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A New CA125-like Antigen (CA602) Recognized by Two Monoclonal Antibodies against a Newly Established Ovarian Clear Cell Carcinoma Cell Line (RMG-II)

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A cell line designated RMG-II was established from the ascites of a patient with ovarian clear cell carcinoma. The chromosomal analysis revealed aneuploidy with a hypertetraploid modal number and 8 marker chromosomes. Radioimmunoassay and immunocytochemical staining showed that RMG-II cells produced some tumor markers such as CA125 and TPA. Two monoclonal antibodies, designated MA602-1 and MA602-6, were generated by immunization of mice with an extract prepared from the culture supernatant of RMG-II cells. The epitopes recognized by these two monoclonal antibodies were proved to differ from the CA125 epitope, but to exist on the molecule bearing CA125. We developed a double-determinant sandwich enzyme immunoassay using these two monoclonal antibodies, and the antigen defined by this assay was termed CA602. CA602 was frequently found in the sera of ovarian cancer patients; the positive rates were 92%, 38%, 60%, and 80% for serous, mucinous, clear cell, and endometrioid ovarian carcinomas, respectively, when the cut-off value was set at 60 U/ml (=mean+3SD of healthy females). CA602 levels in serum were also high in endometriosis patients and in early pregnancy, as is the case for CA125, and the correlation coefficient between CA602 and CA125 was high (r=0.88). Our preliminary evidence suggests that this CA602 assay system has higher sensitivity than the CA125 one.

Key words: Ovarian carcinoma — Monoclonal antibodies — Tumor marker — CA125 — CA602

The necessity for early detection of ovarian cancer and the availability of hybridoma technology[1] have led to the discovery of many tumor markers defined by monoclonal antibodies. Recently, we have found a new ovarian tumor marker, CA54/61, which is characterized by high serum levels in mucinous cystadenocarcinoma and very low false-positive rates in gynecological benign diseases, including endometriosis.[2] Although the positive rate of CA54/61 for ovarian cancer is about 10–20% less than that of CA125,[3,4] the combination of CA54/61 and another tumor marker might be expected to be more useful for ovarian cancer screening than CA54/61 alone, so we tried to find a new ovarian tumor marker which would complement CA54/61, by immunization of mice with a newly established ovarian cancer cell line.

In this paper, we will report the establishment of a new ovarian cancer cell line, RMG-II; the characterization of two monoclonal antibodies raised against RMG-II; and the development and clinical usefulness of a new sandwich EIA system using these two monoclonal antibodies.

MATERIALS AND METHODS

Culture materials and characterization of the cell line
The culture was carried out by the reported methods.[5] In brief, on October, 19th, 1982, ascites from a 53-year-old patient with stage III ovarian carcinoma was used as the starting material. Post-surgical pathological examination revealed that the tumor was a typical clear cell carcinoma. The cells were obtained from ascites by centrifugation, and cultured in Ham's F-12 medium (GIBCO Lab., New York, NY) containing 10% fetal calf serum.

For morphological investigation of the cultured cells, periodic acid-Schiff (PAS)- or Papanicolaou-stained specimens were prepared. Studies of growth curve, doubling time, saturation density, chromosomal analysis, DNA content, and tumorigenicity of RMG-II were carried out as described previously.[6,7]

The localization of CA125 and tissue polypeptide antigen (TPA) in the cultured cells and original tumor was investigated by the immunoperoxidase methods described elsewhere.[8] Anti-CA125 monoclonal antibody (M-Ab) (CIS, Ille, Belgium) and anti-TPA polyclonal antibody (P-Ab) (Sangtec Medical, Broma, Sweden) were used as primary antibodies. CA125 and TPA in 4-

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day culture medium were measured by radioimmunoassay (RIA) with a CA125 kit (Centrocor, Malvern, MA) and a TPA kit (Sangtec Medical).

Antigen preparation To prepare the antigens for immunization and for use in an EIA, we obtained culture supernatants of RMG-II cells grown for about two weeks in serum-free medium, and applied them after concentration to an Ultrogel AcA 22 column (IBF Bio-Technics, Villeneuve La Garenne, France). Material in the void fractions was pooled and termed RMG-II-sptAg. For further purification, RMG-II-sptAg was extracted with perchloric acid at 0.6 *M* final concentration for 30 min at room temperature. The soluble fraction was neutralized to pH 6.5, dialyzed against 50 m*M* Tris-HCl, pH 8.0, then made up to 6 *M* (final) urea and applied to a Sepharose CL-4B column (Pharmacia, Uppsala, Sweden) previously equilibrated with 50 m*M* Tris-HCl (pH 8.0), 6 *M* urea, and 0.1% SDS. The void fractions, named RMG-II-sptAg/4B, were collected and dialyzed against phosphate-buffered saline (PBS). Protein concentration was measured by the method of Lowry *et al.*[9]

Monoclonal antibodies Monoclonal antibodies were generated by the usual method. In brief, BALB/c mice were immunized with 25 μg of RMG-II-sptAg/4B 5 times at 2-week intervals. Four days after the final injection, spleen cells were harvested and fused with mouse P3/×63-Ag-8.U1 myeloma cells by using polyethyleneglycol according to the method of Hales.[10] Hybridomas selectively grown in hypoxanthine-, aminopterin-, and thymidine-supplemented medium were screened for their reactivity to RMG-II-sptAg. The specific antibody-producing hybridomas were cloned by the limiting dilution method.

Two monoclonal antibodies, designated MA602-1 (IgM) and MA602-6 (IgG), were chosen according to the method described below so that they would not have cross-reactivities with one another. The antibodies in the culture supernatants were purified by Ultrogel AcA22 column chromatography followed by salting out with 60% ammonium sulfate.

Enzymatic and $NaIO_4$ treatments Ninety-six-well aminoplates (Sumitomo Bakelite Co., Ltd., Tokyo) were coated (4°C, overnight) with RMG-II-sptAg (1 μg/ml in PBS). After blockage with 0.5% bovine serum albumin (BSA), the RMG-II-sptAg-coated wells were treated with pronase from *Streptomyces griseus* (Boehringer Mannheim GmbH, Mannheim, Germany; 0.8 and 4 U/ml), trypsin from bovine pancreas (Sigma, St. Louis, MO; 0.8 and 4 U/ml) in 100 m*M* Tris-HCl (pH 8.0) containing 5 m*M* $CaCl_2$ for 2 h at 37°C, or $NaIO_4$ (2 or 10 m*M* in 25 m*M* acetate buffer pH 4.5) for 1 h at 4°C. After 5 washes with the wash solution (saline containing 0.005% Tween 20), the treated wells were reacted with horseradish peroxidase (HRP)-labeled MA602-1 (0.5 n*M*), MA602-6 (0.2 n*M*), or OC125 (5 n*M*) antibodies in assay buffer A (10% normal rabbit serum, 0.1% BSA in PBS) for 1.5 h at room temperature. OC125 antibody was kindly donated by Toray-Fuji Bionics (Tokyo). The enzyme reaction was performed as described previously.[2]

Competitive inhibition assay To test the cross reactivity between MA602-1, MA602-6, and OC125 antibodies, a competitive inhibition assay was performed. RMG-II-sptAg-coated wells were prepared as described above. Then HRP-labeled MA602-1 (0.5 n*M*), MA602-6 (0.1 n*M*), or OC125 (5 n*M*) was mixed with another non-labeled competitive antibody (0.1 and 5 n*M*) in assay buffer A, and the mixtures were reacted with the coated RMG-II-sptAg on the well for 1.5 h at room temperature; the enzyme reaction was performed as described above.

Immunoperoxidase staining Formalin-fixed paraffin-embedded tissue specimens of ovarian tumors were stained by the immunoperoxidase method as described previously.[8]

Measurement of CA602 and CA125 levels in sera CA602 antigen as defined by MA602-1 and MA602-6 antibodies was measured by utilization of a double-determinant enzyme immunoassay (EIA) kit (Mochida Pharmaceutical Co., Ltd., Tokyo) according to the manufacturer's protocol. In brief, MA602-1-coated polystyrene beads were incubated with 25 μl of serum sample or RMG-II-sptAg as CA602 standard and 200 μl of HRP-labeled MA602-6 solution. After incubation for 2 h at 37°C, the beads on which MA602-1-CA602-MA602-6 complexes had formed were washed 4 times with wash solution, and enzyme reaction was performed for 20 min with hydrogen peroxide and *o*-phenylenediamine used as the substrate and color developer, respectively. After termination of the enzyme reaction with 3 *M* phosphoric acid, optical density at 492 nm was measured, and the CA602 concentration of sample serum was calculated from the standard curve. Ten nanograms of RMG-II-sptAg measured by this assay was defined as 1 unit of CA602. Precision and accuracy of this CA602 kit were guaranteed by the manufacturer as follows: the intra- and inter-assay coefficients of variation were both less than 7%, 90–110% recovery was shown in the recovery test, and the concentration of CA602 in sera decreased linearly with increasing dilution, with the values obtained being those expected. The detection limit of the CA602 kit was 4 U/ml. Meanwhile, CA125 levels were measured with an immunoradiometric assay kit (Centrocor, Inc., Malvern, PA).

Tissues and sera All the samples of tissues and sera were collected at Keio University Hospital. The diagnosis of each lesion was pathologically confirmed.

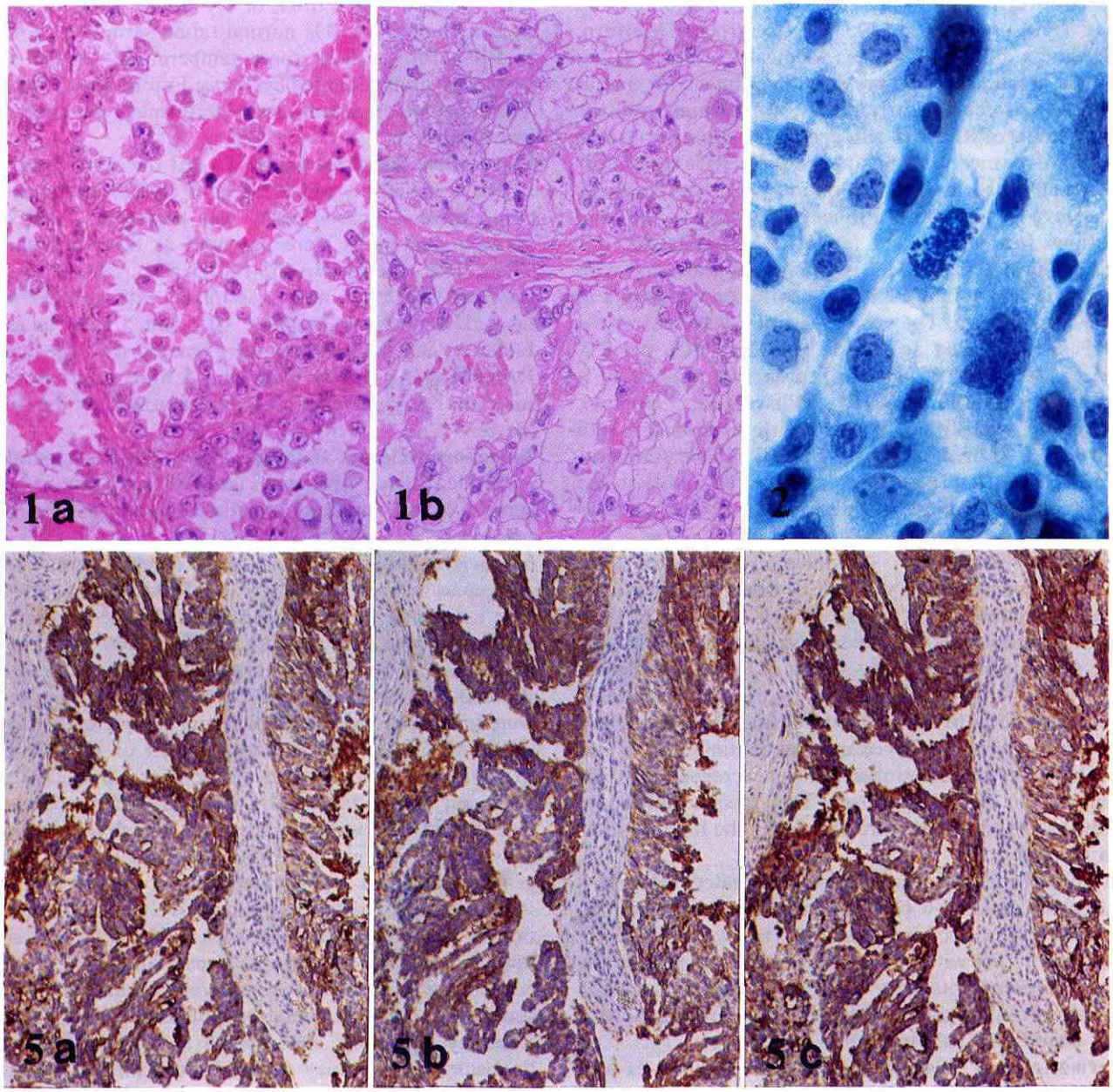

Fig. 1. Hematoxylin- and eosin-stained specimen of the original tumor, which consisted of hobnail-shaped cells (a) and clear cells (b). ×50.

Fig. 2. Papanicolaou staining of RMG-II cells. Spindle- or polygonal-shaped cultured cells appear to be epithelial in nature with a pavement-like arrangement. ×100.

Fig. 5. Immunohistochemical staining of ovarian serous cystadenocarcinoma with MA602-1 (a), MA602-6 (b), and OC125 (c). ×50.

RESULTS

Establishment of a new cell line (RMG-II) and its cell-biological characteristics The original tumor was composed of hobnail-shaped cells with eccentric nuclei and no clear cytoplasm (Fig. 1a) and clear cells with large intracytoplasmic clear areas (Fig. 1b). These cells were arranged in glandular or papillary patterns. From the beginning of culture, no contamination by fibroblasts was observed, and the cultured cells have been growing well

without any interruption for more than 8 years. In monolayer culture, the cells, having nuclei of different sizes, appear as polygons or short spindles of various sizes (Fig. 2). They exhibit a pavement or cobblestone-like arrangement and pile up very often without contact inhibition. PAS-positive substances are present in the cytoplasm and some of them disappear after amylase digestion, proving the existence of glycogen in the cells. The growth curve revealed a doubling time of about 58 h, and saturation density and plating efficiency were 8.0×10^4 cells/cm^2 and 42%, respectively. The number of chromosomes varied widely, showing aneuploidy. The modal chromosome number was hypertetraploid, which is consistent with the result on the DNA index (=2.3) obtained by flow cytometry (data not shown), and G-band staining revealed 8 marker chromosomes (Fig. 3). RMG-II cells have not been heterotransplanted to nude mice.

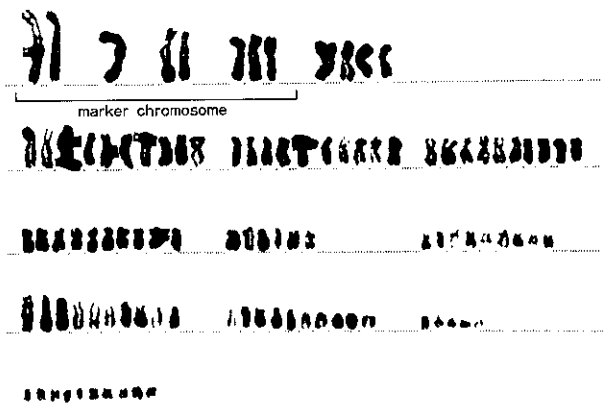

Fig. 3. Karyotype analysis of RMG-II cells. Application of the G-banding method revealed 8 marker chromosomes.

Immunocytohistochemistry revealed that CA125 and TPA were present in the original tumor and in the cultured cells. RIA of these tumor markers found in the culture medium also confirmed their production (CA125: 1240 U/ml/4 days, TPA: 12600 U/1/4 days).

Characterization of epitopes recognized by MA602-1 and MA602-6 MA602-1 and MA602-6 were able to form the sandwich complex not only with each other, but also with OC125 antibody (unpublished results), suggesting that the epitopes of MA602-1, MA602-6, and CA125 exist on the same molecule.

The properties of epitopes recognized by MA602-1 (CA602-1) and MA602-6 (CA602-6) were examined by means of the treatments shown in Table I. The reactivities of MA602-1 and MA602-6 with RMG-II-sptAg were greatly diminished by proteolytic enzyme digestions, while they showed no reduction following $NaIO_4$ treatment, even at 10 m*M*. These results suggest that these antibodies recognize at least the protein part of the antigen molecule.

Since RMG-II cells secrete CA125, the relationship between MA602-1, MA602-6, and OC125 was investigated. As shown in Fig. 4, the results of the competitive inhibition assay suggested that each antibody recognized a different epitope, in view of their minimal cross-reactivities.

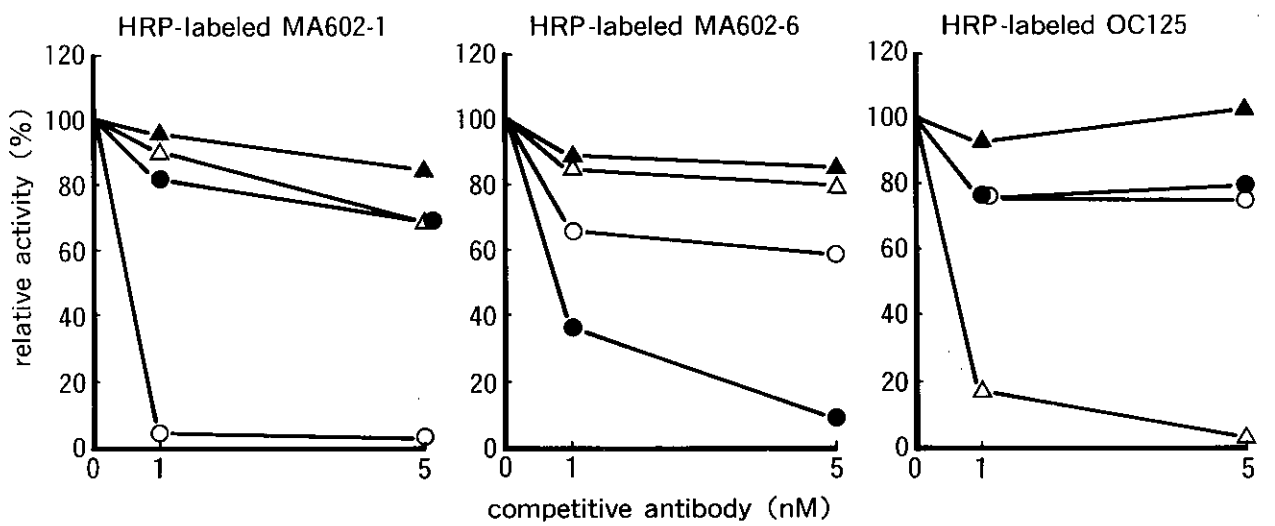

Fig. 4. Result of competitive inhibition assay between MA602-1, MA602-6, and OC125. The competitive antibodies, MA602-1 (○), MA602-6 (●), OC125 (△) and anti hCG β subunit antibody HM70 (▲) as a negative control, were added at the concentration of 1 or 5 n*M* to the HRP-labeled antibodies. Relative activities of bound HRP-labeled antibodies were determined.

Immunohistochemical reactivity of MA602-1, MA602-6, and OC125 Since immunohistochemical staining revealed that the sites positive for MA602-1, MA602-6, and OC125 binding in cancer tissues looked almost the same (Fig. 5), MA602-1 was used to determine the localization of CA602. The immunohistochemical reactivity in each gynecological tumor specimen was evaluated on the basis of both staining intensity and incidence of positive cells. The intensity was graded on an arbitrary scale into (+), (++), and (+++), and the incidence was classified into three grades (less than 10%, 11% to 50%, and more than 51%) according to the percentage of positive cells among all the tumor cells in each section. By combining both intensity and incidence, we divided the reactivity of each specimen into weakly (W), moderately (M), or strongly (S) positive (Table II).

The immunohistochemical reactivity of MA602 is summarized in Table III. Of ovarian benign cystadenomas, one of the 2 cases of the serous type and one of the 3 cases of the mucinous type were weakly positive, giving a total positive rate of 40% (2/5). In the case of the epithelial cystadenocarcinomas, the total positive rate was 89% (16/18). Six out of the 7 serous type, 1 out of the 5 mucinous type, 1 of the 2 clear cell type, and 3 of the 4 endometrioid type showed a moderately or strongly positive reaction. For uterine cervical carcinomas and endometrial carcinomas, the positive rates were 76% (26/34) and 25% (8/32), respectively, but most of them showed weakly positive reactivities (data not shown).

Serum CA602 levels in patients with various gynecological diseases The mean value and standard deviation of CA602 in sera of 126 healthy females were 17.0 U/ml and 14.9 U/ml, respectively; and only one case (1/126) was positive when the cut-off value was set at 60 U/ml (=mean+3SD). The mean value for healthy females in their thirties was the highest (less than 19 years old: 17.1 U/ml, 20–29: 16.1, 30–39: 26.7, 40–49: 23.5, 50–59: 11.6, 60–69: 10.6, over 70: 8.3); and among various stages of the menstrual cycle of 12 healthy women, the mean value was the highest during the menstrual period (data not shown). The positive rate in sera of pregnant women was 25% (21/84). During the course of gestation, all of the positive cases were observed during the 5th to 11th gestational weeks (data not shown).

For CA602 antigen in sera of patients with uterine myoma or ovarian benign tumor except endometrioid cyst, the positive rate was 7% (2/27) or 8% (3/38), respectively, and their mean levels were under the cut-off value. Positive rates in sera of patients with endometriosis such as adenomyosis and ovarian endometrioid cyst were 47% (7/15) and 72% (26/36), respectively, and the mean level in endometrioid cyst patients was very high (406.1 U/ml). In serum samples

Table I. Characterization of Antigen Recognized by MA602-1 and MA602-6

Enzymatic or $NaIO_4$ treatment		Remaining activity after treatment (%)		
		MA601-1	MA602-6	OC125
No treatment		100	100	100
Pronase	0.8 U/ml	23	0	0
	4.0 U/ml	23	0	0
Trypsin	0.8 U/ml	55	96	0
	4.0 U/ml	17	31	15
$NaIO_4$	2 m*M*	113	114	98
	10 m*M*	117	117	100

Table II. Criteria for Immunohistochemical MA602-1 Reactivity

Frequency (%)	Intensity		
	+	++	+++
1–10	±	W	W
11–50	W	M	M
51–100	W	M	S

W: weakly positive. M: moderately positive. S: strongly positive.

Table III. Immunohistochemical MA602-1 Reactivity in Ovarian Tumors

Tissues	Total no.	Reactivity				Positive rate (%)
		±	W	M	S	
Serous cystadenoma	2	1	1	0	0	50
Mucinous cystadenoma	3	2	1	0	0	33
Serous cystadenocarcinoma	7	0	1	5	1	100
Mucinous cystadenocarcinoma	5	1	3	1	0	80
Clear cell carcinoma	2	0	1	1	0	100
Endometrioid carcinoma	4	1	0	3	0	75

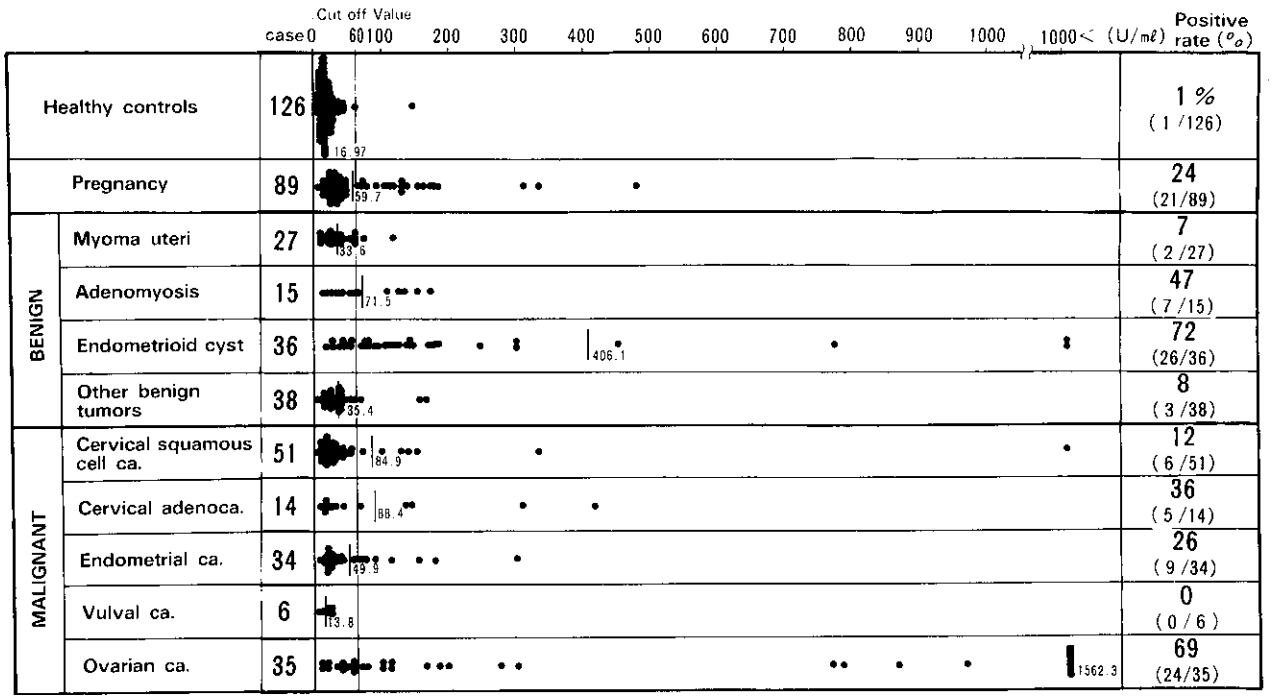

Fig. 6. Serum CA602 levels in various kinds of gynecological benign diseases and cancer patients. The cut-off value was set approximately at the mean+3SD (60 U/ml) of healthy females.

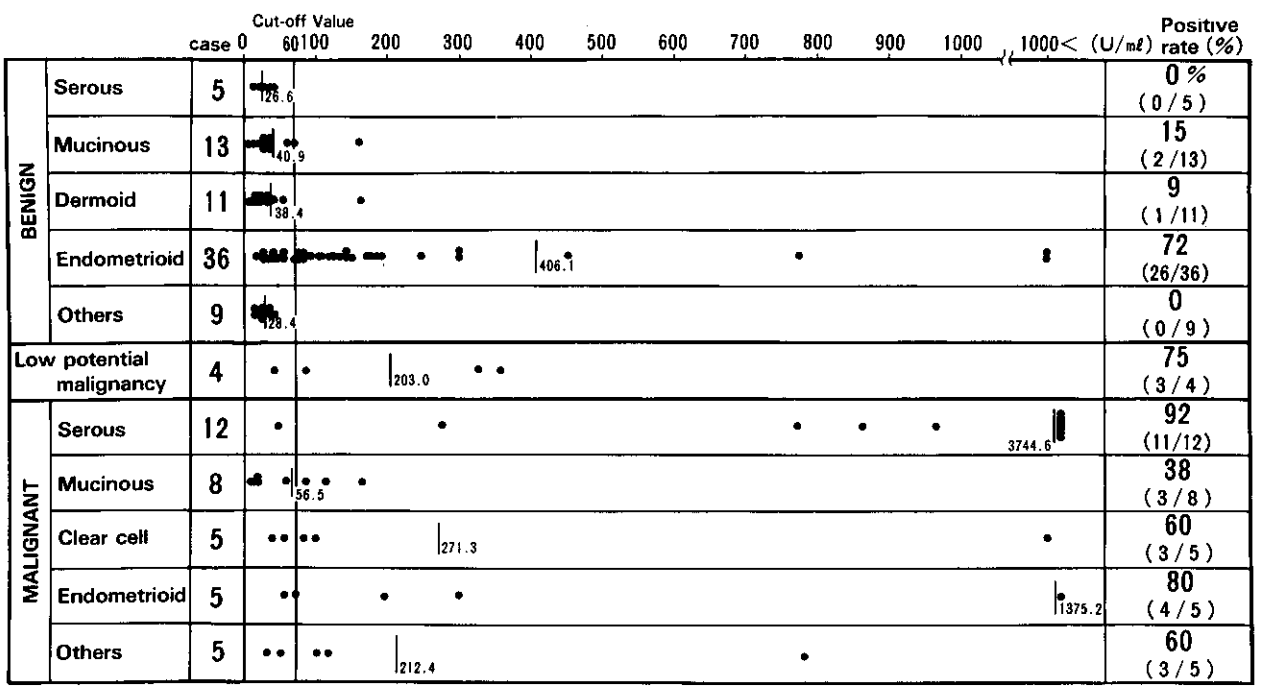

Fig. 7. Serum CA602 levels in various histological types of ovarian tumor. Mean values are indicated by the vertical bar in each row.

from patients with various gynecological carcinomas, the highest positive rate, 69% (24/35), was obtained from patients with ovarian carcinoma. In patients with other carcinomas, the positive rate was less than 30% except for the rate of 36% (5/14) for patients with cervical adenocarcinoma (Fig. 6). Fig. 7 indicates the serum CA602 levels in various kinds of benign and malignant ovarian tumors. Positive rates for serous cystadenocarcinoma (92%), endometrioid carcinoma (80%), and endometrioid cyst (72%) were high. On the other hand, mucinous cystadenocarcinomas showed a much lower positive rate, 38%. This pattern resembles that of CA125 very closely.

Correlation between serum CA602 and CA125 or CA54/61 levels in ovarian tumors Levels of CA602 and CA125 were assayed simultaneously in sera from 48 cases of benign ovarian tumors, 2 of low potential of malignancy, and 20 of malignant ovarian tumors (Fig. 8). A significant correlation was observed between the serum CA602 and CA125 levels (r=0.88). With respect to the relationship between the assay kits, 258 CA602 units of the standard antigen, RNG-II-sptAg, was equal to 108 CA125 units. Simultaneous measurements of CA602 and CA54/61 serum levels were performed preliminarily (data not shown), and these data suggest no correlation between CA602 and CA54/61 levels.

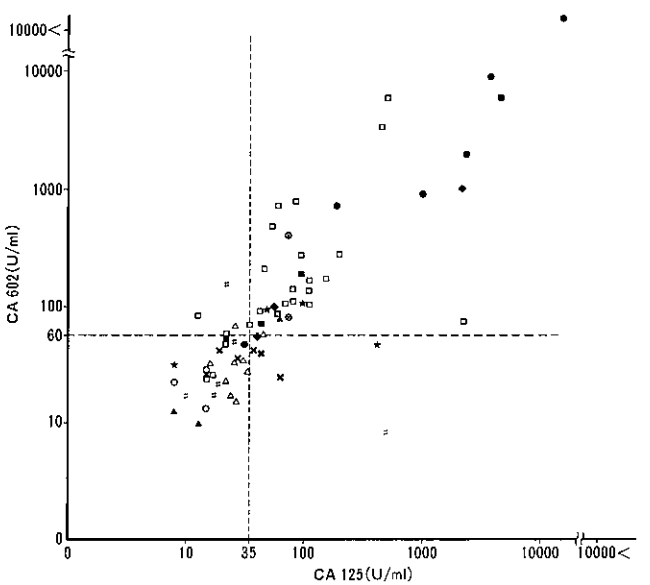

Fig. 8. Correlation between CA602 (Y-axis) and CA125 (X-axis) levels. Each histological type was diagnosed as serous cystadenoma (○), mucinous cystadenoma (△), endometrioid cyst(□), dermoid cyst (#), other benign tumor (×), tumor of low potential malignancy (⊗), serous cystadenocarcinoma (●), mucinous cystadenocarcinoma (▲), endometrioid carcinoma (■), clear cell carcinoma(◆), or other malignant tumors (★).

DISCUSSION

RMG-II cells were identified as a clear cell carcinoma cell line on the basis of the following facts: 1) the RMG-II cells grew well for more than 8 years with no contact inhibition, 2) chromosomal analysis revealed aneuploidy with a hypertetraploid modal number, 3) the original tumor was histologically a typical clear cell carcinoma and there was no evidence of any other kind of malignancy from the pathological examination of the patient, 4) the result of immunocytohistochemical staining of tumor markers showed similar findings between the original tumor and RMG-II cells. Facts 1 and 2 suggest RMG-II cells to be malignant in nature; and from the facts 3 and 4, we conclude that RMG-II originated from an ovarian clear cell carcinoma cell.

The result of the epitope analysis for the two monoclonal antibodies, MA602-1 and MA602-6, suggests that the epitopes recognized by these two antibodies are defined by the protein moiety of the antigen molecule, which includes CA125, but are different from the OC125 epitope and from each other.

Matsuoka *et al.*[11] reported a monoclonal antibody, 130-22, which also recognized a different epitope on the molecule bearing CA125. It will be interesting to investigate the relationships among the epitopes recognized by these four monoclonal antibodies, and these antibodies may be useful for analysis of the CA125 molecule itself. The immunohistochemical studies with MA 602- 1, MA602-6, and OC125 support our above speculation, because the three staining patterns were almost the same and high positive rates of moderate or intense staining were demonstrated for ovarian cancer, especially for serous and endometrioid types.

CA602 and CA125 levels in serum were very similar in the following points. Firstly, CA602 levels fluctuated during the menstrual cycle and gestational weeks in the same manner as CA125.[12] Secondly, the observation of high CA602 levels in serous cystadenocarcinoma and endometriosis, and low levels in mucinous cystadenocarcinoma, is similar to that of CA125 as well.[13] The correlation between CA602 and CA125 (r=0.88) also suggested the similarity of these two markers, so that the epitopes for MA602-1 and MA602-6 may exist on the molecule carrying CA125.

Clinically, CA602 has several weak points such as low positive rate in ovarian mucinous cystadenocarcinoma and relatively high false-positive rate in endometriosis, just like CA125. But, as these deficiencies could be complemented by CA54/61, and simultaneous measurements of CA602 and CA54/61 revealed no correlation between

these two markers, the combination assay of CA602 and CA54/61 should be very useful for ovarian cancer detection.

Furthermore, the double-determinant EIA for detection of CA602 showed high sensitivity, possibly because the two antibodies recognized different epitopes on the CA602 molecule. Although the detection limits of CA602 and CA125 are almost the same (CA602: 4 U/ml, CA125: 5 U/ml guaranteed by manufacturer), the CA602 kit is at least two-fold better than the CA125 one with respect to sensitivity, because 258 CA602 units of RMG-II-sptAg were equal to 108 CA125 units. As a matter of fact, the assay data in Fig. 8 demonstrate good agreement with this result. A preliminary follow-up study for post-operative cancer patients suggested that measurement of very low levels of serum CA602 was reliable due to the high sensitivity of this assay system. Therefore, it seems that the CA602 assay system would be more useful than the CA125 one in terms of the possibility of early detection of ovarian cancers, including recurrence. In conclusion, CA602 may be valuable as a new CA125-like tumor marker.

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