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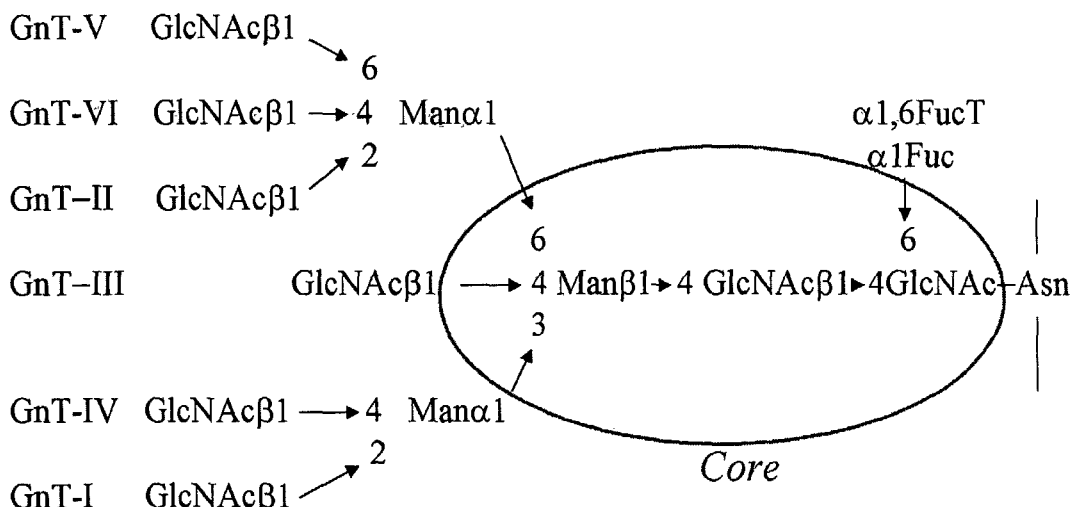
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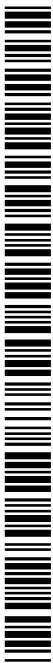
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(54) Title: METHOD FOR THE DIAGNOSIS OF CANCERS BY MEASURING THE CHANGES OF GLYCOSYLATION OF PROTEINS RELATED TO TUMORIGENESIS AND METASTASIS AND KIT FOR DIAGNOSIS OF CANCERS USING THE SAME



(57) Abstract: The present invention relates to a method for diagnosing cancers by measuring proteins associated with tumorigenesis and metastasis, and a diagnostic kit using the same, particularly relates to the method for diagnosing cancers by measuring the changes of glycosylation of proteins and the kit for diagnosis of cancers using the said method. The method and kit of the present invention can effectively be used for the sensitive diagnosis of cancers comprising colon cancer, stomach cancer, lung cancer and liver cancer.



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METHOD FOR THE DIAGNOSIS OF CANCERS BY  
MEASURING THE CHANGES OF GLYCOSYLATION OF  
PROTEINS RELATED TO TUMORIGENESIS AND  
METASTASIS AND KIT FOR DIAGNOSIS OF CANCERS  
5 USING THE SAME

FIELD OF THE INVENTION

The present invention relates to a method for  
diagnosing cancers by measuring proteins associated  
10 with tumorigenesis and metastasis and a diagnostic kit  
using the same, more particularly, relates to a method  
for diagnosing cancers by measuring the changes of  
glycosylation of proteins, especially, the changes of  
N-linked sugar chains of proteins associated with  
15 tumorigenesis and metastasis and a diagnostic kit using  
the same.

BACKGROUND ART OF THE INVENTION

In order to analyze the functions of proteins,  
20 two-dimensional electrophoresis has long been used.  
But recently, proteomics, a post-genome functional  
analyzing method, took the place of it, which was led  
by the development of mass spectrometer like MALDI-TOF  
and the establishment of easy methods for determining  
25 N-terminal amino acid sequences. The proteomics,

though, is limited in use for the research of cancer caused by the result of highly complicated signal transduction, because proteomics is selectively available for the analysis of functions at the one point of time although human body is in dynamic motion. In order to detect a cancer, it is more important to investigate the increased expression or post-translational modification that are resulted from signal transduction than to observe the appearance of new spots by staining. The amounts of proteins that can be detected on the two-dimensional electrophoresis are too small to analyze by simple staining. Observing glycosylation of proteins can help overcome the above problem and correct the errors of analysis based on post-translational modification. When patients group with cancer was compared with control group, it was difficult to find out the difference of spots between the two groups by general electrophoresis, in the meantime, it was possible to draw a clear line between the two groups by analyzing the changes of glycosylation with lectin. It is now called glycomics that is an upgraded analyzing method overcoming difficulties in analysis with proteomics and is characterized by tracing the changes of glycosylation of proteins while post-translational modification is undergoing.

From observing cellular biological changes during tumorigenesis and metastasis process, it is concluded that various kinds of glycoproteins or glycolipids on the surface of cell membrane are induced to go through  
5 "aberrant glycosylation" by the specific signal from oncogene, resulted in the changes of sugar chain that consecutively causes the changes of intercellular adhesion and recognition, resulted in tumorigenesis and metastasis in the event (Hakomori and Kannagi, 1983, *J.*  
10 *Natl. Cancer Inst.*, 71:231-251; Feizi, 1985, *Nature*, 314:53-571). When external stimuli come in, signals are transmitted via oncogene ras, transcription factor ets-1 to stimulate the expression of N-acetylglucosaminyltransferase V (GnT-V). The GnT-V is  
15 an enzyme catalyzing such reaction that N-acetylglucosamine is attached onto the  $\beta$  1,6 site of the basic sugar chain of a glycoprotein and is known to be directly associated with cancer invasion and metastasis (Dennis, et al., 1987, *Science*, 236:582-  
20 5853). As for glycoproteins, basic sugar chain is formed in endoplasmic reticulum (ER) after protein is synthesized, which moves to Golgi apparatus. Then, sugars are added thereto by various glycotransferases resulted from various vital phenomena of cells.  
25 Primary sugar chains are formed by catalyzing of six N-acetylglucosaminyltransferases (I-VI) and especially GnT-V forming  $\beta$  1,6-N-acetylglucosamine sugar chain has

been believed to be deeply associated with tumorigenesis and metastasis. GnT-V is located in Golgi apparatus. This enzyme makes target proteins be secreted to or out of the cell surface by causing the changes of sugar chains. At this time, glycoproteins recognize surface proteins of target cells and then adhere thereto, causing a cancer.

GnT-V was first noticed by the report of Dennis *et al.* in 1987 that the  $\beta$  1,6 branches were remarkably represented as cancer tissues were growing or during metastasis (Dennis, *et al.*, 1987, *Science*, 236:582-5853). A cell surface protein gp130 is one of major target proteins of GnT-V and shows highly metastasis activity when  $\beta$  1,6 N-acetylglucosamine is added. GnT-V knockout mice were established in which GnT-V was deficient in their embryonic stem (ES) cells and to which polyomavirus middle T antigen (referred "PyMT" hereinafter) viral oncogene was introduced in order to induce a cancer. Resultingly, the growth of cancer and metastasis induced by PyMT were remarkably inhibited in GnT-V knockout mice comparing with another normal mice group in which only PyMT was over-expressed (Granovsky, *et al.*, 2000, *Nature Med.*, 6:306-312), and the growth of  $\beta$  1,6 branch caused high metastasis especially in mice with breast cancer. Recent studies support that the GnT-V activity to 33 types of hepatocellular

carcinoma (HCC) tissues is fifty times as high as it's activity to normal tissues and four times as high as to cancer surrounding tissues (Yao, et al., 1998, *J Cancer Res. Clin. Oncol.*, 124:27-307). And high metastatic activity was also confirmed when large intestine cancer cell line WiDr in which GnT-V was over-expressed was introduced into immunodeficient mice in order to induce large intestine cancer or when angiogenesis was investigated by CAM analysis using fertilized eggs (Miyoshi, et al., 2001, unpublished results). Thus, GnT-V is believed to be associated with metastasis and have high metastatic activity regardless of types of tissues. GnT-V enzyme was purified from human lung cancer cells and a mouse kidney, and cDNA cloning and analysis of genomic structure and promoter of the enzyme have been made (Gu, et al., 1993, *J Biochem*, 113:614-619; Soreibah, et al., 1993, *J Biol. Chem.*, 268:15381-15385; Kang, et al., 1996, *J. Biol. Chem.*, 271:26706-26712). The present inventors also reported in a recent study that transcription factor ets-1 was deeply associated with the expression of GnT-V (Ko, et al., 1999, *J. Biol. Chem.*, 274(33):22941-22948). As for large intestine cancer, it now takes the 4<sup>th</sup> highest incidence in both men and women owing to the changes of dietary life into western and the development of the cancer increases continuously. However, there is no way to diagnose colon cancer

accurately except large intestine endoscopy, so far.

Thus, the present inventors detected  $\beta$  1,6-N-acetylglucosamine in which sugars were attached by GnT-V in cancer-induced cells and found out a novel glycoprotein showing the changes of sugar chains by analyzing amino acid sequences with a mass spectrometer. And the present inventors completed this invention by developing a method for diagnosing cancers by measuring the changes of sugar chains of the above protein in test samples and a diagnostic kit for cancers using the same.

#### DETAILED DESCRIPTION OF THE INVENTION

It is an object of the present invention to provide a method for diagnosing cancers by measuring the changes of sugar chains of proteins related to tumorigenesis and metastasis and a diagnostic kit using the same.

To achieve the above object, the present invention provides a method for diagnosing cancers by measuring the changes of sugar chains of proteins related to tumorigenesis and metastasis.

The present invention also provides a diagnostic kit for cancers using the above method.

Further features of the present invention will appear hereinafter.

To achieve the above object, the present invention provides a method for diagnosing cancers by  
5 measuring the changes of sugar chains of proteins related to tumorigenesis and metastasis.

Cancers stated above can be selected from a group consisting of colon cancer, gastric cancer, lung cancer,  
10 liver cancer, uterine cancer, breast cancer and pancreas cancer, but are not limited thereto.

The present invention provides a method for measuring the changes of sugar chain branches of proteins in cancer cells and metastatic cells  
15 representing the changes of  $\beta$  1,6 N-acetylglucosamine, that is the changes of N-linked sugar chains, by comparing them with those in normal cells. GnT-V forming sugar chains of  $\beta$  1,6 N-acetylglucosamine is associated with tumorigenesis and metastasis regardless  
20 of types of tissues, and the sugar chains of  $\beta$  1,6-N-acetylglucosamine can be detected with lectin phytohaemagglutinin-L4 (referred "L4-PHA" hereinafter).

Metastasis is caused by intercellular recognition and adhesion. Glycoproteins associated with  
25 intercellular recognition and adhesion, are on the cell surface or secreted therefrom. Thus, cancers can be diagnosed by detecting primary markers from body fluids

like blood or urine. In the preferred embodiments of the present invention, a colon cancer cell line WiDr, in which GnT-V expression was comparatively low, was used in order to identify specific markers of large intestine cancer, and glycomics was performed using 5 GnT-V/WiDr, a cell line over-expressing GnT-V, as a model system of colon cancer. In this invention, metastatic cancer cell culture solution was analyzed by 2-dimensional electrophoresis. As a result, 2 sheets of gel were obtained. One sheet of gel was stained with Coomassie brilliant blue and the other sheet was treated with lectin blotting. Finally, proteins in which sugar chains were changed in cancer cells and metastatic cells rather than in normal cells were separated (see FIG. 5 and FIG. 6). Some regions having strong dark spots were detected in cancer cells by lectin blot recognizing  $\beta$  1,6 N-acetylglucosamine branch. The proteins corresponding to the spots were confirmed to be associated with tumorigenesis and metastasis and show the changes of sugar chains. 10 15 20

The present inventors cut the spots off, and then cleaved proteins again. Determined amino acid sequence of the peptide using Electrospray Ionization (ESI)/Quadruple Time of Flight (Q-TOF) mass spectrometer, resulting in the confirmation of peptide sequences represented by SEQ. ID. Nos 1-15. Identified the peptide sequences with precise names by comparing 25

that with already established protein database and further analyzed each sequence, molecular weight and isoelectric point (see Table 1).

The above protein was confirmed based on the fact that N-linked sugar chain is located on the Asn in the sequence of Asn-Xaa-Thr/Ser (Varki, et al., 1999, *Essentials of glycobiology*, Cold Spring Harbor Laboratory, New York, USA, pp85-100).

The peptide sequences analyzed above, which are represented by SEQ. ID. No 1 and No 2, are the parts of PDF (prostate-derived factor). In the meantime, PDF is known as one of BMPs (Bone morphogenetic proteins) that is a member of TGF- $\beta$  (transforming growth factor beta) family and is associated with bone development and regeneration by inducing cartilage formation (Paralkar, V.M. et al., 1998, *J Biol. Chem*, 273:13760-13767). And the peptide has 2 well-reserved N-linked sugar chain sites. The above proteins have been found by many research groups in the name of PDF, MIC-1 (macrophage inhibitory cytokine-1), PLAB (placental bone morphogenic protein), GDF-15 (growth/differentiation factor 15), etc.

The peptide sequences analyzed above, which are represented by SEQ. ID. No 3, No 4 and No 5, are known as T cell cyclophilin and also called peptidyl-prolyl cis-trans isomerase. The reserved N-linked sugar chain

can be found on 3 sites on the sequence that is also well known as a constituent for anti-oxidant system.

The peptide sequences analyzed above, which are represented by SEQ. ID. Nos 6-11, were confirmed to be  
5 a new protein by comparing that up with the entire established database. The protein has Asn-Xaa-Ser sequence in its 4<sup>th</sup> peptide, suggesting it probably contains N-linked sugar chain.

The peptide sequences analyzed above, which are  
10 represented by SEQ. ID. No 12 and No 13, are known in many names of galectin binding protein, L3 antigen, Mac-2-binding protein, serum protein 90K, tumor associated antigen 90K, etc, and are detected from the blood of cancer or AIDS patients. The result of  
15 Northern blot was that the protein was expressed much in primary cancer tissues and tumor-associated cell lines, though the expression varied with disease types. The protein was proved to have 7 reserved N-linked sugar chain sites (Ullich, A. et al., 1994, *J Biol. Chem.*, 269:18401-18407).  
20

The peptide sequences analyzed above, which are represented by SEQ. ID. No 14 and No 15, are known as TIMP-1 (tissue inhibitor of matrix metalloproteinase-1) and 4 TIMPs (1-4) have been found so far. As a low  
25 molecular protein, the molecular weights of the proteins are 22K-30K and the proteins have 40-50% homology. TIMP-1 takes the form of train by

glycosylation, because of which the molecular weight of TIMP-1 seems to be high (see FIG. 7). And N-terminal is attached to MMPs, resulting in the inhibition of the activity of matrix metalloproteinase. This protein has  
5 N-linked sugar chains on two sites (Gomls-Reuth, F. et al., 1997, *Nature*, 389:77-81).

Even though two of the above five proteins, galectin binding protein (tumor associated antigen 90K) and TIMP-1, have been known to be directly related to  
10 tumorigenesis and metastasis, clear reports on the changes of sugar chain branches of  $\beta$ 1,6 N-acetylglucosamine induced by cancer and the above two proteins have not been presented yet.

15 The proteins detected out above that reflect the changes of sugar chains in connection with tumorigenesis and metastasis are glycoproteins, and they are on the surface of cells or secreted therefrom, so that cancer can be diagnosed by measuring the  
20 expression and the changes of N-linked sugar chains of the proteins in body fluids like blood or urine.

A method for diagnosing cancers using the proteins related to tumorigenesis and metastasis comprises following two steps: Taking samples from  
25 patients (Step 1); and Measuring the changes of N-linked sugar chains and the expression of proteins related to tumorigenesis and metastasis with the above

samples (Step 2).

The sample of the above Step 1 can be taken from blood or urine, and preferably prepared by the general separation method of serum.

5 As for the measuring method of the above Step 2, every analyzing methods based on the principle of antigen-antibody reaction can be used. Especially, ELISA (enzyme-linked immunosorbent assay) and immunoblot, the most common methods for analyzing  
10 antigen-antibody reaction, are preferably used.

The present inventors provide a method for measuring the changes of N-linked sugar chains and the expression of proteins related to tumorigenesis and metastasis after producing antibodies to the proteins  
15 in order to diagnose cancers.

A method for measuring the changes of N-linked sugar chains and the expression of the proteins by ELISA comprises the following steps:

20 1) Adhering antibody against the protein related to tumorigenesis and metastasis to matrix;

2) Adding serum of a sample to the above matrix to induce reaction, and then washing thereof;

25 3) Adding marked the same antibody or marked L4-PHA for further reaction;

4) After washing the above matrix, adding the secondary antibody conjugated with coloring enzyme or

fluorescent substance thereto for further reaction; and

5) After coloring thereof using coloring substrate solution, measuring optical density with ELISA reader.

5

The matrix of the Step 1 can be selected from a group consisting of nitrocellulose membrane, 96 well plate synthesized with polyvinyl resin, 96 well plate synthesized with polystyrene and slide glass, and 96 well plate was chosen for this invention.

10

For a mark of the above Step 3, a chemical compound (or its derivatives) like biotin can be used. The expression of proteins related to tumorigenesis and metastasis can be analyzed using biotin-labeled the same antibody and the changes of  $\beta$  1,6 N-acetylglucosamine sugar chain branches, the changes of N-linked sugar chains, can be measured using biotin-labeled L4-PHA.

15

Peroxidase or alkaline phosphatase can be used as a coloring enzyme conjugated with antibody of the Step 4, and FITC or RITC can be used as a fluorescent substance. Particularly, peroxydase conjugated antibody was used in this invention.

20

ABTS [2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)], OPD (o-Phenylenediamine) and TMB (Tetramethyl Benzidine) can be used as a coloring substrate solution of the Step 5, and particularly, OPD

25

that was colored by peroxidase was used in this invention.

The method for diagnosing cancers of the present invention is available for mass-analysis of samples  
5 using biological microchip and automatic microarray system.

The present invention also provides a kit for diagnosing cancers by measuring the changes of sugar  
10 chains and the expression of proteins associated with tumorigenesis and metastasis.

Cancers mentioned above include large intestine cancer, stomach cancer, lung cancer, liver cancer, uterine cancer, breast cancer and pancreas cancer, but  
15 are not limited thereto, that is, any kind of cancer can be a target of this invention.

The protein associated with tumorigenesis and metastasis is selected from a group consisting of PDF, peptidyl-prolyl cis-trans isomerase, galectin binding  
20 protein, L3 antigen, Mac-2-binding protein, serum protein 90K, tumor associated antigen 90K, TIMP-1 and proteins containing peptide sequences represented by SEQ. ID. Nos 6-11.

The diagnostic kit of the present invention is  
25 available for qualitative analysis or quantitative analysis of the expression of the above proteins and for measuring the changes of N-linked sugar chain,

exactly the changes of  $\beta$  1,6-N-acetylglucosamine sugar chain branches, for which ELISA can be used. For instance, the diagnostic kit can be provided for the ELISA using 96 well microtiter plate coated with antibody against the above proteins.

The diagnostic kit of the present invention can include antibody against the above proteins, matrix, buffer solution, coloring enzyme or fluorescent substance-labeled secondary antibody, coloring substrate, and especially, L4-PHA to measure the changes of  $\beta$  1,6-N-acetylglucosamine sugar chain branches.

As for the matrix, nitrocellulose membrane, 96 well plate synthesized with polyvinyl resin, 96 well plate synthesized with polystyrene resin, and slide glass can be used. As for the coloring enzyme, peroxidase and alkaline phosphatase can be used. As for the fluorescent substance, FITC and RITC can be used and as for the coloring substrate solution, ABTS (2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)), OPD (o-Phenylenediamine), or TMB (Tetramethyl Benzidine) can be used.

In order to diagnose cancers with the diagnostic kit of the present invention, automatic analyzing method using biological microchip can be used. For example, the diagnostic kit can be composed to prepare a protein chip by fixing the proteins in which sugar

chains are changed by being associated with tumorigenesis and metastasis onto the surface of glass slide and to measure the changes of those sugar chains of the proteins simultaneously. This diagnostic kit  
5 also includes the proteins, buffer solution and L4-PHA, etc.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a diagram showing sugar chains obtained  
10 by the action of 6 glycotransferases related to N-linked sugar chain,

FIG. 2 is a set of diagrams showing the adding process of  $\beta$  1,6 N-acetylglucosamine (GlcNAc $\beta$  1) to  
15 sugar chain by glycotransferase GnT-V,

FIG. 3 is a set of photographs showing the results of staining gels obtained by 2-dimensional electrophoresis of three cell lines (Mock/WiDr, ets-  
20 1/WiDr, GnT-V/WiDr) with Coomassie brilliant blue,

FIG. 4 is a set of photographs showing the results of lectin blotting with gels obtained by 2-dimensional electrophoresis of three cell lines,  
25

FIG. 5 is a set of photographs showing the

results of staining serum-free media derived from three cell lines with Coomassie brilliant blue after 2-dimensional electrophoresis,

5           FIG. 6 is a set of photographs showing the results of lectin blotting on serum-free media derived from three cell lines with L4-PHA after 2-dimensional electrophoresis,

10           FIG. 7 is a graph showing the result of analysis of timp-1 amino acid sequence by ESI/Q-TOF.

#### EXAMPLES

15           Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

20           However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

#### Example 1: Two-dimensional electrophoresis and lectin blotting of cell lines

25           The present inventors performed proteomics with GnT-V/WiDr cell line, considering that cell line as a model system of large intestine cancer. Particularly,

cultured cells first, and then searched out a specific protein group evoking aggravation of cancer by being associated with the enzyme among intracellular and extracellular secreted proteins. Cultured Mock/WiDr, a control group cell line, ets-1/WiDr, a cell line over-expressing ets-1, and GnT-V/WiDr, a cell line over-expressing GnT-V, in RPMI medium (Gibco BRL) supplemented with 10% FCS. For the preparation of an over-expressing cell line, introduced eukaryotic over-expression plasmid (Ko, et al., 1999, *J. Biol. Chem.*, 274(33): 22941-22948), transcription factor ets-1 and glycotransferase GnT-V into the large intestine cancer cell line WiDr (provided by American Type Tissue Culture; ATCC, USA). Treated the cells with G418 (350  $\mu$ g/ml) to correspond to neomycin resistant gene. As colonies were formed, detected ets-1 with Western blot using antibody and GnT-V with Northern blot using cDNA. 2-3 days later, when cells covered 90% of the bottom of culture plate (confluent) in a CO<sub>2</sub> incubator, the cells were washed with PBS (phosphate buffered saline) more than twice to remove remaining serum. Collected the cells with a scraper and washed with PBS. Suspended the cells in 1 ml of PBS and then sonicated thereof with a ultrasonicator (three times, 1 minute each time). Added acetone containing TCA (trichloroacetic acid) to the above crushed cells (final conc. 10%) to precipitate proteins only. Washed thereof with acetone

more than three times to remove remaining TCA and then dried. Dissolved thereof by adding gel loading buffer solution (8 M Urea, 2% Triton X-100, 20 mM DTT, 0.5% carrier ampholyte, Bromophenol Blue dye) and performed  
5 1-dimensional electrophoresis (18 cm drystrip pH 3-10) using Multiphor-II (Pharmarcia).

After equilibrating the obtained 1-dimensional electrophoresis gel with equilibrium buffer solution containing SDS and 2-mercaptoethanol, performed 2-  
10 dimensional electrophoresis on 12% polyacrylamide using Protean II (Bio-Rad). Obtained 2 sheets of gel: One was stained with Coomassie brilliant blue using biosafe staining solution (Bio-Rad) and the other was transferred to PVDF (polyvinylidene difluoride)  
15 membrane using semi-dry transfer (Bio-Rad). Adhered biotin-labeled L-PHA recognizing  $\beta$  1,6 N-acetylglucosamine branch to glycoprotein having the branch, and then attached HRP-labeled streptoavidin thereto. Exposed thereof to film by ECL fluorescent  
20 reaction.

In order to compare with a control group (Mock/WiDr), carried out the experiments with the same condition, that is, performed 1-diminsinal  
25 electrophoresis (separation of isoelectric point) first and then stained with Coomassie brilliant blue (FIG. 3), transferred the proteins of gel to PVDF membrane,

followed by performing lectin blotting using GnT-V-specific L4-PHA (FIG. 4). Performed 2-dimensional electrophoresis more than three times and analyzed the changes of the expression of intracellular protein using computer software (PDQUEST, Bio-Rad). As a result, no protein that showed more than 4-times difference in the expression profile of protein was detected and no significant change was seen in lectin blot (FIG. 4), either.

10

Example 2: Two-dimensional electrophoresis and lectin blotting of serum-free media

The present inventors cultured cell lines in serum-free media, through which investigated the changes of sugar chains. Particularly, cultured Mock/WiDr cell line, ets-1/WiDr cell line and GnT-V/WiDr cell line in RPMI (Gibco BRL) media containing 10% FCS. 2-3 days later, when cells covered around 80% of the bottom of culture plate in a CO<sub>2</sub> incubator washed with PBS more than twice to remove remaining serum. Then, put RPMI medium without serum, further cultured for 48 hours, and obtained culture solution. After concentrating the medium, added acetone (final conc. 10%) containing TCA (trichloro acetic acid), resulting in the precipitation of proteins. Washed thereof with acetone more than 3 times to remove

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remaining TCA, and then dried. Dissolved thereof by adding gel loading buffer solution (8 M Urea, 2% Triton X-100, 20 mM DTT, 0.5% carrier ampholyte, Bromophenol Blue dye) thereto. Performed 1-dimensional electrophoresis and 2-dimensional electrophoresis with the same method as the above Example 1.

After completing 2-dimensional electrophoresis, small amounts of FCS were detected and wide changes of PIM (post translational modification) were expected, so that the expression could hardly be compared with limited software. In the mean time, it was confirmed from the result of L-PHA lectin blot that darker spots were detected in ets-1 over-expressing cell line, comparing with the control group, and the darkest spots were observed in GnT-V over-expressing cell line (FIG. 5 and FIG. 6). Cut 12 spots off among all the above spots and identified 5 spots of them.

Example 3: Sequence analysis of proteins with an ESI/Q-TOF mass spectrometer

In order to identify proteins located in the above 5 spots, the present inventors stained PVDF membrane (lectin blot) that was already exposed to X-ray film with Coomassie brilliant blue and then adhered thereof onto exposed film again to confirm the exact

locations of spots, followed by cutting spots off corresponding to those of a gel stained with Coomassie brilliant blue. After destaining thereof using 30% methanol and 100% acetonitrille, added 10 U of trypsin (Promega) and then cut peptides at 37°C for overnight. 5  
 Extracted the peptides using acetonitrille, freeze-dried thereof with a centrifugal freezing drier, and then determined amino acid sequences using ESI/Q-TOF (Electrospray Ionization/Quadruple Time of Flight) mass spectrometer. Each peptide could be separated by ESI and sequence analysis could be possible by tandom mass. 10

As a result, peptide sequences represented by SEQ. ID. Nos 1-15 were determined by ESI/Q-TOF and identified after being compared with protein database. 15  
 Each sequence, molecular weight (MW) and isoelectric point (pI) were presented below (Table 1).

<Table 1>

No	Sequence	Name	Function	M.W	pI	N-linked glycosylation
a	SEQ.ID. No 1 SEQ.ID. No 2	GDF-15	PLAB/TFG- β family	34168.6	9.79	Y

b	SEQ.ID. No 3 SEQ.ID. No 4 SEQ.ID. No 5	Cyclophilin	Peptidyl-prolyl cis-trans isomerase A	18012.7	7.86	Y
c	SEQ.ID. No 6 SEQ.ID. No 7 SEQ.ID. No 8 SEQ.ID. No 9 SEQ.ID. No 10 SEQ.ID. No 11	Not identified	Not identified	About 20K	About 5.5	Y
d	SEQ.ID. No 12 SEQ.ID. No 13	Galectin 3 binding protein	Galectin binding serum protein	65331.6	5.13	Y
e	SEQ.ID. No 14 SEQ.ID. No 15	TIMP-1	Tissue inhibitor of matrix metallopro teinase	23171.1	8.46	Y

Their characteristics are explained below. The above proteins were confirmed based on the fact that N-linked sugar chain is located on the Asn in the sequence of Asn-Xaa-Thr/Ser (Varki, et al., 1999, *Essentials of glycobiology*, Cold Spring Harbor Laboratory, New York, USA, pp85-100).

A of the above Table 1 containing peptide sequences represented by SEQ. ID. No 1 and No 2, that is PDF (prostate-derived factor), is one of BMP proteins (Bone morphogenetic proteins) of TGF

(transforming growth factor)- $\beta$  family and has two preserved N-linked sugar chain sites. This protein has been searched in many names of not only PDF but also MIC-1 (macrophage inhibitory cytokine-1), PLAB  
5 (placental bone morphogenic protein), GDF-15, PTGFP, etc by many research groups.

B known as T cell cyclophilin containing peptide sequences represented by SEQ. ID. No 3, No 4 and No 5 is also called peptidyl-prolyl cis-trans isomerase. It  
10 has three preserved N-linked sugar chain sites.

C containing peptide sequences represented by SEQ. ID. Nos 6-11 is proved to be a new protein by comparing it with the established databases. It is also believed to have N-linked sugar chain since it has the sequence  
15 of Asn-Xaa-Ser in its forth peptide.

D containing peptide sequences represented by SEQ. ID. No 12 and No 13 has been known in many names such as galectin binding protein, L3 antigen, Mac-2-binding protein, serum protein 90K, tumor associated antigen  
20 90K, etc, and has seven preserved N-linked sugar chain sites.

E containing peptide sequences represented by SEQ. ID. No 14 and No 15 is known as TIMP-1 (tissue inhibitor of matrix metalloproteinase-1) and is an  
25 example of the above timp-1 identified by ESI/Q-TOF (FIG. 7). As shown in FIG. 7, TIMP-1 is shaped as a train formed by glycosylation, by which its molecular

weight seems high. TIMP-1 also has two N-linked sugar chain sites.

Example 4: Measurement of the changes of sugar chain branches or the expression of proteins

5 In order to diagnose cancers by measuring the changes of sugar chains or the expression of proteins identified above, the present inventors measured the changes of sugar chains and the expression of the  
10 proteins using ELISA.

First, obtained cDNA of target protein, which was then cloned into the eukaryotic gene expression vector. Cloned thereof into WiDr cell line. Purified the protein (1 mg each) from the above culture solution.  
15 Mixed thereof with adjuvant, which was injected hypodermically into rabbits, resulting in the preparation of polyclonal antibodies. Adhered the required amount of the antibody to two 96 well plates respectively. Precisely, coated protein A originated  
20 from bacteria to 96 well plate (Maxisorb, Nunc) by 1  $\mu$ g per well under the condition of containing 100  $\mu$ l of 0.1 M sodium carbonate (pH 9.6). Washed thereof with TBS-T (Tris-buffered saline-Tween, 0.2 M Tris-Cl, 0.4 M NaCl, 0.05% Tween-20) and adhered the above prepared  
25 antibody thereto. Washed with TBS-T again and then blocked parts that were not adhered, resulting in the

preparation of a stable antibody system. Consecutive dilution was performed after adding blood or other test samples to the plate for the reproducibility and statistical handling, and then washed thereof with TBS-T three times. At that time, the target protein would be adhered to its specific antibody. The expression of the protein was confirmed by the same antibody labeled with biotin, and the changes of sugar chains of  $\beta$  1,6 N-acetylglucosamine were confirmed by lectin, in which biotin-labeled L-PHA was used. In order to prepare biotin-labeled antibody, dialyzed antibody (5-10 mg/ml) in 250 ml of SBRB (succinimidyl-biotin reaction buffer) at 23°C for 6 hours. Adjusted the concentration of NHS-biotin (N-hydroxysuccinimidyl-biotin) or NHS-LC-biotin (long chain sulfosuccinimidyl 6-(biotinamido) hexanoate derivative biotin) to 2-4 mg/ml in DMSO (dimethylsulfoxide) and mixed the antibody and the biotin solution in the ratio of 1:30 (Ab:biotin), followed by stirring at 37°C. Left thereof for 1 hour to bind biotin and antibody. Prepared biotin-labeled antibody after dialyzing thereof in BBS (Borate buffered saline). For the lectin, commercial biotin-labeled L-PHA was used. Detected biotin-labeled antibody and lectin by using commercial avidine-peroxidase kit. Precisely, added H<sub>2</sub>O<sub>2</sub> and O-phenylamine, a substrate for peroxidase, and measured optical density at 490 nm. Measured the expression of protein

and the changes of glycosylation of  $\beta$  1,6 N-acetylglucosamine in the blood samples of patients with cancer and then compared them with those in normal control group.

5           As a result, the glycosylation extent of  $\beta$  1,6 N-acetylglucosamine in the blood of cancer patients was confirmed to increase 10-20 times as much as that in normal blood.

10

#### INDUSTRIAL APPLICABILITY

As described hereinbefore, a method for diagnosing cancers by measuring the changes of sugar chains of proteins associated with tumorigenesis and metastasis, and diagnostic kit using the same of the  
15 present invention can be effectively used for diagnosing cancers including large intestine cancer.

**What is claimed is**

1. A method for diagnosing cancers comprising the following steps:
  - 5 i) Taking samples from patients; and
  - ii) Measuring the changes of N-linked sugar chains of proteins related to tumorigenesis and metastasis with the above samples.
- 10 2. The method as set forth in claim 1, wherein the sample is taken from blood or urine.
3. The method as set forth in claim 1, wherein the cancer is selected from a group consisting of  
15 large intestine cancer, stomach cancer, lung cancer, liver cancer, uterine cancer, breast cancer and pancreas cancer.
4. The method as set forth in claim 1, wherein the  
20 changes of sugar chains are the changes of sugar chain branches of N-linked  $\beta$  1,6 N-acetylglucosamine.
5. The method as set forth in claim 1, wherein the  
25 protein is selected from a group consisting of PDF, peptidyl-prolyl cis-trans isomerase,

galectin binding protein, L3 antigen, Mac-2-binding protein, serum protein 90K, tumor associated antigen 90K, TIMP-1 and proteins containing peptide sequences represented by SEQ. ID. Nos 6-11.

5  
6. The method as set forth in claim 1, wherein the measuring method is ELISA or the method using antigen-antibody binding response including immunoblotting.

10  
7. A kit for diagnosing cancers by measuring the changes of sugar chains and the expression of the above proteins composed of antibody against protein associated with tumorigenesis and metastasis, matrix, buffer solution, coloring enzyme or fluorescent substance-labeled secondary antibody, coloring substrate and labeled antibody or labeled L4-PHA.

15  
20  
8. The kit as set forth in claim 7, wherein the cancer is selected from a group consisting of large intestine cancer, stomach cancer, lung cancer, liver cancer, uterine cancer, breast cancer and pancreas cancer.

25  
9. The kit as set forth in claim 8, wherein the

cancer is large intestine cancer.

10. The kit as set forth in claim 7, wherein the changes of sugar chains are the changes of sugar chain branches of N-linked  $\beta$  1,6 N-acetylglucosamine.

11. The kit as set forth in claim 7, wherein the protein is selected from a group consisting of PDF, peptidyl-prolyl cis-trans isomerase, galectin binding protein, L3 antigen, Mac-2-binding protein, serum protein 90K, tumor associated antigen 90K, TIMP-1 and proteins containing peptide sequences represented by SEQ. ID. Nos 6-11.

12. The kit as set forth in claim 7, wherein the matrix is selected from a group consisting of nitrocellulose membrane, 96 well plate synthesized by polyvinyl resin, 96 well plate synthesized by polystyrene resin and slide glass.

13. The kit as set forth in claim 7, wherein the coloring enzyme is selected from a group consisting of peroxidase and alkaline phosphatase, and the coloring substrate solution is selected from a group consisting of ABTS[2, 2'-Azino-bis

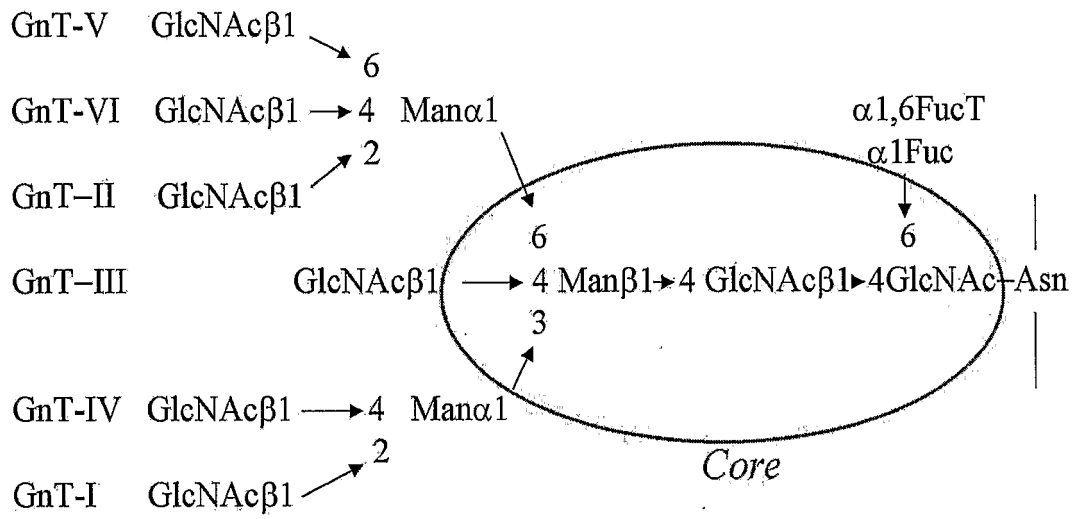
(3-ethylbenzothiazoline-6-sulfonic acid)], OPD (o-Phenylenediamine) and TMB (Tetramethyl Benzidine).

- 5 14. The kit as set forth in claim 7, wherein the labeled antibody is biotin(or its derivatives)-labeled antibody, and the labeled L4-PHA is biotin(or its derivatives)-labeled L4-PHA.

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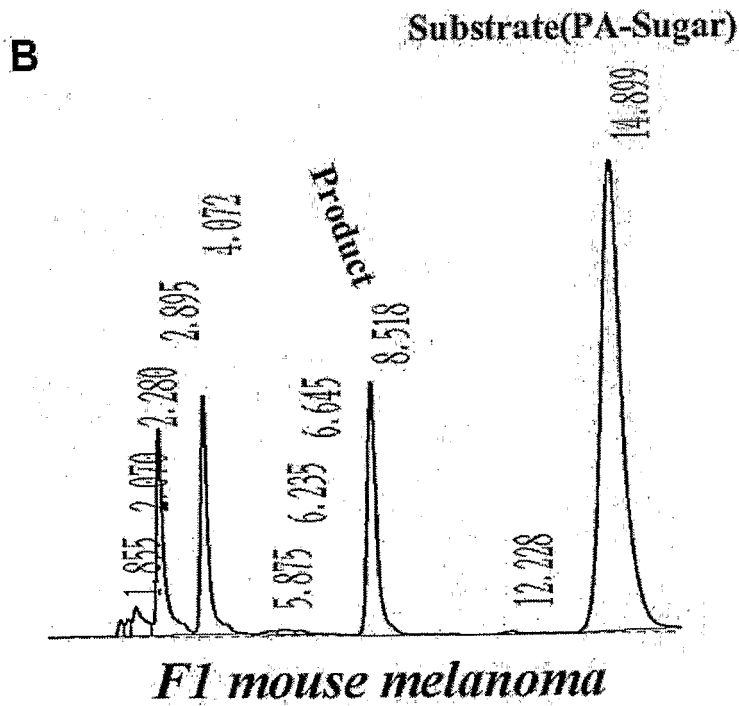
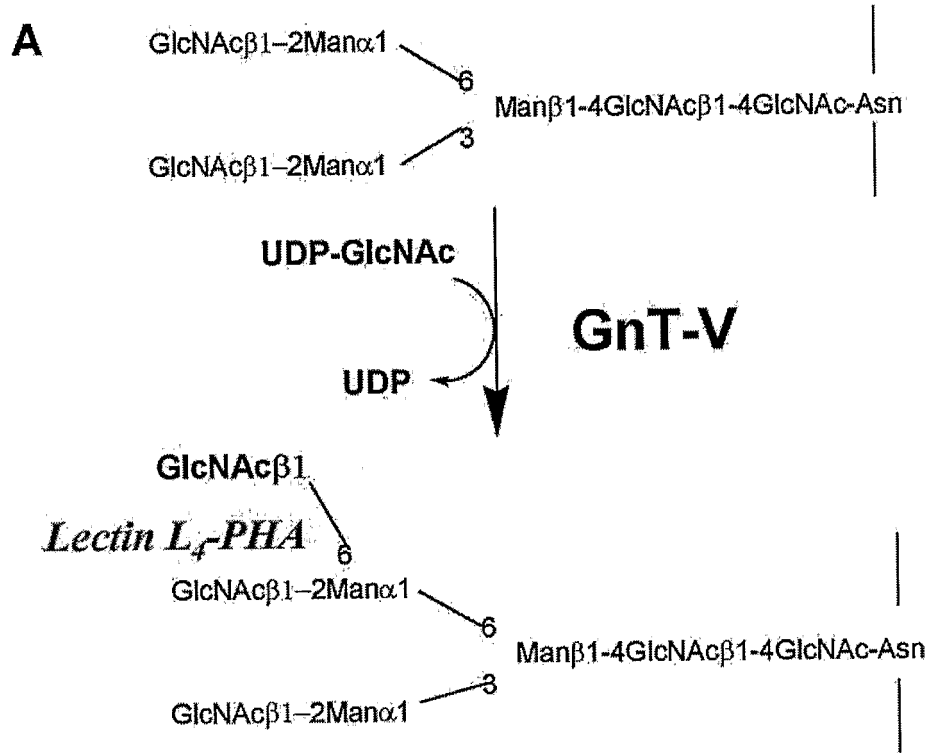
FIGURES

FIG. 1



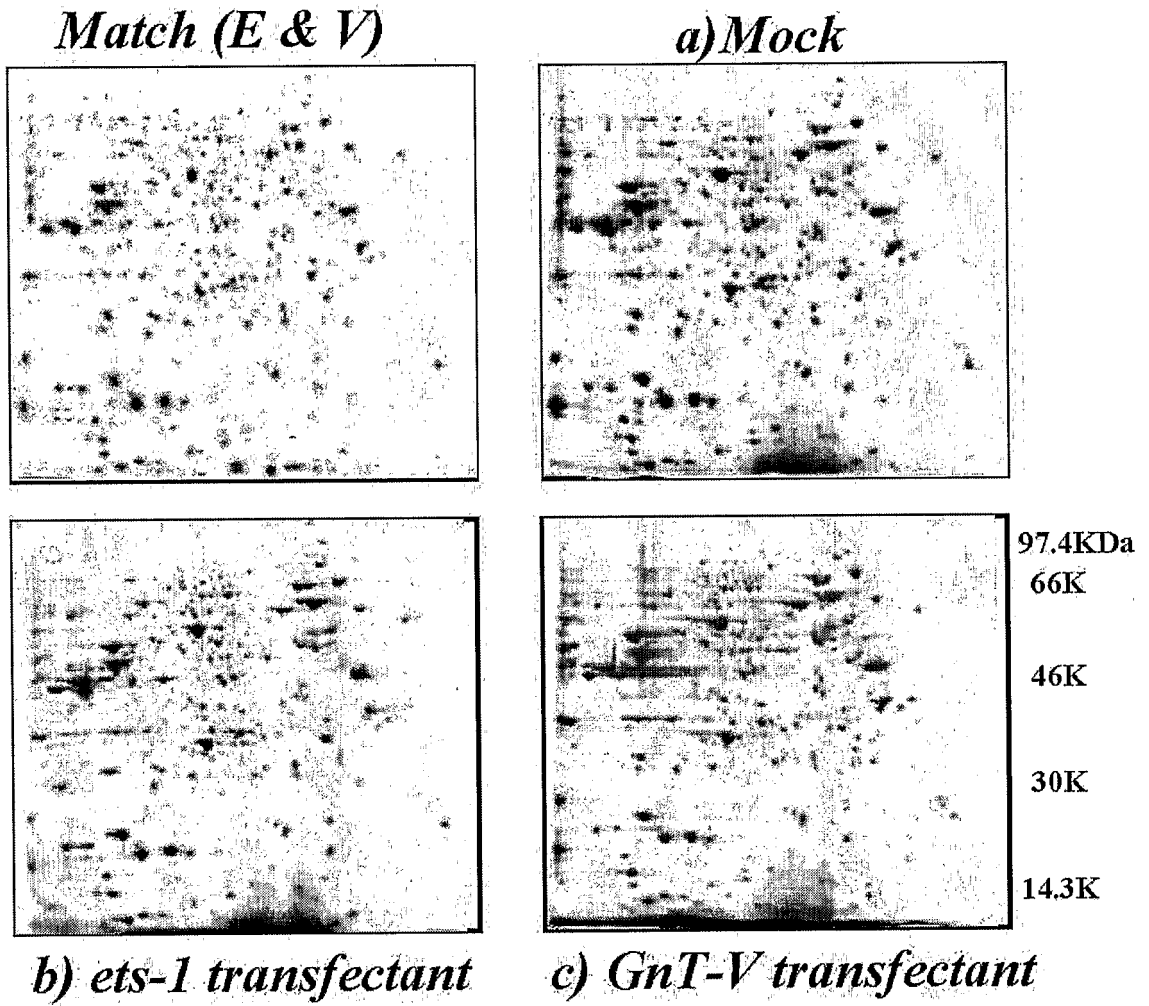
2/7

FIG. 2



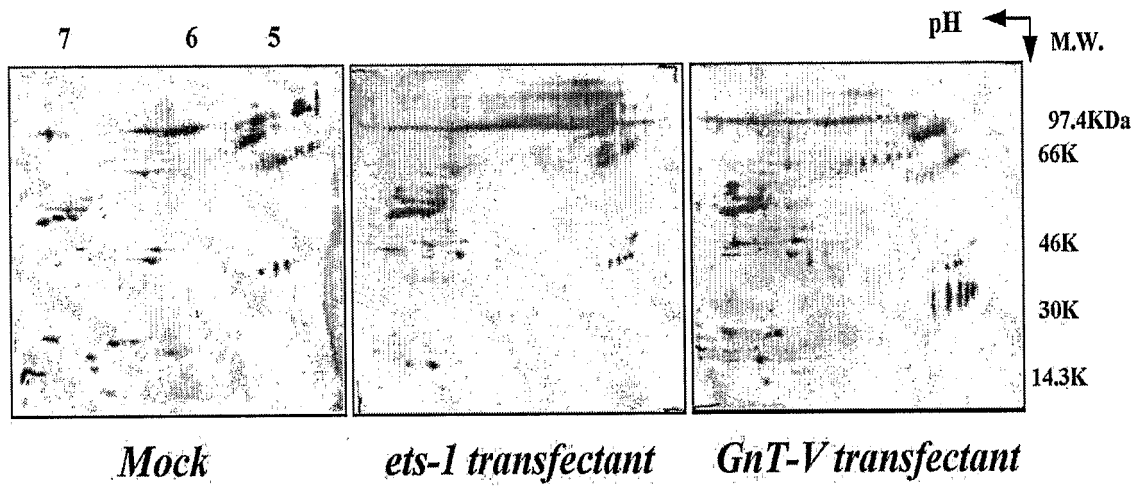
3/7

FIG. 3



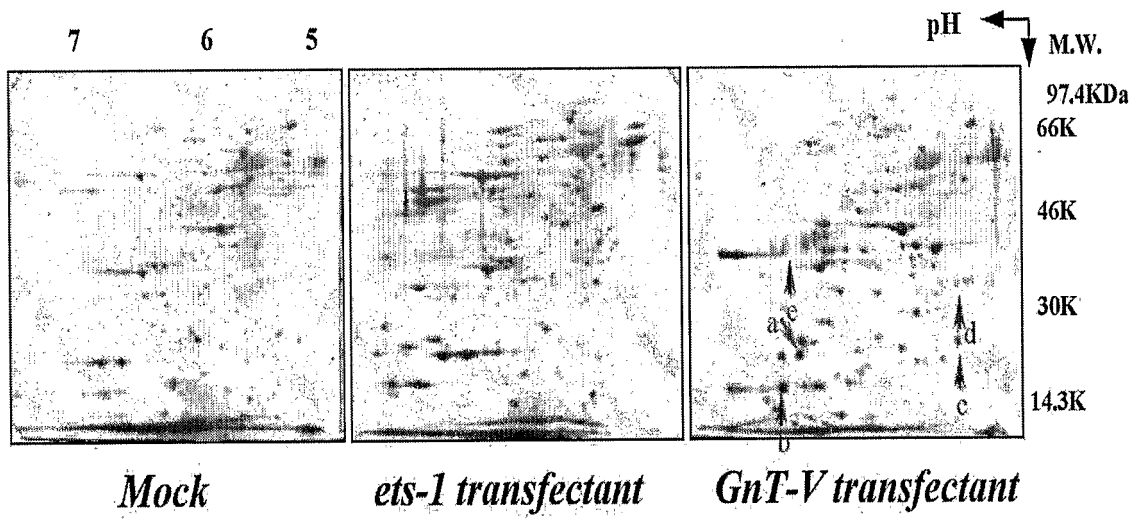
4/7

FIG. 4



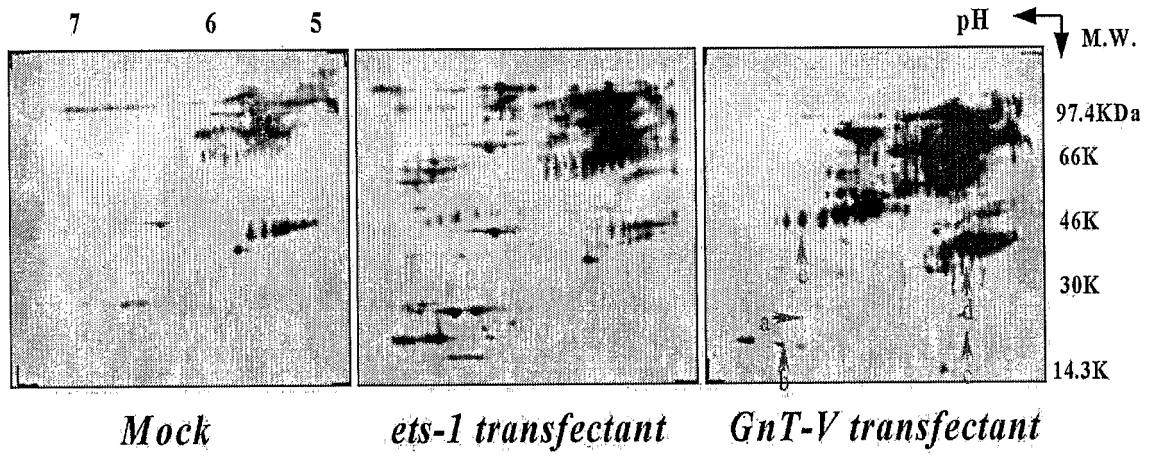
5/7

FIG. 5



6/7

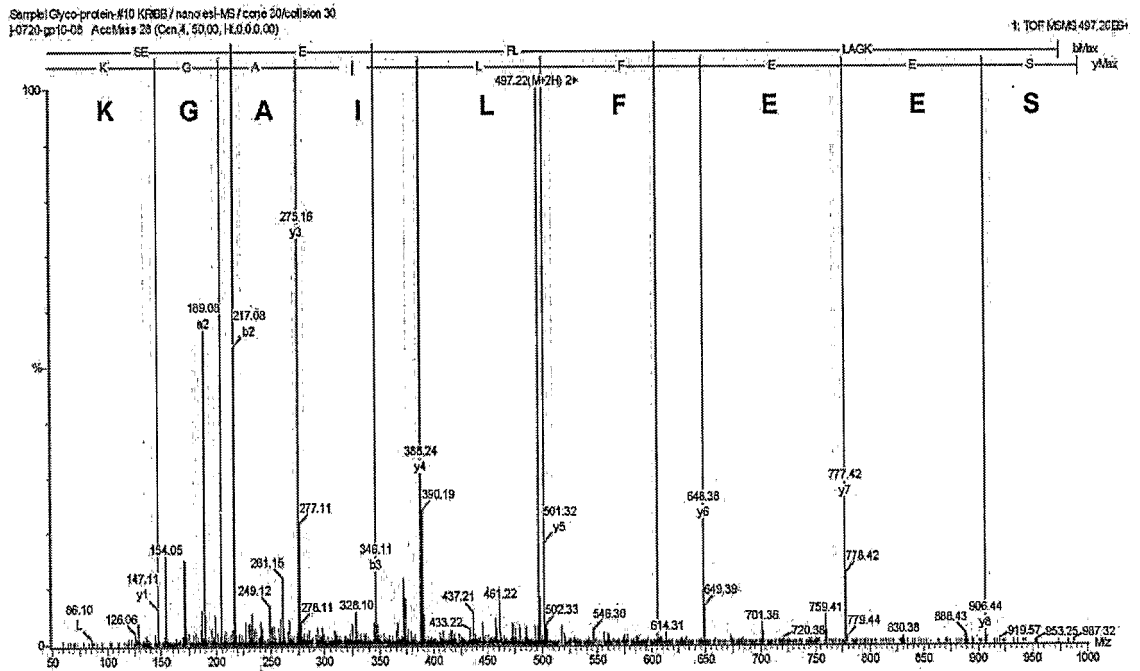
FIG. 6



7/7

FIG. 7

MS/MS Spectrum of Spot 'e'  
SEEFLIAGK (M+H=993.5)



SEQUENCE LISTING

<110> Korea Research Institute of Bioscience and Biotechnology

<120> Method for the diagnosis of cancers by measuring the changes of Glycosylation of proteins related to tumorigenesis and metastasis and kit for diagnosis of cancers using the same

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<150> KR2001-88090

<151> 2001-12-29

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/KR02/02469

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> <b>IPC7 G01N 33/574</b> According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) G01N 33/574, C12N 15/52, C07K 14/47 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Korean Patent and applications for inventions since 1975 Korean Utility models and application for Utility models since 1975 Japanese Utility models and application for Utility models since 1975 Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) NCBI, eKIPASS, USPTO, INSPECT "Glycosylation, Diagnosis of cancer, Lectin, Blot "		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5605807 A (Mount Sinai Hospital Corp.) 25 Feb 1997 -see the claims 1-27 except claim 24-	1, 3, 4, 6, 7, 8, 10
Y	US 5427914 A (Mount Sinai Hospital Corp.) 27 Jun 1995 -see the claims-	1, 3, 4, 6, 7, 8, 10
A	US 4994374 A (Oriental Yeast Co., Ltd.) 19 Feb 1991 -see the whole document-	1, 3, 4, 6, 7, 8, 10
A	KR 1999-0055111 A (KIST) 15 Jul 1999 -see the whole document-	1, 3, 4, 6, 7, 8, 10
A	KR 1999-0069637 A (Kim, Chul-Ho) 06 Sep 1999 -see the whole document-	1, 3, 4, 6, 7, 8, 10
T	Free Radic Res Vol36:827-833 (Ekuni, et al.) Aug 2002 -see the whole document-	1, 3, 4, 6, 7, 8, 10
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <span style="margin-left: 200px;"><input checked="" type="checkbox"/> See patent family annex.</span>		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 23 APRIL 2003 (23.04.2003)		Date of mailing of the international search report 23 APRIL 2003 (23.04.2003)
Name and mailing address of the ISA/KR Korean Intellectual Property Office 920 Dunsan-dong, Seo-gu, Daejeon 302-701, Republic of Korea Facsimile No. 82-42-472-7140		Authorized officer JOO, Young Sik Telephone No. 82-42-481-5995 

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR02/02469

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: 1- 6  
because they relate to subject matter not required to be searched by this Authority, namely:  
Although claims 1- 6 are directed to a diagnostic method for various human cancers, which the International Searching Authority is not required to search under Rule 39.1(iv) of the Regulations under the PCT, the search has been carried out based on the alleged effects of the diagnosis kit.
2.  Claims Nos.:  
because they relate to part of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3.  Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Search Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be established without effort justifying an additional fee, this Authority did not invite payment of any addition fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR02/02469

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5,605,807	1997/02/25	none	
US 5,427,914	1995/06/27	none	
US A 4994374	1991/02/19	WO A1 8902474 JP 1065454 EP A1 334962	1989/03/23 1989/03/10 1989/10/04
KR 1999-0055111	1999/07/15	none	
KR 1999-0069637	1999/09/06	none	

专利名称(译)	通过测量与肿瘤发生和转移相关的蛋白质的糖基化变化来诊断癌症的方法和使用该方法诊断癌症的试剂盒		
公开(公告)号	<a href="#">EP1466179A1</a>	公开(公告)日	2004-10-13
申请号	EP2002793548	申请日	2002-12-28
[标]申请(专利权)人(译)	韩国生命工学研究院		
申请(专利权)人(译)	韩国研究学院生物科学与生物		
当前申请(专利权)人(译)	韩国研究学院生物科学与生物		
[标]发明人	KO JEONG HEON HWANG SOO YOUNG SOHN HOSUNG OH SEJEONG LEE JEONG HWA LEE SANG CHUL YOO JONG SHIN LEE DAE SIL		
发明人	KO, JEONG HEON HWANG, SOO YOUNG SOHN, HOSUNG OH, SEJEONG LEE, JEONG HWA LEE, SANG CHUL YOO, JONG-SHIN LEE, DAE-SIL		
IPC分类号	C07K7/06 C07K7/08 G01N21/78 G01N33/53 G01N33/543 G01N33/574		
CPC分类号	G01N33/57469		
优先权	1020010088090 2001-12-29 KR		
其他公开文献	EP1466179B1 EP1466179A4		
外部链接	<a href="#">Espacenet</a>		

#### 摘要(译)

本发明涉及通过测量与肿瘤发生和转移相关的蛋白质来诊断癌症的方法，以及使用该方法的诊断试剂盒，特别涉及通过测量蛋白质的糖基化变化来诊断癌症的方法和用于诊断癌症的试剂盒。使用所述方法。本发明的方法和试剂盒可有效地用于包括结肠癌，胃癌，肺癌和肝癌的癌症的敏感诊断。