cell-cell adhesion. Its alteration leads to tumor invasion. According to various studies it was determined to be one of the most frequently methylated genes in both GBC and CC. In one study it was found to be 44% [25] and 70% in another [26]. In CC its frequency was reported to be 8% [25]. Cases with carcinoma infiltrating the serosa layer represents the greatest proportion of deceased patients with gene methylation (>80%) [26]. Since CDH13 is an adhesion factor, its role is significant for GBC metastasis.

RUNX3: RUNX3 plays a role in TGF-beta signal pathway. In Chile its methylation frequency was 32% [25] and in Japan it was 22% [28]. Methylation of RUNX3 was more frequent in elderly patients. Environmental factors such as tobacco smoking and Helicobacter pylori infection can accelerate the process of DNA methylation [22]. RUNX3 had 78% methylation in carcinoma of the biliary duct compared with 22.2% in GBC [28]. Hypermethylation combined with hemizygous deletion of the RUNX3 correlates with a significant reduction in expression, and the tumorigenicity of cell lines in nude mice was inversely related to their level of RUNX3 expression [25].

APC: APC is involved in cell migration, adhesion and apoptosis. Its methylayion frequency is 30% and it is one of those genes whose methylation frequency is significantly higher in GBC than in CC [25]. Another study concluded that APC methylation was present in 42% of the US cases but in only 14% of the Chilean tumors [29]. Tumors displaying only papillary or mixed papillary/tubular patterns demonstrated higher frequency of APC methylation than cancers exhibiting just the tubular features (60% versus 13% respectively) [25]. It is a putative TSG and its methylation is frequently positive in GBC and CC. The survival chances of patients with this gene methylation are quite poor [26].

P16INK4A: P16INK4A is a cyclin dependent kinase and has been studied in GBC in various geographic locations. Its frequency is 56% in Chile and USA [23], 60% in Germany [30], 24% in Japan [28] and 15% in China [31]. On contrary two independent studies were done in Chile, and its frequency was observed to be 24% [25] and 20% [26]. In CC its methylation frequency was just 4%. In CC without metaplasia its methylation frequency was found to be 13.3%, 10% in CC with metaplasia, 5.3% in early carcinoma, 44% in subserous cancer and and 26% in serous cancer [26]. Loss of heterozygosity and homozygote deletion are two different pathways of p16 inactivation and have been shown to be combined with hypermethylation of the promoter in GBC. This is a tendency that is also observed in GBC with a loss of expression of up to 62.5% [30,32]. The absence of alterations in p16 (methylation, mutation, loss of heterozygosity in chromosome 9p) in cases of GBC showed a better by and large survival rate [22]. Therefore it can be considered a significant prognostic factor.

RIZ1: RIZ1 is a retinoblastoma protein-interacting zinc finger gene, a putative TSG and a member of a nuclear histone/protein methyltransferase superfamily. In addition to the relatively high frequency of RIZ1 methylation in GBC (about 26%), this gene was significantly linked with increasing depth of invasion and tumor-node-metastasis (TNM) [25]. Its methylation was also higher in GBC compared to CC.

MGMT: MGMT is a methyltransferase whose methylation frequency was 17% amongst Chile population [26]. According to another study, it was concluded to be 13% [23]. Patients with methylated MGMT when treated with alkylating agents, showed better response to the treatment and therefore we can say that such patients have a better chance of survival [26].Therefore prognosis of patients with MGMT methylation is quite favourable, contributing to better survival. A Kaplan-Meier survival analysis showed significant correlations between survival rates and its promoter methylation status [26].

CDH1: CDH1 frequency of methylation was 11% in Japan [28], 38% [25] and 65% [26] in Chile. CDH1 belongs to a family of genes directly related to the processes of tumor invasion and cytoskeleton destabilization. CDH1 expression has been reported in less differentiated tumors. It has been described to be hypermethylated in GBC, with frequencies ranging from 11.1% to 65.2% [22].The methylation of this gene in advanced stage III and IV of GBC was evaluated, demonstrating approximately a frequency of 60% methylation [22]. Loss of E-cadherin expression is an important event for increasing cell proliferation, motility and invasion activity in the progression of GBC and thus can be used as a marker. This gene does not exhibit significant differences in survival rates between the methylated and non-methylated cases, when patient survival is considered [22].

DLC1: DLC1 is a GTPase activating protein. Its frequency of methylation is 39% amongst Chile population [26]. This gene is gradually methylated during progression of cancer from CC without metaplasia (0%) to serous stage of GBC (39.1%). There was a correlation found between the methylation of this gene and survival of patients. Maximaum deceased patients had methylated DLC1 and MGMT genes [26].

Some other genes may also play a role: Reprimo (methylation frequency of 62% [25]) is a cell cycle regulator and SEMA3B (methylation frequency of 62% [33]) induces apoptosis and thus controls cellular proliferation. FHIT (methylation frequency of 66% [33]) plays a role in apoptosis and DNA replication. Their methylation also seems to play an important role in GBC. No correlation was found between survival rates and methylation state for FHIT [34]. In a study eight genes (P73, RAR_2, SOCS-1, DAPK, DcR2, DcR1, HIN1, and CHFR) showed a low frequency (2-14%) of methylation , and no methylation was detected for the six genes, namely TIMP-3, P57, RASSF1A, CRBP1, SYK, and NORE1 [25]. 4% methylation was found for DrC2 in CC [25]. DAPK1 methylation frequency was reported as 21% in early carcinomas, 52% in subserous carcinomas, and 60% in serous carcinomas [26]. The methylation of DAPK1 is likely to be associated with the presence of early morphological alterations of the gallbladder mucosa, since it is not observed in chronic cholecystitis without the presence of metaplasia [26]. In another study very low frequencies in RASSF1A (4/50, i.e 8%) was observed [35]. RASSF1A inhibits the expression of the RAS oncogene, thus acting as a tumor suppressor gene through various pathways, including apoptosis, genomic stability and cell cycle regulation [22]. In the gallbladder, it was found that the methylation in exon 1 of this gene was 36.4% in carcinoma samples, 25.0% in adenoma samples and 8.0% in normal epithelium samples [28,35]. The methylation frequency of this gene in cholangiocarcinoma can reach upto 65%, but inspite of the close anatomical relationship with GBC, has different methylation patterns [22]. The expression was decreased in more than 90% of cancers (40 out of 44) [22]. It was again noted that the methylation frequency of this gene also increased from CC without metaplasia to serous stage carcinoma [26]. Though the difference was not that significant, a

larger sample space can give a better picture if RASSF1A is a potential diagnostic marker to check the chances of progression from CC to GBC. TIMP3 showed a methylation frequency greater than 20% in advanced carcinomas, being less than 10% in early carcinomas and chronic cholecystitis in Gracia et al [26]. TIMP3 is a protein limited to the extracellular matrix that regulates its composition by acting as an inhibitor of metalloproteinase-3 (MMP-3), which is a lithic protein able to degrade the proteins that comprise the extracellular matrix [26]. UCHL which belongs to peptidase C12 family was studied amongst Korean population and its frequency was about 27% [36]. However it was seen that a progressive decrease in the methylation of this gene has been observed, with 84.6% in normal epithelium, 37.5% in adenoma and 27.2% in carcinoma [22]. HPP1 is also involved in TGF- beta signaliing pathway. Its methylation frequency was found to be 20% i.e ten out of fifty patients [25]. However to determine if inactivation of HPP1 plays a role in pathogenesis of GBC, methylation pattern was not checked in normal gall bladder cells since the samples were difficult to obtain. BLU (role in cell cycle) and DUTT1 (role in metastasis and migration) had methylation frequencies of 26% and 22% respectively amongst Chile population [33]. Frequency status of p14 was studied to be 40% in USA [30].

Conclusion

Since genetic instability in the form of MSI has shown to be present in early stages of gallbladder cancer and is also co related with other genetic markers eg. tumor suppressor genes or oncogenes the utilization of MSI as a diagnostic marker is promising. Further studies in context to the fact that whether MSI positive gallbladder cancer follow the classical pathways of gallbladder cancer. If yes, then in what frequency and if not, then whether it forms a new group for gallbladder cancer progression model. Since, MSI is a permanent change in the DNA it may be inherited and thus there is increased chances of cancer being hereditarily transferred to offspring. More intensive research in this regard is required to gain knowledge about gallbladder cancer and to prepare molecules for early detection and therapeutics.

The methylation of RASSF1A, CDH13, APC and DLC1 can be promising biomarkers for GBC. Since CDH13 is important for cell-cell adhesion, its alteration leads to metastasis which is the leading cause of mortality in case of GBC. It is involved in EMT and MET during metastasis. On the other hand CDH13 is also involved in signal transduction. Patients with APC methylation generally have a poor survival chance and therefore as a therapeutic step, this gene can be targeted with de-methylating agents with specificity. Furthermore APC is involved in other cancers as well. The APC protein is a negative regulator controlling Beta-catenin concentrations and also interacts with E-cadherin, which are involved in cell adhesion. Mutations in the APC gene mainly results in colorectal cancer [37]. DLC1 is said to be a candidate tumor suppressor gene for human liver cancer, as well as for prostate, lung, colorectal, and breast cancers. The main function of DLC1 is its Rho-GAP activity. It enhances activated GTP-bound Rho-GTPases' specifically, RhoA and Cdc42, to convert their GTP into GDP, thus leaving them inactive. Rho-GTPases are members of the Ras superfamily, and are involved in cell adhesion and cell polarity. Thus it finally leads the way to further migration of tumor cells. It regulates apoptosis as well. It is also responsible for negatively regulating angiogenesis. Its loss/inactivation results in upregulation of VEGF through EGFR-MEK-HIF1 signalling pathway. VEGF upregulation is a highly significant event in prostate cancers wherein DLC1 is downregulated. Thus, this strongly suggests that its loss may serve as a "second hit" in inducing angiogenesis in a paracrine fashion during tumor progression [38,39]. Thus an anti-angiogenic agent may upregulate DLC1 and thus help in preventing metastasis and angiogenesis as well as enhance apoptosis. It has been also shown to induce the expression of E-cadherin (regulated by CDH1 gene) in prostrate cancers through Rho pathway [39]. By this way it also contributes to EMT. DLC1 can be a potential diagnostic marker for CC and GBC. Patients with CC can be checked for the methylation of this gene and then prognosis can be determined based on the level of its expression. Then the chances of progression from CC to advanced carcinoma can be estimated by the data. There have been satisfactory results with epigenetic therapy and more therapeutic approaches are being evaluated.

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