Histological phenotype is correlated with the wall-invasion pattern of gallbladder adenocarcinoma

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ABSTRACT

Gallbladder carcinoma (GBC) is one of the most aggressive malignancies, and frequently shows vascular invasion and lymph node metastasis. Our previous study has classified the wall-invasion pattern of GBC into two groups, *i.e.*, infiltrative growth type (IG type) and destructive growth type (DG type). The DG type was significantly associated with poor clinical outcome. In this study, we analyzed the relationship between the wall-invasion pattern and the histological phenotype of GBC, using 61 surgically-resected primary gallbladder adenocarcinomas. Histologically, the 61 cases were classified into the biliary (44 cases, 72.1%), gastric foveolar (13 cases, 21.3%), and intestinal (4 cases, 6.6%) types. Biliary type frequently exhibited MUC1, but less frequently showed MUC2, MUC5AC, and MUC6. The biliary type and MUC1 expression were significantly correlated with DG type wall-invasion pattern ($P = 0.020$ and $P < 0.001$, respectively). In conclusion, histological phenotype and mucin expression were thought to be indicators of aggressiveness of GBC.

Gallbladder carcinoma (GBC) is the most common malignancy of the biliary tract and the fifth most common malignancy of the digestive system. GBC is a relatively uncommon neoplasm in the majority of countries, and its incidence rate shows marked geographic and ethnic variation. Japan and several Latin American countries, such as Chile, Mexico and Bolivia, have the highest incidences of GBC in the world (11, 27). In Japan, GBC is responsible for 1.25% and 3.49% of cancer death in male and female, respectively. The number of surgically resected cases of GBC has recently increased because of advances in imaging diagnosis and operative procedures. However, GBC is a highly lethal disease since it is usually diagnosed at advance stage (5).

The 5-year survival rate of patients with GBC is 10–30% despite of surgical resection. Moreover, the majority of patients have frequent recurrences after the surgery, and pursue the chemotherapy or radiotherapy treatment when recurrences happened after the surgery (2, 15).

Several previous studies have demonstrated high risks of disease progression, using surgically-resected GBC cases, and histological grade, depth of wall infiltration, and lymph node status have been determined to be clinicopathological prognostic factors (6, 22). In addition, we have classified the wall-invasion pattern of GBC into two groups, *i.e.*, infiltrative growth type (IG type) and destructive growth type (DG type) (18–20). The DG type was significantly associated with lymph node metastasis and poor prognosis. In this study, we analyzed the relationship between the wall-invasion pattern and the histological phenotype of GBC, using 61 surgically-resected primary gallbladder adenocarcinomas.

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MATERIALS AND METHODS

Patient and tissue samples. A total of 61 cases of GBC were retrieved from archival files of Hirosaki University Hospital (Aomori, Japan), in the period between 2000 from 2012. We examined 61 patients (18 males and 43 females) with a mean age of 68.9 years (range, 44–92 years) who received curative resection and lymph node resection. GBC were tumors invading beyond the gallbladder wall, *i.e.* tumors greater than or equal to pT2, according to the TNM classification (24). The present study followed the principles of the World Medical Association Declaration of Helsinki 1964.

Histopathological and immunohistochemical examinations. The gallbladder tissue specimens for histological analysis were routinely fixed with formalin, embedded in paraffin, thin-sectioned, and stained with hematoxylin and eosin (H&E). We divided GBC into two wall invasion pattern groups, IG type and DG type. IG type, cancer cells show infiltrative growth in the muscle layer (through the intermuscular space) without muscle layer destruction. DG type, cancer cells invade the subserosal layer with destruction of the muscle layer. The DG type was accompanied by a stromal desmoplastic reaction characterized by active irregular fibrosis due to tumor invasion. Furthermore, according to the WHO classification (1), histological phenotype of the GBC was divided into three groups as follows: (a) Biliary type: the carcinoma was composed of short or long tubular glands lined by cells that vary in height from cuboidal to tall columnar, superficially resembling biliary epithelium; (b) Gastric foveolar type: the carcinoma was composed of tall columnar cells with basally oriented nuclei and abundant mucin-containing cytoplasm; and (c) Intestinal type: the carcinoma was composed of tubular glands closely resembling those of colonic adenocarcinomas, or consisted of glands lined predominantly with goblet cells, and usually with a variable number of neuroendocrine and Paneth cells. We used a term “metaplastic type” as the “gastric foveolar type plus intestinal type.” The degree of venous invasion was

classified as: v0, no venous invasion; v1, minimal venous invasion, *i.e.* 1 or 2 foci of venous invasion in one histological section; v2, moderate venous invasion, *i.e.* 3 or 4 foci; and v3, severe venous invasion with 5 or more foci. The degree of lymphatic invasion was classified as: ly0, no lymphatic invasion; ly1, mild lymphatic invasion; ly2, moderate lymphatic invasion; and ly3, severe lymphatic invasion. The degree of perineural invasion was classified as: ne0, no perineural invasion; ne1, mild perineural invasion; ne2, moderate perineural invasion; and ne3, severe perineural invasion. The subserosal invasion patterns were classified into three groups, according to Classification of Biliary Tract Carcinoma (8, 20), *i.e.* INF α (Infiltration alpha): the tumor shows expanding growth and a distinct border from the surrounding tissue; INF β (Infiltration beta): the growth fashion and invasive pattern are between those of INF α and INF γ (Infiltration gamma); and INF γ : the tumor shows infiltrating growth and a distinct border from the surrounding tissue.

Immunohistochemistry. Representative histological specimens at the deepest invaded area of the gallbladder carcinoma lesions were selected for immunohistochemistry. Four μ m thick paraffin sections were mounted on silane-coated glass slides. Immunohistochemical examination was performed on deparaffinized sections using the standard avidin-biotin-peroxidase complex (ABC) method with an automated immunostainer (BenchMark XT; Ventana Medical System, Tucson, AZ, USA). We used MUC1, MUC2, MUC5AC and MUC6 to clarify mucin expression of GBC. The antibodies used are listed in Table 1.

Histological and immunohistochemical evaluation. Two investigators (TT and KH) simultaneously assessed the immunohistochemical results without any patient's clinicopathological data. Luminal membranous immunoreactivities of the tumor were judged as positive for MUC1, and cytoplasmic immunoreactivities as positive for MUC2, MUC5AC and MUC6. According to the above immunohistochemical expression of each mucin, the cases were divid-

Table 1 Antibodies for immunohistochemistry

Antigen	Monoclonal/polyclonal	Clone	Dilution	Source
MUC1	Monoclonal, mouse	Ma696	1 : 50	Novocastra
MUC2	Monoclonal, mouse	Ccp59	1 : 50	Novocastra
MUC5AC	Monoclonal, mouse	CLH2	1 : 100	Novocastra
MUC6	Monoclonal, mouse	CHL5	1 : 100	Novocastra

ed into two groups; a negative group in which < 10% of tumor cells were stained, and a positive group in which $\geq 10\%$ were stained.

Statistics. Univariate statistical analysis was performed using the Pearson’s chi-square test or Fisher’s exact test. Differences were considered to be significant if the *P*-value was less than 0.05. All statistical analyses were performed using the R (<http://www.r-project.org>).

RESULTS

Wall-invasion pattern and clinicopathological factors of GBC

Scheme/microscopic findings of wall-invasion pattern and mucin expression are shown in Fig. 1 and Table 2, respectively. Of the 61 cases of GBC, 32 cases (52.5%) were IG type, and 29 cases (47.5%) were DG type. All of the DG type GBC showed strong MUC1 expression, while MUC2, MUC5AC, and MUC6 were less frequently detected in the DG

Fig. 1 Scheme and histological findings of advanced GBC invasion pattern. In IG type (A and C), cancer cells show infiltrative growth in the muscle layer (through the intermuscular space) without muscle layer destruction. In DG type (B and D), cancer cells invade the subserosal layer with destruction of the muscle layer. The DG type was accompanied by a stromal desmoplastic reaction which is active and irregular fibrosis due to the tumor invasion.

Table 2 Mucin expression of gallbladder adenocarcinoma

	MUC1	MUC2	MUC5AC	MUC6
GBCs (total n = 61)	70.5%	14.8%	41.0%	37.7%
Wall-invasion pattern				
IG type (n = 32)	43.8%	28.1%	62.5%	59.4%
DG type (n = 29)	100%	0%	17.2%	13.8%
Histological phenotype				
Biliary type (n = 44)	77.3%	11.4%	27.3%	22.7%
Gastric foveolar type (n = 13)	61.5%	15.4%	84.6%	84.6%
Intestinal type (n = 4)	25.0%	50.0%	50.0%	50.0%

GBCs: gallbladder carcinomas, IG type: infiltrative growth type, DG type: destructive growth type.

type. The IG type GBC frequently expressed MUC1 (43.8%), MUC2 (28.1%), MUC5AC (62.5%), and MUC6 (59.4%), but not in all cases. The results of univariate analysis between wall-invasion pattern and clinicopathological factors are summarized in Table 3. Age, gender and histological differentiation exhibited no significant differences. The DG pattern significantly increased lymphatic invasion, venous invasion, lymph node metastasis and neural invasion. The wall-invasion pattern was also associated with spread pattern and histological phenotype.

Histological phenotype and clinicopathological factors of GBC

Representative findings of histological phenotype are demonstrated in Fig. 2. Of the 61 cases of GBC, 44 cases (72.1%) were biliary type, and 17 cases (27.9%) were metaplastic type; *i.e.* 13 (21.3%) were gastric foveolar type, and 4 (6.6%) were intestinal

type. Histological phenotype and mucin expression of GBC are summarized in Table 2 and in Fig. 3. The biliary type GBC frequently showed strong MUC1 expression (77.3%, 34/44), while MUC2, MUC5AC, and MUC6 were less frequently detected in the biliary type. The gastric foveolar type frequently expressed MUC1 (61.5%, 8/13) as well as MUC5AC and MUC6 (84.6%, 11/13; and 84.6%, 11/13, respectively). The results of univariate analysis between histological phenotype pattern and clinicopathological factors are summarized in Table 4. The biliary type was significantly correlated with the DG type wall-invasion, but was not statistically associated with the other clinicopathological factors.

DISCUSSION

In the present study, we investigated the wall-invasion pattern and histological phenotype of GBC,

Table 3 The wall-invasion pattern and clinicopathological features of gallbladder adenocarcinoma

Clinicopathological features	Number of patients	Wall-invasion pattern		Rate of DG pattern (%)	P-value
		IG	DG		
Age					0.053
< 65	18	6	12	66.7	
≥ 65	43	26	17	39.5	
Gender					0.417
Male	18	8	10	55.6	
Female	43	24	19	44.2	
Histological differentiation					0.095
well, mod	44	26	18	40.9	
por, others	17	6	11	64.7	
Lymphatic invasion					0.003*
ly0, 1	31	22	9	29.0	
ly2, 3	30	10	20	66.7	
Venous invasion					0.032*
v0, 1	36	23	13	36.1	
v2, 3	25	9	16	64.0	
Neural invasion					0.016*
ne0, 1	37	24	13	35.1	
ne2, 3	24	8	16	66.7	
LN metastasis					0.003*
+	30	10	20	66.7	
–	31	22	9	29.0	
Spread pattern					0.003*
INF α , β	35	24	11	31.4	
INF γ	26	8	18	69.2	
Phenotype					0.020*
Biliary type	44	19	25	56.8	
Metaplastic type	17	13	4	23.5	

IG: infiltrative growth type, DG: destructive growth type, well: well differentiated adenocarcinoma, mod: moderately differentiated adenocarcinoma, por: poorly differentiated adenocarcinoma, ly: lymphatic invasion, v: venous invasion, ne: neural invasion, INF α , β and γ : the mode of subserosal infiltration. *Statistically significant: $P < 0.05$.

Fig. 2 Three morphological subtypes of GBC (hematoxylin and eosin). (A) Biliary type: tumor was composed of short or long tubular glands lined by cells that vary in height from cuboidal to tall columnar, superficially resembling biliary epithelium. (B) Gastric foveolar type: tumor was composed of tall columnar cells with basally oriented nuclei and abundant mucin-containing cytoplasm, and (C) Intestinal type: tumor was composed of tubular glands closely resembling those of colonic adenocarcinomas, and consisted of glands lined predominantly with goblet cells.

Fig. 3 Representative mucin expression images of GBCs. A case of biliary type (A)–(D). A case of gastric foveolar type (E)–(H) and a case of intestinal type (I)–(L). Immunohistochemical staining of MUC1 (A, E, I), MUC2 (B, F, J), MUC5AC (C, G, K), and MUC6 (D, H, L). Biliary type tumor (A)–(D) shows MUC1 positive, MUC2, MUC5AC and MUC6 negative. Gastric foveolar type tumor (E)–(H) shows MUC5AC positive, MUC1, MUC2 and MUC6 negative. Intestinal type tumor (I)–(L) shows MUC2 and MUC6 positive, MUC1 and MUC5AC negative.

Table 4 *Histological phenotype and clinicopathological features of adenocarcinoma*

Clinicopathological features	Number of patients	Histological phenotype		P-value
		Biliary	Metaplastic	
Age				0.524
< 65	18	14	4	
≥ 65	43	30	13	
Gender				0.207
Male	18	15	3	
Female	43	29	14	
Histological differentiation				0.268
well, mod	44	30	14	
por, others	17	14	3	
Lymphatic invasion				0.715
ly0, 1	31	23	8	
ly2, 3	30	21	9	
Venous invasion				0.574
v0, 1	36	25	11	
v2, 3	25	19	6	
Neural invasion				0.177
ne0, 1	37	29	8	
ne2, 3	24	15	9	
Spread pattern				0.195
INF α , β	35	23	12	
INF γ	26	21	5	
LN metastasis				0.715
+	30	21	9	
–	31	23	8	
Wall-invasion pattern				0.020*
IG	32	19	13	
DG	29	25	4	

well: well differentiated adenocarcinoma, mod: moderately differentiated adenocarcinoma, por: poorly differentiated adenocarcinoma, ly: lymphatic invasion, v: venous invasion, ne: neural invasion, INF α , β and γ : the mode of subserosal infiltration, IG: infiltrative growth type, DG: destructive growth type.

*Statistically significant: $P < 0.05$.

using 61 surgically-resected primary gallbladder adenocarcinomas. GBC cases were classified into the biliary, gastric foveolar, and intestinal types. The biliary type and MUC1 expression were significantly correlated with the DG type wall-invasion pattern. This is the first report to demonstrate biliary type/MUC1 expression as indicators of aggressiveness of GBC.

Several studies have reported prognostic significance of histological phenotype and mucin expression in GBC (3, 9, 10, 21, 25). However, the correlation between the histological phenotype/mucin expression and wall-invasion pattern has not yet clarified. Mucin is a heterogeneous group of high molecular weight glycoproteins with many carbohydrate side chains. The mucin shares the common features of having an extensive tandem repeat region, and a peptide domain containing a high per-

centage of serine and threonine (4, 12, 16). Recently, 19 human mucin genes have been assigned to the MUC gene family. MUC1, located in 1q21–23, is a transmembrane mucin and is expressed in pancreato-biliary cancer, and may function as an anti-adhesion molecule that inhibits homotypical cell aggregation and adhesion to the extracellular matrix, promoting cell invasion (14). MUC2, MUC5AC and MUC6, which are clustered within the 11p15 locus, are expressed in intestinal goblet cells, gastric foveolar cells and pyloric gland cells (23, 26), respectively. Histological phenotype of GBC is thought to modulate the biological behavior of carcinoma cells such as tumor progression.

We previously clarified that the DG type wall-invasion pattern was significantly associated with poor differentiation, aggressive infiltration, and decreased postoperative survival in terms of its histo-

logical differentiation, lymphatic invasion, venous invasion, lymph node status, neural invasion, and mode of subserosal infiltration (18–20). In present study, both histological phenotype and MUCs expression were significantly correlated with wall-invasion pattern of GBC. Several studies have demonstrated that different histological phenotypes expressed different mucins; *i.e.* mucin expression on carcinoma cells representing a metaplastic phenotype was qualitatively and quantitatively different from those of non-metaplastic phenotype (7, 13, 17). Therefore, the biliary type and MUC1 expression were thought to be significantly correlated with DG type, *i.e.* the more aggressive wall-invasion pattern. In conclusion, histological phenotype and mucin expression were thought to be indicators of aggressiveness of GBC.

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