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Regulation of Metastasis-Suppressive Gene *Nm23*-H1 on Glycosyl-transferases Involved in the Synthesis of Sialyl Lewis Antigens

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Abstract By using reverse transcriptase-polymerase chain reaction (RT-PCR), the mRNA expressions of three families of glycosyltransferases involved in the synthesis of sialyl Lewis antigens were determined in H7721 human hepatocarcinoma cell line before and after the transfection of metastasis-suppressive gene *nm23*-H1. These glycosyltransferases included α1,3fucosyltransferase (α1,3FucT)-III, -IV, -VI, -VII, and -IX, α2,3-sialyltransferase (ST3Gal)-I, -II, -III, and -IV as well as *O*-glycan core 2 β1,6 *N*-acetylglucosaminyltransferase (C2GnT)-I and -II. In mock cells transfected with the vector, the expression-order of α1,3FucTs was IV > VI > III > VII > IX, that of ST3Gals was IV > I > II > III, and that of C2GnT was I > II. *Nm23*-H1 downregulated the mRNA expressions of all five subtypes of α1,3FucT and -I, -III, -IV subtypes of ST3Gal, but not ST3Gal-II and C2GnT-I, II. On the other hand, the expressions of cell surface sialyl Lewis X (SLe^x) and α2,3 sialyl residues were decreased on *nm23*-H1 transfected cells as detected with monoclonal antibody of SLe^x and enzyme-labeled lectins, respectively. Since SLe^x was reported to be a metastasis-associated glycan structure, the reduced expressions of SLe^x and some enzymes related to its synthesis may be one of the mechanisms to explain the metastasis-suppressive effect of *nm23*-H1. J. Cell. Biochem. 94: 1248–1257, 2005.

Key words: human hepatocarcinoma cell line; metastasis-suppressive gene *nm23*-H1; sialyl Lewis X; α1,3 fucosyltransferase; sialyltransferase; core 2 β1,6 *N*-acetylglucosaminyltransferase

Nm23 is a highly conserved gene family in eukaryotes, which was first characterized by Steeg et al. [1988]. Up to date, eight different genes (*nm23*-H1 to *nm23*-H8) have been identified in human [Lacombe et al., 2000]. The *nm23*-H1 was first discovered in the members of the gene family [Gilles et al., 1991] and demonstrated to have anti-metastatic properties in various models of human and animal cancers. The gene is located on chromosome 17q 21, which codes an 18.5 kDa protein containing 166 amino acid residues. It was reported that the expression of *nm23*-H1 was reduced in numerous types of cell lines with highly metastatic potential [Kantor et al., 1993; Baba et al., 1995; Fukuda et al., 1996]. On the other hand, *nm23*-H1 expression has shown an inverse correlation with lymph node metastasis and patient mortality in many cancers, such as breast [Hirayama et al., 1991; Tokunaga et al., 1993], cervical [Marone et al., 1996], and primary liver cancer [Fujimoto et al., 1998].

It was reported by our laboratory [Guo et al., 2000] that transfection of the cDNA of *nm23*-H1 into H7721 human hepatocarcinoma cell line resulted in the downregulation of *N*-acetylglucosaminyltransferase V (GnT-V) as well as its product GlcNAcβ1,6Manα1,6-branching

Abbreviations used: Man, mannose; Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; SA, sialic acid; SLe^x, sialyl Lewis X; SLe^a, sialyl Lewis A; $SDLe^x$, sialyl dimeric (difucosyl) Lewis X; Fuc T, fucosyltransferase; ST, sialyltransferase; CMP, cytidine monophosphate; ST3Gal, CMP-*N*-acetylneuraminate: β-galactoside α2,3-sialyltransferase; ST6Gal, CMP-*N*-acetylneuraminate: β-galactoside α2,6-sialyltransferase; C2GnT, core 2 β1,6 *N*-acetyl-glucosaminyltransferase; FITC, fluorescein isothiocynate; FACS, fluorescence activated cell spectra; RT-PCR, reverse transcriptase-polymerase chain reaction; MAA, Maackia amurensis agglutinin; SNA, Sambucus nigra agglutinin, HRP, horseradish peroxidase; PBS, phosphate buffered saline.

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structure in asparagine-linked (*N*-) glycans on the cell surface. Concomitantly, the in vitro metastatic potential, including cell adhesion to laminin, chemotaxic cell migration through transwell, and invasion through matrigel, was reduced [Guo et al., 2000]. GnT-V and GlcNAc β1,6- branch in *N*-glycans were well documented to associate with cancer metastasis [Dennis et al., 1999; Taniguchi et al., 1999]. The down-regulation of β1,6-GlcNAc branch on cell surface *N*-glycans is one of the mechanisms to explain the suppression of cancer metastasis by *nm23*-H1 [Guo et al., 2000]. We have also found that a sialyl Lewis sugar antigen, sialyl Lewis X (SLe^x) [SAα2,3Galβ1,4 (Fuc α1,3) GlcNAc-], is another glycan structure related to cancer metastasis. It was evidenced by the findings that cell surface SLe^x, but not sialyl Lewis A (SLe^a) [SAα2,3Galβ1,3 (Fuc α1,4) GlcNAc-] and sialyl dimeric (difucosyl) Lewis X ($SDLe^x$) [SAα2,3Galβ1,4 (Fuc α1,3) GlcNAcβ1,3Galβ1,4 (Fucα1,3) GlcNAcβ1,3-] was upregulated by the transfection of metastasis-promoting gene, *c-erb*B2/*neu* [Liu et al., 2001a]. In addition, surface SLe^x was increased after the cells were treated with epidermal growth factor or phorbol ester [Liu et al., 2001b] and decreased after the treatment of *all-trans* retinoic acid or cyclic AMP [Liu et al., 2001b]. The above-mentioned in vitro metastatic potential was positively proportional to the expression of surface SLe^x, and could be inhibited by using monoclonal antibody to block the surface SLe^x, but the antibodies to SLe^a and $SDLe^x$ showed no or very little effect [Liu et al., 2001a,b]. These sialylated Lewis antigens are mainly located at the terminal of cell surface glycans and are reported to be the ligands of E- or P-selectin expressed on the surface of vascular endothelial cells [Takada et al., 1993; Varki, 1994]. They could mediate the adhesion of malignant cells to vascular endothelium at the beginning of metastasis process. However, the effect of *nm23*-H1 on the expressions of sialyl Lewis antigens has not been elucidated.

The sialylation and fucosylation steps in the synthesis of sialyl Lewis antigen are catalyzed by α2,3 sialyltransferase (α2,3ST) and α1,3 fucosyltransferase (α1,3FucT), respectively. Human fucosyltransferase (FucT) family includes three subfamilies, α1,2FucT, α1,3FucT, and α1,6 FucT [Narimatsu, 1998]. α1,3FucT subfamily is the main glycosyltransferase responsible for the synthesis of Lewis antigens. α1,2FucT participates in the synthesis of ABO blood group antigens and Lewis antigens Y and B; both Lewis Y and B contain two fucosyl residues with α1,2 and α1,3 (or α1,4) linkages. Up to date, six α1,3FucTs have been cloned [Kukowska-Latallo et al., 1990; Lowe et al., 1991; Weston et al., 1992a,b; Sasaki et al., 1994; Kudo et al., 1998a]. Four of them, α1,3 FucT-III, -V, -VI, -VII efficiently fucosylate sialylated acceptors and produce sialyl Lewis antigens, while α1,3FucT-IV and -IX prefer neutral acceptors and usually form non-sialyl Lewis antigens as their products. α1,3FucT-III has both α1,3 and α1,4 fucosylation activities, which can synthesize both α1,3 fucosyl-containing Le^x or SLe^x, and α1,4 fucosyl-containing SLe^a (so α1,3FucT-III is also called α1,3/α1,4 FucT-III). On the contrary, α1,3 FucT-VII catalyzes the synthesis of SLe^x only. The gene of α1,3FucT-V has been reported to be a silent gene and is rarely expressed in tissues where other α1,3 FucTs were effectively expressed [Narimatsu, 1998].

Sialyltransferases are divided in four specific subfamilies based on their substrate specificities and the positions of the newly formed sialyl linkages in the products [Tsuji, 1996, 1998]. α2,3 sialyltransferase (ST3Gal) transfers the sialyl group from CMP-*N*-acetyl-neuraminate (CMP-SA) to the galactosyl (Gal) residue of glycans, forming an α2,3 linkage. ST6Gal also transfers sialyl group from CMP-SA to the Gal residue of glycans in glycoproteins, but produces an α2,6 linkage, its main substrate is *N*-glycans. ST6GalNAc subfamily transfers sialyl group to the *N*-acetylgalactosaminyl (GalNAc) residue in Ser/Thr-linked (*O*-) glycans or glycolipids and forms an α2,6 linkage. ST8Sia subfamily transfers sialyl group to another sialyl residue at the terminal of glycans to create an α2,8 linkage. ST3Gal can be further divided into five subtypes. The substrates of types I and II are the Galβ1,3GalNAc- sequence on *O*-glycans and glycolipids, respectively, while the preferred substrates of ST3Gal-III and -IV are Galβ1,3GlcNAc- (precursor of SLe^a) and Galβ1,4GlcNAc- (precursor of SLe^x), respectively. Type V was evidenced as a ganglioside GM3 synthase.

In addition, core 2 β1,6-*N*-acetylglucosaminyltransferase (C2GnT) responsible for the branching of *O*-glycans has been reported to be an important enzyme for the synthesis of SLe^x precursor [Nakamura et al., 1998], since SLe^x

is predominantly located at the terminal of the branched *O*-glycan. C2GnT is a family of glycosyltransferase that catalyzes the synthesis of the core 2 and core 4 of *O*-glycans. These enzymes transfer the GlcNAc group from UDP-GlcNAc to the innermost GalNAc residue in the sequence of Galβ1,3GalNAc-Ser/Thr (precursor of core 2) or GlcNAcβ1,3GalNAc-Ser/Thr (precursor of core 4) and produce a branching GlcNAcβ1,6GalNAc-linkage. At least three C2GnT subtypes were discovered [Fukuda and Yeh, 2002; Fukuda et al., 2002]. Among them, C2GnT-I and -III synthesize core 2 only, while C2GnT-II synthesizes both core 2 and core 4. C2GnT-II also shows an activity of IGnT, which catalyzes the GlcNAcβ1,6- branching of the outer chain on glycans to form the structure of I antigen.

In this investigation, the effects of *nm23*-H1 on the mRNA expressions of α1,3FucTs, α2,3STs, and three sialyl Lewis antigens were studied in order to explore whether these glycosyltransferases and antigens mediate the metastasis-suppressing effect of *nm23*-H1. The subtypes III, IV, VI, VII, and IX of α1,3FucT were chosen to investigate the effect of *nm23*-H1 cDNA. The mRNA expressions of ST3Gal subtype I–IV were concomitantly studied in *nm23*-H1 transfected cells. In addition, C2GnT-I and -II were also selected as two representatives in C2GnT family with different substrate specificities to study their regulation by *nm23*-H1.

MATERIALS AND METHODS

Materials

H7721 cell line was obtained from the Institute of Cell Biology, Academic Sinica (Shanghai, China). RPMI 1640 and DMEM mediums were purchased from Gibco/BRL (Carlsbad, CA). KM93 (anti-SLe^x) and FH6 (anti-$SDLe^x$) monoclonal antibodies (mAb) were the products of Seikagaku Company in Japan and kindly provided by Dr. K. Furukawa at Tokyo Metropolitan Institute of Gerontology. CA 19-9 (anti-SLe^a mAb) and the primers of α1,3FucTs, ST3Gals, ST6Gal, and C2GnTs were gifted from Dr. Narimatsu in National Institute of Advanced Industrial Science and Technology (AIST) of Japan. Maackia amurensis agglutinin (MAA) and sambucus nigra agglutinin (SNA) lectins, fluorescein isothiocynate (FITC)-conjugated second antibodies (goat anti-mouse IgM or IgG), and horseradish peroxidase (HRP) were purchased from Sigma (Buffalo, NY). TRIzol and AMV reverse transcriptase were from Promega. Other reagents, including Taq enzyme, RNAase inhibitor, dNTP, oligo (dT)-18 were commercially available in China.

The plasmid Bluescript(+)/*nm23*-H1 was obtained from the Institute of Liver Cancer affiliated to our University. The construction of plasmid pcDNA3/*nm23*-H1 was performed according to our published method [Guo et al., 2000]. Bluescript(+)/*nm23*-H1 plasmid was cut with *Bam*HI restriction enzyme, and the product, *nm23*-H1 cDNA (0.74 kb), was separated and recovered on agarose electrophoresis. After characterization and quantification, the cDNA was inserted into the multiple cloning site of the linearized eukaryotic expression plasmid pcDNA3 vector (5.4 kb) by using *Bam*HI enzyme and T4 DNA ligase. The reconstructed plasmid, pcDNA/*nm23*-H1, was transferred into H7721 cells using electroporation method (250 V/0.4 cm and 1,000 μF). The transfected H7721 cells were incubated in RPMI 1640 medium containing G418 (0.8 mg/ml). Neomycin-resistant cells were obtained after 2–3 weeks and recloned by serial dilution. The pcDNA/*nm23*-H and vector transfected stable cell lines were named *nm23*-H/H7721 and "Mock-transfected," respectively. The *nm23*-H1/H7721 cells were characterized by the significant increase in *nm23*-H1 mRNA as compared with the mock-transfected cells [Guo et al., 2000].

Cell Culture and Treatment

Cells were cultured at 37°C, 5% CO_2 in RPMI-1640 medium containing 10% fetal calf serum, penicillin, and streptomycin as previously described [Liu et al., 2001a,b].

Determination of the Expression of Glycosyltransferase mRNAs With RT-PCR

Total RNA was extracted from cells using TRIzol according to the protocol provided by Promega. Complementary DNAs (cDNAs) were synthesized with oligo(dT)-18 primer and AMV reverse transcriptase from 3 μg of the total RNA. The reverse transcriptase-polymerase chain reaction (RT-PCR) was performed in a volume of 50 μl containing 5 μl cDNA, 0.2 μM primer pair of different glycosyltransferases or β-actin (internal standard), PCR buffer (10 mM Tris-HCl pH 8.3/50 mM KCl/5 mM $MgCl_2$), 0.2 μM of each dNTP, and 1 IU Taq polymerase. The cDNAs were subjected to denaturation at 95°C

for 5 min, followed by 28 cycles of PCR. Each cycle included denaturation at 94°C for 1 min, annealing at 61.5°C for 1 min, and elongation at 72°C for 1 min. Finally, the samples were further incubated for elongation at 72°C for 10 min and 4°C for 5 min. After RT-PCR, 10 μl products were applied to 2% agarose gel electrophoresis. The amplified DNA bands were scanned and the photos were analyzed with NIH Image software. The semi-quantitative data were obtained by the intensity ratios of glycosyltransferase bands to the β-actin band. The sequences of the primers were reported in the references as follows [Bierhuizen and Fukuda, 1992; Kudo et al., 1998b; Yeh et al., 1999].

FucT-III	F: 5′-CCTCCCGACAGGACACCACTCC-3′ R: 5′-GCGTCCGTACACGTCCACCTGG-3′
FucT-IV	F: 5′-GAGAGGCTCAGGCCGTGCTTTT-3′ R: 5′-GCAGGAGCCCAATTTCGGGCAC-3′
FucT-VI	F: 5′-AATGGGTCCCGCTTCCCAGACAG-3′ R: 5′-GCGTCCGTACACGTCCACCTTG-3′
FucT-VII	F: 5′-CACCTCCGAGGCATCTTCAACTG-3′ R: 5′-CGTTGGTATCGGCTCTCATTCATG-3′
FucT-IX	F: 5′-CAGCTGGGATCTGACTAACTTACC-3′ R: 5′-CCACATGAATGAATGAATCAGCTGG-3′
ST3Gal-I	F: 5′-TATGGGCCTGAGATAGACAGTCAC-3′ R: 5′-GATCCGGATTTTATTGATGGAGGC-3′
ST3Gal-II	F: 5′-GAGAACATGGATCTTCCACCGG-3′ R: 5′-GGCTGGGTTGTAGATCTGGACC-3′
ST3Gal-III	F: 5′-ATGGAGGCGTTCTTGCCAACAAG-3′ R: 5′-ATGCGAACGGTCTCATAGTAGTG-3′
ST3Gal-IV	F: 5′-TTGAACAATGCCCCAGTGGCTGG-3′ R: 5′-TCTTGGGAGACATTATGGCCTGAC-3′
ST6Gal-I	F: 5′ GAGAGCATTAGGACCAAGGCTGG 3′ R: 5′ACGTCAGTCTTGCGCTTGGATGG 3′
C2GnT-I	F: 5′-GCAATGAGTGCAAACTGGAAGT-3′ R: 5′-AATTGCCCGTAATGGTCAGTGTT-3′
C2GnT-II	F: 5′-TCAGGGGTCACCCGAGGGGACCAAG-3′ R:5′-CTCCACCTCTTCTTTGCTCAGTGG-3′
β-actin	F: 5′-GATATCGCTGCGCTCGTCGTCGAC-3′; R: 5′-CAGGAAGGAAGGCTGGAAGAGTGC-3′

Detection of Lewis Antigens With Flow Cytometry

The cells (1×10^6) were detached with 2 mM ethylenediamine tetraacetate, washed, and resuspended in phosphate buffered saline (PBS) containing 1% bovine serum albumin, then incubated with 1:50 diluted monoclonal antibodies against sialyl Lewis antigens (KM93, FH6, and CA19-9 for SLe^x, SDLe^x, and SLe^a, respectively) for 30 min at 4°C. After being washed twice, the cells were incubated for 45 min at 4°C with 1:200 diluted FITC-conjugated goat antibodies against mouse IgM (for KM93 and FH6) or IgG (for CA 19-9). Then the cells were washed again and 1×10^4 cells/sample were subjected to flow cytometry for fluorescence analysis [Guo et al., 2004]. A "(−) Control" sample without first antibody was set up in each run to normalize the assay condition. Fluorescence activated cell spectra (FACS) were drawn automatically, and the left- or right-shift of the curve or its peak indicated the decrease or increase of the mean fluorescence intensity (MFI), respectively, as indicated by the "M1" bar in the figures. Quantitative data were expressed as the relative MFI.

Analysis of α2,3 Sialyl and α2,6 Sialyl Residues on Cell Surface With HRP-Lectin Staining

Cells coated on the microscopic cover glasses were washed with PBS and fixed with 4% polyformaldehyde for 30 min, followed by treatment with 3% H_2O_2 at 37°C for another 30 min. Then the cells were blocked with normal goat serum at 37°C for 20 min, treated with 1:50 dilute HRP labeled lectin (HRP-MAA or HRP-SNA), and incubated at 37°C for 90 min (PBS was added instead of HRP-lectin in the negative control sample). After the cover glasses were put at room temperature overnight, the cells were stained by 0.05% diaminobenzene (DAB)/0.03 H_2O_2, and finally treated with gradient ethanol, xylol, and neutral gel sequentially for microscope observation. Image analysis was used for quantification of the staining intensities and expressed as relative light index (set the index of Mock-MAA sample as 100%). The lectin–HRP conjugates were prepared by a routine method in our laboratory [Wilson and Nakane, 1978].

RESULTS

Effect of *nm23*-H1 on the Expressions of α1,3 FucT Subtypes

The products of α1,3FucT-III, -IV, -VI, -VII, and IX were 521, 516, 534, 497, and 530 bp, respectively, and the product of β-actin was 789 bp (Fig. 1); these lengths were the same as the products reported in the literatures when the same primers were used [Kudo et al., 1998a,b]. In mock-transfected cells, the expression-order of α1,3FucT mRNAs was IV > VI > III > VII > IX. Among them, the expression of α1,3FucT-IX mRNA was extremely low. After *nm23*-H1 transfection, the mRNA expressions of α1,3FucT-III, -IV, -VI, and -VII were decreased very significantly, ca. 22%–40% of those

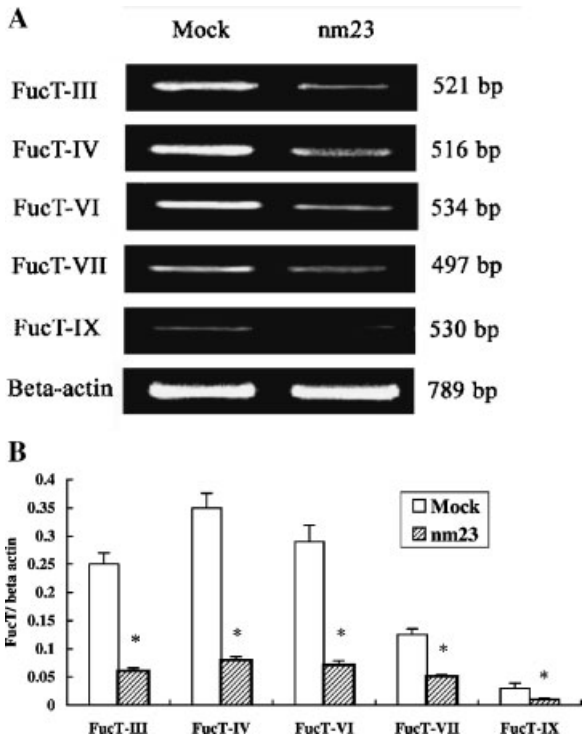

Fig. 1. Alteration of the mRNA expressions of five α1,3FucT subtypes in *nm23*-H1 transfected H7721 cells. **A**: Profiles of RT-PCR. **B**: Semi-quantification of A. Relative absorbance of each α1,3FucT mRNA was calculated from the intensity ratio of each α1,3FucT band to β-actin band (internal standard). $^*P < 0.01$ compared with the ''Mock'' group (FucT-III: 0.25 ± 0.02 vs. 0.06 ± 0.006; FucT-IV: 0.35 ± 0.026 vs. 0.08 ± 0.008; FucT-VI: 0.29 ± 0.029 vs. 0.07 ± 0.009; FucT-VII: 0.125 ± 0.01 vs. 0.05 ± 0.004; FucT-IX: 0.03 ± 0.009 vs. 0.01 ± 0.002). Three experiments were performed for both Mock and *nm23*-H1 transfected cells. Mock: H7721 cells transfected with vector pcDNA3, nm23: H7721 cells transfected with plasmid pcDNA/*nm23*-H1, FucT: α1,3 fucosyltransferase. The experimental procedure was described in Materials and Methods.

in mock-transfected cells ($P < 0.01$), and the mRNA of α1,3FucT-IX was reduced to a negligible level ($P < 0.01$).

Effect of *nm23*-HI on the Expressions of ST3Gal Subtypes

As indicated in Figure 2A,B, the RT-PCR products of ST3Gal-I, -II, -III, -IV were 543, 483, 529, and 503 bp, also in agreement with the reported lengths [Kudo et al., 1998b]. In "Mock" cells, the expression-order of ST3Gal mRNAs was IV > I > II > III. After *nm23*-H1 transfection, the mRNA of ST3Gal-I and -III were obviously decreased to 16.1% and 19.2%, respectively, of those in "Mock" cells ($P < 0.01$), while the mRNA of ST3Gal-IV was reduced to 57.7% of that in "Mock" sample ($P < 0.01$). In contrast,

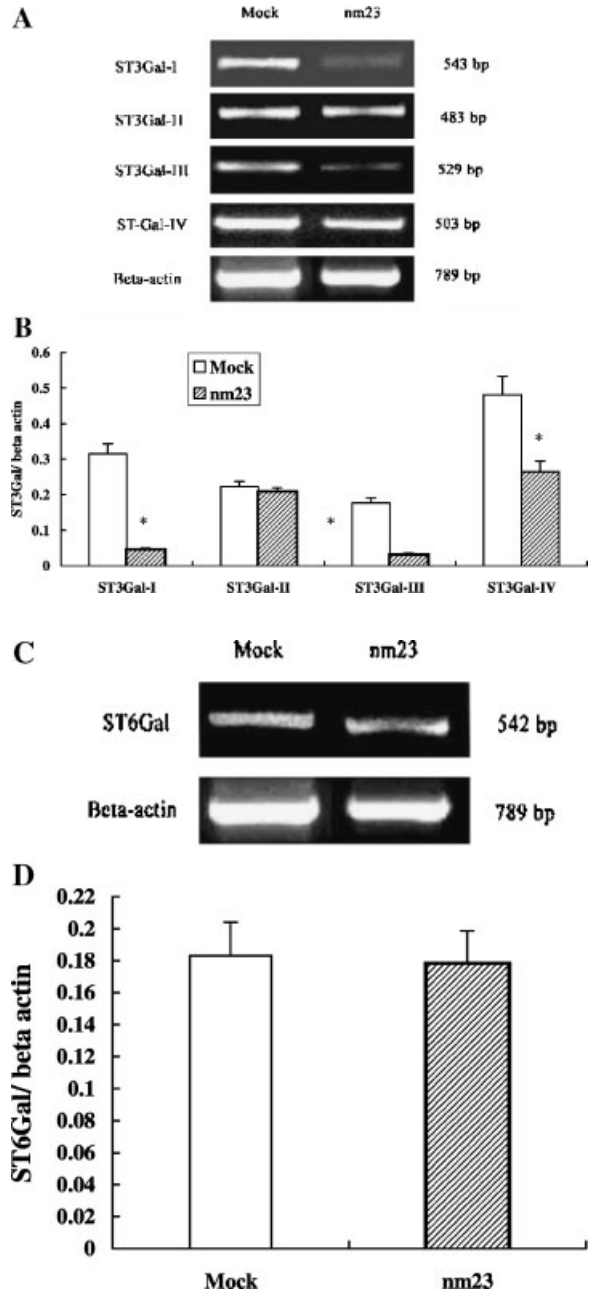

Fig. 2. Alteration of the mRNA expressions of four ST3Gal subtypes and ST6Gal-1 in *nm23*-H1 transfected H7721 cells. **A**: Profiles of RT-PCR of ST3Gals. **B**: Semi-quantification of ST3Gals. **C**: Profiles of RT-PCR of ST6Gal-I. **D**: Semi-quantification of ST6Gal-I. Relative absorbance of each ST3Gal and ST6Gal-I mRNAs was calculated from the intensity ratios of ST3Gal (or ST6Gal) bands to β-actin band (internal standard). $^*P < 0.01$ compared with the ''Mock'' group (ST3Gal-I: 0.315 ± 0.029 vs. 0.045 ± 0.005; ST3Gal-II: 0.223 ± 0.015 vs. 0.208 ± 0.012; ST3Gal-III: 0.178 ± 0.013 vs. 0.031 ± 0.005; ST3Gal-IV: 0.481 ± 0.51 vs. 0.265 ± 0.03). Three experiments were performed for both Mock and *nm23*-H1 transfected cells. ST3Gal: α2,3-sialyltransferase; ST6Gal: α2,6-sialyltransferase.

ST3Gal-II was unchanged after *nm23*-H1 transfection.

In order to study whether the expression change of ST in *nm23*-H1 transfected H7721 cells was specific for ST3Gal subfamily, the mRNA expression of ST6Gal as a control glycosyltransferase for ST3Gal was also studied after transfection of *nm23*-H1. To date, two subtypes, ST6Gal-I and ST6Gal-II, were reported [Krzewinski-Recchi et al., 2003]. The ubiquitously expressed ST6Gal-I was chosen for study. Figure 2C,D showed that ST6Gal-I (its RT-PCR product was 542 bp) was unchanged in *nm23*-H1 transfected cells.

Effect of *nm23*-H1 on the Expressions of C2GnT Subtypes

The products of C2GnT-I and C2GnT-II were 692 and 180 bp, respectively, which were also equal to the reported lengths using the same primers [Bierhuizen and Fukuda, 1992; Yeh et al., 1999]. In "Mock" cells, the expression of C2GnT-I was slightly higher than that of C2GnT-II. However, both enzymes were not altered after the transfection of *nm23*-H1 (Fig. 3).

Effect of *nm23*-H1 on the Expressions of Sialyl Lewis Antigens on Cell Surface

The mock-transfected H7721 cells expressed an apparent amount of SLe^x, but very few SLe^a and SDLe^x sugar antigens on the surface (Fig. 4). The expression of SLe^x antigen on *nm23*-H1transfected cells was decreased to 66.6% of the "Mock" value ($P < 0.01$), while SLe^a and SDLe^x were almost not changed.

Effect of *nm23*-H1 on the Amount of α2,3 and α2,6 Sialyl Residues on Cell Surface Glycans

The decreased ST3Gal expression resulted in the alteration in the amount of α2,3 sialyl residue and its ratio to α2,6 residue on the cell surface glycans. This was evidenced by the findings shown in Figure 5. By using HRP labeled MAA and SNA as the specific probes of α2,3- and α2,6-linked sialyl residues, respectively [Broekaert et al., 1984; Knibbs et al., 1991], it was found that the total staining intensity of HRP-MAA was lower than that of HRP-SNA on the mock-transfected cells. After *nm23*-H1 was transfected, the color intensity of both HRP-MAA (α2,3 SA) and HRP-SNA (α2,6 SA) were obviously reduced to 31.5% and 36.3%

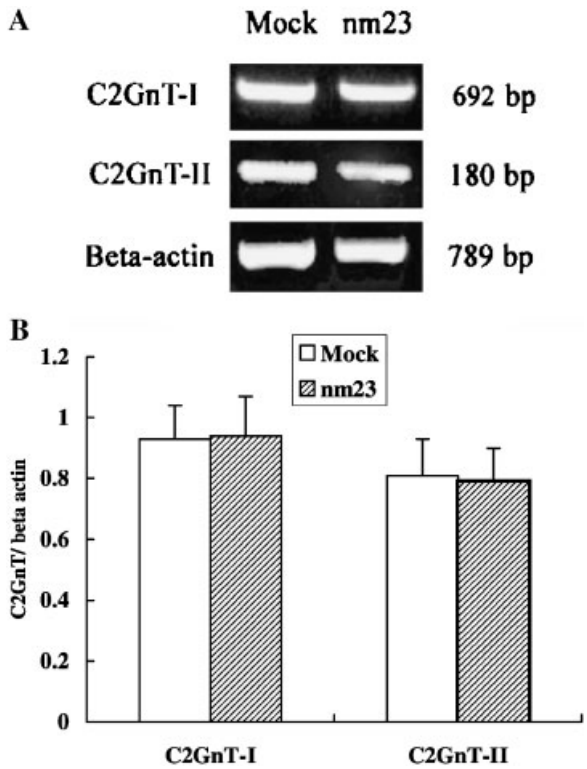

Fig. 3. Alteration of the mRNA expressions of C2GnT-I and C2GnT-II in *nm23*-H1 transfected H7721 cells. **A**: Profiles of RT-PCR. **B**: Semi-quantification of A. Relative absorbance of each C2GnT mRNA was calculated from the intensity ratios of each C2GnT band to β-actin band (internal standard). Three experiments were performed for both Mock and *nm23*-H1 transfected cells. C2GnT: Core 2 β1, 6-*N*-acetylglucosaminyltransferase.

of the "Mock" level ($P < 0.01$) when measured by Image analysis.

DISCUSSION

In the previous and present studies of our laboratory, it was discovered that *nm23*-H1 downregulated the transcription of many glycosyltransferase genes, including GnT-V [Guo et al., 2000], α1,3FucTs, and ST3Gals, and these effects were related to its anti-metastatic function. We have observed that *nm23*-H1 regulated not only the genes of glycosyltransferases but also the expressions of other proteins. By using immunoassay, the marker of hepatocyte differentiation, albumin, in the culture medium of *nm23*-H1 transfected cells was increased to 169.5% of the value in the medium of the mock-transfected cells. Conversely, the marker of hepatocyte carcinogenesis, α-fetal protein, was decreased to 24.8% of the level in the medium of the mock cells. These results indicate that *nm23*-H1 is not only a metastasis-suppressor

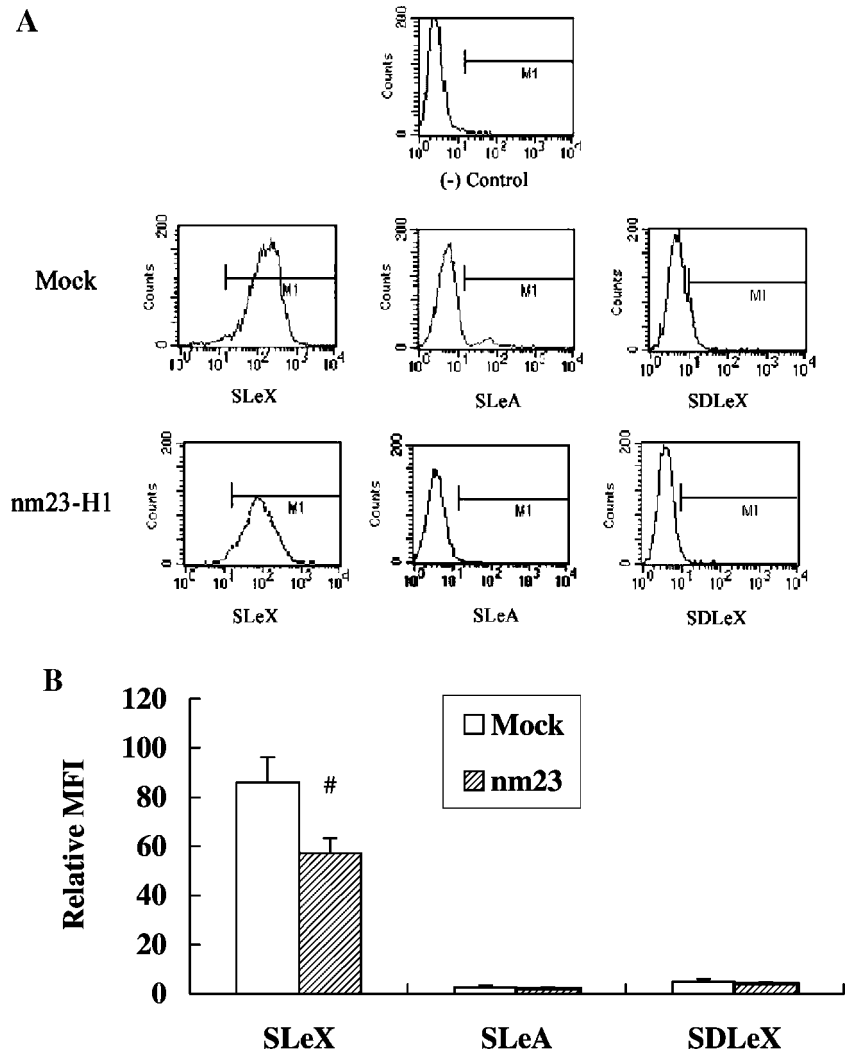

Fig. 4. Expression of surface sialyl Lewis antigens on *nm23*-H1 transfected H7721 cells. **A**: Fluorescence activated cell spectra (FACS) of three sialyl Lewis antigens. **B**: Relative expressions (relative MFI) of sialyl Lewis antigens. $^{*}P < 0.01$ compared with the ''Mock'' group (SLe^x: 85.9 ± 10.5 vs. 57.2 ± 6.1; SLe^a: 2.25 ± 0.9 vs. 1.81 ± 0.72; $SDLe^x$: 4.92 ± 1.1 vs. 3.85 ± 0.81). Three experiments were performed for both Mock and *nm23*-H1 transfected cells. SLe X, sialyl Lewis X (SLe^x); SLe A, sialyl Lewis A (SLe^a); SDLe X, sialyl dimeric (difucosyl) Lewis X ($SDLe^x$).

gene but also a differentiation gene for H7721 cells.

Most of the human nm23 proteins were recognized as nucleoside diphosphate (NDP) kinase [Lacombe et al., 2000]. This enzyme removes the terminal phosphate from nucleoside triphosphate (NTP) to autophosphorylate its own histidine 118 (histidine kinase activity), then transfers the phosphate to NDP to recreate NTP [Wallet et al., 1990]. The molecular mechanism underlying the role of nm23 protein in the different cell processes is elusive and the object of extensive study. Lombardi and Mileo [2003] indicated that nm23-H1 was a multifunctional protein and could not merely be regarded as an NDP kinase. Its enzyme activity was not required for metastasis-suppression, differentiation, and gene transcription. For example, an nm23 protein mutated at the active center of NDP kinase, H118F, still promoted the neural growth factor (NGF)-induced differentiation of PC12 cells via the transcription of some differentiation-related genes [Kimura et al., 2003]. Postal et al. [2000] have reported that nm23 protein is able to recognize and bind to the nuclease-hypersensitive transcriptional element of DNA in a sequence-specific manner, and subsequently induces a DNA structural transformation. This property is influenced by the positive or negative modulators of DNA

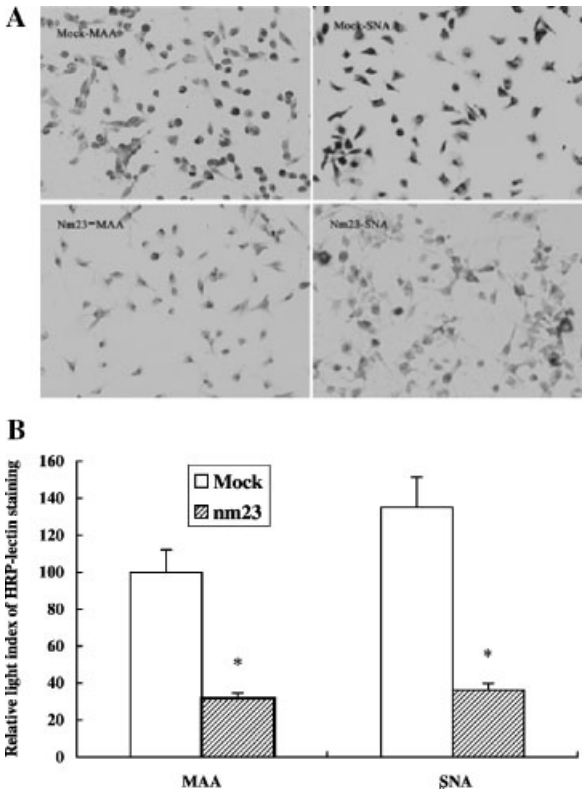

Fig. 5. Alteration of α2,3 sialyl and α2,6 sialyl residues of surface glycans on *nm23*-H1 transfected H7721 cells. **A**: Cell stained with horseradish peroxidase (HRP) labeled lectin (maackia amurensis agglutinin (MAA) or sambucus nigra agglutinin (SNA)). **B**: Image analysis for quantification of A and expressed as relative light index (set the light index of "Mock" sample stained with HRP-MAA as 100%). $^*P < 0.01$ compared with the "Mock" group (MAA: 100 ± 12.0 vs. 31.5 ± 3.7; SNA: 135.1 ± 16.2 vs. 36.3 ± 4.3). Three experiments were performed for both Mock and *nm23*-H1 transfected cells.

transcription. The catalytically important His 118 of NDP kinase is not required for its DNA binding.

In mock-transfected H7721 cells, the mRNAs of α1,3FucT-IV, -VI, and -III were relatively highly expressed, but those of α1,3FucT-VII and -IX were expressed very low or negligible, respectively. Therefore, it can be speculated that the SLe^x is mainly synthesized by α1,3FucT-VI, since α1,3FucT-IV hardly synthesizes sialyl Lewis antigens [Lowe et al., 1991], and the efficiency of α1,3FucT-VI in the synthesis of SLe^x is 6.4 times and 1.5 times those of α1,3FucT-III and -VII, respectively [Togayachi et al., 1999]. However, α1,3FucT-III and -VII may also participate in SLe^x synthesis. α1,3FucT-VI was also reported to be the enzyme responsible for the synthesis of SDLe^x [Weston et al., 1992b]. The incompatibility between the higher expression of α1,3FucT-VI and very low expression of SDLe^x was unresolved. It is possible that the β1,3-*N*-acetylglucosaminyltranferase (β1,3GnT) required for the synthesis of SDLe^x precursor (Galβ1,4GlcNAcβ1,3Galβ1,4GlcNAcβ1,3-) is limited in H7721 cells, and the negligibly expressed α1,3FucT-IX may be needed in the double fucosylation steps during SDLe^x synthesis [Kudo et al., 1998a; Narimatsu, 2002]. α1,3FucT-III is the only α1,3FucT for the synthesis of SLe^a; however, the rate-limiting enzyme for SLe^a synthesis is not α1,3FucT-III, but β1,3-galatosyltransferase 5 (β3GalT-5), which synthesizes the precursor of SLe^a, Galβ1,3GlcNAc sugar chain [Issiki et al., 1999]. The expression of β3GalT-5 activity is in accordance with the expression of SLe^a antigen in many cell lines. It is probably that the expression of this enzyme in H7721 cells is very low, so the synthesis of SLe^a precursor and SLe^a antigen is limited. In addition, the low expression ST3Gal-III, the main enzyme for transferring sialyl residue to SLe^a precursor, may be another reason for the low expression of SLe^a.

In *nm23*-H1 transfected H7721 cells, all of the α1,3FucTs that were assayed in the present investigation were downregulated, especially α1,3FucT-VI and -III. These results were consistent with the decreased expression of surface SLe^x. Therefore, the decrease of α1,3FucTs and the consequent reduction of SLe^x synthesis were implicated in the metastasis-suppressive action of *nm23*-H1. Similarly, the downregulation of ST3Gal-I, -III, and -IV may be the other mechanism for the decreased cell surface expression of SLe^x; hence, ST3Gal was also associated with the *nm23*-H1 effect on metastatic potential. We have reported that the removal of sialyl residues on the surface of H7721 cells resulted in the dramatic decrease of metastasis-related phenotypes, indicating that surface sialyl residues were crucial for the metastatic potential [Zhang et al., 2002]. On the other hand, the unaltered expression of C2GnT-I and -II suggested that these two glycosyltransferases, unlike α1,3FucT and ST3Gal, were not regulated by *nm23*-H1. The reason is unknown and is being investigated.

It was interesting to find in our laboratory that the mRNA expressions of α1,3FucT-IV, -VI, and -VII as well as ST3Gal-I, -II, -III, and -IV were upregulated by the metastasis-promoting

oncogene *H-ras*. As a consequence, the surface expression of SLe^x was also enhanced by the transfection of *H-ras* (unpublished data). These observations provide evidences from the opposite side that α1,3FucT and ST3Gal are associated with the metastasis of hepatocarcinoma cells. However, C2GnT-I and -II were also unchanged in *H-ras* transfected cells.

In mock-transfected H7721 cells, the sum of the expressions of ST3Gal (I–IV) mRNAs was far higher than the expression of ST6Gal-I mRNA, but the amount of α2,3 linked SA on the cell surface glycans was less than that of α2,6 linked SA (Fig. 5). This contradiction may be partially explained by the finding that α2,6 SA is not only synthesized by ST6Gal, but also by ST6GalNAc family [Tsuji, 1996, 1998]. The latter family consists of I–VI subtypes, being the major enzyme family for the α2,6-sialylation of *O*-glycans. Therefore, the sum of the activities of ST6GalNAc-I–VI plus ST6Gal-I and -II may exceed the sum of ST3Gal activity (I–IV), resulting in the higher content of α2,6 SA residue on the cell surface.

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REFERENCES

Baba H, Urano T, Okada K, Furuka K, Nakayama E, Tanaba H, Iwasaki K, Shiku H. 1995. Two isotypes of murine nm23/nucleoside diphosphate kinase, nm23-M1 and nm23-M2, are involved in metastatic suppression of a murine melanoma line. Cancer Res 55:1977–1981.

Bierhuizen MF, Fukuda M. 1992. Expression cloning of a cDNA encoding UDP-GlcNAc: Gal β1-3-GalNAc-R (GlcNAc to GalNAc) β1-6 GlcNAc transferase by gene transfer into CHO cells expressing polyoma large tumor antigen. Proc Natl Acad Sci USA 89:9326–9330.

Broekaert WF, Nsimba-Lubaki M, Peeters B, Peumans WJ. 1984. A lectin from elder (*Sambucus nigra* L.) bark. Biochem J 221:163–169.

Dennis JW, Granovsky M, Warren CE. 1999. Glycoprotein glycosylation and cancer progression. Biochim Biophys Acta 1473:21–24.

Fujimoto Y, Ohtake T, Nishimori H, Ikuta K, Ohhira M, Ono M, Kohgo Y. 1998. Reduced expression and rare genomic alteration of *nm23*-H1 in human hepatocarcinoma and hepatoma cell line. J Gastroenterol 33:368–375.

Fukuda M, Yeh JC. 2002. Core 2 β6-*N*-acetylglucosaminyltransferase-II. In: Taniguchi N, Honke K, Fukuda M, editors. Handbook of glycosyltransferases and related genes. Tokyo: Springer-Verlag Press. pp 143–150.

Fukuda M, Ishii A, Yasutomo Y, Shimada N, Ishikawa N, Hanai N, Nagata N, Irimura T, Nicolson GL, Kimura N. 1996. Decreased expression of nucleoside diphosphate kinase α isoform, an *nm23*-H2 gene homology, is associated with metastatic potential of rat mammary adenocarcinoma cells. Int J Cancer 65:531–537.

Fukuda M, Schwientek T, Clausen H. 2002. Core 2 β6-*N*-acetylglucosaminyltransferase-I and -III. In: Taniguchi N, Honke K, Fukuda M, editors. Handbook of glycosyltransferases and related genes. Tokyo: Springer-Verlag Press. pp 133–142.

Gilles AM, Presecan E, Vonica A, Lascu I. 1991. Nucleoside diphosphate kinase from human erythrocytes. J Biol Chem 266:8784–8789.

Guo HB, Liu F, Zhao JH, Chen HL. 2000. Down-regulation of *N*-acetylglucosaminyl-transferase V by tumorigenesis- or metastasis-suppressor gene and its relation to metastatic potential of human hepatocarcinoma cells. J Cell Biochem 79:370–385.

Guo P, Zhang Y, Shen ZH, Zhang XY, Chen HL. 2004. Effect of *N*-acetylglucosaminyltransferase V on the expressions of other glycosyltransferases. FEBS Lett 562: 93–99.

Hirayama R, Sawai S, Takagi Y. 1991. Positive relationship between expression of the anti-metastatic factor (*nm23* gene product or nucleoside diphosphate kinase) and good prognosis in human breast cancer. J Natl Cancer Inst 82:1249–1250.

Issiki S, Togayachi A, Kudo T, Nishihara S, Watanabe M, Kobata T, Kitajima M, Shiraishi H, Sasaki K, Andoh T, Narimatsu H. 1999. Cloning, expression, and characterization of a novel UDP-galactose:β-*N*-acetylglucosamine β1,3-galactosyltransferase (β3Gal-T5) responsible for synthesis of type I chain in colorectal and pancreatic epithelia and tumor cells derived therefrom. J Biol Chem 274:12499–12507.

Kantor JD, Mccormick B, Steeg PS, Zetter BR. 1993. Inhibition of cell mobility after *nm23* transfection of human and murine tumor cells. Cancer Res 53:1971–1973.

Kimura N, Shimada N, Ishijima Y, Fukuda M, Takagi Y, Ishikawa N. 2003. Nucleoside diphosphate kinase in mammalian signal transduction systems: Recent development and perspective. J Bioenerg Biomembr 35: 41–47.

Knibbs RN, Goldstein IJ, Ratcliffe RM, Shibuya N. 1991. Characterization of the carbohydrate binding specificity of the leukoagglutinating lectin from Maackia amurensis. Comparison with other sialic acid-specific lectins. J Biol Chem 266:83–88.

Krzewinski-Recchi MA, Julien S, Juliant S, Teintenier-Lelievre M, Samyn-Petit B, Montiel MD, Mir AM, Cerutti M, Harduin-Lepers A, Delannoy P. 2003. Identification and functional expression of a second human beta-galactoside alpha2,6-sialyltransferase, ST6Gal II. Eur J Biochem 270:950–961.

Kudo K, Ikehara Y, Togayachi A, Kaneko M, Hiraga T, Sasaki K, Narimatsu H. 1998a. Expression, cloning, and

characterization of a novel murine α1,3-fucosyltransferase, mFuc-TIX, that synthesis the Lewis X (CD15) epitope in brain and kidney. J Biol Chem 273:26729–26738.

Kudo T, Ikehara Y, Togayachi A, Morozumi K, Watanabe M, Nakamura M, Nishihara S, Narimatsu H. 1998b. Up-regulation of a set of glycosyltransferase genes in human colorectal cancer. Lab Invest 78:797–811.

Kukowska-Latallo JF, Larsen RD, Nair RP, Lowe JB. 1990. A cloned human cDNA determines expression of a mouse stage-specific embryonic antigen and the Lewis blood group (α1,3/1,4) fucosyltransferase. Genes Develop 4: 1288–1303.

Lacombe ML, Molon L, Munier A, Mehus JG, Lambeth DO. 2000. The human nm23/nucleoside diphosphate kinases. J Bioenerg Biomembr 32:247–258.

Liu F, Qi HL, Zhang Y, Zhang XY, Chen HL. 2001a. Transfection of *c-erb*B2/*neu*gene up regulates the expression of sialyl Lewis X, α1,3 fucosyltransferase VII and metastatic potential in human hepatocarcinoma cell line. Eur J Biochem 268:3501–3512.

Liu F, Qi HL, Chen HL. 2001b. Regulation of differentiation- and proliferation-inducers on Lewis antigens, α-fucosyltransferase, and metastatic potential in hepatocarcinoma cells. Brit J Cancer 268:3501–3512.

Lombardi D, Mileo AM. 2003. Protein interactions provide new insight into nm23/nucleoside diphosphate kinase functions. J Bioenerg Biomembr 35:67–71.

Lowe JB, Kukowska-Latallo JF, Nair RP, Larsen RD, Marks RM, Marcher BA, Kelly RJ, Ernst LK. 1991. Molecular cloning of a human fucosyltransferase gene that determines expression of the Lewis X and VIM-2 epitopes but not ELAM-1-dependent cell adhesion. J Biol Chem 266:17467–17477.

Marone M, Scambia G, Ferrandina G, Ghiannitelli C, Benedetti-Panici P, Iacovella S, Leone A, Mancuso S. 1996. *Nm23* expression in endometrial and cervical cancer inverse correlation with lymph node involvement and myometrial invasion. Brit J Cancer 74:1063–1068.

Nakamura M, Kudo T, Marimatsu H, Furukawa Y, Kikuchi J, Asakura S, Yang W, Iwase S, Hatake K, Miura Y. 1998. Single Glycosyltransferase, Core 2 $\beta 1 \rightarrow 6$ -*N*-acetylglucosaminyl transferase, regulates cell surface sialyl-Le^x expression level in human pre-B lymphocytic leukemia cell line KM3 treated with phorbol ester. J Biol Chem 273:26779–28789.

Narimatsu H. 1998. Human fucosyltransferases, tissue distribution of blood group antigens, cancer associated antigens, and fucosyltransferase. Protein Nucleic Acid Enzyme (in Japanese) 48:2394–2403.

Narimatsu H. 2002. α3-fucosyltransferase-IX (FUT9). In: Taniguchi N, Honke K, Fukuda M, editors. Handbook of glycosyltransferases and related genes. Tokyo: Springer-Verlag Press. pp 252–258.

Postal EH, Berberich SJ, Rooney JW, Kaetzel DM. 2000. Human nm23/nucleoside diphosphate kinase regulates gene expression through DNA binding to nuclease-hypersensitive transcriptional elements. J Bioenerg Biomembr 32:277–284.

Sasaki K, Kurata K, Funayama K, Nagata M, Watanabe E, Ohta S, Hanat N, Nishi T. 1994. Expression cloning of a novel α1,3-fucosyltransferase that is involved in biosynthesis of the sialyl Lewis X carbohydrate determinants in leukocytes. J Biol Chem 269:14730–14737.

Steeg PS, Bevilacqua G, Kopper L, Thorgeirsson UP, Talmadge IE, Liotta LA, Sobel ME. 1988. Evidence for a novel gene associated with low tumor metastatic potential. J Natl Cancer Inst 80:200–204.

Takada A, Ohmori K, Yoneda T, Tsuyuoka K, Hasegawa A, Kiso M, Kannagi R. 1993. Contribution of carbohydrate antigens sialyl Lewis A and sialyl Lewis X to adhesion of human cancer cells to vascular endothelium. Cancer Res 53:354–361.

Taniguchi N, Miyoshi E, Ko JH, Ikeda Y, Ihara Y. 1999. Implication of *N*-acetylglucosaminyl-transferases III and V in cancer: Gene regulation and signaling mechanism. Biochim Biophys Acta 1455:287–300.

Togayachi A, Kudo T, Ikehara Y, Iwasaki H, Nishihara S, Andoh T, Higashiyama M, Kodama K, Nakamori S, Narimatsu H. 1999. Up-regulation of Lewis enzyme (FucTIII) and plasma-type α1,3 fucosyltransferase (FucTVI) expression determines the augmented expression of sialyl Lewis X antigen in non-small cell lung cancer. Int J Cancer 83:70–79.

Tokunaga Y, Urano T, Furukawa K. 1993. Reduced expression of *nm23*-H1, but not of *nm23*-H2, is concordant with the frequency of lymph node metastasis of human breast cancer. Int J Cancer 55:66–71.

Tsuji S. 1996. Molecular cloning of and functional analysis of sialyltranferases. J Biochem 120:1–13.

Tsuji S. 1998. Sialyltransferase superfamily: Structure and function. Protein, Nucleic Acid Enzyme 48:2338–2348 (in Japanese).

Varki A. 1994. Selectin ligands. Proc Natl Acad Sci USA 91:7390–7397.

Wallet V, Mutzel R, Troll H, Barzu O, Wurster B, Veron M, Lacombe ML. 1990. Dictyostelium nucleoside diphosphate kinase highly homologous to Nm23 and Awd proteins involved in mammalian tumor metastasis and Drosophila development. J Natl Cancer Invest 82:1199–1202.

Weston BW, Nair RP, Jarsen RD, Lowe JB. 1992a. Isolation of a novel human α1,3 fucosyl transferase gene and molecular comparison to the human Lewis blood group α(1,3/1,4) fucosyltransferase gene, synthetic, homologous, nonallelic genes encoding enzymes with distinct acceptor substrate specificity. J Biol Chem 267:4152–4160.

Weston BW, Smith PL, Kelly RJ, Lowe JB. 1992b. Molecular cloning of a fourth member of a human α(1,3) fucosyltransferase gene family. Multiple homologous sequences that determine expression of the Lewis X, sialyl Lewis X, and difucosyl sialyl Lewis X epitopes. J Biol Chem 267:24575–24584.

Wilson MB, Nakane PK. 1978. Immunofluorescence and related staining techniques. In: Knapp W, Holubar K, Wick G, editors. Elsevier: Amsterdam. pp 215–224.

Yeh JC, Ong E, Fukuda M. 1999. Molecular cloning and expression of a novel beta-1, 6-*N*-acetylglucosaminyl-transferase that forms core 2, core 4, and I branches. J Biol Chem 274:3215–3221.

Zhang Y, Zhang XY, Liu F, Qi HL, Chen HL. 2002. The roles of terminal sugar residues of surface glycans in the metastatic potential of human hepatocarcinoma. J Cancer Res Clin Oncol 128:617–620.