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ORIGINAL ARTICLE

Microsatellite instability in gallbladder carcinoma

Andrea P. Moy · Mohammad Shahid · Cristina R. Ferrone · Darrell R. Borger · Andrew X. Zhu · David Ting · Vikram Deshpande

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Abstract The genetic abnormalities involved in the pathogenesis of gallbladder carcinoma (GBC) remain unclear. Microsatellite instability (MSI) has been described in many carcinomas, but little is known about the significance of mismatch repair in gallbladder carcinogenesis. Additionally, methylation status of long interspersed element-1 (LINE-1), a surrogate marker of global DNA methylation, has defined distinct subsets of other cancer types but has not been explored in GBC. Immunohistochemical expression of MSH2, MSH6, MLH1, and PMS2 and LINE-1 mRNA in situ hybridization was evaluated in 67 primary and 15 metastatic GBCs from 77 patients. Amplification of human epidermal growth factor receptor 2 (*HER2*) was evaluated by fluorescence in situ hybridization. Genotyping for 24 genes involved in carcinogenesis was performed using a multiplex PCR-based platform. MSI was present in 6 of 77 GBCs (7.8 %). Loss of MSH2/MSH6 was detected in five cases and loss of MLH1/PMS2 in one case. MSI status was not associated with Lynch syndrome, tumor grade, extracellular mucin, or tumor-infiltrating lymphocytes. There was no significant difference in mean overall survival of patients with and without MSI. Strong LINE-1 staining was identified in none of the GBC with MSI and in 36 of 69 (52 %) of those without MSI ($p=0.005$), suggesting that LINE-1 in the former cohort was hypermethylated. All MSI tumors were negative for *HER2* amplification, and *TP53* and *NRAS* mutations were only found in GBC without MSI. MSI was identified in a minority of GBC cases. The strong correlation between global DNA methylation as measured by LINE-1 and loss of mismatch repair proteins suggests that methylation may account for the loss of these proteins. These hypermethylated tumors appear to represent a genetically unique cohort of gallbladder neoplasms, and the data suggests that demethylating agents may have a therapeutic value in this class of tumors.

Keywords Gallbladder carcinoma · Microsatellite instability · Mismatch repair · LINE-1

A. P. Moy · V. Deshpande (✉)
Pathology Service, Massachusetts General Hospital and Harvard Medical School, 55 Fruit Street, Warren 256, Boston, MA 02114, USA
e-mail: vdeshpande@partners.org

M. Shahid · C. R. Ferrone
Department of Surgery, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, USA

D. R. Borger · A. X. Zhu · D. Ting
Division of Oncology, Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, USA

Introduction

Gallbladder carcinoma (GBC) is the most common malignancy affecting the biliary tract. The incidence varies worldwide with the greatest numbers of cases occurring in India and Chile. GBC is often diagnosed at advanced stages when surgical resection is no longer an option. Thus, although relatively rare in the USA, GBC remains a highly aggressive cancer with limited therapeutic options and a poor prognosis [1].

Despite advances in molecular pathology, the molecular pathogenesis of GBC remains poorly defined. Mutations in *KRAS* have been identified in gallbladder dysplasia and carcinoma in the setting of abnormal junction of the pancreatic and bile ducts [2–4]. Activation of the mitogen-activated protein kinase or the phosphoinositide 3-kinase signaling pathways via mutation in one of many involved genes appears to be important in the pathogenesis of GBC associated with cholelithiasis [4–13]. However, the frequency with which these

mutations occur and the relationship between these mutations in GBC remain to be clarified.

There is much variation in the literature regarding the frequency with which microsatellite instability (MSI) is present in GBC. Using either polymerase chain reaction (PCR)-based techniques or immunohistochemistry (IHC) for mismatch repair (MMR) proteins, prior studies have reported MSI in a subset of GBC, ranging from 0 to 40 % of cases [6–8, 14–23]. Of note, a higher prevalence of MSI has been reported in GBC cases from patients with abnormal junction of the pancreatic and bile ducts [24]. Few studies have also noted MSI within dysplastic lesions of the gallbladder, suggesting that MSI occurs early in gallbladder carcinogenesis [17, 18]. It is important to note, however, that these investigations evaluated populations with a high prevalence of GBC; no studies have evaluated the frequency of MSI in the North American populations.

Some prior studies have evaluated clinical and histopathologic features of tumors with and without MSI. Roa et al. [18] found no difference in tumor grade, tumor stage, and survival in patients with tumors with and without MSI. Only one small study evaluated the morphologic features of GBC with MSI and found that one of two cases showed evidence of mucinous differentiation on histologic evaluation; both tumors with MSI occurred in patients with no history of Lynch syndrome [7]. Additionally, despite reports that MSI and mutations in select genes, including *KRAS* and *BRAF*, were mutually exclusive, the relationship between microsatellite status and the mutational profile of GBC remains unclear [6–8, 15, 20, 21].

In order to better characterize the role of MSI in gallbladder carcinogenesis, we evaluated protein expression of MMR genes by IHC in primary and metastatic tumors and assessed the prevalence of Lynch syndrome in the cohort of patients with MSI. We also correlated histologic features with MSI and the relationship of MSI status with the mutational profile, as analyzed by a multiplex PCR-based platform. In addition, we correlated GBC MSI status with expression of long interspersed nuclear element-1 (LINE-1), a surrogate marker of global methylation status. LINE-1 is a retrotransposon that accounts for approximately 18 % of the human genome [25]. Lack of methylation of LINE-1 is believed to account for much of the genomic hypomethylation observed in human cancer, such that it serves as a surrogate marker for global DNA methylation status [26, 27].

Materials and methods

Case selection

The study was approved by the Massachusetts General Hospital (Boston, MA) Institutional Review Board. Cases from 1988 to 2012 were retrospectively selected from a search of the surgical pathology files. A total of 80 cholecystectomy specimens were evaluated, of which 67 contained GBC (4 with concurrent high-grade dysplasia (HGD) and 1 from a patient with subsequent metastatic GBC), 8 contained HGD only, and 7 contained pyloric gland adenomas (PGAs; 2 with HGD and 1 from a patient with concurrent GBC). Additionally, 15 cases of metastatic GBC (including 4 from patients whose primary lesion was also studied here) were collected. Overall, specimens from 92 patients were evaluated in this study. Clinical data, including patient sex and age at the time of diagnosis, history of cigarette smoking, clinical stage of disease at the time of diagnosis, and survival information, was collected from each patient.

Tissue microarray construction

All cases were fixed in 10 % buffered formalin and embedded in paraffin by standard procedures. Selected tissue blocks were obtained for tissue microarray (TMA) construction. For each representative lesion, a 0.3-cm in diameter core, as outlined on review of the corresponding hematoxylin and eosin stained slide, was removed from the tissue blocks, for a total of 101 cores (67 primary GBCs, 15 metastatic GBCs, 12 HGDs, and 7 PGAs) and used to construct the TMA. Each TMA also included sections of liver parenchyma, including biliary epithelium, as controls.

Histologic evaluation

Hematoxylin and eosin stained sections of the primary and metastatic GBC cases were reviewed to assess the histologic features, specifically tumor grade, the presence of extracellular mucin, and tumor-infiltrating lymphocytes (TILs). Tumor grade was defined as well differentiated, moderately differentiated, or poorly differentiated based upon the percentage of the solid component of the tumor (<25, 25–75, or >75 %, respectively). The presence of TILs was defined as ≥5 intraepithelial lymphocytes per high power field.

Immunohistochemical analysis

Protein expression of four MMR proteins, MSH2, MSH6, MLH1, and PMS2, was evaluated by immunohistochemical staining of 5-μm-thick sections cut from the paraffin-embedded TMAs. The immunohistochemical stains were performed using the Leica Bond III auto-stainer (Leica Biosystems, Buffalo Grove, IL), according to the manufacturer's protocol. After deparaffinization, monoclonal antibodies against hMLH1 (Leica Biosystems), hMSH2 (Leica Biosystems), hMSH6 (Leica Biosystems), and hPMS2 (Becton Dickinson Biosciences, San Jose, CA) were applied to the tissue sections. Slides were counter-stained with hematoxylin. The presence of MSI was defined as complete loss of

nuclear staining for one or more of the four MMR proteins. Adjacent normal mucosa, stromal cells, and inflammatory cells with intact nuclear staining served as internal positive controls. The stained slides were reviewed by two authors (APM and VD), and cases were scored as either positive or negative. In cases with loss of protein expression, whole

Table 1 Mutations interrogated by the SNaPshot tumor genotyping assay

Gene	Amino acid - cDNA residue	Gene	Amino acid - cDNA residue	Gene	Amino acid - cDNA residue
AKT1	E17 - 49G	*GNAQ*	Q209 - 626A	*NOTCH1*	L1575 - 4724T L1601 - 4802T
APC	R1114 - 3340C Q1338 - 4012C R1450 - 4348C T1556fs - 4666_4667insA	GNAS	R201 - 601C R201 - 602G	*NRAS*	G12 - 34G G12 - 35G G13 - 37G G13 - 38G Q61 - 181C Q61 - 182A Q61 - 183A
BRAF	G466 - 1397G G469 - 1406G L597 - 1789C L597 - 1790T V600 - 1798G V600 - 1799T	HRAS	G12 - 34G G12 - 35G G13 - 37G G13 - 38G Q61 - 181C Q61 - 182A Q61 - 183G	*PIK3CA*	R88 - 263G E542 - 1624G E545 - 1633G E545 - 1634A Q546 - 1636C Q546 - 1637A H1047 - 3139C H1047 - 3140A G1049 - 3145G
CTNNB1	D32 - 94G D32 - 95A S33 - 98C G34 - 101G S37 - 109T S37 - 110C T41 - 121A T41 - 122C S45 - 133T S45 - 134C	*IDH1*	R132 - 394C R132 - 395G	*PTEN*	R130 - 388C R173 - 517C R233 - 697C K267fs - 800delA
EGFR	G719 - 2155G G719 - 2156G T790 - 2369C L858 - 2573T L861 - 2582T E746_A750 - 2235_2249del E746_A750 - 2236_2250del	*IDH2*	R140 - 418C R140 - 419G R172 - 514[a] R172 - 515G	*RET*	M918 - 2753T
EML4-ALK	L1196 – 4493C	*KIT*	D816 - 2447A	*TP53*	R175 - 524G G245 - 733G R248 - 742C R248 - 743G R273 - 817C R273 - 818G R306 - 916C
FGFR3	Y373 - 1118A	*KRAS*	G12 - 34G G12 - 35G G13 - 37G G13 - 38G Q61 - 181C Q61 - 182A Q61 - 183A A146 - 436G A146 - 437C	*EGFR*[a]	exon 19 deletions exon 20 insertions/deletions
GNA11	Q209 - 626A	*MAP2K1(MEK1)*	Q56 - 167A K57 - 171G D67 - 199G	*ERBB2 (Her2)*[a]	exon 20 insertions

[a] Sizing assays (insertions/deletions)

sections stained with antibody corresponding to the particular protein with lost expression were studied to confirm this finding.

Molecular analysis

Twenty-three gallbladders (21 with GBC, 1 with HGD, and 1 with a PGA) were genotyped for hot spot mutations in 24 cancer genes with SNaPshot, a previously described mutation assay (Applied Biosciences; Table 1). In brief, DNA was isolated from formalin-fixed, paraffin-embedded tissues and subjected to a multiplexed PCR and single-base extension reaction platform in order to generate fluorescently labeled signals. These SNaPshot products were then analyzed via capillary electrophoresis in order to detect the presence of selected hot spot mutations [28].

Fluorescence in situ hybridization analysis

Fluorescence in situ hybridization (FISH) was performed on deparaffinized, protease-treated, 5-μm-thick sections cut from the TMA to evaluate copy number changes in the human epidermal growth factor receptor 2 (*HER2*) gene. Dual-colored FISH with a probe specific to the *HER2* gene and a copy number control probe that recognizes centromere 17 (CEP17) were hybridized according to the manufacturer's protocol (Abbott Molecular). A case was considered positive for low-level *HER2* amplification if the *HER2*/CEP17 signal ratio was 1.8–2.2 and for high-level *HER2* amplification if the *HER2*/CEP17 signal was greater than 2.2. The cases were evaluated for *HER2* overexpression by two authors.

LINE-1 in situ hybridization analysis

In situ hybridization (ISH) using an RNA probe aligned to open reading frame 1 of LINE-1 was performed using the QuantiGene® ViewRNA technology (Affymetrix, Santa Clara, CA). ISH staining was scored as follows: weak—the intensity of the stain was similar to the background lymphocytes and stroma; and strong—the intensity of the stain was greater than the signal in stromal cells and lymphocytes.

Statistical analysis

Statistical analysis was done using the SPSS (model 21) for Macintosh. The chi-square test and unpaired Student's *t* test were used for categorical data and continuous variables, respectively. Survival was compared using the Kaplan-Meier analysis and log rank test. Statistical significance was set at $p=0.05$.

Results

MSI immunohistochemistry

By IHC, MSI was present in specimens from 6 of 77 (7.8 %) patients with GBC (Fig. 1 and Table 2). Concurrent loss of expression of MSH2 and MSH6 was seen in five cases. The loss of mismatch proteins identified initially on the tissue microarray was confirmed by evaluating whole sections. Isolated loss of PMS2 occurred in one case; the metastatic lesion corresponding to this primary GBC showed loss of both PMS2 and MLH1. Neither HGD (without concomitant carcinoma) nor PGA had loss of expression of MMR proteins. Notably, all adjacent areas of normal gallbladder mucosa showed positive staining for all MMR proteins.

Clinical features

Among the patients with GBC with and without MSI, 66.7 and 64.8 % were female, respectively. The mean age of the patients with MSI was 70.8 years while that for patients with microsatellite stable (MSS) GBC was 67.7 years. Four of 5 (80.0 %) patients with MSI and 21 of 51 (41.2 %) patients with MSS GBC were non-smokers ($p=0.15$); smoking history was not available for the remainder of patients. There was no significant difference in the stage at initial presentation of patients with and without MSI (Table 3). Of note, none of the patients with MSI had a personal history of malignancy. There was no significant difference in estimated mean overall survival between patients with and without MSI (34.0±19.0 and 34.0±8.0 months, respectively; $p=0.93$).

Morphologic evaluation

All studied GBC cases were adenocarcinomas (Table 4). There was no association between MSI status and tumor grade ($p=0.60$). Furthermore, there was no correlation between MSI status and the presence of extracellular mucin or TILs ($p=0.664$ and $p=0.640$, respectively; Table 4).

Correlation of MSI status with molecular data

Mutation status

SNaPshot testing revealed a mutation in two of the six (33.3 %) GBC cases with MSI: one case harbored a mutation in *KRAS* and the other a mutation in *PIK3CA*. The remaining four GBC cases with MSI (66.7 %) were wild type for hot spot mutations in 24 genes evaluated by SNaPshot. Of the 15 cases of MSS GBC that were analyzed, 4 (26.7 %) showed mutations in one of the following genes: *TP53*, *NRAS*, *KRAS*, or *PIK3CA*; the remaining 11 MSS GBC cases that were tested revealed no mutations. Overall, there was no significant

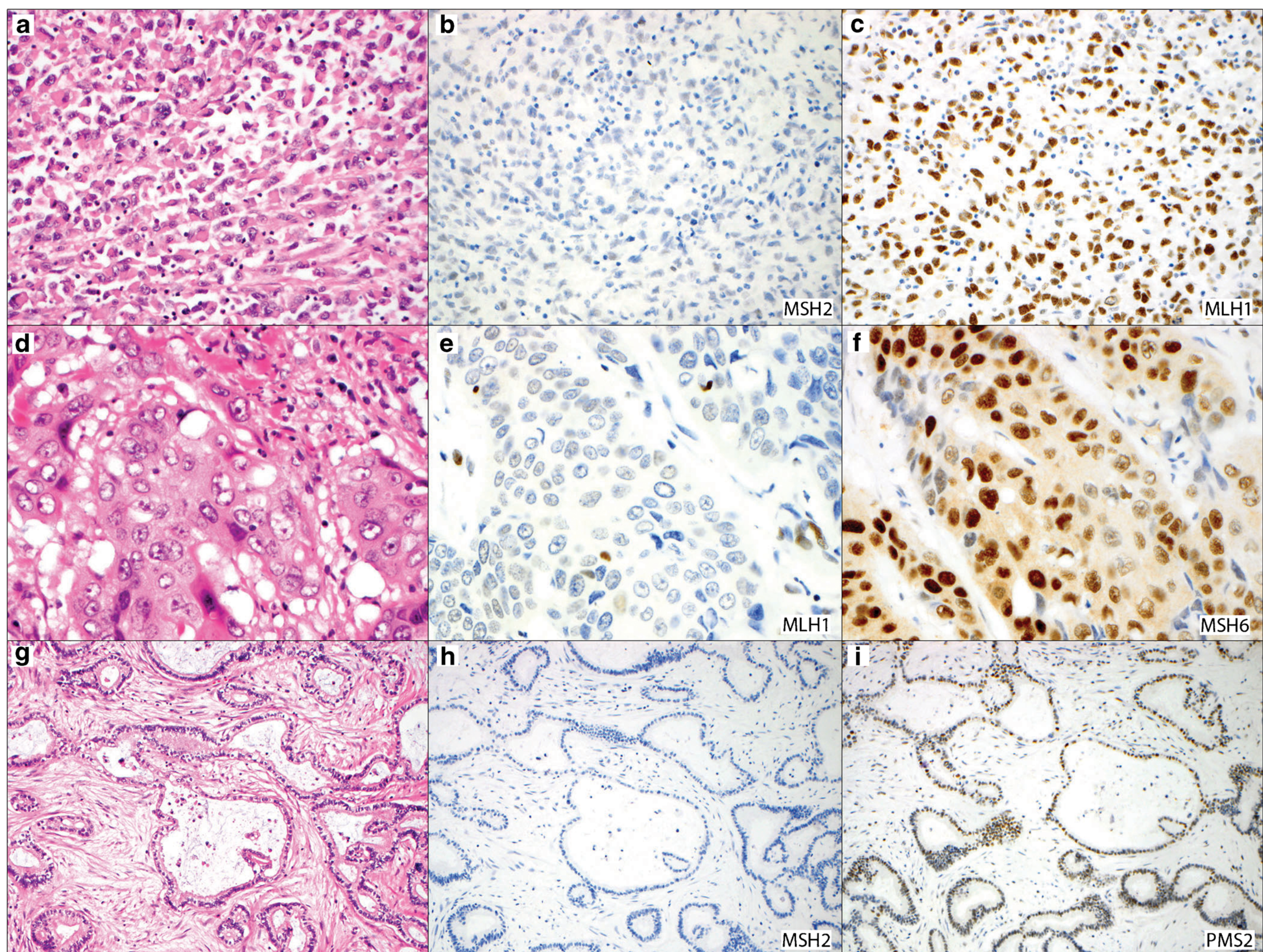

Fig. 1 Poorly differentiated adenocarcinoma (**a**) showing loss of MSH2 (**b**) and MSH6 (not shown) with preserved expression of MLH1 (**c**) and PMS2 (not shown). Poorly differentiated adenocarcinoma (**d**) showing loss of MLH1 (**e**) and PMS2 (not shown) with preserved expression of MSH6 (**f**) and MSH2 (not shown). Adenocarcinoma (**g**) showing loss of MSH2 (**h**) and MSH6 (not shown) with preserved expression of MLH1 (not shown) and PMS2 (**i**)

difference in the number of mutations identified in GBCs with and without MSI. A *PIK3CA* mutation and a *CTNNB1* (*β-catenin*) mutation were identified in one HGD and one PGA, respectively.

HER2 status

None of the GBC with MSI had overexpression of *HER2* by FISH. Conversely, 4 of 71 (5.6 %) MSS GBC cases had *HER2* amplification—2 cases had low-level amplification and 2 cases had high-level amplification. All HGD and PGA lacked *HER2* amplification.

Table 2 MMR protein immunohistochemistry results in GBC

Immunohistochemistry result	Number of cases (n=77)
Loss of MSH2 and MSH6	5
Loss of PMS2 only[a]	1

[a] The metastatic lesion corresponding to this primary GBC showed loss of both PMS2 and MLH1

Correlation of MSI status with LINE-1 expression

LINE-1 ISH was evaluated in 6 cases with MSI and 69 MSS cases. None of the six patients with MSI showed strong LINE-1 staining. In contrast, 36 (52.2 %) cases without MSI showed strong LINE-1 staining (p=0.004; Fig. 2).

Discussion

To date, this is the only study from the USA and the largest study overall to evaluate the role of MSI in GBC. We identified MSI in 7.8 % of GBCs. Prior studies report much variation in the prevalence of MSI within GBC [6–8, 14–23]. Many

Table 3 Clinical features at initial presentation and survival of patients with GBC with and without MSI

Features		MSI (*n*=6)	MSS (*n*=71)	*p* value
Female		4 (66.7 %)	46 (64.8 %)	NS
Male		2 (33.3 %)	25 (34.7 %)	NS
Non-smokers[a]		4 (80.0 %)	21 (41.2 %)	NS
Average age		70.8 years	67.7 years	NS
Stage	0	0	3 (4.3 %)	NS
	I	1 (16.7 %)	5 (7.0 %)	NS
	II	3 (50.0 %)	10 (14.1 %)	NS
	IIIA	0	16 (22.5 %)	NS
	IIIB	1 (16.7 %)	8 (11.3 %)	NS
	IVA	0	7 (9.9 %)	NS
	IVB	1 (16.7 %)	21 (29.6 %)	NS
Estimated mean overall survival		34.0±19.0 months	34.0±8.0 months	NS

MSI microsatellite instability, *MSS* microsatellite stable, *NS* not significant

[a] Smoking history was available from 5 MSI GBC patients and 51 MSS GBC patients

of these studies evaluated small numbers of patients, and several of these were from regions of the world with a high prevalence of GBC. The variation in the frequency of MSI in GBC may thus represent differences in the underlying pathogenesis of carcinoma in the gallbladder.

Molecular testing for MSI relies on the identification of changes in length of a panel of microsatellites by PCR-based techniques within a tumor as compared to normal tissue. The Bethesda guidelines define microsatellite-high (MSI-H) as MSI within two or more of five microsatellite loci and microsatellite-low (MSI-L) as MSI within one microsatellite locus. Chang et al. [14] studied 32 cases of GBC and 11 cases of gallbladder dysplasia and found MSI by PCR-based testing in only 1 GBC. Kim et al. [6] identified MSI by PCR-based techniques in only one of five cases of gallbladder dysplasia; notably, the carcinoma adjacent to this dysplasia showed no MSI and all 15 GBC cases and 3 adenomas studied showed no MSI. Both of these Korean studies conclude that MSI plays a very limited role in gallbladder carcinogenesis. In contrast, Yanagisawa et al. [17] found MSI in 7 of 17 (41 %; 1 MSI-H and 6 MSI-L) patients with GBC and MSI-L in 9 of 30 (41 %) patients with chronic cholecystitis, suggesting that MSI plays an early role in the pathogenesis of GBC.

Immunohistochemistry is now the most commonly used (and widely considered the gold standard) modality to determine MSI status. Unlike IHC, PCR-based techniques are both labor-intensive and technically demanding. Furthermore, unlike with PCR-based methods, evaluation of MSI status by IHC reveals the particular MMR protein that is defective. Prior studies of colon and endometrial carcinoma have demonstrated that IHC is as sensitive as PCR-based analysis in the detection of MSI in tumors [29–32]. Roa et al. [18] studied a series of 59 cases of GBC and found that 10 % of tumors were MSI-H when evaluated with a PCR-based assay and negative for staining of at least one MMR protein by IHC. In this study, we used IHC analysis to evaluate MSI status in gallbladder tumors and found MSI in 7.8 % of GBC cases, supporting earlier studies that suggest that MSI plays an important role in the pathogenesis of a subgroup of GBC. Interestingly, one GBC with MSI in this study showed isolated loss of PMS2 in the primary tumor and loss of both MLH1 and PMS2 in the metastasis. Prior studies in colorectal carcinoma have demonstrated genetic diversity among primary and metastatic tumors, including discordant expression of MMR proteins [33, 34]. We speculate that the preservation of MLH1 in the primary tumor may be related to a somatic mutation in MLH1 resulting in degradation of the PMS2 protein. However, the abnormal MLH1 protein may nevertheless be recognized on immunohistochemistry. Methylation of MLH1 may constitute the second hit, resulting in loss of MLH1 in the metastatic tumor.

Table 4 Histopathologic features of GBC with and without MSI

Features		MSI (*n*=6)	MSS (*n*=71)	*p* value
Tumor grade	Well differentiated	0	6 (8.5 %)	NS
	Moderately differentiated	4 (66.7 %)	51 (71.8 %)	NS
	Poorly differentiated	2(33.3 %)	13 (18.3 %)	NS
Extracellular mucin		3 (50.0 %)	25 (35.2 %)	NS
TILs		2 (50.0 %)	16 (22.5 %)	NS
Extracellular mucin and TILs		0	4 (5.6 %)	NS
LINE-1 ISH[a]	0 (absent or minimal nuclear staining)	2 (33.3 %)	3 (4.3 %)	0.004
	1 (moderate intensity with partial nuclear staining)	4 (66.7 %)	30 (43.4 %)	
	2 (strong reactivity of entire nucleus)	0	36 (52.2 %)	

MSI microsatellite instability, *MSS* microsatellite stable, *LINE-1* long interspersed element-1, *ISH* In situ hybridization, *NS* not significant

[a] Of 6 GBC cases with MSI and 69 GBC cases with MSS

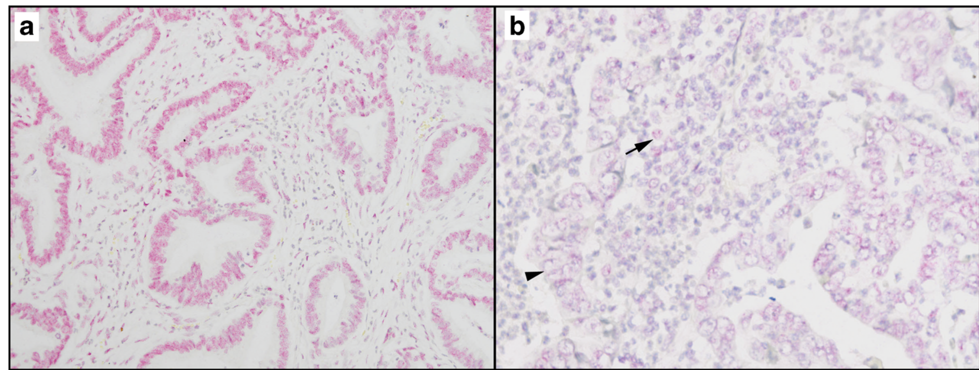

Fig. 2 Gallbladder adenocarcinoma with strong expression of LINE-1 in tumor cells (**a**). The intensity of LINE-1 reactivity in the tumor cells (*arrow head*) is similar to the reactivity in stroma cells and lymphocytes (*arrow*) (**b**)

Clinical testing of tumors for defective MMR protein function and MSI status has two potential endpoints. First, it allows for the identification of patients with Lynch syndrome who are at increased risk for developing other malignancies of the gastrointestinal tract, such as colorectal, gastric, and small intestinal, and malignancies of other organs, most commonly endometrial carcinoma. These patients benefit from surveillance to detect Lynch-syndrome-associated malignancies. Furthermore, their family members may benefit from genetic testing to determine mutation status. In colonic carcinomas, the loss of MSH2 function is generally associated with a germline mutation in *MSH2* and Lynch syndrome. In endometrial carcinoma, the observations are similar to that seen in colonic carcinoma, such that gene mutation analysis is recommended when loss of expression of MSH2 and MSH6 is observed. However, while loss of expression of MSH2 represents the most common cause of MSI in GBC in this study, none of the studied patients had clinical evidence of Lynch syndrome (i.e., a personal or family history of Lynch-syndrome-associated malignancy). Therefore, MSI in GBC does not seem to be a manifestation of Lynch syndrome. Second, the identification of tumors with MSI is important as these tumors may show distinct clinical and pathological features and distinctly different sensitivity to chemotherapy [35–44]. This is the first study to evaluate the clinical and morphologic features of gallbladder tumors in the context of MSI. In other organ systems, tumors with MSI exhibit particular clinical and histologic features. For example, in colonic carcinoma, MSI more commonly occurs in younger patients with right-sided tumors as compared to left-sided tumors. Histologic evaluation of colonic tumors with MSI is characterized by poor differentiation, signet ring and medullary growth patterns, mucinous differentiation, tumor-infiltrating lymphocytes, a dense, Crohn's-like inflammatory host response, and the absence of "dirty necrosis" [45, 46]. Similarly, the presence of TILs and peritumoral lymphocytes appears to be a sensitive indicator of MSI in endometrial carcinoma [47]. However, in this study, we found no association between tumor grade, the presence of extracellular mucin or TIL, and MSI status. As this was a retrospective study of GBC from the last three decades, we were unable to assess the impact of MSI on response to chemotherapy.

In colorectal carcinoma, tumors with MSI have been associated with a less aggressive clinical course and an improved survival rate [38, 48]; the association of MSI and survival in endometrial carcinoma remains controversial. Some studies show a negative relationship between the presence of MSI and prognostic factors, including higher histological grade, depth of invasion, and the presence of lymphovascular invasion [49–51], while others have shown a positive correlation between the presence of MSI and prognostic factors [52] or survival [53]. Still, other studies have found no significant correlation [54]. In our study, similar to the findings in a prior study of MSI in GBC [18], MSI status had no impact on survival, although the sample size for the MSI group was small.

Prior studies of GBC have attempted to correlate the presence of MSI with the presence of mutations in select genes [6–8, 15, 20, 21]. However, this is the first study to explore the relationship of MSI status and genotype, as evaluated on a large scale with a multiplex PCR-based platform. Amplification of *HER2* and mutations in *NRAS* and *TP53* were identified only in MSS GBC. However, mutations in *PIK3CA* and *KRAS* were identified in both groups of patients with GBC. In colorectal carcinoma, there is a well-characterized relationship between MSI status and mutational phenotype. Colorectal tumors with MSI have a "mutator phenotype" as they develop mutations in tumor suppressor genes that contain microsatellites, including transforming growth factor beta receptor type II, insulin-like growth factor receptor type II, and *BAX*. Additionally, mutations in *BRAF* are seen in 40–50 % of sporadic MSI cases [55–58] but are not seen in colorectal carcinoma associated with Lynch syndrome, such that the presence of a *BRAF* mutation virtually excludes Lynch syndrome in colorectal carcinomas with MSI. In this analysis, there was no significant correlation between mutational status and GBC. Of note, recent whole exome and targeted sequencing approaches identified mutations in a higher proportion of GBCs [59, 60]. Our targeted approach yielded mutations in a lower proportion of genes as well as a lower incidence of

mutations in particular genes, such as p53. It is possible that genetic variations observed in these studies reflects geographic variation as our study, unlike the others, evaluated only tumors from patients in the USA. Additionally, we evaluated a limited number of hot spot mutations in a limited number of genes by SNaPshot and FISH analysis that were performed such that other mutations of possible relevance were not interrogated. A whole exome sequencing effort would be required to thoroughly address the issue of geographic difference in mutation profile of GBCs.

The methylation status of LINE-1, which makes up approximately 18 % of the human genome, has been shown to be a useful marker of global DNA methylation status [25–27], such that decreased LINE-1 expression correlates with the global DNA hypermethylation. This analysis is generally performed using traditional methylation platforms. However, novel ISH platforms allow for the robust evaluation of mRNA in paraffin-embedded tissue. In this study, taking advantage of the advances in chromogenic ISH technology, we assessed LINE-1 RNA ISH as a marker of global methylation status. This ISH platform offers significant advantages over the currently available methylation assays by allowing the distinction of tumor cells and stromal cells; traditional methylation assays reflect the methylation status of the tumor cells as well as stromal cells and lymphocytes, unless microdissection techniques have been employed.

We found a correlation between LINE-1 expression within tumor cells and MSI status: no cases of GBC with MSI had strong LINE-1 RNA expression suggesting that these cases showed a global hypermethylator phenotype. The strong correlation between LINE-1 reactivity and MSI status suggest that the mechanism underlying the loss of mismatch repair proteins is methylation. However, the possibility that somatic mutations are involved in the silencing of mismatch repair genes [61] cannot be entirely excluded. This data is consistent with the observation made in colorectal carcinoma that hypermethylation of genes implicated in the MSI pathway correlates with global hypermethylation status [62]. Thus, hypermethylated GBCs may represent a distinct pathway in gallbladder carcinogenesis: a paradigm that parallels colorectal carcinoma [63–65]. Additionally, in line with prior studies that there is decreased DNA methylation in smokers as compared to non-smokers [66–68], we found that patients with MSI-positive tumors were more likely to be non-smokers. Overall, this data provides additional insight regarding the hypermethylated pathways of gallbladder carcinogenesis and may have implications for guiding therapy. As it is now widely accepted that epigenetic dysregulation, including alteration of DNA methylation profiles, is involved in cancer development and progression, therapeutic approaches utilizing demethylating agents, including 5-azacytidine and decitabine, have been extensively studied in many tumor types and are currently FDA-approved for clinical use in the treatment of myelodysplastic syndromes. Our findings open up the prospect of investigating demethylating agents for the therapy of this class of gallbladder carcinomas.

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