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Cancer-associated quantitative differences in antigen levels of sialosyl Lewis^x in stool from patients with colorectal carcinomas

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A microenzyme-linked immunosorbent assay system employing monoclonal antibody SNH3 was developed for the detection of sialosyl Lewis^x antigen in stool extracts from 80 patients with colorectal cancer, 13 patients with colorectal non-malignant disorders and 90 normal subjects. Sialosyl Lewis^x antigen was detected in 35% of stool extracts from cancer patients but only in 7.7% and 2.2% of those from non-malignant patients and normal subjects respectively. Hemoglobin in the same stool samples was detected in 37.5% of cancer patients, 15.4% of non-malignant patients and 5.6% of normal subjects. The appearance of sialosyl Lewis^x antigen in stool was not necessary correlated with that of hemoglobin, and overall 61.3% of cancer patients were detected by the combination of the two assays. The combination assay was also impressive in early detection of colorectal cancer (Dukes' A, 52%; Dukes' B, 57.1%). Therefore, the assay for sialosyl Lewis^x antigen in stool would be useful for detecting colorectal cancer.

Keywords: cancer-associated carbohydrate antigen, cancer screening, colorectal cancer, fecal antigen, tumor marker

Introduction

A stage-specific embryonic antigen, SSEA-1, is found on the cell surface of murine embryos beginning at the eight-cell stage [1]. This antigenic determinant was demonstrated to be a carbohydrate antigen carrying Le^x-hapten, Galβ1,4[Fucα1,3]GlcNAcβ1-R [2, 3]. The sialosyl Le^x (SLX) structure, NeuAcα2,3Galβ1,4[Fucα1,3]GlcNAcβ1-R, has been shown to be specifically expressed on the developing gland cells during the formation of bronchial glands in human lung embryos [4]. SLX and related antigens have also been observed in various cancers [5].

The detection of hemoglobin in stool, which is based on the detection of bleeding in gastrointestinal tissues, has been utilized as a screening test for colorectal cancer [6, 7]. The bleeding, however, is caused not only by cancer proliferation but also by other disorders, such as hemorrhoids. Recently, *ras* oncogene mutation was identified in stool from patients with colorectal cancer using the polymerase chain reaction technique, however the method is complicated and unsuitable as a screening test [8].

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SLX and related antigens have been demonstrated to be widely distributed in colorectal cancer but are rarely found in normal colorectal tissues and hyperplastic polyps [9, 10]. Recently, it has been demonstrated that the expression of these antigens in colorectal cancer might be associated with cancer metastasis [11, 12]. Furthermore, the SLX molecule has been shown to be a carbohydrate ligand for E-selectin (ELAM-1), expressed in the activated endothelial cells of blood vessel walls and essential for cell adhesion between endothelial cells and cells carrying the SLX ligand, such as granulocytes and some cancer cells in the bloodstream [13].

We focused on SLX antigen in stool and developed an enzyme-linked immunosorbent assay (ELISA) system for this antigen with the aid of SNH3 MAb. The aim of present study was to examine whether the SLX antigen might be detected in stool samples from patients with colorectal cancer differently from patients with non-malignant colorectal disorders and from normal subjects. In order to evaluate SLX as a marker to detect colorectal cancer, the results of the examination were compared with those of the fecal hemoglobin test and the serum CEA test using the same stool samples.

Materials and methods

Monoclonal antibody

The SNH3 MAb was generated against a purified glycosphingolipid that has an SLX antigenic structure, as described previously [14]. The MAb was purified from ascites by the sequential use of a Baker Bond ABx ion-exchange column (J.T. Baker Chemical, Phillipsburg, NJ, USA) and a Superose 6 gel filtration column (Sigma, St Louis, MO, USA).

Stool samples

A summary of the characteristics of the patients tested in this study is given in Table 1. Ninety stool samples from normal subjects were collected from outpatients who were negative for bowel disorders by examination. These samples were randomly collected from outpatients of different sex and age group. Thirteen samples were collected from patients with non-malignant colorectal disorders, consisting of nine patients with polypectomies and four patients with active inflammatory bowel disorders (three ulcerative colitis and one Crohn's disease). Eighty stool samples were collected from patients with colorectal cancer: more than 60% of these samples were from patients with early-stage (Dukes' A or B) colorectal cancer and from patients with rectal cancer. Most of the stool samples from cancer patients and all of the normal stool samples were from Sameshima Hospital (Kagoshima, Japan). Some stool samples from cancer patients were kindly donated by Drs Katsuro Kuroki and Hirooki Yoshii. All samples from cancer patients used in this study were collected from patients who had not undergone any surgical procedure to remove tumors. The stool samples were stored below −70°C until use.

Table 1. Characterization of the samples

		No. of sample (%)
Normal ($n = 90$)	Male	53 (58.9)
Average age, 53.4 years	Female	37 (41.1)
Non-malignant ($n = 13$)	Male	8 (61.5)
Average age, 50.1 years	Female	5 (38.5)
Cancer ($n = 80$)	Male	53 (66.3)
Average age 50.7 years	Female	27 (33.8)
Dukes' stage	A	28 (35.0)
	B	21 (26.3)
	C	20 (25.0)
	D	11 (13.8)
Tumor site	Ascending colon	2 (2.5)
	Transverse colon	5 (6.3)
	Descending colon	6 (7.5)
	Sigmoid colon	11 (13.8)
	Rectum	49 (61.3)
	Unknown	7 (8.8)

Serum CEA test

Fifty-eight serum samples were collected from the same colorectal cancer patients who provided the stool samples. The CEA levels in serum were measured by IMx automatic analyzer (Abott, Abott Park, IL, USA).

Extraction of glycoproteins from stool

Five hundred milligrams of each stool sample was added to 1 ml of 20 mM Tris–HCl buffer, pH 8.2, containing 1% Triton X-100 and 0.02% sodium azide and mixed rigorously by vortex mixer. The mixture was heated for 20 min at 80°C and centrifuged for 30 min at 4°C. The supernatant was pooled and stored at −70°C until use.

Preparation of standard antigen for ELISA
Approximately 1 kg of stool from infants born no more than a week prior to collection (donated by Dr Hirokazu Chikakiyo) was extracted with 0.6 M perchloric acid and centrifuged at 8000 r.p.m. for 30 min at 4°C. The supernatant was pooled and neutralized with 10 M NaOH in an ice bath. The extract was concentrated by ultrafiltration and dialyzed against distilled water. The concentrated extract was filtrated through a 0.22-mm membrane filter, then stored at −70°C until use. The solution of standard antigen was prepared by diluting each antigen at a dilution ratio suitable for the ELISA system.

ELISA protocol for sialosyl Le^x antigen
Each well of the 60-well Terasaki microtiter trays (Robbins Science, Sunnyvale, CA, USA) was coated overnight with 10 μl of purified SNH3 MAb at a concentration of 20 μg/ml in 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.02% thiomerosal (PBS). After washing with PBS containing 0.05% Tween 20 (washing buffer), the wells were blocked overnight with 15 μl of PBS containing 0.5% human serum albumin (HSA) and 10% sorbitol per well. The trays were dried at 25°C after aspirating the blocking buffer and stored at −70°C until use. The trays were washed with washing buffer before use, then 5 μl of PBS containing 25 mM EDTA, 0.5% HSA and 0.05% Tween 20 (assay buffer) was added to each well. Five microliters of sample or standard antigen was added to the assay buffer in each well and incubated overnight at 25°C. After washing the wells with washing buffer, 8 μl of horseradish peroxidase-conjugated SNH3 MAb at a predetermined dilution was added to each well followed by incubation for 2 h at 25°C. The trays were washed with washing buffer, then 5 μl of color reagent, 0.04% *o*-phenylendiamine dihydrochloride and 1.5% H_2O_2 in 0.1 M citrate phosphate buffer, pH 5.0, was added to each well for color development. After incubation for 15 min at room temperature, the color development was stopped by adding 5 μl of 2.5 M H_2SO_4 to the wells. The absorbance of each well at 488 nm was measured with a microreader manufactured for the 60-well microtiter trays (model TR-200, Dynatech Laboratories, Alexandria, VA, USA) and the antigen concentration of samples was calculated with a second-order polynomial formula derived from the net absorbances resulting from the standard antigen. The antigen level was expressed in an arbitrary unit (unit/ml) by comparison with the standard antigen (Figure 1).

Assay performance
Inter-assay reproducibility was tested with six replicates and intra-assay reproducibility was demonstrated by duplicating the assay five times using two different stool samples from normal subjects. Samples whose antigen levels were calculated to be above the assay range were measured again after dilution with assay buffer.

Fecal hemoglobin test
The hemoglobin test was performed on part of each sample using OC-Hemodia kits (Eiken, Tokyo, Japan) at each hospital prior to shipping.

Gel filtration analysis of sialosyl Le^x antigen in stool
A Sephacryl S-300 HR column (1.0 × 47 cm) equilibrated with 50 mM borate buffer, pH 8.6, containing 0.5 M NaCl and 0.02% sodium azide (elution buffer) was prepared. One milliliter of the stool extract which showed the highest level of SLX and 1 ml of adequately diluted solution of standard antigen (stool extracts from newborn babies) were applied to this column and eluted with the same buffer. The SLX, CEA and protein concentration in each fraction were measured as described previously [15]. The standard proteins used were bovine thyroglobulin (669 000), horse spleen apoferritin (443 000), sweet potato β-amylase (200 000) and bovine serum albumin (66 000) (Sigma, St Louis, MO, USA).

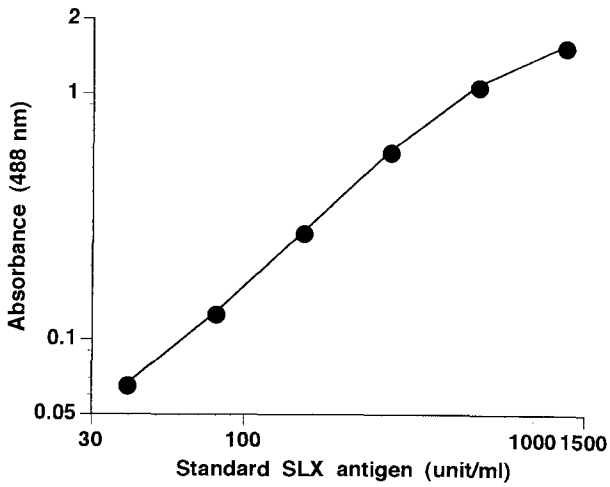

Figure 1. Standard curve of sialosyl Le^x antigen. The absorbance was calculated after subtracting blank values from each assay measured at 488 nm.

Results

Analytical studies

As shown in Figure 1, the absorbance was found to increase dose dependently with the concentration of SLX antigen from 40 to 1280 units/ml. In order to evaluate the assay precision, inter- and intra-assay reproducibility were examined using two different samples, and the precision was demonstrated to be adequate (Table 2).

Antigen levels of sialosyl Le^x in stool extracts

Twenty-eight of 80 samples from patients with colorectal cancer showed elevated levels of SLX antigen, and seven of them showed extremely high levels (Figure 2). More than 67% of these positive samples were, however, found to be negative by the hemoglobin test. In most of the samples from patients with benign diseases (12/13) and from normal subjects (88/90) the level was below the minimum detectable limit of the antigen. The only positive sample from the patients with benign diseases was from a patient with Crohn's disease, and the result of the hemoglobin test was also positive.

Table 2. Inter- and intra-assay precision of SLX antigen in human stool extracts

Sample	SLX antigen (unit/ml)		
	Mean	SD	CV (%)
Inter-assay ($n = 6$)			
A	74.8	8.72	11.6
B	351.5	19.11	5.4
Intra-assay ($n = 5$)			
A	74.4	1.61	2.2
B	339.4	18.91	5.6

Positive rates for fecal sialosyl Le^x antigen, fecal hemoglobin and serum CEA

A summary of all the results is given in Table 3. Of the total colorectal cancers tested, almost identical positive ratios were obtained by the fecal SLX, fecal hemoglobin and serum CEA tests. However, the positive ratio obtained by the SLX test was highest in the early stages of cancer (Dukes' A and B). No significant association was found between antigen levels and stage of cancer. Although the detection ratio was only 25%, the fecal SLX test in this study was found to be the most useful test for detection of cancer of the ascending, transverse and descending colon. On the other hand, 54.5% of the sigmoid colon cancers and 40.8% of rectal cancers were detected by fecal hemoglobin test and fecal SLX test respectively. In the combination, these two fecal tests detected more than 50% of patients with early cancer (Dukes' A and B) and more than 70% of patients with advanced cancer (Dukes' C and D) (61.3% of the total cancer patients).

Gel filtration analysis of sialosyl Le^x antigen in stool

As shown in Figure 3, SLX antigen extracted from the stool of both newborn babies and cancer patients was recovered in the void volume of the Sephacryl S-300 column. Therefore, the molecular weight of SLX antigen in both samples was estimated to be more than 1000 kDa.

Discussion

Changes in the expression of glycoconjugates have been observed in a variety of cancers, and aberrant glycoproteins and glycolipids, which are absent or minimal in normal tissues, are expressed in various cancer tissues [16–18]. SLX antigen, one of the cancer-associated antigens, has been found

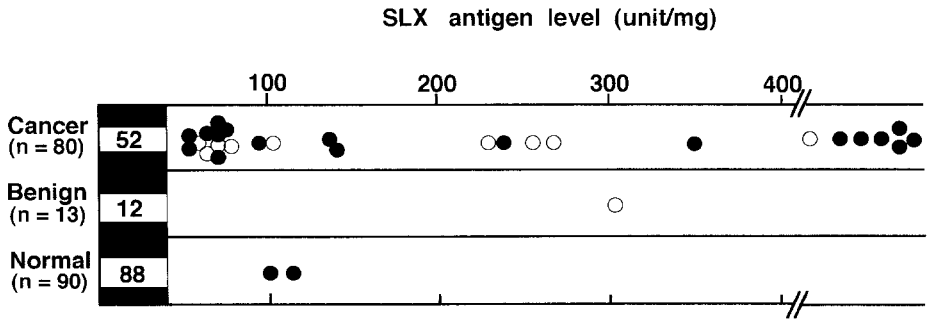

Figure 2. Fecal sialosyl Le^x antigen levels in patients and normal subjects. Samples negative (●) and positive (○) by fecal hemoglobin test.

Table 3. Comparison of positive rates for fecal sialosyl Le^x antigen, fecal hemoglobin and serum CEA according to Dukes' stage and the location of colorectal cancer

	Serum test		Fecal test			
	No. of samples tested	No. of positive samples (%)	No. of samples tested	No. of positive samples (%)		
		CEA		Hemoglobin	SLX	Combined[a]
Cancer (total)	58	19 (32.8)	80	30 (37.5)	28 (35.0)	49 (61.3)
Dukes'						
A	19	3 (15.8)	28	7 (25.0)	8 (32.0)	13 (52.0)
B	17	4 (23.5)	21	5 (23.8)	8 (38.1)	12 (57.1)
C	13	7 (53.8)	20	13 (65.0)	8 (40.0)	16 (80.0)
D	9	5 (55.6)	11	5 (45.5)	4 (36.4)	8 (72.7)
Ascending transverse and descending colon	10	2 (20.0)	12	1 (7.7)	3 (25.0)	4 (33.3)
Sigmoid colon	6	3 (50.0)	11	6 (54.5)	2 (18.2)	6 (54.5)
Rectum	39	13 (33.3)	49	19 (38.8)	20 (40.8)	32 (65.3)
Unknown	3	1 (33.3)	8	0 (0.0)	2 (25.0)	2 (25.0)
Non-malignant		ND	13	2 (15.4)	1 (7.7)	2 (15.4)
Normal		ND	90	5 (5.6)	2 (2.2)	9 (10.0)

[a]Combined assay of fecal hemoglobin and sialosyl Le^x antigen.
ND, not determined.

in serum, and elevation of the antigen level has also been demonstrated in serum from cancer patients [15].

Recently, α1,3-L-fucosyltransferase, which is responsible for the synthesis of SLX and related antigens, was found in sera from patents with various cancers, and elevated activity of this enzyme was associated with the presence of tumors [19, 20]. Elevated levels of antigens and enzyme activities in serum, such as SLX and α1,3-L-fucosyltransferase, seem to be a result of secretion or release from tumor cells, but in some cases the secretion and release are not enough to cause elevated levels in serum.

On the other hand, stool could be a useful tool for detecting antigens secreted or released from gatrointestinal and colorectal tissues. In fact, elevated levels of CEA have been observed in stool extracts from colorectal cancer patients, and the fecal CEA test has been demonstrated to be more useful than the serum CEA test for detecting colorectal cancer [21, 22].

The fecal hemoglobin test is one of the most sensitive diagnostic tests for colorectal cancer. However, no bleeding or intermittent bleeding has been reported in the early stages of colorectal cancer [23], whereas the presence of SLX antigen and related antigens has been observed in 70–80% of colorectal cancer tissues [9, 10], and it is clear that the appearance of SLX antigen in the stool samples is not dependent upon bleeding. In this study, we demonstrate for the first time not only the cancer-associated presence of the SLX antigen in the stool samples, but a higher detection rate of the antigen at the early stages of cancer.

Using the procedure described in the study it was found to be easy to extract SLX antigen from stools, but the antigen was hardly extracted without incubating the samples for 20 min at 80°C (data not shown). The molecule containing the SLX antigen in the stool was thought to be a mucin-like glycoprotein which had been previously found in stool samples from newborn babies, as well as in sera from cancer patients, and in the medium of cultured cancer cells [15, 19, 24]. Compared with antigen levels in serum or plasma, the antigen levels in stool samples might be affected not only by the fluidity of the sample taken, but also by the particular part of the stools sampled. It might be difficult therefore to assay the antigen levels precisely. Further improvement in the preparation of fecal samples could help to increase the diagnostic sensitivity and specificity of the test and decrease the number of false-negatives. Multiple sampling on different dates might also help, as recommended in the fecal hemoglobin test.

The diagnostic specificity, as well as the sensitivity, was increased in the combined assays for two

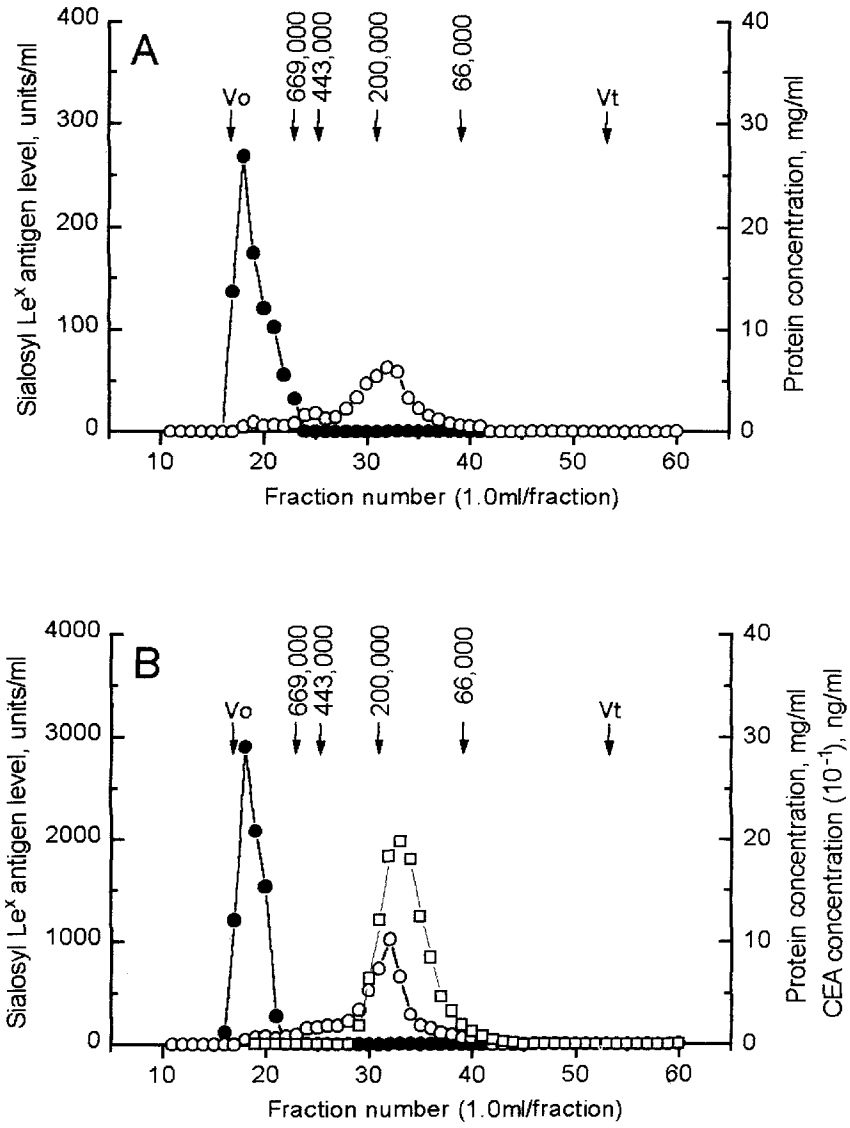

Figure 3. Gel filtration analysis of sialosyl Le^x antigen in stool from newborn babies (A) and a patient with colorectal cancer (B). Levels of SLX (●), CEA (□) and protein concentration (○).

independent antigens, fecal SLX and hemoglobin, in the same stool sample. In fact, more than 50% of patients with early-stage cancer and more than 70% of patients with advanced-stage cancer could be detected by the combined assay.

In conclusion, levels of the SLX antigen in fecal samples could be useful for the screening of colorectal cancer, particularly in the early stages. To elucidate further the clinical potential of fecal SLX level, an increased number of samples are being assayed.

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