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Original contribution

Gastric hepatoid adenocarcinomas are a genetically heterogenous group; most tumors show chromosomal instability, but MSI tumors do exist☆

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Summary The Cancer Genome Atlas Research Network classified gastric adenocarcinoma into four molecular subtypes: (1) Epstein-Barr virus–positive (EBV), (2) microsatellite-instable (MSI), (3) chromosomal instable (CIN), and (4) genomically stable (GS). The molecular subtypes of gastric hepatoid adenocarcinomas are still largely unknown. We analyzed 52 hepatoid adenocarcinomas for the expression of surrogate markers of molecular subtypes (MLH1, p53, and EBER in situ hybridization) and some biomarkers (p21, p16, Rb, cyclin D1, cyclin E, β-catenin, Bcl-2, IMP3, ARID1A and HER2), and mutations of *TP53*, *CTNNB1*, *KRAS*, and *BRAF*. We analyzed 36 solid-type poorly differentiated adenocarcinomas as a control group. Hepatoid adenocarcinomas were categorized as follows: EBV group (EBER-positive), no cases (0%); MSI group (MLH1 loss), three cases (6%); "CIN or GS" (CIN/GS) group (EBER-negative, MLH1 retained), 49 cases (94%). In the CIN/GS group, most of the tumors (59%) had either p53 overexpression or *TP53* mutation and a coexisting tubular intestinal-type adenocarcinoma component (90%), suggesting that most hepatoid adenocarcinomas should be categorized as a true CIN group. Hepatoid adenocarcinomas showed relatively frequent expressions of HER2 (score 3+/2+: 21%/19%). Hepatoid adenocarcinomas showed shorter survival, more frequent overexpressions of p16 (67%) and IMP3 (98%) than the control group. None of hepatoid adenocarcinomas had *KRAS* or *CTNNB1* mutations except for one case each, and no hepatoid adenocarcinomas had *BRAF* mutation. In conclusion, gastric hepatoid adenocarcinomas are a genetically heterogenous group. Most hepatoid adenocarcinomas are "CIN," but a small number of hepatoid adenocarcinomas with MSI do exist. Hepatoid adenocarcinomas are characterized by overexpressions of p16 and IMP3.

☆ Conflicts of interest/disclosures: the authors declare that there are no conflicts of interest to disclose.

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1. Introduction

Hepatoid adenocarcinoma is an extrahepatic malignant tumor defined by morphologic similarity to hepatocellular carcinoma [1-3]. The stomach is the most common site of hepatoid adenocarcinoma [4,5]. Tubular and enteroblastic adenocarcinoma components often coexist with gastric hepatoid adenocarcinoma [6-10]. Primary gastric hepatoid adenocarcinoma is known to be prognostically unfavorable due to extensive vascular invasion and frequent liver metastases [4,11]. Alpha-fetoprotein (AFP), glypican 3, SALL4, HepPar-1 and Arginase-1 are known to be diagnostic markers for hepatoid adenocarcinoma [12-16].

In 2014, The Cancer Genome Atlas (TCGA) categorized gastric adenocarcinomas into four subtypes based on molecular analyses: (1) Epstein-Barr virus (EBV) positive, (2) microsatellite instability (MSI) with loss of MLH1 function, (3) genomically stable (GS), and (4) chromosomal instability (CIN) with frequent *TP53* mutation [17]. Epstein-Barr virus–encoded small RNA (EBER) in situ hybridization (EBER-ISH) and immunohistochemical staining of MLH1 and p53 have been reported to be useful as surrogate markers of molecular subtyping [18]. In addition, amplification of the ERBB2 gene, which encodes the human epidermal growth factor receptor 2 (HER2) protein, is most commonly present in CIN subtype [17], and ARID1A alteration is frequently seen in the MSI and EBV subtypes [17,19]. Although previous studies demonstrated the correlation between molecular subtypes and histological types [17,20,21], the molecular subtypes of hepatoid adenocarcinoma have not been fully clarified.

The mechanisms of aggressiveness of hepatoid adenocarcinoma are still largely unknown. The immunohistochemical status of cell-cycle regulators and apoptosis modulators such as p21, p16, Rb, cyclin D1, cyclin E and Bcl-2 were shown to be related to proliferation and differentiation in gastric cancer [22-27]. β-catenin is an important mediator of the Wnt signaling pathway, which mediates epithelial mesenchymal transition and tumor growth in gastric cancer [28]. One of the oncofetal proteins, IMP3, was associated with poor prognosis in gastric cancer [29-31]. Gastric hepatoid adenocarcinoma has not been a focus of the attention for these biomarkers.

In this study, we attempted to systematically broaden our understanding of the molecular features of gastric hepatoid adenocarcinoma by analyzing surrogate markers of molecular subtypes (MLH1, p53, and with EBER-ISH) and cell-cycle markers/biomarkers (p21, p16, Rb, cyclin D1, cyclin E, Bcl-2, ARID1A, HER2, β-catenin, and IMP3), using a relatively large number of hepatoid adenocarcinomas. We also analyzed *TP53*, *KRAS*, *BRAF* and *CTNNB1* mutations.

2. Materials and methods

2.1. Case selection

Gastric hepatoid adenocarcinoma was morphologically defined as a tumor composed of large polygonal eosinophilic hepatocellular carcinoma-like cells arranged in a solid or sheet-like pattern (Fig. 1A), based on the World Health Organization system [32]. Hyaline globule and canalicular structures are known to be morphological features of hepatoid adenocarcinomas (Fig. 1B and C) [4,9]. Solid-type poorly differentiated adenocarcinomas without hepatoid morphology were selected as a control group. Poorly differentiated adenocarcinomas with non-solid diffuse growth, signet-ring cell carcinomas, carcinomas with lymphoid stroma, and neuroendocrine carcinomas were not included in our control group.

We collected 52 cases of hepatoid adenocarcinoma with hepatoid morphology and 36 cases of solid-type poorly differentiated adenocarcinoma without hepatoid morphology in this study, based on the above definition. Fifty of the 52 hepatoid adenocarcinomas (96%) showed the positive expression of at least one of the following diagnostic markers: AFP, glypican-3, SALL4, HepPar-1 and Arginase-1 (Supplementary Table 1). These samples were histologically diagnosed at the Department of Anatomic Pathology of Kyushu University and its affiliated hospitals between 1979 and 2016. All patients had undergone curative resection, without preoperative chemotherapy or radiation therapy. The research protocol was approved by the Kyushu University Medical Human Investigation Committee (Institutional Review Board no. 29-240).

2.2. Clinicopathological assessment

The clinical characteristics of all cases were recorded, including patient age and sex, tumor location, tumor size, invasion depth, lymphatic permeation, venous invasion, lymph node metastasis, liver metastasis, and coexisting tubular or enteroblastic adenocarcinoma component (Fig. 1D and E). "Enteroblastic adenocarcinoma component" was defined as the presence of cuboidal or columnar carcinoma cells with clear cytoplasm resembling primitive gut.

2.3. Immunohistochemistry and in situ hybridization

Representative formalin-fixed and paraffin-embedded (FFPE) blocks were cut into 4-μm-thick slices. The antibodies used for immunohistochemistry (IHC) are summarized in Table 1. For this staining, we used a polymer-based detection system (Envision+; Dako, Carpinteria, CA). After deparaffinization, rehydration, inhibition of endogenous peroxidase, and antigen retrieval, the sections were exposed to the primary antibodies. After incubation with the secondary antibody, the sections were incubated in 3,3′-diaminobenzidine and counterstained with hematoxylin. We counted the proportion of positive cells (labeling index) for each antibody and defined the cutoffs in reference to previous reports (Table 1) [22,26-29,33,34]. The Ruschoff/Hofmann method was used to score HER2 IHC staining; 0 (negative): no reactivity or membranous reactivity in ≥10% of tumor cells; 1+ (negative): faint/barely perceptible membranous reactivity in ≥10% of tumor cells; 2+ (equivocal): weak-to-moderate,

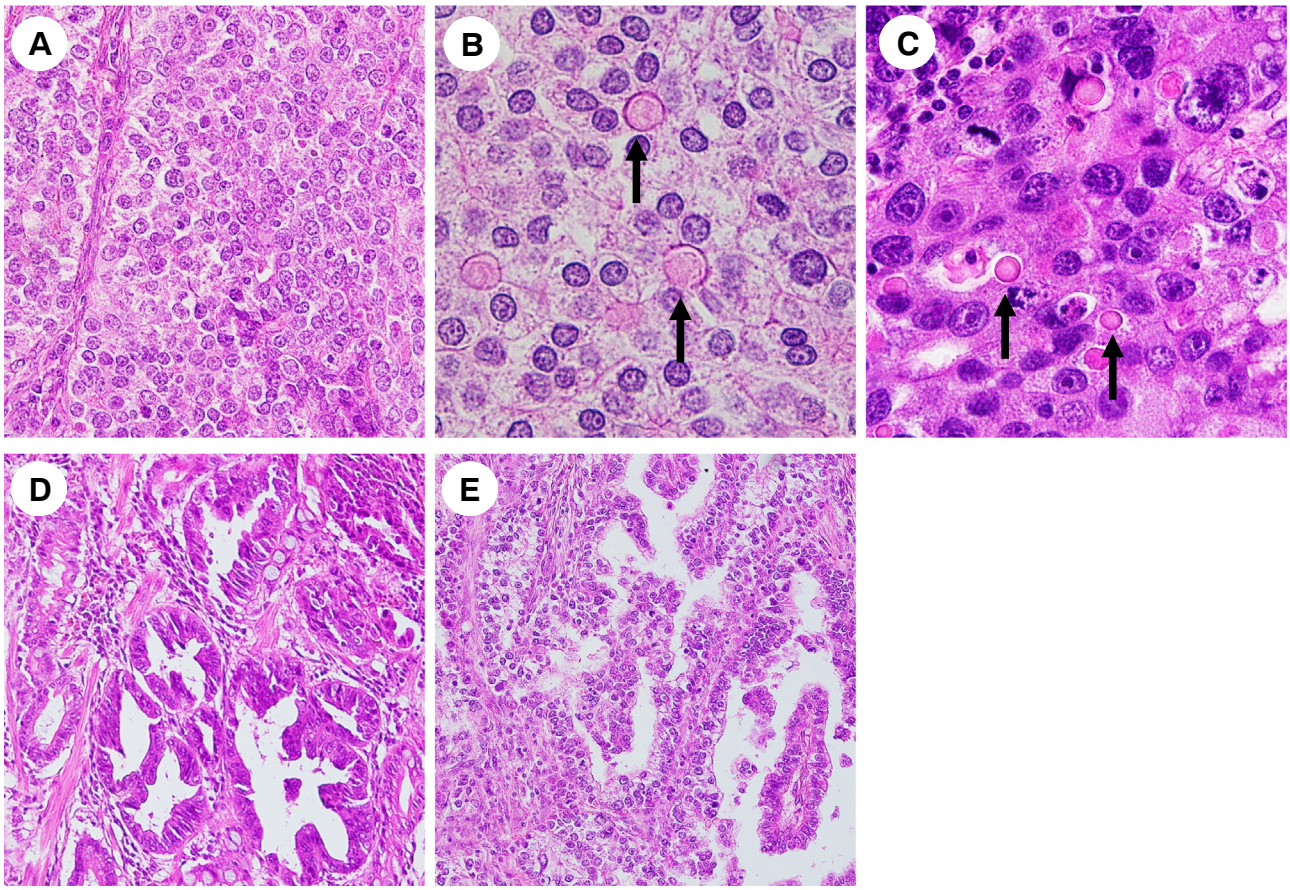

Fig. 1 Representative histologic findings of gastric hepatoid adenocarcinomas. A, Hepatoid adenocarcinoma composed of tumor cells with eosinophilic cytoplasm in a solid growth pattern. B, Canalicular structure (arrows). C, Hyaline globules (arrows). D, Coexisting tubular adenocarcinoma component at the superficial area. E, Coexisting enteroblastic adenocarcinoma component composed of cuboidal or columnar cells with clear cytoplasm. A, D, and E: Original magnification ×200; B and C: ×400.

complete, basolateral, or lateral membranous reactivity in ≥10% of tumor cells; and 3+ (positive): strong, complete, basolateral, or lateral membranous reactivity in ≥10% of tumor cells [35]. An EBER probe (#Y5200, Dako) was detected using the PNA ISH Detection Kit (#K5201, Dako). Identifiable nuclear staining for EBER was interpreted as a positive result.

To estimate the molecular subtypes of the hepatoid adenocarcinomas, we stratified all of the cases into three groups

Table 1 Primary antibodies used for immunohistochemical staining

Antibody	Clone	Source	Dilution	Localization	Cut-off
p53	DO-7	Calbiochem	1:500	Nuclear	>70%
p21	EA10	Calbiochem	1:100	Nuclear	>5%
p16	E6H4	Roche MTM Laboratories	1:1	Nuclear/cytoplasm	>70%
Rb	G3–245	BD Bioscience	1:50	Nuclear	<20%
Cyclin D1	SP-4	Thermo Fisher Scientific	1:20	Nuclear	>10%
Cyclin E	HE12	Oncogene Research Products	1:100	Nuclear	>10%
Bcl-2	124	DAKO	1:100	Cytoplasm	>25%
β-catenin	14/β-catenin	BD Bioscience	1:100	Nuclear	>10%
MLH1	G168–15	BD Bioscience	1:50	Nuclear	Complete loss
ARID1A	Rabbit polyclonal	SIGMA	1:500	Nuclear	Complete loss
IMP3	clone 49.1	DAKO	1:50	Cytoplasm	>10%
HER2	Rabbit polyclonal	DAKO	1:250	Cell membrane	>10%

NOTE. HER2 score; 0 (negative): no reactivity or membranous reactivity in ≥10% of tumor cells; 1+ (negative): faint/barely perceptible membranous reactivity in ≥10% of tumor cells; 2+ (equivocal): weak-to-moderate, complete, basolateral, or lateral membranous reactivity in ≥10% of tumor cells; and 3+ (positive): strong, complete, basolateral, or lateral membranous reactivity in ≥10% of tumor cells.

Table 2 Sequences of primers for the PCR

Gene	Exon	F-primer	R-primer
TP53	Exon 5	CTCTTCCTACAGTACTCCCCTGC	CTCCGTCATGTGCTGTGACT
		GTGCAGCTGTGGGTTGATT	GCCCCAGCTGCTCACCATCGCTA
	Exon 6	GATTGCTCTTAGGTCTGGCCCCT	CTTAACCCCTCCTCCCAGAG
	Exon 7	CTTGGGCCTGTGTTATCTCC	AGGGTGGCAAGTGGCTCCTGAC
	Exon 8	TGGTAATCTACTGGGACGGA	TAACTGCACCCTTGGTCTCC
CTNNB1	Exon 3	GAAAAGCGGCTGTTAGTCAC	GAGAAAATCCCTGTTCCCAC
KRAS	Exon 2	GGTACTGGTGGAGTATTTGA	CTGTATCGTCAAGGCACTCT
BRAF	Exon 15	CCTTTACTTACTACACCTCA	CATCCACAAAATGGATCCAG

based on the staining results by reference to the TCGA algorithm [17,18]: EBER-positive cases were placed in the EBV group; of the remaining cases, the MLH1-lost cases were placed in the MSI group, and the remaining cases were placed in the "CIN or GS" ("CIN/GS") group.

2.4. Mutational analysis

Polymerase chain reaction (PCR) and a Sanger sequencing analysis were carried out to assess the mutational status of *TP53, CTNNB1, KRAS*, and *BRAF*. Genomic DNA was

Table 3 Comparison of clinicopathological characteristics between hepatoid adenocarcinomas and solid-type poorly differentiated adenocarcinomas

	Hepatoid adenocarcinomas	Solid-type poorly differentiated adenocarcinomas	*P*
	n = 52	n = 36	
Age			.0174 *
Mean (range)	69.1 (49-87)	74.1 (55-90)	
Sex			.4811
Male	38 (73%)	23 (64%)	
Female	14 (27%)	13 (36%)	
Location			.7946
Upper	10 (19%)	8 (22%)	
Mid	18 (35%)	14 (39%)	
Lower	24 (46%)	14 (39%)	
Size			.1242
Mean (range), cm	5.9 (2.0-16.3)	6.9 (1.2-16.5)	
Invasion depth			1
pT1b-2	19 (37%)	13 (36%)	
pT3-4	33 (63%)	23 (64%)	
Lymphatic permeation			.8271
(−)	22 (42%)	14 (39%)	
(+)	30 (58%)	22 (61%)	
Vascular invasion			.0333 *
(−)	11 (21%)	16 (44%)	
(+)	41 (79%)	20 (56%)	
Lymph node metastasis			.3623
(−)	15 (29%)	14 (39%)	
(+)	37 (71%)	22 (61%)	
Liver metastasis			
(−)	29 (56%)	30 (83%)	.0106 *
(+)	23 (44%)	6 (17%)	
Tubular component			.5386
(−)	6 (12%)	6 (17%)	
(+)	46 (88%)	30 (83%)	
Enteroblastic component			<.0001 *
(−)	18 (35%)	36 (100%)	
(+)	34 (65%)	0 (0%)	

* Significant.

extracted from paraffin-embedded tissue using a QIAamp DNA FFPE Tissue Kit (Qiagen, Tokyo) according to the manufacturer's instructions. If the quality of the DNA or the level of PCR amplification was insufficient for a mutational analysis, the cases were excluded from the molecular study. The primer sequences are summarized in Table 2.

2.5. Statistical analysis

We assessed statistical differences between the groups using the Mann-Whitney U test, the χ^2 test, or Fisher's exact test. Survival data were assessed by the Kaplan-Meier method and tested for significance between the groups with the log-rank test. All calculations were performed using JMP software ver. 13.0 (SAS Institute, Cary, NC). $P < .05$ was considered significant.

3. Results

3.1. Clinicopathological features of the hepatoid adenocarcinomas and solid-type poorly differentiated adenocarcinomas

The clinicopathological features of hepatoid adenocarcinomas and solid-type poorly differentiated adenocarcinomas are summarized in Table 3. Hepatoid adenocarcinomas showed frequent lymphatic permeation (58%), vascular invasion (79%), lymph node metastasis (71%), and liver metastasis (44%). Hepatoid adenocarcinomas frequently coexisted with a tubular component (88%), an enteroblastic component (65%), or both components (50/52, 96%). Compared to the solid-type poorly differentiated adenocarcinomas, vascular invasion and liver metastasis were both significantly more frequent in hepatoid adenocarcinomas ($P = .0333$, $P = .0106$, respectively).

3.2. Immunohistochemistry and in situ hybridization

3.2.1. Molecular subtyping and biomarker/cell-cycle marker expressions

The results of the immunohistochemistry and in situ hybridization are summarized in Table 4. Representative immunohistochemical images are provided in Fig. 2. None of the 52 hepatoid adenocarcinomas showed EBER positivity. Three hepatoid adenocarcinomas (6%) showed MLH1 loss, suggesting MSI. Tumor infiltrating lymphocytes and Crohn's-like reaction were observed in one hepatoid adenocarcinoma with MLH1 loss (Fig. 3). p53 overexpression was frequently seen in hepatoid adenocarcinomas (46%). Based on these results of surrogate markers of molecular subtyping, we categorized hepatoid adenocarcinomas as follows: the EBV group (EBER-positive), no cases (0%); the MSI group (MLH1 loss), three cases (6%); the CIN/GS group (EBER-negative, MLH1 retained), 49 cases (94%) (Fig. 4). As for HER2 expression, 11 of 52 hepatoid adenocarcinomas (21%) showed positive expression (score 3+), and 10 of 52

Table 4 Summary of immunohistochemical and ISH results of hepatoid adenocarcinomas and solid-type poorly differentiated adenocarcinomas

	Hepatoid adenocarcinomas	Solid-type poorly differentiated adenocarcinomas	*P*
	n = 52	n = 36	
EBER-positive (ISH)	0 (0%)	0 (0%)	-
MLH1 loss	3 (6%)	15 (42%)	<.001 *
p53 overexpression	24 (46%)	11 (31%)	.1388
p21 overexpression	14 (27%)	17 (47%)	.0695
p16 overexpression	35 (67%)	8 (22%)	<.001 *
Rb loss	3 (6%)	0 (0%)	.2665
Cyclin D1 overexpression	21 (40%)	22 (61%)	.0823
Cyclin E overexpression	19 (37%)	11 (31%)	.65
β-catenin nuclear expression	9 (17%)	3 (8%)	.3455
Bcl-2 overexpression	0 (0%)	0 (0%)	-
IMP3 overexpression	51 (98%)	29 (81%)	.0072 *
ARID1A loss	1 (2%)	11 (31%)	<.001 *
HER2 expression			.106
Positive (score 3+)	11 (21%)	3 (8%)	
Equivocal (score 2+)	10 (19%)	2 (6%)	
Negative (score 1+ or 0)	31 (60%)	31 (86%)	

NOTE. HER2 expressions were classified as either positive (score 3+) or not (score 0-2+), and the statistical analysis was performed using the χ^2 test.
Abbreviation: ISH, in situ hybridization.
* Significant.

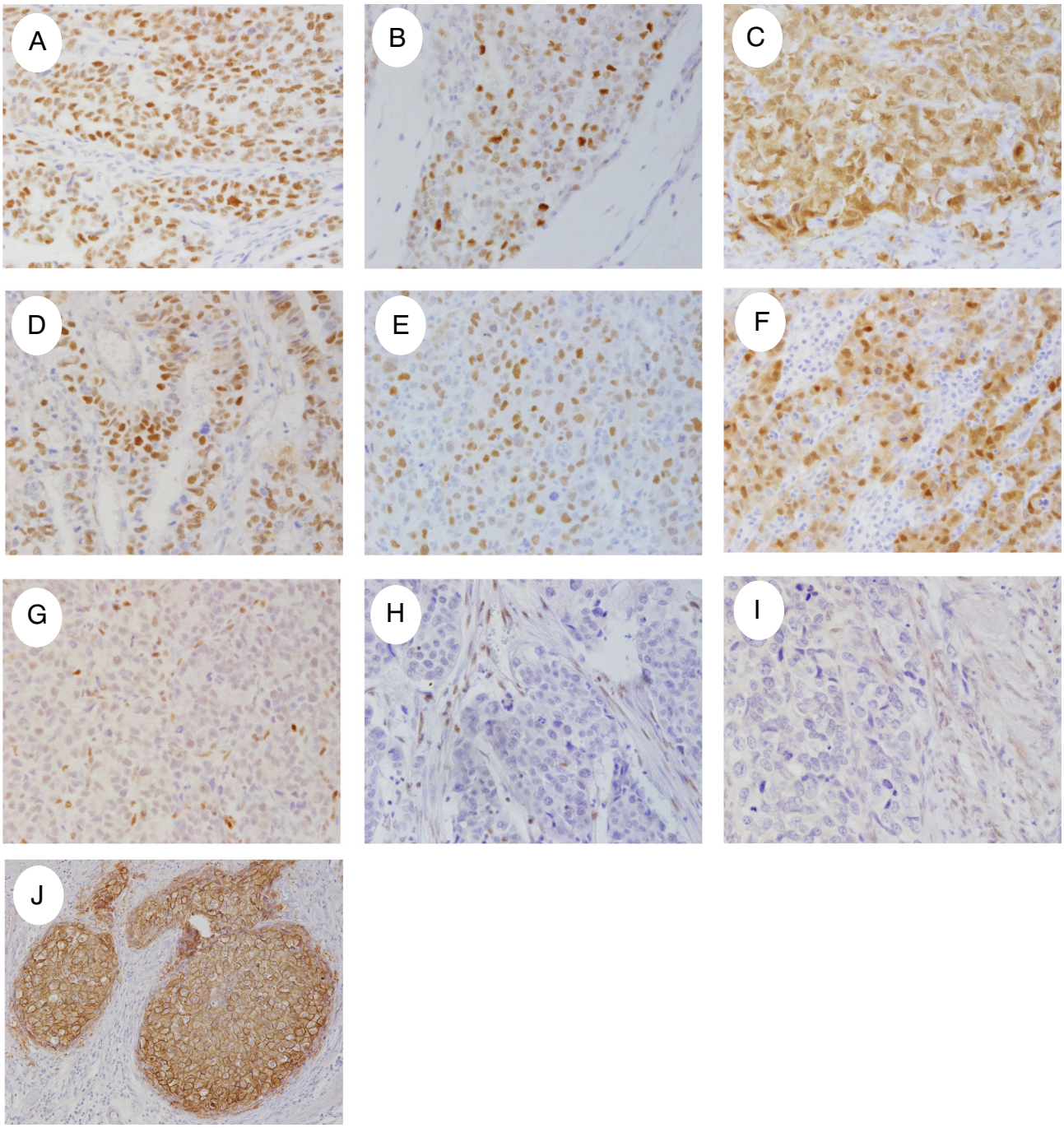

Fig. 2 Representative images of immunohistochemical staining in hepatoid adenocarcinomas. A, p53 overexpression. B, p21 overexpression. C, p16 overexpression. D, Cyclin D1 overexpression. E, Cyclin E overexpression. F, β-Catenin nuclear expression. G, Rb loss. H, ARID1A loss. I, MLH1 loss. J, HER2 positive expression (HER2 score 3+). A-I, Original magnification ×200.

hepatoid adenocarcinomas (19%) showed equivocal expression (score 2+).

Compared to the solid-type poorly differentiated adenocarcinomas, hepatoid adenocarcinomas showed significantly more frequent overexpressions of p16 and IMP3 ($P < .001$, $P = .0072$, respectively), and significantly less frequent losses of MLH1 and ARID1A ($P < .001$, $P < .001$, respectively). Hepatoid adenocarcinomas showed relatively more frequent positive expressions of HER2 than solid-type poorly differentiated adenocarcinomas, but it did not reach the significance ($P = .106$). There was no significant difference in p21, Rb, cyclin D1, cyclin E, β-catenin, or Bcl-2 between

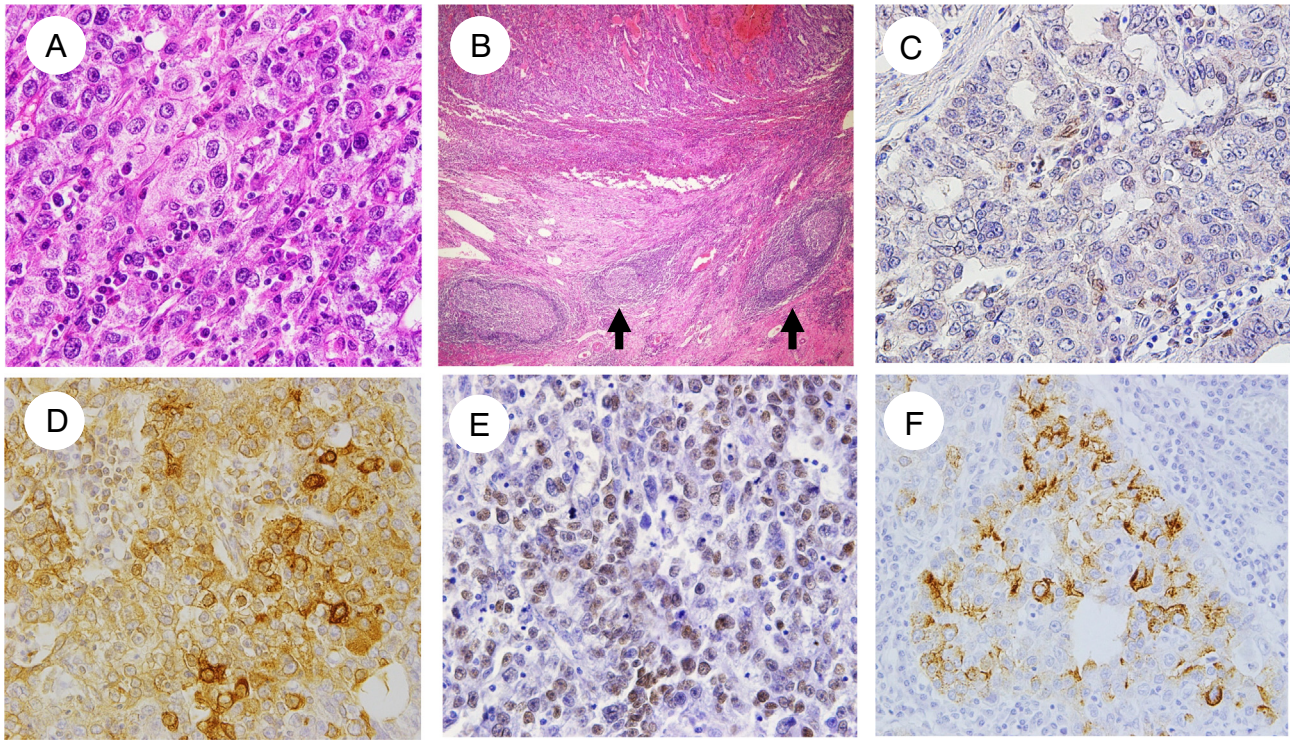

Fig. 3 Representative images of one case of hepatoid adenocarcinoma with MSI. A, Tumor-infiltrating lymphocytes (TILs). B, Crohn's-like reaction (arrows). C, MLH1 loss of tumor cells. D, Positive expression of AFP. E, Positive expression of SALL4. F, Positive expression of Glypican-3. A, C-F, Original magnification ×200; B, ×40.

hepatoid adenocarcinomas and solid-type poorly differentiated adenocarcinomas.

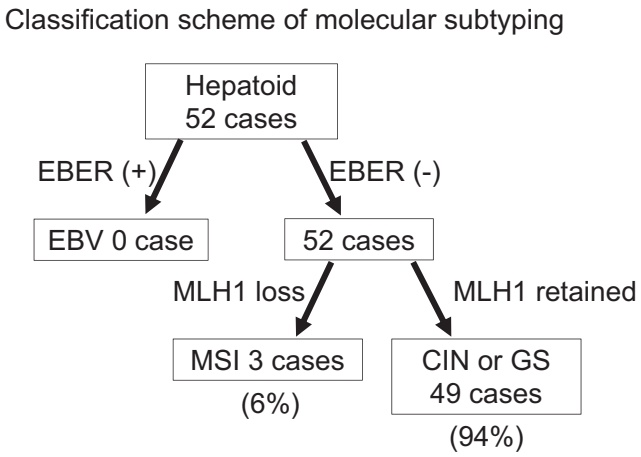

Fig. 4 Classification scheme of molecular subtyping based on the expressions of surrogate markers. None of the 52 hepatoid adenocarcinomas showed EBER positivity (EBV group). MLH1 loss was observed in three hepatoid adenocarcinomas (6%), and these cases were classified as the MSI group. The remaining 49 hepatoid adenocarcinomas were classified as the CIN/GS group.

3.2.2. Comparison of the hepatoid, enteroblastic, and tubular adenocarcinoma components

The immunohistochemical status in each histological component (hepatoid, enteroblastic, and tubular adenocarcinoma components) in hepatoid adenocarcinomas is summarized in Table 5. There was no significant difference in the immunohistochemical status of each antibody among the three histological components (Table 5).

3.2.3. Correlation of the expression level of each marker

In hepatoid adenocarcinomas, there were significant correlations between p53 overexpression and p16 overexpression (P = .0069), between p53 overexpression and cyclin E overexpression (P = .0038), and between p16 overexpression and cyclin E overexpression (P = .0017), between p16 overexpression and HER2 positive expression (P = .0092), and there was a significant inverse correlation between p16 overexpression and cyclin D1 overexpression (P = .0029). Rb loss was seen in only three hepatoid adenocarcinomas, and all three of these tumors showed p16 overexpression. However, the correlation did not reach significance (P = .5423).

In solid-type poorly differentiated adenocarcinomas, there were significant correlations between p16 overexpression and cyclin E (P = .0003) and between MLH1 loss and ARID1A loss (P = .0024), and there were significant inverse

Table 5 Summary of immunohistochemical and ISH results of hepatoid, enteroblastic and tubular components

	Hepatoid	Enteroblastic	Tubular
	n = 52	n = 23	n = 32
EBER-positive (ISH)	0 (0%)	0 (0%)	0 (0%)
MLH1 loss	3 (6%)	1 (4%)	1 (3%)
p53 overexpression	24 (46%)	9 (39%)	17 (53%)
p21 overexpression	14 (27%)	10 (43%)	13 (40%)
p16 overexpression	35 (67%)	16 (70%)	21 (66%)
Rb loss	3 (6%)	1 (4%)	2 (6%)
Cyclin D1 overexpression	21 (40%)	9 (39%)	8 (25%)
Cyclin E overexpression	19 (37%)	10 (43%)	13 (43%)
β-catenin nuclear expression	9 (17%)	1 (4%)	2 (6%)
Bcl-2 overexpression	0 (0%)	0 (0%)	0 (0%)
IMP3 overexpression	51 (98%)	22 (96%)	31 (97%)
ARID1A loss	1 (2%)	0 (0%)	1 (3%)
HER2 expression			
Positive (score 3+)	11 (21%)	7 (30%)	8 (25%)
Equivocal (score 2+)	10 (19%)	4 (17%)	6 (19%)
Negative (score 1+ or 0)	31 (60%)	12 (52%)	18 (56%)

NOTE. There was no significant difference in immunohistochemical status of each antibody among the three histological components.

correlations between p53 overexpression and MLH1 loss (P = .0112) and between p16 overexpression and MLH1 loss (P = .0114).

3.3. Genetic analysis

The results of our mutational analyses are summarized in Tables 6 and 7. Representative sequencing results are shown in Fig. 5. Tumors with an insufficient quality of DNA or level of PCR amplification were excluded from this study. *TP53* mutations were observed in hepatoid adenocarcinomas (Tables 6 and 7). The *TP53* mutations were not always consistent with p53 overexpression shown by immunohistochemistry (Table 7). *KRAS* mutation and *CTNNB1* mutation were observed in only one hepatoid adenocarcinoma each (Tables 6 and 7). No *BRAF* gene mutation was observed in hepatoid adenocarcinomas (Table 6).

Of the 49 hepatoid adenocarcinomas with the CIN/GS molecular subtypes, 29 tumors (59%) showed either p53 overexpression or *TP53* mutation, and 44 tumors (90%) coexisted with tubular (intestinal-type) adenocarcinoma components, suggesting a true "CIN subtype." The results of the gene mutation profiles of solid-type poorly differentiated adenocarcinomas were roughly similar to those of hepatoid adenocarcinomas (Table 6).

3.4. Prognosis after surgery

The patients with hepatoid adenocarcinomas showed significantly shorter overall survival (OS) periods than those with solid-type poorly differentiated adenocarcinomas (P = .0479) (Fig. 6). There were no significant correlations between each immunohistochemical status and prognosis in hepatoid adenocarcinomas.

Table 6 Summary of genetic alterations of hepatoid adenocarcinomas and solid-type poorly differentiated adenocarcinomas

	Hepatoid adenocarcinomas	Solid-type poorly differentiated adenocarcinomas
TP53	12/44 (27%)	6/33 (18%)
TP53 exon 5	7/38 (18%)	4/32 (13%)
TP53 exon 6	1/32 (3%)	1/21 (5%)
TP53 exon 7	2/33 (6%)	0/28 (0%)
TP53 exon 8	4/33 (12%)	0/29 (0%)
KRAS	1/38 (3%)	2/29 (7%)
BRAF	0/30 (0%)	0/29 (3%)
CTNNB1	1/33 (3%)	0/24 (0%)

Table 7 Detailed results of genetic alterations

Gene	Exon	Codon	Nucleotide change	Mutation type	Amino acid change	Histological type	p53 overexpression	Molecular subtype
TP53	5	152	C>T	Missense	P→S	Hepatoid	–	CIN or GS
TP53	5	165	C>T	Nonsense	Q→X	Hepatoid	–	CIN or GS
TP53	5	151	C>A	Missense	P→T	Hepatoid	–	CIN or GS
TP53	5	179	A>T	Missense	H→L	Hepatoid	–	CIN or GS
TP53	5	152	C>A	Missense	P→T	Hepatoid	–	CIN or GS
TP53	5	151	C>A	Missense	P→H	Hepatoid	+	CIN or GS
TP53	5	180	G>A	Missense	E→K	Hepatoid	+	CIN or GS
TP53	5	173	G>A	Missense	V→M	Hepatoid	–	CIN or GS
TP53	6	205	A>G	Missense	T→C	Hepatoid	–	CIN or GS
TP53	7	258	A>G	Silent	G→G	Hepatoid	+	CIN or GS
TP53	7	252	T>C	Missense	L→P	Hepatoid	–	CIN or GS
TP53	8	273	G>A	Missense	R→H	Hepatoid	–	CIN or GS
TP53	8	275	G>A	Missense	C→Y	Hepatoid	+	CIN or GS
TP53	8	277	G>A	Missense	C→Y	Hepatoid	+	CIN or GS
TP53	8	302	G>A	Missense	G→A	Hepatoid	–	CIN or GS
KRAS	2	13	C>A	Silent	G→G	Hepatoid	–	CIN or GS
CTNNB1	3	36	C>G	Missense	S→C	Hepatoid	+	CIN or GS
TP53	5	165	G>A	Missense	G→Q	Solid-type	–	CIN or GS
TP53	5	162	T>A	Missense	I→N	Solid-type	–	CIN or GS
TP53	5	155	C>T	Missense	T→I	Solid-type	–	MSI
TP53	5	175	G>A	Missense	A→H	Solid-type	+	CIN or GS
TP53	6	215	G>T	Missense	S→I	Solid-type	+	CIN or GS
KRAS	2	12	G>A	Missense	G→A	Solid-type	+	CIN or GS
KRAS	2	12	G>A	Missense	G→A	Solid-type	–	MSI

Abbreviations: C, cytosine; T, thymine; G, guanine; A, adenine. A, alanine; C, cysteine; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine. M, methionine; N, asparagine; P, proline; Q, Glutamine; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine; X, stop codon; Hepatoid, hepatoid adenocarcinoma, Solid-type, solid-type poorly differentiated adenocarcinoma CIN, chromosomal instability, GS, genomically stable, MSI, microsatellite instability.

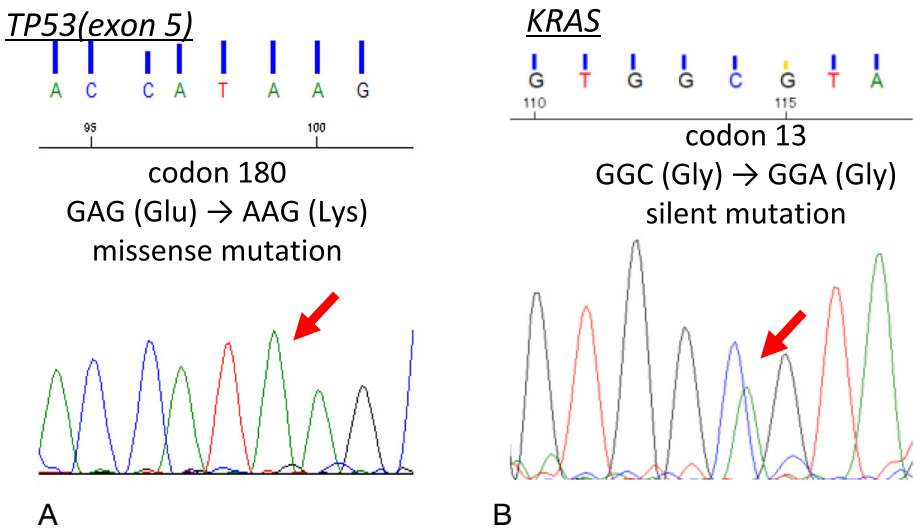

Fig. 5 Sequencing results of *TP53* and *KRAS* gene mutation in hepatoid adenocarcinomas. A, Sequencing shows the substitution of GAG to AAG at codon 180 in *TP53* gene exon 5 (arrow), causing an amino acid change from glutamic acid to lysine. B, Sequencing shows the substitution of GGC to GGA at codon 13 in *KRAS* gene exon 2 (arrow), causing no amino acid change.

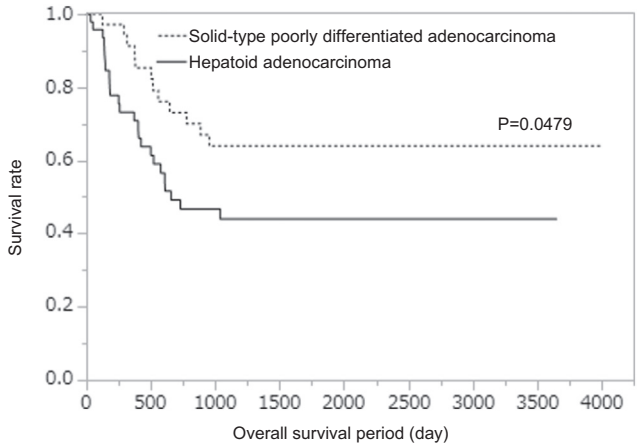

Fig. 6 Survival analysis of hepatoid adenocarcinomas and solid-type poorly differentiated adenocarcinomas. The patients with hepatoid adenocarcinomas showed significantly worse prognoses compared to the patients with solid-type poorly differentiated adenocarcinoma (P = .0479).

4. Discussion

The present analyses increased our understanding of the molecular features of gastric hepatoid adenocarcinoma. Regarding molecular subtypes, none of 52 hepatoid adenocarcinomas showed EBER positivity. To our best knowledge, no previous study examined the EBV infection status of hepatoid adenocarcinomas.

In an earlier investigation, none of 15 hepatoid adenocarcinomas had MSI-high status [36]. In the present study, we examined a relatively large number of hepatoid adenocarcinomas and observed a few tumors with MLH1 loss (3/52, 6%). Interestingly, one case of hepatoid adenocarcinoma with MLH1 loss showed tumor-infiltrating lymphocytes and Crohn's-like reaction (Fig. 3), both of which are features of gastric cancer with MSI [37,38]. To the best of our knowledge, hepatoid adenocarcinomas with MLH1 loss were first identified in the present study.

The remaining cases of hepatoid adenocarcinoma were CIN or GS (Fig. 4). In the CIN/GS group, most of hepatoid adenocarcinomas had either p53 overexpression or *TP53* mutation (29/49, 59%) and a coexisting tubular adenocarcinoma component (44/49, 90%). Tubular adenocarcinomas correspond to the intestinal type in Lauren's classification [39]. Intestinal-type histology and frequent *TP53* mutations are features of CIN [17]. In addition, a feature of GS is diffuse morphology with non-solid growth or signet-ring cells [40], whereas none of the present study's hepatoid adenocarcinomas coexisted with diffuse-type adenocarcinoma. We therefore believe that most of hepatoid adenocarcinomas classified as CIN or GS are actually CIN. We should admit the fact that CIN/GS subtypes could not be clearly separated in this study because the somatic number aberrations were not directly investigated.

Not only hepatoid adenocarcinomas but also gastric adenocarcinomas with enteroblastic differentiation are considered a characteristic histologic type of AFP-producing cancers [6,10,13]. It has been reported that hepatoid adenocarcinomas, adenocarcinomas with enteroblastic differentiation, and other gastric cancers with positive expressions of AFP, glypican-3, CLDN6, or SALL4 showed frequent *TP53* mutation or p53 overexpression and little association with EBV infection or mismatch repair deficiency [9,10,36,41,42], suggesting that most of these gastric cancers are CIN. Although those findings are similar to our present results, those studies included only small numbers of conventional hepatoid adenocarcinomas. We examined the molecular subtypes of hepatoid adenocarcinomas by using a relatively large number of cases, and the results of our analyses demonstrated a heterogenous genetic background (most of the hepatoid adenocarcinomas were CIN, but a small population of hepatoid adenocarcinomas were MSI).

Previous studies showed that frequent positive expression of HER2 was seen in hepatoid adenocarcinomas (25%-43%) [43,44]. In our study, hepatoid adenocarcinomas showed relatively frequent positive expressions of HER2 compared to the control group. These present results suggest not only the effectiveness of trastuzumab for hepatoid adenocarcinomas, but also an association between hepatoid adenocarcinomas and the CIN subtype because it is well known that most HER2-amplified gastric cancers belong to the CIN subgroup [17].

ARID1A alteration is frequent in the EBV and MSI subtypes, and rare in the CIN subtype in gastric cancers [17,19,45]. In the present study, ARID1A loss was very rarely seen in hepatoid adenocarcinomas (1/52, 2%). The low frequency of ARID1A loss is consistent with our hypothesis that most hepatoid adenocarcinomas are CIN.

As for cell-cycle markers, Rb-p16 pathway abnormality was reported to be associated with poor prognosis in gastric cancers [22,26]. Takizawa et al showed frequent p16 overexpression (56%) and Rb loss (56%) in colorectal neuroendocrine carcinomas (NECs) [33], which are known to be biologically aggressive tumors, suggesting that Rb-p16 pathway disruption may contribute to the promotion of proliferative activity in NECs. In our study, p16 overexpression was frequent in hepatoid adenocarcinomas (67%), but Rb loss was rare (6%), unlike colorectal NECs. Although there was no significant correlation between p16 overexpression and Rb loss in the present hepatoid adenocarcinomas, there were significant correlations among the overexpressions of p16, p53, and cyclin E in hepatoid adenocarcinomas. Frequent disruption of cell-cycle checkpoints such as p16 may contribute to the aggressive behavior of hepatoid adenocarcinomas.

IMP3 is an oncofetal protein that is involved in carcinogenesis, cell proliferation, and tumor development in some neoplasms [46-48]. IMP3 overexpression was shown to be associated with vascular invasion, perineural invasion, nodal metastasis, depth of invasion, and poor prognosis in gastric cancers [29-31]. In the present study, IMP3 overexpression

was significantly more frequent in the hepatoid adenocarcinomas compared to the control group. IMP3 may contribute to the aggressive behavior of hepatoid adenocarcinomas.

As for our mutational analyses other than *TP53*, *KRAS* mutation and *CTNNB1* mutation were seen in one case each. No cases showed *BRAF* mutation. A previous study showed none of 15 hepatoid adenocarcinomas harbored *KRAS* mutation [36]. The *CTNNB1* and *BRAF* mutation status in hepatoid adenocarcinomas had not been examined in any prior study, to our knowledge. In this context, we surmise that most hepatoid adenocarcinomas do not harbor *KRAS*, *CTNNB1*, or *BRAF* mutations.

Regarding histology, hepatoid adenocarcinomas in our study showed the frequent coexistence of enteroblastic or tubular components, which is consistent with previous reports [6-8,10,12,13]. Our comparison of each histological component revealed that there was no significant difference in the immunohistochemical status of each biomarker. In addition, Akiyama et al showed identical patterns of chromosome X inactivation, *TP53* mutation, and loss of heterozygosity between tubular adenocarcinoma and hepatoid adenocarcinoma [36]. These findings suggested that hepatoid adenocarcinomas are clonally identical to coexisting tubular adenocarcinoma or enteroblastic adenocarcinoma components.

We should note that some investigators categorized hepatoid adenocarcinomas as solid-type gastric adenocarcinomas with enteroblastic differentiation [10]. In the present study, we focused on hepatoid adenocarcinomas according to the WHO classification [32], and we excluded adenocarcinomas with enteroblastic differentiation without a hepatoid component and other AFP-producing gastric cancers in order to clarify the molecular features of conventional hepatoid adenocarcinomas. Further investigation is necessary to reach a consensus regarding the correct classification of AFP-producing cancers, by comparing the molecular features of hepatoid and non-hepatoid tumors.

As for a comparison with hepatocellular carcinomas, TCGA categorized them into three subtypes, one of which was characterized by chromosomal instability and frequent *TP53* mutations [49]. Hence, gastric hepatoid adenocarcinomas seem to resemble hepatocellular carcinomas not only in morphology but also in molecular features. Some investigators reported that SALL4 was useful to distinguish hepatoid adenocarcinomas from hepatocellular carcinomas because no hepatocellular carcinomas showed positive expressions of SALL4 [12,50]. However, others reported hepatocellular carcinomas occasionally showed positive expressions of SALL4 [51,52]. Therefore, diagnosis of hepatoid adenocarcinomas is still challenging especially in cases of hepatic metastasis, and it merits further investigations.

In conclusion, hepatoid adenocarcinomas are a genetically heterogenous group. Most hepatoid adenocarcinomas probably have chromosomal instability, but approx. 6% of them have microsatellite instability. EBV infection is not associated with hepatoid adenocarcinomas. High frequency of HER2 positive expression has the therapeutic significance. Overexpressions of p16 and IMP3 may be associated with the aggressive behavior of hepatoid adenocarcinomas.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humpath.2019.03.006.

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