Shimane J. Med. Sci., Vol.29 pp.37-52, 2012

Characteristics and Malignant Potential of Gastric Cancer Which Produce AFP and CA 19-9, in Reference to Pathohistological Findings

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（Received May 29, 2012; Accepted October 5, 2012）

Some clinical cases have been reported on α-fetoprotein（AFP）and carbohydrate antigen 19-9（CA 19-9）, which gastric cancers produce, a disease whose clinical prognosis is poor. However, there are no reports identifying whether the prognosis of gastric cancer producing AFP or that producing CA 19-9 is poorer. Thus, the main purpose of the present study is to determine the malignant potential of gastric cancer producing AFP and gastric cancer producing CA 19-9. The patients were selected from cancer patients undergoing surgery for gastric carcinoma at the First Department of Surgery, Shimane Medical University, between July 1984 and December 2000. The preoperative serum AFP and CA 19-9 levels were measured, and the resected specimens were examined macroscopically and microscopically. There were 12 patients with preoperative serum AFP levels over 20 ng/ml（Group A: gastric cancer which produce AFP）ranging from 48ng/ml to 22,790 ng/ml, and all 12 patients demonstrated positive immunoreactivity for AFP. On the contrary, there were 10 patients with serum CA 19-9 values over 37 U/ml（Group B: gastric cancer which produce CA 19-9）ranging from 60 IU/ml to 138,900 IU/m, and all 10 patients demonstrated positive immunoreactivity for CA19-9. The 92 patients with normal preoperative serum levels of AFP and CA 19-9, which were immunohistochemically negative for those two tumor markers, were selected as a control group（Group C: gastric cancer which do not produce AFP and CA 19-9）. Because all patients of groups A and B were in clinical Stage III or IV, 39 in the clinical Stage III or IV of group C were selected as a control to evaluate the prognosis after surgery, collect pathohistological findings, and determine the malignant potential of the cancer cells. The malignant potential of the cancer cells of the three groups was studied by measuring the argyrophilic nuclear organizer region（Ag-NOR）score and area, DNA ploidy pattern, and 4c exceeding rate（4cER）, which is defined as the percentage of cells with an aneuploid nuclear DNA content over the tetraploid（4c）. The Ag-NOR score and area of group A were significantly（$P<0.05$）higher than those of the two other groups. Moreover, the incidence of the aneuploid pattern and 4cER of group A were significantly（$P<0.05$）higher than those of the other two groups. From the viewpoint of the pathological analysis of the resected specimens, the incidence of poorly differentiated adenocarcinoma and postoperative liver metastasis was higher, and vascular and lymphatic infiltrations were prominent in group A. Meanwhile, in group B, vascular and lymphatic infiltrations were also prominent, and there was a relationship between the serum CA 19-9 level and lymph node metastasis at operation. After a 5-year follow-up, the accumulative rate of group A and B was smaller than that of Stage IV of group C, and the prognosis after surgery in group A was the worst of all. We attribute this poor prognosis in groups A and B to the extremely high malignant potential of the cancer cells. In conclusion, multimodality therapy using chemotherapy, radiation, or a combination of both must be performed to prevent liver metastasis or recurrence in local regional lymph nodes after gastrectomy in patients with AFP and CA 19-9 producing gastric cancer.

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Key words: AFP producing gastric cancer, CA 19-9 producing gastric cancer, Ag-NORs score and area, DNA ploidy pattern, Malignant potential

INTRODUCTION

AFP is a fetal serum protein produced mainly in the fetal liver and yolk sac. [1] After birth, AFP rapidly disappears and is undetected in the serum of healthy individuals. However, AFP often reappears in the serum of patients with hepatoma and germ cell tumors, such as yolk sac tumors. [2] The serum level of AFP has been revealed to be elevated in other tumors, most of which are gastric cancers. [3, 4] The incidence of AFP-producing gastric cancer has been reported 1.3-15% [5, 6], and it is approximately 5% of gastric cancer patients in Japan. The criteria for diagnosing AFP producing gastric cancer depend on positive staining of the primary lesions because of the uncertainty of the serum AFP level. Several diseases, such as hepatitis, liver cirrhosis, and HCC demonstrate high serum AFP levels that are unrelated to gastric cancerous lesions. On the other hand, there is an AFP producing gastric cancer with a normal serum AFP level. It was reported that some early gastric cancer patients had a normal serum AFP, yet, immunostaining of the gastric cancer lesions was strongly positive. [7] So it is important to examine immunohistochemically an massive existence of AFP in the gastric cancer lesions, if we define an AFP producing gastric cancer. Consequently, without staining all of the gastric specimens, this indication "AFP non-producing gastric cancer" is usually quite skepical.

Meanwhile, CA 19-9 was first isolated in 1979, and it is the only available serum biomarker for pancreatic cancer or bile duct cancer. [8] CA 19-9 is a high-molecular-weight mucin, defined by a monoclonal antibody reacting with a repetitive carbohydrate epitope of the heterogeneous family of mucins, [8] with a carbohydrate structure (sialylated-lacto-N-fucopentose II) whose antigenic determinant is a sialized substance from the Lewis blood group. [9] An elevated serum CA 19-9 level has been found in patients with pancreatic and bile duct carcinoma, as well as in patients with colorectal, gastric, and hepatic neoplasias. [11, 12] Atkinson et al. [13] demonstrated immunoreactivity for CA19-9 in gastric cancer cells, and they reported that 89% of these cells were immunohistochemically positive. Previously, we demonstrated that about 66% of the gastric cancer was immunohistochemically positive for CA 19-9. [14] Whether gastric cancer produces CA 19-9 depends on positive immunohistochemical staining of the primary lesions. Clinical reports have indicated that the prognoses of both AFP and CA 19-9 producing gastric cancer are bad. [5, 6, 11, 12] However, there are no explanations for the poor prognosis of those cancers or for the malignant potential of the gastric cancer cells.

Thus, the main purposes of the present study are to ascertain the malignant potential of AFP and CA 19-9 producing gastric cancer cells by measurement of their Ag-NORs score and area and to analyze the DNA ploidy. An additional goal is to clarify the relationship between the prognosis and the biological malignant potential of the three groups.

PATIENTS AND METHODS

Present study is based on the analysis of data among the patients who developed gastric cancer and underwent gastric carcinoma surgical treatment at the first department of surgery, Shimane Medical University between July 1984 and December 2000. To all patients, preoperative serum AFP and CA 19-9 levels were examined, and microscopic histopathological findings of the resected specimens were examined. There were 12 patients with preoperative serum AFP level over 20 ng/ml (AFP producing gastric cancer group: group A) ranging from 48ng/ml to 22,790 ng/ml and all 12 patients demonstrated positive immunoreactivity for AFP. On the contrary, there were 10 patients with serum CA 19-9 values over 37 U/ml (CA 19-9 producing gastric cancer group: group B) ranging from 60 U/ml to 33,700 IU/m, and all 10 patients demonstrated positive immunoreactivity for CA19-9. 92 gastric cancer patients were selected as a control group (AFP and CA 19-9 non producing gastric cancer: group C). These exhibited normal preoperative serum AFP and CA 19-9 levels and these two tumor markers were immunohistochemically negative. Because both in group A and B, the clinical stages were Stage III or IV, in order to compare the malignant potentials of cancer cells of the three groups and the pathological findings of the resected specimens, 39

patients of clinical Stage III or IV in group C were selected as a control. Inclusion criteria was the presence of gastric carcinoma confirmed by histopathological study of the lesion extirpated with curative or palliative intention. The Ethics Committee of the institution approved this study.

Of 114 patients included in this study, 68(60.0%) were females and 46 (40.0%) were males, which gives a male/female ratio of 1.47: 1. Mean age was 74.6 ± 11.5 years (37 to 88 years). Those patients with resectable lesions located in the antropyloric region were submitted to D2 standard subtotal curative gastrectomy. Patients with resectable lesions in the medium and proximal third of the stomach were submitted to D2 standard total curative gastrectomy. Patients presenting penetration of the lesion into the gastric serosa on macroscopic evaluation underwent total gastrectomy, with extirpation of the spleen and pancreas tail.

1) Immunohistochemical staining for AFP and CA 19-9

For the immunohistochemical study, the specimens were fixed in a 10% neutral buffered formaldehyde solution and embedded in paraffin. The formalin-fixed, paraffin-embedded tissue blocks of the gastric tumors were sliced into 4μm sections. They were then stained with hematoxylin and eosin (H&E) and examined histologically and the same deparaffinized sections were used for the immunohistochemical investigation of AFP and CA19-9 expression. The immunohistochemical analyses were performed using the streptoavidin-biotin method. Sections were dewaxed in xylene, passed through ethanol, and incubated with 0.3% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity. Sections were washed in phosphate-buffered saline (PBS), and 10% normal rabbit serum was applied for 20 minutes to reduce nonspecific antibody binding. AFP, a mouse monoclonal antibody purchased from the DAKO Corporation (Copenhagen, Denmark), was diluted 1: 100 and reacted with tissue specimens at room temperature for 2 hours. The specimens were washed three times with PBS. The sections were incubated with biotinylated rabbit antimouse immunoglobulin G at a dilution of 1: 100 for 30 minutes follows by three washes. Slides were reacted with a streptoavidin-biotin peroxidase reagent for 30 minutes at a dilution of 1: 100 and washed with PBS three times. Finally, the slides were reacted in PBS containing diaminobezidine and 1% hydrogen peroxidase for 10minutes, counterstained with methylgreen, and mounted. Hepatocellular carcinoma tissues containing AFP were used as positive controls. Meanwhile, immunostaining of CA19-9 were done by the peroxidase antiperoxidase method using a polyclonal antibody against CA19-9 (DAKO Inc., Tokyo, Japan) as the primary antibody and the color reaction for peroxidase was developed with 0.02%3, 3-diaminobenzidine tetrahydrochloride.

2) Investigation of clinicopathological finding among the resected gastric specimens

In the present study, clinicopathological factors, such as clinical stage, histological type of tumor, tumor size, lymph node metastasis, vascular invasion, and tumor differentiation were investigated by use of resected gastric specimens in each groups. The macroscopic stage, the histological diagnosis of the primary tumor, the depth of invasion, the extent of lymph node metastasis was determined according to TNM (tumor-nodes-metastases) classification. Tumors were classified according to the 5th International Union Against Cancer (UICC) [15].

3) Ag-NOR staining technique and counting procedure

Same sections used for immunostaining of AFP and CA19-9 were cut at a thickness of 4μm from routinely processed paraffin blocks. These sections were dewaxed in xylene and dehydrated through a graded ethanol series to deionized water. The Ag-NOR staining solution was prepared by dissolving gelatin in 1% aqueous formic acid at a concentration of 2%; this solution was mixed at a 1: 2 volume ratio with a 150% (w/v) aqueous silver nitrate solution to provide the final working solution. This solution was poured over the tissue sections, which were left for 20-30 minutes at a room temperature in the dark. The silver colloid was washed off with deionized water, and the sections were dehydrated to xylene and mounted in a synthetic medium. Ag-NORs were firmly stained as black nuclear dots of

various sizes. The number and area of Ag-NOR nuclear dots in cancer and normal cells were counted by computerized analysis system, such as Analysis Program in the CAS 200 Image Analysis System (CAS 200 Image Analysis System).

4) DNA analysis technique and counting procedure

Likewise Ag-NOR staining, sections containing cancer lesion were cut at a thickness of 4μm section from routinely processed paraffin blocks and then dewaxed and rehydrated. Then, the sections were stained with Feulgen using a quantiative DNA-staining kit (Cell Analysis system, Inc., Elmhurst, IL), a commercially available staining and reagent system. All slides were studied using the Quantitative Ploidy Analysis Program in the CAS 200 Image Analysis System (Cell Analysis System, Inc., Lombard, IL). At least 200 structurally identified neoplastic cell nuclei encountered in a systematic screening of each cancer were analyzed in each specimen, and DNA histograms were generated for each cell population. As a control, the DNA content of normal cells at the G0/G1 phase was defined as 7.18pg using a DNA calibration slide supplied by the CAS 200 Image Analysis System. The DNA ploidy pattern of the neoplastic cells was calculated, and the 4c exceeding rate (4c ER), defined as the percentage of cells with an aneuploid nuclear DNA content over 4c, was also figured in each group.

5) Statistical analysis

Statistical differences in the AgNORs score and area, and 4c ER in each group were analyzed by Student's t-test, then percentage of DNA ploidy pattern and pathological factors were evaluated by the chi-square test. Overall survival rate was determined by Kaplan-Meier method and were compared using the log-rank test. $P<0.05$ was defined significant difference.

RESULTS

The characteristics of the patients' preoperative serum AFP levels, immunoreactivity for AFP, and tumor size in group A are shown in Table 1. The immunohistochemical expression of AFP was demonstrated in all specimens of group A. The macroscopic view of the resected AFP producing gastric cancer is shown in Fig. 1. The macroscopic view of this tumor was not different from a usual IIc-like gastric cancer. However, serum AFP level of this patient was extremely high, so this tumor was demonstrated in this figure. And this gastric cancer was histologically composed of poorly differentiated adenocarcinoma, and immunohistochemical staining for AFP of the gastric biopsy of this case is shown in Fig. 2. AFP was stained partially in the cytoplasm of cancer cells. In group A, the depths of carcinoma invasion into the gastric wall were T_2 in 3 patients, T_3 in 8, and T_4in 1. Liver metastasis, identified during surgery, had occurred in only 1 patient. Macroscopic lymph node metastases were recognized in all 12 patients, peritoneal dissemination was detected in only 1 patient, and distant metastasis was not recognized at all. The clinical stages according to the histology were III and IV, and the incidence of postoperative liver metastases were extremely high (8/12: 66.7%). AFP producing gastric cancers were divided into two histological subgroups: one with well-differentiated adenocarcinomas, which consisted of papillary adenocarcinomas, and, the other with poorly differentiated adenocarcinomas, signet-ring cell carcinomas, and mucinous adenocarcinomas. All patients presented venous infiltration, and 11 patients presented lymphatic infiltration (Table 2 A). The mean preoperative AFP level was 5418.6±7683.0 ng/ml (Table 1). The preoperative mean serum AFP level of the patients with gastric lesions ≧ 5 cm in diameter(n=6) was 9511.7±8744.2 ng/ml, which was significantly ($P<0.05$) higher than that of patients with gastric lesions < 5 cm (422.5±537.4 ng/ml: n=6).

Meanwhile, the characteristics of the patients' preoperative serum CA 19-9 levels, immunoreactivity for CA 19-9, and tumor size in group B are shown in Table 1. The macroscopic view of the resected specimen of the CA 19-9 producing gastric cancer of which serum CA 19-9 level of this tumor is extremely high is shown in Fig. 3. And CA 19-9 is stained diffusely in the cytoplasm of cancer cells in an immunohistochemical study (Fig. 4a, b). The depths of carcinoma invasion into the gastric wall in Group B were T_1 in 1 patient, T_2 in 4, T_3 in 4,

and T_4 in 1. After surgery, all 10 patients presented venous and lymphatic infiltrations with a clinico-pathological stage of III or IV. From microscopic findings, almost all tumors consisted of well-differentiated adenocarcinomas and papillary adenocarcinomas (Table 2A). The preoperative mean serum CA 19-9 level of the patients with gastric lesions ≧ 4cm (n=4) in diameter was 12,117.5±13,385.7 ng/ml, which was higher than that in gastric lesions <4cm (299.3±220.4 ng/ml: n=6), although there was no significant difference. With regard to the macroscopic findings of group C, the depths of carcinoma invasion into the gastric wall were T_1 in 23 patients, T_2 in 35, T_3 in 30, and T_4 in 4. Forty patients were Stage I (A, B); 21, Stage II; 18, Stage III (A, B); and 6, Stage VI. Their pathological findings are shown in Table 2. Of 92 patients in group C, 38 (41.3%) presented venous infiltration, and 45 (48.9%) presented lymphatic infiltration by the histological examination (Table 2A). The precise analysis of venous and lymphatic infiltration in three groups is shown in Table 2 B. Massive venous (v2, v3) and lymphatic (ly2, ly3) infiltrations were recognized in groups A and B. In

Table 1. The characteristics of the patients of group A and B. This table shows sex, age, preoperative serum AFP level, CA 19-9 level, immunoreactivity for AFP and CA 19-9 and tumor size of group A and B.

AFP producing gastric cancer (groupA n=12)

Sex	Age	preoperativ serum AFP level	immnoreactivity	tumor size
F	69	10500ng/ml	strong	10.8×9.8cm
F	73	147ng/ml	weak	2.5×1.2cm
M	67	3730ng/ml	strong	8.4×6.5cm
M	53	324ng/ml	moderate	4.5×3.5cm
F	63	870ng/ml	moderate	8.5×6.8cm
F	84	18900ng/ml	strong	12.5×6.5cm
M	66	48ng/ml	weak	3.2×2.8cm
F	67	220ng/ml	weak	3.6×2.5cm
M	78	186ng/ml	weak	2.8×2.2cm
M	74	22790ng/ml	strong	23.5×18.9cm
F	83	1610ng/ml	moderate	4.5×3.5cm
M	48	280ng/ml	moderate	5.5×4.5cm
mean±SD		5418.6± 7683.0ng/ml		

CA19-9 producing gastric cancer (groupB n=10)

Sex	Age	preoperativ serum CA19-9 level	immnoreactivity	tumor size
M	56	33700U/ml	strong	12.5×9.5cm
M	74	390U/ml	moderate	2.5×1.8cm
F	69	12900U/ml	strong	8.6×5.4cm
M	35	1250U/ml	moderate	4.5×4.6cm
F	68	620U/ml	moderate	5.2×4.3cm
M	76	86U/ml	weak	1.8×0.8cm
F	77	240U/ml	weak	1.2×0.6cm
M	82	720U/ml	moderate	2.6×2.2cm
F	76	60U/ml	weak	1.8×1.2cm
F	77	300U/ml	weak	2.8×1.4cm
mean±SD		5026.6± 10257.7U/ml		

group C, venous and lymphatic infiltrations were not prominent in the early stage, but they were frequently spotted in the advanced stage. In evaluating the survival rate after surgery, many of the patients in group A died following liver metastasis or peritoneal dissemination within two years after surgery even if curative resections were performed. Three patients died of local recurrence by lymph node metastasis within five years of surgery even with curative resection in group B. There was one patient in group A who survived 14 years. The cumulative survival rate following surgery is shown in Fig. 5. The rate in Groups A and B were lower than those in Stages III and IV in Group C.

Table. 2A. Operative findings and pathohistological findings of group A, B and C.

Macroscopic findings at operation	AFP producing gastric cancer (group A n=12)	CA19-9 producing gastric cancer (group B n=10)	AFP,CA19-9 non-producing gastric cancer (group C n=92)
T_1	0/12 (0%)	1/10 (10.0%)	23/92 (26.1%)
T_2	3/12 (25.0%)	4/10 (40.0%)	35/92 (38.0%)
T_3	8/12 (66.7%)	4/10 (40.0%)	30/92 (32.6%)
T_4	1/12 (8.3%)	1/10 (10.0%)	4/92 (4.3%)
N(-)	2/12 (16.7%)	1/10 (10.0%)	52/92 (56.5%)
N(+)	10/12 (83.3%)	9/10 (90.0%)	40/92 (43.5%)
H(-)	10/12 (83.3%)	10/10 (100%)	89/92 (96.7%)
H(+)	1/12 (8.3%)	0/10 (0%)	3/92 (3.3%)
P(-)	10/12 (83.3%)	9/10 (90.0%)	89/92 (96.7%)
P(+)	1/12 (8.3%)	1/10 (10.0%)	3/92 (3.3%)
M(-)	12/12 (100%)	10/10 (100%)	90/92 (97.8%)
M(+)	0/12 (0%)	0/10 (0%)	1/92 (1.1%)
stage I A	0/12 (0%)	0/10 (0%)	18/92 (19.6%)
stage I B	0/12 (0%)	0/10 (0%)	14/92 (15.2%)
stage II	0/12 (0%)	0/10 (0%)	21/92 (22.8%)
stage III A	2/12 (16.7%)	5/10 (50.0%)	13/92 (14.1%)
stage III B	8/12 (66.7%)	4/10 (40.0%)	20/92 (21.7%)
stage IV	2/12 (16.7%)	1/10 (10.0%)	6/92 (6.5%)
past ope liver metastasis	8/12 (66.7%)	0/10 (0%)	2/92 (2.2%)
Microscopic findings (histologic findings)			
pap	4/12 (33.3%)	2/10 (20%)	13/92 (14.1%)
tub1	0/12 (0%)	2/10 (20%)	21/92 (22.8%)
tub2	2/12 (16.7%)	2/10 (20%)	22/92 (23.9%)
por1	0/12 (0%)	1/10 (10%)	14/92 (15.2%)
por2	12/12 (100%)	1/10 (10%)	6/92 (6.5%)
sig	2/12 (16.7%)	1/10 (10%)	8/92 (8.7%)
muc	1/12 (8.3%)	1/10 (10%)	6/92 (6.5%)
lymphatic infiltration			
ly(-)	1/12 (8.3%)	0/10 (0%)	47/92 (51.1%)
ly(+)	11/12 (91.7%)	10/10 (100%)	45/92 (48.9%)
Venous infiltration			
v(-)	0/12 (0%)	0/10 (0%)	54/92 (58.7%)
v(+)	12/12 (100%)	10/10 (100%)	38/92 (41.3%)
mean gastric cancer size	23.9±17.8cm	7.8±4.3cm	8.6±4.6cm

Table. 2B. The precise investigation about venous and lymphatic infiltrations of the three groups.

AFP producing gastric cancer (group A n=12)

	ⅠA	ⅠB	Ⅱ	ⅢA	ⅢB	Ⅳ
lymphatic infiltration						
ly 0						
ly 1					1/12	
ly 2				1/12	4/12	1/12
ly 3				1/12	3/12	1/12
Venous infiltration						
v 0						
v 1						
v 2					2/12	
v 3				2/12	6/12	2/12

CA19-9 producing gastric cancer (group B n=10)

	ⅠA	ⅠB	Ⅱ	ⅢA	ⅢB	Ⅳ
lymphatic infiltration						
ly 0						
ly 1						
ly 2				3/10	2/10	
ly 3				2/10	2/10	1/10
Venous infiltration						
v 0						
v 1						
v 2				3/10	3/10	
v 3				2/10	2/10	1/10

AFP,CA19-9 non-producing gastric cancer (group C n=92)

	ⅠA (n=18)	ⅠB (n=14)	Ⅱ (n=21)	ⅢA (n=13)	ⅢB (n=20)	Ⅳ (n=6)
lymphatic infiltration						
ly 0	18/18	14/14	13/21			
ly 1			8/21	1/13	6/20	1/6
ly 2				12/13	13/20	4/6
ly 3					1/20	1/6
Venous infiltration						
v 0	18/18	14/14	16/21			
v 1			5/21	5/13	6/20	
v 2				8/13	12/20	4/6
v 3					2/20	2/6

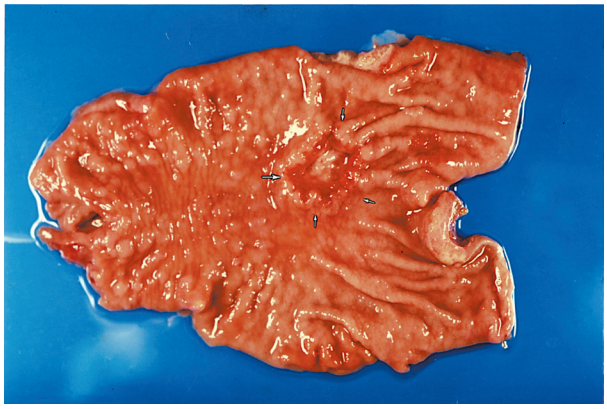

Fig. 1. Macroscopic view of the AFP producing gastric cancer is demonstrated (mucosal side). This is a *0-IIc* like advanced type. Preoperative serum AFP level of this patient was extremely high (10500 ng/ml). To this patient, distal gastrectomy with lymph nodes dissection and gastroduodenostomy was performed.

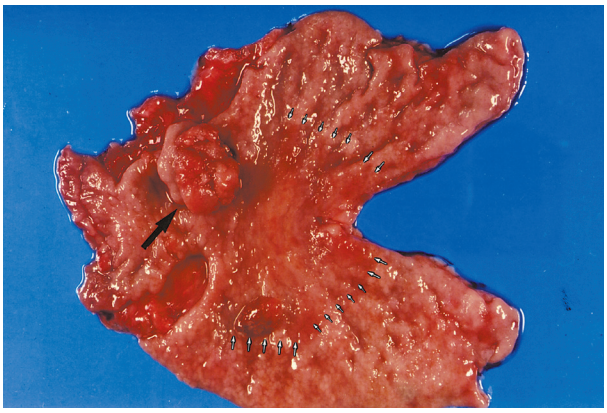

Fig. 3. Macroscopic view of the CA19-9 producing gastric cancer is shown. This cancer is a wide IIc type combined with Bormann I cancer (→). Preoperative serum level of CA 19-9 of this patient was 12,900 U/ml. To this patient, distal gastrectomy with lymph nodes dissection was performed and reconstructed by gastrodudenostomy.

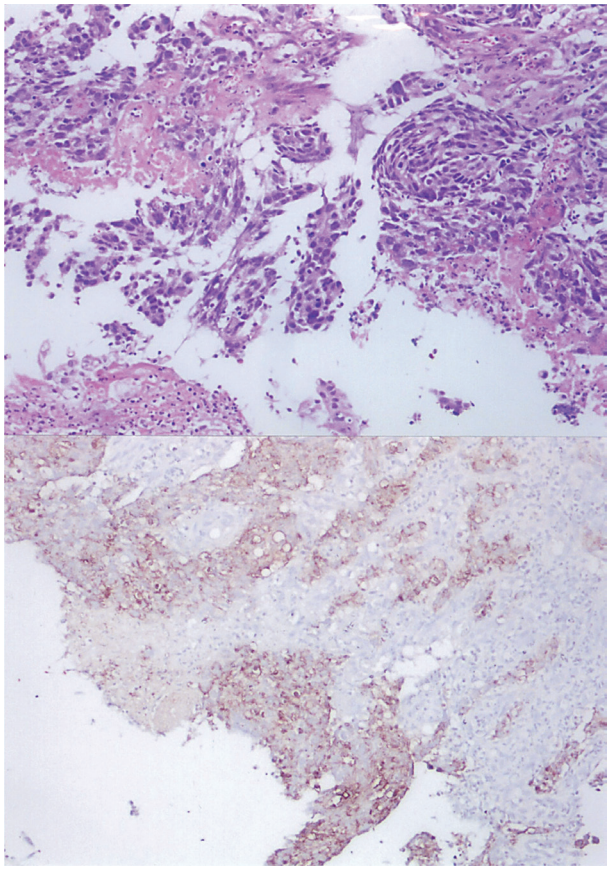

Fig. 2. The tumor was composed of poorly differentiated adenocarcinoma. The histological finding of the gastric biopsy of this AFP producing gastric cancer is shown at upper side (H.E staining: ×200). And immunohistochemical staining of this tissue shows a scattered localization of AFP at cytoplasm of the cancer cells (×200).

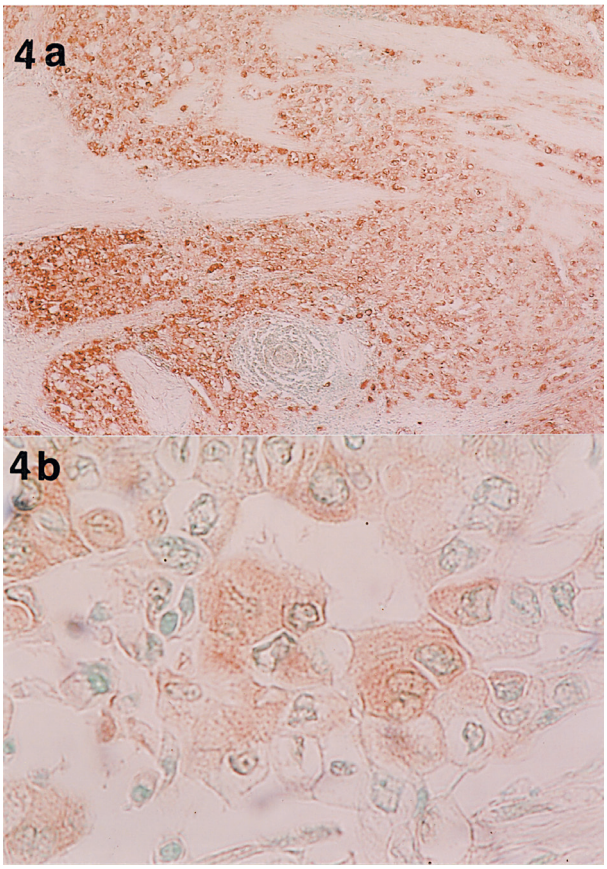

Fig. 4. Immunohistochemical examination of this CA 19-9 producing gastric cancer shows a diffuse localization of CA 19-9 in the gastric cancer cells (×40: upper side, 4a) And precise examination of the cancer cells of the CA 19-9 producing gastric cancer shows strongly positive staining at the cytoplasm (×200: lower side, 4b).

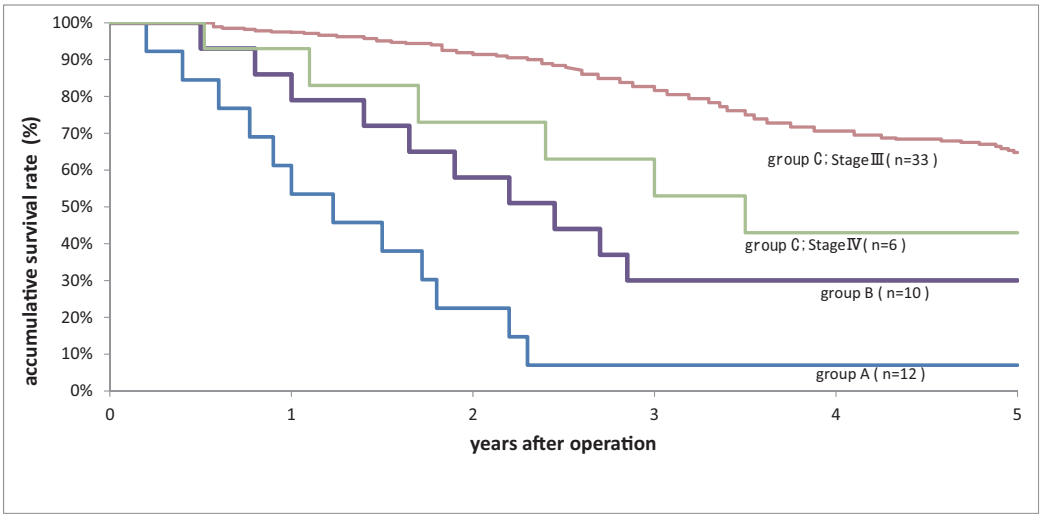

Fig. 5. Postoperative 5-year follow up survival rate of each group. (Kaplan-Meier method).
The accumulative survival rate was worst in group A of three groups and that of group B was worse than that of group C by log-rank test.

In the three groups, Ag-NORs were strongly stained as black nuclear dots of various sizes in normal cells and gastric cancer cells (Fig 6). These black dots were analyzed with the CAS 200 Image Analysis System, and the mean score and area of Ag-NORs were calculated (Fig 7). The Ag-NORs in the normal gastric epithelium were were small and round. The mean score and area of the Ag-NORs in normal gastric epithelium examined in all 114 patients were 1.65±0.23 and 1.58±0.32μm^2, respectively. On the other hand, Ag-NORs were larger and irregular; the mean value of the Ag-NORs score and area of the cancer cells in group A (n=12) were 11.99±2.19 and 7.88±1.57μm^2, respectively. Those of group B (n=10) were 8.56±1.98 and 5.87±1.34μm^2, respectively. Furthermore, those of group C (Stage IIIA, B, or IV ;n=39) were 6.48±1.09 and 4.11±1.22μm^2, respectively. Both the Ag-NORs score and area of group A were significantly (P=0.038) higher than those of the two other groups (Table 3). Those of group B were significantly (P=0.043) higher than those of group C (Stage IIIA, B or IV). The DNA ploidy pattern and 4c ER of cancer cells of the three groups were measured by the CAS 200 Image Analysis System (Fig. 8). The incidence of the aneuploidy pattern of group A (n=12) was significantly (P=0.045) higher than that of the two other groups. The 4c ER of groups A (n=10), B (n=12), and C (Stage III or IV ;n=39) were 26.88±7.07%, 14.07±5.09%, and 7.01±3.44%, respectively. The 4c ER of group A was significantly (P=0.046) higher than that of the two other groups (Table 4). Those of group B were significantly (P=0.047) higher than those of group C (Stage III or IV).

DISCUSSION

Worldwide, gastric cancer is the fourth most common and the second cause of cancer death. [16] It shows extensive tumor invasion and early spread to metastatic sites. [17] Several serum tumor markers have been used in the diagnosis of gastric cancer. Some clinical investigations demonstrated that increased levels of classic tumor markers, such as AFP, CA 19-9, and (carcinoembryonic antigen) CEA, correlated with the clinicopathological features of gastric cancer [4, 5, 18]. Among them, the organs of the gastrointestinal (GI) tract and malignancies of the GI tract, pancreaticobiliary, pulmonary, and urogenital organs have also been reported to produce AFP. [4, 5] It has been reported that gastric carcinomas have a close relationship with hepatomas because both the stomach and

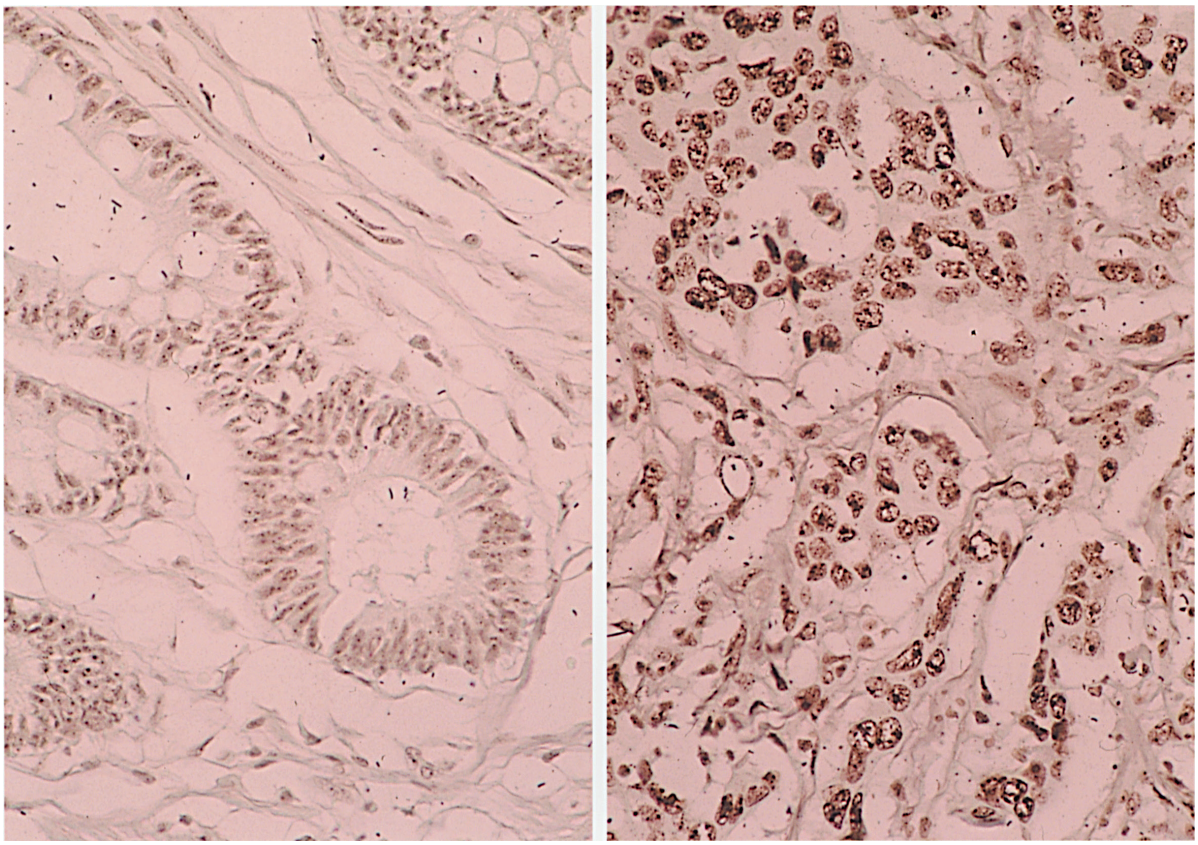

Fig. 6. Ag-NORs staining of the normal gastric epithelial cells (left side: ×100) and gastric cancer cells (right side×100) are showed. In normal gastric epithelial cells, 1 or 2 small sized and round shaped black dots were recognized in the nucleus. Meanwhile, many large sized and irregular shaped black dots were recognized in the nucleus of poorly differentiated adenocarcinoma. *Numbers* of Ag-NORs were recognized in the nucleus of the cancer cells.

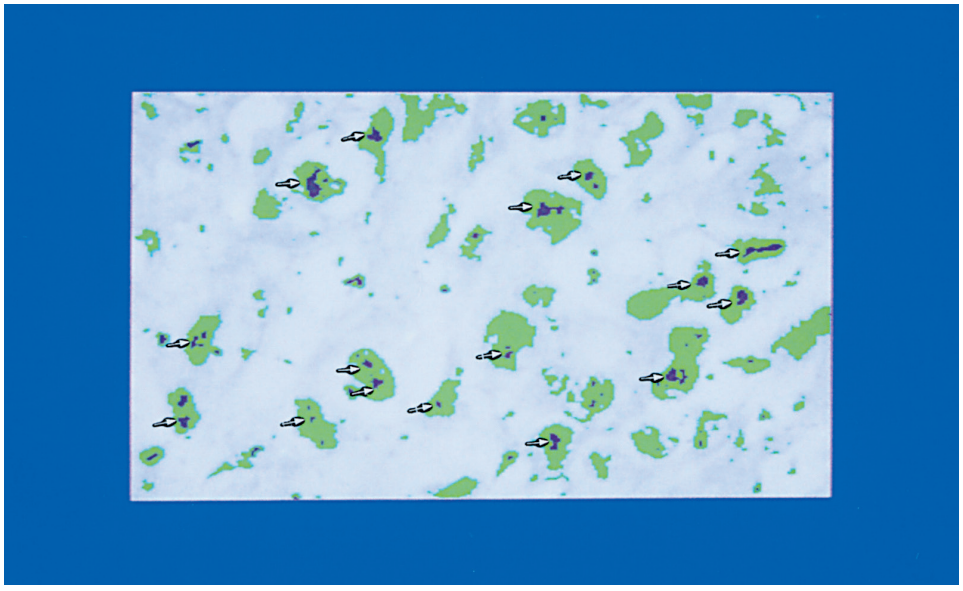

Fig. 7. The schema of identification process of the Ag-NORs score and area by Cell Analysis System.
Arrow pointed out Ag-NORs by Cell Analysis System.

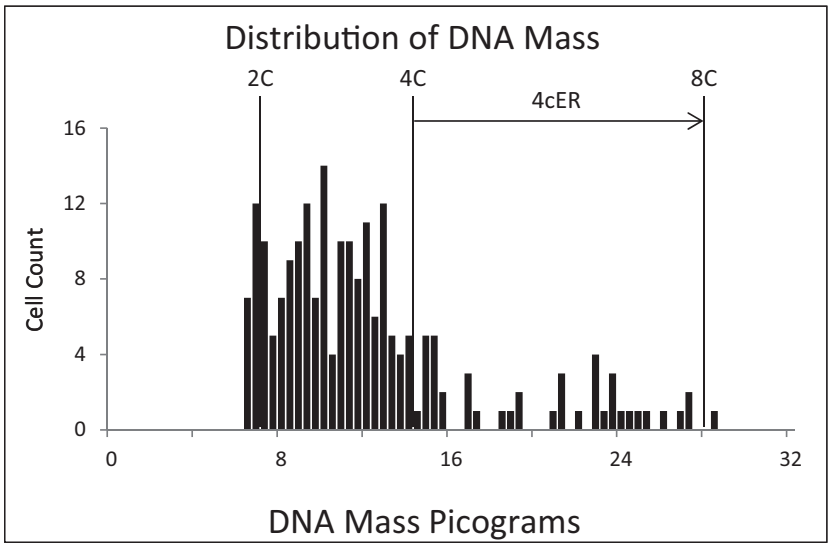

Fig. 8. This figure is a typical DNA histogram of group A. This figure demonstrates an aneuploid pattern of DNA mass and 4c ER is 26.5%. 4cER is demonstrated by arrow (→).

Table. 3. Comparison of Ag-NOR score and area in each group and normal gastric epithelial cells.

	Ag-NORs score and area			
	AFP producing gastric cancer (group A;n=12)	CA19-9 producing gastric cancer (group B;n=10)	AFP,CA19-9 non-producing gastric cancer (group C;n=39)	normal gastric epitherial cell (n=114)
	a	b	c	d
Ag-NORs score	11.99±2.19	8.56±1.98	6.48±1.08	1.65±0.23
	e	f	g	h
Ag-NORs area	7.88±1.57 μm^2	5.87±1.34 μm^2	4.11±1.22 μm^2	1.58±0.32 μm^2
	a vs b p<0.05	e vs f p<0.05		
	a vs c p<0.05	e vs g p<0.05		
	b vs c p<0.05	f vs g p<0.05		
	a,b,c vs d p<0.01	e,f,g vs h p<0.01 (by student`s t-test)		

Table. 4. The percentage of DNA aneuploidy, diploid pattern and 4c rate(%) in the DNA histogram in each group and normal gastric epithelial cells.

	DNAploidy pattern and 4cER(%)			
	AFP producing gastric cancer (group A;n=12)	CA19-9 producing gastric cancer (group B;n=10)	AFP,CA19-9 non-producing gastric cancer (group C;n=39)	normal gastric epitherial cell (group D;n=114)
	a	b	c	
1) Aneuploid pattern	11/12 (91.7%)	7/10 (70.0%)	6/39 (15.4%)	0/114 (0.0%)
	d	e	f	
2) Diploid pattern	1/12 (8%)	3/10 (30.0%)	33/39 (84.6%)	114/114 (100%)
3) 4cER(%)	26.88±7.07 %	14.67±5.09 %	7.01±3.44 %	0 %
	a vs b p<0.05	d vs e p<0.01		
	a vs c p<0.01	d vs f p<0.01		
	b vs c p<0.05	e vs f p<0.05 (by chi-square test)		

the liver are derived from the primitive foregut of the embryo. Therefore, in some primary gastric carcinomas, disturbances of differentiation occur. So it is not surprising that some gastric carcinomas contain foci of hepatocellular differentiation. [19, 20] These gastric carcinoma cells tend to produce AFP in great amounts, as do most neoplastic hepatocytes. [21] Kodama et al. [22] reported that there are two histological types of AFP producing gastric cancers of the stomach. The first is a histologically hepatoid type, characterized by polygonal cells arranged in solid nests or sheets with scattered large pleomorphic or multinucleated giant cells, and the second is histologically the well-differentiated tubular type or papillary type with a clear cytoplasm without mucin or glycogen. The poor prognosis of AFP producing gastric cancer may be attributed to the production of AFP because AFP has an immunosuppressive effect. [2] A normal serum AFP level alone cannot identify a tumor as an AFP producing tumor because Chang et al. [23] reported a patient who had a normal serum AFP, yet showed strongly positive immunohistochemical staining of AFP. Consequently, the term AFP producing gastric cancer can be clinically used for gastric specimens that are immunohistochemically stained. In the present study, of 12 patients in group A, all were immunohistochemically stained in the cytoplasm of cancer cells of the stomach. Five patients with a serum AFP level above 1,000ng/ml were strongly stained, but 3 patients (3/12: 25.0%) with a serum AFP level below 50 ng/ml were weakly stained. At the same time, Kodama and his associates [22] reported that there was a close relationship between elevated serum AFP levels and an increased number of AFP-positive tumor cells. Furthermore, Alpert et al. [4] demonstrated that the serum AFP levels reflected the degree of tumor growth in the host.

Meanwhile, in 1982, Atkinson et al. demonstrated immunoreactivity for CA19-9 in gastric cancer cells, and they reported that 89% of these cells were immunohistochemically positive. [13] At the same time, we reported that approximately 65% of gastric cancer cells showed positive immunoreactivity for CA 19-9. In the present study, it was indicated that the preoperative serum CA 19-9 levels in advanced stages were higher than those in the early stages in group B. Joypaul et al. [24] reported that there was a difference between the mean value of serum CA 19-9 levels for early and advanced tumors. Other authors have also shown that the advanced stages of gastric carcinoma were more frequent in patients with high serum CA 19-9 levels. [25] They reported that the CA 19-9 antigen from neoplastic cells was not released into the blood stream during the initial stages of the carcinoma; however, they might enter circulation when lymphatic vessels or veins are invaded as neoplasia spreads. [25, 26] This hypothesis can explain the finding of more elevated serum CA 19-9 levels in more advanced gastric neoplastic lesions. On the contrary, Wobbes et al. [25] found no association between the stage of the disease and immunopositivity for CA19-9 and reported that the values of the serum CA 19-9 levels in gastric carcinoma might not influence staging or the degree of cellular differentiation of neoplasias. Kodera et al. [26] also found a significant number of lymph node metastasis in patients with elevated serum CA19-9 and verified that neoplasias that extended beyond the muscle layer were more frequent in patients with elevated serum CA 19-9 levels. Furthermore, Tomasich et al., [27] in a study involving 144 patients, assessed the association between serum CA 19-9 levels, depth of invasion into the gastric wall, and staging of gastric carcinoma. Thus, these results could reflect that the values of CA 19-9 serum levels in gastric carcinoma might be related to the morphological aspects of neoplasias, which influence staging, and the degree of cellular differentiation of neoplasias.

Clinically, the patient numbers of AFP and CA 19-9 producing gastric cancer are small, and the prognosis of those cancers is believed to be extremely poor. Furthermore, there is little basic research concerning the malignant potential of these cancers. From the above, it is necessary to identify the malignant potential of AFP and CA19-9 producing gastric cancer and to explore the difference of the malignant biological potential between AFP and CA 19-9 producing gastric cancer. Furthermore, it is necessary to investigate whether there is a significant difference between the malignant potential of AFP producing gastric cancer and CA19-9 producing gastric cancer and gastric cancer not producing

AFP or CA 19-9.

Recently, there have been many reports about the biological malignant potential of cancer cells using several methods. [28, 29] Analyzing the DNA content of neoplastic cells in gastric cancer fields, Kimura and Yonemura [29] proposed that the DNA ploidy pattern was a useful prognostic indicator of advanced gastric cancer. In addition, they suggested that an aneuploid tumor might reflect the malignant potential of advanced gastric cancer; however, they noted that it was difficult to explain the malignant potential of gastric cancer on the basis of the DNA ploidy pattern alone because some patients with a diploid tumor had poor prognoses even after curative surgery. On the other hand, a silver technique for identifying Ag-NORs has been applied to various cancerous lesions. [30, 31, 32] The assessment of the Ag-NORs score demonstrates that this score is greater in neoplastic cells than in corresponding normal tissue. It is strictly related to cell proliferative activity and increases during the G1-phase, reaching its peak during the S-phase in the cell cycle. Accordingly, this DNA and Ag-NORs analysis is reliable for evaluating biologically the malignant potential of carcinoma cells. However, evaluation of the malignant potential of the present three groups must include a comparison of patients in the same clinical stage. Thirty-nine Stage III or IV patients were selected as a control group in group C because all patients were in groups A and B were in Stage III or IV.

From the viewpoint of the survival rate, it was demonstrated that the prognosis of the patients in groups A or B was worse than that of Stage III or IV patients in group C. Furthermore, it was also demonstrated that most patients in group A or B had complications resulting from a high incidence of microscopic infiltration into the lymphatic system and veins. To resolve these problems, the reason that the prognosis of groups A and B was worse than that for group C must be determined; therefore, Ag-NOR and DNA analyses were undertaken in the present study. In the present study, the Ag-NOR score and area of groups A and B were significantly higher than those of group C. Group A, especially, was significantly higher than group B, and the incidence of the aneuploid pattern of groups A and B was significantly higher than that in group C. Moreover, in an investigation of the 4c ER percentage in the DNA histogram, it was demonstrated that the 4c ER of groups A and B was significantly higher than that of group C and that of group A was significantly higher than that of group B. These results confirmed that the biologically malignant potentials of carcinoma cells of group A and B were much higher than those of group C. The proliferative activities of cancer cells of group A were especially high. This phenomenon might reflect an lower survival rate after surgery, as shown in the present study.

The malignant potential of gastric cancer cells in group A was then increased in accordance with tumor growth, eventually resulting in early liver metastasis even if curative surgery was performed. Meanwhile, it was also demonstrated that the Ag-NORs score and area, also an aneuploid pattern including 4c ER in the DNA histogram of group B were significantly larger than those in the 39 Stage III or IV patients in group C. These facts clearly showed that the malignant potential of gastric cancer cells of group A was higher than that of group B and that the high malignant potential of group A was associated with the low survival time following surgery. At the same time, the tumor malignancy in group B was higher than that in the 39 patients of group C.

In the present study, there was a patient in group C that had early gastric cancer with high Ag-NORs scores and areas, along with high 4cER. This patient with an extremely high Ag-NORs score (8.83) and high 4cER (14.8%) developed lymph node metastasis and peritoneal dissemination at postoperative 15 months. This suggests some concern about early recurrence in a patient with a high Ag-NOR score and high 4cER, even if they are in an early clinical stage. These facts strongly suggest that, if the intensity of the lymphatic and venous infiltrations is strong, evaluation by both measuring the serum AFP and CA 19-9 and immunohistochemical study of those two tumor markers may be necessary to determine the characteristics of gastric cancer.

In conclusion, the present study shows that the evaluation of cellular kinetics by the Ag-NORs score and area and the analysis of the DNA ploidy

pattern provide important prognostic information in addition to that obtained from clinical and pathological obsrvation in gastric cancer patients. Furthermore, gastric cancers that are immunohistochemically positive for AFP or CA19-9 have a high and large Ag-NORs score and area. Their prognoses are poorer than those in individuals with typical gastric cancer. Accordingly, preoperative assessment of AFP and CA19-9 must be done routinely, and immunostaining analysis of gastric cancer cells for AFP and CA19-9 must also be performed because prognostic information can be obtained.

Despite the development of advanced treatment over the last decade, gastric cancer is among the most difficult to treat, generally with a poor outcome. A multidisciplinary approach is now a widely accepted standard of treatment for operable diseases, although marked variation occurs worldwide. In Japan, postoperative flioropyrimidine chemoradiation or perioperative combination chemotherapy is commonly used, although a neoadjuvant approach with chemotherapy or chemoradiation may also be used for cancers of the cardia or cardia-esophageal junction. Postoperative recurrence of gastric cancer is most likely due to occult metastatic disease in the tumor bed and distant sites; hence, multimodality approaches using chemotherapy, radiation, or a combination of both have been evaluated in the last few decades in an attempt to improve outcomes following surgery. Regardless of the differences in these approaches, a benefit is usually seen from adding systemic chemotherapy, thereby reinforcing the concept that operable gastric cancer is in fact a local presentation of systemic diseases with occult micrometastatic disease at the time of diagnosis, leading to relapse and poor survival after radical surgery. In the present study, AFP and CA 19-9 producing gastric cancers have close connections with microscopic venous and lymphatic infiltrations, eventually leading to early liver metastasis after surgery. From the viewpoint of the tumor characters, it is essential to have an exact assessment of cancer malignant potential by not only investigation of microscopic pathological findings but also evaluation of the malignant potential of cancer cells by the methodology of the Ag-NORs score and area and the DNA ploidy pattern. Currently, there is not a specific tumor marker for gastric cancer. As a result, it is necessary to measure the preoperative serum AFP and CA 19-9 level and to analyze the malignant potential of the cancer cells of the resected gastric cancer. These trials help with the identification of efficacious therapies that permit an individualized approach for the treatment of patients and improve the generally poor prognosis of this aggressive disease.

REFERENCES

1) Gitlin D, Perricelli A and Gitlin GM (1972) Synthesis of alpha-fetoprotein by liver, yolk sac, and gastrointestinal tract of the human conceptis. *Cancer Res* 32: 979-982.

2) Abelev GI (1968) Production of embryonal serum α-globulin by hepatomas: Review of experimental and clinical data. *Cancer Res* 28: 1344-1350.

3) Purves LR, Bersohn I and Geddes EW (1970) Srum alpha-fetoprotein and primary cancer of the liver in man. *Cancer* 25: 1261-1270.

4) Alpert E, Pinn VW and Isselbacher KJ (1971) Alpha-fetoprotein in a patient with gastric carcinoma metastatic to the liver. *N Eng J Med* 285: 1058-1060.

5) Bierfeld JL, Scheiner MI, Shultz DR, Moral MD and Rogers AI (1973) Alpha-fetoprotein and carcinoembryonic antigen in a case of gastric carcinoma metastatic to the liver. *Am J Dig Dis* 18: 517-520.

6) Motoyama T, Saito K, Iwafuchi M and Watanabe H (1985) Endormal sinus tumor of the stomach. *Acta Pathol Jpn* 35: 497-505.

7) Ishikura H, Fukasawa Y, Ogasawara K, Natori T, Tsukada Y and Aizawa M (1985) An AFP producing gastric carcinoma with features of hepatic differentiation; a case report. *Cancer* 56: 840-848.

8) Goonetilleke KS and Sirwardena AK (2007) Systemic review of carbohydrate antigen (CA 19-9) as a biochemical marker in the diagnosis of pancreatic cancer. *Eur J Surg Oncol* 33: 266-270.

9) Kannagi R (2003) Molecular mechanism for cancer-associated induction of sialyl Lewis X and sialyl Lewis A expression-the Warburg effect revisited. *Glycoconjugate Journal* 20: 353-364.

10) Shiro T, Seki T, Naitoh Y, Inoue K and Okamura A (1993) A correlation of argyrophilic nucleolar organizer regions with stages of hepatocellular carcinoma. *Cancer* 71: 44-49.

11) Kochi M, Fujii M, Kanamori N, Kaiga T, Kawakami T, Aizaki K, Kasahara M, Mochizuki F, Kasakura Y and Yamagata M (2000) Evaluation of serum CEA and CA 19-9 levels as prognostic factors in patients with gastric cancer. *Gastric Cancer* 3: 177-186.

12) Tsuneyoshi M (2002) Malignant transformation of gastric hyperplastic polyps: alteration of phenotypes, proliferative activity, and p53 expression. *Hum Pathol* 33: 1016-1022.

13) Atkinson BF, Ernst CS, Herlyn M, Steplewski Z, Sears HF and Koprowski H (1982) Gastrointestinal cancer associated antigen in innmunoperoxidase assay. *Cancer Res* 42: 4820-4823.

14) Nagami H, Tamura K, Kin S, Yano S, Seshimo T, Sumi S and Nio Y (1994) Correlation between argyrophilic nucleolar organizer regions and immunostaining for carbohydrate antigen 19-9 in human gastric cancers. *Ann Cancer Res and Ther* 3: 113-116.

15) Jass JR and Sobin LH (1989) WHO international histological classification of tumors. In: Histological typing of intestinal tumours. 2nd ed. Springer-Verlag, Berlin.

16) Parkin DM, Bray F, Ferlay J and Pisani P (2002) Global cancer statics. *CA Cancer J Clin* 55: 74-108.

17) Lochhead P and El-Omar EM (2008) Gastric cancer. *Br Med Bull* 85: 87-100.

18) Marta LZ, Barbara M, Mariusz G, Boguslaw K and Maciej S (2011) Comparison between clinical singnificance of serum proinflammatory proteins (IL-6 and CRP) and classic tumor markers (CEA and CA19-9) in gastric cancer. *Clin Exp Med* 11: 89-96.

19) Tsung SH (1975) Alpha-fetoprotein in lung cancer metastatic to the liver. *Arch Pathol* 99: 267-269.

20) Inada T, Imura J, Ogata Y, Shimamura K, Ando J, Ozawa I, Matsui J, Hishimura S, Shimizu H, Kotake K and Koyama Y (1993) The clinicopathological characteristic and proliferative activity of alpha-fetoprotein producing gastric cancer. *Jpn J Gastroenterol Surg* 26: 979-983.

21) Kubo T, Sowa Y, Nishimura M, Kato Y, Chung Y. S, Yoshino H and Umemiya K (1989) Clinicopathological study of primary gastric cancer with high serum levels of a-fetoprotein. *Jpn J Gastroenterol Surg* 22: 1761-1767.

22) Kodama T, Kameya T, Hirota T, Shimasato Y, Ohkura H, Mukojima T and Kitaoka H (1981) Production of alpha-fetoprotein, normal serum proteins, and human chronic gonadotropin in stomach cancer. Histologic and immunohistochemical analyses of 35 cases. *Cancer* 48: 1647-1655.

23) Chang YC, Nagasue N, Abe S, Taniura H, Dhar DK and Nakamura T (1992) Comparison between the clinicopathological features of AFP-positive and AFP-negative gastric cancer. *Cancer* 87: 321-325.

24) Joypaul B, Browning M, Newman E, Byrne D and Cuschieri A (1995) Comparison of serum CA 72-4 and CA 19-9 levels in gastric cancer patients and correlation with recurrence. *Am J Surg* 169: 595-599.

25) Wobbes T, Thomas CGM, Segers MFG and Nagengast FM (1992) Evaluation of seven tumor markers (CA 50, CA 19-9 TruQuanant, CA 19-9, CA 125, carcinoembryonic antigen and tissue polypeptide antigen) in patients with gastric cancer. *Eur J Surg Oncol* 21: 379-384.

26) Kodera Y, Yamamura Y, Torii A, Uekusa K, Hirai T, Yasui K, Morimoto T, Kato T and Kito T (1996) The prognostic value of preoperative serum levels of CEA and CA 19-9 in patients with gastric cancer. *Am J Gastroenterol* 91: 49-53.

27) Tomasich FDS, Augusto VC, Luz MA, Dias LAN and Kato M (2002) Correlaçâo entre os marcadoerestumorais CEA e CA 19-9 e a profundidate de invasão no cancer gàstrico. *Rev Bras Cancerol* 48: 57-62.

28) Nagami H, Tamura K, Kin S, Yano S, Seshimo T, Sumi S and Nio Y (1996) Nuclear organizer regions in invasive ductal carcinoma of the pancreas: Quantitative and qualitative evaluation in predicting biological potential and prognosis. *J Exp Clin Cancer Res* 15: 71-76.

29) Kimura H and Yonemura Y (1991) Flow

cytometric analysis of nuclear DNA content in advanced gastric cancer and its relationship with prognosis. *Cancer* 67: 2588-2593.

30）Ploton D, Bobichon H and Adnett JJ（1982）Ultrastructural localization of NOR in nuclei of human breast cancer tissues using a one-step Ag-NORs staining method. *Biol Cell* 43: 229-232.

31）Dervan PA, Gilmartin LG, Loftus BM and Carney DN（1989）Breast carcinoma kinetics; argyrophilic nuclear organizer region counts correlate with Ki-67 scores. *Am J Clin Pathol* 92: 401-407.

32）Nagami H,Tamura K,Kin S, Yano S, Seshimo T, Sumi S and Nio Y（1996）Relationship between the expression of neuron specific enolase and argyrophilic nucleolar organizer regions in human pancreatic carcinomas. *J Exp and Clin Cancer Res* 15: 123-127.