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Down-Regulation of N-Acetylglucosaminyltransferase V by Tumorigenesis- or Metastasis-Suppressor Gene and Its Relation to Metastatic Potential of Human Hepatocarcinoma Cells

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Abstract The effects of transfection of the metastasis suppressor gene *nm23*-H1 and cell-cycle related tumor-suppressor gene *p16* on the activity of N-acetylglucosaminyltransferase V (GnT-V) and their relations to cancer metastatic potential were investigated. After transfection of *nm23*-H1 into 7721 human hepatocarcinoma cells and A549 human lung cancer cells, the activities of GnT-V were decreased by 28%–42% in the cells. In contrast, when *p16* was transfected into these two cell lines, the decrease of GnT-V activity was only observed in A549 cells. This was probably to be due to the obvious expression of *p16* gene in parental 7721 cells and the deletion of *p16* in A549 cells. The decrease of GnT-V mRNA was only observed in *nm23*-H1-transfected cells, but not in *p16*-transfected A549 cells, suggesting that these two genes regulated GnT-V via different mechanisms. Horseradish peroxidase (HRP)-lectin staining showed that the 7721 cells transfected with *nm23*-H1 or the A549 cells transfected with *p16* displayed a decreased intensity with HRP–leucoagglutinating phytohemagglutinin and increased intensity with HRP–concanavalin A, indicating the decline of β1,6 N-acetylglucosamine branching structure on the asparagine-linked glycans of cell-surface and intracellular glycoproteins. The *nm23*-H1 transfected 7721 cells also displayed some changes in metastasis-related phenotypes, including the increase in cell adhesion to fibronectin (Fn), the decline in cell adhesion to laminin (Ln), and the decreased cell migration and invasion through matrigel. Transfection of antisense GnT-V cDNA into 7721 cells resulted in a decrease of GnT-V activity, an increase of cell adhesion to Fn or Ln, and a decrease in cell migration and invasion through matrigel. These phenotypes bore similarity to those of the 7721 cells transfected with *nm23*-H1. Our findings indicate that the down-regulation of GnT-V by *nm23*-H1 contributes to the alterations in metastasis-related phenotypes, and is an important molecular mechanism of metastasis suppression mediated by *nm23*-H1. J. Cell. Biochem. 79:370–385, 2000.

Key words: human hepatocarcinoma cells; metastasis- or tumorigenesis-suppressor gene; N-acetyllucosaminyltransferase V (GnT-V); antisense GnT-V cDNA; metastasis-related phenotypes

Malignant transformation is highly associated with the alterations in the structure of cell-surface glycans. In many instances, alterations in the surface glycans cause significant changes in the growth, adhesion, and migration of the cells [Finne et al., 1989; Kawano et al., 1993; Hakomori ,1996]. One of the common alterations in the glycosylation of cell-surface

Abbreviations used: Abs, absorbance; AP, 2-aminopyridine; BSA, bovine serum albumin; ConA, concanavalin A; DAB, diaminobenzene; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; FCS, fetal calf serum; Fn, fibronectin; GADPH, 3-phosphoglyceraldehyde dehydrogenase; GlcNAc, N-acetylglucosamine; GnT, N-acetylglucosaminyltransferase; HPF, high-power field; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; Ln, laminin; L-PHA, leucoagglutinating phytohemagglutinin; Man, mannose; MES, 2-(N-morpholino)-ethanesulfonic acid; NDP, nucleoside diphosphate; N-glycan, asparagine-linked glycans; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride.

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glycoproteins that has been documented for many years concerns the significant increase in the levels of asparagine-linked glycans (N-glycans) containing β1,6-N-acetylglucosamine (GlcNAc) linked to the α1,6- mannose (Man) of the trimannosyl core. This GlcNAcβ1,6Man structure is synthesized by N-acetylglucosaminyltransferase V (GnT-V, E.C.2,1,4,155), located in the Golgi apparatus. GnT-V is a key enzyme in the processing of multiantennary N-glycans during the synthesis of glycoproteins [Brockhausen et al., 1988]. GnT-V is well known as a tumorigenesis and metastasis-associated enzyme, since its product is the preferred substrate for the synthesis of poly-acetyllactosamine (repeating Galβ1,4GlcNAc-β1,3-). The GlcNAcβ1,6Man branch, as well as the poly-acetyllactosamine–containing glycans, are closely associated with the metastatic potential of many cancers [Dennis and Laferté, 1987, 1989; Dennis et al., 1987; Fernandes et al., 1991]. The alteration in β1,6 GlcNAc branch on the cell surface has been implicated in the modulation of cell adhesion and migration, which were also closely related to the metastasis potential of many cancers [Aznavoorian et al., 1993; Demetriou et al., 1995; Hakomori, 1996]. GnT-V is over-expressed in many malignant tumors, such as mammary, hepatic, and pancreatic cancer [Dennis and Laferté, 1989; Fernandes et al., 1991; Nan et al., 1998; Yao et al., 1998]. In our laboratory, it was found that GnT-V showed the highest activity at G_2/M phase of cell cycle and was related to the proliferation of 7721 human carcinoma cells [Guo et al., 2000a]. On the other hand, the oncogene is commonly associated with carcinogenesis and metastasis. Recently, we found that the over-expressions of *H-ras* and *v-sis* up-regulated GnT-V and changed some of the metastasis-related phenotypes in 7721 human hepatocarcinoma cells. These included the decline of cell adhesion to fibronectin (Fn), the enhancement of cell adhesion to laminin (Ln), as well as the increased invasiveness of the cells through matrigel [Guo et al., 2000b]. The above observations suggested that the alterations of cell adhesion and invasion induced by oncogenes were closely related to the up-regulation of GnT-V, and GnT-V might be involved in the metastasis of some cancers.

nm23 is a specific gene family related to the nonmetastatic behaviors of cancer. It was first characterized in 1988 [Steeg et al., 1988]. The gene is located on chromosome 17q21, codes an 18.5-kDa protein containing 166 amino acids with nucleoside diphosphate kinase and protein-histidine kinase activities, as well as serine autophosphorylation activity [De La Rosa et al., 1995]. The first member of the gene family was *nm23*-H1[Gilles et al., 1991]. *nm23*-H1 was demonstrated to have anti-metastatic properties in numerous human and animal cancer models and lower-expressed in many types of cell lines with highly metastatic potential [Steeg et al., 1988; Baba et al., 1995; Kantor et al., 1993; Fukuda et al., 1996]. The levels of *nm23*-H1 protein or RNA have shown an inverse correlation with lymph node metastasis and patient survival in a number of studies on human breast cancer [Hirayama et al. 1991; Tokunaga et al. 1993].

On the other hand, Kamub et al. [1994] and Nobori et al. [1994] independently reported a cell cycle-related tumor suppressor gene, *p16* (also known as *MTS1*). The product of *p16* exerts its function by competing with cyclin D1 in binding to CDK4, preventing the activity of CDK4, resulting in the decrease of phosphorylated Rb, a protein encoded by retinoblastoma-related gene. The lower phosphorylated Rb combines with the transcription factor, which regulates the expression of some protein participating in the control of cell cycle. As a consequence, the cell cycle is arrested at the G_0/G_1 phase and cell proliferation is inhibited [Serrano et al., 1993; Tam et al., 1994]. It has been demonstrated that *p16*, acting as a tumor suppressor gene, prevents cellular transformation by *H-ras*, [Serrano et al., 1995]. Kamb et al. [1994] examined 290 different tumor cell lines and found that 75% have *p16* deletion or mutation, more than the mutation of *p53* [Kamb et al., 1994]. Point mutations and small deletions are common in the cases with pancreatic adenocarcinoma, esophageal carcinoma, biliary tract cancer, and in families with hereditary susceptibility to melanoma and pancreatic cancer [Serrano et al., 1996]. Among 20 cases with non-small lung cancer, *p16* deletions were identified in the chromosome DNA of six cases [Wasihimi et al., 1995]. Recently, Zhang et al. [1999] reported that the cell growth was decreased in A549, a lung adenocarcinoma cell line with deletion of *p16*, and the cells were arrested at the G_0/G_1 phase after transfection with *p16* cDNA.

nm23-H1 and *p16* represent different kinds of tumor-suppressor genes. The former is a metastasis-suppressor gene, and the latter is a tumor-suppressor gene. To further elucidate the correlation between GnT-V and metastatic potential, the effects of *nm23*-H1 or *p16* transfection on the GnT-V expression and β1,6 GlcNAc branch on N-glycans in 7721 human hepatocarcinoma cells and A549 lung adenocarcinoma cells were investigated. The relation between GnT-V and the metastasis-related phenotypes, including cell adhesion, migration, and invasion were simultaneously studied in 7721 cells. Meanwhile, the alterations in GnT-V activity and metastasis-related phenotypes of 7721 cells transfected with antisense GnT-V cDNA were also observed in 7721 cells to further examine whether reduced GnT-V expression plays a causative role in tumor metastasis. Our studies were more focused on the 7721 cell line, and A549 was selected as a control cell to compare with the 7721 cell because 7721 shows a high expression of *p16* and A549 is a *p16*-deleted cell line.

MATERIALS AND METHODS

Materials

The human 7721 hepatocarcinoma cell line and A549 pulmonary adenocarcinoma cell line were obtained from the Institute of Cell Biology, Academic Sinica. The pcDNA3 mock-transfected cells were kindly provided by Prof. Gu at the gene center of our university. The plasmid Bluescript(+)/*nm23*-H1 and Bluescript(+)/*p16*, as well as the 7721 cell line transfected with *p16* cDNA and A549 cell line transfected with *nm23*-H1 cDNA were obtained from the Institute of Liver Cancer affiliated with our university. The plasmid pcDNA3FluHuTV (pcDNA3/GnT-V) was a gift from Prof. M. Pierce at the University of Georgia. The cDNA of 3-phosphoglyceraldehyde dehydrogenase (GAPDH) was obtained from Prof. I. Shimizu at Tokushima University. Rabbit anti-human p16 antibody and mouse anti-human nm23-H1 monoclonal antibody were purchased from Santa Cruz. RPMI 1640 medium, matrigel and Trizol were purchased from Gibco/BRL. DNA restriction endonucleases and random primer labeling kit were from Promega. Hybond-N^+ nylon membrane, Hybond-polyvinylidene difluoride (PVDF) membrane, enhanced chemiluminescence (ECL) assay kit and [α-^{32}P]-dATP were from Amersham Corp. UDP-GlcNAc, GlcNAc, 2-(N-morpholino)-ethanesulfonic acid (MES), N-(2-hydroxyethyl)-piperazine-N-(2-ethanesulfonic acid) (HEPES), horseradish peroxidase (HRP)-labeled lectin, neuraminidase (*clostridium perfringens*), Fn, Ln, and poly-L-lysine were the products of Sigma. The Insert (transwell) and 96-well culture plates were obtained from NUNC Company. The acceptor substrate for GnT assay, fluorescence (aminopyridine, AP)-labeled biantennary N-glycan ($GlcNAc_2Man_3$-$GlcNAc_2$-AP) was prepared in our laboratory as described previously [Ju et al., 1995]. Other reagents were commercially available in China.

Cell Culture and Treatment

Cells from line 7721 or A549 (10^6 cells/flask) were cultured at 37°C, 5% CO_2 in RPMI-1640 medium containing 10% bovine calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The medium was changed daily as previously described by our laboratory [Ai et al., 1995].

Construction of Plasmid pcDNA3/*nm23*-H1 and pcDNA3/*p16*

The construction of plasmid pcDNA3/*nm23*-H1 and pcDNA3/*p16* were according to our published method [Liu et al., 2000]. Plasmid of Bluescript(+)/*nm23*-H1 and Bluescript(+)/*p16* were cut with *Bam*HI and *Hin*dIII/*Xho* restriction enzymes, respectively, and the product, cDNA of *nm23*-H1 (0.74 kb) or *p16* (0.56 kb), was separated with and recovered from agarose electrophoresis. After characterization and quantification, each cDNA was inserted into the multiple cloning site of the linearized eukaryotic expression plasmid pcDNA3 vector (5.4 kb) using *Bam*HI or *Hin*dIII/*Xho*, then treated with T4 DNA ligase to ligate the plasmids.

Transfection of Constructed Plasmid into Cells

The constructed plasmids or vector pcDNA3 were transfected to 7721 or A549 cells using a standard electroporation method [Ausybel et al, 1995]. Briefly, after cells were washed three times with phosphate-buffered saline (PBS) and resuspended in PBS, then 0.8×10^7 cells in 0.8 ml were transfected with 25 μg plasmid or vector by electroporation using Gene Pulser II

(Bio-Rad) at 250 V per 0.4 cm and 1,000 μF. The transfected cells were diluted to 10.8 ml with PRMI-1640, and transferred to RPMI 1640 medium containing G418 (0.8 mg/ml) after 24 h. Neomycin-resistant cells were obtained after two to three weeks and recloned by serial dilution. Finally, more than four positive clones of pcDNA3/*nm23*-H1–transfected 7721 (*nm23*-H1/7721) and pcDNA3/*p16*-transfected A549 (*p16*/A549) cells were established and numbered.

Preparation of GnT-V cDNA and Construction of Antisense GnT-V cDNA

Plasmid of pcDNA3/GnT-V was cut with *Kpn* I and *Xba* I. The product, cDNA (2.3 kb) of GnT-V, was separated with and recovered from agarose electrophoresis, then characterized, quantitated, and used as a probe for Northern blot. The cDNA of antisense GnT-V was constructed as previously reported [Guo et al., 1999]. About a 0.8-kb fragment near the 5′ end of GnT-V cDNA was cut out from pcDNA3FluHuTV with *Eco*R I. This fragment was selected, purified, and then subcloned into pcDNA3, which was also cut with *Eco*R I. The direction of the inserted fragment (0.8 kb) can be determined by *Bam*H I digestion of the recombinant plasmid (*Bam*H I mapping), because there was a *Bam*H I site near the 3′ end of the inserted fragment, and about 0.6 kb (if inserted in normal direction) or 0.2 kb (if inserted in inverse direction) apart from the upstream of another *Bam*H I site located in multiple cloning sites of pcDNA3 vector. After the plasmid with inversely inserted fragment (0.8 kb) was verified by *Bam*H I digestion, this plasmid was selected, purified, and named pcDNA3/GnT-V-AS.

Determination of mRNA of *p16, nm23*-H1 and GnT-V

The isolation of cell RNA and Northern blot of RNA were carried out according to the method described by Sagerstrom and Sive [1996]. Briefly, total cell RNA was extracted with Trizol, followed by formaldehyde denatured electrophoresis, transferred to Hybond-N^+ nylon membrane, and prehybridized. Hybridization was performed using full-length cDNA of *p16*, *nm23*-H1, or GnT-V as the probes. The probes were labeled with [α-^{32}P]-dATP using random primer labeling kit from Promega according to the instruction described in the manual. The cDNA of GAPDH was labeled with the same method and used as an intrinsic standard during the Northern blot. After exposure of the membrane under X-ray film, the intensity of each hybridized band was quantified using a densitometer.

Western Blot of nm23-H1 and p16 Proteins

The cells were homogenized in 0.1 M MES buffer, pH 6.5, and centrifuged with 1,000*g* at 4°C for 15 min. The protein concentration of the supernatant was determined by the Lowry method [Lowry et al., 1951]. Western blotting was performed according to a modified method of Marone et al. [1996]. Aliquots of 50-μg protein samples were separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto a PVDF membrane, which was then blocked with 5% fat-free dry milk in 1 × TBST (0.1 M Tris, 0.15 M NaCl, 0.05% Tween 20, pH 7.4). The membrane was treated with a 1:200 dilution of anti-nm23-H1 or anti-p16 antibody in 5% fat-free dry milk in PBS, followed by incubation with 1:200 diluted HRP-labeled second antibody (anti-mouse IgM or anti-rabbit IgG), then the staining was performed with the ECL reagent.

Enzyme Preparation and Assay of GnT-V

The cells were homogenated in 50 mmol/liter maleate buffer, pH6.5, containing sucrose and phenylmethylsulfonyl fluoride, and centrifuged at 700*g* for 30 min. The supernatant was centrifuged at 105,000*g* for 1 h, and the precipitate was extracted with 1% Triton X-100 and centrifuged at 105,000*g* for 1 h again. The second supernatant was used for the assay of GnT-V according to a routine method in our laboratory [Ju et al., 1995]. Briefly, 50 μl reaction mixture, pH 6.25 contained 20 μl enzyme, 0.1 mol/liter MES, 0.3 mmol/liter acceptor substrate ($GlcNAc_2Man_3GlcNAc_2$), 0.02 mmol/L UDP-GlcNAc, 0.2 mol/L GlcNAc and 2% Triton X-100. After incubation at 37°C for 5 h and stopped by heating, the samples were centrifuged at 5,000 rpm and an aliquot (20 μl) of each sample was applied to reverse-phase high-performance liquid chromatography (HPLC) with a TSK-gel ODS C_{18} column. All the samples were assayed in duplicate. The activity of GnT-V was calculated according to the peak areas of the product in the sample and the standard with known concentration, and

expressed as pmol of GlcNAc transferred per hour per milligram protein.

Cell Staining with HRP-Lectin Complex

Cells coated on the microscopic plates were treated with PBS for 10 min and neuraminidase (0.03 unit) for 5 h at 37°C to remove the sialic acids at the terminal of glycans. After washing with PBS, 0.3% H_2O_2 and 1% bovine serum albumin (BSA) were added sequentially and incubated for 30 min at each step. After washing, the plates were incubated with 4 μg/ml HRP–leucoagglutinating phytohemagglutinin (L-PHA) complex and incubated for 2 h at 37°C, then stained with diaminobenzene (DAB). Finally the plates were treated with gradient alcohol, xylol, and neutral gel sequentially for microscopic observation [Guo et al., 1999].

Assay of Cell Adhesion to Fn and Ln

Cell adhesion experiment was according to the methods described by Busk et al. [1992]. In brief, the wells of the culture plate were coated with 0.1 ml of different concentrations of Fn or Ln. Additional 1 mg/ml poly-L-lysine and 1% BSA were coated for each two wells as maximal and minimal adhesion controls, respectively. The plate was incubated at 37°C for 1 h, and blocked by 1% BSA at 37°C for 0.5 h after washing. Cells (1×10^5) were added to each coated well and incubated for 2 h at 37°C, followed by staining with crystal violet after two washings, then the absorbance (Abs) at 595 nm was measured. The cells adhered to the coated wells was calculated as follows. The data were expressed as the mean of triplicate wells.

$$\frac{\text{Abs (experimental wells)} - \text{Abs (mean of BSA-coated wells)}}{\text{Abs (mean of poly-L-lysine wells)} - \text{Abs (mean of BSA-coated wells)}}$$

Determination of Cell Migration and Invasion

The chemotactic cell migration assay was performed using 24-well transwell units with an 8-μm pore size polycarbonate filter according to the method of Mensing et al. [1983]. Each lower compartment of the transwell contained 600 μl of 0.5% fetal calf serum (FBS) as chemoattractant, or 0.5% BSA as negative control in Dulbecco's modified Eagle's medium (DMEM). Cells (2×10^4) in 0.1 ml DMEM–0.1% BSA were added into the upper compartment of the transwell unit and incubated for 6 h at 37°C in a humidified atmosphere containing 5% CO_2. The cells were then fixed with glutaraldehyde and stained with crystal violet. Then the numbers of cells that had migrated to the lower side of the polycarbonate filter were counted in eight high-powered fields (HPFs) (×200). The data were expressed by the mean value of cells per HPF four times with two independent experiments.

The procedure reported by Repesh [1989] for the chemotactic cell invasion test was the same as in the chemotactic cell migration assay, except that the upper side of polycarbonate filter was coated with 0.1 ml (20 μg per filter) of matrigel in cold DMEM to form a continuous thin layer. The added cells were 1×10^5 in 0.1ml, and the incubation time was prolonged to 36 h. Cells were stained and counted as described above, and the number of cells invaded to the lower side of the filter was a measure of the invasive activity of the cells.

RESULTS

Characterization of nm23- and p16-Transfected Cells

The pcDNA3/*p16*-transfected 7721 cells (*p16*/7721) and pcDNA3/*nm23*-H1–transfected A549 cells (*nm23*-H1/A549) were already characterized in the Institute of Liver Cancer before we obtained the cells. It has been found that 7721 cell line expresses an obvious amount of p16 mRNA, so it is not a suitable cell line for the transfection and investigation of the effect of *p16* cDNA. However, nm23-H1 mRNA was lowly expressed in the parental and mock-transfected A549 cells, and the expressions were increased after *nm23*-H1 transfection (data not shown). The characterization of pcDNA3/*nm23*-H1–transfected 7721 cells (*nm23*-H1/7721) and pcDNA3/*p16*-transfected A549 cells (*p16*/A549) were carried out in our laboratory. Northern blot for the determination of nm23-H1 or p16 mRNA (Fig. 1) and Western blot combined with immunodetection for the estimation of nm23-H1 or p16 protein (Fig. 2) were used.

As shown in Fig. 1, there was a relatively low expression of nm23-H1 mRNA (0.74 kb) in the parental or mock-transfected 7721 cells, but the expression was higher in the *nm23*-H1/7721 cells. Among four clones of *nm23*-H1/7721, clone 3 showed the highest and clone 4

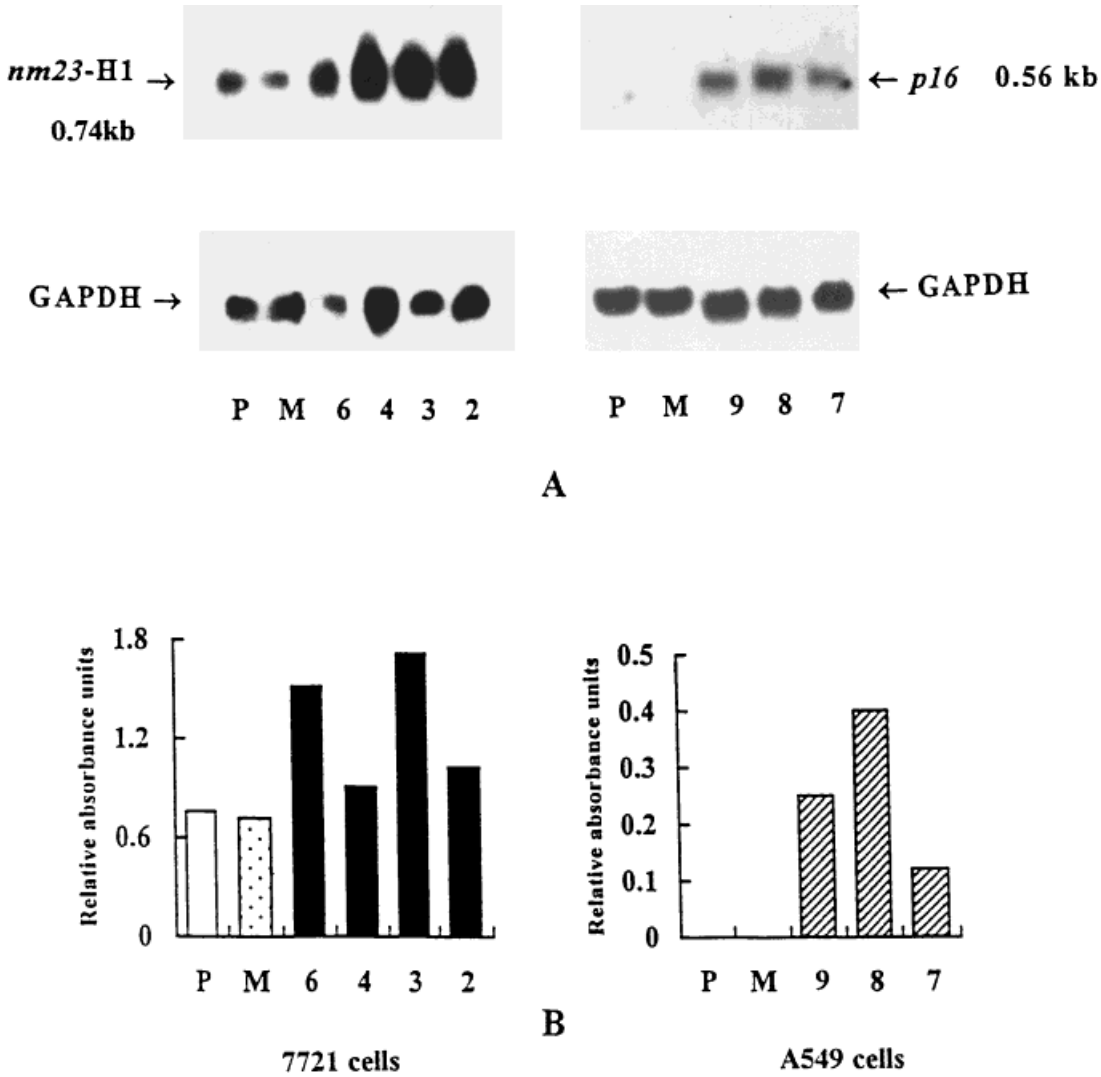


Fig. 1. Characterization of transfected cells with determination of nm23-H1 and p16 mRNAs. **A:** Northern blot of mRNA of *nm23*-H1 or *p16*. **B:** Densitometric analysis. P, parental cells; M, mock-transfected cells; 6, 4, 3, 2, different clones of 7721 cells transfected with pcDNA3/*nm23*H1; 9, 8, 7, different clones of A549 cells transfected with pcDNA3/*p16*. The RNA was extracted with Trizol and the Northern blot procedure was described in Materials and Methods.

showed the lowest content of nm23-H1 mRNA. The relative absorbance units (the ratio of the absorbance of nm23-H1 to the absorbance of GAPDH intrinsic standard) in clone 3 was more than double the value of the mock-transfected 7721 cells, when determined with quantitative densitometric method. In the A549 cells, it was found that there was almost no expression of p16 mRNA (0.56 kb) in the parental and mock-transfected A549 cells, whereas the expressions were high in three different clones of *p16*/A549. The expression in clone 8 was the highest and clone 7 was the lowest. This finding indicated that the *p16* is deleted in A549 cells, and the transfection of pcDNA3/*p16* to A549 cells was effective.

Fig. 2 showed that the nm23-H1 protein (18.5 kDa) was also higher in *nm23*-H1/7721 than in the mock-transfected cells. Clone 3 was again the highest and clone 4 was the lowest. This result was compatible with the expression of nm23-H1 mRNA in *nm23*-H1/7721 cells. On the other hand, in accordance with the p16 mRNA, the p16 protein (16 kDa) in *p16*/A549 cells was also only present in *p16*/A549 cells, and was negligible in the parental and mock-

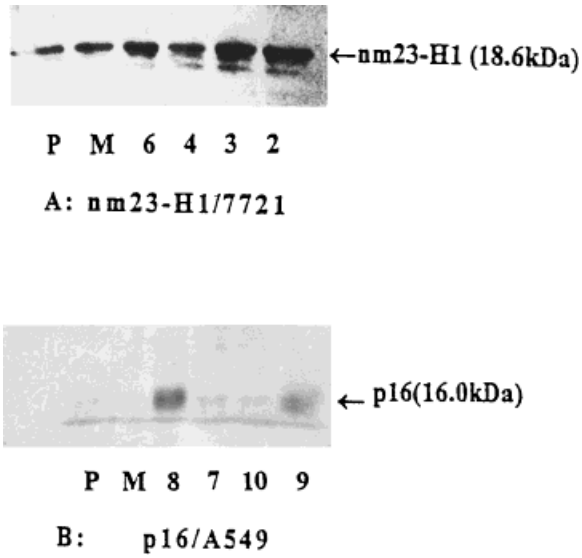


Fig. 2. Characterization of transfected cells with determination of nm23-H1 and p16 proteins. P, parental cells; M, mock-transfected cells; 6, 4, 3, 2, different clones of 7721 cells transfected with pcDNA3/*nm23*H1; 8, 7, 10, 9, different clones of A549 cells transfected with pcDNA3/*p16*. The proteins were determined using Western blot as described in Materials and Methods.

TABLE I. Activities of GnT-V in *nm23*-H1– and *p16*-Transfected Cells[a]

Cell line	Activity (pmol/h mg)	Relative activity (%)
7721	268.9 ± 12.4	—
Mock (pcDNA3/7721)	265.0 ± 9.6	100.0
nm23-H1/7721(clone3)	153.7 ± 11.5*	58.0
nm23-H1/7721(clone4)	191.9 ± 12.6*	72.4
p16/7721	267.9 ± 8.6	101.1
A549	113.6 ± 6.8	—
Mock (pcDNA3/A549)	110.7 ± 5.4	100.0
nm23-H1/A549	71.1 ± 6.5*	64.2
p16/A549(clone8)	77.7 ± 7.1*	70.1
p16/A549(clone7)	92.4 ± 8.7	83.5

[a]Data were expressed as mean value ± SD of three independent experiments. The enzyme activities were determined with HPLC method using fluorescent substrate. Mock, 7721 or A549 cells mock-transfected with pcDNA3 vector; *nm23*-H1/7721, 7721 cells transfected with pcDNA3/*nm23*-H1; *p16*/7721, 7721 cells transfected with pcDNA3/*p16; nm23*-H1/A549, A549 cells transfected with pcDNA3/*nm*23-H1; *p16*/A549, A549 cells transfected with cDNA3/*p16;*
*$P < 0.05$ compared with mock cells.

transfected cells. The amount of p16 protein in clone 8 was higher than in clone 9 and clone 7.

Effects of *p16* and *nm23*-H1 Transfection on the Activity of GnT-V

The activities of GnT-V in different cell lines were assayed by using a reverse-phase HPLC method to separate the fluorescent product from the substrate described in Materials and Methods. As shown in Table 1, the specific activities of GnT-V in mock-transfected 7721 (pcDNA3/7721) and A549 (pcDNA3/A549) cells did not change significantly as compared with the counterpart parental cells. The GnT-V activities in *nm23*-H1–transfected 7721 cells (*nm23*-H1/7721) were down-regulated to 58.0% and 72.4% of the mock cell level in clone 3 and clone 4, respectively; however, it was not changed in the *p16*-transfected 7721 cells (*p16*/7721). This finding confirmed the high expression of *p16* in the parental 7721 cells, and indicated that the transfection of exogenous *p16* could not further increase the effect of endogenous *p16* on GnT-V activity. When A549 cells were transfected with *nm23*-H1 (*nm23*-H1/A549), GnT-V activity was decreased by 36%. In contrast to 7721 cells, the activity of GnT-V in *p16*-transfected A549 cells (*p16*/A549) was decreased by 30% or 16.5% in clone 8 and clone 7, respectively, compared with the mock-transfected cells. The lower activities in clone 3 of *nm23*-H1/7721 and clone 8 of *p16*/A549 were compatible with the higher expression of *nm23*-H1 in clone 3 and *p16* in clone 8, respectively.

Effects of *p16* and *nm23*-H1 Transfection on the Expression GnT-V mRNA

The mRNA of GnT-V in the *nm23*-H1–transfected cells and *p16*-transfected cells was also determined with Northern blot, followed by densitometric analysis. Similar to the result of GnT-V activity, the GnT-V mRNA in mock-transfected cells was also not altered when compared with counterpart parental cells. The decrease of GnT-V mRNA expression was observed in *nm23*-H1/7721 and *nm23*-H1/A549 cells when compared with the corresponding mock-transfected cells (Fig. 3, left panels), which was consistent with the decrease of GnT-V activity in these two cell lines. However, the GnT-V mRNA in *p16*-transfected 7721 cells (*p16*/7721) and A549 cells (*p16*/A549) were not altered (Fig. 3, right panels).

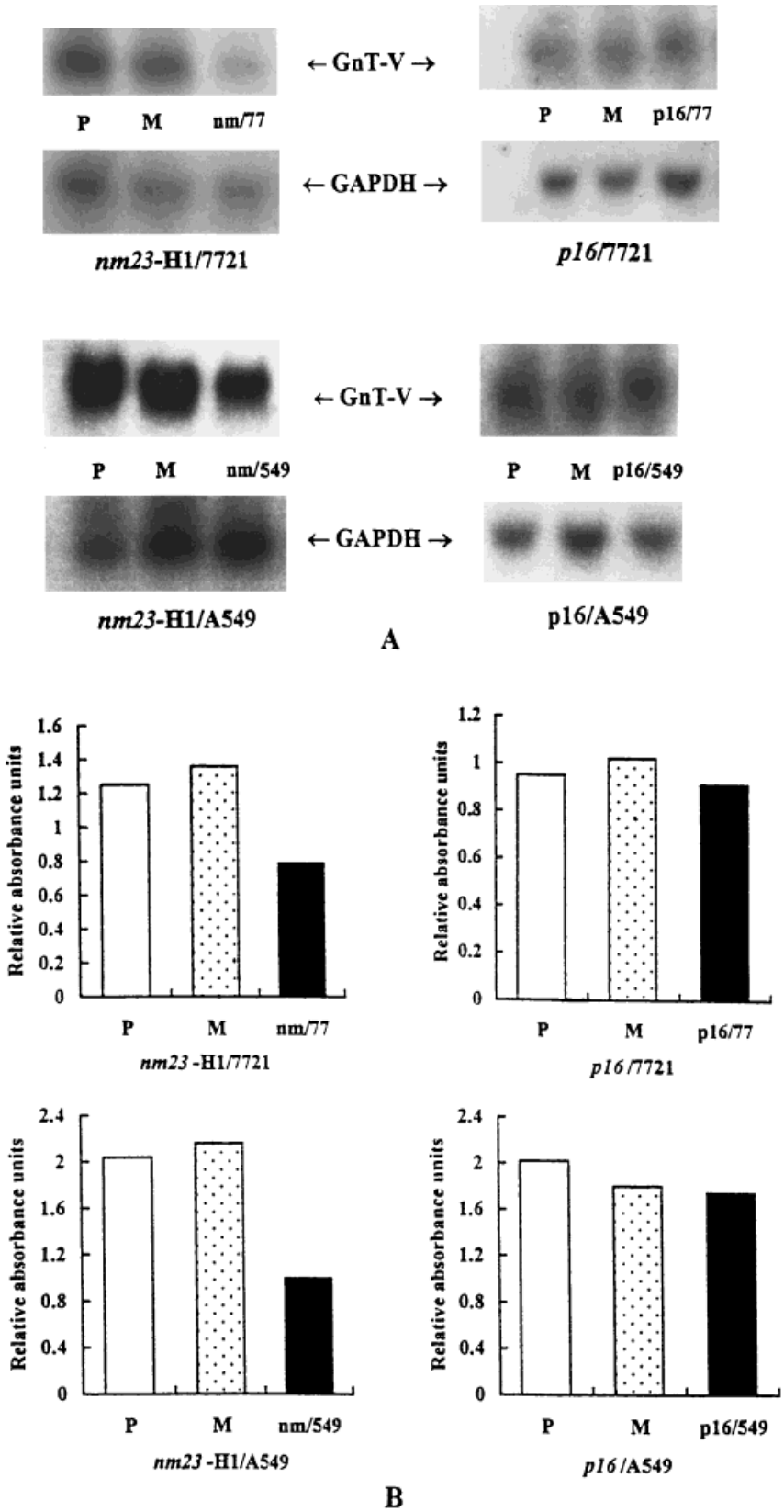


Fig. 3. Determination of GnT-V mRNA in *nm23*-H1– and *p16*-transfected 7721 and A549 cells. **A:** Northern blot of GnT-V mRNA. **B:** Densitometric analysis. P, parental cells; M, mock-transfected cells, nm/77, *nm23*-H1/7721 cells (clone3); p16/77, *p16*/7721 cells; nm/549, *nm23*-H1/A549 cells; p16/549, *p16*/A549 cells (clone 8). The experimental procedure was the same as described under Figure 1.

The unchanged GnT-V mRNA in *p16*/A549 cells was different from the result of decreased GnT-V activity in this cell line.

Effects of *p16* and *nm23*-H1 Transfection on the Structure of Glycoprotein N-Glycans

The alterations in the structure of N-glycans were observed in the *p16*- and *nm23*-H1–transfected cells with HRP-labeled lectins as probes, followed by DAB staining and image analysis of the color intensity. As compared with the mock-transfected cells, the intensities of the N-glycans on *nm23*-H1/7721 cells (Fig. 4) or *p16*/A549 cells (Fig. 5) were obviously decreased with HRP–L-PHA staining ($P < 0.05$) and increased with HRP–concanavalin A (ConA) staining ($P < 0.05$), indicating the decreased content of β1,6 GlcNAc branch in N-glycans of surface and intracellular glycoproteins [Osawa and Tsuji, 1987].

Alterations in Cell Adhesion of *nm23*-H1–Transfected 7721 Cells

Cell adhesion was determined by using Fn- or Ln-coated wells of the culture plates. The adhesion of mock-transfected cells to both Fn and Ln were increased in a concentration-dependent manner with the coated Fn or Ln. After *nm23*-H1 transfection, the cell adhesions to Fn displayed a tendency to increase, and became significant when the concentrations of coated Fn were 10 μg/ml ($P < 0.05$) and 30 μg/ml ($P < 0.01$). In contrast, the adhesions of *nm23*-H1–transfected cells to Ln were decreased as compared with the mock-transfected cells, and also became significant when the concentrations of coated Ln were 10 μg/ml ($P < 0.05$) and 30 μg/ml ($P < 0.01$) (Fig. 6).

Alterations in Cell Migration and Invasion of *nm23*-H1–Transfected 7721 Cells

Cell migration and invasion were performed using transwell with a polycarbonate filter and matrigel-coated polycarbonate filter, respectively, as detailed in Materials and Methods. No changes were observed in the cell migration and invasion of 7721 cells after transfection with pcDNA3 vector. However, the cell migration (Fig. 7A) and invasion through matrigel (Fig. 7B) of *nm23*-H1–transfected 7721 cells were decreased by 48% and 52%, respectively ($P < 0.05$).

Alterations in GnT-V Activity of 7721 Cells Transfected with Antisense GnT-V cDNA

To elucidate whether the above-mentioned alterations in cell adhesion, migration, and invasion of *nm23*-H1–transfected cells were the consequences of decreased GnT-V activity, the GnT-V activity was examined in 7721 cells transfected with antisense GnT-V cDNA (GnT-V-AS/7721). Compared with mock pcDNA3/7721 cells, the GnT-V activity in GnT-V-AS/7721 cells was decreased to 46.1% of the level in mock cells, as shown in Fig. 8.

Alterations in Cell Adhesion of 7721 Cells Transfected with Antisense GnT-V cDNA

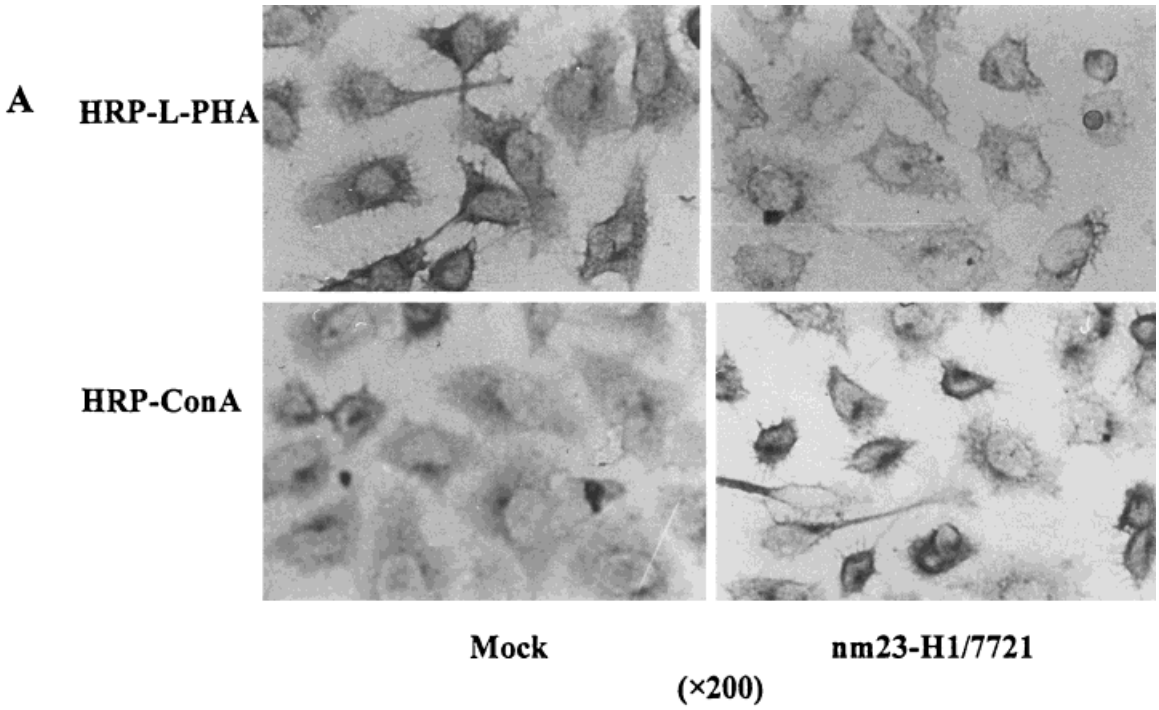
Similar to the *nm23*-H1–transfected 7721 cells, both the adhesion of mock-transfected and GnT-V-AS–transfected cells to Fn or Ln also showed obvious concentration-dependence with the coated Fn or Ln. In GnT-V-AS/7721 cells, the cell adhesions to Fn (>3 μg/ml) displayed a significant increase ($P < 0.05$ or $P < 0.01$) when compared with the mock 7721 cells (Fig. 9). This result was also similar to that of the *nm23*-H1–transfected cells. However, cell adhesion to Ln was increased in GnT-V-AS/7721 cells when the concentrations of Ln were more than 10 μg/ml. This finding was opposite to the decreased cell adhesion to Ln in *nm23*-H1/7721 cells.

Alterations in Cell Migration and Invasion of Antisense GnT-V cDNA Transfected 7721 Cells

The cell migration and invasion of 7721 cells after transfection with pcDNA3 vector were not changed, whereas the levels of both cell migration (Fig. 10A) and invasion through matrigel (Fig. 10B) were significantly decreased in GnT-V-AS–transfected 7721 cells, being 56.7% and 33.3% of the values of mock-transfected cells. These results were almost identical to those in the *nm23*-H1–transfected cells.

DISCUSSION

In our study, the expressions of GnT-V in *nm23*-H1– or *p16*–transfected 7721 hepatocarcinoma cells and A549 lung carcinoma cells were investigated. Our findings revealed that the over-expression of *nm23*-H1 not only down-regulated the activity of GnT-V, but also decreased the expression of GnT-V mRNA in both cell lines. These results revealed that the metastasis-suppressor gene, *nm23*-H1, regu-



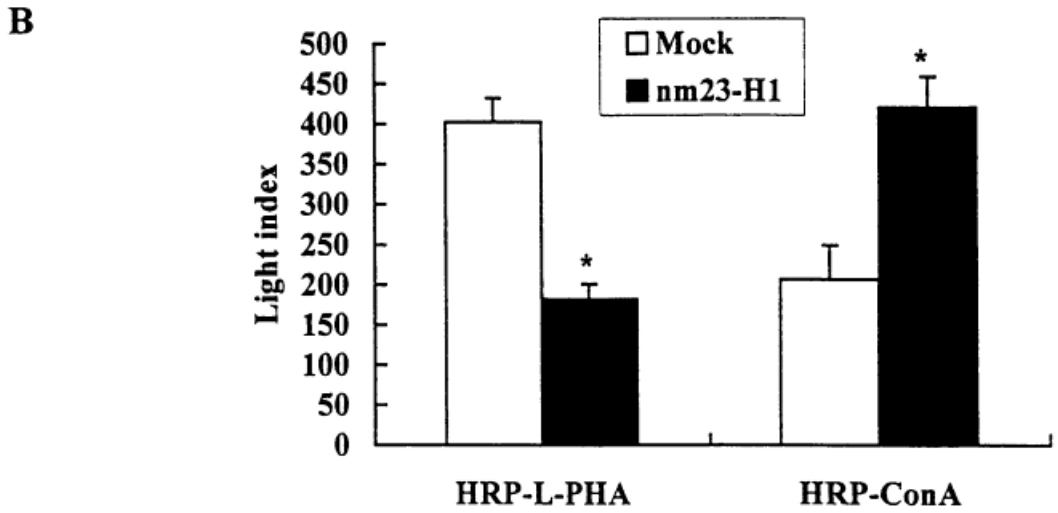
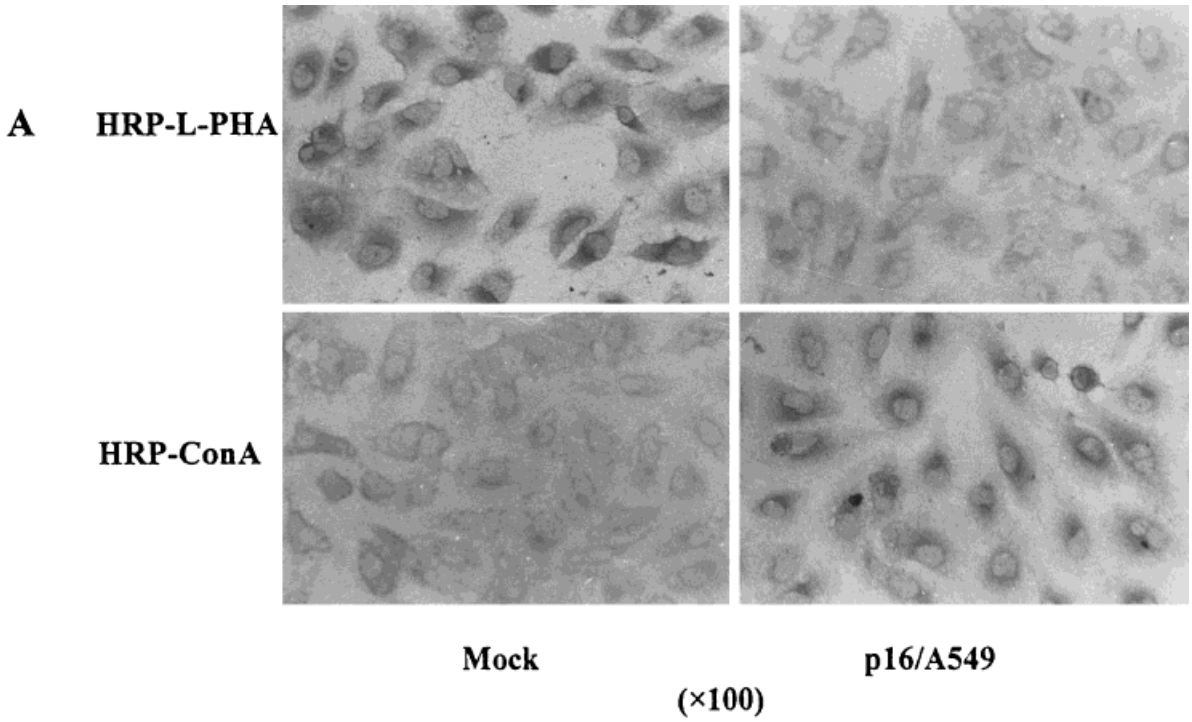


Fig. 4. HRP-lectin staining of cell N-glycans in *nm23*-H1/7721 cells. **A:** HRP-lectin staining. **B:** Image analysis. Mock, mock-transfected 7721 cells; nm23-H1/7721, 7721 cells transfected with pcDNA3/*nm23*-H1 (clone3). Cells were treated with HRP-L-PHA or HRP-ConA lectin followed by DAB taining as described in Materials and Methods. $^*P < 0.05$ compared with mock cells.



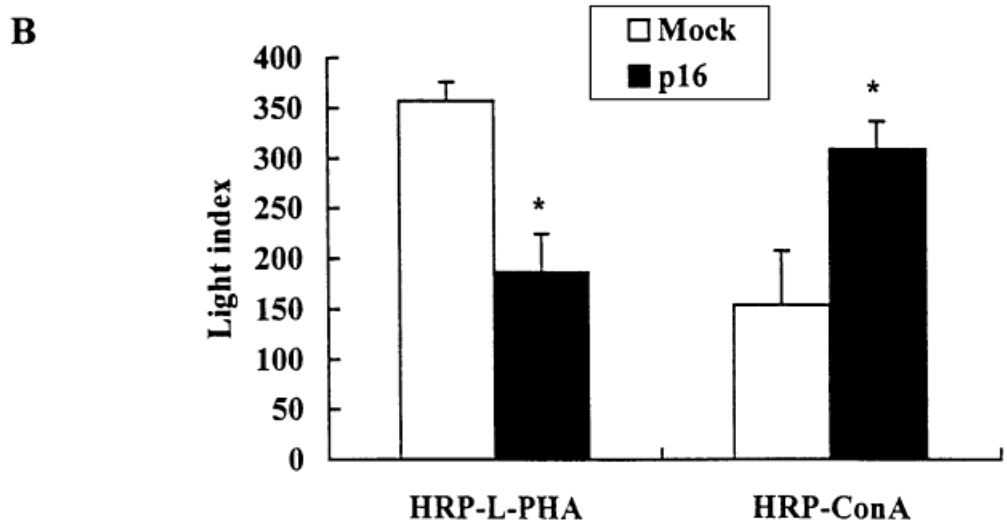
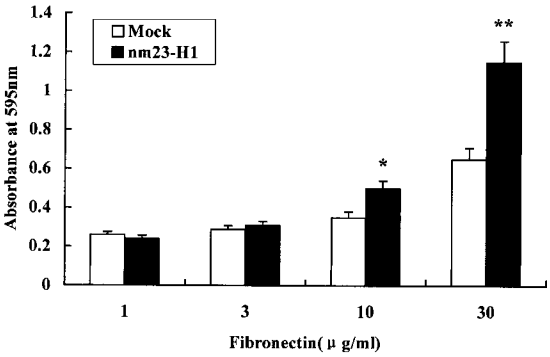


Fig. 5. HRP-lectin staining of cell N-glycans in *p16*/A549 cells. **A:** HRP-lectin staining. **B:** Image analysis. Mock, mock-transfected A549 cells; p16/A549, A549 cells transfected with pcDNA3/*p16* (clone 8). The experimental procedure was the same as described for Figure 4. $^*P < 0.05$ compared with mock cells.



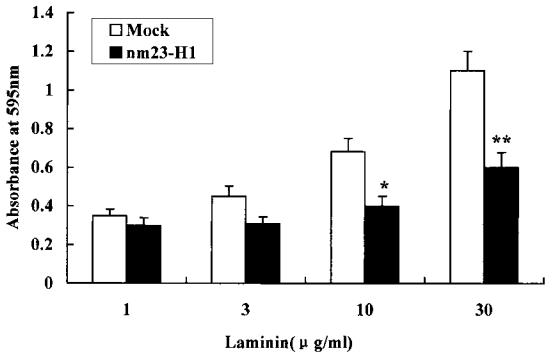
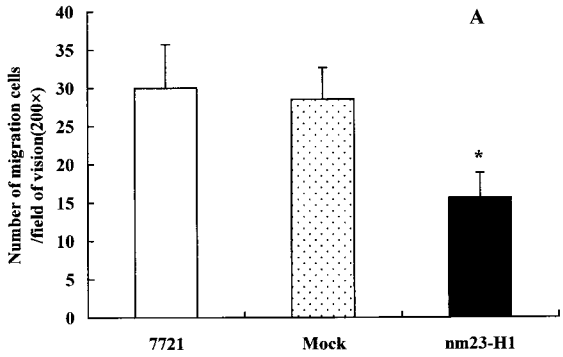


Fig. 6. Adhesions of 7721 cells to Fn and Ln after transfection with *nm23*-H1 cDNA. Mock, mock-transfected 7721 cells; nm23-H1, cells transfected with pcDNA3/*nm23*-H1 (clone 3). The procedure of the experiment was described in Materials and Methods. $*P < 0.05$ compared with mock cells; n = 3. $**P < 0.01$ compared with mock cells; n = 3.



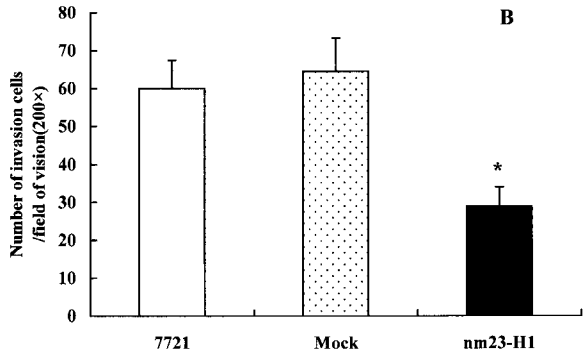


Fig. 7. Migration and invasion of 7721 cells after transfection with *nm23*-H1 cDNA. **A:** Migration. **B:** Invasion through matrigel. 7721, parental cells without any transfection; mock, mock-transfected 7721 cells; nm23-H1, cells transfected with pcDNA3/*nm23*-H1 (Clone 3). The experiments were performed using 0.5% FBS as described in Materials and Methods. $*P < 0.05$ compared with mock cells; n = 8.

lates GnT-V, a metastasis-associated enzyme. In contrast to *nm23*-H1, *p16* transfection decreased the GnT-V activity in A549 cells only, but not in 7721 cells, and showed no effect on the expression of GnT-V mRNA in either cell line. One of the reasons was probably owing to the obvious expression of *p16* in 7721 cells as evidenced by Western blotting performed in the Liver Institute of our university (data not shown). It confirmed the result reported by Biden et al. [1997] concerning the hepatocellular carcinoma from an Australian population. The high expression of *p16* in parental 7721 cells, therefore, might not show any additional response to the exogenous transfected *p16* cDNA on the expression of GnT-V mRNA and activity. The reason of high expression of *p16* in 7721 human hepatocarcinoma cell is unknown, but this cell line is known as α-fetal protein positive and hepatitis B virus negative with a medium potential of metastasis. However, the effectiveness of *p16* on GnT-V activity and ineffectiveness of *p16* on GnT-V mRNA in *p16*-deleted A549 cells might be due to the different mechanisms of *nm23*-H1 and *p16* on the regulation of GnT-V. GnT-V was regulated by *nm23*-H1 via the decrease of gene transcription and the protein synthesis of GnT-V; whereas the regulation of *p16* on GnT-V might be via a post-translational mechanism, such as indirect phosporylation and dephosphorylation. The latter is similar to the regulation of the GnT-V activity in cell cycle by CDKs [Guo et al., 2000a].

The down-regulation of GnT-V activity in *nm23*-H1/7721 and *p16*/A549 cells should result in the decreased content of β1,6 GlcNAc branch on the N-glycans of the cells. Our find-

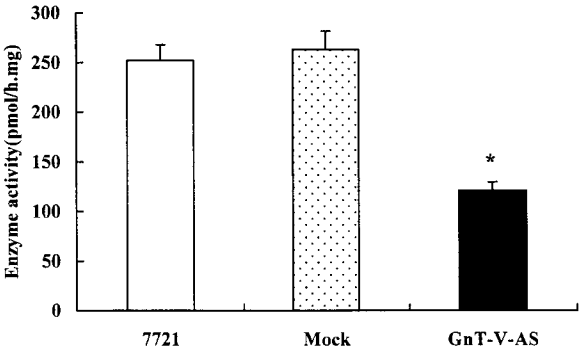
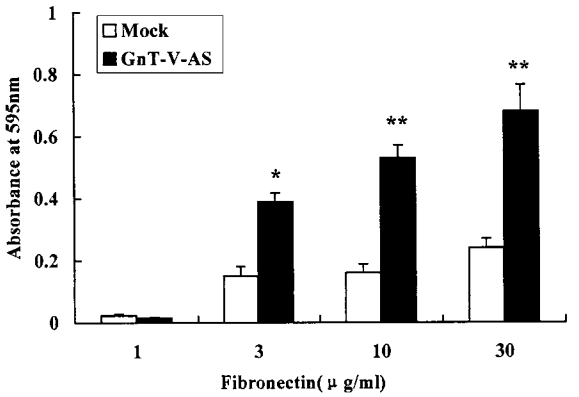


Fig. 8. Down-regulation of GnT-V activity in 7721 cells transfected with antisense GnT-V cDNA. 7721, parental cells without any transfection; mock, mock-transfected 7721 cells; GnT-V-AS, cells transfected with pcDNA3/antisense GnT-V cDNA. The procedure of the experiment was the same as described in Table I. $*P < 0.05$ compared with mock cells; n = 5.



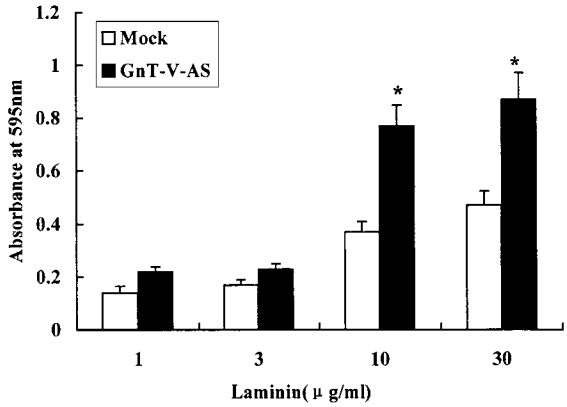


Fig. 9. Adhesions of 7721 cells to Fn and Ln after transfection with antisense GnT-V cDNA. Mock, mock-transfected 7721 cells; GnT-V-AS, cells transfected with pcDNA3/antisense GnT-V cDNA. The procedure of the experiment was the same as described in Figure 6. $*P < 0.05$ compared with mock cells; n = 3. $**P < 0.01$ compared with mock cells; n = 3.

ings showed that the staining intensities of cell glycoproteins, especially cell surface glycoproteins, in these two cell lines were decreased with glycoproteins HRP–L-PHA and increased with HRP–ConA. These findings indicated that the β1,6 GlcNAc branching structure of glycoprotein N-glycans was declined in the cells [Osawa and Tsuji, 1987], which was in agreement with the decrease of GnT-V activity.

It has been found in the present study that both *nm23*-H1 and *p16* may function as negative factors in the regulation of GnT-V. The down-regulation of GnT-V by *nm23*-H1 or *p16* was specific, since we found an up-regulation of GnT-V after transfection of a metastasis-promoting gene *H-ras*, or tumor-promoting gene, *v-sis* [Guo et al., 2000b]. Therefore, it is reasonable to infer that GnT-V activity might be down-regulated by metastasis-associated gene *nm23*-H1 and tumor-suppressor gene *p16*.

Hsu et al. [1997] reported that *nm23* and *p16* might be coregulated and their expressions correlated with the metastatic behavior in CH27 cells, a human lung squamous carcinoma cells line. *p16* was identified as a member of *nm23*/nucleoside diphosphate (NDP) kinase family based on its amino acid sequence homology, NDP kinase activity, and the degree of recognition by anti-human NDP kinase A antibody. This antibody also coimmunoprecipitated with Hsc70 chaperone and NDP kinase from human HepG2 cells. Therefore, *p16* may be a unique *nm23*/NDP kinase that functions as an accessory protein for cytosolic Hsc70 in eukaryotes [Leung and Hightower, 1997]. These indicated that there was a close link between *nm23*-H1 and *p16* in their roles on tumorigenesis and metastasis suppression.

Tumor metastasis is a complex process involving numerous sequential steps in which tumor cells must detach from the primary tumor, enter blood vessels, escape from the attack of the host immune system, and finally adhere to and invade the target organs. The metastasis-potential of transformed cells can be assayed in vivo by inoculation of the modified (substance treated or gene transfected) cells into nude mice or other immunopermissive animals to observe the number of metastatic foci in the lung. However, the ability of

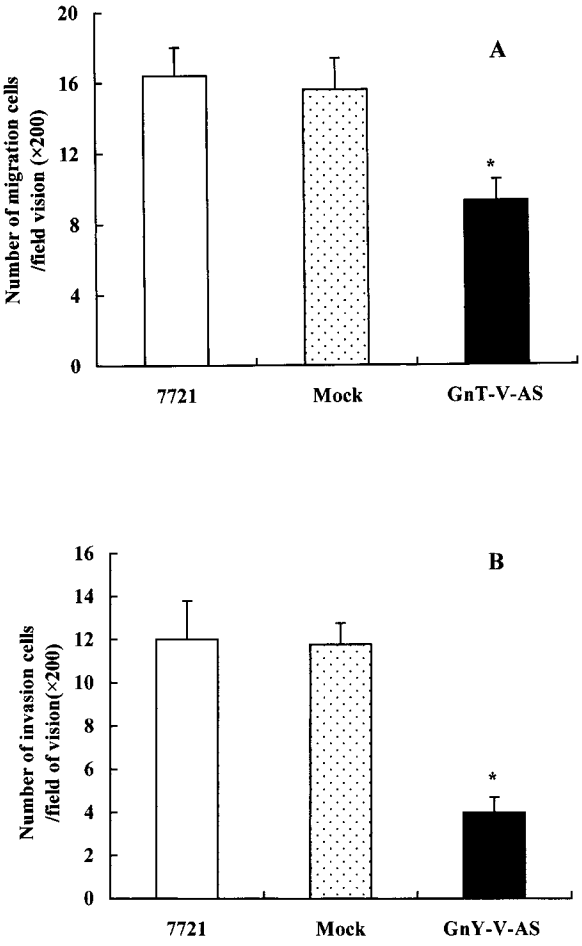


Fig. 10. Migration and invasion of 7721 cells after transfection with antisense GnT-V cDNA. **A:** Migration. **B:** Invasion through matrigel. 7721, parental cells without any transfection; mock, mock-transfected 7721 cells; GnT-V-AS, cells transfected with pcDNA3/antisense GnT-V cDNA. The experiments were performed using 0.5% FBS as described in Figure 7. $*P < 0.05$ compared with mock cells; n = 8.

cell adhesion, cell migration, and invasion are good indexes for the estimating of metastasis-potential ex vivo and are widely used in many laboratories. The results of these ex vivo experiments are always in accordance with the in vivo assays [Welch, 1997].

Our findings revealed that the decrease in GnT-V activity and β1,6 GlcNAc branch on the cell surface might contribute to the decreased metastasis-related phenotypes in 7721 cells. In addition, altered cell-cell and cell-extracellular matrix (including collagen, Fn, Ln, etc.) interactions and adhesion are also important in the acquisition of metastasis-related phenotypes, leading to the increase of migration and invasion abilities. Some integrins, especially the integrins bound to Fn or Ln, on the cell surface play major roles in the above process [Aznavoorian et al., 1993; Demetriou et al., 1995; Hakomori, 1996]. Their functions, in general, were highly controlled by N-glycosylation [Zheng et al., 1994]. In this investigation, it was found that the cell adhesion to Fn and Ln was also affected by the ectopically expressed *nm23*-H1 in 7721 cells, indicating that the decrease in the β1.6 GlcNAc branch on N-glycans by down-regulation of GnT-V activity may significantly affect the functions of integrins on the cell surface. One of the mechanisms of the altered adhesion of *nm23*-H1–transfected 7721 cells to Fn might result from the change of surface integrin expression, such as α5β1, or its glycan structure. On the other hand, the changes in interactions of cell-cell and cell-extracellular matrix may also affect the migration of tumor cells [Hakomori, 1996], as we found that over-expression of *nm23*-H1 markedly decreased the migration of 7721 cells. The invasion of 7721 cells through matrigel was also suppressed by the transfection of *nm23*-H1. It is likely to be due to the decrease of cell migration and the down-regulation of some proteases by *nm23*-H1, which destroy the membrane composition. These metastasis-related alterations were just opposite to those induced by over-expression of *H-ras* and *v-sis* in 7721 cells, as it was found in our laboratory [Guo et al., 2000b], further supporting that GnT-V might be involved in the metastasis of cancer.

To further elucidate whether the alterations in metastasis-related phenotype in *nm23*-H1 transfected cells resulted directly from the down-regulation of GnT-V activity, we constructed antisense GnT-V cDNA and transferred it into 7721 cells. The transfectant GnT-V-AS/7721 cells showed decrease of cell growth and acceleration of cell death in serum-free medium. An increase of susceptibility to apoptosis determined by Flow cytometry, cell fluorescent staining, and TUNEL method was observed in GnT-V-AS/7721 cells [Guo et al., 1999]. In the present study, we found a significant decrease in GnT-V activity after transfection of antisense GnT-V cDNA into 7721 cells. Cell adhesion to Fn or Ln was increased, whereas cell migration and invasion through matrigel were decreased in GnT-V-AS/7721 cells. These alterations were specific, and just opposite to the changes after the sense GnT-V

cDNA was transfected into the same 7721 cells, which showed decreased adhesion to Fn and Ln, and increased migration and invasion of the cells transfected with sense GnT-V cDNA (unpublished data). The phenotypic changes in the antisense GnT-V–transfected 7721 cells bore a close similarity to those of the 7721 cells transfected with *nm23*-H1, except the cell adhesion to Ln, which was decreased in *nm23*-H1/7721 cells but increased in GnT-V-AS/7721 cells. The difference between *nm23*-H1 and antisense GnT-V–transfected 7721 cells in adhesion to Ln revealed that the effects of two exogenous gene transfections on the biologic phenotypes of cells were not totally identical with each other. *nm23*-H1, as a tumor-related gene, might have a wider influence upon the cells than the GnT-V gene. These results strongly suggested that the induced changes of biologic phenotypes by the over-expression of *nm23*-H1 were at least partially mediated by the down-regulation of GnT-V. In other words, the down-regulation of GnT-V and its product may represent one of the mechanisms by which *nm23*-H1 suppresses the metastasis of cancers. The functions of GnT-V might be mediated by its product, β1,6 GlcNAc branch in N-linked glycans, as a structural inhibitory factor for cell adhesion to Fn and Ln, and as a promoting factor of cell migration and invasion. GnT-V is, therefore, closely related to cancer metastasis and its down-regulation is an important molecular mechanism of metastasis inhibition. However, the mechanisms of GnT-V action on the cell adhesion to Fn and Ln, as well as on cell migration and invasion, were not resolved and remain to be further studied.

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