Regional Differences in Gallbladder Cancer Pathogenesis: Insights from a Comparison of Cell Cycle-Regulatory, PI3K, and Pro-Angiogenic Protein Expression

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ABSTRACT

Background. The variable incidence of gallbladder cancer (GBCA) suggests regional pathogenetic differences. This study compares cell cycle-regulatory, angiogenesis-related, and PI3K pathway protein expression in GBCAs from three continents.

Methods. Immunohistochemical expression of several proteins was assessed, correlated with clinicopathologic variables, and compared among centers from Chile (Fundación Arturo López Pérez [FALP]), Japan (Yokohama City University [YCU]), and the United States (Memorial Sloan-Kettering Cancer Center [MSKCC]). Hierarchical clustering was used to partition the data based on protein-expression and treatment center.

Results. Tissue from 117 patients (MSKCC = 76; FALP = 22; YCU = 19) was analyzed. Mdm2 overexpression was seen only at MSKCC ($p < 0.0001$). Absence of p21 ($p = 0.03$) and VEGFR2 ($p = 0.018$) were more common and p27 expression was less frequent ($p = 0.047$) in tumors from YCU. Ki-67 labeling index in YCU tumors (median = 10) was two-thirds lower than at other centers. On hierarchical clustering analysis, all YCU patients

($p = 0.017$) and those with early tumors ($p = 0.017$) clustered separately from MSKCC. Median disease-specific survival after curative intent (R0) resection was 27 months and was similar among centers ($p = 0.9$). Median disease-specific survival of patients with early tumors was 28.4 months and was higher at YCU (not reached, $p = 0.06$).

Conclusions. Cell cycle-regulatory protein expression patterns of YCU tumors differed from those treated at FALP and MSKCC. The differential clustering of protein expression and survival in patients with early tumors suggest regional differences in pathogenesis and disease biology.

Gallbladder cancer (GBCA) is an uncommon malignancy with poor prognosis and variable incidence worldwide, although the common feature among all patients is advanced disease at diagnosis.^{1–8} Epidemiological and molecular studies suggest that GBCA may arise and progress through different pathways, a hypothesis supported by a recent comparison among centers from Japan, the United States, and Chile revealing disease-related differences based on national origin.^{9–12}

Whether these differences reflect regional variation in pathogenesis is uncertain but plausible based on prior work. For example, K-ras mutations are frequent in patients with anomalous pancreaticobiliary duct junction, a relatively common risk factor in Japan, but are rare in

GBCA associated with adenomas.^{13–15} Additionally, deregulatory mutations in the TP53 gene are seen in both Japan and Chile, but the spectrum of mutations varies according to location.^{16–19}

The expression patterns of certain cell cycle-regulatory, pro-angiogenic, and PI3K pathway proteins are important predictors of clinical behavior in biliary tract cancers.^{20–22} Most studies, however, include small numbers of patients, and few, if any, include a comprehensive assessment in the same cohort. This study examines expression of key elements of these pathways in GBCA patients treated at centers on three continents. The primary objective was to assess for differences in expression patterns among groups, which might suggest regional differences in pathogenesis.

METHODS

Subjects and Data Collection

After institutional review board approval from Instituto Oncológico Fundación Arturo López Pérez (FALP, Santiago, Chile), Yokohama City University (YCU, Yokohama, Japan), and Memorial Sloan-Kettering Cancer Center (MSKCC, New York, NY), patients with GBCA treated from 1994 to 2009 and with adequate archived tissue were identified. Demographics, laboratory values, procedures, perioperative outcome, histopathology and staging, follow-up, and survival data were recorded. The approach to evaluation and treatment of GBCA at each center has been described.^{12,23,24} Incidental gallbladder cancer (IGBCA) was defined as an unsuspected tumor from a specimen removed for presumed benign disease. All cases of IGBCA were re-reviewed to confirm diagnosis and T stage. All patients with tumor invasion to at least the muscularis propria layer (T1b) and without distant metastases were offered reoperation and definitive resection.²³

Pathologic Examination

All archived hematoxylin and eosin (HE)-stained slides were reviewed by study pathologists: staging was based on the 7th edition of the American Joint Committee on Cancer, Cancer Staging Manual.²⁵ Tissue microarrays were created by identifying tumor and nontumor tissue on HE-stained slides; an automated tissue arrayer (Beecher ATA-27) was used to procure triplicate 0.6-mm cores from the corresponding paraffin block.²¹ HE-stained sections were examined to ensure specimen integrity. Immunostaining was performed using standard streptavidin–biotin immunoperoxidase techniques. Primary antibodies were placed overnight at 4 °C. Mouse antihuman monoclonal antibodies to mutant or wild-type p53 (clone D07, 1:500;

Dako), Bcl2 (clone 124, 1:200; Dako), Ki-67 (clone 30–9, 1:100; Ventana), p21^{Waf-1} (clone 57, 1:100; Oncogene Science), p27^{Kip-1} (clone SX53G8, 1:2,000; Dako), Mdm2 (clone IF2, 1:4,000; Calbiochem), CD1 (clone SP4, 1:25; Labvision), VEGF (clone sc-7269, 1:200; Santa Cruz), VEGFR2 (clone 55B11, 1:600; cell signaling), CD31 (clone JC70A, 1:700; Dako), CD34 (clone QBEnd 10, 1:1,000; Dako), PTEN (clone 6H2.1, 1:50; Dako), and polyclonal antibody phospho-S6 ribosomal protein (clone 2215L, 1:100; Cell Signaling) were used. After washing, a biotinylated antimouse secondary antibody (1:500; Vector) was added, followed by phosphate-buffered saline washing and a peroxidase-conjugated streptavidin (1:500; Dako). The secondary antibody and streptavidin were placed for 1 h at ambient temperature. Diaminobenzidine served as the chromogen, and hematoxylin was the nuclear counterstain. Positive controls were p53 (bladder transitional-cell carcinoma), Mdm2 (colon adenocarcinoma), Ki-67, Bcl2, and p21^{Waf-1} (lymphoma), p27^{Kip-1} (prostate adenocarcinoma), CD1 (breast adenocarcinoma), VEGF (colon carcinoma), VEGFR2 (clear cell carcinoma), CD34 (colon), CD31 and Phospho-S6 ribosomal protein (tonsil), and PTEN (ovarian carcinoma).

Immunostaining Analysis

Stained sections from tissue microarrays were evaluated and graded by three pathologists (JT, EV, and DK) using the same criteria. All sections were graded for intensity on a scale of 0–4 (0 = absence of staining at all magnifications; 1 = staining not observed at 10× magnification but positive on 40×; 2 = mild staining observed at 10× magnification; 3 = staining easily observed at 10×; 4 = intense staining observed at 4×) and percentage of tumor cell staining (p53, p21, p27, Bcl2, Mdm2, CD1, VEGF, VEGFR2, and phospho-S6 ribosomal protein); for these antibodies, criteria for positive staining included intensity grade ≥ 2 or ≥ 20 % positive tumor cells. Ki-67 was graded only by the percentage of stained cells. Microvessel density was assessed by counting microvessels stained with CD31 and CD34. Nonneoplastic tissue was the internal control for each antibody. Only staining of GBCA cells was scored; nonspecific staining or staining of other cell types was discounted. Immunoreactivity of PTEN was evaluated semiquantitatively. The surrounding microenvironment was used as a positive internal control and internal reference for scoring. The cytoplasmic and/or nuclear immunoreaction score of tumor cells was based on intensity: a score of 2 was positive, a score of 1 was weak, and a score of 0 was negative. Loss of PTEN was considered only in cases defined as negative, whereas cases with no staining of surrounding tissue were discarded as not evaluable.²⁶

The results were dichotomized into positive or negative for each antibody, except Ki-67, CD31, CD34, and PTEN. Values for Ki-67 represented the average percentage of positively stained cells; CD31 and CD34 were expressed as average number of positive microvessels identified; PTEN was considered either present or lost. For p53, p21, Bcl2, Mdm2, CD1, VEGF, VEGFR2, and phospho-S6 ribosomal protein, criteria for positive staining were an intensity of 2 and ≥ 20 % of cells reactive; p27 was positive if intensity was ≥ 1 and ≥ 10 % cells reactive. These reference values are consistent with those used in prior studies.^{21,27–32}

Statistical Analysis

Results are presented as proportions for categorical variables or as mean $\pm$ SD and median (range) for continuous variables. Continuous and categorical variables were compared using the Student's *t* test and the χ^2 test, respectively. Surgical mortality was defined as death resulting from postoperative complications at any time after surgery. Survival analysis was performed in all patients and in patients with early GBCA (T1 + T2). Survival probabilities were calculated using the Kaplan–Meier method and compared with log-rank test. Disease-specific survival (DSS) was defined as the interval between the date of operation and the date of last follow-up or death. Curative intent (R0) resection was defined as free of microscopic disease at all margins, whereas R1- or R2-resection had microscopic or macroscopic disease after definitive resection, respectively, at one or more margin. Univariate proportional hazards regression identified clinicopathologic factors predictive of DSS after an R0-resection; a multivariate model was performed using proportional hazards regression and including variables significant at the 20 % level on univariate analysis. Only patients with tumor differentiation typically seen in GBCA (adenocarcinoma, squamous/adenosquamous carcinoma) were included in the survival, uni- and multivariate analyses. χ^2 test was used to compare the prevalence of positive staining across centers (Student's *t* test for Ki-67, CD31, and CD34, which were dichotomized according to median value). All tests were two-sided, $p < 0.05$. Hierarchical clustering with complete linkage and binary distance was performed to partition the data based on protein expression and then compared with center of origin, excluding patients with incomplete immunostaining data. The same analysis was repeated for T1/T2 versus T3/T4. The rationale for this is that differences in pathogenesis are likely to be more apparent in early stage disease. In each subset, partitions were redefined based on the subset by hierarchical clustering only on that subset. R functions hclust and dist (version 2.12) were used for analysis (www.r-project.org). Statistical analysis was performed with SPSS 19.0, SAS 9.2, and R 2.9.

RESULTS

Demographics and Treatment

The analysis included 117 patients treated for GBCA between 1994 and 2009 at MSKCC ($N = 76$), FALP ($N = 22$), or YCU ($N = 19$). Demographic, operative, and perioperative data are summarized in Table 1. Ethnic distribution differed among centers, with greater heterogeneity at MSKCC, although Caucasians still represented the large majority ($N = 58$, 76.3 %). Cholelithiasis was more common at FALP and MSKCC compared with YCU ($p < 0.0001$), and IGBCA ($N = 46$, 39.3 %) was far less common at YCU ($p < 0.0001$), consistent with prior observations.¹²

R0-resections ($N = 71$, 60.7 %) were more common at MSKCC and YCU compared with FALP ($p = 0.027$). R0-resection procedures included segmentectomy 4B/5 in 41 patients, extended hepatectomy in 22, pancreaticoduodenectomy combined with 4B/5 liver segmentectomy in 3, and cholecystectomy alone in 5. These procedures were combined with porta hepatis lymphadenectomy (except in patients treated with cholecystectomy alone), and in selected patients were combined with bile duct resection, adjacent organ resection, or vascular reconstruction. Fifty-three (45.3 %) patients received perioperative adjuvant therapy, which was not different among the centers (Table 1).

Pathologic Characteristics

A total of 109 patients had invasive disease; depth of invasion was unknown in three patients (Table 1). There were more T2 tumors in patients from FALP compared with the other centers ($p = 0.001$). One hundred three (88 %) patients had at least one lymph node resected, and this was more common at MSKCC (90.8 %) and YCU (94.8 %) compared with FALP (72.7 %; $p = 0.03$). The median number of lymph nodes resected was 3.5 (range, 0–55). Sixty-four (54.7 %) patients had positive nodes; 39 (33.3 %) had negative nodes; and 14 (12 %) had none evaluated histologically. The median number of positive nodes for all patients was 1 (range, 1–43) and was higher at YCU ($p = 0.019$). Note that two patients with neuroendocrine tumor were excluded from survival, uni-, and multivariate analyses.

Immunostaining Analysis

Immunostaining data are shown in Table 2. p21, Mdm2, p27, and VEGFR2 expression were significantly less common in tumors from YCU. In fact, neither p21, Mdm2, nor VEGFR2 was detectable in tumors from Japanese

TABLE 1 Patient demographics, treatment, and pathology results

	Total <i>N</i> = 117 (%)	FALP <i>N</i> = 22 (%)	YCU <i>N</i> = 19 (%)	MSKCC <i>N</i> = 76 (%)	<i>p</i>
Gender					0.9
Female	75 (64.1)	15 (68.2)	12 (63.2)	48 (63.2)	
Male	42 (35.9)	7 (31.8)	7 (36.8)	28 (36.8)	
Age (years)					0.056
Median (range)	65 (28–91)	60 (41–91)	63 (48–85)	69 (28–90)	
Mean $\pm$ SD	65.4 $\pm$ 11.4	60.3 $\pm$ 12.3	65.8 $\pm$ 10.2	66.8 $\pm$ 11.1	
Race					<0.0001
Caucasian	58 (49.6)	0	0	58 (76.3)	
Hispanic	26 (22.2)	21 (95.5)	0	5 (6.6)	
Asian-Japanese	19 (16.2)	0	19 (100)	0	
African-American	3 (2.6)	0	0	3 (3.9)	
Pacific Islander	2 (1.7)	0	0	2 (2.6)	
Asian-American	3 (2.6)	0	0	3 (3.9)	
Indian	1 (0.9)	0	0	1 (1.3)	
Native American	1 (0.9)	1 (4.5)	0	0	
Unknown	4 (3.4)	0	0	4 (5.3)	
Gallstones					<0.0001
Yes	94 (80.3)	22 (100)	9 (47.4)	63 (82.9)	
No	23 (19.7)	0	10 (52.6)	13 (17.1)	
Incidental					<0.0001
Yes	46 (39.3)	12 (54.5)	0	34 (44.7)	
No	71 (60.7)	10 (45.5)	19 (100)	42 (55.3)	
Curative resection					0.027
Yes	71 (60.7)	8 (36.4)	14 (73.7)	49 (64.5)	
No	46 (39.3)	14 (63.6)	5 (26.3)	27 (35.5)	
Main procedure					0.002
Segmentectomy 4B/5	49 (41.9)	7 (31.8)	9 (47.4)	33 (43.4)	
Segmentectomy 4B/5 + PDD	4 (3.4)	1 (4.5)	2 (10.5)	1 (1.3)	
Extended hepatectomy	28 (23.9)	0	6 (31.6)	22 (28.9)	
Cholecystectomy (noncurative)	27 (23.1)	12 (54.5)	0	15 (19.7)	
Cholecystectomy (curative)	5 (4.3)	1 (4.5)	2 (10.5)	2 (2.6)	
Nonresection	4 (3.4)	1 (4.5)	0	3 (3.9)	
Hepatic resection					<0.0001
Yes	81 (69.2)	8 (36.4)	17 (89.5)	56 (73.7)	
No	36 (30.8)	14 (63.6)	2 (10.5)	20 (26.3)	
Bile duct resection					<0.0001
Yes	56 (47.9)	3 (13.6)	14 (73.7)	39 (51.3)	
No	61 (52.1)	19 (86.4)	5 (26.3)	37 (48.7)	
Adjacent organ resection					0.1
Yes	15 (12.8)	0	3 (15.8)	12 (15.8)	
No	102 (87.2)	22 (100)	16 (84.2)	64 (84.2)	
Adjuvant therapy					0.1
Yes	53 (45.3)	6 (27.3)	11 (57.9)	36 (47.4)	
No	64 (54.7)	16 (72.7)	8 (42.1)	40 (52.6)	
T stage					0.001
1	5 (4.3)	1 (4.5)	1 (5.3)	3 (3.9)	
2	41 (35)	14 (63.6)	5 (26.3)	22 (28.9)	

TABLE 1 continued

	Total N = 117 (%)	FALP N = 22 (%)	YCU N = 19 (%)	MSKCC N = 76 (%)	p
3	63 (53.8)	6 (27.3)	9 (47.4)	48 (63.2)	
4	5 (4.3)	0	4 (21.1)	1 (1.3)	
Unknown	3 (2.6)	1 (4.5)	0	2 (2.6)	
Histology					0.3
Adenocarcinoma	106 (90.6)	21 (95.5)	17 (89.5)	68 (89.5)	
Adenosquamous	6 (5.1)	1 (4.5)	0	5 (6.6)	
Squamous	2 (1.7)	0	0	2 (2.6)	
Neuroendocrine	2 (1.7)	0	1 (5.3)	1 (1.3)	
Undifferentiated	1 (0.9)	0	1 (5.3)	0	
Differentiation					<0.0001
Well	16 (13.7)	1 (4.5)	10 (52.6)	5 (6.6)	
Moderate	61 (52.1)	15 (68.2)	5 (26.3)	41 (53.9)	
Poor	40 (34.2)	6 (27.3)	4 (21.1)	30 (39.5)	
Lymphovascular invasion (n = 88)					0.2
No	42 (47.7)	5 (45.5)	6 (31.6)	31 (53.4)	
Yes	46 (52.3)	6 (54.5)	13 (68.4)	27 (46.6)	
Perineural invasion (n = 81)					0.8
No	30 (37)	4 (40)	8 (42.1)	18 (34.6)	
Yes	51 (63)	6 (60.0)	11 (57.9)	34 (65.4)	
Lymph node stage					0.03
Negative	39 (33.3)	4 (18.2)	4 (21.1)	31 (40.8)	
Positive	64 (54.7)	12 (54.5)	14 (73.7)	38 (50)	
Unknown	14 (12)	6 (27.3)	1 (5.3)	7 (9.2)	
Lymph nodes resected					<0.0001
Median (range)	3.5 (0–55)	4 (0–13)	13 (0–55)	3 (0–17)	
Mean ± SD	6.6 ± 9.5	4.6 ± 4	19 ± 17	3.9 ± 3.7	
Positive lymph nodes					0.019
Median (range)	1 (1–43)	2 (1–7)	2 (1–43)	1 (1–6)	
Mean ± SD	1.6 ± 4.2	1.3 ± 1.8	4 ± 9.6	1 ± 1.5	
TNM stage					0.02
1	3 (2.6)	0	1 (5.3)	2 (2.6)	
2	10 (8.5)	4 (18.2)	2 (10.5)	4 (5.3)	
3a	21 (17.9)	0	1 (5.3)	20 (26.3)	
3b	37 (31.6)	7 (31.8)	7 (36.8)	23 (30.3)	
4a	3 (2.6)	0	2 (10.5)	1 (1.3)	
4b	43 (36.8)	11 (50)	6 (31.6)	26 (34.2)	

PDD pancreaticoduodenectomy

patients, whereas Mdm2 was detected only in tumors from patients treated at MSKCC ($N = 50$, 70.4 %; $p < 0.0001$). Additionally, the Ki67 index was strikingly lower in cases from YCU (median, 10; range, 0–65) compared with FALP (median, 30; range, 1–70) and MSKCC (median, 31; range, 1–94), although this difference did not reach significance ($p = 0.07$). There were no location-related differences in the staining patterns of the other proteins analyzed (Table 2).

Protein Expression and Clinicopathologic Variables

PTEN was more commonly expressed in patients with IGBCA ($p = 0.03$) and in the absence of metastases ($p = 0.04$). Similarly, phospho-S6 ribosomal protein was more often expressed in patients with IGBCA ($p = 0.04$), T2 tumors ($p = 0.009$), and absence of perineural invasion ($p = 0.02$). p21 expression correlated with CD1 ($p < 0.001$) and Mdm2 ($p = 0.01$) expression. Ki-67 index correlated

TABLE 2 Immunohistochemistry results

	Total <i>N</i> = 117 (%)	FALP <i>N</i> = 22 (%)	YCU <i>N</i> = 19 (%)	MSKCC <i>N</i> = 76 (%)	<i>p</i>
BCL2 (<i>n</i> = 103)					0.3
Negative	95 (92.2)	16 (100)	16 (94.1)	63 (90)	
Positive	8 (7.8)	0	1 (5.9)	7 (10)	
p53 (<i>n</i> = 104)					0.5
Negative	75 (72.1)	12 (75)	14 (82.4)	49 (69)	
Positive	29 (27.9)	4 (25)	3 (17.6)	22 (31)	
p21 (<i>n</i> = 104)					0.03
Negative	80 (76.9)	12 (80)	17 (100)	51 (70.8)	
Positive	24 (23.1)	3 (20)	0	21 (29.2)	
p27 (<i>n</i> = 101)					0.047
Negative	70 (69.3)	6 (42.9)	14 (82.4)	50 (71.4)	
Positive	31 (30.7)	8 (57.1)	3 (17.6)	20 (28.6)	
CD1 (<i>n</i> = 102)					0.1
Negative	64 (62.7)	7 (46.7)	14 (82.4)	43 (61.4)	
Positive	38 (37.3)	8 (53.3)	3 (17.6)	27 (38.6)	
Mdm2 (<i>n</i> = 104)					<0.0001
Negative	54 (51.9)	16 (100)	17 (100)	21 (29.6)	
Positive	50 (48.1)	0	0	50 (70.4)	
VEGF (<i>n</i> = 102)					0.5
Negative	36 (35.3)	6 (37.5)	4 (23.5)	26 (37.7)	
Positive	66 (64.7)	10 (62.5)	13 (76.5)	43 (62.3)	
VEGFR2 (<i>n</i> = 102)					0.018
Negative	97 (95.1)	13 (81.3)	17 (100)	67 (97.1)	
Positive	5 (4.9)	3 (18.8)	0	2 (2.9)	
PTEN (<i>n</i> = 88)					0.7
Lost	20 (19.4)	3 (18.8)	2 (11.8)	15 (21.4)	
Present	68 (66)	12 (75)	12 (70.6)	44 (62.9)	
Noninterpretable	15 (14.6)	1 (6.2)	3 (17.6)	11 (15.7)	
P-S6 (<i>n</i> = 98)					0.1
Negative	66 (67.3)	7 (46.7)	12 (70.6)	47 (71.2)	
Positive	32 (32.7)	8 (53.3)	5 (29.4)	19 (28.8)	
Ki 67 (<i>n</i> = 100)					0.07
Median (range)	23.5 (0–94)	30 (1–70)	10 (0–65)	31 (1–94)	
Mean ± SD	31.3 ± 25.9	28 ± 27	20 ± 22	35 ± 26	
CD31 (<i>n</i> = 99)					0.4
Median (range)	17 (0–48)	19 (5–46)	19 (0–33)	16 (0–48)	
Mean ± SD	17.7 ± 10.3	20.7 ± 9.8	18 ± 9.5	17 ± 10.5	
CD34 (<i>n</i> = 100)					0.1
Median (range)	17 (0–54)	22 (10–36)	21 (0–47)	15 (0–57)	
Mean ± SD	19 ± 11.5	22.3 ± 7.8	22 ± 13	17.5 ± 11.5	

with expression of Mdm2 ($p = 0.04$), PTEN ($p = 0.01$), and phospho-S6 ribosomal protein ($p = 0.04$); CD34-positive microvessel count was associated with expression of phospho-S6 ribosomal protein ($p = 0.002$).

Hierarchical Clustering

Notable differences in protein expression were observed among the groups when all patients (excluding 2 with

TABLE 3 Hierarchical clustering partitions

	Total <i>N</i> (%)	FALP <i>N</i> (%)	YCU <i>N</i> (%)	MSKCC <i>N</i> (%)	<i>p</i> *
A) Center					
All patients					0.017
Partition 1	36	3 (8.3)	3 (8.3)	30 (83.3)	
Partition 2	40	9 (22.5)	10 (25)	21 (52.5)	
R0-resection					0.546
Partition 1	29	3 (10.3)	10 (34.5)	16 (55.2)	
Partition 2	18	2 (11.1)	1 (5.6)	15 (83.3)	
B) T stage					
T 1 + 2					0.017
Partition 1	20	8 (40)	5 (25)	7 (35)	
Partition 2	10	1 (10)	0	9 (90)	
T 3 + 4					0.859
Partition 1	29	2 (6.9)	6 (20.7)	21 (72.4)	
Partition 2	15	1 (6.7)	2 (13.3)	12 (80)	

* Fisher’s exact test

FIG. 1 Expression profile of all patients (a) and patients with T1/T2 GBCA (b). Each row represents 1 patient; columns indicate the immunostaining results (positive = yellow; negative = red). Center of

origin is indicated in the shaded column (red FALP, blue YCU, green MSKCC). The column dendrogram indicates patterns of protein clustering, and the row dendrogram indicates clustering by center

neuroendocrine tumors) were analyzed; partition 1 included a significantly higher number of MSKCC patients ($N = 30$, 83.3 %) compared with partition 2 ($N = 21$, 52.5 %), and the majority of YCU patients ($N = 10$, 77 %) were located in partition 2 ($p = 0.017$). Most FALP patients ($N = 9$, 75 %) also were mainly located in partition 2 (Table 3; Fig. 1a).

To better assess for possible site-specific differences in pathogenesis, the staining patterns in patients with early

lesions were compared with those with more advanced tumors (T1/T2 vs. T3/T4). Early T-stage tumors showed significant differences, with partition 1 including an equal distribution of patients treated at FALP ($N = 8$, 40 %), MSKCC ($N = 7$, 35 %), and YCU ($N = 5$, 25 %) compared with partition 2 where almost all patients ($N = 9$, 90 %) were treated at MSKCC and did not include patients treated at YCU ($p = 0.017$; Table 3; Fig. 1b). By contrast, the distribution of patients with advanced disease (T3/T4)

FIG. 2 **a** Disease-specific survival (DSS) among all patients with T1/T2 GBCA. **b** DSS among patients with T1/T2 GBCA treated with R0-resection. Survival analysis did not include two patients with neuroendocrine tumor

did not differ between partitions when center of origin was analyzed (Table 3).

Survival

Median follow-up for all patients (excluding 2 with neuroendocrine tumors) was 14 months (range, 0.4–109) and was similar among centers ($p = 0.2$). Median DSS was 15.6 months (range, 1–109), with an actuarial DSS of 17.6 % at 5 years, and this was similar among sites ($p = 0.6$). Median DSS after complete resection ($N = 69$) was 27 months (range, 2–109; 30 % at 5 years) and was similar among centers: MSKCC (median, 28.1 months; range, 1–108), FALP (median, 21.1 months; range, 5.5–94.2), and YCU (median, 27 months; range, 3–60; $p = 0.9$).

Median DSS of all T1/T2 patients ($N = 44$) was 28.4 months (95 % confidence interval (CI), 16.9–39.9) with an actuarial DSS of 71.6 % at 1 year and 28 % at 5 years. There was a trend toward significant difference in DSS in YCU patients (not reached) compared with MSKCC (median, 21.6 months) and FALP (median, 19.6 months; $p = 0.06$). Median DSS of T1/T2 patients treated with R0-resection ($N = 30$) was 52 months (95 % CI, 38.2–65.9) with an actuarial DSS of 79.4 % at 1 year and 43.1 % at 5 years: MSKCC (median, 37.8 months), FALP (median, 19.6 months), and YCU (not reached; $p = 0.1$; Fig. 2a, b).

Univariate and Multivariate Analysis of DSS

Outcome analysis was limited to R0-resections. Univariate analysis of clinicopathologic variables is shown in Table 4. On multivariate analysis, higher T stage,

lymphovascular invasion, and CD1 expression predicted worse survival. When the analysis was limited to T1/T2 tumors, the presence of gallstones was the only predictor of poor outcome (20.1 months with versus not reached without gallstones, $p = 0.012$). When treatment center was included as a variable, DSS was strikingly better in YCU patients, although this difference did not achieve significance ($p = 0.1$; Fig. 2b).

DISCUSSION

Gallbladder cancer is a rare and enigmatic disease with the highest incidence in India and some countries of South America and Asia, such as Chile and Japan, respectively.^{1,2,33} By contrast, the United States is a low-incidence area, although Native Americans of the Southwest have a high risk.³⁴ The dysplasia-carcinoma sequence, related to chronic inflammation, and adenoma-carcinoma progression, after development of a polyp, are two distinct pathways associated with GBCA.^{11,19} It has been suggested that local factors may be linked with this typical demographic distribution. Specifically, a strong association between gallstones and GBCA has been found in Chile and in high-risk areas of the United States, whereas other risk factors appear uncommon.⁴ Also, while pancreaticobiliary maljunction is rarely reported in Chile or the United States, it is an important GBCA risk factor in Japan.^{6,13,35}

We recently reported several differences in clinical and pathological variables in patients treated at three centers in the United States, Japan, and Chile.¹² However, these data may not necessarily represent definitive evidence of differences between centers or countries of origin, and molecular studies are necessary to validate these disparities. The molecular markers used in this analysis were

TABLE 4 Uni- and multivariate analyses for clinicopathologic variables associated with DSS

Variable	Total	Number failed	1-year survival	5-year survival	Median survival	<i>p</i> log-rank	<i>p</i> multivariate	Hazard ratio	95 % CI
Center						0.9			
FALP	8	4	0.62	0.46	21.1				
YCU	13	7	0.69	0.35	25				
MSKCC	48	32	0.77	0.29	28.1				
Gender						0.19			
Female	46	28	0.77	0.37	28.1				
Male	23	15	0.66	0.09	25				
Age (*, years)						0.1			
≤68	34	19	0.78	0.36	43.8				
>68	35	24	0.69	0.24	21.1				
Race						0.9			
Caucasian	37	25	0.76	0.29	28.2				
Hispanic	11	7	1	0	13.2				
Asian-Japanese	13	7	0.69	0.35	19				
African-American	1	1	1	0.50	–				
Pacific Islander	1	1	0.63	0.34	16.7				
Asian-American	2	1	–	–	–				
Indian	1	0	1	0	31.8				
Unknown	3	1	0.74	0.28	28.4				
Gallstones						0.09			
Yes	53	36	0.69	0.25	24.1				
No	16	7	0.87	0.52	–				
Incidental diagnosis						0.24			
Yes	27	17	0.84	0.32	44.3				
No	42	26	0.67	0.33	21.1				
Hepatic resection						0.19			
Yes	64	41	0.7	0.27	24.6				
No	5	2	1	0.66	88.8				
Bile duct resection						0.06			
Yes	41	29	0.68	0.21	19.0				
No	28	14	0.81	0.42	44.4				
Extra organ resection						0.7			
Yes	10	5	0.76	0.38	31.8				
No	56	35	0.74	0.30	28.1				
Histology						<0.0001			
Adenocarcinoma	62	37	0.79	0.32	28.1				
Adenosquamous	5	4	0.40	0.20	11.8				
Squamous	1	1	0	0	4.7				
Grade						0.25			
Well	13	6	0.76	0.44	43.8				
Moderate	29	17	0.81	0.34	25				
Poor	27	20	0.64	0.19	17.7				
T stage						0.009	0.02	0.37	0.16–0.88
T1 + T2	30	13	0.79	0.43	52				
T3 + T4	39	30	0.69	0.20	16.7				
N stage						0.48			
Positive	38	24	0.72	0.32	24.1				
Negative	30	18	0.75	0.30	31.8				

TABLE 4 continued

Variable	Total	Number failed	1-year survival	5-year survival	Median survival	<i>p</i> log-rank	<i>p</i> multivariate	Hazard ratio	95 % CI
M stage						0.23			
Yes	12	10	0.58	0.25	17.2				
No	57	33	0.77	0.31	28.4				
TNM stage						0.17			
1 + 2	56	32	0.77	0.32	28.4				
3 + 4	13	11	0.61	0.23	19				
Lymphovascular invasion						0.04	0.01	0.38	0.17–0.82
Yes	30	24	0.58	0.09	24.1				
No	26	14	0.91	0.42	31.8				
Perineural invasion						0.05			
Yes	31	23	0.65	0.15	19.0				
No	21	11	0.85	0.36	47.9				
Adjuvant treatment						0.9			
Yes	34	23	0.79	0.29	27.0				
No	35	20	0.68	0.31	24.6				
BCL2						0.37			
Positive	5	3	0.8	0.6	67.9				
Negative	57	37	0.71	0.26	24.1				
p53						0.92			
Positive	16	12	0.73	0.2	28.4				
Negative	47	29	0.73	0.33	19.1				
p21						0.47			
Positive	11	8	0.63	0.27	13.3				
Negative	51	33	0.75	0.28	27				
p27						0.13			
Positive	21	15	0.61	0.13	21.1				
Negative	40	25	0.78	0.34	28.1				
Cyclin D1						0.01	0.03	2.57	1.07–6.1
Positive	19	8	0.89	0.41	52				
Negative	43	32	0.65	0.22	19				
Mdm2						0.2			
Positive	29	17	0.84	0.3	28.4				
Negative	34	24	0.63	0.27	21.1				
VEGF						0.5			
Positive	40	26	0.76	0.33	27				
Negative	20	13	0.61	0.22	13.3				
PTEN						0.16			
Lost	13	10	0.69	0.18	17.7				
Present	40	22	0.75	0.35	42.1				
PS6						0.004			
Positive	16	4	0.79	0.68	–				
Negative	45	36	0.69	0.17	19.1				

* Dichotomized around the median (68; range, 28–86)

chosen to evaluate pathways possibly related with the pathogenesis of GBCA and to facilitate comparison among centers of origin.

The present study found several differences among the centers when expression of specific molecular markers and their pathways were compared. In particular, cell

cycle-regulatory proteins, specifically expression of p21, p27, and Mdm2, were significantly different in Japanese patients; indeed, neither p21, nor Mdm2 were expressed in any patient in this subgroup, and immunohistochemistry detection of p53 also was lower. Mutations of TP53 gene and accumulation of its protein product lead to increased expression of p21 and CD1.^{36,37} Alternatively, wild-type p53 can be inhibited through direct interaction with the Mdm2 oncoprotein. Thus, for the Japanese patients in the present study, inactivation of p53—either directly or indirectly—was an uncommon pathogenetic event compared with patients from Chile and the United States. These disparities also may explain the clinical differences among the groups and the final survival rates. Patients treated at YCU had a significantly lower incidence of tumors associated with cholelithiasis but had more tumors arise from polyps. Indeed, Japanese patients had a higher incidence of well-differentiated tumors. Site-specific differences in angiogenesis-related and PI3K pathway markers were less striking.^{38,39} Activation of the PI3K pathway is associated with aggressive tumor biology. Loss of the tumor suppressor PTEN, which is associated with PI3K activation, increased cell proliferation, and reduced apoptosis, was lowest in YCU patients and nearly 50 % lower compared with MSKCC.⁴⁰ Overexpression of p-S6, a marker of AKT activation, was seen in one-third of patients, more than 50 % of FALP patients, compared with less than 30 % of patients from YCU and MSKCC.⁴¹ Although neither marker was significantly different with respect to treatment center, p-S6 was significantly associated with earlier T-stage tumors, suggesting that PI3K pathway activation is a proximal pathogenetic change.

The clustering analysis accounts for variations in protein expression across treatment centers, and the results suggest the possibility of different disease biology among Japanese patients in this study. When all patients were included, two groups emerged: the first partition included more than 80 % of patients treated at MSKCC, whereas the second included a variable proportion from three centers, but YCU patients were almost exclusively located in this second group. These differences persisted when the analyses were repeated in patients with early lesions, where one would expect any such differences would be more evident. The survival analysis, suggesting better DSS in patients with early GBCA (T1/T2) treated at YCU, and the markedly lower Ki67 labeling index in the entire Japanese cohort compared with the other two sites, both support this argument.

This analysis has several limitations, not the least of which is the small sample size, even though large in comparison to other published studies of this type. Furthermore, although several differences were identified in YCU patients, suggesting differences in pathogenesis and

outcome compared with other sites, it cannot be assumed that they will extend to and reflect each center's respective national population. Whereas this may be true, the possibility that the differences are related to regional differences within each country must be considered. Definitive conclusions must await confirmation in larger series.

In summary, the findings of this study suggest regional differences in GBCA pathogenesis. Differences in tumor biology were suggested when patients with early tumors treated in Japan and the United States were compared. These disparities are supported by differential clustering based on molecular marker expression and the suggestion of a survival difference in patients with early tumors. Additionally, inactivation of p53 was uncommon in Japanese patients.

Disclosures None.

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