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From Cell Biology to Tissue Engineering

Immunohistochemical expression of mucin antigens in gallbladder adenocarcinoma: MUC1-positive and MUC2-negative expression is associated with vessel invasion and shortened survival

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Summary. Mucins play pivotal roles in influencing cancer biology, for example affecting carcinoma invasion, aggressiveness and/or metastatic potential. Our aim is to investigate the significance of expression profiles of two mucins in particular, MUC1 and MUC2, their correlations with various clinicopathological features, and prognosis in gallbladder adenocarcinoma (GBAC). We performed immunohistochemistry from patients with surgically resected GBAC, using antibodies against mucin core proteins MUC1/DF3 and MUC2/Ccp58 in 81 paraffin-embedded tumor samples. MUC1 or MUC2 expression was considered to be high when ≥20% or 10% of the GBAC cells showed positive staining, respectively. High MUC1 expression was revealed to have a significant relationship to the presence of pathologically lymphatic and vascular invasion, and regional lymph node metastasis. By contrast, high MUC2 expression showed a significant correlation with pathologically perineural invasion, T stage ≥3, and post-operative recurrence. Moreover, MUC1 showed significantly positive co-expression and potentially complementary correlations with MUC2. Multivariate analyses demonstrated that the high MUC1 expression group had significantly shorter disease-specific survival times. However, the combination of both high MUC1 and MUC2 expression did not predict worse outcome in GBACs. Therefore, although each mucin has a somewhat important role in the pathogenesis of GBAC progression, MUC1 can independently predict vessel invasion and poor prognosis in patients with GBAC. The detection of MUC1 might well offer a useful parameter for providing clinical management and treatment against postsurgical GBACs.

Key words: Gallbladder adenocarcinoma (GBAC), MUC1, MUC2, Vessel invasion, Disease-specific survival

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Introduction

Gallbladder cancer is one of the most lethal malignancies worldwide; and approximately 19,000 new cases are diagnosed and more than 15,000 patients die of this disease each year in Japan alone (http://ganjoho.jp/professional/index.html, 2013), with much less than 15% of a five-year overall survival rate (Donohue, 2001; Misra et al., 2003). It is not only the fifth most common cancer of the gastrointestinal tract, but the most frequent neoplasm found among all biliary tract cancers (Misra et al., 2003). Gallbladder adenocarcinoma (GBAC) is the most common of these, accounting for more than 90% of the histopathological type of gallbladder cancer (Manfredi et al., 2000; Bal et al., 2015). GBACs often pose a great diagnostic challenge to clinicians, due to their vague symptomatology, nonspecific imaging findings and/or grossly misleading appearances (Bal et al., 2015). Moreover, once GBAC develops in the organ (which, in particular, lacks the layer of lamina

muscularis mucosa), it most likely will tend to invade the adjacent organs, such as liver or stomach, ever when not only it was diagnosed accurately at early stage, but also it found incidentally (Misra et al., 2003; Albores-Saavedra et al., 2010). It is thus likely that more than 70% of GBAC cases could be inoperable or unresectable in an unexpectedly and previously undiscovered advanced stage upon diagnosis, resulting in much worse prognosis (Donohue, 2001; Misra et al., 2003). Furthermore, rates of long-term survival are rare even after radical cholecystectomy, considered to provide the only chance of a cure, because postoperative early relapses (local or distant) occur frequently. In fact, various clinicopathological characteristics, including the depth of tumor invasion (i.e., pathological tumor stage), presence of vessel invasion and lymph node metastases, have been reportedly proposed as prognostic indicators, despite inconsistent results and conclusions to date (Manfredi et al., 2000; Bal et al., 2015), whereas molecular and genetic factors still remain to be fully uncovered. On the basis of those backgrounds, it is critical to predict which GBAC patients are prone to develop recurrence/metastases and will have a high mortality rate before and after surgery, although practically accurate and significant biomarkers are under evaluation and are unknown (Bal et al., 2015).

It is well known that glycosylation is a major type of post-translational modification of most secretory and cellular proteins, resulting in alterations of the physicochemical properties and biological activities of these proteins (Brockhausen, 1999). The initiation and progression of malignant neoplasms are significantly associated with not only frequent aberrant glycosylation but subsequent alterations in cell-surface carbohydrate antigens (CAs), inducing changes in the functions of glycoproteins and transforming cellular phenotypes (Brockhausen, 1999). Mucin-type O-glycosylation in the Golgi apparatus encompasses diverse classes of glycoproteins and mucins, high molecular weight glycoproteins, which constitute up to 80% of the total amount of CAs in mammals (Brockhausen, 1999; Hollingsworth and Swanson, 2004). Indeed, mucins are aberrantly expressed in many carcinomas, closely related to the presentation of shortened irregular glycan structures, easily affecting cell differentiation, adhesion, invasion and/or metastasis and resulting in aggressive behaviours (Hollingsworth and Swanson, 2004; Yonezawa et al., 2008).

To date, more than 20 distinct members of the human mucins have been identified, and classified into two categories: membrane-bound mucins, including MUC1, MUC3 and MUC4; and gel forming secreted mucins, including MUC2, MUC5AC and MUC6 (Yonezawa et al., 2011). Interestingly, mucins show a relatively tissue-specific expression and display variably deregulated expression patterns of one or more types of them in various cancers (Ho et al., 1995; Yonezawa et al., 2008). Our collected data have indicated that, MUC1 and MUC2, in particular, would be significantly useful for evaluating a large number of carcinomas of the digestive system, including stomach (Utsunomiya et al., 1998), esophagus (Sagara et al., 1999), pancreas (Osako et al., 1993; Saitou et al., 2005), intrahepatic bile duct (Higashi et al., 1999, 2012), breast carcinoma (Matsukita et al., 2003) or bile duct tumor (Tamada et al., 2002, 2006). However, to the best of our knowledge, there are very few studies regarding possible detailed correlations between MUC1 and MUC2 expressions in gallbladder carcinoma, especially GBAC, and associated clinicopathological features, including vessel invasion or the patient's outcome. By contrast, Takagawa group has previously revealed that the group of immunohistochemically strong MUC1 and weak MUC2 expression had a significantly worse overall survival time, despite the limitations of assessing a smaller cohort (Takagawa et al., 2005). In addition, the expression of MUC1 or MUC4 might reportedly be an independent prognostic marker for patients with postoperatively pathological tumor stage 2 GBAC (Kawamoto et al., 2001) and/or all stage GBAC (Yamato et al., 1999; Kashiwagi et al., 2001; Lee et al., 2012), respectively.

In the present study, our aim is to clarify the relationship of the immunohistochemical expression profiles of mucins, especially focusing on MUC1 and MUC2, in larger samples of collected postsurgical GBACs with their clinicopathological factors, including the patient's prognosis. We show for the first time that, in patients with postoperative GBAC, a high MUC1 expression has a significantly close correlation with the presence of vessel invasion, lymph node metastasis and shortened disease-specific survival (DSS), and that MUC1 might be a promising biomarker for the clinical management and treatment of postoperative GBACs. While a high MUC2 expression could be a useful adjunctive aid for identifying worse clinicopathological features (such as perineural invasion involvement, depth of invasion, postsurgical recurrence and shorter DSS), evaluating the expression especially of MUC1 might be useful for guiding the clinical management of postsurgical GBAC patients.

Materials and methods

Patients

Surgically resected GBAC tissues were studied in the present study. Pathological reports were reviewed to identify patients who underwent simple cholecystectomy or radical cholecystectomy for GBAC between 1991 and 2003 at the files of the Department of Pathology, Faculty of Medicine and Kagoshima University. Three patients who suffered perioperative deaths, defined as death during the patient's initial hospitalization or within 30 days of surgery, were excluded from the study. A total of 81 patients with available follow-up data comprised the cohort of this retrospective study, after further excluding those with the following characteristics: (a) other prior or concomitant malignant tumors; (b) coexisting medical

problems of sufficient severity to shorten life expectancy and (c) treatment with adjuvant chemotherapies or radiotherapies prior to the surgery. All materials in this article were approved by the Ethical Committee of Kagoshima University Hospital (28-66). The duration of survival (DSS) was defined as the interval from the date of surgery to death or the most recent hospital visit, without the patients who died from causes other than GBAC.

Pathological examination

Three pathologists examined all resected specimens to confirm their histopathological features. The pathologic findings were described using the TNM Classification of Malignant Tumors, 7th edition, published by the International Union Against Cancer (Sobin et al., 2009). All GBACs were graded based on the three-tiered histological grading system from The World Health Organization (WHO) classification of tumours of the digestive system (Albores-Saavedra et al., 2010); and a grade of G2 or higher was considered to indicate a high-grade tumor. Clinical information was gathered from the patients' records, and no patients had a biopsy specimen obtained from the GBAC before surgery. The duration of survival was the time from the date of surgery until the patient's death or most recent clinic visit. Patients were followed-up and evaluated postoperatively at approximately three to six month intervals, using thoracic and abdominal CT scans and/or measurements of tumor marker levels. Additional examinations, including brain CT, MRI and bone scintigraphy, were performed if any symptoms or signs of recurrence were recognized. The formalin-fixed, paraffin-embedded tissue blocks came from our Department of Pathology, Field of Oncology. Normal human tissue was taken from non-tumor portions of the surgically-resected specimens, and then stained with haematoxylin and eosin (H&E), combined elastica Masson trichrome (E-M) or were subjected to immunohistochemical analyses of sequential sections. The E-M and immunohistochemical CD31 (DAKO, Glostrup, Denmark; diluted 1:1), Podoplanin (D2-40; DAKO; diluted 1:1) and S-100 protein (DAKO; diluted 1:20) staining clearly revealed whether there was vascular invasion (v), lymphatic invasion (ly) and perineural invasion (ne), respectively.

Preparation of antibodies against mucins and secondary antibody

Immunohistochemistry for various mucins was performed by using the following established antibodies. For the immunohistochemical staining of each mucin, we used human cancer cells of well to moderately differentiated adenocarcinoma of the pancreas, or human non-carcinomatous epithelium of the pancreas and stomach, appropriately, as positive controls (Yonezawa et al., 2008). The profile of all these antigens is summarized in Table 1. Next, biotinylated affinity-purified horse anti-mouse IgG and avidin-biotinylated horseradish peroxidase complex (ABC) were purchased from Vector Laboratories (Burlingame, CA) as the Vectastain Elite ABC kit.

Immunohistochemistry of tissue samples

Immunohistochemical staining was performed by an immunoperoxidase method using the ABC complex as described previously (Saitou et al., 2005; Tamada et al., 2006). Each section was deparaffinized with xylene. Endogenous peroxidase was blocked by incubating the sections in 0.3% hydrogen peroxidase in absolute methanol at room temperature for 30 min. After hydration in decreasing concentrations of ethanol in water, the sections were washed in 0.01 mol/L PBS (pH 7.4).

The sections were washed twice with PBS. Then, 2% horse or goat serum in PBS was applied for 30 min at room temperature to prevent nonspecific staining. In the staining using each antibody, the sections were incubated with dilutions of the primary antibodies of mucin in PBS with 1% bovine serum albumin for 16 hr at 4°C. The sections were washed twice with PBS, incubated with the biotinylated secondary antibody of horse anti-mouse IgG, and washed twice with PBS. All sections then received ABC for 30 min. After washing with PBS twice, the sections were finally reacted with diaminobenzidine substrate for 10 min for visualization, rinsed with tap water, counterstained with hematoxylin, and mounted. Reaction products were not present when non-immune serum or PBS was used instead of the primary antibodies.

Evaluation of the immunohistochemical results by scoring

The immunoreactivity for mucins in each case was assessed semi-quantitatively by evaluating the proportion of positive cells compared to the total GBAC cells. To assess the membranous, apical and intracytoplasmic MUC1 and MUC2 expressions, positive areas that were ≥20% and 10% of the total were

Table 1. List of antibodies including mucins used in the present study.

Antigens	Antibodies	Sources of antibodies	Dilution
MUC1 (core peptide)	DF3	Toray-Fuji Bionics	1:50
MUC2 (core peptide)	Ccp58	Novocastra	1:200
MUC4 (core peptide)	8G7	generated by one of authors (S. K. B)	1:3000
MUC5AC (core peptide)	CLH2	Novocastra	1:100
MUC6 (core peptide)	CLH5	Novocastra	1:100
HIK1083	M-GGMC-1	Kanto Cemical Co.	1:50
CD31	JC70A	DAKO	1:1
Podoplanin	D2-40	DAKO	1:1
S-100	S-100	DAKO	1:20

considered to be highly positively-stained, respectively. We selected and validated these immunohistochemical cut-off scores, based on the performance of a receiver operating characteristic (ROC) curve analysis (Hanley, 1989; Harada et al., 2016). Finally, all patients were divided into two groups based on each mucin expression as follows: high, when the MUC1, MUC2, MUC4, MUC5AC, MUC6 or HIK1083 staining was ≥20, 10, 5, 30, 10, 10%, respectively, and low, when the staining was less than that. The distribution of the staining for mucins in the GBAC and the adjacent non-neoplastic epithelium in each case was also assessed semi-quantitatively and compared.

All histological and immunohistochemical slides were evaluated by two independent observers (certified surgical pathologists in our department; T.H. and S.Y.) using a blind protocol design (the observers were blinded to the clinicopathological data). The agreement between the observers was excellent (more than 90% agreement rate) for all antibodies investigated as measured by the interclass correlation coefficient. For the few (less than 1%) instances of disagreements, a consensus score was determined by third board-certified pathologists (M.H.) in our department (Kawatsu et al., 2014; Harada et al., 2016).

Statistical analysis

The significance of correlations was determined by the Fisher's exact test or χ^2 test, where appropriate, in order to assess the relationships between the immunohistochemical expression levels and the clinicopathological features (Harada et al., 2016). Survival curves were plotted with the Kaplan-Meier method and compared with the log-rank test. Hazard ratios and 95% confidence intervals (95% CI) were estimated using univariate or multivariate Cox proportional hazard models. All statistical tests were two-tailed, with values of $P<0.05$ considered to be significant. All of the above statistical analyses were performed with the EZR (Saitama Medical Center, Jichi Medical University, Japan) graphical user interface for the R software program (The R Foundation for Statistical Computing, version 2.13.0) (Kanda, 2013; Harada et al., 2016). More precisely, it is a modified version of R commander (version 1.6-3) designed to add the statistical functions that are frequently used in biostatistics.

Results

Clinicicopathological features

Clinicopathological features of 81 patients with GBAC who were able to be evaluated are summarized in Table 2. The range of age at surgery was 44-91 years (average and median were 72 years and 73 years, respectively). The range of tumor size was 5-105 mm (median was 30 mm). At diagnosis, 18 patients (22.2%) had lymph node metastases and two (2.5%) had distant

Table 2. The GBAC patients' clinicopathological characteristics.

Characteristic	Patients (n=81)
Age (years)	
Average	72
Median	73
Range	44-91
<65 years	18
≥65 years	63
Sex	
Male	27
Female	54
Stone	
(-)	56
(+)	25
Size (mm)	
Average	36.5
Median	30
Range	5-105
<35 mm	48
≥35 mm	33
Months after surgery	
Average	44.7
Median	36.0
Range	1-149
Differentiation	
well	45
moderately	26
poorly	10
T stage	
T0	12
T1	6
T2	52
T3	9
T4	2
Lymphatic invasion	
(-)	45
(+)	36
Vascular invasion	
(-)	48
(+)	33
Perineural invasion	
(-)	58
(+)	23
Lymph node metastasis	
(-)	63
(+)	18
Distant metastasis	
(-)	79
(+)	2
TNM stage (UICC 7th)	
stage 0	12
stage I	6
stage II	41
stage IIIA	3
stage IIIB	17
stage IVA	0
stage IVB	2
Recurrence	
(-)	56
(+)	25

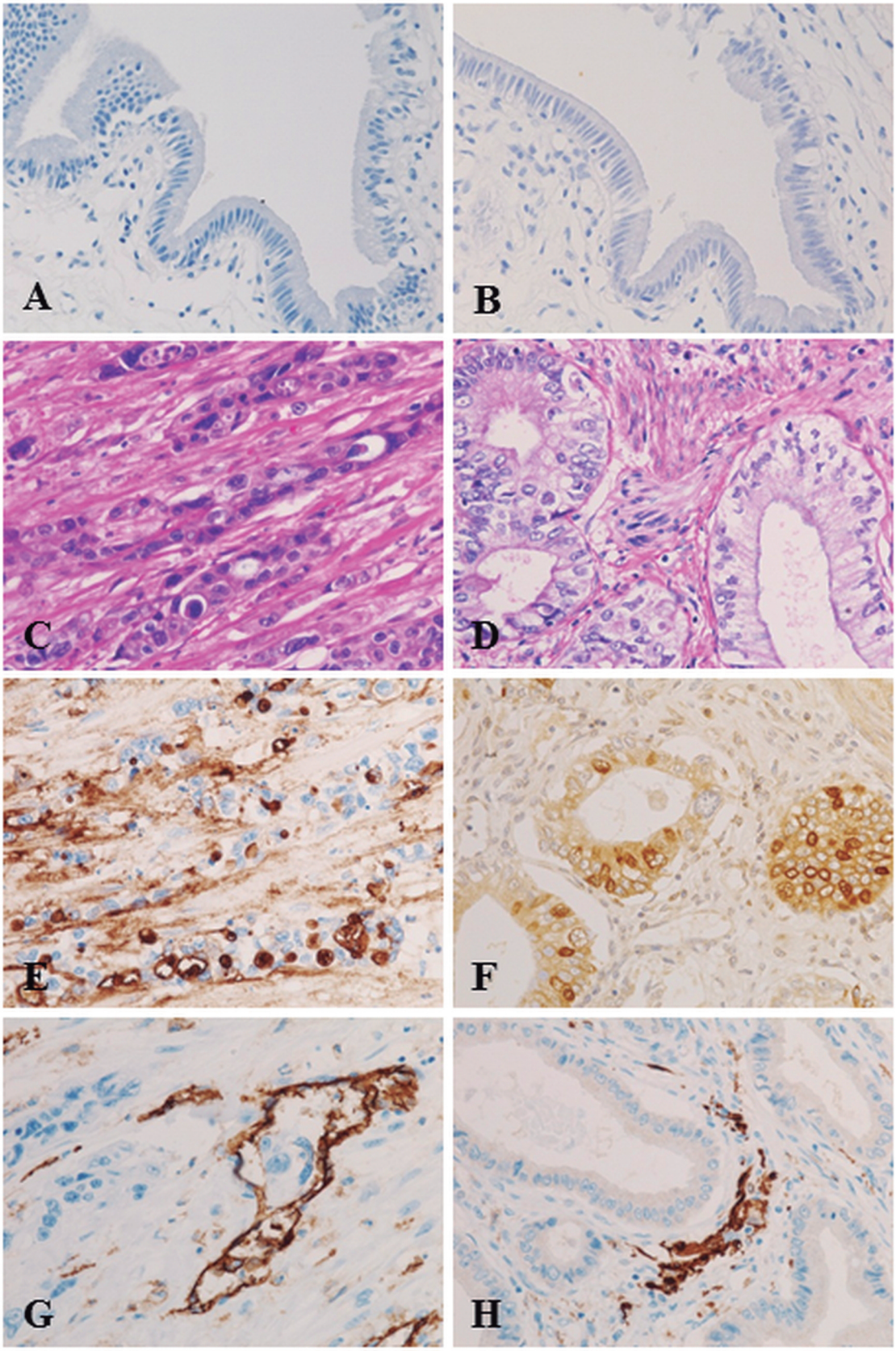

Fig. 1. Representative images of the immunohistochemical staining of both MUC1 and MUC2 in the non-neoplastic epithelium and GBAC. Both MUC1 **(A)** and MUC2 **(B)** expressions were not detectable in the adjacent non-neoplastic epithelium. High-grade adenocarcinoma (G2 to G3) showed membranous and apical MUC1 expression pattern (**C**, H&E; **E**, MUC1/DF3), with vessel invasion (**G**, CD31). Low-grade adenocarcinoma (G1) showed intracytoplastic MUC2 expression pattern (**D**, H&E; **F**, MUC2/Ccp58), with perineural invasion (**H**, S-100 protein). H&E: hematoxylin and eosin. x 40

metastases. The tumor grading included 45 well differentiated (G1; 55.6%), 26 moderately differentiated (G2; 32.1%) and 10 poorly differentiated adenocarcinomas (G3; 12.3%). The majority of the patients (n=41; 50.6%) had stage II disease according to the UICC criteria. Post-surgical follow-up data was available for all 81 patients (average: 44.7 months; range: 1-149 months). The median DSS was 36.0 months, and their DSS rate was 76.5% at 1 year and 37.0% at 5 years.

Mucins expressions in normal gallbladder tissues and GBAC specimens

MUC1 and MUC2 expressions were not detectable in the adjacent non-neoplastic epithelium (Fig. 1A,B). Representative GBACs show Fig. 1C,D. Fig. 1E shows representative expression pattern of MUC1, and Fig. 1F shows representative expression pattern of MUC2. MUC1-high expression was in 42 cases (51.9%). MUC2-high expression was in 13 cases (16.0%). The profiles of mucin expression patterns and clinicopathological features are shown in Table 3.

Association of mucins expression, especially of MUC1 and MUC2, with the clinicopathological features

The relationship between MUC1 or MUC2 expression and clinicopathological features is summarized in Table 4. MUC1-high expression was strongly correlated with lymphatic permeation, vessel invasion and regional lymph node metastasis (P<0.001, <0.001, and 0.03, respectively), but not with high tumor grade (G2 and G3), depth of invasion (i.e., T classification), or ne (P>0.05) in the overall cohort (Table 4). Besides, there were no significant differences between age, gender, presence of gallbladder stone, post-surgical recurrence and distant metastasis between MUC1 low and high groups. Moreover, MUC1-high expression in both membranous and apical patterns was immunohistochemically evident especially in the vessel invasion of GBAC components, as shown respectively by the D2-40, E-M and CD31 staining (Fig. 1G). Post-surgical DSS of GBAC patients with MUC1-high expression (25.0 months) was significantly shorter than those with MUC1-low expression (55.5 months) (P=0.02, Fig. 2A).

In contrast, MUC2 showed only intracytoplasmic immunohistochemical expression (Fig. 1F). MUC2-high expression was significantly correlated with ne, T classification 3 and 4, and post-surgical recurrence (P=0.04, 0.01, and 0.02 respectively) (Table 3 and Fig. 1). MUC2-high expression was not related to the age, gender of the patients, presence of stone, ly, v, high tumor grade, regional lymph node or distant metastases (P>0.05) (Table 4). In fact, MUC2-high expression was conspicuous in ne of invasive GBAC components, as clearly demonstrated by S-100 protein staining (Fig. 1H). Post-surgical median DSS of GBAC patients with MUC2-high expression was significantly shorter than those with MUC2-low expression (36.0 months) (P=0.04, Fig. 2B).

On the other hand, regarding the profiles of the other mucins (MUC4, MUC5AC, MUC6 and HIK1083)' associations with the clinicopathological characteristics including DSS, there was no significant difference between patients with low and high expressions (data not shown) (P>0.05).

Correlations between high MUC1 and MUC2 expression

There was a significant relationship between the immunohistochemical mucins expression patterns (P=0.03, r=0.27) (Fig. 1, Table 4), with high MUC1 expression showing a significantly positive rate of co-expression with high MUC2 expression (Table 4). When

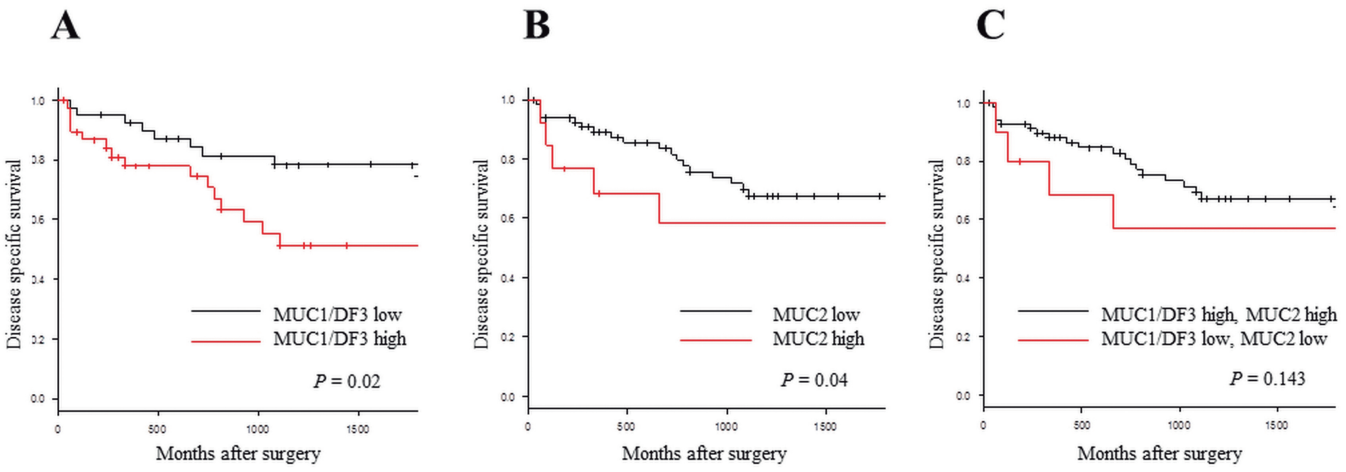

Fig. 2. A, B. Kaplan-Meier curves of the disease-specific survival (DSS) in patients with GBACs after surgery according to the MUC1 **(A)** or MUC2 **(B)** expression. **C.** Kaplan-Meier curves of the DSS in patients with GBACs after surgery according to the combination of both high MUC1 and MUC2 expressions.

Table 3. The detailed relationships among the mucins expression and each patient's variables.

No.	Sex	Age (years)	Stone	Size (cm)	Months after surgery	Grade	ly	v	ne	T stage	N stage	M stage	TNM stage	recurrence	DF3	MUC2	MUC4	MUC5AC	MUC6	HIK1083
1	F	63	−	3.0	25	G2	+	+	+	2	0	0	II	+	high	low	low	high	high	high
2	F	67	+	2.0	149	G1	−	−	−	2	0	0	II	−	low	low	low	low	high	high
3	F	60	−	2.0	31	G1	+	−	−	2	1	0	IIIB	+	high	low	low	high	low	low
4	F	78	+	0.5	96	G2	−	−	−	2	0	0	II	−	low	low	low	high	high	low
5	F	70	+	4.0	120	G1	+	+	−	2	1	0	IIIB	−	high	high	high	low	low	low
6	F	86	+	2.5	111	G3	+	+	+	4	0	1	IVB	−	high	high	high	high	low	low
7	F	63	−	5.0	74	G2	−	−	−	2	0	0	II	+	high	high	low	low	high	high
8	F	64	+	2.5	26	G1	+	+	−	2	0	0	II	+	high	low	low	high	high	low
9	M	47	−	1.0	128	G2	−	−	−	is	0	0	0	−	high	low	low	low	high	high
10	M	68	−	3.5	91	G1	+	+	+	3	1	0	IIIB	−	high	high	high	high	high	low
11	F	64	−	4.0	27	G1	+	+	−	2	1	0	IIIB	+	high	low	low	high	high	low
12	F	85	+	2.0	9	G1	+	+	−	2	1	0	IIIB	+	high	low	high	high	high	low
13	F	62	−	7.0	122	G2	+	−	+	2	0	0	II	−	low	low	high	low	high	low
14	F	56	−	3.5	119	G2	+	−	−	2	1	0	IIIB	−	high	low	high	low	low	low
15	F	75	−	2.0	65	G2	+	+	+	3	1	0	IIIB	+	low	low	high	high	high	low
16	M	69	−	7.5	4	G2	+	+	+	3	1	0	IIIB	+	high	high	high	high	high	low
17	F	64	−	7.0	24	G2	+	+	+	3	1	0	IIIB	−	low	low	low	low	low	high
18	F	70	−	3.0	60	G1	−	−	−	2	0	0	II	−	low	low	low	low	low	low
19	F	72	−	2.7	67	G1	−	−	−	2	0	0	II	−	low	low	high	low	low	low
20	F	63	+	9.0	100	G1	−	−	−	2	0	0	II	−	low	low	low	high	low	high
21	F	83	−	2.5	88	G3	+	+	+	2	0	0	II	−	low	low	high	low	low	high
22	M	68	−	1.8	101	G3	+	+	+	2	0	0	II	−	high	low	low	low	low	low
23	F	72	−	6.0	100	G1	−	−	−	2	0	0	II	−	low	low	high	low	high	high
24	F	66	−	5.0	98	G2	−	−	−	is	0	0	0	−	low	low	low	low	high	high
25	M	82	−	3.0	70	G2	+	−	+	2	0	0	II	+	high	high	high	low	low	high
26	M	70	+	1.5	97	G1	−	−	−	1b	0	0	I	−	low	low	high	high	high	high
27	F	76	+	2.0	60	G2	+	+	−	2	1	0	IIIB	+	low	high	low	high	low	low
28	F	75	−	4.0	8	G3	+	+	−	3	1	0	IIIB	+	high	low	high	high	high	low
29	F	64	−	5.0	2	G1	−	−	−	2	0	0	II	+	high	low	low	low	low	low
30	F	84	+	3.0	1	G1	−	−	−	is	0	0	0	−	low	low	low	low	high	high
31	F	60	−	8.0	36	G2	+	+	+	3	1	0	IIIB	+	low	low	low	low	low	low
32	F	68	+	1.5	88	G1	−	−	−	2	0	0	II	−	low	low	low	low	high	high
33	F	65	−	1.3	88	G1	−	−	−	is	0	0	0	−	low	low	low	low	high	high
34	F	76	+	1.8	80	G1	−	−	−	2	0	0	II	−	low	low	low	low	high	low
35	M	83	−	5.0	1.5	G3	+	+	+	2	1	0	IIIB	+	high	low	low	low	low	low
36	M	82	−	3.0	45	G1	−	−	−	2	0	0	II	−	low	low	low	low	high	low
37	F	91	+	7.0	9	G1	−	−	−	2	0	0	II	−	high	low	high	low	high	high
38	F	55	+	10.5	16	G2	+	+	−	2	0	0	II	+	low	low	low	low	high	high
39	F	84	+	2.5	27	G1	−	−	−	2	0	0	II	−	high	low	high	low	high	low
40	M	70	+	5.0	73	G2	+	+	−	2	0	0	II	−	low	low	low	high	high	low
41	M	67	−	3.5	75	G1	−	−	−	1b	0	0	I	−	low	low	low	high	high	low
42	M	44	−	2.0	71	G1	+	−	−	2	0	0	II	−	low	low	high	high	low	low
43	F	63	+	3.5	68	G1	+	+	−	2	0	0	II	−	high	low	low	high	high	high
44	F	58	−	1.0	60	G2	−	−	−	is	0	0	0	−	low	low	low	low	high	high
45	F	77	−	7.0	34	G1	+	+	+	2	1	0	IIIB	+	high	low	low	low	low	low
46	F	65	−	2.0	62	G1	−	−	−	is	0	0	0	−	low	low	low	high	low	low
47	F	78	+	7.0	59	G1	−	−	−	2	0	0	II	−	low	low	low	low	high	high
48	F	80	+	5.0	2	G2	+	+	−	3	0	0	IIIA	+	high	high	high	high	high	high
49	M	77	−	1.5	22	G1	−	−	−	1b	0	0	I	+	low	low	low	low	high	low
50	F	80	+	5.0	36	G1	−	−	−	is	0	0	0	−	low	low	high	low	high	high
51	F	81	+	4.0	37	G1	+	+	+	2	1	0	IIIB	+	high	low	high	low	low	low
52	F	70	+	3.0	41	G1	+	−	−	2	0	0	II	−	high	low	high	high	high	low
53	M	70	+	0.5	20	G2	−	−	−	is	0	0	0	−	low	low	low	low	high	high
54	M	77	+	1.5	52	G1	−	−	−	is	0	0	0	−	low	low	low	high	high	low
55	F	84	−	6.0	11	G2	−	−	−	2	0	0	II	+	high	high	low	high	high	high
56	F	74	−	8.0	48	G1	−	−	−	2	0	0	II	−	high	low	low	low	low	low
57	M	77	−	1.0	14	G3	−	+	+	2	0	0	II	+	low	low	low	low	low	low
58	F	73	+	7.0	2	G2	−	+	+	2	0	0	II	+	high	low	low	high	high	low
59	M	75	+	3.0	42	G1	−	−	−	2	0	0	II	−	high	low	high	low	low	low
60	M	73	−	8.0	27	G2	−	−	−	2	0	0	II	−	low	low	low	low	low	low
61	M	78	+	2.6	37	G2	+	+	−	2	1	0	IIIB	−	high	low	low	high	low	low
62	M	72	+	2.0	12	G2	−	−	−	1b	0	0	I	−	low	low	low	high	high	high
63	F	67	−	1.5	38	G1	−	−	−	1b	0	0	I	−	low	low	low	high	high	low
64	F	84	+	2.0	40	G1	−	−	−	is	0	0	0	−	low	low	low	low	high	high
65	F	58	−	7.0	1	G3	+	+	+	4	1	1	IVB	−	high	low	high	high	low	low
66	M	75	−	2.5	3	G1	−	−	+	3	0	0	IIIA	+	low	high	high	low	low	low
67	M	83	−	7.0	2	G3	−	−	−	3	0	0	IIIA	+	low	low	low	low	low	low
68	M	71	+	2.0	22	G2	−	−	+	2	0	0	II	+	high	high	low	low	high	low
69	M	78	−	2.5	23	G1	+	+	−	2	0	0	II	−	high	low	low	low	high	low
70	F	74	−	3.0	11	G3	−	−	−	2	0	0	II	+	low	low	low	low	high	high
71	M	74	+	1.8	18	G1	−	−	−	is	0	0	0	−	low	low	low	low	low	low
72	M	74	+	9.0	15	G1	−	+	−	2	0	0	II	−	high	low	low	low	high	low
73	F	78	−	2.0	12	G1	+	+	−	2	0	0	II	−	low	low	high	high	high	high
74	M	73	−	4.5	11	G3	+	+	−	2	0	0	II	−	high	low	low	high	high	high
75	M	70	−	2.0	13	G2	+	+	+	2	0	0	II	−	high	low	low	low	low	high
76	F	69	−	0.9	12	G1	−	−	−	1b	0	0	I	−	low	high	low	high	high	low
77	F	50	−	3.0	10	G1	−	−	−	is	0	0	0	−	high	low	low	high	high	low
78	F	88	−	3.0	8	G1	−	−	+	2	0	0	II	−	high	low	high	high	high	high
79	F	79	−	2.5	3	G1	+	+	−	2	0	0	II	−	high	low	low	low	high	low
80	F	85	+	4.0	7	G1	−	−	+	2	1	0	IIIB	−	low	low	high	high	high	high
81	F	84	−	2.7	6	G2	+	+	+	2	0	0	II	−	high	high	high	high	high	high

Table 4. Detailed correlations between each high MUC1 and MUC2 expression and the clinicopathological variables

Variables	Total	(%)	MUC1 expression Low (n=42)	MUC1 expression High (n=39)	P value
Age					
<65 years	18	22.2	7	11	0.29
≥65 years	63	77.8	35	28	
Sex					
Male	27	33.3	14	13	1.0
Female	54	66.7	28	26	
Stone					
(-)	48	59.3	24	24	0.82
(+)	33	40.7	18	15	
Size					
<35 mm	48	59.3	28	20	0.18
≥35 mm	33	40.7	14	19	
Differentiation					
well	45	55.5	25	20	0.51
moderately, poorly	36	44.5	17	19	
T stage					
T0, T1, T2	70	86.4	37	33	0.75
T3, T4	11	13.6	5	6	
Lymphatic invasion					
(-)	45	55.5	32	13	<0.001
(+)	36	44.5	10	26	
Vascular invasion					
(-)	48	59.3	33	15	<0.001
(+)	33	40.7	9	24	
Perineural invasion					
(-)	58	71.6	34	24	0.08
(+)	23	28.4	8	15	
Lymph node metastasis					
(-)	63	77.8	37	26	0.03
(+)	18	22.2	5	13	
Distant metastasis					
(-)	79	97.5	42	37	0.23
(+)	2	2.5	0	2	
Recurrence					
(-)	56	69.1	33	23	0.09
(+)	25	30.9	9	16	
MUC2 expression					
low	68	83.4	39	29	0.03
high	13	16.6	3	10	

Variables	Total	(%)	MUC2 expression Low (n=68)	MUC2 expression High (n=13)	P value
Age					
<65 years	18	22.2	17	1	0.28
≥65 years	63	77.8	51	12	
Sex					
Male	27	33.3	22	5	0.75
Female	54	66.7	46	8	
Stone					
(-)	48	59.3	40	8	1.0
(+)	33	40.7	28	5	
Size					
<35 mm	48	59.3	40	8	1.0
≥35 mm	33	40.7	28	5	
Differentiation					
well	45	55.5	41	4	0.07
moderately, poorly	36	44.5	27	9	
T stage					
T0, T1, T2	70	86.4	62	8	0.01
T3, T4	11	13.6	6	5	
Lymphatic invasion					
(-)	45	55.5	40	5	0.23
(+)	36	44.5	28	8	
Vascular invasion					
(-)	48	59.3	42	6	0.36
(+)	33	40.7	26	7	
Perineural invasion					
(-)	58	71.6	52	6	0.04
(+)	23	28.4	16	7	
Lymph node metastasis					
(-)	63	77.8	54	9	0.47
(+)	18	22.2	14	4	
Distant metastasis					
(-)	79	97.5	67	12	0.3
(+)	2	2.5	1	1	
Recurrence					
(-)	56	69.1	51	5	0.02
(+)	25	30.9	17	8	
MUC1 expression					
low	42	51.9	39	3	0.03
high	39	48.1	29	10	

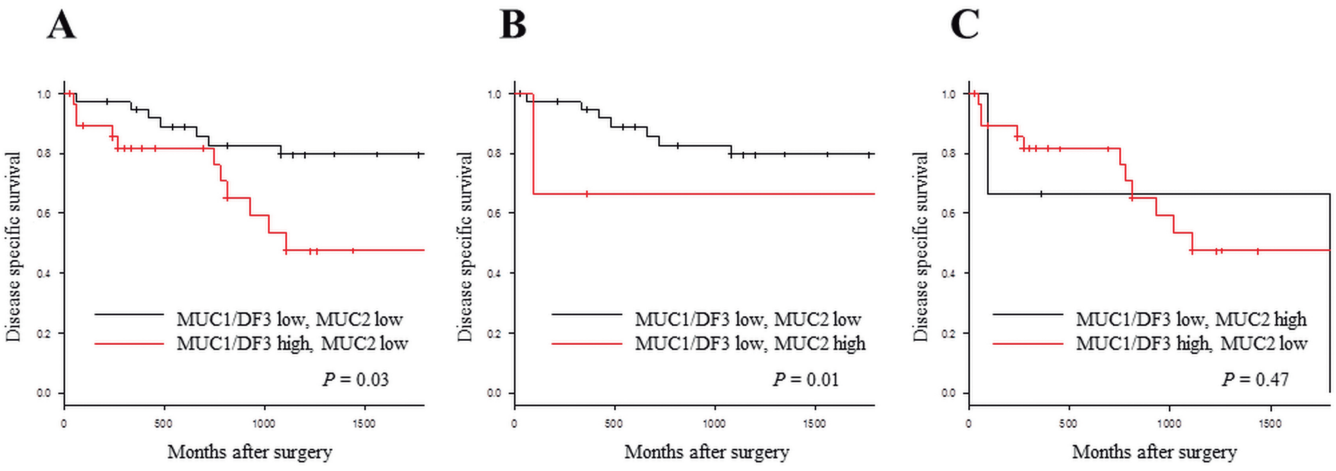

Fig. 3. Kaplan-Meier curves of the DSS in patients with GBACs after surgery, when the patients were split into groups based on the MUC1 and MUC2 expressions (high or low): (i) low and low vs. high and low **(A)** or low and high **(B)**; and (ii) high and low vs. low and high **(C)**.

the patients were divided into groups based on their MUC1 and MUC2 expression patterns, defined as high or low positivity, respectively, their immunoprofiles were 48.2% low and low (39 cases); 35.8% high and low (29 cases); 3.7% low and high (3 cases) and 12.3% high and high (10 cases). High MUC1 and high MUC2 expression patterns were closely associated with ne, high ≥G2 tumor grade, and pathological T stage ≥3 (data not shown) (P=0.03, 0.02 and 0.03, respectively), However, the GBAC patients with both the high MUC1 and MUC2 profile did not show a significantly shorter postoperative median DSS (36.0 months) than that of the other groups (46.0 months) (P=0.15, Fig. 2C). Next, the DSS of the GBAC patients, divided into two groups: (i) low and low vs. high and low (Fig. 3A) or low and high (Fig. 3B); and (ii) high and low vs. low and high (Fig. 3C); then the Kaplan-Meier method was used to further examine the associations of these groupings with the survival. The DSS of the GBAC patients showed statistically significant differences (Fig. 3A: P=0.03; Fig. 3B: P=0.01, respectively) in the former (i) classification approach, but not in the latter approach (ii) (P=0.47; Fig. 3C), thus indicating that there were potentially complementary, but not competitive, correlations between MUC1 and MUC2.

MUC1 expression represents a significant independent prognostic indicator for GBAC

To assess whether mucins expression was an independent predictor of the postoperative DSS, a Cox proportional hazards model was created in a forward fashion including only covariates that had statistically significant correlations with the DSS, using an inclusion threshold of P<0.05 (Table 5). A univariate analysis showed that the presence of v, ne, depth of invasion

Table 5. The results of the univariate and multivariate analyses of survival in 81 patients with GBAC, according to the clinicopathological variables and each high MUC1 and high MUC2 expression.

Variables	No. of patients (%)	Univariate			Mutivariate		
		HR	95 %CI	P value	HR	95 %CI	P value
Age							
<65	18 (22.2)	1					
≥65	63 (77.8)	0.64	0.29-1.44	0.28			
Sex							
Male	27 (33.3)	1					
Female	54 (66.7)	1.09	0.47-2.48	0.85			
Stone							
(-)	56 (69.1)	1					
(+)	25 (30.9)	0.56	0.25-1.29	0.17	0.57	0.22-1.43	0.23
Size							
<35	48 (59.3)	1					
≥35	33 (40.7)	2.03	0.95-4.33	0.07			
Differentiation							
well	45 (55.5)	1					
moderately, poorly	36 (44.5)	2.37	1.09-5.19	0.03	2.46	1.06-5.70	0.04
T stage							
Tis, T1, T2	70 (86.4)	1					
T3, T4	11 (13.6)	4.49	2.00-10.02	<0.001			
Lymphatic invasion							
ly (-)	63 (77.8)	1					
ly (+)	18 (22.2)	2.16	0.99-4.72		0.1	0.02-0.51	0.006
Vascular invasion							
v (-)	48 (59.3)	1					
v (+)	33 (40.7)	3.8	1.70-8.49	0.001	6.77	1.49-30.75	0.01
Perineural invasion							
ne (-)	58 (71.6)	1					
ne (+)	23 (28.4)	2.83	1.33-6.03	0.007	1.25	0.45-3.50	0.67
lymph node involvement							
N (-)	63 (77.8)	1					
N (+)	18 (22.2)	3.55	1.66-7.56	0.001	4.34	1.36-13.89	0.01
MUC1 expression							
low	42 (51.9)	1					
high	39 (48.1)	2.42	1.11-5.30	0.03	2.46	1.05-5.76	0.04
MUC2 expression							
low	68 (83.4)	1					
high	13 (16.6)	2.335	1.02-5.36	0.04	1.78	0.73-4.33	0.21

(equal to or more than T3), lymph node metastasis, high MUC1 and high MUC2 expressions, were significant predictors of a poorer survival (P=0.001, 0.007, <0.001, 0.001, 0.03 and 0.04, respectively). Furthermore, a multivariate analysis demonstrated that, after correction for confounding variables, immunohistochemically high MUC1 expression remained an independent prognostic indicator for the DSS (P=0.04), in addition to ly, v, lymph node involvement and high tumor grade (P=0.006, 0.01, 0.01 and 0.04, respectively) (Table 5).

Discussion

The present findings in a relatively large cohort of GBAC indicate for the first time that (1) MUC1-high expression is significantly correlated with invasive/aggressive behaviours, manifesting as pathological vessel invasion, and lymph node metastasis; (2) MUC1-high expression has significantly positive co-expression with MUC2; and (3) MUC1-high expression is a powerful and independent negative indicator of DSS in patients with postoperative GBAC, and by extension, as a novel prognostic marker for the disease, whereas MUC2-high expression is not. By contrast, MUC2-high expression can demonstrate a significant association with perineural invasion, depth of invasion corresponding to ≥ pathological tumor stage 3, and postoperative recurrence, but MUC1 is not. Taken together, each mucin plays an important role in the pathogenesis of GBAC progression, and MUC1 and MUC2 might be expressed simultaneously, but function separately in a potentially complementary or reciprocal manner, reminiscent of crosstalk. Here, we can actually show that the combination of MUC1 and MUC2 expression predicts insignificantly worse outcome in GBACs, whereas reveals significant difference of the further DSS time analyses in a double-weak (MUC1-low and MUC2-low) vs. either-high expression group (MUC1-high or MUC2-high) classification approach, as previously shown (Kawatsu et al., 2014; Takeda et al., 2014; Kimura et al., 2015). Despite that, the present study would contain some limitations in its interpretation: (a) merely a cohort study at a single institution; and (b) no detailed molecular analyses.

Similar to the previously collected data, our cohort study shows that more than 30% of the patients experience postoperative recurrence (local or distant) and approximately 25% and 65% of them die within the first one and five years after surgery, respectively (Manfredi et al., 2000; Bal et al., 2015). In fact, there have been no reliable predictors of the progressive potential of GBAC to date. In that sense, MUC1 expression patterns in surgical specimens of primary GBAC might allow clinicians to select patients with regard to postoperative adjuvant therapies, possibly including specific anti-MUC1 antibodies, and the prediction of appropriate clinical postoperative courses, especially in the early phase. Furthermore, the adjacent non-neoplastic epithelial cells display absence of MUC1 expression, suggesting that MUC1 has a potentially crucial role in acquired gallbladder carcinogenesis through aberrant secretion of shortened and/or irregular O-linked glycans, mucins. We can thus propose that MUC1 could also be a specific diagnostic tumor marker for GBAC and MUC1 could be shed into body fluids; therefore, it can be a quantitative soluble marker. Besides, the MUC1 core peptide might be an ideal therapeutic target with minimal risk of side effects, even though our laboratory has reported that positive expression of MUC1 is found in many organs (Yonezawa et al., 2011). Nevertheless, the utility of anti-MUC1 antibodies as therapeutic modalities requires much further study in the future.

Further supporting our results, other groups have reported that the positive expression of MUC1 plays a pivotal role in worsening the clinical course of postoperative GBAC patients through the induction of poor differentiation, tumor growth, invasiveness and metastatic potential in GBAC (Yamato et al., 1999; Kim et al., 2012; Xiong et al., 2012). Indeed, these obtained results with regard to MUC1 accord with our serial studies of several other carcinomas in stomach (Utsunomiya et al., 1998), esophagus (Sagara et al., 1999), pancreas (Osako et al., 1993; Saitou et al., 2005), intrahepatic bile duct (Higashi et al., 1999, 2012), breast (Matsukita et al., 2003) or bile duct tumor (Tamada et al., 2002, 2006). Also, our data are in agreement with in vitro studies of cultured cells with overexpression of MUC1, resulting in anti-adhesive effects and leading to vessel permeation (Ligtenberg et al., 1992; Yonezawa et al., 2008). Furthermore, the immunohistochemically intra-cytoplasmic, depolarized or deregulated, expression pattern of MUC1 has reportedly been dominant in the GBAC cells of pathologically advanced tumor stage (Ghosh et al., 2005). In fact, we found high MUC1 expression in both membranous and apical, intracytoplasmic, patterns, especially at the invasive fronts including vessel invasion, most likely being related to tumor aggressiveness of GBAC. The present findings are considered to be in line with our recent studies in terms of the expression of polypeptide N-acetylgalactosaminyltransferases (GalNAc-Ts) in several types of carcinoma (Li et al., 2011; Kitada et al., 2013; Harada et al., 2016), since the various GalNAc-Ts are well known to be reliable markers for aberrant O-linked glycans, mucins, in not only carcinogenesis but invasiveness and aggressiveness of carcinomas. However, further in-depth experiments and analyses are needed to clarify these results.

Furthermore, we herein demonstrated, for the first time, that high MUC2 expression could be a useful adjunctive aid for identifying worse clinicopathological features, such as perineural invasion, depth of invasion, postsurgical recurrence and shorter DSS, in patients with GBACs, in addition to the high MUC1 expression. However, these observations are in marked disagreement with other groups' studies of immunohistochemistry. They report that the patient group with strong MUC1

and weak MUC2 expression had a significantly shorter postoperative survival period, and this outcome is partly associated with lower proliferative activities (Yamato et al., 1999; Takagawa et al., 2005). They also report and that although MUC2 expression is likely linked to better survival without any statistical significance, MUC4-positive and MUC2-negative groups showed a significantly worse outcome (Lee et al., 2012). These discrepancies could be due to (i) the size of cohort, (ii) the heterogeneity of GBAC, (iii) arbitrary methods to select and validate the immunohistochemical cut-off scores for MUC2, and (iv) various glycoforms for the MUC2 core protein antigen, such as underglycosylated, sialylated, and fully glycosylated forms (Yonezawa et al., 2008, 2011), at least in part. Despite these considerations, in this context, it should be noted that the combination of these two mucins should effectively predict higher clinicopathological aggressiveness of the tumor or a poor outcome in patients with postoperative GBAC, even though the combination of both high MUC1 and MUC2 expressions cannot significantly show worse DSS in GBACs in the present study. Further follow-up in much larger cohorts of GBAC patients, together with detailed molecular investigation, will be necessary to confirm the comprehensive relationships between the MUC1 and MUC2 core peptides.

In conclusion, the present cohort study demonstrates, for the first time, that high expression of MUC1 but not MUC2 is an independent, novel and reliable marker for a poor prognosis in GBAC patients with surgical treatment. Our data collectively indicate that immunohistochemically high expression of MUC1 and/or MUC2 in GBAC (1) has a significantly close relationship with more invasive/aggressive behaviours, manifesting as vessel invasion, together with advanced tumor stage and post-operative recurrence; (2) shows significantly positive co-expression; and (3) potentially regulates the progression of GBAC in a complementary or reciprocal manner. Finally, evaluating the expression especially of MUC1 might well be useful for guiding the clinical management of postsurgical GBAC patients.

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