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(54) **METHOD OF EXAMINING COLON CANCER AND COLON ADENOMA**

VERFAHREN ZUR UNTERSUCHUNG VON KOLONKARZINOM UND KOLONADENOM

METHODE DE DEPISTAGE DES CANCERS ET DES ADENOMES DU COLON

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Description

Field of the Invention

[0001] The present invention relates to a method for examining human colorectal cancers and colorectal adenomas, and to antibodies and examination reagents thereof.

Prior Art

[0002] Since the number of colorectal cancer patients is increasing year by year, a method for early detection is necessary. Although, immunological fecal occult blood test and various tumor markers are used at present for the examination of colorectal cancers, these methods do not have satisfactory positive rates. Namely, the positive rate of immunological fecal occult blood test used for the examination of colorectal cancers is 50-60%. As to the tumor markers of colorectal cancers, carcino-embryonic antigen (CEA), CA19-9, STX, which are used for examining the therapeutic effect and for monitoring recurrence, are not satisfactory as tumor markers for early detection of colorectal cancers.

[0003] The colon is divided into right-half and left-half drawing a line at the flexure coli and colorectal cancers in right-half result frequently in pseudo-positive by fecal occult blood test using an anti-hemoglobin antibody, which is pervasively used for the detection of colorectal cancers. Therefore, development of a test method contributing to increased early diagnosis rate particularly of the right-half colorectal cancers is expecting. As to the laboratory diagnosis of colorectal adenomas, which is regarded as the birthplace of colorectal cancers, suitable methods of detecting are not available and people rely inevitably on endoscopy at the present time. Simple method of diagnosing colorectal adenomas will be beneficial to classify patients into groups necessary and unnecessary for endoscopy, and contribute to early detection of colorectal cancers.

[0004] Although the sulfation of sugar residues is active in a normal large bowel, it is known to be remarkably reduced in colorectal cancers.

IZAWA M ET AL ("Expression of sialyl 6-sulfo Lewis X is inversely correlated with conventional sialyl Lewis X expression in human colorectal cancer." CANCER RESEARCH 1 MAR 2000, vol. 60, no. 5, 1 March 2000 (2000-03-01), pages 1410-1416, XP002475645 ISSN: 0008-5472 [A]), discloses that both 3'-sulfation of galactose and 6-sulfation of N-acetylglucosamine (hereinafter referred to as <GlcNAc>), which are abundant in the colorectum, are reduced in colorectal cancers. A number of GlcNAc-6-sulfotransferase isozymes have been known in colorectal cancer tissues and in non-cancer colorectal tissues of patients, and I-GlcNAc6ST is significantly decreased in course of carcinogenesis, which leads to the reduced sulfation of sugar residues in colorectal cancer, while, GlcNAc6ST-1, one of the isozymes in a normal colorectum, does not show significant changes in the level in course of carcinogenesis.

SEKO A ET AL ("Biochemical differences between two types of N-acetylglucosamine:-->6sulfotransferases in human colonic adenocarcinomas and the adjacent normal mucosa: specific expression of a GlcNAc:-->6sulfotransferase in mucinous adenocarcinoma." GLYCOBIOLOGY SEP 2000, vol. 10, no. 9, September 2000 (2000-09), pages 919-929, XP002475644 ISSN: 0959-6658 [A]) discloses that HEC-GlcNAc6ST, another isozyme, increases significantly in colorectal cancer.

[0005] HEC-GlcNAc6ST, which increases in colorectal cancers, synthesizes 6-sulfated GlcNAc and carries out sulfation of GlcNAc in various sugar residues. Therefore, there are a huge variety of the structures of intra-cellularly synthesized sugar residues and their antigenicity. Since GlcNAc6ST-1 and I-GlcNAc6ST also synthesize 6-sulfatedGlcNAc, only the fact that 6-sulfatedGlcNAc is synthesized from HEC-GlcNAc6ST cannot be used as a specific method for diagnosis of colorectal cancer. However, it is known from SEKO A ET AL previous and SEKO A ET AL ("Ectopic expression of a GlcNAc 6-O-sulfotransferase, GlcNAc6ST-2, in colonic mucinous adenocarcinomas", Glycobiology, Irl Press, GB, vol. 12, no. 6, 2002, pages 379-388, XP002982000 ISSN: 0959-6658 [A]) that the substrate selectivity of GlcNAc6ST-1 and I-GlcNAc6ST is more specific than that of HEC-GlcNAc6ST. Therefore, certain 6-sulfated sugar residues might be produced by HEC-GlcNAc6ST, but not by GlcNAc6ST nor by I-GlcNAc6ST. However, an actual system for diagnosis of colorectal cancers has not been established.

[0006] STREETER P R ET AL ("Immunohistologic and functional characterization of a vascular addressin involved in lymphocyte homing into peripheral lymph nodes." The Journal Of Cell Biology Nov 1988, vol. 107, no. 5, November 1988 (1988-11), pages 1853-1862, XP002475642 ISSN: 0021-9525) discloses that MECA-79 antibody reacts with HEV antigens in Peyer's patches and blocks binding of lymphocytes to peripheral lymph node HEV.

On the other hand, YEH, J.C. ET AL (Cell 105: 957-969, 2001) discloses that the monoclonal antibody MECA-79, is known to react with chemically synthesized GlcNAc6-sulfated sugar residues. Moreover, the antigens recognizable by the antibody (MECA-79) are reported to emerge on the cell surface, when a mouse gene encoding HEC-GlcNAc6ST enzyme is transduced into CHO cells (hamster ovary cells).

BRUEHL R E ET AL (Minimal sulfated carbohydrates for recognition by L-selectin and the MECA-79 antibody." The Journal Of Biological Chemistry 20 Oct 2000, vol. 275, no. 42, 20 October 2000 (2000-10-20), pages 32642-32648,

XP002475643 ISSN: 0021-9258) discloses that MECA-79 binds to sulphated carbohydrates that act as HEV-expressed ligands on endothelial cells.
KIMURA N ET AL ("Proceedings Of The National Academy of Sciences of USA, National Academy Of Science, Washington, DC, US, vol. 96, April 1999 (1999-04), pages 4530-4535, XP002942391 ISSN: 0027-8424 [A]) discloses the reconstitution of functional 1-selectin ligands on a cultured human endothelial cell line by co-transfection of α1,3fucosyltransferase VII and newly cloned GlcNAcβ:6-sulfotransferase cDNA.
UCHIMURA K ET AL (Journal Of Biological Chemistry, American Society Of Biolochemical Biologists, Birmingham" US, vol. 277, no. 6, 8 February 2002 (2002-02-08), pages 3979-3984, XP002981999 ISSN: 0021-9258 [A]) discloses the specificities of N-acetylglucosamine-6-O-sulfotransferases in relation to L-selectin ligand synthesis and tumor-associated enzyme expression.
JIN KYU LEE ET AL (Biochemical And Biophysical Research Communications, Academic Press Inc. Orlando, FL, US, vol. 2, no. 263, September 1999 (1999-09), pages 543-549, XP002942393 ISSN: 0006-291X) discloses the cloning and characterization of a mammalian n-acetylglucosamine-6-sulfotransferase that is highly restricted to intestinal tissue.
However, it has not been known whether the antigens are recognizable by MECA-79 antibody emerged on human cancer cells.

Problems to be solved by the Invention

[0007] The present invention provides an *in vitro* method for examining colorectal cancer and colorectal adenoma.

Means to solve the Problems

[0008] The present inventors discovered that there are significant differences in the distribution of GlcNAc-6-sulfotransferase isozymes, sulfation enzymes of sugar residues, between non-cancer colorectal tissues and colorectal cancer tissues or colorectal adenoma tissues, as a result of investigations. Then the inventors found that colorectal cancers and adenomas could be detected specifically by assaying 6-sulfated sugar residues, which are synthesized only by HEC-GlcNAc6ST, but not by GlcNAc6ST-1 nor by I-GlcNAc6ST, in tissues of patients and in fecal samples. Previously, many antibodies such as AG223 (Biochem. (Tokyo), 124:670-678,1998), G152, G72, AG97, AG107, AG273, G2706, G27011, G27039 (all above, J.Biol.Chem.,273:11225-11233,1998) are known as those reacting with GlcNAc-6-sulfated sugar residues. Meanwhile, MECA-79 antibody (Pharmingen, catalog No. 09961D, distributor: Becton Dickinson), which is commercially available as immunological homing receptor of lymphocytes, is known to react in some way with GlcNAc-6-sulfated sugar residues (BRUEHL R E ET AL).
The inventors made a search for an antibody, which have little reactivity to such cells as normal colorectal epithelial cells expressing GlcNAc-6-sulfated sugar residues, but have strong reactivity to cells with such GlcNAc-6-sulfated sugar residues as expressed in cancer cells, by screenings these antibodies. Then the obtained antibodies were assayed against samples from patients and were shown to be highly positive to colorectal cancer cells. The above results lead to completion of the present invention.
[0009] According to one aspect of the present invention there is provided an *in vitro* method for examining colorectal cancer and colorectal adenoma comprising assaying the reactivity of an antibody to tissues, body fluid or feces of patients, or extracts thereof, wherein said antibodies react with such antigen that is present in cells expressing HEC-GlcNAc6ST gene encoding GlcNAc-6-sulfotransferase and that is absent or almost absent in cells expressing GlcNAc6ST-1 or I-GlcNAc6ST gene, wherein said antigen comprises the sugar residues expressed by the following formula:

R1-Gal β1-3/4(SO_3-6) GlcNAc β1-R2,

where R1 represents sugar residues added by other enzymes and is not limited in structure, Gal β represents β galactose, GlcNAc β represents β N-acetylglucosamine, Gal β1-3/4 represents binding of 1 position of Gal β and 3 position and/or 4 position of GlcNAc β, (SO_3-6) represents addition of a sulfate group to 6 position of GlcNAc β, R2 represents -3GalNAc α, -3Gal β or -2Manα and binds to 1 position of GlcNAc β.
The antigen may be present in cells transduced with HEC-GlcNAc6ST gene and may be absent or almost absent in cells transduced with GlcNAc6ST-1 gene or I-GlcNAc6ST gene.
[0010] As the antibody, MECA-79 antibody (Phamingen, catalog No. 09961D, shown in Figures 6 and 7) is preferably used.
According to a second aspect of the present invention there is provided an *in vitro* method for examining colorectal cancer and colorectal adenoma comprising assaying the reactivity of MECA-79 antibody or its equivalent with tissues, body fluid, feces or extract thereof of test subjects.
[0011] Furthermore, the present invention is any of said methods comprising the reaction of a labeled probe to said

antibody and qualitative or quantitative assay of the label. The preferable method for examination comprises fixing the antigens present in tissues, body fluid or feces or extracts thereof of patients to a membrane, reacting with the antibody, reacting with a labeled probe and detecting the label. It is preferable to insert washing procedures appropriately between the above processes. Said probe includes anti-human-IgG, antibody, protein G, protein A, and protein L. These probes are usually labeled. Said labels include a radioactive isotope (125I) and enzymes (peroxidase, alkaline phosphatase). An antibody with enzyme may involve observation of a change (i.e. color change) by the reaction between the enzyme and the substrate.

[0012] According to a third aspect of the present invention there is provided an antibody (excluding MECA-79 antibody) reacting specifically with an antigen carrying sugar residues, which is present in cells expressing HEC-GlcNAc6ST gene and is absent or almost absent in cells expressing GlcNAc6ST-1 or GlcNAc6ST gene, wherein said sugar residues are expressed by the following general formula:

R1-Gal β1-3/4(SO_3-6) GlcNAc β1-R2,

where R1 represents sugar residues added by other enzymes and is not limited in structure, Gal β represents β galactose, GlcNAc β represents β N-acetylglucosamine, Gal β1-3/4 represents binding of 1 position of Gal β and 3 position and/or 4 position of GlcNAc β, (SO_3-6) represents addition of a sulfate group to 6 position of GlcNAc β, R2 represents -3GalNAc α, -3Gal β or -2Manα and binds to 1 position of GlcNAc β.

[0013] According to a fourth aspect of the present invention there is provided an antibody (excluding MECA-79 antibody) reacting specifically with an antigen carrying sugar residues, which is present in cells transduced with HEC-GlcNAc6ST gene and is absent or almost absent in cells transduced with GlcNAc6ST-1 or GlcNAc6ST gene, wherein said sugar residues are expressed by the following general formula:

R1-Gal β1-3/4(SO_3-6) GlcNAc β1-R2,

where R1 represents sugar residues added by other enzymes and is not limited in structure, Gal β represents β galactose, GlcNAc β represents β N-acetylglucosamine, Gal β1-3/4 represents binding of 1 position of Gal β and 3 position and/or 4 position of GlcNAc β, (SO_3-6) represents addition of a sulfate group to 6 position of GlcNAc β, R2 represents -3GalNAc α, -3Gal β or -2Manα and binds to 1 position of GlcNAc β.According to a fifth aspect of the present invention there is provided an antibody (excluding MECA-79 antibody) reacting specifically with an antigen carrying sugar residues, which are present in tissues, body fluid or feces of patients with colorectal cancer and colorectal adenoma and expressed by the following formula:

R1-Gal β1-3/4(SO_3-6) GlcNAc β1-R2,

where, R1 represents sugar residues added by other enzymes and is not limited in structure Gal β represents β galactose, GlcNAc β represents β N-acetylglucosamine, Gal β1-3/4 represents binding of 1 position of Gal β and 3 position and/or 4 position of GlcNAc β, (SO_3-6)represents addition of a sulfate group to 6 position of GlcNAc β, R2 represents -3GalNAc α, -3Gal β or -2Manα and binds to 1 position of GlcNAc β.

According to a sixth aspect of the present invention there is provided an *in vitro* method for examining colorectal cancer and colorectal adenoma comprising assaying the reactivity of an antibody to tissues, body fluid or feces of patients, or extracts thereof, wherein said antibodies react with an antigen comprising the sugar residues expressed by the following formula:

R1-Gal β1-3/4(SO_3-6) GlcNAc β1-R2,

where, R1 represents sugar residues added by other enzymes and is not limited in structure, Gal β represents β galactose, GlcNAc β represents β N-acetylglucosamine, Gal β1-3/4 represents binding of 1 position of Gal β and 3 position and/or 4 position of GlcNAc β, (SO_3-6) represents addition of a sulfate group to 6 position of GlcNAc β, R2 represents -3GalNAc α, -3Gal β or -2Manα and binds to 1 position of GlcNAc β.Brief Description of the Drawings

Figure 1 shows the results of flowcytometric analysis using MECA-79 antibody on colorectal cancer cells (COL0201 cells) and on normal colorectal epithelial cells (SW480 cells treated with Tricostatine A). [a] shows the enzyme specificity of colorectal cancer cells (COL0201 cells) and normal colorectal epithelial cells (SW480 cells). [b] shows the results of flowcytometric analysis on the reactivity of anti-6-sulfated sugar residues antibody with the above 2 kinds of cells. The ordinate shows the cell frequency (the number of cells) and the abscissa axis shows the fluorescence (Arbitrary Unit).

Figure 2 shows the result of the flowcytometric analysis on the reactivity of MECA-79 antibody with cells transduced with HEC-GlcNAc6ST gene, GlcNAc6ST-1 gene or I-GlcNAc6ST gene. The ordinate shows the cell frequency (the number of cells) and the abscissa axis shows the fluorescence (Arbitrary Unit).

Figure 3 shows the photographs of tissues stained with MECA-79 antibody of a colorectal cancer patient. Ca shows a colorectal cancer tissue and N shows non-cancer colorectum tissue.

Figure 4 shows the photographs of tissues of colorectal cancer and normal colorectum of a patient stained with MECA-79 antibody. Ca shows cancer tissues and N shows non-cancer colorectum tissues in a to c. N shows a non-adenomatous colorectum tissue and A shows adenomatous cells in d. The black part (brown in color) shows the presence of antigens recognizable by the antibody and the gray part (faint blue in color) shows control staining with methylene blue.

Figure 5 shows the reactivity of MECA-79 antibody with fecal extracts of patients. Each row from above shows colorectal cancer 8 cases, colorectal adenoma 8 cases, benign 8 cases and healthy control 8 cases, respectively.

Figure 6 shows a catalog of MECA-79 antibody (Pharmingen, catalog No. 09961D).

Figure 7 shows a catalog of MECA-79 antibody (Pharmingen, catalog No. 09961D).

Detailed Description of the Invention

[0014] The structure of 6-sulfated sugar residues, which are little synthesized by GlcNAc6ST-1 or by I-GlcNAc6ST, but are synthesized only by HEC-GlcNAc6ST is expressed as the following general formula:

R1-Gal β1-3/4 (SO_3-6) GlcNAc β1-R2.

[0015] GlcNAcβ, which is the substrate of GlcNAc6-sulfotransferase in a body, is carried by various sugar residue carrier. R2 shows the carrier.
HEC-GlcNAc6ST has been known to transfer sulfate residues to all kinds of GlcNAcβ1-R2 previously tested according to both our research and other people's research. In contrast, GlcNAc6ST-1 and I-GlcNAc6ST transfer sulfate residues only to such GlcNAcβ-R2 as accompanied with a specific form of R2. The case, which HEC-GlcNAc6ST but not GlcNAc6ST-1 nor I-GlcNAc6ST can transfer sulfate residues, is known as a case when R2 is -3GalNAcα (the structure after sulfation is SO_3-6GlcNAcβ1-3GalNAcα), a case when R2 is -3Galβ (the structure after sulfation is SO_3-6GlcNAcβ1-3Galβ) and a case when R2 is -2Manα (the structure after sulfation is SO_3-6GlcNAcβ1-2Manα). In the examination method of the present invention, a specific antibodies to any of the three cases and antibodies cross reacting to all three sugar residues may be usable.
[0016] GlcNAc-6-sulfotransferase adds sulfate group to distal GlcNAc of sugar residues and synthesizes 6-sulfated GlcNAc (i.e. SO_3-6GlcNAc) intra-cellularly. However, after synthesis of distal 6-sulfated GlcNAc of sugar residues, the modified sugar residues are further added sugar residues (R1) by other enzyme groups intra-cellularly, and a large variety of the structure and antigenicity of the sugar residues are finally synthesized and produced from cells. Generally the structure added to 6-sulfated GlcNAc is Galβ1-4 and Galβ1-3 (referred to as Galβ1-3/4). Moreover, it is known that NeuAc α2-3/6, SO_3-3/6, and Fuc α1-2/3/4 are added to the 6-sulfated GlcNAc. The R1 part is added after the synthesis of 6-sulfated GlcNAc by GlcNAc-6-sulfotransferase. Therefore, R1 part is not related to the substrate specificity of such GlcNAc-6-sulfotransferases as HEC-GlcNAc6ST, GlcNAc6ST-1 and I-GlcNAc6ST.
[0017] The antigens with the above sugar residues are present in cancer tissues obtained from colorectal cancer patients by biopsy or by surgical operation, and present in such samples as serum, ascites and feces containing the antigens. Also, the antigen may be easily extracted from these samples using phosphate buffered saline. Also, the antibody against this sugar residue antigen could be obtained by known arts producing antibodies (e.g. Methods in Enzymology, 312: 160-179, 2000; Methods in Molecular Biology, 199: 203-218, 2002 et al.).
[0018] The GlcNAc-6-sulfated sugar residues detected by the present invention are positive not only in colorectal cancers but also in colorectal adenomas, which are regarded as the birthplace of colorectal cancers. Therefore, using the sugar residues as a target of screening test, a group of patients with colorectal adenomas, for whom endoscopic examination or follow-up is required, could be detected. Compared with the occult blood test with anti-hemoglobin antibody, which is currently used for screening of colorectal cancers, the present method has higher yield of detection of colorectal adenomas. Also, 6-sulfated sugar residues are originally abundant in right-half of colorectum, if a colorectum is separated in right-half and left-half, therefore the above method of diagnosis is particularly useful for the diagnosis of colorectal cancers and colorectal adenomas generated in right-half colorectum. Since colorectal cancers in right-half

are not frequently positive by fecal occult blood test using anti-hemoglobin antibody, concomitant use of the present method may contribute to increased yield of positive diagnosis in right-half colorectal cancers.
The following examples are provided to illustrate the present invention, but are not intended to limit the scope thereof.

Example 1

[0019] Gene expression of GlcNAc-6-sulfotransferase isozymes was examined by RT-PCR on human-derived colorectal cancer cells and on normal colorectal epithelial cells. In the RT-PCR analysis, PCR primers for detection of the expression of HEC-GlcNAc6ST gene (Genebank, AF131235) are synthetic oligonucleotides of SEQ ID NO. 1 for upper strand side and those of SEQ ID NO. 2 for lower strand side (Tm=59°C), those for GlcNAc6ST-1 gene (Genebank, AB011451) are synthetic oligonucleotides of SEQ ID NO. 3 for upper strand side and those of SEQ ID NO. 4 for lower strand side (Tm=62°C) and those for I-GlcNAc6ST gene (Genebank, AF176838) are synthetic oligonucleotides of SEQ ID NO. 5 for upper strand side and those of SEQ ID NO. 6 for lower strand side (Tm=60°C).
The results are shown in Figure 1a. COL0201 cells are typical cells showing colorectal cancer pattern, which expresses strongly HEC-GlcNAc6ST gene and little GlcNAc6ST-1 and I-GlcNAc6ST genes. TSA-SW480 cells treated SW480 cells with tricostatinA are typical cells showing normal epithelial pattern, which little expresses HEC-GlcNAc6ST gene, but significantly expresses GlcNAc6ST and I-GlcNAc6ST genes.
[0020] Then, a number of anti6-sulfated sugar residues antibody were screened based on the reactivity to the above two kinds of cells. In other words, antibodies, which react well with COLO201 cells and not react with TSA-SW480 cells, were searched. The screening of the reactivity between cells and antibodies was performed by flowcytometric analysis by use of FACScan (Becton Dickinson) stained cells with indirect fluorescent antibody method (the first antibody 1.0 μg/ml, 4°C, 30 min, the second antibody rabbit anti-rat IgM antibody (Zymed Laboratories), 4°C, 30 min). A typical result of the analysis is shown in Figure 1b. MECA-79 antibody (Pharmingen, catalog No. 09961D, Distributor: Becton Dickinson) showed strong reactivity with COL0201 cells, but showed little reactivity with TSA-SW480 cells. The above result showed that MECA-79 antibody is a preferable antibody for diagnosis of colorectal cancers. While, G72 antibody used as a control (J. Biol. Chem., 273: 11225-11233, 1998) reacted significantly with both COLO201 cells and TSA-SW480 cells and is not appropriate for diagnosis of colorectal cancers.

Example 2

[0021] In this example, cells transduced with HEC-GlcNAc6ST gene, GlcNAc6ST-1 gene or I-GlcNAc6ST gene were prepared. Then flowcytometric analysis for these cells was performed using MECA-79 antibody.
For the preparation of cells transduced with HEC-GlcNAc6ST gene, the gene (Genebank, AF131235) inserted into pCDNA3.1 vector was used. For those with GlcNAc6ST-1 gene, the gene (Genebank, AB011451) inserted into pIRES1hygro vector was used. For those with I-GlcNAc6ST gene, the gene (Genebank, AF176838) inserted into pCDNA3.1 vector was used. The flowcytometric analysis was performed as in Example 1.
The results are shown in Figure 2. MECA-79 antibody reacted strongly with those cells transduced with HEC-GlcNAc6ST, but reacted with slightly those cells transduced with GlcNAc6ST gene or I-GlcNAc6ST gene.

Example 3

[0022] Colorectal cancer tissues derived from patients (31 cases) were stained with immunohistological staining using MECA-79 antibody. For immunohistological staining, frozen sections with 10 μm thick were used, 1.0 μg/ml MECA-79 antibody was used as the first antibody, and a reagent kit (Vectastain) of Vecta Co. containing anti-rat IgM antibody was used as the second antibody according to the protocol of the company.
The results are shown in Figure 3. The antibody does not react with non-cancer colorectal mucosa (N) (0 case/31 ca31 cases, 32 %). Positive reaction rate is higher for cancers in right-half colorectum (60%) and lower for those in left-half colon (19%).
[0023] A typical stained photographs are shown in Figure 4.

Figure 4a shows the stained photograph of a colorectal cancer tissue (ca) and a colorectal non-cancer tissue (N) of a patient. The colorectal cancer tissue is strongly stained but the non-cancer tissue is little stained.
Figure 4b shows the photograph of the same tissue stained using AG107 antibody. Since AG107 antibody reacts with general GlcNAc-6-sulfated sugar residues, the non-cancer tissue (N) was stained well much more than the cancer tissue (Ca) in contrast to 4a and GlcNAc-6-sulfated sugar residues only could not be used for the specific detection of cancer tissues. Namely, use of such antibody as MECA-79, which detects the specific GlcNAc-6-sulfated sugar residues abundant in colorectal cancers and colorectal adenomas, could be applied for the specific detection.
Figure 4c shows the example of the expression of the sugar residues in a colorectal cancer tissue derived from

right-half colorectum. That the cancer tissue is strongly stained shows strong expression of the sugar residues. Figure 4d shows the expression in an adenomatous polyposis coli. N shows non-adenomatous colorectal tissue and A shows adenomatous cells. The adenomatous part of A is well stained by MECA-79 antibody. GlcNAc-6-sulfated sugar residues, which could be detected by MECA-79 antibody, are expressed abundantly in non-cancer adenomatous polyposis as well as in cancer tissues. Adenomatous polyposis coli, which is benign by itself but is regarded as the birthplace of colorectal cancer, could be detected credibly.

Example 4

[0024] In this example, an enzyme-linked immunosorbent assay, which is a simple qualitative test and uses MECA-79 antibody for the reaction with fecal extracts of colorectal cancer patient, confirmed the emergence of GlcNAc-6-sulfated sugar residues in feces of patients. Sometimes sugar residue antigens are decomposed by enzymes secreted from fecal bacterium and could not be detected in feces. Therefore, the confirmation is important for the practicability of the present invention.
0.1 g human feces was dispersed in 1 ml fecal extraction buffer (10 mM PBS, 1% BSA, pH 7.5), centrifuged at 8,000g for 15 min at 4°C, and the supernatant was recovered. To 40 μl supernatant, 120 μl extraction buffer was added and the sample was prepared. The sample was blotted to a PVDF membrane (Immobilon, Milipor, Lot K2JN2659B) by suction and the membrane was reacted with MECA-79 antibody, rabbit anti-rat IgM antibody, POD labeled goat anti-rabbit IgG antibody, and avidin-biotin complex solution, sequentially, after blocked for nonspecific reactions, and stained in NTB solution.
The results are shown in Figure 5.
Positive results are 4 cases in 8 cases of colorectal cancers (50 %) and 4 cases in 8 cases of colorectal adenomas (50 %). For benign disorders cases, 1 case gives a slight positive result and almost all cases of normal healthy subjects are negative.

SEQUENCE LISTING

[0025]

<110> Japan Science and Technology Agency

<120>

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Claims

1. An *in vitro* method for examining colorectal cancer and colorectal adenoma comprising assaying the reactivity of an antibody to tissues, body fluid or feces of patients, or extracts thereof, wherein said antibodies react with such antigen that is present in cells expressing HEC-GlcNAc6ST gene encoding GlcNAc-6-sulfotransferase and that is absent or almost absent in cells expressing GlcNAc6ST-1 or I-GlcNAc6ST gene, wherein said antigen comprises the sugar residues expressed by the following formula:

R1-Gal β1-314(SO_3-6) GlcNAc β1-R2,

where R1 represents sugar residues added by other enzymes and is not limited in structure, Gal β represents β galactose, GlcNAc β represents β N-acetylglucosamine, Gal β1-3/4 represents binding of 1 position of Gal β and 3 position and/or 4 position of GlcNAc β, (SO_3-6) represents addition of a sulfate group to 6 position of GlcNAc β, R2 represents -3GalNAc α, -3Gal β or -2Manα and binds to 1 position of GlcNAc β.

2. The method of claim 1, wherein said antigen is present in cells transduced with HEC-GlcNAc6ST gene and is absent or almost absent in cells transduced with GlcNAc6ST-1 gene or I-GlcNAc6ST gene.

3. The method of any one of claims 1 or 2, wherein said antibody is MECA-79 antibody (Pharmingen, catalog No. 09961D).

4. An *in vitro* method for examining colorectal cancer and colorectal adenoma comprising assaying the reactivity of MECA-79 antibody or its equivalent with tissues, body fluid, feces or extract thereof of test subjects.

5. The method of any one of claims 1 to 4 comprising reacting a labeled probe to said antibody and assaying the label qualitatively or quantitatively.

6. An antibody (excluding MECA-79 antibody) reacting specifically with an antigen carrying sugar residues, which is present in cells expressing HEC-GlcNAc6ST gene and is absent or almost absent in cells expressing GlcNAc6ST-1 or GlcNAc6ST gene, wherein said sugar residues are expressed by the following general formula:

R1-Gal β1-3/4(SO_3-6) GlcNAc β1-R2,

where R1 represents sugar residues added by other enzymes and is not limited in structure, Gal β represents β galactose, GlcNAc β represents β N-acetylglucosamine, Gal β1-3/4 represents binding of 1 position of Gal β and 3 position and/or 4 position of GlcNAc β, (SO_3-6) represents addition of a sulfate group to 6 position of GlcNAc β, R2 represents -3GalNAc α, -3Gal β or -2Manα and binds to 1 position of GlcNAc β.

7. An antibody (excluding MECA-79 antibody) reacting specifically with an antigen carrying sugar residues, which is present in cells transduced with HEC-GlcNAc6ST gene and is absent or almost absent in cells transduced with GlcNAc6ST-1 or GlcNAc6ST gene, wherein said sugar residues are expressed by the following general formula:

R1-Gal β1-3/4(SO_3-6) GlcNAc β1-R2,

where R1 represents sugar residues added by other enzymes and is not limited in structure, Gal β represents β galactose, GlcNAc β represents β N-acetylglucosamine, Gal β1-3/4 represents binding of 1 position of Gal β and 3 position and/or 4 position of GlcNAc β, (SO_3-6) represents addition of a sulfate group to 6 position of GlcNAc β, R2 represents -3GalNAc α, -3Gal β or -2Manα and binds to 1 position of GlcNAcβ.

8. An antibody (excluding MECA-79 antibody) reacting specifically with an antigen carrying sugar residues, which are present in tissues, body fluid or feces of patients with colorectal cancer and colorectal adenoma and expressed by the following formula:

R1-Gal β1-3/4(SO_3-6) GlcNAc β1-R2,

where, R1 represents sugar residues added by other enzymes and is not limited in structure Gal β represents β galactose, GlcNAc represents β N-acetylglucosamine, Gal β1-3/4 represents binding of 1 position of Gal β and 3 position and/or 4 position of GlcNAc β, (SO_3-6) represents addition of a sulfate group to 6 position of GlcNAc β, R2 represents -3GalNAc α, -3Gal β or -2Manα and binds to 1 position of GlcNAc β.

9. An *in vitro* method for examining colorectal cancer and colorectal adenoma comprising assaying the reactivity of an antibody to tissues, body fluid or feces of patients, or extracts thereof, wherein said antibodies react with an antigen comprising the sugar residues expressed by the following formula:

R1-Gal β1-3/4(SO_3-6) GlcNAc β1-R2,

where, R1 represents sugar residues added by other enzymes and is not limited in structure, Gal β represents β galactose, GlcNAc β represents β N-acetylglucosamine, Gal β1-3/4 represents binding of 1 position of Gal β and 3 position and/or 4 position of GlcNAc β, (SO_3-6) represents addition of a sulfate group to 6 position of GlcNAc β, R2 represents -3GalNAc α, -3Gal β or -2Manα and binds to 1 position of GlcNAc β.

Patentansprüche

1. *In vitro*-Verfahren zur Untersuchung von kolorektalem Krebs und kolorektalem Adenom, umfassend die Untersuchung der Reaktivität eines Antikörpers mit Geweben, Körperflüssigkeiten oder Faeces von Patienten, oder Auszügen daraus, wobei die Antikörper mit einem solchen Antigen reagieren, das in Zellen vorkommt, die das Gen HEC-GlcNAc6ST exprimieren, welches für GlcNAc-6-Sulfotransferase kodiert, und das in Zellen, die das Gen GlcNAc6ST-1 oder I-GlcNAc6ST exprimieren, nicht oder praktisch nicht vorkommt, wobei das Antigen die Zuckerreste umfasst, die durch die folgende Formel ausgedrückt werden:

   R1-Gal β1-3/4(SO_3-6) GlcNAc β1-R2,

   wobei R1 Zuckerreste darstellt, die durch andere Enzyme hinzugefügt werden und in seinem Aufbau nicht eingeschränkt ist, Gal β β-Galaktose darstellt, GlcNAc β β-N-Acetylglucosamin darstellt, Gal β1-3/4 die Bindung der Position 1 von Gal β und der Position 3 und/oder der Position 4 von GlcNAc β darstellt, (SO_3-6) die Anfügung einer Sulfatgruppe an die Position 6 von GlcNAc β darstellt, R2 -3GalNAc α, -3Gal β oder -2Manα darstellt und an Position 1 von GlcNAc β bindet.

2. Verfahren nach Anspruch 1, wobei das Antigen in Zellen vorkommt, die mit dem Gen HEC-GlcNAc6ST transduziert sind, und in Zellen, die mit dem Gen GlcNAc6ST-1 oder dem Gen I-GlcNAc6ST transduziert sind, nicht oder praktisch nicht vorkommt.

3. Verfahren nach einem der Ansprüche 1 oder 2, wobei der Antikörper der Antikörper MECA-79 (Pharmingen. Katalog Nr. 09961D) ist.

4. *In vitro*-Verfahren zur Untersuchung von kolorektalem Krebs und kolorektalem Adenom, umfassend die Untersuchung der Reaktivität des Antikörpers MECA-79 oder dessen Äquivalents mit Geweben, Körperflüssigkeiten oder Faeces von Patienten, oder Auszügen daraus.

5. Verfahren nach einem der Ansprüche 1 bis 4, umfassend die Umsetzung einer markieren Sonde mit dem Antikörper und die qualitative oder quantitative Untersuchung der Maserung.

6. Antikörper (mit Ausnahme des Antikörpers MECA-79), der spezifisch mit einem Antigen reagiert, das Zuckerreste aufweist und in Zellen vorkommt, die das Gen HEC-GlcNAc6ST exprimieren, und in Zellen, die das Gen GlcNAc6ST-1 oder GlcNAc6ST exprimieren, nicht oder praktisch nicht vorkommt, wobei die Zuckerreste durch die folgende allgemeine Formel ausgedrückt werden:

   R1-Gal β1-3/4(SO_3-6) GlcNAc β1-R2,

   wobei R1 Zuckerreste darstellt, die durch andere Enzyme hinzugefügt werden und in seinem Aufbau nicht eingeschränkt ist, Gal β β-Galaktose darstellt, GlcNAc β β-N-Acetylglucosamin darstellt, Gal β1-3/4 die Bindung der Position 1 von Gal β und der Position 3 und/oder der Position 4 von GlcNAc β darstellt, (SO_3-6) die Anfügung einer Sulfatgruppe an die Position 6 von GlcNAc β darstellt, R2 -3GalNAc α, -3Gal β oder -2Manα darstellt und an Position 1 von GlcNAc β bindet.

7. Antikörper (mit Ausnahme des Antikörpers MECA-79), der spezifisch mit einem Antigen reagiert, das Zuckerreste aufweist und in Zellen vorkommt, die mit dem Gen HEC-GlcNAc6ST transduziert sind, und in Zellen, die mit dem Gen GlcNAc6ST-1 oder GlcNAc6ST transduziert sind, nicht oder praktisch nicht vorkommt, wobei die Zuckerreste durch die folgende allgemeine Formel ausgedrückt werden:

   R1-Gal β1-3/4(SO_3-6) GlcNAc β1-R2,

   wobei R1 Zuckerreste darstellt, die durch andere Enzyme hinzugefügt werden und in seinem Aufbau nicht einge-

schränkt ist, Gal β β-Galaktose darstellt, GlcNAc β β-N-Acetylglucosamin darstellt, Gal β1-3/4 die Bindung der Position 1 von Gal β und der Position 3 und/oder der Position 4 von GlcNAc β darstellt, (SO_3-6) die Anfügung einer Sulfatgruppe an die Position 6 von GlcNAc β darstellt, R2 -3GalNAc α, -3Gal β oder -2Manα darstellt und an Position 1 von GlcNAc β bindet.

8. Antikörper (mit Ausnahme des Antikörpers MECA-79), der spezifisch mit einem Antigen reagiert, das Zuckerreste trägt, welche in Geweben, Körperflüssigkeiten oder Faeces von Patienten mit kolorektalem Krebs oder kolorektalem Adenom vorkommen und durch die folgende allgemeine Formel ausgedrückt werden:

R1-Gal β1-3/4(SO_3-6) GlcNAc β1-R2,

wobei R1 Zuckerreste darstellt, die durch andere Enzyme hinzugefügt werden und in seinem Aufbau nicht eingeschränkt ist, Gal β β-Galaktose darstellt, GlcNAc β β-N-Acetylglucosamin darstellt, Gal β1-3/4 die Bindung der Position 1 von Gal β und der Position 3 und/oder der Position 4 von GlcNAc β darstellt, (SO_3-6) die Anfügung einer Sulfatgruppe an die Position 6 von GlcNAc β darstellt, R2 -3GalNAc α, -3Gal β oder -2Manα darstellt und an Position 1 von GlcNAc β bindet.

9. *In vitro*-Verfahren zur Untersuchung von kolorektalem Krebs und kolorektalem Adenom, umfassend die Untersuchung der Reaktivität eines Antikörpers mit Geweben, Körperflüssigkeiten oder Faeces von Patienten, oder Auszügen daraus, wobei die Antikörper mit einem Antigen reagieren, das die Zuckerreste umfasst, die durch die folgende Formel ausgedrückt werden:

R1-Gal β1-3/4(SO_3-6) GlcNAc β1-R2,

wobei R1 Zuckerreste darstellt, die durch andere Enzyme hinzugefügt werden und in seinem Aufbau nicht eingeschränkt ist, Gal β β-Galaktose darstellt, GlcNAc β β-N-Acetylglucosamin darstellt, Gal β1-3/4 die Bindung der Position 1 von Gal β und der Position 3 und/oder der Position 4 von GlcNAc β darstellt, (SO_3-6) die Anfügung einer Sulfatgruppe an die Position 6 von GlcNAc β darstellt, R2 -3GalNAc α, -3Gal β oder -2Manα darstellt und an Position 1 von GlcNAc β bindet,

Revendications

1. Procédé in vitro pour examiner un cancer colorectal et un adénome colorectal, comprenant la réalisation d'un essai de réactivité d'un anticorps sur des tissus, un fluide corporel ou des selles de patients, ou sur des extraits de ceux-ci, dans lequel ledit anticorps réagit avec un antigène qui est présent dans des cellules exprimant le gène HEC-GlcNAc6ST codant pour la GlcNAc-6-sulfotransférase et qui est absent ou quasiment absent dans des cellules exprimant le gène GlcNAc6ST-1 ou I-GlcNAc6ST, ledit antigène comprenant les résidus de sucre exprimés par la formule suivante :

R1-Gal β1-3/4(SO_3-6)GlcNAc β1-R2,

dans laquelle R1 représente des résidus de sucre ajoutés par d'autres enzymes et n'est pas limité en structure, Gal β représente le β-galactose, GlcNAc β représente la β-N-acétylglucosamine, Gal β1-3/4 représente une liaison de Gal β en position 1 et de GlcNAc β en position 3 et/ou en position 4, (SO_3-6) représente l'addition d'un groupement sulfate sur la position 6 de la GlcNAc β, R2 représente -3GalNAc α, -3Gal β ou -2Manα et se lie à la GlcNAc β au niveau de la position 1,

2. Procédé selon la revendication 1, dans lequel ledit antigène est présent dans des cellules transduites avec le gène HEC-GlcNAc6ST et est absent ou quasiment absent dans des cellules transduites avec le gène GlcNAc6ST-1 ou I-GlcNAc6ST.

3. Procédé selon l'une quelconque des revendications 1 ou 2, dans lequel l'anticorps est l'anticorps MECA-79 (Pharmingen, numéro de référence 09961D).

4. Procédé in vitro pour examiner un cancer colorectal et un adénome colorectal, comprenant la réalisation d'un essai de réactivité de l'anticorps MECA-79 ou de son équivalent sur des tissus, un fluide corporel, des selles ou des extraits de ceux-ci provenant de sujets d'essai.

5. Procédé selon l'une quelconque des revendications 1 à 4, comprenant la réaction d'une sonde marquée avec ledit anticorps et la réalisation d'un essai qualitatif ou quantitatif du marqueur.

6. Anticorps (à l'exception de l'anticorps MECA-79) réagissant spécifiquement avec une antigène portant des résidus de sucre, qui est présent dans des cellules exprimant le gêne HEC-GlcNAc6ST et qui est absent ou quasiment absent dans des cellules exprimant le gène GlcNAc6ST-1 ou GlcNAc6ST, dans lequel lesdits résidus de sucre sont exprimés par la formule générale suivante :

R1~Gal β1-3/4(SO_3-6) GlcNAc β1-R2,

dans laquelle R1 représente des résidus de sucre ajoutés par d'autres enzymes et n'est pas limité en structure, Gal β représente le β-galactose, GlcNAc β représente la β-N-acélylglucosamine, Gal β1-3/4 représente une liaison de Gal β en position 1 et de GlcNAc β en position 3 et/ou en position 4, (SO_3-6) représente l'addition d'un groupement sulfate en position 6 de la GlcNAc β, R2 représente -3GalNAc α, -3Gal β ou -2Manα et se lie à la GlcNAc β au niveau de la position 1.

7. Anticorps (à l'exception de l'anticorps MECA-79) réagissant spécifiquement avec un antigène portant des résidus de sucre, qui est présent dans des cellules transduites avec le gène HEC-GlcNAc6ST et qui est absent ou quasiment absent dans des cellules transduites avec le gène GlcNAc6ST-1 ou GlcNAc6ST, dans lequel lesdits résidus de sucre sont exprimés par la formule générale suivante :

R1-Gal β1-3/4(SO_3-6) GlcNAc β1-R2,

dans laquelle R1 représente des résidus de sucre ajoutés par d'autres enzymes et n'est pas limité en structure, Gal β représente le β-galactose, GlcNAc β représente la β-N-acétylglucosamine, Gal β1-3/4 représente une liaison de Gal β en position 1 et de GlcNAc β en position 3 et/ou en position 4, (SO_3-6) représente l'addition d'un groupement sulfate en position 6 de la GlcNAc β, R2 représente -3GalNAc α, -3Gal β ou -2Manα et se lie à la GlcNAc β au niveau de la position 1.

8. Anticorps (à l'exception de l'anticorps MECA-79) réagissant spécifiquement avec un antigène portant des résidus de sucre, qui sont présents dans des tissus, un fluide corporel ou des selles de patients atteints d'un cancer colorectal et d'un adénome colorectal et qui sont exprimés par la formule suivante :

R1-Gal β1-3/4(SO_3-6) GlcNAc β1-R2,

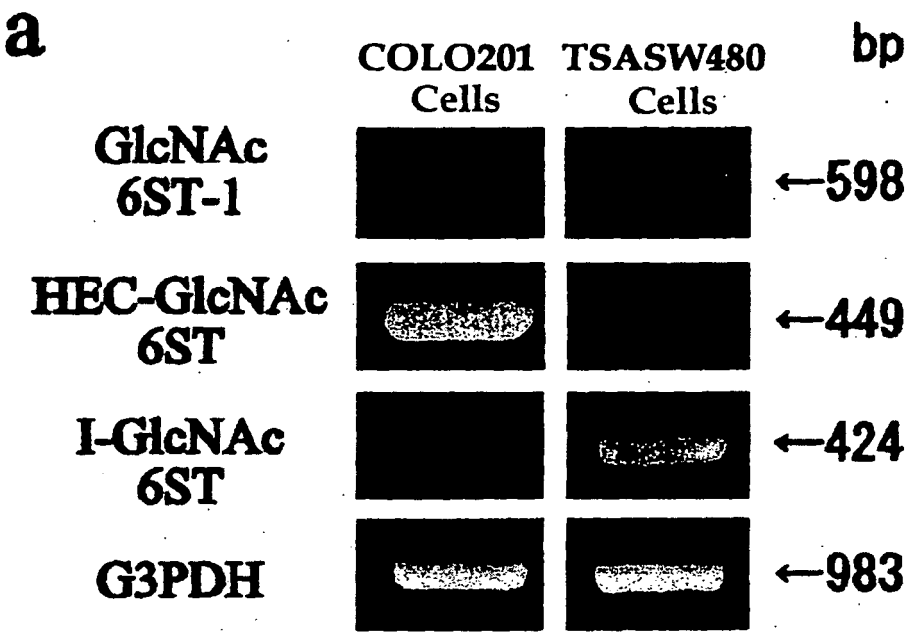
dans laquelle R1 représente des résidus de sucre ajoutés par d'autres enzymes et n'est pas limité en structure, Gal β représente le β-galactose, GlcNAc β représente la β-N-acétylglucosamine, Gal β1-3/4 représente une liaison de Gal β en position 1 et de GlcNAc β en position 3 et/ou en position 4, (SO_3-6) représente l'addition d'un groupement sulfate en position 6 de la GlcNAc β, R2 représente -3GalNAc α, -3Gal β ou ~2Manα et se lie à la GlcNAc β au niveau de la position 1.

9. Procédé in vitro pour examiner un cancer colorectal et un adénome colorectal, comprenant la réalisation d'un essai de réactivité d'un anticorps sur des tissus, un fluide corporel ou des selles de patients, ou des extraits de ceux-ci, dans lequel ledit anticorps réagit avec un antigène comprenant les résidus de sucre exprimés par la formule suivante :

R1-Gal β1-3/4(SO_3-6) GlcNAc β1-R2,

dans laquelle R1 représente des résidus de sucre ajoutés par d'autres enzymes et n'est pas limité en structure, Gal β représente le β-galactose, GlcNAc β représente la β-N-acétylglucosamine, Gal β1-3/4 représente une liaison de Gal β en position 1 et de GlcNAc β en position 3 et/ou en position 4, (SO_3-6) représente l'addition d'un groupement sulfate en position 6 de la GlcNAc β, R2 représente -3GalNAc α, -3Gal β ou -2Manα et se lie à la GlcNAc β au niveau de la position 1.

Figure 1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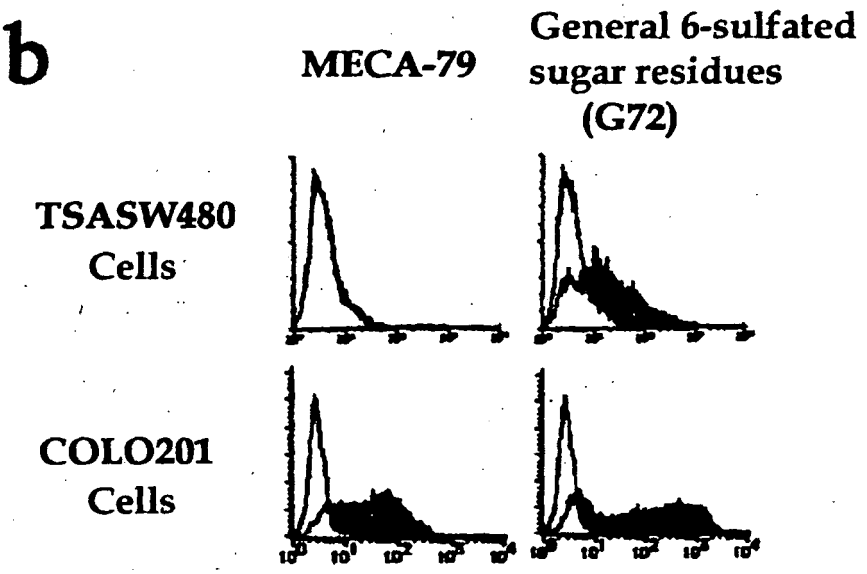
←983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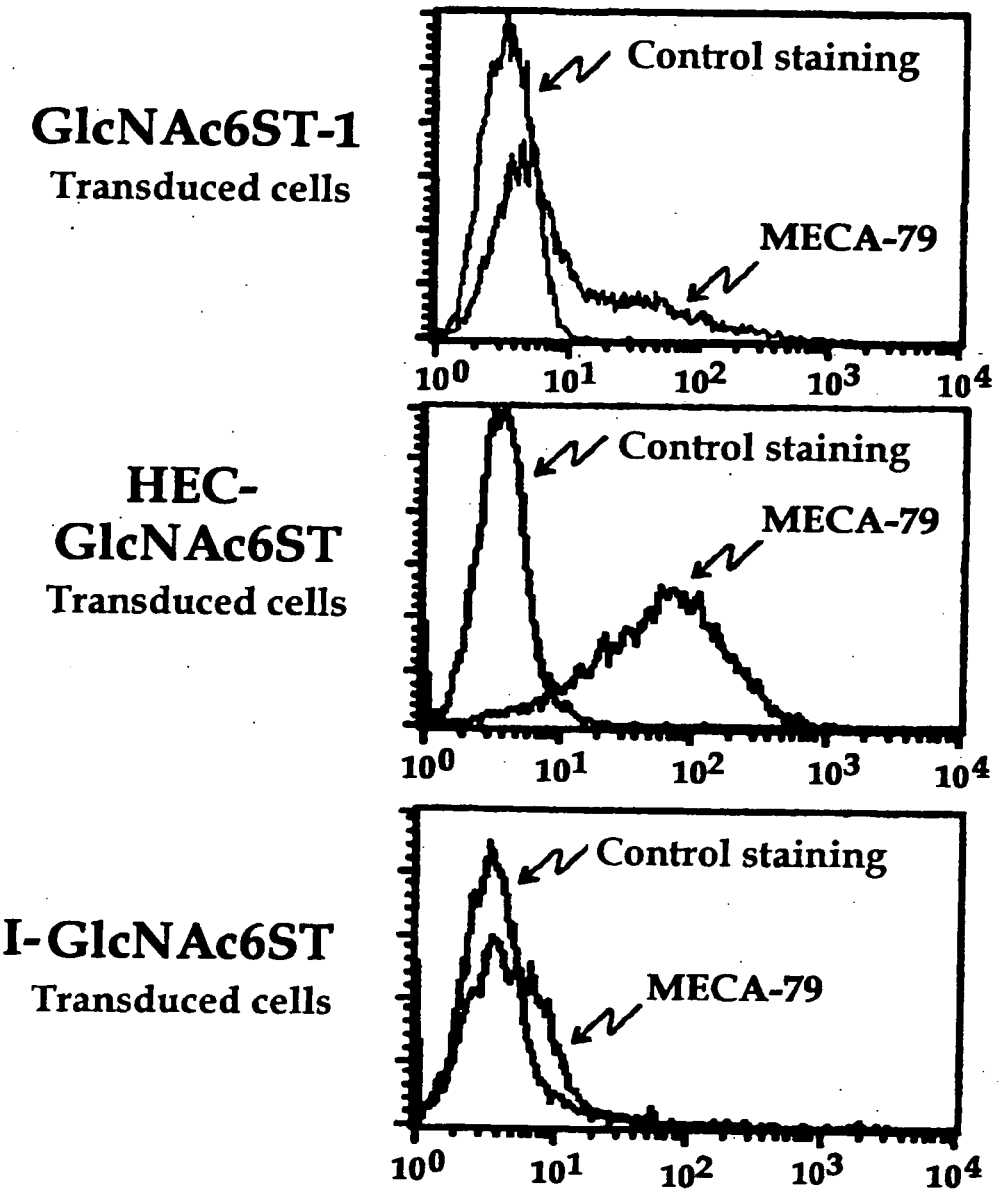
MECA-79
General 6-sulfated sugar residues (G72)
TSASW480 Cells
COLO201 Cells

Figure 2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Control staining
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10^4
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Transduced cells
Control staining
MECA-79
10^0
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10^2
10^3
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Control staining
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10^1
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10^3
10^4

Figure 3

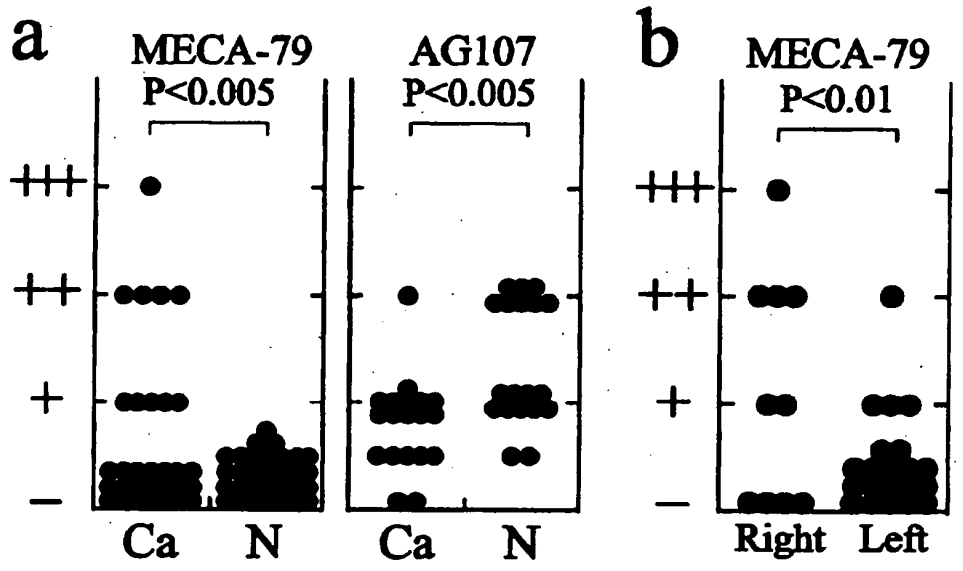

Figure 4

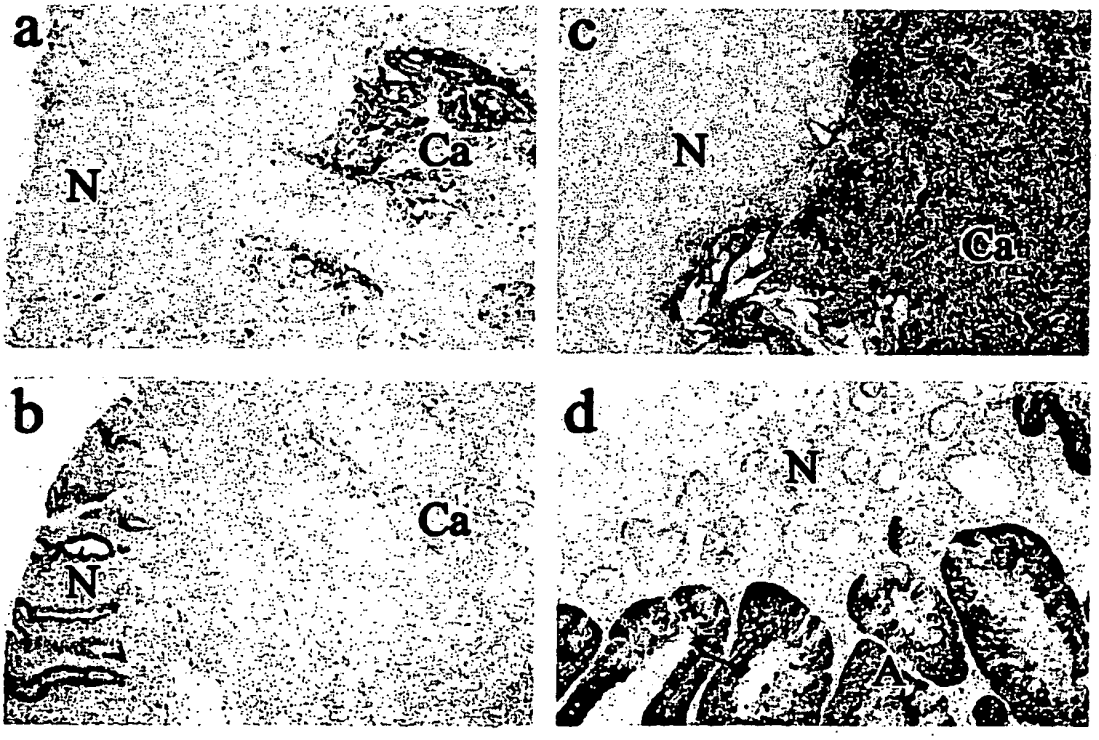

Figure 5

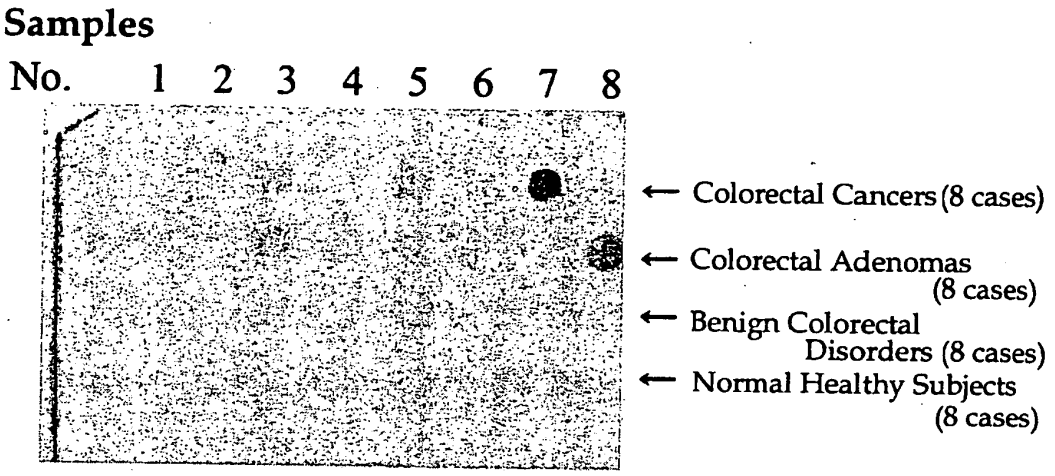
Samples
No. 1 2 3 4 5 6 7 8
← Colorectal Cancers (8 cases)
← Colorectal Adenomas (8 cases)
← Benign Colorectal Disorders (8 cases)
← Normal Healthy Subjects (8 cases)

Figure 6

BD PharMingen Technical Data Sheet

Page 1 of 2

PURIFIED RAT ANTI-MOUSE PNAd CARBOHYDRATE EPITOPE (CD62L Ligand) MONOCLONAL ANTIBODY

PRODUCT INFORMATION

Catalog Number:	**553863** (Was: 09961D), **0.5 mg**
Description:	Purified anti-mouse PNAd Carbohydrate Epitope (CD62L Ligand)
Clone:	MECA-79
Immunogen:	Collagenase-dispersed BALB/c lymph node stroma[1]
Isotype:	Rat (Wistar) IgM, κ
Contents:	Purified immunoglobulin in 10 mM phosphate buffer, pH 7.2 with 500 mM NaCl and 0.09% (w/v) sodium azide.

SPECIFICITY

The MECA-79 antibody reacts with sulfate-dependent carbohydrate epitopes of peripheral lymph node addressin (PNAd).[2] The MECA-79-reactive antigen is closely associated with the carbohydrate ligands for L-selectin (*e.g.*, CD34, GlyCAM-1, MAdCAM-1), which are expressed on high endothelial venules (HEV) in lymphoid tissues and at sites of chronic inflammation.[1,2,3,4,5,6] Cross-reactivity with human,[3,4] ovine,[7] bovine,[7] primate,[7] and porcine[8] tissues has been observed. MECA-79 antibody inhibits L-selectin– dependent lymphocyte and platelet homing to lymph nodes *in vivo*[1,9] and *in vitro* adhesion to lymphoid tissue HEV[1,4] and immobilized PNAd.[3,9,10]

PREPARATION AND STORAGE

The antibody was purified from tissue culture supernatant by affinity chromatography. The antibody solution should be stored undiluted at 4°C.

USAGE

This antibody has been tested by immunohistochemical staining (IHC) of citrate-pretreated formalin-fixed paraffin-embedded sections (5 - 20 µg/ml) to assure specificity and reactivity. Other reported applications include IHC of acetone-fixed frozen sections,[1,4,5] immunoprecipitation[2,3] western blot analysis,[10] and *in vitro* and *in vivo* adhesion blocking.[1,3,4,9,10] **Since applications vary, each investigator must determine dilutions appropriate for individual use.**

Caution: Sodium azide is a reversible inhibitor of oxidative metabolism; therefore, antibody preparations containing this preservative agent must not be used in cell cultures nor injected into animals. Sodium azide may be removed by washing stained cells or plate-bound antibody or dialyzing soluble antibody in sodium azide-free buffer. Since endotoxin may also affect the results of functional studies, we recommend the NA/LE™ (No Azide/Low Endotoxin) antibody format for *in vitro* and *in vivo* use.

REFERENCES

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Figure 7

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