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Establishment of an Enzyme Immunoassay Using Monoclonal Antibody (HG1-219) and Its Application for the Diagnosis of Hepatocellular Carcinoma

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A monoclonal antibody (MoAb HG1-219) against a human gastric cancer cell line (HuG-1) and its shedding antigen (HG1-219 Ag) was generated and a solid-phase sandwich enzyme immunoassay (EIA-219) was developed. The mean serum HG1-219 Ag concentration in normal individuals was 30.5±14.5 U/ml measured by EIA-219. When the mean+3 SD of the antigen concentration in normal individuals was used as a cut-off level, 4.3% (2/47) of patients with chronic hepatitis, 9.1% (4/44) of cirrhotic patients and 37.5% (18/48) of patients with hepatocellular carcinoma (HCC) had HG1-219 Ag above the cut-off value. The positive rates of α-fetoprotein (AFP) (>400 ng/ml) and protein induced by vitamin K absence or antagonist-II (PIVKA-II) for HCC were 26.7% (12/45) and 33.3% (12/36), respectively. There was no significant correlation between HG1-219 Ag and AFP or PIVKA-II in patients with HCC. The combination assay of EIA-219, AFP and PIVKA-II for HCC gave the positive rate of 75% (27/36). The effect of periodic acid on the HG1-219 Ag and the inhibition of EIA-219 by CA 19-9 suggest that the epitope of HG1-219 Ag is a suger chain similar to CA 19-9.

Key words: HuG-1 cell line — Monoclonal antibody HG1-219 — Hepatoma — Carbohydrate antigen

Among many tumor markers so far proposed, α-fetoprotein (AFP) and an abnormal prothrombin termed "protein induced by vitamin K absence or antagonist-II (PIVKA-II) or acarboxyprothrombin" are thought to be reasonably reliable markers for hepatocellular carcinoma (HCC). Since the concentration of AFP is fairly elevated in benign hepatic diseases such as acute hepatitis, chronic hepatitis and liver cirrhosis, 400 ng/ml of AFP has been used as a cut-off level for a specific diagnosis of HCC.[1,2] In our previous study,[3] about 43% of the patients with HCC had a serum AFP level above 400 ng/ml. This positive rate is not sufficiently high to be useful for HCC screening.

On the other hand, since Liebman *et al.*[4] reported that approximately 91% (69/76) of patients with HCC had an elevated level of PIVKA-II in their sera measured by RIA, many studies dealing with PIVKA-II have been reported.[5–7] Recently, the positive rate of this marker in patients with HCC was reported to be 46.2 to 52.9%[6,7] indicating that a considerable number of patients with HCC can not be detected by PIVKA-II. Therefore, the development of other tumor markers for HCC is still needed.

In the present study, we report the establishment of an enzyme immunoassay (EIA) using monoclonal antibody (MoAb) against HuG-1 cell line[8] and its shedding antigen, and its clinical application for the diagnosis of HCC.

MATERIALS AND METHODS

Samples Forty-eight patients with HCC (43 with liver cirrhosis and 5 with chronic hepatitis), 44 patients with liver cirrhosis (LC) and 47 patients with chronic hepatitis (CH) were investigated in this study. Diagnoses were made on the bases of clinical, laboratory, and radiological findings and were confirmed by histological examination of specimens obtained at liver biopsy or autopsy. Twenty-one normal controls were also investigated.

Assays of AFP and PIVKA-II AFP and PIVKA-II were measured with a commercially available radioimmunoassay kit (normal value; < 25 ng/ml) (Alpha-fetoprotein RIA BEAD, Dinabott, Tokyo) and an enzyme immunoassay kit (normal value; < 0.1 U/ml) (Eisai Co. Ltd., Tokyo), respectively.

Cell line HuG-1 cell line was established in our laboratory[8] from a gastric cancer patient and has been maintained in RPMI 1640.

Preparation of shedding antigen from HuG-1 cells HuG-1 cells (5×10^6/ml) were cultured in serum-free RPMI 1640 medium for 24 h. The used medium was passed through a 0.22 μm filter and dialyzed against phosphate-buffered saline (PBS). The dialyzed sample was used as the shedding antigen of HuG-1 cells.

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Immunization and somatic cell hybridization BALB/c mouse was immunized with 3×10^6 HuG-1 cells by four subcutaneous injections at 2-week intervals, followed by a subcutaneous injection of the shedding antigen (32 μg of protein amount) emulsified with Freund's complete adjuvant. Two weeks later, the mouse was immunized with 500 μl (32 μg of protein) of the shedding antigen by intravenous injection and 3×10^6 HuG-1 cells by subcutaneous injection simultaneously. Three days later, the splenocytes of the mouse were fused with mouse myeloma cell line P3-X63-Ag.8U1(P3U1) by the method of Köhler and Milstein.[9] Then hybridomas were selected by enzyme-linked immunosorbent assay (ELISA) as described below and cloned by the limited dilution technique.

ELISA for the selection of the hybridomas Each well of a 96-well plate was coated with the shedding antigen (10 μg of protein) in 10 m*M* carbonate-bicarbonate buffer (pH 8.5). The culture supernatant of each hybridoma was added to each well and incubated at 37°C for 2 h. After the plate had been washed, horse-radish peroxidase(HRP)-labeled goat anti-mouse immunoglobulin antibody (Organon Teknika Corp., West Chester, PA) in buffer A (PBS containing 25% Block Ace) (Yukijirushi Nyugyo Co. Ltd., Tokyo) was added to each well, followed by incubation at 37°C for 2 h. The plate was washed, then the substrate solution (0.02% H_2O_2 and *o*-phenylenediamine in 100 m*M* citrate buffer, pH 5.5) was added, and the mixture was incubated at 37°C for 10 min. The reaction was stopped by adding 4 *N* H_2SO_4, and the resulting solution in each well was measured at 492 nm.

Immunofluorescence staining of HuG-1 cells Immunofluorescence staining of HuG-1 cells or normal human lymphocytes was performed using culture supernatant of the hybridomas according to the method of Nezu *et al.*[10]

Preparation and purification of monoclonal antibody The hybridoma cells (2×10^6), which were confirmed to produce immunoglobulin reacting with HuG-1 cells or the shedding antigen, were injected intraperitoneally into mineral-oil-treated, 8-week-old female BALB/c mice to produce malignant ascites. Seven days later, the ascites was aspirated, passed through a 0.45 μm filter and applied to a Sepharose CL-6B column (2.6×70 cm) which had been equilibrated with 10 m*M* Tris-HCl, pH 7.4, containing 500 m*M* NaCl, 5 m*M* EDTA and 0.02% NaN_3. Immunoglobulin fraction was collected and stored at 4°C until used.

Sandwich-type EIA Each well of a 96-well plate was incubated with 100 μl of MoAb (1 μg of protein/well) in 100 m*M* carbonate-bicarbonate buffer (pH 9.5). After the plate had been blocked with buffer A, 100 μl of shedding antigen solution or 4-fold-diluted human serum with buffer B (buffer A + 50 μg/ml of unrelated mouse immunoglobulin M) was added to each well, followed by incubation at 4°C overnight. After the plate had been washed, 100 μl of biotin-conjugated MoAb, prepared according to the method of Hsu *et al.*,[11] was added and the plate was incubated at 37°C for 2 h. After the plate had been washed, 100 μl or avidin-peroxidase complex (Peroxidase standard PK-4000, Vector Laboratories, Inc., Burlingame, CA) was added to the plate, followed by incubation at room temperature for 30 min. The plate was washed and then the substrate solution (0.02% H_2O_2 and 0.15% *o*-phenylenediamine) was added to each well and the optical density of the resulting solution was measured at 492 nm.

Treatment of HuG-1 cells and the shedding antigen with periodic acid, sialidase, or fucosidase Deparaffinized sections of HuG-1 cells were incubated with 1% periodic acid at 37°C for 30 min, 0.2 U/ml of sialidase (from *Streptococcus* 6646K, Seikagaku Kogyo, Tokyo) in 100 m*M* acetate buffer, pH 6.5, containing 10 m*M* $CaCl_2$ and 0.05% bovine serum albumin at 37°C for 2 h, or 0.2 U/ml fucosidase (from *Fusarium oxysporum*, Sumitomo Seika Co. Ltd., Tokyo) in 50 m*M* citrate buffer, pH 4.5, at 37°C for 2 h. After the sections of HuG-1 cells had been washed with distilled water, each section was stained using the avidin-biotin-peroxidase complex method according to the method of Hsu *et al.*[11]

Shedding antigen-coated plate was treated with periodic acid (0.5%) at room temperature for 1 h, sialidase (0.1 U/ml) in 100 m*M* acetate buffer, pH 6.5, containing 20 m*M* $CaCl_2$ at 37°C for 2 h or fucosidase (0.1 U/ml) in 50 m*M* citrate buffer, pH 4.5, at 37°C for 2 h. After the plate had been washed, EIA was performed, as described above, to determine the reactivity of the MoAb to the modified shedding antigen.

Inhibition test For the inhibition test, 50 μl (5 ng of protein) of MoAb and 50 μl of CA 19-9, CA 125 (Scripps Laboratories, San Diego, CA), or L-fucose at the concentration illustrated in Fig. 5 or 6 were added to the shedding antigen-coated plate simultaneously, followed by incubation at room temperature for 2 h. Then EIA was performed, as described above, to measure the reactivity of the residual antibody.

RESULTS

Selection of hybridomas Following the cell fusion, supernatants from 960 hybrid clones grown in HAT-selective medium were tested by ELISA in order to select clones producing specific MoAb against the shedding antigen. Fifty-four clones were selected at this step. Next, 26 hybrid clones were chosen based on the reactivity of their antibodies with HuG-1 cells but not with normal human lymphocytes. Then eleven clones that can react with the shedding antigen at low concentration (3.9–15 ng/ml)

on serial dilution were chosen by means of our ELISA. Among 11 clones, one clone that reacted strongly with normal human serum was omitted. Finally, two clones, termed HG1-28 and HG1-219, were saved by the immunohistochemical method on paraffin sections of human hepatocellular carcinoma and gastric carcinoma tissues. MoAb HG1-219 was used in the following investigations.

Sandwich EIA A typical standard curve of our sandwich EIA (EIA-219) is shown in Fig. 1. In this assay system, 1 U/ml of HG1-219 antigen (HG1-219 Ag), which is the lower limit of detection, is defined as 3.9 ng/ml of the antigen.

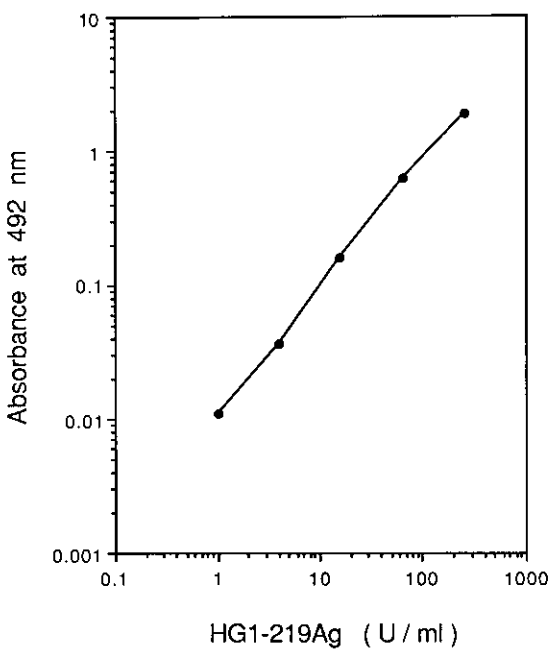

Fig. 1. Standard curve of EIA-219.

Application of EIA-219 to the diagnosis of hepatocellular carcinoma The mean serum HG1-219 Ag concentration in normal individuals was 30.5±14.5 U/ml, as shown in Fig. 2. When the mean+3 SD of the antigen concentration in normal individuals was used as a cut-off level, 4.3% (2/47) of patients with chronic hepatitis, 9.1% (4/44) of cirrhotic patients and 37.5% (18/48) of patients with HCC had HG1-219 Ag concentrations above the cut-off value. So, the sensitivity of EIA-219 for HCC is 37.5% and the specificity of this assay is 91% in patients with LC or 96% in patients with CH. In gastric cancer and lung cancer patients, the positivity rates for this antigen were 27.3% (3/11) and 4.5% (1/22), respectively.

Relationship between HG1-219 Ag concentration and the size of HCC We investigated the relationship between HG1-219 Ag concentration and the size of HCC for eighteen patients who had levels of HG1-219 Ag above the cut-off value. As shown in Fig. 3, HG1-219 Ag concentration seemed to increase in proportion to the tumor mass.

Correlation between HG1-219 Ag concentration and AFP or PIVKA-II level in sera of patients with HCC There was no significant correlation between HG1-219 Ag and AFP or PIVKA-II. HG1-219 Ag also showed no significant correlation with CEA or CA 19-9 levels (not shown).

Positive rate of each tumor marker for HCC and the result of combination assay of these markers The positive rates of AFP and PIVKA-II for HCC were 26.7% and 33.3%, respectively (Table I). As no significant correlation was observed between HG1-219 Ag and AFP or PIVKA-II, we examined combination assays. When

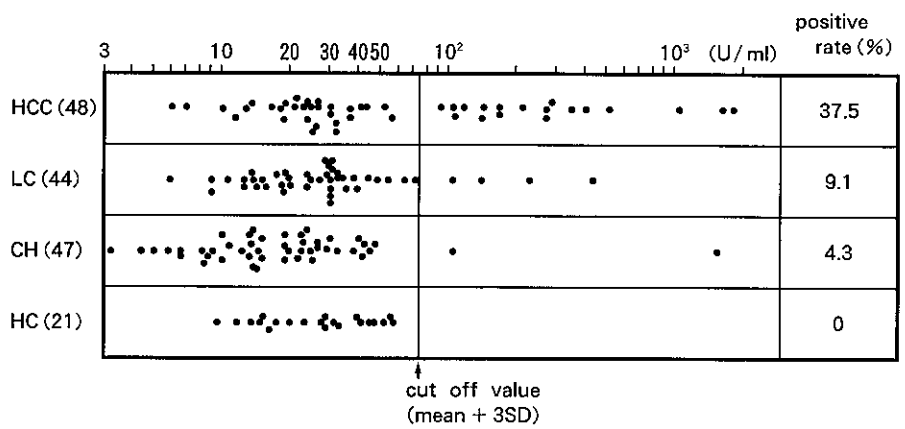

Fig. 2. Positive rate of HG1-219 antigen evaluated by EIA-219.

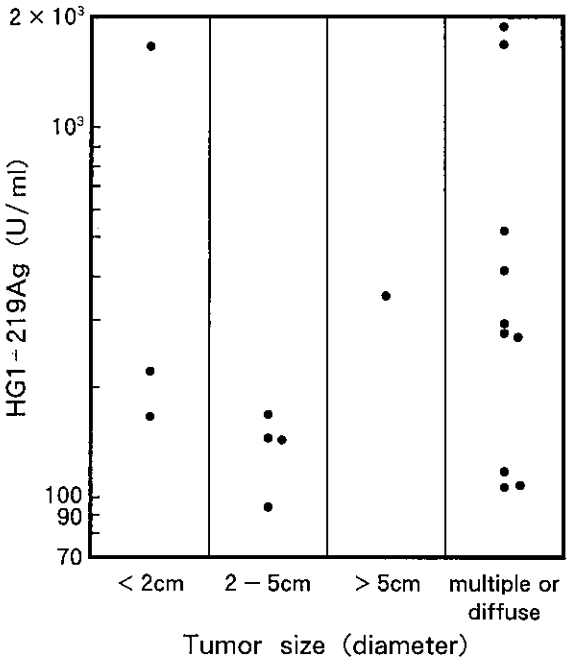

Fig. 3. Relationship between HG1-219 Ag concentration and the size of HCC. The tumor size was measured by ultrasonography

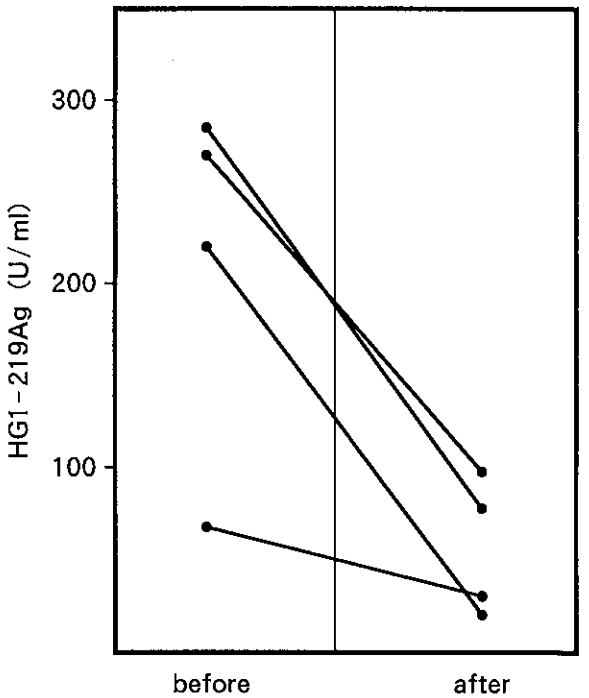

Fig. 4. Effect of therapy of hepatocellular carcinomas on serum levels of HG1-219 Ag.

Table I. Combination Assay

Assay system	Positive rate
(1) EIA-219	18/48 (37.5%)
(2) PIVKA-II	12/36 (33.3%)
(3) AFP (>400 ng/ml)	12/45 (26.7%)
(4) (1)+(2)	24/36 (66.7%)
(5) (1)+(3)	26/45 (57.8%)
(6) (2)+(3)	15/36 (41.7%)
(7) (1)+(2)+(3)	27/36 (75%)

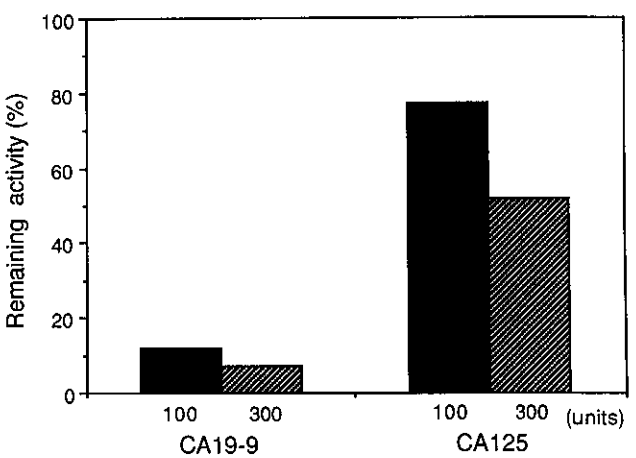

Fig. 5. Effect of CA 19-9 or CA 125 on the binding activity of MoAb HG1-219 to the shedding antigen.

EIA-219 and AFP were used in combination, 57.8% (26/45) of the patients with HCC had elevated levels of either marker or both. On the other hand, combination assay using EIA-219 and PIVKA-II gave a positive rate of 66.7% (24/36) for the patients with HCC, while that of AFP and PIVKA-II gave a positive rate of 41.7% (15/36) for HCC. The combination assay of EIA-219, PIVKA-II and AFP gave the highest positivity rate (75%, 27/36) for the detection of HCC (Table I).

On the other hand, the specificities of AFP and PIVKA-II in patients with LC were 100% (42/42) and 96.7% (29/30), respectively. The specificity of the combination assay using these three markers was 90% (27/30) in patients with LC. As AFP and PIVKA-II were tested in only a small number of patients with CH, we did not determine the specificity for both markers in patients with CH.

Effects of therapy of HCC on serum HG1-219 antigen

As shown in Fig. 4, the elevated levels of serum HG1-219

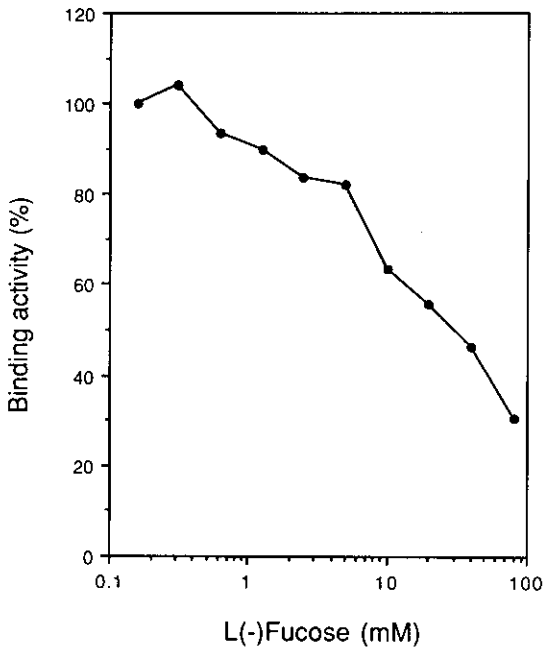

Fig. 6. Effect of L-fucose on the binding activity of MoAb HG1-219 to the shedding antigen.

in four patients with HCC before treatment decreased after transcatheter arterial embolization or percutaneous ethanol infusion therapy, although the number of patients is very small. This result may indicate that HG1-219 antigen is produced in cancer cells.

Characterization of the epitope of HG1-219 antigen

HuG-1 cells treated with periodic acid lost reactivity with MoAb HG1-219 (not shown). This result indicates that the epitope of this antigen recognized by the MoAb HG1-219 is a sugar chain. Then, we examined whether EIA-219 can be blocked by CA 19-9 or CA 125, well-known cancer-associated sugar-chain antigens.[12, 13] CA 19-9 blocked 90% of the reactivity of the antigen with the antibody. CA 125, however, had only a slight effect on EIA-219 (Fig. 5). EIA-219 was not affected by treatment with sialidase (not shown), but L-fucose (40 m*M*) produced a 50% inhibition of EIA-219 (Fig. 6). These results indicate that the epitope of HG1-219 Ag is a sugar chain similar to CA 19-9. Furthermore, L-fucose seemed to bind to or near the epitope of HG1-219 Ag.

DISCUSSION

A new gastric cancer cell line, HuG-1, was established and has been maintained in our laboratory.[8] This cell line has continued to produce CA 19-9, CEA, TPA and a hybrid (placental/intestinal) form of alkaline phosphatase. Therefore, the shedding antigen in the spent medium of HuG-1 cells used as the immunogen in the present study was expected to contain many antigenic substances including CA 19-9, CEA, TPA and a hybrid form of alkaline phosphatase. We tried to produce MoAb using HuG-1 cells and the shedding antigen in the spent medium and succeeded in the production of a new MoAb, HG1-219. Judging from the present data, MoAb HG1-219 seemed to react with a sugar-chain antigen similar to CA 19-9. We measured serum levels of CA 50, another cancer-associated sugar-chain antigen already reported,[14] for three hepatoma patients who all had serum HG1-219 Ag concentrations above 1000 U/ml. Serum levels of CA 50 were within the normal range in all of them. Therefore, HG1-219 Ag seems to differ from CA 50. In our preliminary study, HG1-219 Ag was purified by affinity chromatography using Sepharose 4B coupled with MoAb HG1-219. The molecular weight of HG1-219 Ag was estimated to be more than 200,000 by SDS-PAGE. Although several high-molecular-weight carbohydrate antigens[12–17] detected by MoAbs have been suggested to be useful for cancer diagnosis, most of the epitopes detected by these MoAbs were sensitive to neuraminidase. As HG1-219 Ag was not affected by neuraminidase treatment, this antigen did not seem to contain sialic acid in the epitope. Some information is available on sugar chains without sialic acid in their epitopes.[18, 19] Adachi *et al.*[18] reported a carbohydrate antigen without terminal sialic acid on macromolecular glycoproteins from gastrointestinal cancers. HG1-219 Ag seemed to be more sensitive to periodic acid than the Ag reported by Adachi *et al.* However, as the methods used were different from each other, further characterization of both antigens is needed. Another asialocarbohydrate antigen (antigen YH 206) reported by Hinoda *et al.*[19] seemed to be an adenocarcinoma-associated antigen of mucin type. This antigen was completely different from CA 19-9 in elution profile on affinity chromatography with MoAb YH 206. As the present results showed that HG1-219 Ag was similar to CA 19-9, HG1-219 Ag may be different from Ag YH 206.

As HG1-219 Ag was derived from a gastric cancer cell line (HuG-1), we expected that HG1-219 Ag could be detected in sera of patients with gastric cancer. But our preliminary study showed that serum HG1-219 Ag in only 3 (27.3%) of 11 patients with gastric cancer was elevated above the cut-off level. This positive rate was not satisfactory. Then we tried to measure serum HG1-219 Ag level in patients with HCC or lung cancer, obtaining the results described here. Further mass screenings will be required to clarify the specificity of HG1-219 Ag for HCC.

Patients with liver cirrhosis, especially that caused by hepatitis B and C viruses, frequently develop HCC. In

the present study, among 48 hepatoma patients, 7 patients were positive for HB_s Ag, 18 were positive for anti-hepatitis C virus antibody (HCV Ab) detected with the HCV ELISA test (Ortho Diagnostic Systems, Tokyo), 4 were negative for both virus markers and the remaining 19 cases were not examined for HCV Ab. None of the 48 hepatoma patients investigated had had either alcoholic liver diseases or autoimmune hepatitis as judged from their previous history. HG1-219 Ag was positive in 3 (43.0%) out of 7 patients positive for HB_s Ag and in 8 (44.4%) of 18 patients positive for HCV Ab. The rest of the patients had a 30.4% (7/23) positive rate for HG1-219 Ag. Thus, the emergence of HG1-219 Ag in patients with HCC did not seem to have a specific relationship with hepatitis B virus or hepatitis C virus infection.

On the other hand, five of 48 hepatoma patients were complicated with CH. Among these five patients, two patients had elevated levels of HG1-219 Ag in their sera (286 and 106 U/ml). This result indicated that the positivity rate for HG1-219 Ag in hepatoma patients with CH was 40%, suggesting that this antigen is almost equally expressed in hepatoma patients with or without LC.

Recently, ultrasonography (USG) and computed tomography (CT) have contributed to the detection of small HCC, but the imaging diagnosis of HCC still has some limitations. For instance, it is very difficult to find diffuse-type HCC by USG or CT scan, and HCC emerging in the subdiaphragmatic area is difficult to pick up. Therefore, any tumor markers suggesting the emergence of HCC should be very useful for the detection of HCC. AFP and PIVKA-II are accepted to be good markers for HCC, but their low positive rate makes them of limited value for detecting HCC, as shown in the present study. HG1-219 Ag did not show any significant correlation with either AFP or PIVKA-II. The combination assay of EIA-219 with PIVKA-II gave a better result (Table I). But the combination assay using all three markers (EIA-219, PIVKA-II and AFP) gave the best result. In the present study, HG1-219 Ag was positive in three cases of HCC smaller than 2 cm in diameter, and this may suggest the importance of HG1-219 Ag measurement in patients suspected to have HCC. These three markers (HG1-219 Ag, AFP and PIVKA-II) had relatively low positive rates for HCC when each of them was used individually as a tumor marker (Table I). But, the combination assay using these three markers could contribute effectively to the detection of small HCC in combination with USG and CT scan. Further investigation is needed to clarify the epitope of HG1-219 Ag and to ascertain whether measurement of HG1-219 Ag can be useful in monitoring HCC.

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