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Evaluation of the Malignant Potential of the α-Fetoprotein (AFP)-Producing Gastric Cancer

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α-fetoprotein (AFP) gastric cancer is defined as a gastric cancer partially consisting of tumor cells producing AFP.

In the present study, we investigated the malignant potential of AFP producing gastric cancer (n=11) and AFP-non-producing gastric cancer (n=73).

The immunohistochemical expression of AFP was demonstrated in all specimens of AFP producing gastric cancer. However, it was not shown in all specimens of AFP non-producing gastric cancer. From the viewpoint of clinical pathology, the incidence of low-differentiated adenocarcinoma and liver metastasis was higher in AFP producing gastric cancer. The argyrophilic nuclear organizer region (Ag-NOR) score and area of AFP producing gastric cancer were 3.28±1.19 and 4.68±1.57μm^2, respectively, significantly (P<0.05) higher than those of AFP non-producing gastric cancer. On the other hand, the incidence of the aneuploid pattern of AFP producing gastric cancer was 80.0%, significantly (P<0.05) higher than that of AFP non-producing gastric cancer. Moreover, the 4c exceeding rate (4cER), which is defined as the percentage of cells with an aneuploid nuclear DNA content over the tetraploid (4c) of AFP producing gastric cancer was 23.07±7.97%, significantly (P<0.05) higher than that of AFP non-producing gastric cancer.

In conclusion, the poor prognoses of patients with AFP producing gastric cancer may be attributed to the extremely malignant potential of neoplastic cells.

Key words: AFP producing gastric cancer, Ag-NOR score and area, DNA ploidy pattern, biological malignant potential

INTRODUCTION

α-fetoprotein (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 (2,3). The serum level of AFP has been revealed to be elevated in other tumors, most of them gastric cancers (4,5). Whether the AFP is produced by the gastric cancer or the regenerative hepatocytes around the metastatic foci remains unknown. However, the prognosis of patients diagnosed with AFP producing gastric cancer is extremely poor. Matsunou *et al.* concluded that AFP producing gastric carcinomas were differentiated in the fetal intestine, which is a blastomatous characteristic, and that those tumors develop in association with intestinal metaplasia (8).

Recently, the measurement of cell proliferative activity has been shown to be effective in determining the malignant potential of various carcinomas; thus,

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the activity has been measured with the use of nuclear organizer region (Ag-NOR)-associated proteins, DNA ploidy analysis, Ki-67, or bromodeoxyuridine (BrdU) immunostaining (9,10,11). There have been many reports on the cell proliferative activity of gastric cancer cells; however, there have been few on the malignant potential of AFP producing gastric cancer. We strongly suspect that there is a striking difference between the tumor characteristics of AFP producing gastric cancer and those of AFP non-producing gastric cancer. Therefore, in the present study, we investigated the malignant potential of AFP producing gastric cancer and AFP non-producing gastric cancer by means of Ag-NOR and DNA analysis of cancer cells.

PATIENTS AND METHODS

For the present study, 84 patients were selected from among those who underwent primary gastric carcinoma resection at Shimane Medical University from 1988 to 1996. The patients ranged in age from 26 to 74 years (average, 62.2); 35 were male and 49, female. The preoperative serum AFP values of all patients were examined by radioimmunoassay, and patients were classified into two groups according to those values. If the serum AFP level was in excess of 20 ng/ml, the patient was placed in the AFP-positive group. If it was under 20 ng/ml, the patient was placed in the AFP-negative group. In our study, the AFP-positive group consisted of 12 patients with serum AFP values ranging from 48ng/ml to 14,700ng/ml.

AFP staining

The specimens were fixed in a 10% neutral buffered formaldehyde solution and embedded in paraffin. They were then stained with hematoxylin and eosin (H&E) and examined histologically. They were also examined immunohistochemically to determine the presence of the tumor-associated antigen AFP. 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 followed 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 10 minutes, counterstained with methyl green, and mounted. Hepatocellular carcinoma cell tissues containing AFP were used as positive controls. The first antibody was replaced with a sodium phosphate buffer for negative controls.

Ag-NOR technique and counting procedure

Sections were cut at a thickness of 3μ 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 give 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 strongly stained as black nuclear dots of various sizes. The number of Ag-NOR nuclear dots was also calculated with an image analyzer.

DNA analysis

For DNA analysis, the tumor area within the paraffin-embedded material was identified by a pathologist using hematoxylin and eosin-stained reference sections. One Four-μm sections were cut, dewaxed, and rehydrated. Then, the sections were stained with Feulgen using a quantitative 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 calculated. In the present study, we investigated clinicopathological factors, such as clinical stage, tumor size, lymph node metastasis, perivascular invasion, and tumor differentiation, in both AFP producing gastric cancer and AFP non-producing gastric cancer. Furthermore, the Ag-NOR index (score and area) and DNA parameters (ploidy pattern and 4c ER) in both AFP producing gastric cancer and AFP non-producing gastric cancer were investigated.

Statistical analysis

All values were expressed as the mean±standard deviation. Statistical analysis was conducted using the Student's t-test or the χ^2 square test. $P<0.02$ was considered statistically significant.

RESULTS

The immunohistochemical expression of AFP was demonstrated in all specimens of the AFP-positive group. AFP was stained diffusely in the cytoplasm of each carcinoma cell (Fig 1). In the present study, the clinicopathological factors of the AFP-positive and AFP-negative groups were examined. In this study, tumors were divided into two histological subgroups; one was that of well-differentiated adenocarcinomas, which consisted of papillary adenocarcinomas, and the other was that of low-differentiated adenocarcinomas, which consisted of poorly differentiated adenocarcinomas, signet ring cell carcinomas, and mucinous adenocarcinomas. There was no significant difference between the two groups except that the incidence of low differentiated adenocarcinomas in the AFP-positive group was higher than that in the AFP-negative group. Significant differences existed with respect to venous invasion between the two groups. Moreover, the incidence of synchronous liver metastasis in the AFP-positive group was significantly higher. In addition, the incidence of advanced stage according to the histological stage (Stage Ⅲ or Ⅳ) in the AFP-positive group was significantly higher than that in the AFP-negative group. Many of the patients in the AFP-positive group died of liver metastasis or peritoneal dissemination within two years of surgery even if they had undergone curative resection.

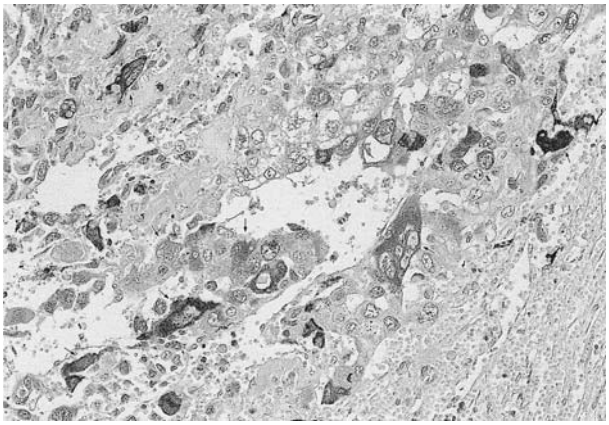

Fig. 1. Immunohistochemical expression of AFP of specimens surgically resected in the case of AFP producing gastric cancer. (→)

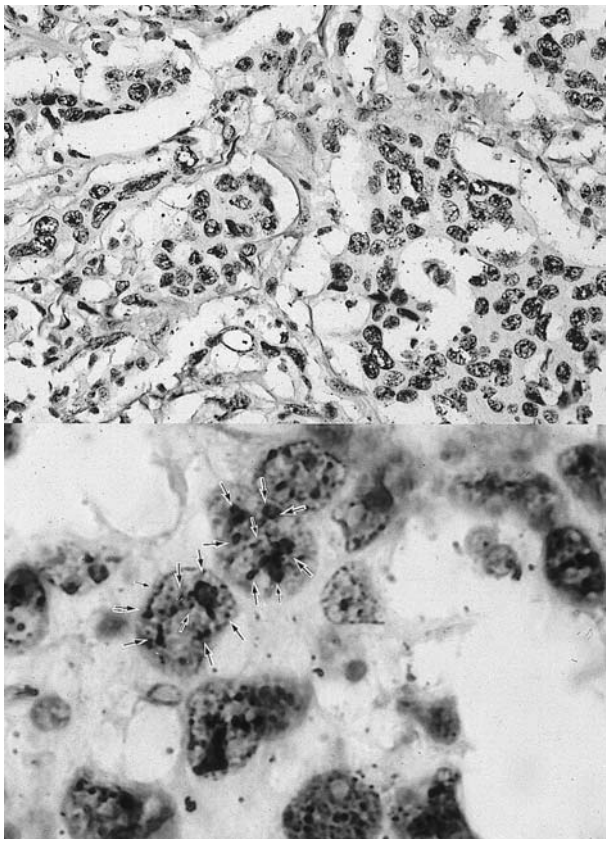

Fig. 2. Argyophilic nuclear organizer regions (Ag-NORs) (→) are shown as black dots in the nucleus of a carcinoma cell (upper;×100, lower;×400).

In all specimens, Ag-NORs were strongly stained as black nuclear dots of various sizes in normal and cancerous cells (Fig 2). The mean values of the Ag-NOR score and area in normal gastric epithelium examined in all 84 patients were 1.85±0.21 and 1.54 ±0.32μm^2. On the other hand, the mean value of the Ag-NOR score of the neoplastic cells of AFP producing gastric cancer was 3.28±1.19, significantly ($p<0.05$) higher than that of AFP non-producing gastric cancer. On the contrary, the mean value of the Ag-NOR area of the neoplastic cells of AFP producing gastric cancer was 4.68±1.57μm^2, significantly ($p<0.05$) higher than that of AFP non-producing gastric cancer (Table 1). From the viewpoint of the DNA ploidy pattern of neoplastic cells, the incidence of the aneuploidy pattern of AFP producing gastric cancer was significantly ($p<0.05$) higher than that of AFP non-producing gastric cancer. The 4c ER of AFP producing gastric cancer was 23.07±7.97%, significantly ($p<0.05$) higher than that of AFP non-producing gastric cancer. Table 2 shows the DNA ploidy pattern and 4c ER of neoplastic cells in both AFP producing and AFP non-producing gastric cancer.

Table 1: There was no siginificant difference between the AFP positive group and the AFP negative group in low differentiated adenocarcinoma. But, in venous invasion, there was significant difference between the two groups in level v2 and v3. Also, significant difference was seen in liver metastasis between the two groups. In macroscopic staging, there was significant difference between the two groups in stages Ⅲ and Ⅳ. Lastly, between the two groups, there was significant difference in death cases within two years after operation.

	AFP positive group (n=12)		AFP negative group (n=72)		
well differentiated adenocarcinoma	4 cases (33.3 %)	N.S	30 cases (41.7 %)		
low differentiated adenocarcinoma	8 cases (66.7 %)		42 cases (58.3 %)		
venous invasion	v0 0 –		v0 55 cases (76.4 %)		
	v1 0 –		v1 11 cases (15.3 %)		
	v2 4 cases (33.3 %)	100% ※	v2 4 cases (5.6 %)	8.4%	
	v3 8 cases (66.7 %)		v3 2 cases (2.8 %)		
lymphatic invasion	ly0 2 cases (16.7 %)		28 cases (38.9 %)		
	ly1 7 cases (58.3 %)		31 cases (43.1 %)		
	ly2 2 cases (16.7 %)		11 cases (15.3 %)		
	ly3 1 cases (8.3 %)		2 cases (2.8 %)		
liver metastasis (synchronously or unsynchronously)	10 cases (83.3 %)	※	3 cases (4.2 %)		
macroscopic staging					
Stage Ⅰ	0 –		28 cases (38.9 %)		
Stage Ⅱ	0 –		38 cases (52.8 %)		
Stage Ⅲ	2 cases (16.7 %)	100% ※	6 cases (8.3 %)	8.3%	
Stage Ⅳ	10 cases (83.3 %)		0 –		
Death cases within two years after operation	11/12 cases (91.7 %)	※	3/72 cases (4.2 %)		

※ χ^2 square test
$p<0.01$

Table 2: The mean value of Ag NOR count and area of the neoplastic cell were significantly higher than those of normal gastric epithelium. Moreover, those of AFP producing gastric cancer were significantly higher than those of AFP non-producing gastric cancer.

	Normal gastric epithelium (n=84)		AFP producing gastric cancer (n=12)		AFP non-producing gastric cancer (n=72)
Ag NOR score	1.85±0.21	※	3.28±1.19	※	2.45±0.98
Ag NOR area (μm^2)	1.54±0.32	※	4.68±1.11	※	2.68±1.12

※ $p<0.001$ by Student's t-test

DISCUSSION

AFP is a normal product of the fetal liver and yolk sac. At present, it is commonly used for diagnosing hepatocellular carcinoma. However, 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 (13,14). The incidence of AFP producing gastric cancer is reported to be 1.3-1.5% (15), occurring in about 5% of gastric cancer patients in Japan. It has been reported that gastric carcinomas have a close relationship with hepatomas because both the stomach and the liver are derived from the primitive foregut of the embryo. Therefore, in some primary gastric carcinomas, disturbances of differentiation occur. Thus, it is not surprising that some gastric carcinomas contain foci of hepatocellular differentiation. These gastric carcinoma cells tend to produce AFP in great amounts, as do most neoplastic hepatocytes (7). They can also produce some other substances which normal hepatocytes produce, including albumin, alpha-1 antitrypsin, and transferin (16). Kodama *et al.* (16) reported that there are two histological types of AFP producing gastric cancers of the stomach; the first is the medullary type, characterized by polygonal cells arranged in solid nests or sheets with scattered large pleomorphic or multinucleated giant cells, and the second is the well-differentiated papillary or tubular type, which has a clear cytoplasm without mucin or glycogen. Furthermore, these researchers pointed out that the medullary type somewhat resembles liver cell carcinoma in morphology. These histological characteristics correspond to those of hepatoid adenocarcinomas of the stomach. Compared with hepatoid adenocarcinomas of the stomach, which have high serum AFP levels, gastric carcinomas, which have relatively low serum AFP levels, may be categorized as a slightly different entity. The histological features of those tumors are, in general, well-differentiated adenocarcinomas of the intestinal type. Gastric mucosal cells with intestinal metaplasia can produce AFP as effectively as embryonal intestinal mucosal cells can. Therefore, many gastric carcinomas with relatively low AFP levels may show intestinal rather than hepatic differentiation. It should be noted, therefore, that not all gastric carcinomas with AFP production are hepatoid adenocarcinomas of the stomach.

The criteria for diagnosing AFP producing gastric cancer depend on positive staining of the primary lesions by immunohistochemical methods. Several diseases, such as hepatitis, liver cirrhosis, and hepatocellular carcinoma, show high levels of serum AFP that are not related to the gastric lesion. On the other hand, a normal serum AFP level alone cannot identify a tumor as an AFP producing tumor. Chang and his associates (17) reported the case of a patient who had a normal serum AFP, yet showed strongly positive immunohistochemical staining. Consequently, the term, AFP producing gastric cancer, can be clinically used for gastric specimens that are immunohistochemically stained. In the present study, all twelve cases with a high serum level of AFP were immunohistochemically stained; however, a shadow of staining was recognized only in the cytoplasm of carcinoma cells of the stomach, indicating that each carcinoma cell may have produced AFP in it. Moreover, seven cases with a serum AFP level above 2,000ng/ml were strongly stained, and two cases with a serum AFP level below 100ng/ml were weakly stained (data not shown). Kodama and his associates (16) reported that the coexistence of an elevated serum AFP and a significant number of AFP-positive tumor cells was recognized in some cases. However, an overall quantitative correlation between the serum AFP levels and degree of immunohistochemical AFP positivity was not demonstrated. On the other hand, Alpert (4) *et al.*, using human liver cell carcinomas xenotransplanted to athymic nude mice, demonstrated that the storage of AFP in a tumor and its release into the blood stream were well balanced and that the serum AFP levels reflected the degree of tumor growth in the host. These results were in agreement with our present result and revealed that the intensity of immunohistochemical staining tended to correlate with the serum AFP levels.

Chang *et al.* (17) advocated that the variables demonstrating a significant difference between AFP producing gastric cancer and AFP non-producing gastric cancer were venous and lymphatic invasion, which led to an advanced stage and metastases. They reported the case of a patient with AFP producing gastric cancer with a tumor thrombus along the splenic,

inferior mesenteric, and portal veins; this patient had multiple liver metastases, suggesting that early venous invasion may contribute to the high incidence of liver metastasis. The poor prognosis and high incidence of liver metastasis imply that AFP producing gastric cancer is quite different from the usual type of gastric cancer. Indeed, the prognosis of AFP producing gastric cancer is clinically poor, although the exact reason for this is not known. Accordingly, it is necessary to investigate why the prognosis for this type of cancer is poor in relation to its biological malignant potential.

In recent years, there have been many reports about the biological malignant potential of cancer cell using several methods (18,19,20). Analyzing the DNA content of neoplastic cells in gastric cancer fields, Kimura and Yonemura proposed that the DNA ploidy pattern was a useful prognostic indicator of advanced gastric cancer and 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 a poor prognosis even after curative surgery (20). On the other hand, a silver technique for identifying Ag-NORs has been applied to various cancerous lesions (21,22). The assessment of the Ag-NOR score is particularly interesting in cancer research because it is well known that this score is greater in neoplastic cells than in corresponding normal tissues. The Ag-NOR score is strictly related to cell proliferative activity and increases during the G1-phase, reaching its peak during the S-phase in the cell cycle. From these facts, it is strongly confirmed that DNA analysis and Ag-NOR analysis are reliable methods to evaluate the biological malignant potential of carcinoma cells. In our study, the incidence of the aneuploid pattern in AFP producing gastric cancer was significantly higher than that in AFP non-producing gastric cancer. Moreover, we investigated the 4c ER in the DNA histogram of all patients and found that the 4c ER in AFP producing gastric cancer was significantly higher than that in AFP non-producing gastric cancer. This result confirmed that the proliferative activity of carcinoma cells of AFP producing gastric cancer was extremely high compared to that of the usual type of gastric cancer (AFP non-producing gastric cancer). On the other hand, Ag-NOR analysis demonstrated that the Ag-NOR score and area were significantly higher in AFP producing gastric cancer than in AFP non-producing gastric cancer. These results also indicated that the malignant potential of carcinoma cells in AFP producing gastric cancer was worse and that this phenomenon might reflect a poor survival rate after surgery, as shown in the present study. Kubo *et al.* (23) reported that AFP producing gastric cancer showed a high incidence of the aneuploid pattern, which suggested that AFP production was one of the malignant phenotypes in gastric cancer. Similarly, Inada *et al.* (24) analyzed the labeling index of the proliferating cell nucleolar antigen of both AFP producing gastric cancer and AFP non-producing gastric cancer. They reported that its percentage in AFP gastric cancer was significantly higher than that in AFP non-producing gastric cancer. The present results are in agreement with the previous report, and we clearly observe a definite difference in the malignant potential of AFP producing gastric cancer and AFP non-producing gastric cancer.

The poor prognosis of AFP producing gastric cancer may be further attributed to the production of AFP because AFP has an immunosuppressive effect (2,15). The presence of alpha 1 antitrypsin (AAT) and the production of AFP by tumor cells might endow hepatoid adenocarcinomas with an invasive character by the additive effect of these substances.

Few reports on the degree of malignancy of gastric cancer have been based on DNA analysis and Ag-NOR staining. The present result that AFP producing gastric cancer had a high 4c ER provides a novel finding in this field.

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