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Original Article

Sialic acid specific lectins from *Episesarma tetragonum* (Decapoda, Grapsidae): isolation, purification and characterization

R. Viswambari Devi*, M. R. Basil Rose, P. D. Mercy

Department of Zoology, Holy Cross College, Nagercoil-629 004, Tamil Nadu, India.

Abstract: Two sialic acid specific lectins *Episesarma tetragonum* agglutinin–1 and 2 were purified from the hemolymph of the Mangrove crab, *Episesarma tetragonum*. The major lectin was purified using CNBr-activated sepharose 4B conjugated to fetuin. N-acetyl glucosamine containing buffer was used for elution. The hemagglutination activity of purified lectin was inhibited by glycoproteins containing Siaα, 2-3Galβ, 1-4 GlcNAc linkages. On SDS-PAGE, the molecular weight of calcium dependent lectin was observed to be 70 kDa. Lectin had the maximum activity at a wide range of pH (6.5 – 9.5) and temperature (0 - 40 °C). The physicochemical characteristics of the minor agglutinin showed that its hemagglutinating activity was calcium dependent, optimum at pH 8 – 9.5 and temperature 0 – 37 °C. The only potent inhibitor of minor lectin was bovine submaxillary mucin. An attempt was also made to purify minor lectin by affinity chromatography using bovine submaxillary mucin coupled to CNBr-activated sepharose 4B column. The lectin was eluted with elution buffer containing ethylene diamine tetra acetate. Strong inhibition of purified minor lectin by bovine submaxillary mucin and non-inhibitory action of de-O-acetylated bovine submaxillary mucin suggested that the lectin was O-acetyl sialic acid specific.

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Introduction

Invertebrates lack an adaptive immune system, but have developed efficient innate immune systems to defend themselves against foreign materials. Molecular structures and functions of various defense components that participate in immune processes are being discovered. New molecules such as fibrinogen-related proteins (FREPs) are being uncovered that might have the potential to recognize and attack specific pathogens, while the roles of better studied molecules continue to expand. This challenges the idea that invertebrates are adequately served by broad-spectrum pathogen recognition proteins. Lectins are one such protein that is involved in defense and various biological phenomena. Lectins may recognize a part of a sugar, a whole sugar, their glycosidic linkages or a sequence of sugars. This property of lectins has moved their efficacy to humans in various biological applications (Devi et al., 2010). Among invertebrates, arthropods are the major source of such sialic acid specific lectins. It is interesting to note that some of the agglutinins of arthropods are capable of binding specifically to sialic acids, the family of sugars not synthesized by protostomian invertebrates (Warren, 1963). Sialic acids discriminating against agglutinins of arthropods will definitely be of immense value in identifying sialylated tumor-associated antigens. Careful search for these may provide lectins with unique specificity for different kinds of sialic acids and their glycosidic linkages. Therefore in the present study, our aim is to isolate and purify

* Corresponding author: R. Viswambari devi
E-mail address: viswambaridevi@gmail.com
Tel: +919941635133

significant sialic acid specific lectins from the hemolymph of *Episesarma tetragonum*, which may be added to the library of potential diagnostic tools for identifying sialyl epitopes in pathogenic bacteria, and malignant tumor cells.

Materials and methods

Collection of samples: The mangrove crab, *Episesarma tetragonum* was collected for the study from the mangrove and fresh water regions of Manakudy, Kanyakumari district, Tamil Nadu. Hemolymph was collected from fresh, uninjured non-autotomized crabs. The hemolymph of small crabs (5-15 g) was drawn using a sterile 1.0 ml syringe and 22 gauge needles through the arthrodial membrane at the base of third walking leg. For large crabs (20-60 g), after cutting the dactylus, the hemolymph was allowed to bleed directly into centrifuge tubes placed on ice and, was allowed to clot for 15 minutes. The hemolymph was then centrifuged to separate the serum which was stored at -20°C.

Hemagglutination assay: The mammalian blood samples were collected directly in sterile modified Alsevier's medium. Before use, the erythrocyte types were suspended and washed three times with Tris Buffered Saline and resuspended in the same buffer to give 1.5% v/v cell suspension for agglutination assays. Hemagglutination titer is defined as the reciprocal of the highest dilution of the test sample showing visual agglutination of the test erythrocytes.

Cross adsorption assay: The adsorption assays carried out using dog, horse and rat erythrocytes in this study were essentially the same as those carried out by Mercy and Ravindranath (1992). Hemagglutination inhibition (HAI) assay was performed to test the ability of various glycoproteins and sugars (mono and oligosaccharides) to inhibit agglutination. Hemagglutination inhibition titer was reported as the reciprocal of the lowest dilution of inhibitors giving complete inhibition of agglutination after 1 hour.

HA assays to determine physicochemical parameters: The physicochemical properties were determined by hemagglutination assays with serum samples under conditions of varying pH, temperature, bivalent cation of diverse concentration, EDTA and some chemical agents.

Affinity purification of Episesarma tetragonum agglutinin–1 (EtA-1): Clarified serum (70 ml) was applied to 3.5 ml of fetuin coupled to cyanogen bromide activated sepharose 4B in an econo column (Bio-Rad) previously equilibrated with TBS at 4°C. The elution of lectin was done with elution buffer that contained 100 mM GlcNAc and collected 1 ml fractions on ice in polypropylene tubes containing 10 µl of 100 mM Calcium Chloride at a rate of 0.3 ml/minute. The fractions were vortexed immediately after collection and kept on ice. Fractions containing lectin were pooled on the same day and dialyzed against 1 mM $CaCl_2$, at 4°C for 3 hours and the dialysate was then aliquoted, lyophilized (speed-vac, Sawant), and stored at -20°C.

Sialidase treatment of dog erythrocytes: A reaction mixture (total 1.0 ml) containing 10% washed dog erythrocytes in PBS-BSA (pH 7.0) and 140 milliunits of neurminidase of *Clostridium perfringens* (Sigma type X) was incubated at 37°C for 4 hours. Neuraminidase treated cells were washed with PBS-BSA three times and pelleted by low speed centrifugation. HA assays were performed against the desialylated erythrocytes using purified lectin, EtA-1.

Sialidase treatment of sialoglycoprotein: Asialo fetuin was prepared by incubating 2 mg of glycoprotein (fetuin) with 0.1 unit of *Clostridium perfringens* sialidase (Sigma type X) in 400 µl of 5 mM acetate buffer, pH 5.5 for 2 hours at 37°C. As a control, fetuin was treated similarly without sialidase. HAI assay was performed with purified lectin for sialidase treated and untreated fetuin against 1.5% dog erythrocyte suspension.

Polyacrylamide gel electrophoresis: SDS-polyacrylamide 12.5% slab gel electrophoresis was performed according to Laemmli (1970).

Purification of EtA-2: Clarified serum (50 ml) was applied to 1.5 ml of BSM coupled to cyanogen bromide activated Sepharose 4B in an econo column

Table 1. Purification of EtA-1 from the native hemolymph of *E. tetragonum*.

Step	Sample	Volume (ml)	Protein (mg)	Total activity (HA units)	Specific activity (HA units/mg)	Purification fold
1	Crude	70	530	1.792×10^5	338	1
2	Clarified sample	30	57	3.84×10^4	673	2
3	Purified using formalinized dog RBC	10	1.2	5.12×10^4	4.26×10^4	63
4	Purified using fetuin – agarose affinity column	6	0.04	3×10^4	7.68×10^5	1141

Table 2. HAI assay of EtA-1 by sialoglycoproteins.

Glycoproteins (N=5)	Nature of sialic acid	HAI	Minimum concentration for inhibition µg/ml	Relative inhibitory potency%
Fetuin	NeuGc	256	19.531	100
Transferrin	NeuGc	128	39.062	50
Porcine thyroglobulin	NeuGc	128	39.062	50
α-acid glycoprotein	NeuGc	16	312.5	6.25
PSM	NeuGc	8	625	3.125
Apotransferrin	-	4	1250	1.5625
Bovine thyroglobulin	NeuGc	4	1250	1.5625
BSM	NeuAc/NeuGc	0	0	0
Lactoferrin	NeuAc	0	0	0

(Bio-Rad) previously equilibrated with TBS at 4°C. The elution of lectin was done with elution buffer that contained 10 mM EDTA and collected 1 ml fractions on ice in polypropylene tubes containing 10 µl of 100 mM Calcium Chloride at a rate of 0.3 ml/minute. The fractions were vortexed, dialyzed, lyophilized and stored at -20°C.

De-O-acetylated preparation of glycoproteins: De-O-acetylation of sialic acids was performed following the procedures of Sarris and Palade (1979) and Schauer (1982).

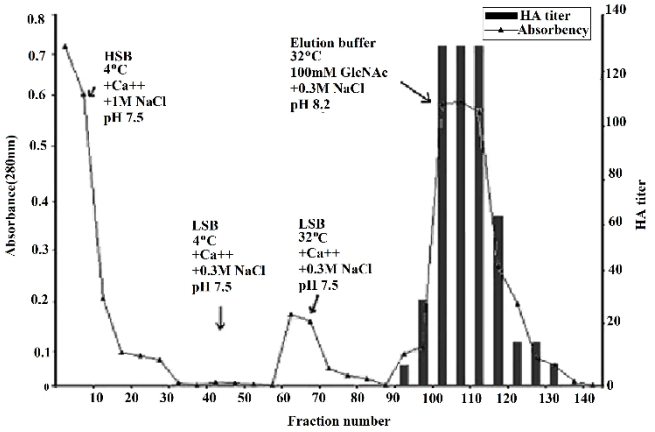

Figure 1. Fetuin Affinity elution profile-Purification of EtA-1.

Results

Identification of agglutinins of different specificity: The presence of naturally occurring agglutinins in the serum of *E. tetragonum* was detected using a panel of 15 mammalian erythrocyte types. HA titer with the different species of erythrocytes can be graded as follows: Dog > Albino rat = Mice = Horse > Buffalo = Cat > Human B = O > A = Rabbit = Goat = Donkey. The HA titer was high (HA = 64) with dog erythrocytes, was moderate (HA = 16) with albino rat, mice and horse erythrocytes followed by cat and buffalo (HA = 8). Results of cross adsorption studies showed that serum adsorbed with dog and rat erythrocytes completely lost the agglutinating activity towards all the three erythrocytes tested. But the serum adsorbed with horse erythrocytes retained the agglutinating activity with dog erythrocytes even after four adsorptions and thus indicating the presence of heteroagglutinins, namely *E. tetragonum* agglutinin-1 (EtA-1) specific for dog erythrocytes and *E. tetragonum* agglutinin-2 (EtA-2) specific for horse erythrocytes.

Purification of EtA-1: The specific activity of the purified lectin increased about 1141 folds from 673 to 7.68 x 105 HA unit/mg of protein (Table 1, Fig. 1). An analysis of purified EtA-1 on SDS-PAGE in

Table 3. Effect of neuraminidase treatment of glycoprotein on HAI of EtA-1.

Glycoprotein treatment	HAI titer with dog erythrocytes
Fetuin (without sialidase)	256
Fetuin + Sialidase (3 hours)	128
Fetuin + Sialidase (6 hours)	8
Fetuin + Sialidase (12 hours)	8
Fetuin + Sialidase (18 hours)	8
Fetuin + Sialidase (20 hours)	8

Table 4. Inhibition of EtA-1 hemagglutination by various sugars.

Sugars (N=5)	HAI	Minimum concentration for inhibition µg/ml	Relative inhibitory potency%
GlcNAc	64	1.5625	100
Galactose	64	1.5625	100
NeuGc	32	3.125	50
Maltose	16	6.25	25
Lactose	16	6.25	25
ManNAc	8	12.5	12.5
GalNAc	0	0	0
NeuAc	0	0	0
Trehalose	0	0	0

Table 5. Purification of EtA-2 from the native hemolymph of *E. tetragonum*.

Step	Sample	Volume (ml)	Protein (mg)	Total activity (HA units)	Specific activity (HA units/mg)	Purification fold
1	Crude	125	3125	1.6×10^5	51.2	1
2	Clarified sample	50	250	4×10^3	16	3
3	Purified	2	0.07	160	2000	125

Table 6. Hemagglutination inhibition titer of crude and purified EtA-2 by BSM.

Glycoprotein	HAI titer	Minimum conc. required for inhibition (µg /ml)	Relative inhibitory potency (%)
BSM	Crude 512	9.7	100
	Purified 8192	0.6103	100

the presence of 2-mercaptoethanol revealed a major band at molecular weight of 70 kDa (Fig. 2).

Physicochemical properties of EtA-1: The HA activity of the agglutinin was sensitive to pH and temperature. The HA was stable between pH 6.5 - 9.5 and at temperature ranging from 10 – 40°C. Among the cations tested, calcium ions did not have any effect on HA titer, however magnesium at 10 – 0.1 mM concentration reduced the HA titer to 32 while at 100 mM concentration gave the normal HA titer. Though calcium ions were not required for HA, the metal ion chelator – EDTA showed a different action towards the HA activity. At very low concentrations (1 – 0.01 mM) the HA activity decreased (HA = 32). Concentrations ranging from 1 – 5 mM, the HA activity increased one fold from the normal HA (128) and at concentrations from 10 - 20 mM there was sudden decrease in HA activity, after which the HA activity was completely lost. The agglutinating activity was completely inhibited by chloroform while the activity was greatly inhibited by incubation with denaturing agents such as HCl and NaOH.

Binding specificity of purified EtA-1: With a view to ascertain the nature of the binding specificity of purified lectin, hemagglutination assays were

Table 7. HAI of purified EtA-2 of *E. tetragonum* by BSM before and after de-O-acetylation and desialylation.

S.No	Glycoprotein treatment	HAI titer
1	**De-O-acetylation**	
	BSM untreated	8192
	BSM + 0.04 N NaOH, 4° C, 45 minute	0
	BSM + 0.4 N NaOH, 4° C, 45 minute	0
2	**Desialylation**	
	BSM (without sialidase)	8192
	BSM + sialidase (3 hour)	2048
	BSM + sialidase (6 hour)	1024
	BSM + sialidase (12 hour)	512
	BSM + sialidase (18 hour)	512
	BSM + sialidase (20 hour	128

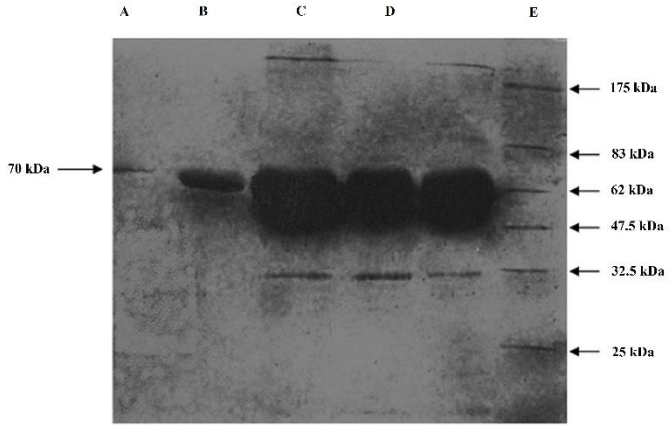

Figure 2. SDS-PAGE of purified lectin EtA-1. Lane A, fetuin-agarose purified lectin; Lane B, formalinised dog erythrocyte adsorbed purified lectin; Lane C, protein from crude serum; Lane D, protein from crude serum after ultracentrifugation; Lane E, standard of known molecular weight (Prestained, broad range marker, Bio-Rad laboratories).

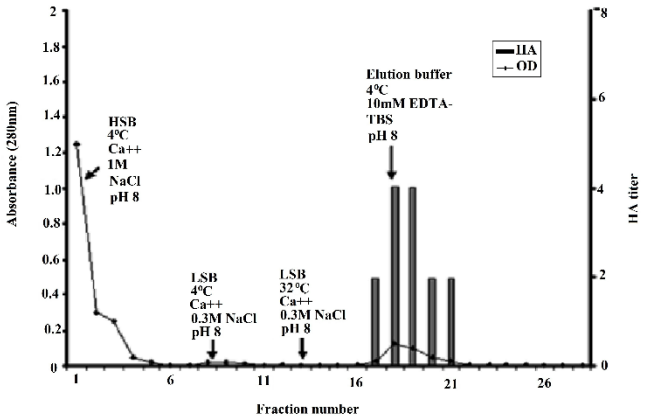

Figure 3. BSM – Affinity elution profile-Purification of EtA-2.

performed with a variety of sialoglycoproteins. The inhibitory potency of EtA-1 was as follows: Fetuin > porcine thyroglobulin = transferrin > α-acid glycoprotein > PSM > apotransferrin = bovine thyroglobulin (Table 2). The purified EtA-1 showed a remarkable inhibitory potency with fetuin containing sialic acids with α, 2-3 linkages. On the other hand, BSM and lactoferrin failed to inhibit the HA activity of EtA-1. To further define the possible role of sialic acids as potent inhibitor of lectin, the sialoglycoprotein, fetuin was enzymatically modified and its derivative was examined for HAI. Sialidase treatment of fetuin reduced its inhibitory properties at 20 hours (Table 3). In order to find out if EtA-1 was NeuAc or NeuGc specific, free sialic acids NeuAc and NeuGc were tested as inhibitors of hemagglutination. NeuAc did not inhibit hemagglutination whereas NeuGc inhibited (Table 4). However NeuGc linked glycoproteins were inhibitorier than free NeuGc. Galactose that showed very low inhibitory potency with crude agglutinin was a potent inhibitor of purified EtA-1.

Purification of EtA-2: Both the clarified serum, which had passed through the affinity matrix and the effluent collected during subsequent washing of this matrix with high salt buffer and low salt buffer, did

not show hemagglutination activity against horse erythrocytes. This indicated that lectin in the serum was adsorbed by the affinity matrix. When elution buffer with 10 mM EDTA was passed through the column, peaks at 280 nm absorbance coincided with agglutinating activity against horse erythrocytes emerged from the affinity matrix (Table 5, Fig. 3).

Physicochemical properties of EtA-2: The HA was stable between pH 8 – 9.5 and at temperature ranging from 10 - 35°C. Among the cations tested calcium ions did not have any effect on HA titer, however magnesium at 0.1 – 10 mM concentration reduced the HA titer to 16 while at 100 mM concentration gave the normal HA titer. Though calcium ions were not required for HA, the metal ion chelator – EDTA showed a different action towards the HA activity similar to EtA-1.

Binding specificity of EtA-2: It was interesting to note that only BSM was the potent inhibitor of purified EtA-2. It gave a hemagglutination inhibition (HAI) titer of 8192, when compared to crude agglutinin that gave only 512 HAI titer (Table 6). No inhibitors that inhibited EtA-1 were capable of inhibiting EtA-2. Base treatment, specific for hydrolysis of the O-acetyl groups of sialic acids without cleavage of peptide bonds (Schauer, 1982), completely reduced the ability of BSM to inhibit hemagglutination. Sialidase treatment of BSM reduced its inhibitory properties at 20 hours (Table 7).

Discussion

The serum of mangrove crab, *E. tetragonum* possess two naturally occurring agglutinins, the major agglutinin, EtA-1 specific for dog erythrocytes and the minor agglutinin, EtA-2 specific for horse erythrocytes. The heterogeneity of agglutinin in these crustaceans gives important support to the fact that, agglutinins as defense molecules for recognition of "non-self" are the part of an invertebrate immune system. EtA-1 shared some common properties of other crustacean agglutinins namely pH and thermal sensitivity and calcium dependency. Purified agglutinin might yield precise information on its sugar specificity and would be of great interest for biomedical applications. Glycoprotein inhibition experiments provide valuable information pertaining to the sialyl oligosaccharides preferred by EtA-1. The potent inhibitor of EtA-1 was fetuin. Fetuin, the major glycoprotein in calf serum contain carbohydrates distributed between an equal numbers of N- and O-glycosidically linked units. Fetuin contains sialic acid α, 2-3 and sialic acid α, 2-6 in a 2:1 ratio. The second potent inhibitors were porcine thyroglobulin and bovine transferrin. Porcine thyroglobulin is a major glycoprotein synthesized in the thyroid gland. The carbohydrate moiety of porcine thyroglobulin was shown to consist of a complex type (unit B-type) oligosaccharides being linked to asparagine residues (Arima et al., 1972). Unit B-type oligosaccharides of the glycoprotein were of triantennary and biantennary complex type. In triantennary oligosaccharides, the sialic acid residues were not localized on certain specific branches but distributed on all three branches. α, 2-3 linked sialic acid residues were exclusively located on the terminal of the branch arising from C-4 of the branching α-mannose residue, whereas α, 2-6 residues occupied terminals of the other branches. The natural position of α, 2-3 linked sialic acid residue of triantennary complex type sugar chain was similar to that observed to exist in glycoprotein fetuin (Nilsson et al., 1979; Baenziger and Fiete, 1979). But the ratio of α, 2-3 linked sialic acid to α, 2-6 linked sialic acid in triantennary complex type of thyroglobulin was 1:2 and this explained the higher reactivity of fetuin to EtA-1 than thyroglobulin. Clear information pertaining to sialyl linkages of bovine transferrin and α-acid glycoprotein are not available. They are also known to possess sialic acid α, 2-3 Gal β1-4 GlcNAc β1 residues (Vliegenthart et al., 1982). This corroborates the specificity of EtA-1 towards sialic acid α, 2-3 Gal β1-4 GlcNAc β1 residues. This can be further assured by the non-inhibitory potency of BSM which contains a terminal sialic acid sequence of NeuAc α, 2-6 GalNAc (Gottschalk and Graham, 1959; Graham and Gottschalk, 1960). The affinity of

EtA-1 to NeuGc was also reflected in the glycoprotein inhibition study. These investigations obviously demonstrate that EtA-1 exhibits high affinity towards sialoglycoconjugates possessing terminal NeuGc α, 2-3 linked to penultimate galactose residues. To the best of our knowledge, EtA-1 is the first known arthropodan lectin with sialic acid α, 2-3 Gal β1-4 GluNAc-R specificity.

BSM was the only potent inhibitor of EtA-2. All other glycoproteins which are found to possess NeuGc or NeuAc were non inhibitory. The most common type of glycosidic linkages involving sialic acid in BSM is α-2, 6 GalNAc. Although the terminal oligosaccharide sequence of BSM is NeuAc α-2, 6 GalNAc, a major fraction (>50%) of the sialic acid is O-acetylated (Graham, 1966). Since EtA-2 is not inhibited by any other sugars and glycoproteins, it is of clear evidence that EtA-2 has unique specificity to O-acetyl sialic acid and not to NeuAc α-2, 6 GalNAc fraction of BSM. Hemagglutination inhibition tests thus revealed BSM, which contains mainly 9-O-acetyl and 8, 9-di-O-acetyl-N-acetyl NeuAc (Schauer, 1982), as the most potent inhibitor of the agglutinin. De-O-acetylation of BSM by base treatment specifically hydrolyses O-acetyl groups of sialic acids without cleavage of peptide bonds (Sarris and Palade, 1979; Schauer, 1982). In the present study, the inhibitory potency of BSM was completely abolished, after de-O-acetylation. This observation further reveals the specificity of EtA-2 for O-acetyl sialic acid.

From our studies the most important prerequisite for EtA-1 binding to glycoconjugate seems to be Sia(NeuGc)α, 2-3Galβ, 1-4 GluNAc linkages rather than NeuGc per se. Considering the importance of sialic acids in cell sociology, lectins which specifically recognize terminal sialic acid residues are potentially useful as analytical tools in studying the biological functions of sialoglycoconjugates. These lectins, along with monoclonal antibodies raised against sialoglycoconjugates, have been used in the detection, affinity purification, cytochemical localization and quantification of such glycoconjugates (Varki, 1997). Lectins that recognize linkages or modifications of sialic acid are thus indispensable as reagents in biochemical research and diagnostic analysis. Carbohydrate residues of the membrane glycoproteins can be detected using lectins due to their binding specificity to carbohydrates. Lectins, therefore have gained an importance in the field of cancer research (Sherwani et al., 2003). It is believed that an elevated level of α, 2-3-linked sialylation increases the metastatic potentials of tumor cells (Dennis et al., 1986). The increase of α, 2-3-linked sialic acids with increased branching of glycans is considered to accompany hepatocarcinoma (Montreuil et al., 1997). Tumor associated antigen sialyl Lewis x (sLx) 1 contains a sialic acid α, (2-3) galactose moiety and has been implicated in inflammation and cancer metastasis (Tyrrell et al., 1991; Kannagi et al., 2004). Hence EtA-1 that can recognize sialic acid α, 2-3 linkages can be used in detection and quantification of those glycoconjugates accompanying hepatocarcinoma, inflammations and cancer metastasis. Thus there is no doubt that EtA-1 is a valuable diagnostic tool for identifying the sialoconjugates in normal and malignant tissues.

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