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The pathobiological behaviors and prognosis associated with Japanese gastric adenocarcinomas of pure WHO histological subtypes

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Summary. Japan is a high-risk region for gastric carcinoma with a comparatively early stage and favorable prognosis. To clarify the pathobiological behaviors and prognosis of Japanese gastric adenocarcinoma, we analyzed the clinicopathological characteristics of different WHO subtypes of carcinomas. The expression of ki-67, CPP32, p53, FHIT, maspin, parafibromin, GRP78, GRP94, EMMPRIN, VEGF, P-GSK3ß-ser^9, fascin, cortactin, Arp2, Arp3 MUC-2, MUC-5AC and MUC-6 was examined using immunohistochemistry and tissue microarrays. The majority of cases were well-, poorly-, or moderately-differentiated subtype, whereas the minority were papillary or signet ring cell carcinoma (SRC). Patients with poorly-differentiated or SRC carcinoma were predominantly young and female. Poorly-differentiated and mucinous carcinomas were larger, with deeper invasion, more venous or lymphatic invasion, frequent lymph node involvement and peritoneal dissemination, or higher staging. The SRC group exhibited weaker expression of ki-67, CPP32, p53, parafibromin, GRP78, GRP94, P-GSK3ß-ser^9, VEGF or cortactin. The moderately-differentiated subtype exhibited lower expression of FHIT and Arp3 positivity. The poorly-differentiated group showed weaker expression of CPP32, EMMPRIN, MUC-2, MUC-5AC, and MUC-6. Survival analysis indicated that the patients with poorly-differentiated or mucinous subtypes had a lower cumulative survival rate than those with papillary, well-, moderately-differentiated, or SRC carcinomas (P<0.05). The age, invasive depth, lymphatic invasion, peritoneal dissemination, and WHO classification were independent prognostic factors for carcinoma patients (P<0.05). It was suggested that poorly-differentiated and mucinous subtypes are more aggressive and of unfavorable prognosis among Japanese gastric carcinomas. Lower levels of proliferation and apoptosis, as well as alterations in tumor suppressor genes, mucin production and ER stress protein played important roles in the pathogenesis of poorly-differentiated and SRC carcinomas.

Key words: Gastric carcinoma, WHO histological classification, Pathobiological behaviors, Prognosis

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Introduction

Gastric cancer is the fourth most common cancer and ranks as the world's second leading cause of cancer mortality behind lung cancer, despite a sharp worldwide decline in both its incidence and mortality since the second half of the 20th century. It continues to be a major health problem because of the slow decrease in incidence in Asia and high mortality of diagnosed gastric carcinoma in the West, even though sophisticated diagnostic and operative techniques are widely applied in clinical practice (Kelley and Duggan, 2003; Zheng et al., 2007b). Generally, prognosis of the patients with gastric carcinoma is very dependent on such morphological features as anatomic site, clinical and pathological extent of disease, tumor size, histological type, grading, and growth pattern. Among them, it is of general difficulty and importance to clarify the clinicopathological significance and biological characteristics of a practical and reliable histological

classification of gastric carcinoma.

Although its morphological features vary substantially with the individual patients, gastric carcinoma is a malignant tumor originating from the same gastric epithelium and the most commonly histological form of stomach cancer is adenocarcinoma (Zheng et al., 2007b). Lauren (1965) classified gastric carcinomas into intestinal, diffuse and mixed (unclassified) subtypes in term of morphological features and growth pattern of tumor. The decrease in intestinal-type tumors largely accounts for the declining trends of stomach cancer incidence, suggesting larger influences of environmental risk factors on intestinal-type cancer than diffuse-type cancer (Zheng et al., 2007b). Preneoplastic lesions, such as intestinal metaplasia, are often associated with intestinal-type cancer, but are infrequently associated with diffuse-type cancer. Both observations suggest that the two histological types develop through distinct pathways (Zheng et al., 2008d). In 1974, the WHO classification categorizes adeno-carcinoma into papillary adenocarcinoma, well-, moderately-, or poorly- differentiated adenocarcinoma, mucinous adeno-carcinoma, signet ring cell carcinoma (SRC) and undifferentiated carcinoma only according to the histomorphological features (Hamilton and Aaltonen, 2000). Goseki et al. (1992) described a novel grading system based on tubular differentiation and intracellular mucin, namely tubular well-differentiated with poor mucus in cytoplasm; tubular well-differentiated with rich mucus in cytoplasm; tubular poorly-differentiated with poor mucus in cytoplasm; tubular poorly-differentiated with rich mucus in cytoplasm. Based on the growth pattern, gastric cancers were recognized as the expanding and infiltrating subtypes by Ming (1977) and classified into expanding, nest and diffuse subtypes by Zhang et al. (1964). With the criteria proposed by Japanese Nakamura (1968), gastric carcinomas are divided into differentiated and undifferentiated ones, but it is very difficult to classify the moderately-differentiated and mucinous adenocarcinoma with this approach. Although the WHO classification only emphasizes the morphological appearance of gastric carcinoma, and therefore is not suitable for clarifying the questions of histopathogenesis, it is easy to establish its relationship with other grouping approaches due to its detailed categorization. For example, the intestinal-type principally includes papillary, well-differentiated, moderately-differentiated or mucinous adenocarcinoma without SRC cells, whereas diffuse-type one mainly consists of the poorly- differentiated adenocarcinoma, SRC carcinoma, undifferentiated adenocarcinoma of WHO classification (Zheng et al., 2007b). Therefore, WHO classification is widely applied in the clinicopathological practice.

Japan is a high-risk area of gastric carcinoma worldwide and Japanese gastric carcinoma is characterized as follows: (1) more frequent at the distal stomach; (2) often detected at an early stage (nearly 50%); (3) mostly restricted to the elderly population; (4) comparatively better prognosis (Innoue and Tsugane, 2005). These epidemiological characteristics were attributable to lifestyle modification such as salt reduction and increased fruit and vegetable intake, together with avoidance of smoking and counter-measures against *H. pylori* infection (Crew and Neugut, 2006). It has been reported that diagnostic criteria for gastric carcinoma were different worldwide. Until now, there has been no report describing the pathobiological behaviors of WHO histological subtypes despite its wide application in clinical work. Therefore, the present study for the first time aimed to clarify the clinicopathological and prognostic characteristics of Japanese gastric adenocarcinoma with pure histological type according to WHO classification. Subsequently, a series of molecular pathological markers were also designed and screened to explore the biological behaviors of these pure adenocarcinomas, ranging from cell proliferation and apoptosis (Ki-67, CPP32, p53, FHIT, maspin, parafibromin, GRP78, GRP94, and P-GSK3ß-Ser^{9}), cell mobility(fascin, Cortactin,Arp2, and Arp3), angiogenesis (EMMPRIN and VEGF), and mucins (MUC-2,MUC-5AC,and MUC-6) in combination with tissue microarray and immunohistochemistry.

Materials and methods

Subjects

A total of 814 gastric carcinomas were collected from surgical resection in Takaoka Citizen Hospital and Kouseiren Takaoka Hospital between 1993 and 2006. Among them, the 517 patients with pure histological diagnosis went into our investigation due to long follow-up time. The patients with gastric carcinoma were 366 men and 151 women (29~91 years, mean=67.1 years). Among them, 183 cases have carcinomas accompanied by lymph node metastasis. None of these cases underwent chemotherapy, radiotherapy and adjuvant treatment before surgery. All patients gave their informed consent for the use of tumor tissue specimens for clinical research and the University Ethical Committee approved the research protocol. All subjects were followed-up by consulting their case documents and telephone interviews.

Pathology

All tissue specimens were fixed in 10% neutralized formalin, embedded in paraffin and cut into 4 μm sections. These sections were stained by hematoxylin-and-eosin (HE) to confirm their histological diagnosis and other microscopic characteristics. The staging for each gastric carcinoma was evaluated according to the Internationale Contre le Cancer (UICC) system indicating the extent of tumor spread (Sobin and Wittekind, 2002). Histomorphological architecture of the tumors was expressed according to WHO classification (Hamilton and Aaltonen, 2000). Furthermore, tumor

size, depth of invasion, lymphatic and venous invasion were determined.

Tissue microarray (TMA)

Representative areas of solid tumors were identified in HE stained sections of the selected tumor cases and a two mm-in-diameter tissue core per donor block was punched out and transferred to a recipient block with a maximum of 48 cores using a Tissue Microarrayer (AZUMAYA KIN-1, Japan). Four-μm-thick sections were consecutively incised from the recipient block and transferred to poly-lysine-coated glass slides. HE staining was performed on TMA for confirmation of tumor tissue.

Immunohistochemistry

Consecutive sections were dewaxed with xylene, dehydrated with alcohol, and subjected to antigen retrieval by irradiating in target retrieval solution (TRS, DAKO, Carpinteria, UAS) for 15 min in a microwave oven (Oriental rotor Lmt. Co. Tokyo, Japan). Five percent bovine serum albumin (BSA) was then applied for 15 min to prevent non-specific binding. The sections were incubated with primary antibodies for 15 min, then treated with the anti-mouse or anti-rabbit Envison-PO (DAKO, USA) antibodies for 15 min. All incubations were performed in the microwave oven for intermittent irradiation as described previously (Kumada et al., 2004). After each treatment, the slides were washed with TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20) three times for 1 min. The primary antibodies are summarized in Table 1. All immunostaining was visualized with 3,3'-diaminobenzidine (DAB) and counterstained with Mayer's hematoxylin. Omission of the primary antibody was used as a negative control.

Table 1. Primary antibodies used in this study.

Names	Source	Company	Dilution
Ki-67	Rabbit	DAKO, Carpinteria, USA	1:25
CPP32	Rabbit	DAKO, USA	1:150
p53	Mouse	DAKO, USA	1:100
FHIT	Rabbit	Neomarkers, Fremont, USA	1:200
Maspin	Mouse	Novocastra, Newcastle upon Tyne, UK	Ready-to-use
Parafibromin	Mouse	Santa Cruz, USA	1:200
GRP78	Goat	Santa Cruz, USA	1:50
GRP94	Goat	Santa Cruz, USA	1:50
P-GSK3,-Ser^9	Rabbit	SAT, USA	1:300
EMMPRIN	Mouse	Novocastra, UK	1:100
VEGF	Rabbit	Labvision, Fremont, USA	1:50
fascin	Mouse	Neomarkers, Fremont, USA	Ready-to-use
Cortactin-421	Rabbit	Applied Biological Materials Inc., Canada	1:50
Arp2	Rabbit	Santa Cruz, USA	1: 50
Arp3	Rabbit	Santa Cruz, USA	1: 50
MUC-2	Mouse	Novocastra, UK	1:100
MUC-5AC	Mouse	Novocastra, UK	1:100
MUC-6	Mouse	Novocastra, UK	1:100

The immunoreactivity to ki-67, p53 and parafibromin was localized in the nucleus, CPP32, FHIT, GRP78, GRP94, P-GSK3ß-ser^9, VEGF, fascin, cortactin, Arp2, Arp3, MUC-2,MUC-5AC, MUC-6 in the cytoplasm, EMMPRIN was seen in the cytoplasm and the plasma membrane, and maspin in the plasma membrane and in the nucleus. These molecules were strongly expressed in the gastric carcinomas (Figure 1). All evaluations were performed blindly by two independent observers (Zheng and Takano). The inconsistent data were confirmed by both persons until final agreements were reached.

Statistical analysis

The statistical evaluation was performed using Fisher's exact possibility to differentiate the rates. Kaplan-Meier survival plots were generated and comparisons between the survival curves were made with the log-rank statistic. The Cox proportional hazards model was employed for multivariate analysis. $P<0.05$ was considered to represent a statistically significant difference. The SPSS 10.0 software program was employed to analyze all data.

Results

Clinicopathological characteristics of different histological types of gastric adenocarcinomas

Among 517 cases of pure gastric adenocarcinoma investigated in this study, the positive rates of papillary, well-differentiated, moderately-differentiated, poorly-differentiated, mucinous and SRC carcinoma were 1.2% (6 cases), 34.6% (179), 13.7% (71), 41.4% (214), 1.5% (8) and 7.5% (39) respectively. As summarized in Table 2, the patients with poorly-differentiated or SRC carcinoma were younger than those with well- or moderately-differentiated adenocarcinoma ($P<0.05$). In addition, females predominated in the poorly-differentiated and SRC groups ($P<0.05$). The poorly-differentiated and mucinous adenocarcinomas showed larger size, deeper invasion and more frequent lymph node involvement than papillary, well-differentiated, moderately differentiated and SRC carcinomas ($P<0.05$). The well-differentiated group showed less frequent venous invasion than the poorly-differentiated group ($P<0.05$) and lower positive rates of peritoneal dissemination than the mucinous carcinoma group ($P<0.05$). The papillary subtype exhibited smaller size compared with the poorly- differentiated and mucinous subtypes ($P<0.05$). SRC carcinomas showed higher staging and less frequent lymphatic invasion than moderately differentiated ones. The frequency of venous invasion for moderately- or poorly-differentiated adenocarcinomas was higher than for SRC carcinomas ($P<0.05$), and peripheral dissemination was more common in moderately- or poorly-differentiated adenocarcinomas than in mucinous ones ($P<0.05$). The

Table 2. The clinicopathological features of gastric carcinomas according to WHO histological subtype classification.

Clinicopathological features	Pap n (%)	Well n (%)	Mod n (%)	Por n (%)	Muc n (%)	SRC n (%)
Age (years)						
<65	3(50.0)	62(34.6)	20(28.2)	102(47.7)[a]	3(37.5)	23(59.0)[a]
≥ 65	3(50.0)	117(65.4)	51(71.8)	112(52.3)	5(62.5)	16(41.0)
Sex						
Male	4(66.7)	138(77.1)	52(73.2)	143(66.8)	6(75.0)	23(59.0)
Female	2(33.3)	41(22.9)	19(26.8)	71(33.2)[b]	2(25.0)	16(41.0)[b]
Tumour size (cm)						
<4	5(83.3)	138(77.1)	45(63.4)	73(34.1)	1(12.5)	29(74.4)
≥ 4	1(16.7)	41(22.9)[e]	26(36.6)	141(65.9)[c]	7(87.5)[c]	10(25.6)
Depth of invasion						
T_{is-1}	4(66.7)	140(78.2)	47(66.2)	57(26.6)	1(12.5)	34(87.2)
T_{2-4}	2(33.3)	39(21.8)	24(33.8)	157(73.4)[c]	7(87.5)[c]	5(12.8)[d]
Lymphatic invasion						
-	4(66.7)	150(83.8)	52(73.2)	101(47.2)	1(12.5)	36(92.3)
+	2(33.3)	29(16.2)	19(26.8)	113(52.8)[c]	7(87.5)[c]	3(7.7)[d]
Venous invasion						
-	6(100.0)	169(94.4)	63(88.7)	179(83.6)	7(87.5)	39(100.0)
+	0(0.0)	10(5.6)	8(11.3)	35(16.4)[b]	1(12.5)	0(0.0)[e]
Lymph node metastasis						
-	4(66.7)	151(84.4)	53(74.6)	89(41.6)	2(25.0)	35(89.7)
+	2(33.3)	28(15.6)	18(25.4)	125(58.4)[c]	6(75.0)[c]	4(10.3)
Liver metastasis						
-	6(100.0)	179(100.0)	69(97.2)	209(97.7)	8(100.0)	39(100.0)
+	0(0.0)	0(0.0)	2(2.8)	5(2.3)	0(0.0)	0(0.0)
Peritoneal dissemination						
-	6(100.0)	179(100.0)	70(98.6)	208(97.2)	6(75.0)[d]	38(97.4)
+	0(0.0)	0(0.0)	1(1.4)	6(2.8)[a]	2(25.0)[b]	1(2.6)
UICC staging						
0-I	4(66.7)	145(81.0)	48(67.6)	73(34.1)	3(37.5)	34(87.2)
II-IV	2(33.3)	34(19.0)	23(32.4)	141(65.9)[c]	5(62.5)[a]	5(12.8)[f]

Pap: papillary adenocarcinoma; Well: well-differentiated adenocarcinoma; Mod: moderately- differentiated adenocarcinoma; Por: poorly-differentiated adenocarcinoma; Muc: mucinous adenocarcinoma; SRC: signet ring cell carcinoma; n: number; %: positive rate. [a]: more than Well and Mod; [b]: more than Well; [c]: more than Pap, Well, Mod and SRC; [d]: less than Mod; [e]: less than Mod and Por; [f]: less than Mod, Por and Muc.

Table 3. The distribution of different molecules in gastric carcinomas according to WHO histological subtype classification.

Biological Markers	Pap		Well		Mod		Por		Muc		SRC	
	Tn	Pn(%)	Tn	Pn(%)	Tn	Pn(%)	Tn	Pn(%)	Tn	Pn(%)	Tn	Pn(%)
Ki-67	3	3(100.0)	138	107(77.5)	66	54(81.8)	137	105(76.6)	2	2(100.0)	18	10(55.6)[a]
CPP32	2	2(100.0)	143	102(71.3)	63	42(66.7)	137	71(51.8)b	2	2(100.0)	19	8(42.1)[b]
P53	3	2(66.7)	144	96(66.7)	65	40(61.5)	136	59(43.4)	2	2(100.0)	19	3(15.8)[d]
FHIT	3	2(66.7)	147	56(38.1)	67	15(22.4)[b]	138	45(32.6)[c]	2	1(50.0)	20	4(20.0)
Maspin	3	2(66.7)	146	54(37.0)	64	25(39.1)	138	42(30.4)	2	1(50.0)	19	2(10.5)[c]
Parafibromin	3	1(33.3)	142	89(62.7)	65	47(72.3)	140	36(25.7)	2	0(0.0)	18	3(16.7)[d]
GRP78	3	3(100.0)	137	94(68.6)	62	47(75.8)	133	85(63.9)	2	1(50.0)	18	6(33.3)[d]
GRP94	3	2(66.7)	128	74(57.8)	61	52(85.2)	132	81(61.4)	2	2(100.0)	15	4(26.7)[d]
GSK3ß-ser^9	3	2(66.7)	151	70(46.4)	63	38(60.3)	130	66(50.8)	2	1(50.0)	17	3(17.6)[d]
CD147	3	2(66.7)	139	81(58.3)	61	32(52.5)	136	42(30.9)f	2	0(0.0)	19	6(31.6)
VEGF	3	3(100.0)	140	100(71.4)	64	45(70.3)	136	74(54.4)	2	1(50.0)	18	4(22.2)[d]
Fascin	3	1(33.3)	150	35(23.3)	65	22(33.8)	132	36(27.3)	2	1(50.0)	13	2(15.4)
Cortactin	3	2(66.7)	151	78(51.7)	67	40(59.7)	134	69(51.5)	2	1(50.0)	12	3(25.0)[a]
Arp2	1	1(100.0)	116	59(50.9)	58	37(63.8)	109	62(56.9)	1	1(100.0)	14	5(35.7)
Arp3	3	2(66.7)	116	99(85.3)	58	56(96.6)[e]	103	87(84.5)	2	2(100.0)	12	10(83.3)
MUC-2	2	0(0.0)	146	65(44.5)	67	36(53.7)	144	37(25.7)[c]	2	2(100.0)	19	6(31.6)
MUC-5AC	3	2(66.7)	142	83(58.5)	66	43(65.2)	137	63(46.0)[f]	2	1(50.0)	19	17(89.5)
MUC-6	3	2(66.7)	140	72(51.4)	64	37(57.8)	131	42(32.1)[c]	2	0(0.0)	17	7(41.2)

Note: Pap: papillary adenocarcinoma; Well: well-differentiated adenocarcinoma; Mod: moderately- differentiated adenocarcinoma; Por: poorly-differentiated adenocarcinoma; Muc: mucinous adenocarcinoma; SRC: signet ring cell carcinoma; Tn: total number; Pn: positive number; %: positive rate. [a]: less than Mod; [b]: less than Well; [c]: less than Well and Mod; [d]: less than Well, Mod and Por; [e]: more than Well and Por; [f]: less than Well, Mod and SRC.

staging was higher in SRC carcinomas than in moderately-, poorly-differentiated or mucinous adenocarcinomas (P<0.05).

Immunohistochemical analysis of different histological subtypes of gastric adenocarcinomas

As shown in Table 3, the SRC group showed lower positive rates of ki-67 and cortactin expression than the moderately-differentiated group (P<0.05). The moderately-differentiated subtype exhibited lower FHIT expression than the well-differentiated subtype (P<0.05), and less Arp3 positivity than the well- or poorly-differentiated subtypes (P<0.05), which weakly expressed MUC-2 and MUC-6 in comparison with well-differentiated and moderately- differentiated subtypes (P<0.05), and displayed more immunoreactivity to maspin than SRC subtypes (P<0.05). The SRC subtype exhibited weaker expression of p53, parafibromin, GRP78, GRP94, P-GSK3ß-ser^{9} and VEGF than the well-, moderately- or poorly-differentiated subtypes (P<0.05). The poorly-differentiated group showed less positivity to EMMPRIN and MUC-5AC than the well-, moderately-differentiated or SRC groups (P<0.05).

Patients' outcome with different histological subtypes of gastric adenocarcinomas

Follow-up information for 517 carcinoma patients was used for a period ranging from five days to 9.15 years (mean=49.8 months). Figure 2 shows the survival curves stratified according to WHO histological classification. Kaplan-Meier analysis indicated that the patients with poorly- differentiated or mucinous

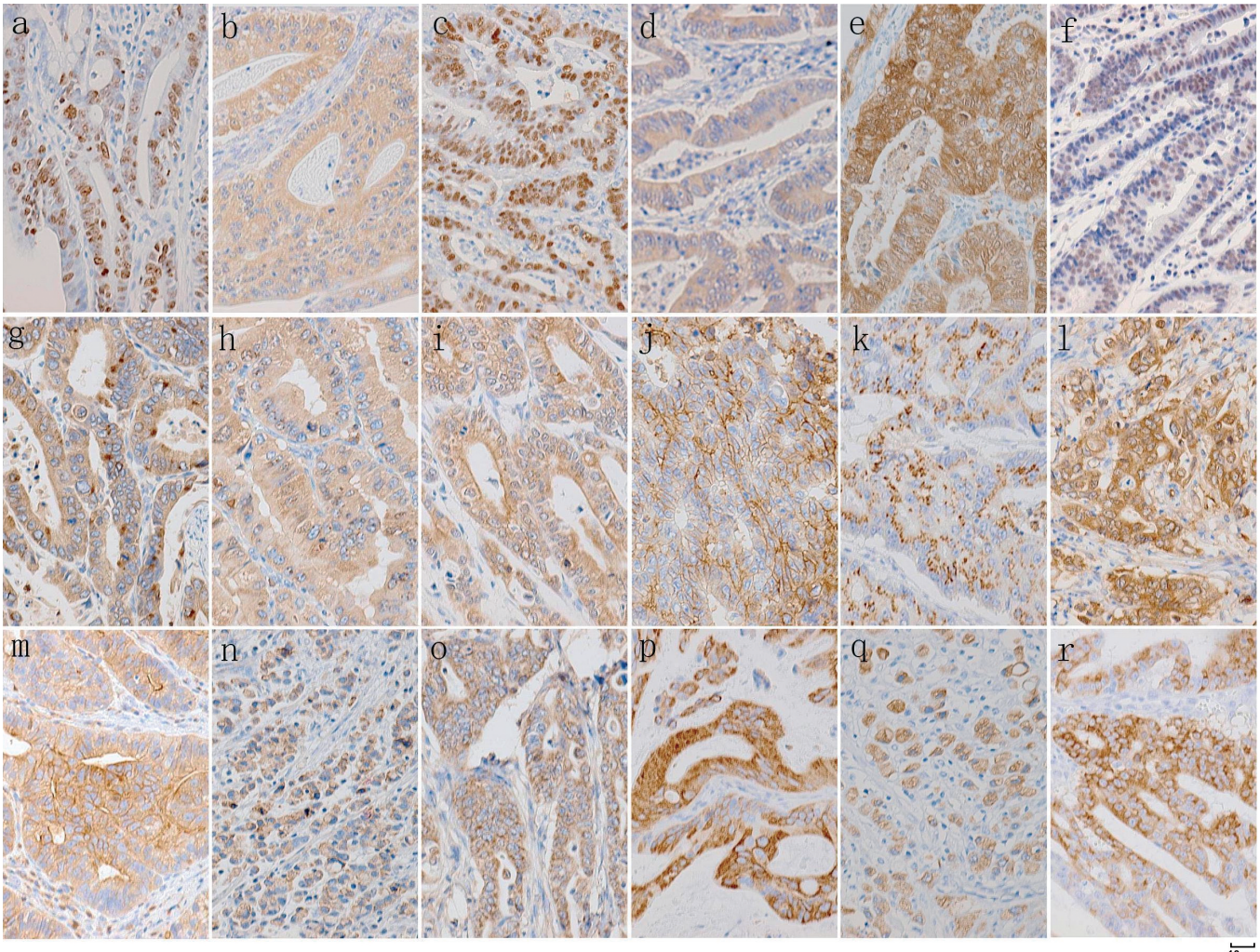

Fig. 1. Immunostaining of TMA of gastric carcinomas. The immunoreactivity to ki-67 **(a)**, p53 **(c)** and parafibromin **(f)** was localized in the nucleus. CPP32 **(b)**, FHIT **(d)**, GRP78 **(g)**, GRP94 **(h)**,P-GSK3ß-ser^{9} **(i)**, VEGF**(k)**, fascin **(l)**, cortactin **(m)**, Arp2 **(n)**, Arp3 **(o)**, MUC-2 **(p)**,MUC-5AC **(q)**, MUC-6 **(r)** were localized in the cytoplasm. CD147 **(j)** was seen in the cytoplasm and the plasma membrane. Maspin **(e)** was seen in the plasma membrane and in the nucleus. These molecules were strongly expressed in the gastric carcinomas examined in this study. x 100

Table 4. Multivariate analysis of clinical variables for gastric carcinomas.

Clinicopathological parameters	Relative risk (95%CI)	P value
Sex (Female/male)	1.137 (0.783-1.652)	0.500
Age (≥ 65years)	1.728 (1.080-20767)	0.023
Tumor size (≥ 4cm)	1.376 (0.852-2.220)	0.192
Depth of invasion (Tis,1/T2,3)	4.375 (1.880-10.181)	0.001
Lymphatic invasion (-/+)	1.774 (1.203-2.614)	0.004
Venous invasion (-/+)	0.915 (0.588-1.425)	0.695
Lymph node metastasis (-/+)	1.655 (0.928-2.953)	0.088
Peritoneal dissemination (-/+)	2.712 (1.840-3.995)	0.000
Liver metastasis (-/+)	1.708 (0.690-4.230)	0.247
UICC staging (O-I/II-IV)	0.976 (0.441-2.158)	0.952
WHO classification (Pap, Wel, Mod & SRC /Por & Muc)	1.291 (1.088-1.531)	0.003

CI: confidence interval; Pap: papillary adenocarcinoma; Well: well-differentiated adenocarcinoma; Mod: moderately-differentiated adenocarcinoma; Por: poorly-differentiated adenocarcinoma; Muc: mucinous adenocarcinoma; SRC: signet ring cell carcinoma

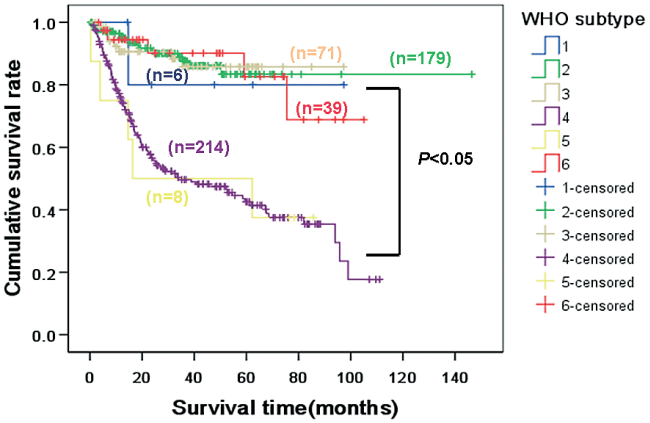

Fig. 2. Relationship between WHO subtype classification and prognosis for gastric adenocarcinoma patients. Kaplan-Meier curves of cumulative survival rate for patients with gastric adenocarcinoma according to WHO histological classification. 1, Papillary adenocarcinoma; 2, Well-differentiated adenocarcinoma; 3, Moderately- differentiated adenocarcinoma; 4, Poorly-differentiated adenocarcinoma; 5, Mucinous adenocarcinoma; 6, Signet ring cell carcinoma.

adenocarcinoma had a lower cumulative survival rate than those with papillary, well-, moderately-differentiated or SRC carcinomas (P<0.05). We divided the favorable and poor prognostic histological types into two groups for Cox's proportional hazard analysis. Multivariate analysis demonstrated that age, invasive depth, lymphatic invasion, peritoneal dissemination, and WHO classification (P<0.05), but not sex, tumor size, venous invasion, UICC staging, lymph node or liver metastasis, were independent factors for the prognosis of gastric carcinoma patients (Table 4).

Discussion

In the present study, we collected data on gastric adenocarcinoma with pure histological diagnosis from general hospitals in Japan. Among these cases, the larger subtypes were the well-differentiated (34.6%) and poorly-(41.4%) differentiated carcinomas, whereas the smaller subtypes were the papillary, mucinous, and SRC carcinomas. This finding was similar to a Japanese report (Kaneko and Yoshimura, 2001). With regard to the pathogenic age, the patients with poorly-differentiated and SRC carcinomas were mostly young women compared with other types. Poorly-differentiated and mucinous subtypes always exhibited larger size, deeper invasion, frequent lymphatic and venous invasion, more lymph node metastasis and peritoneal dissemination, and higher staging, whereas the papillary, well-differentiated and SRC carcinomas displayed early stage, rare invasion and metastasis. This was in agreement with other reports (Adachi et al., 2000; Kunisaki et al., 2006). Sugihara et al. (1985) suggested that SRC forms more frequently in the intramucosal section than the extramucosal section of the lesion. The health insurance system can provide an easy method of early diagnosis of gastric cancer and, actually, half of gastric carcinoma cases in Japan are of early staging at the time of diagnosis (Hyung et al., 2002).

To clarify the phenotypes/genotypes of different WHO histological subtypes of gastric carcinomas, the expression of Ki-67, CPP32, and p53 were examined by immunostaining. Here, poorly-differentiated and SRC carcinomas showed lower levels of Ki-67 and CPP32 expression, and lower frequency of p53, parafibromin, or maspin. This is in agreement with our previous reports (Yu et al., 2007; Zheng et al., 2007b, 2008b,d) because both histological groups are of the diffuse type according to Lauren's classification. The Ki-67 antigen is present in the nuclei of cells undergoing proliferation and should be regarded as a good marker for cell proliferation (Zheng et al., 2006b). CPP32 is responsible for the cleavage of poly (ADP-ribose) polymerase (PARP) and reflects the apoptotic level as a key protease in the cascade reaction of the apoptotic pathway (Zheng et al., 2003). Taken together, we might conclude that disrupted proliferation and apoptosis were present in both subtypes with poor differentiation. Shinohara et al. (1996) also concluded that the apoptotic index labeled by terminal digoxigenin-labeled dUTP nick-end labeling(TUNEL) was significantly higher in well-differentiated and moderately differentiated tumors than in poorly differentiated tumors, supporting our results.

To better understand the proliferative and apoptotic mechanisms characteristic of the subtypes we investigated, we detected p53, FHIT, parafibromin, maspin, glucose-related protein (GRP) 78, GRP94, phosphorylated glycogen synthase kinase 3ß-ser^{9} (P-GSK3ß-ser^{9}) using immunohistochemistry. P53, maspin and parafibromin as tumor suppressor genes play important roles in regulating the balance between the proliferation and apoptosis of cancer cells (Zheng et al., 2006b, 2007a; Zheng et al., 2008b). Glycogen synthase kinaseß-3, (GSK3ß), a serine/threonine protein kinase, also does it and can be inactivated via ser-9

phosphorylation (Zheng et al., 2007a). GRPs are ubiquitously expressed in endoplasmic reticulum (ER), able to assist in protein folding and assembly, and involved in ER stress (Zheng et al., 2008a). Our data indicated low expression of p53, parafibromin and maspin in the SRC carcinomas. Based on the context, we speculated that down-regulation or loss of tumor suppressor genes was possibly involved in the observed altered proliferation and apoptosis. The decreased expression of GRP78, GRP94 and GSK3ß-ser^9 might contribute to the morphological and biological alteration of SRC carcinomas as well.

Tumor growth depends on angiogenesis, while its metastasis has been closely linked to cell adhesiveness, motility, and deformability (Zheng et al., 2006c; Li et al., 2008a). *In vivo* and *in vitro* evidence has indicated that EMMPRIN and VEGF are involved in angiogenic processes in malignancies (Zheng et al., 2006c). In addition, the actin-related protein (Arp) 2/3 complex has been identified as a regulator of actin polymerization, and cortactin and fascin could bind to actin protein (Zheng et al., 2008c; Li et al., 2008a). Here, we observed weaker expression of VEGF in the SRC subtype than in the well-, moderately- and poorly-differentiated subtypes. The poorly-differentiated group showed less positivity to EMMPRIN than the well-differentiated, moderately-differentiated or SRC groups. This suggests that SRC and poorly-differentiated carcinomas have weaker angiogenesis abilities, especially in the case of SRC because most of the SRC carcinomas were in the early stage in our cases. Furthermore, we found that the moderately-differentiated group showed a higher positive rate of cortactin expression than the SRC group and exhibited more Arp3 positivity than the well- or poorly-differentiated groups, indicating that the aggressive behavior of moderately-differentiated adenocarcinomas is closely linked with higher cellular mobility.

Mucins are are heavily glycosylated, with many oligosaccharide side chains linked to a protein backbone. The mucin components of the gastric gel layer function as a protective and lubricating factor against luminal acid, proteolytic enzymes, and carcinogens causing DNA damage (Zheng et al., 2006a). Qualitative and quantitative alteration of mucin expression in preneoplastic and neoplastic lesions suggests potential roles in neoplastic processes, as reviewed by Cozzi et al. (2005). Furthermore, numerous pieces of evidence indicate a close association between aberrant mucin expression and aggressive behavior of malignancies (Aihara et al., 2004; Cozzi et al., 2005; Zheng et al., 2006a; Li et al., 2008b). Here, SRC expressed more MUC-5AC, but less MUC-2 and MUC-6. This trend was consistent with a previous report (Li et al., 2008b). MUC-2, MUC-5AC, MUC-6 are markers for intestinal goblet cells, superficial epithelium, and gastric pyloric gland cells, respectively (Cozzi et al., 2005; Zheng et al., 2006a; Li et al., 2008b). This combined evidence indicates that SRC carcinomas heterogeneously produce mucins, which determines its biological behavior.

Gastric adenocarcinomas originate in the gastric mucosal epithelium. The WHO subtype classification is based on the most unfavorable microscopic elements present, which in order of increasing danger are tubular, papillary, mucinous, SRC, and undifferentiated elements. However, the prognosis also depends on such risk factors for gastric carcinoma as invasion and metastasis. Here, we analyzed the correlation of WHO classification with survival rate for 517 patients with pure gastric adenocarcinoma. The results revealed lower survival rates for patients with poorly-differentiated or mucinous subtypes than for those with the other subtypes. Here, papillary, well-differentiated, moderately-differentiated and SRC carcinomas showed close links with independent prognostic factors for gastric carcinoma, including lighter invasion, less lymphatic node involvement or peritoneal dissemination. This was attributable to the comparatively aggressive behavior of poorly-differentiated and mucinous adenocarcinoma. Multivariate analysis demonstrated that age, invasive depth, lymphatic invasion, peritoneal dissemination, and WHO classification were independent factors for the prognosis of gastric carcinoma patients, consistent with previous findings (Roy et al., 1998).

In summary, patients with poorly-differentiated or SRC carcinoma were young and of female predominance in Japan. Poorly-differentiated or mucinous carcinomas showed larger size, deeper invasion, more venous or lymphatic invasion, frequent lymph node involvement, high rate of peritoneal dissemination, and/or higher staging. The converse was true for well-differentiated and SRC subtypes. The lower levels of proliferation and apoptosis, as well as alterations in tumor suppressor genes, ER stress-associated protein, and mucin production played important roles in the pathogenesis of poorly-differentiated and SRC carcinomas. WHO classification is an independent prognostic factor for Japanese gastric carcinomas. The molecular mechanisms underlying morphological characteristics of the different WHO histological subtypes of gastric carcinoma warrant further investigation in the future.

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