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Establishment of OVS_1 and OVS_2 Monoclonal Antibodies Recognizing Human Ovarian Mucinous Cystadenocarcinoma

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SUMMARY Two newly established murine monoclonal antibodies (MAbs), OVS_1 and OVS_2, to human ovarian mucinous cystadenocarcinoma were further characterized for diagnostic efficacy. The specific SA-1 antigen, purified from the tumor extract was identified as a glycoprotein of 29 kDa. A double determinant biotin-streptavidin alkaline phosphatase immunoassay system, containing OVS_1 and OVS_2 MAbs was used to determine the SA-1 levels in serum. The OVS_1 MAb was used as a first antibody because of its high specificity of 96% while OVS_2 MAb, with a lower specificity of 8% but greater sensitivity of 78%, was chosen as a second antibody. Matched sera of 64 healthy controls and 90 patients with definite diagnoses of 25 benign diseases, 14 nonovarian cancer and 51 ovarian cancer, were simultaneously measured together with CA 125 values. At cut-off levels of 220 and 360 units/ml, the SA-1 test showed 63% and 43% positive rates respectively in all types of ovarian cancer, compared to 65% and 57% positive rates for CA 125 at cut-off levels of 35 and 60 units/ml, respectively. Sensitivity for SA-1 at 220 units/ml cut-off level in mucinous ovarian cancer was 75% and increased significantly to 85% when the test was combined with CA 125 at 35 units/ml cut-off level. Furthermore, The combination of both tests significantly increased the positive rates to 86% in all types of early stage ovarian cancer. The data suggested that the SA-1 antigen detected by OVS_1 and OVS_2 MAbs may be a useful ovarian cancer marker with the advantage of more sensitivity in early stage of the disease, especially the mucinous type as compared to CA 125.

Ovarian cancer is one of the most lethal gynecological malignancies because the tumor is often severely advanced by the time of clinical diagnosis.[1,2] Early stage disease is usually asymptomatic and most conventional investigations at present, such as routine laboratory tests and ultrasonography, have not proven to be helpful.[2] Many tumor markers have recently been developed for serum diagnosis such as CEA, CA 19-9, STN, CA 546 and CA 125. Among these, CA 125 has been shown to have the most clinical promise.[3-12] However, the positive rate of CA 125 in sera from patients with mucinous ovarian tumors is rather low (about 50%). In addition, these tests show some cross positivity with benign conditions.[13-15] Therefore, two new monoclonal antibodies (MAbs), OVS_1 and OVS_2, were developed in our laboratory.[16] These MAbs have greater specificity and sensitivity to mucinous cystadenocarcinoma, the occurrence of which is prevalent in Thailand.[17, 18]

By fusing the murine myeloma cell line NSI/I-Ag 4-1 with spleen cells from mice immunized with fresh human ovarian mucinous cystadenocarcinoma, OVS_1 and OVS_2 MAbs were selected. From the immunohistological staining results on 80 frozen and paraffin sections from 20 normal individuals and 60 patients,[16] OVS_1 MAb showed 96% specificity and 67% sensitivity to mucinous cystadenocarcinoma with no cross reaction to normal, benign or non-ovarian cancer tissues. OVS_2 MAb revealed 8% specificity and 78% sensitivity to mucinous ovarian cancer with some cross reaction to normal, benign, and non-ovarian cancer tissues. The

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results confirmed the highly specific and moderately sensitive recognition of OVS_1 antigen presented on cancer tissues. Since the nature of most ovarian cancer cells is highly malignant, leading to early invasion and metastasis, the presence of their antigens released in serum is likely. If we could determine the specific antigen levels in serum and evaluate them as tumor markers, it would be beneficial to cancer patients.

In this report, we further characterize the antigen specific to OVS_1 MAb, termed SA-1. Then, using a double determinant enzyme immunological assay system, we determined SA-1 levels in sera of ovarian cancer patients compared to control groups.

MATERIALS AND METHODS

Monoclonal antibodies

OVS_1 and OVS_2 MAbs were purified from mouse ascites by salting out with 50% ammonium sulfate followed by affinity chromatography on Protein A Sepharose 4B (Pharmacia Fine Chemicals, Piscataway).[19] Their immunoglobulin subclass was shown to be IgG1, kappa by using a Mab-ID EIA kit (Zymed Laboratory, Inc, San Franciscoo, CA, USA).

Antigen preparation

Each surgical tumor specimen, serous and mucinous cystadenocarcinoma, together with endometrioid cancer, was separately extracted by sonication or 0.5% triton X-100 in PBS.[14,20] The crude extract from mucinous cystadenocarcinoma showing best reactivity to both MAbs, was further purified by 3 subsequent affinity chromatographic steps, concanavalin A Sepharose 4B.[21] The purified SA-1 antigen specific to OVS_1 MAb was further charaterized by SDS PAGE, Western blot and ELISA.[19,22-23]

Human sera

The panel of sera included 64 healthy controls, ages matched with patients ranging from 17 to 76 years old. All patient sera were collected from those admitted in Siriraj Hospital with histologically confirmed definite diagnosis. Twenty-five sera were obtained from non-malignant diseases, including myoma (5), endometriosis (3), mucinous cystadenoma (3), ectopic pregnancy (1), procedentia uteri (1), diabetes mellitus (2), heart diseases (5), and other diseases (5). Fourteen sera were from non-ovarian cancer patients, (one or two cases of carcinoma of cervix, endometrium, liver, colon, rectum, pancreas, leukemia and other cancers). Fifty-one sera were from ovarian cancer patients with 20 cases of mucinous cystadenocarcinoma and another 31 cancers of non-mucinous - type including of serous cystadenocarcinoma (15), endometrioid (8), clear cell (5), dysgerminoma (2) and granulosa tumor (1). Among all ovarian cancer as classified by FIGO staging,[25] 11 were early stage I to II disease, another 40 were late stage III to IV. All patients with malignancy were newly diagnosed without having received any surgical or chemotherapeutic treatment. All sera were separated immediately after being drawn and kept at -20°C before assay.

Assay system

A Two-step, double determinant biotin-streptavidin alkaline phosphatase immunoassay system containing OVS_1 MAb as the immobilized antibody, and OVS_2 MAb conjugating biotin was used.[19,24] To each well (Nunc-Immuno Module Maxisorp F16, Intermed Nunc, Kamstrup, Denmark), 0.5 μg of OVS_1 MAb was coated and kept at 4°C overnight. After removal of the excess MAb the non-specific binding sites were blocked by 1% bovine serum albumin at room temperature for 2 hours. The wells were washed 3 times with PBS, then the SA-1 extract of varying concentration from 6.25 to 200 μg or 50 μl of serum were incubated at 37 °C for 2.5 hours. After adding 100 μl of 1/100 dilution of OVS_2 MAb conjugated biotin (1.5 μg of protein), the reaction was kept at 37°C for 2.5 hours, then 100 μl of 1/5,000 dilution of streptavidin conjugated alkaline phosphatase (Sigma Chemical Compamy, St. Louis, Mo, USA was added. The incubation was carried out at 37°C for 15 minutes followed by the enzymatic reaction with 100 μl substrate solution. Five mg tablet of para-nitrophenyl phosphate (Sigma Chemical Compamy, St. Louis, MO,USA was dissolved in 5 ml of substrate buffer, (100 ml of substrate buffer containing 0.2 M Na_2CO_3, 0.2 M $NaHCO_3$, and 0.5 M $MgCl_2$ with the ratio of 9:16:0.1). The color reaction was read at 405 nm to set the standard curve for the measurable range of the antigen levels from 3.125 to 200 units. Serum containing 1 unit/ml of SA-1 is equivalent to 1 μg/ml of SA-1 extract.

All sera were assayed with the same batch of reagents especially the aliquot of OVS_1 and OVS_2 MAbs and reference antigens. The recoveries from adding 3 different concentrations of antigens into 4 sera from ovarian cancer patients, were 90 to 110%. The coefficient of intra-assay variation from 6 simultaneous measurements of 3 standards by using the same reagents ranged from 6.54 to 15.88%.

CA 125 values were simultaneously measured in all sera with radioimmunoassay kits (Centocor, Malvern, PA) in order to compare the result with SA-1 levels.

RESULTS

From SDS PAGE of the purified antigen extract, we could not demonstrate the antigen band by Coomassie blue R-250 staining, in spite of many bands seen in crude tumor extract and in bound fraction of concanavalin A column (Con-A Ag). The experiment was done many times with the same result. When we increased sensitivity by using the silver staining technique, the purified antigen band was visualized and confirmed by Western blot analysis as a glycoprotein of 29 kDa with designated SA-1 (Fig. 1). The same pattern of SA-1 was also detected from Western blot analysis of 3 μg protein/lane of crude mucinous extract (data not shown).

The high specificity of OVS_1 MAb to SA-1 was shown by ELISA to have no cross reaction with other MAbs (antimyoma and antihepatoma), or other antigens (normal ovarian and normal serum antigens) (Table 1). The nonrelated MAbs and the normal antigens were prepared in our laboratory.

SA-1 values in healthy subjects

The cut-off values of SA-1 were set at two levels, 220 units/ml (mean + 1 SD of healthy females) and 360 units/ml (mean + 2 SD of healthy females). The positive rate was 19% (12/64) at the cut-off level of 220 units/ml, or 3% (2/64) at the cut/off level of 360 units/ml (Fig. 2, Table 2).

SA-1 values in women with non-malignant diseases

The sera from non-malignant diseases, as described, were assayed for SA-1 level. The positive rate was 28% (7/25) at the cut-off level of 220 units/ml, or 4% (1/25) at the cut-off level of 360 units/ml (Fig. 2, Table 2).

SA-1 values in patients with non-ovarian cancer

The positive rate at 220 units/ml cut-off level was 21% (3/14) and at the 360 units/ml cut-off level was 7% (1/14) (Fig. 2, Table 2).

SA-1 values in patients with ovarian cancer

The positive rate at 220 units/ml cut-off level was 63% (32/51) for all types, 75% (15/20) for mucinous cystadenocarcinoma. At 360 units/ml cut-off level, the positive rate was 43% (22/51) for all types, 50% (10/20) for non-mucinous cystadenocarcinoma (Fig. 2, Table 2).

Simultaneous measurement of CA 125 in healthy subjects and patients of the same groups

In healthy group, CA 125 was positive in 27% (17/64) at the cut-off level of 35 units/ml or 3% (2/64) at the cut-off level of 60 units/ml (Fig. 3, Table 2)

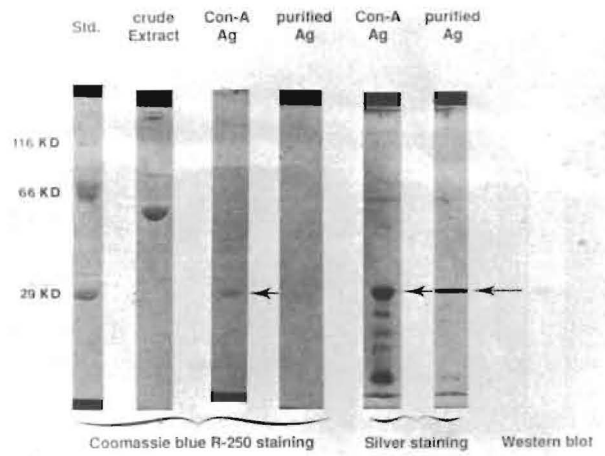

Fig.1. Demonstration of the purified SA-1 antigen as a 29 kDa glycoprotein by SDS-PAGE and Western blot analysis. The amount of other proteins loaded in each lane was adjusted to 3-4 μg, except 0.3 μg for purified antigen.

Table 1 Specific reaction of OVS_1 MAb and SA-1 antigen without cross reaction to other MAbs or antigens read by spectrophotometer at 405 nm (0.5 μg protein of each antigen was coated to react with approximately 5 μg protein of each antibody)

Antibody \ Antigen	Normal Ovary	Normal serum	Purified SA-1	Bovine serum albumin
Unrelated MAb I (anti-myoma)	0.1405	0.1150	0.1165	0.0880
OVS_1 MAb	0.0935	0.1025	1.0640	0.0925
Normal mouse serum	0.0950	0.0990	0.093	0.0920
Unrelated MAb II (anti-hepatoma)	0.1070	0.1155	0.1005	0.1015
Background	0.1055	0.1065	0.1075	0.1111

In the benign diseases group, CA 125 was positive in 40% (10/25) at the cut-off level of 35 units/ml or 24% (6/25) at the cut-off level of 60 units/ml (Fig. 3, Table 2).

In patients with non-ovarian cancer, the positive rate for CA 125 was 36% (5/14) at 35 units/ml cut-off level and 29% (4/14) at 60 units/ml cut-off level.

In patients with ovarian cancer, the positive rate at 35 units/ml cut-off level was 65% (33/51) for all types, 50% (10/20) for mucinous cystadenocarcinoma, and 74% (23/31) for non-mucinous cystadenocarcinoma. At 60 units/ml cut-off level, the positive rate was 57% (29/51) for all types, 45% (9/20) for mucinous cystadenocarcinoma and 65% (20/31) for non-mucinous cystadenocarcinoma (Fig. 3, Table 2)

Combining the assay for CA 125 and SA-1 at the cut-off level of 35 and 220 units/ml, respectively, the positive rate was increased to 86% (44/51) for all types of ovarian cancer, 85% (17/20) for mucinous cystadenocarcinoma and 87% (27/31) for non-mucinous cystadenocarcinoma (Table 2)

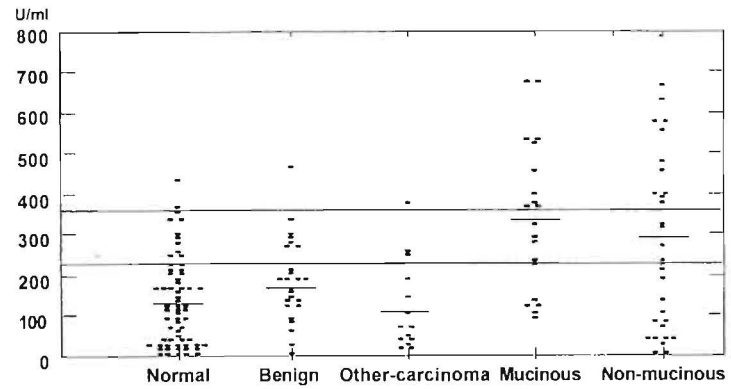

Fig.2 SA-1 mean values in 64 healthy controls, 25 benign diseases, 14 non-ovarian cancers, 20 mucinous and 31 non-mucinous cancers were 132.51, 187.00, 118.79, 338.27 and 281.30 U/ml, respectively. Positive rates among the corresponding groups at 220 and 360 U/ml cut-off values were 19%, 27%, 21%, 75%, 55% and 3%, 4%, 7%, 50% and 39%, respectively.

Table 2 Simultaneous measurement of SA-1 and CA-125 in healthy females and patients. (Sens = Sensitivity, proportion of ovarian cancer patients with SA-1 values higher than cut-off levels. Spec = Specificity, proportion of healthy controls with SA-1 values lower than cut-off levels)

Type	Cases	SA-1 Cut-off values		CA-125 Cut-off values		Combine SA-1, CA-125 (220/35 U/ml)
		220 U/ml	360 U/ml	35 U/ml	60 U/ml	
	% Sens (spec)	% Sens (spec)	% Sens (spec)	% Sens (spec)	% Sens (spec)	
Healthy control	64	19	3	19	3	35
Benign diseases	25	28	4	40	24	62
Non-ovarian cancer	14	21	7	36	29	50
Ovarian cancer	51	63 (81)	43 (97)	65 (73)	57 (97)	86 (60)
Mucinus	20	75	50	50	45	85
Non-mucinous	31	55	39	74	65	87

Fig. 4 and Table 2 showed the sensitivity (proportion of patients with positive values among those with ovarian cancer), the specificity (proportion of cases with negative values among all healthy normal) and the diagnostic efficiency (sensitivity x specificity) of SA-1 and CA 125 at various cut-off levels. The sensitivity of both markers fell as the cut-off level was higher, with a cmplementary increase in specificity. At the cut-off values of 220 units/ml for SA-1 and 35 units/ml for CA 125, the sensitivity of each was 63% and 65% while the specificity was 81% and 73% whereas the diagnostic efficiency was 0.51 and 0.47 for SA-1 and CA 125, respectively. In addition, cut-off levels of 360 units/ml for SA-1 and 60 units/ml for CA 125 were compared, giving sensitivities of 43% and 57%, with the same specificity of 97% and diagnostic efficiency of 0.42 and 0.55 for SA-1 and CA 125, respectively.

The positive rates in early and late stages of ovarian cancer were shown in Table 3. At 220 units/ml cut-off level of SA-1, the results in early stages of all types, mucinous and non-mucinous, were 73, 67 and 80%, respectively, while in late stages they were 60, 79 and 50%, respectively. In comparison with the results from CA 125 at 35 units/ml cut-off level the positive rates of each corresponding group at early stages were 36, 17, and 60%, and at late stages were 73, 64 and 77%, respectively. Positive rates from the combining of both tests in early stages of mucinous type were comparable to the test for SA-1 alone, but in late stages of the three groups the rates were increased to 88, 93, and 85% respectively. Statistical analysis [26] of all data showed significantly increased sensitivity of SA-1 only when combined with CA 125 test in ovarian cancer with all types, early stages, and all mucinous types.

DISCUSSION

SA-1 antigen as specifically detected by OVS_1 MAb was shown to be a glycoprotein of 29 KDa. The tumor antigen of this molecular weight is different from other antigens previously identified.[27]

The mean level of SA-1 in serum of healthy controls 132. 51 units/ml was less than the level of 187 units/ml in patients with benign diseases. The level was slightly lower in non-ovarian cancer, 118.79 units/ml, but markedly increased in all overian cancer, 303.66 units/ml, especially in mucinous type, 338.27 units/ml (Fig. 2)

The values of CA 125 in the same group showed similar patterns in healthy female, beingn diseases, and

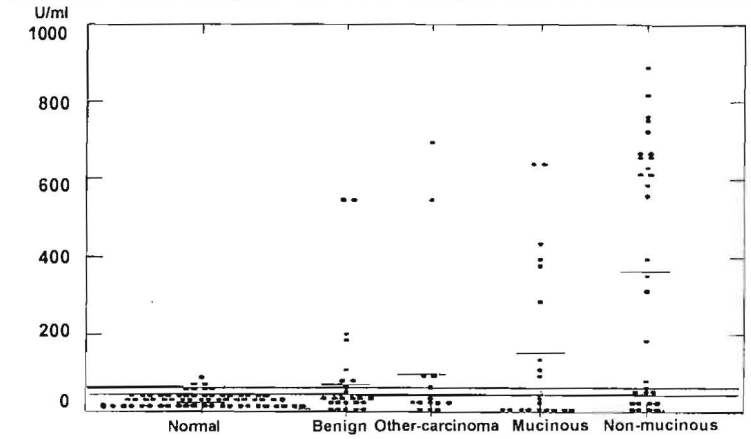

Fig. 3. CA 125 mean values performed simultaneously with SA-1 values in 64 healthy controls, 25 benign diseases, 14 non-ovarian cancers, 20 mucinous and 31 non-mucinous ovarian cancers were 22.86, 86.00, 116.80, 162.40 and 361.30 U/ml, respectively. Positive rates at 35 and 60 U/ml cut-off levels among the corresponding groups were 19%, 38%, 36%, 50%, 74%, and 3%, 27%, 29%, 45% and 65%, respectively.

Table 3 Comparison between the sensitivity from SA-1 and CA 125 in ovarian cancers at early and late stages

	%Sensitivity from		
	SA-1 (220 U/ml)	CA125 (35 U/ml)	Combination
Ovarian cancers all types	63 (32/51)	65 (33/51)	86 (44/51)[b,c]
Early stage	73 (8/11)	36 (4/11)	82 (9/11)[c]
Late stage	60 (24/40)	73 (29/40)	88 (35/40)[b]
Mucinous type	75 (15/20)	50 (10/20)	85 (17/20)[c]
Early stage	67 (4/6)	17 (1/6)	67 (4/6)
Late stage	79 (11/14)	64 (9/14)	93 (13/14)
Non mucinous type	55 (17/31)	74 (23/31)	87 (27/31)[b]
Early stage	80 (4/5)	60 (3/5)	100 (5/5)
Late stage	50 (13/26)	77 (20/26)[a]	85 (22/26)[b]

Statistically significant between the sensitivity of
SA-1 and CA 125[a]
SA-1 and combination[b] and
CA-125 and combination[c]

non-ovarian cancer. Contrary to SA-1, the CA 125 mean value was increased twice in the non-mucinous type compared with the mucinous type (361.36 and 162.42 units/ml) (Fig. 3). Fig. 5, Box plot shows the results and compares the distribution of SA-1 and CA 125 values among each group at 10^{th}, 25^{th}, 50^{th}, 75^{th} and 90^{th} percentile. Values above the 90^{th} and below the 10^{th} percentile were plotted as points.[28]

At the 2 cut-off levels of 220 and 360 units/ml, the SA-1 measurement was slightly less sensitive (63 and 43%) but more specific (81 and 97%) compared to CA 125 at 35 and 60 units/ml cut-off levels (Table 2). The cut-off level of 220 units/ml for SA-1 was selected as it gave a high diagnostic efficiency of 0.51 shown in Fig. 4. Interestingly at a 220 units/ml cut-off level for SA-1 and 35 units/ml cut-off level for CA 125, the increased positive rate in mucinous ovarian cancer for SA-1 was 75% compared to 50% from simultaneous test of CA 125 and also from most ovarian cancer markers at present.

This is beneficial, since the occurrence of mucinous ovarian cancer is more prevalent in Thailand.[17,18] However, for the non-mucinous type, the SA-1 value showed a lower positive rate of 55% while the value for CA 125 was 74%. When combining all positive results from SA-1 and CA 125, the rates were statistically increased to about 86% for all types of ovarian cancer,mucinous and non-mucinous.

Special attention was given to the positive rate in early and late stages of all ovarian cancers. There was no statistically significant difference among single tests for SA-1 and CA 125 in early stages of all ovarian cancer. When combinining both tests, the positive rates were significantly increased in early stages of the disease. Although the number of patients with early stages was small, the data suggested that SA-1 values were more positive in early stages of ovarian cancer (73%) compared to CA 125 (36%) (Table 2). Our preliminary results suggest the SA-1 as recognized by OVS_1 and OVS_2 MAbs could be used as tumor marker for ovarian cancer. The test gave low positive rates for healthy females, benign disease, and non-ovrian cancer, and moderate sensitivity for all types of ovarian cancer, especially mucinous type. Simultaneously with measurement of CA 125, these data suggest that SA-1 will be of clinical value as a new tumor marker to supplement some of the disadvantages of CA 125 in view of its low positive rate for mucinous type cancers and early stages of ovarian cancer. More data in large scale studies of patients from several institutes, including the correlation of these values with clinical evidence, will need to be required to confirm this proposal.

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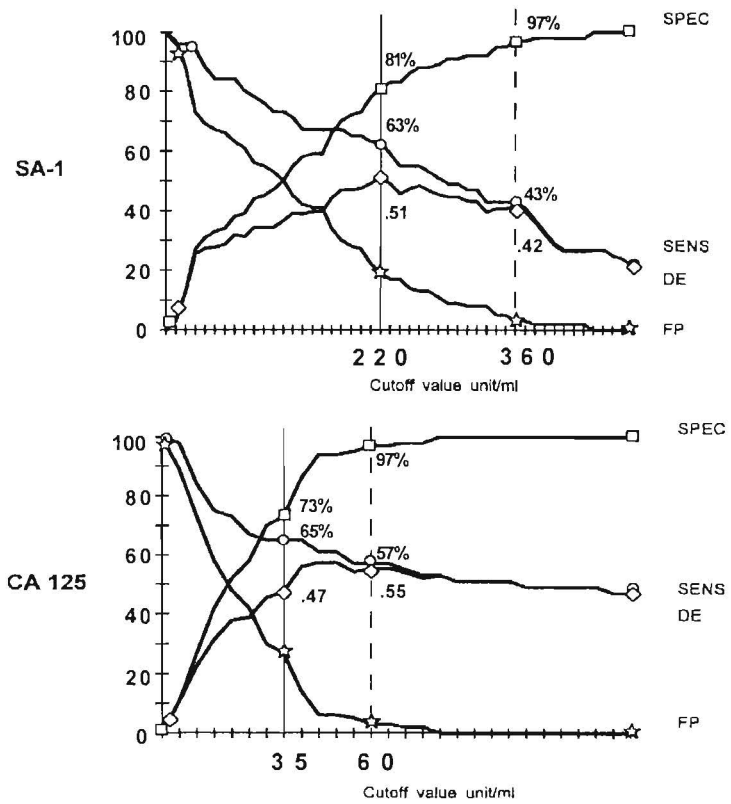

Fig. 4. Comparison of the sensitivity (SENS ○), specificity (SPEC □), diagnostic efficiency (DE ◇), and false positive rate (FP ☆) of SA-1 and CA 125 among normal controls and patients with ovarian cancer.

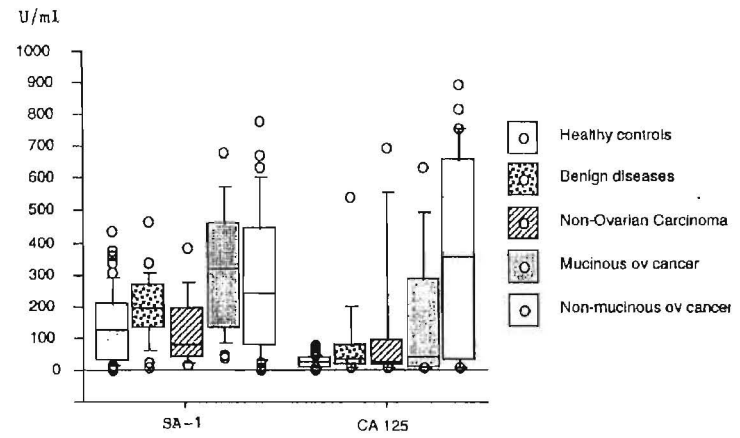

Fig. 5. Box plot compared simultaneous measurement of SA-1 and CA 125 levels at 10^{th}, 25^{th}, 50^{th}, 75^{th} and 90^{th} percentile in healthy controls and patients (shown in lines). Levels aboove 90^{th} and below 10^{th} percentile were plotted as points. The values of both antigens in healthy controls, benign diseases and non-ovarian cancer, showed similar pattern of low levels. Contrary to CA 125, the SA-1 values in ovarian cancer were higher in mucinous types than non-mucinous.

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