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Sandwich Radioimmunometric Assay with Murine Monoclonal Antibody, NCC-ST-439, for Serological Diagnosis of Human Cancers

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Sandwich radioimmunometric assay (RIA) for a new tumor-associated carbohydrate antigen defined by a monoclonal antibody (MoAb), NCC-ST-439, was developed and the antigen levels were determined in sera of normal donors, and patients with various malignant and non-malignant disorders. In normal donors, 97.0% (226/233) of sera were antigen-negative (less than 12 units/ml) except for 7 serum samples from young females. In patients with malignant disorders, 34.2% (82/240) were antigen-positive, in particular 64.0% (16/25) of patients with pancreatic carcinoma, 66.7% (16/24) of patients with recurrent colorectal carcinoma and 54.5% (6/11) of patients with recurrent breast carcinoma. In patients with non-malignant disorders, 6.0% (7/116) were antigen-positive. The positive rate in benign hepatobiliary disorders, including gallstones, hepatitis and liver cirrhosis, was especially low at 4.3% (1/23). We concluded that determination of serum NCC-ST-439 antigen would be useful in serodiagnosis of carcinoma patients.

Key words: Monoclonal antibody — NCC-ST-439 — Sandwich radioimmunometric assay — Tumor-associated carbohydrate antigen

Biochemical studies have revealed the structural alteration of carbohydrates of cell membrane glycolipids, glycoproteins and cell secretory products accompanying neoplastic transformation. Many monoclonal antibodies raised against cancer cells have been shown to react with these altered carbohydrates.[1-3] MoAb[*4] NCC-ST-439 was prepared by Hirohashi *et al.*, using a human gastric carcinoma xenograft St-4 as an immunogen.[4-6] NCC-ST-439 antigen is expressed in various kinds of carcinoma tissues and in a limited range of normal tissues including salivary gland and kidney as a high-molecular-weight glycoprotein, and it is increased in sera and body fluids of carcinoma patients. We have developed a solid-phase sandwich RIA in which NCC-ST-439 is used both as a catcher antibody and an ^{125}I-labeled tracer antibody, and the antigen values in the sera of normal donors and patients with various malignant and non-malignant disorders have been measured. In this report we describe our assay method and the results of measurement.

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*[4] Abbreviations: MoAb, monoclonal antibody; RIA, radioimmunometric assay; PBS, phosphate-buffered saline (0.01*M* sodium phosphate buffer, pH 7.4, containing 0.85% sodium chloride); BSA, bovine serum albumin; MES, 2(N-morpholino)-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; CEA, carcinoembryonic antigen; CV, coefficient of variation; TAE, transarterial embolization; SD, standard deviation.

Materials and Methods

NCC-ST-439 Antibody The generation, characterization and reactivity of the murine MoAb NCC-ST-439 (IgM) have been described previously.[4, 6] The antibody was purified from ascitic fluid of BALB/c mice inoculated with hybridoma cells, using gel chromatography.

Preparation of Immunoreagents Purified NCC-ST-439 antibody was radioiodinated by the lactoperoxidase method using Enzymobead™ (Bio-Rad Laboratories, Richmond, Calif.), according to the manufacturer's recommended procedure.[7] Specific activity of ^{125}I-labeled NCC-ST-439 antibody ranged from 3 to 4 Ci/g. Polystyrene beads, 6.35 mm in diameter, were prepared as the solid-phase material.[8] Four hundred polystyrene beads were incubated in 50 ml of 50m*M* sodium bicarbonate

buffer, pH 8.4, containing 1 mg of NCC-ST-439 antibody for 1 week at 4°, After a 1-week incubation, the beads were washed three times with PBS, and stored in PBS containing 1% BSA and 0.2% sodium azide for up to one month at 4°.

Assay Standards Gastric carcinoma xenograft St-15[5]) was minced, homogenized and lyophilized, then dissolved in PBS and gel-chromatographed using CL-6B (Pharmacia). The void volume fractions were collected as assay standards. Quantities of NCC-ST-439 antigen were expressed in arbitrary units.

Assay Conditions Experimental samples were assayed in duplicate with a "forward sandwich" RIA. We mixed in a reaction tray 20 μl of sera, standards, or positive controls with 200 μl of pH 6.0 buffer (per liter, 50 ml of normal equine serum, 50m*M* 2(N-morpholino)ethanesulfonic acid (MES), 0.1*M* sodium chloride, 1 g of sodium azide and 1m*M* ethylenediaminetetraacetic acid (EDTA)). One antibody-coated bead was added to each reaction well and samples were incubated at 25° for 3 hr. Beads were subsequently washed three times with distilled water, and 150,000 cpm of ^{125}I-labeled NCC-ST-439 antibody in 200 μl of the pH 6.0 buffer was added. After incubation of the samples at 25° for 3 hr, the beads were washed three times and transferred to the test tubes, and their levels of radioactivity were counted with a gamma counter. An assay standard curve was constructed by plotting NCC-ST-439 antigen concentrations per ml vs. cpm. Experimental results were converted into units of NCC-ST-439 antigen per ml by comparison with this standard curve.

Samples Serum samples from healthy blood bank donors were obtained at the Blood Center, Keio University Hospital. All of these donors qualified as blood donors, were negative when tested for hepatitis B surface antigen, and had normal liver function. Patients' samples were obtained at the Department of Surgery, Keio University Medical School. All of these samples were coded and stored at −40° for four to thirty weeks until assayed.

Determination of Carcinoembryonic Antigen (CEA) and Carbohydrate Antigen (CA 19-9) In 135 serum samples, CEA and CA 19-9 were also assayed in parallel with NCC-ST-439 antigen. CEA was determined using a conventional polyclonal sandwich RIA kit (Dainabot Co., Ltd., Tokyo), and CA 19-9 was determined by using a sandwich RIA kit (Centocor, Inc., Melvern, Pa.). For CEA, the cut-off value was set at 2.5 ng/ml, while that for CA 19-9 was 37 units/ml.

Results

Standard Curve The RIA results for assay standards showed an upward convex curve from 0 to 200 units/ml. The coefficients of variation (CV) for the standard antigens ranging from 0 to 200 units/ml averaged less than 4.51%, indicating good accuracy over the entire range of the standard curve. The intraassay CVs for control sera, which had high (82.6 units/ml), medium (24.1 units/ml), and low (10.6 units/ml) antigen concentrations, were 2.29%, 4.54% and 4.86%, respectively. The interassay CVs for the same control sera were 6.03%, 2.19% and 8.97%, respectively, indicating sufficient reproducibility.

NCC-ST-439 Antigen in Normal Donor Sera In order to determine the normal range, 233 serum samples from normal healthy donors were assayed. The mean antigen concentration was 5.72 units/ml, the standard deviations (SD) was 2.98 units/ml, and the mean plus 2SD was 11.68 units/ml. The cut-off value was therefore set at 12 units/ml. Of the 233 cases examined, 7 cases (3.0%) had an

Table I. Age and Sex Distributions of NCC-ST-439 Antigen in Normal Donor Sera

Age group (yr)	Male		Female	
	No. tested	Mean±SD	No. tested	Mean±SD
–20	17	4.77±2.57	8	8.76±3.36
21–30	64	5.34±2.66	33	6.39±3.70
31–40	49	5.44±2.54	12	7.30±3.27
41–50	23	5.74±2.80	10	5.94±3.71
51–	11	4.80±2.44	6	5.09±3.30
Total	164	5.33±2.60[a)]	69	6.63±3.56[a)]

a) Statistically significant ($P<0.01$).

antigen concentration higher than 12 units/ml. The maximum value observed was 16.46 units/ml and all of these false-positive cases were found to be young females below the age of 30.

Age and Sex Distributions of NCC-ST-439 Antigen in Normal Donors The age and sex distributions of these donors were analyzed (Table I). The average for female donors was 6.63 units/ml, and that for male donors, 5.33 units/ml. Female donors showed a significantly higher antigen concentration than males ($P<0.01$). The age distribution with respect to antigen concentration was relatively uniform in males, but females below the age of 40 showed increased antigen concentration. However, in donors over the age of 41, the antigen concentration did not differ with age or sex.

NCC-ST-439 Antigen in Sera of Carcinoma Patients Among 240 carcinoma patients examined, 82 (34.2%) were antigen-positive (Fig. 1). Positive rates were 64.0% (16/25) for pancreatic carcinoma, 66.7% (16/24) for recurrent colorectal carcinoma, and 54.5% (6/11) for recurrent breast carcinoma. These three entities showed relatively high positive rates. In spite of the fact that this antibody was originally prepared against gastric carcinoma, the sensitivity for gastric carcinoma was not particularly high, i.e., 24.4% (11/45) in primary cases and 30.8% (4/13) in recurrent cases, although very high antigen concentrations were observed sporadically. The positive rates ranged from 26.3 to 37.5% in other types of carcinoma, such as carcinoma of the esophagus, liver, and extrahepatic biliary system.

NCC-ST-439 Antigen in Sera of Patients with Non-malignant Disorders One hundred and sixteen patients with non-malignant disorders were examined (Fig. 2). Seven patients

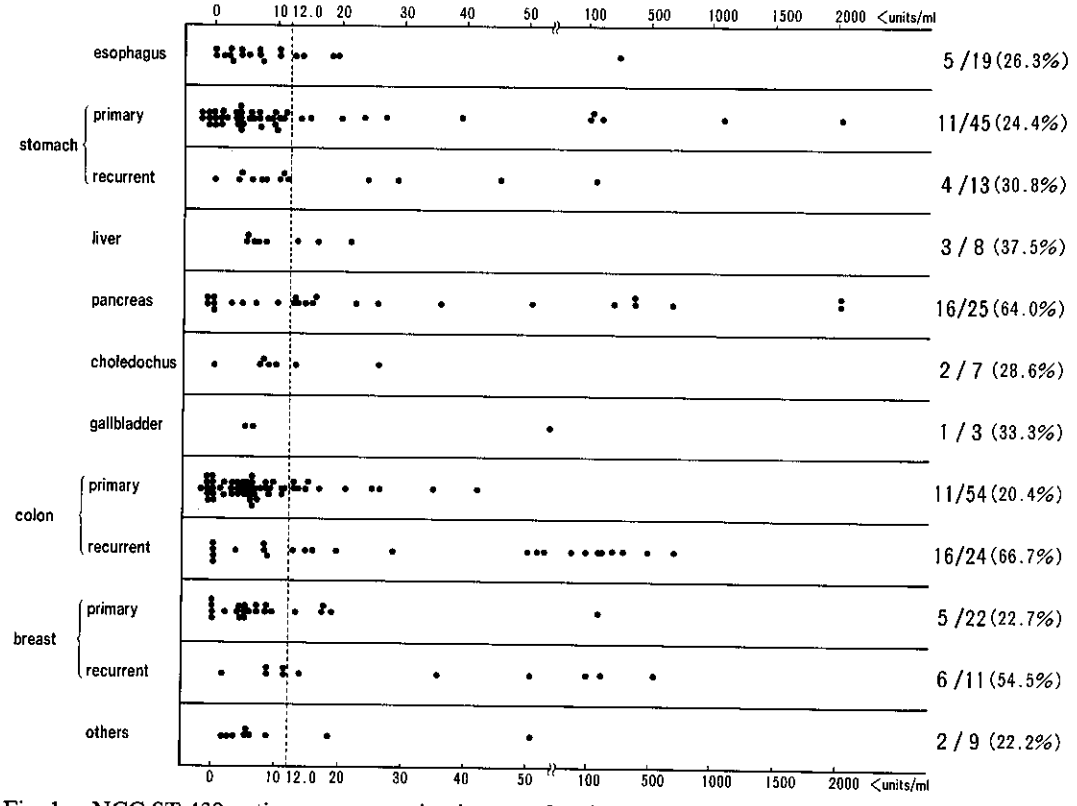

Fig. 1. NCC-ST-439 antigen concentration in sera of patients with malignant disorders. The positive rate was 34.2% (82/240). The category of others included six cases of leiomyosarcoma, one case of malignant melanoma, one case of rhabdomyosarcoma and one case of carcinoid tumor.

(6.03%) were antigen-positive. These included 2 patients with chronic pancreatitis, and 1 patient each with fulminant hepatitis, colonic polyp, breast fibroadenoma, anal fissure and endometriosis of the rectum. Antigen values for these false-positive cases were relatively low; they did not exceed 20 units/ml. In particular, among 23 patients with

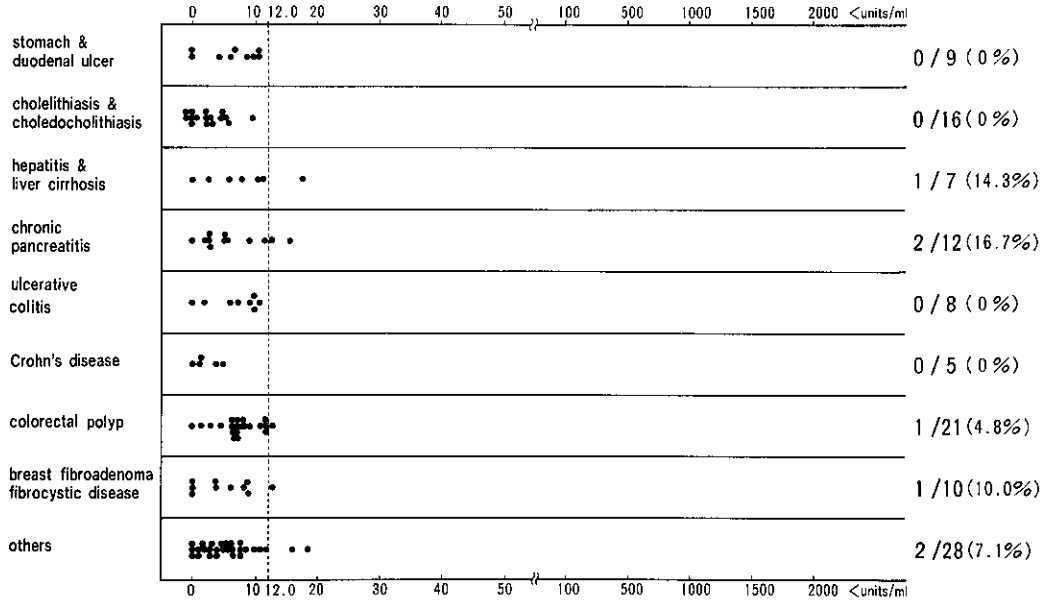

Fig. 2. NCC-ST-439 antigen concentration in sera of patients with non-malignant disorders. The false-positive rate was 6.03% (7/116).

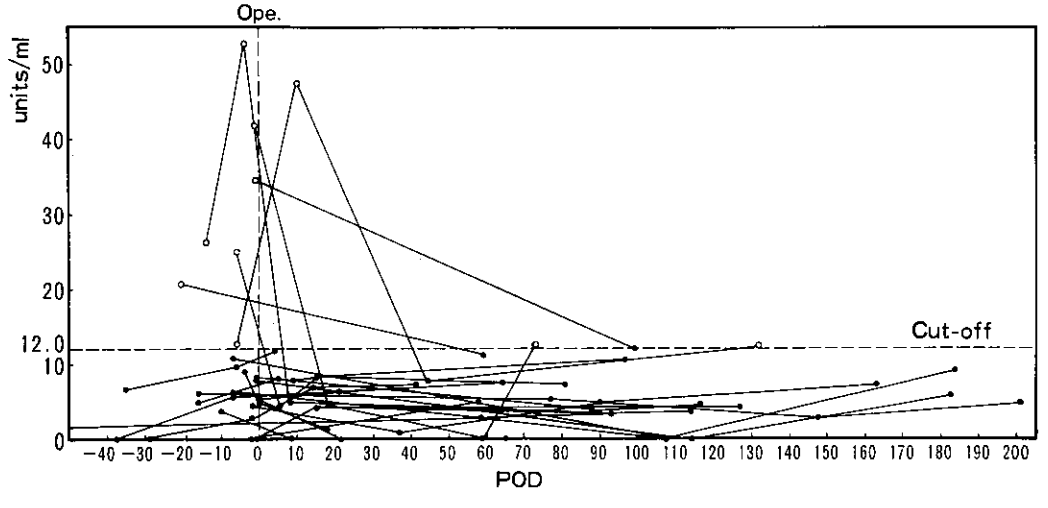

Fig. 3. Serial determination of NCC-ST-439 antigen concentration in sera of patients with colorectal carcinoma, determined before and after curative resection. Clear circles represent values above the cut-off value; solid circles represent values below the cut-off value.

benign hepatobiliary disorders such as gallstones, hepatitis and liver cirrhosis only 1 patient (4.3%) had a false-positive value.

Serial Determinations of NCC-ST-439 Antigen in Sera of Patients with Colorectal Carcinoma In 19 patients with primary colorectal carcinoma, NCC-ST-439 antigen levels were determined serially before and after surgery (Fig. 3). Six patients were antigen-positive before surgery and in all of these positive cases the antigen concentration decreased to below the cut-off value after curative resection. One patient showed a transient increase on the 10th day postoperatively, followed by a decrease to within the normal range at the 44th day after surgery. Two patients showed a modest reincrease to above the cut-off value after surgery, and these patients are at present under close observation. However, there has been no sign of recurrence for twelve months postoperatively.

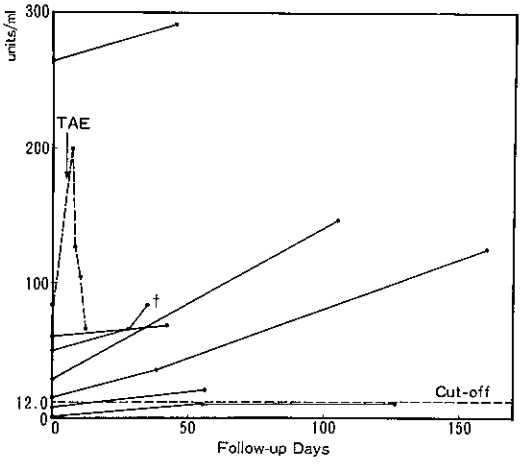

Fig. 4. Serial determination of NCC-ST-439 antigen concentration in sera of patients with recurrent colorectal carcinoma. The dashed line shows a case in which transarterial embolization was performed.

Serial Determinations of NCC-ST-439 Antigen in Sera of Patients with Recurrent Colorectal Carcinoma In eight patients with recurrent colorectal carcinoma, serial examinations were also performed (Fig. 4), and the antigen concentration was observed to increase with the progress of disease. One patient had a low antigen concentration at first examination, but as the disease progressed, the concentration increased to above the cut-off value. The dashed line in Fig. 4 represents a case of multiple liver metastases in which transarterial embolization (TAE) was performed. The antigen level showed a transient increase on the second day after TAE, but decreased to below the preoperative value by the seventh day after TAE.

Comparison of NCC-ST-439 with CEA and CA 19-9 In 135 serum samples, NCC-ST-439 antigen was assayed in parallel with CEA and CA 19-9 (Table II). The positivity rates of NCC-ST-439 were 5.6% (1/18) in benign disorders, 21.4% (3/14) in carcinoma of the esophagus, 27.6% (8/29) in carcinoma of the stomach, 33.3% (5/15) in carcinoma of the pancreas, 30.0% (12/40) in carcinoma of the colorectum and 26.3% (5/19) in carci-

Table II. Comparison of Positive Rates of NCC-ST-439, CEA and CA19-9 in the Sera of Patients with Malignant and Non-malignant Disorders

	No. tested	No. cases positive in		
		NCC-ST-439	CEA	CA19-9
Malignant disorders	117	33 (28.2)[a]	53 (45.3)	35 (29.9)
Carcinoma of				
esophagus	14	3 (21.4)	3 (21.4)	4 (28.6)
stomach	29	8 (27.6)	10 (34.5)	6 (20.7)
pancreas	15	5 (33.3)	7 (46.7)	9 (60.0)
colorectum	40	12 (30.0)	28 (70.0)	15 (37.5)
breast	19	5 (26.3)	5 (26.3)	1 (5.3)
Non-malignant disorders	18	1 (5.6)	3 (16.7)	3 (16.7)

a) The value in parenthesis is the percentage with respect to total cases tested.

noma of the breast. In benign disorders, NCC-ST-439 showed only one false-positive case, while there were 3 false-positive cases for CEA and CA 19-9. Of the categories examined, the positive rate of CEA was highest (70.0%) in colorectal carcinoma, while that of CA 19-9 was highest (60.0%) in carcinoma of the pancreas. Compared with these two tumor markers, the positive rate of NCC-ST-439 is between 20 and 30% in each category, suggesting that this antigen is relatively nonspecific and wide-ranging among the various types of carcinoma.

Discussion

In sera of normal donors, the level of NCC-ST-439 antigen was relatively low, about 97% (226/233) of sera having an antigen value of under 12 units/ml. Only 7 donors showed an antigen value of over 12 units/ml, and these false-positives were all females under 30 years of age. Moreover, among donors over 41 years old, who are supposed to carry a greater risk of carcinoma, the antigen levels lay within a relatively narrow range and did not differ either by age or sex. The result indicates that the cut-off value of 12 units/ml was appropriate. In young female donors, the antigen concentration tended to show a modest increase. The maximum antigen value was 16.45 units/ml and 7 out of 41 samples from females under 30 years of age were antigen-positive. These samples were assayed repeatedly at various dilution ratios and it was confirmed that these modest increases were not due to nonspecific reaction. However, since the increase of NCC-ST-439 antigen in young females was marginal, it was difficult to obtain enough of the antigen for biochemical analysis to know if the molecular properties of the antigen from young females were identical with those of the antigen from carcinoma patients. We also observed modest increases of the NCC-ST-439 antigen and CA 19-9 in sera of pregnant women (unpublished data). It is thus possible that the NCC-ST-439 antigen level may be influenced by changes in hormonal status, such as those due to the menstrual cycle, and further investigation will be required regarding this finding.

In non-malignant disorders, the positive rate for NCC-ST-439 antigen was 6.03% (7/116) and none of the false-positive cases had a high antigen value of over 20 units/ml. The results suggest that only a very low level of nonspecific reaction exists in this assay system and that the cancer specificity of NCC-ST-439 antibody is high. It was shown that the NCC-ST-439 antigen level was frequently increased in sera of carcinoma patients, particularly those with carcinomas of the pancreas, colon and breast. In addition, the antigen level was also increased in sera of some patients with carcinomas of the esophagus, stomach, liver, and extrahepatic biliary system. Although this antibody was prepared using a gastric carcinoma xenograft as the immunogen, NCC-ST-439 antigen is not specific for gastric carcinoma, but is relatively common to a wide variety of carcinomas. The value of NCC-ST-439 antigen changed according to disease status, decreasing after surgery and increasing after recurrence of carcinoma. These findings suggest that NCC-ST-439 antigen could be a useful tumor marker for the evaluation of therapeutic effects and the prediction of carcinoma recurrence.

Although CEA is the most clinically popular tumor marker, it is known that the serum CEA value is increased not only in cases of cancer but also in heavy smokers and various benign disorders such as infection of the upper respiratory tract, hepatitis and liver cirrhosis.[9] Determinations of serum CEA value are undoubtedly a very useful means of following up carcinoma patients. However, it is often difficult to decide whether a result is false-positive or not in cases which show a modest increase in the CEA value to just above the cut-off value. On the other hand, measurement of NCC-ST-439 antigen was shown to have a relatively low false-positive rate in non-malignant diseases, especially in hepatobiliary disorders. This suggests that the use of NCC-ST-439 in combination with CEA and CA 19-9 could be of value in reducing the rate of false-positive results. The low false-positive rate in hepatobiliary disorders could be explained by the absence of NCC-ST-439 antigen in intrahepatic and extrahehatic bile duct epithelium.[4]

In developing the assay system for NCC-ST-439 antigen, we considered sandwich RIA to be appropriate. Although most MoAb-defined tumor-associated antigens have been measured initially by binding inhibition

assay,[10, 11] sandwich RIA is a more popular assay method in clinical laboratories. Generally, the assay procedure involved is easier, it has a wider range and allows more samples to be handled with less effort than the binding inhibition assay. However, in our sandwich RIA, the fact that NCC-ST-439 is an IgM antibody made the development of the assay relatively difficult. When iodinated by a routine method using chloramine T, IgM antibody becomes fragile and easily inactivated. The lactoperoxidase method, on the other hand, is a more gentle radiolabeling technique which avoids injury and inactivation of the molecule, although the resulting specific activity is lower in comparison with that obtained by the chloramine T method.

Many MoAbs against human carcinomas have been produced and reported. Of these MoAbs, NS 19-9 was prepared against a colon carcinoma,[12] DU-PAN-2 against a pancreatic carcinoma,[13] and NCC-ST-439 and CSLEX1 against gastric carcinoma.[4, 14] The antigens defined by these monoclonal antibodies share close similarities. The epitopes recognized by these four MoAbs are all carbohydrates and have a common terminal sialic acid residue,[4, 6, 13–15] and these four antigens are secreted into the circulation of carcinoma patients as a heavily glycosylated protein macromolecule.[6, 10, 14, 16] However, in spite of such similarities, the distributions of the respective antigens are different. Simultaneous measurements of these multiple carbohydrate antigens in sera of carcinoma patients should help to reveal the intrinsic heterogeneity of carcinoma cells and make the serodiagnosis of carcinoma more effective in clinical studies.

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