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ORIGINAL PAPER

F. Liu · Y. Zhang · X.-Y. Zhang · H.-L. Chen

Transfection of the *nm23*-H1 gene into human hepatocarcinoma cell line inhibits the expression of sialyl Lewis X, α1,3 fucosyltransferase VII, and metastatic potential

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Abstract *Purpose*: The expressions of Lewis antigens, α1,3 fucosyltransferase (α1,3 FucT)-VII, and the metastatic potential of the 7721 human hepatocarcinoma cell line after the transfection of the cDNA of *nm23*-H1, a known metastasis-suppressive gene, were studied using mock cells as the control, which were transfected with the pcDNA3 vectors. *Methods*: Cell adhesion to human umbilical vein epithelial cells (HUVEC), chemotaxic cell migration through transwells, and invasion through matrigel were selected as the metastasis-related phenotypes to assess the metastatic potential at the cell level. *Results*: The results showed that the expression of SLe^x was high, while the expression of Le^x, $SDLe^x$, and SLe^a were very low on the surface of the mock cells. After transfection of *nm23*-H1, the expressions of SLe^x, α1,3 FucT-VII, and the cell adhesion to HUVEC, as well as cell migration and invasion were simultaneously decreased in all three clones of *nm23*-H1-transfected cells. Among different clones, the decreased expressions of SLe^x and α1,3 FucT-VII were roughly correlated to each other, and also, in general, proportional to the ability of cell adhesion to HUVEC, cell migration, and invasion. The expressions of these metastasis-related phenotypes were lowest in clone 3 and highest in clone 4. Only the specific monoclonal antibody to SLe^x (KM93) significantly abolished the cell adhesion, migration, and invasion, while other monoclonal antibodies against $SDLe^x$ or Le^x and SLe^a only slightly inhibited or entirely failed to inhibit the above-mentioned phenotypes. However, the rate of cell growth was not changed after the transfection of *nm23*-H1, and the ability of colony formation on the soft agar was only decreased in one clone. *Conclusions*: These findings reveal that the down-regulation of α1,3 FucT-VII and its product, SLe^x, is one of the mechanisms to explain the metastasis-suppressive effect of the *nm23*-H1 gene.

Keywords Human hepatocarcinoma cells · *nm23*-H1 metastasis suppressive gene · Metastatic potential · Lewis antigens · α1,3 Fucosyltransferase

Abbreviations SA sialyl · Gal galactose · GlcNAc N-acetylglucosamine · Man mannose · Fuc fucose · Le^x Lewis X [Galß1,4 (Fuc α1,3) GlcNAc-] · SLe^x sialyl Lewis X [SAα2,3Galß1,4 (Fuc α1,3) GlcNAc-] · SLe^a sialyl Lewis A [SAα2,3Galß1,3 (Fucα1,4) GlcNAc-] · $SDLe^x$ sialyl dimeric (difucosyl) Lewis X [SAα2,3Galß1,4 (Fuc α1,3) GlcNAcß1,3Galß1,4 (Fucα1,3) GlcNAcß1,3-] · FucT fucosyltransferase · HRP horseradish peroxidase · FITC fluorescein isothiocyanate · GAPDH 3-phosphoglyceraldehyde dehydrogenase · HUVEC human umbilical vein endothelial cells · TNF-α tumor necrosis factor-α · ECL enhanced chemiluminescence · FCS fetal calf serum · BSA bovine serum albumin · EDTA ethylene diamine tetraacetate · PBS phosphate buffered saline · HPF high performance field

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F. Liu · Y. Zhang · X.-Y. Zhang · H.-L. Chen (✉)
Key Laboratory of Glycoconjugate Research,
Ministry of Health, Department of Biochemistry,
Fu-Dan University Medical Center,
Shanghai 200032, China
E-mail: hlchen@shmu.edu.cn
Tel.: +86-21-64041900/ext. 2223
Fax: +86-21-64039987

Introduction

Nm23 is a gene family related to the non- or low metastatic behavior of cancers, which was first characterized by Steeg et al. in 1988 (Steeg et al. 1988). The gene is located on chromosome 17q21, which codes an 18.5 kDa protein containing 166 amino acids with nucleoside diphosphate kinase and protein-histidine kinase activities, as well as the activity of serine autophosphorylation (De La Rosa et al. 1995). The *nm23*-H1 gene was discovered as the first member of the gene family

(Gilles et al. 1991), and demonstrated to have anti-metastatic properties in many human and animal cancer models. On the other hand, *nm23*-H1 was less expressed in numerous types of cell lines with highly metastatic potential (Steeg et al. 1988; Baba et al. 1995; Fukuda et al. 1996; Kantor et al. 1993). The levels of *nm23*-H1 mRNA or protein have 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).

In our laboratory, it was reported that transfection of the cDNA of *nm23*-H1 into the 7721 human hepatocarcinoma cell line resulted in the down-regulation of a glycosyltransferase, N-acetylglucosaminyltransferase V (GnT-V), and its product, GlcNAcβ1,6Manα1,6-branching structure in asparagine-linked (N-) glycans, on the cell surface (Guo et al. 2000a). This GlcNAc β1,6-branch was well documented as a cancer metastasis-related structure (Dennis et al. 1987; Dennis and Laferte 1987, 1989; Fernandes et al. 1991; Guo et al. 2000b). The down-regulation of 1,6 β-GlcNAc branch on surface N-glycans is one of the mechanisms for the suppression of cancer metastasis by *nm23*-H1 (Guo et al. 2000a).

We considered that other glycan structures might also participate in the metastatic process. It was reported that sialylated and fucosylated N-acetyllactosamine structures (named Lewis antigens or Lewis epitopes), such as SLe^x and SLe^a, are involved in the process of metastasis (Hakomori 1996). Lewis antigens are located mainly on O-glycans and glycolipids. These oligosaccharide antigens serve as the ligands of E- or P-selectin expressed on the surface of vascular endothelial cells, and mediate the adhesion of malignant cells to vascular endothelium by combining with E-/P-selectin, which is the initial step of malignant cells to penetrate through the vessels before metastasis (Varki 1994; Takeda et al. 1993; Hakomori 1996). Clinical statistics and experimental results also indicate that patients with cancer cells strongly expressing SLe^x or SLe^a have a significantly higher risk to develop hematogenous metastasis, and these factors eventually affect the overall prognosis of the patients (Nakamori et al. 1993; Jorgensen et al. 1995; Ogawa et al. 1994). Therefore, sialyl Lewis antigen is another kind of metastasis-related glycan on the cell structure.

The synthesis of SLe^x and SLe^a in cancer cells is regulated by a set of glycosyltransferases. The last step of their synthesis, fucosylation, is catalyzed by α1,3 fucosyltransferases (α1,3 FucTs). Six subtypes of human α1,3 FucTs have been cloned. Four of them (α1,3 FucT-III, V, VI, and VII) efficiently fucosylate sialylated acceptors (Kukowska-Latallo et al. 1990; Weston et al. 1992a, 1992b; Sasaki et al. 1994), while α1,3 FucT-IV, and α1,3 FucT-IX prefer neutral acceptors (Lowe et al. 1991; Kudo et al. 1998). α1,3/1,4 FucT-III is the only α FucT having both α1,3 and α1,4 fucosylation activities which can synthesis both α1,3 fucosyl containing Le^x or SLe^x, and α1,4 fucosyl containing SLe^a (Kukowska-Latallo et al. 1990), while α1,3 FucT-VII only catalyzes the synthesis of SLe^x (Sasaki et al. 1994). There are many accounts in the literature reporting that the expressions of α1,3 FucTs are positively related to the metastasis potency of cancers, and negatively related to the prognosis of the patients (Ito et al. 1997; Ogawa et al. 1997).

The 7721 cell line expresses a moderate amount of *nm23*-H1 (Liu et al. 2000). Three clones (Clone 4, 3, and 2) of *nm23*-H1 cDNA-transfected 7721 cells were established in our laboratory. These positive clones stably expressed different levels of *nm23*-H1 mRNA and protein, which were highest in clone 3 and lowest in clone 4 (Guo et al. 2000a). In the present investigation, the changes in the expressions of SLe^x and α1,3 FucT-VII of these clones and their relation to the metastatic potential of 7721 cells, including the cell adhesion to human umbilical vein endothelial cells (HUVEC), chemotaxic cell migration and invasion, were studied. The cells used as control were mock-transfected with the pcDNA3 vector of *nm23*-H1 cDNA.

Materials and methods

The 7721 hepatocarcinoma cell line was obtained from the Institute of Cell Biology, Academic Sinica. HUVEC (2–3 passages after isolation) was provided by the Department of Anatomy in our University. RPMI 1640 and DMEM medium, TRIzol and matrigel were purchased from Gibco/BRL. Plasmid containing cDNA of α1,3 FucT-VII and monoclonal antibody (mAb) CA19–9 (anti-SLe^a) were a gift from Dr. Narimatsu at Soka University of Japan. KM93 (anti-SLe^x mAb) and FH6 (anti-$SDLe^x$ mAb) were kindly provided by Dr. Ando at Tokyo Metropolitan Institute of Gerontology and Dr. Hakomori at University of Washington respectively. CD15 (anti-Le^x mAb) and HRP-labeled antibodies to mouse- and rabbit IgG were purchased from Dako. The cDNA of 3-phosphoglyceraldehyde dehydrogenase (GAPDH) was a gift from Dr. Shimizu at Tokushima University. TNF-α, FITC-conjugated goat antibodies against mouse IgM and IgG were purchased from Sigma. A random primer labeling kit was from Promega. Hybond-N+ nylon membrane, a ECL+plus Western blotting detection system, and [α-^{32}P]-dATP were from Amersham. Insert (transwell) and 24-well cell culture plates were obtained from NUNC Company. Other reagents were commercially available in China.

Cell culture

Cells were cultured at 37 °C, 5% CO_2 in RPMI-1640 medium containing 10% FCS, penicillin, and streptomycin as previously described by our laboratory (Guo et al. 2000a, Liu et al. 2001a).

Detection of Lewis antigens with flow cytometry

The cells (1×10^6) were detached with 2 mmol/l EDTA, washed and re-suspended in PBS containing 1% BSA, then incubated with different monoclonal antibodies of Lewis antigens (1:50 dilution) for 30 min at 4 °C. After two washes, the cells were incubated for 45 min at 4 °C with 1:200 dilution of FITC-conjugated goat antibodies against mouse IgM (for CD15, KM93, and FH6) or IgG (for CA19–9). Then the cells were subjected to fluorescence analysis performed on a FACStar Plus model 50H flow-cytometric apparatus after suitable washing. Fluorescence activated cell spectra were drawn automatically, and the left- or right-shift of the curve or its peak indicated the decrease or increase of the mean fluores-

cence intensity, respectively, as shown by the "positive" bar in the figures. Quantitative data were expressed as the mean fluorescence intensities of the positive cells in three independent and repeatable experiments.

Total RNA extraction and Northern blot analysis of α-1,3 FucT-VII

Total RNA was extracted from cells using TRIzol according to the protocol provided by Promega. Northern blot analysis was carried out according to the method described in our previous paper (Liu et al. 2000, 2001a). In brief, total RNA (30 μg) was separated by formaldehyde denatured electrophoresis, then transferred to Hybond-N+ nylon membrane, and pre-hybridized for 4–6 h at 65 °C in 0.2 mol/l sodium phosphate buffer (pH7.4)/ 1 mmol/l EDTA/ 1%BSA/7%SDS/ 15% formamide. Hybridization was performed at 65 °C for 16–20 h in the same hybridization solution containing [α-^{32}P] labeled probe of α-1,3 FucT-VII. The hybridized membrane was washed 3–5 times with 40 mmol/l sodium phosphate buffer (pH 7.4)·1%SDS·1 mmol/l EDTA for 30 min at 65 °C, followed by autoradiography. The intensities of the mRNA bands were quantified by densitometric scanning and compared with the intensities of GAPDH bands on the same membrane, which was re-hybridized by GAPDH probe. The magnitude of expression was indicated as the ratio of the intensity (absorbance units) of the αFucT-VII band to the intensity of the GAPDH band.

The probe used for detection of α1,3 FucT-VII was the full-length cDNA of α1,3 FuT-VII. The GAPDH mRNA was determined with GAPDH cDNA for intrinsic control. These probes were labeled with [α ^{32}P]-dATP using random primer labeling kit from Promega according to the instruction described in the manual.

Assay of cell adhesion to HUVEC

Assay of cell adhesion to HUVEC was carried out according to the method reported by Takada et al. (Takada et al. 1993) with minor modification. Chiefly, HUVEC were coated on a 96-well plate and stimulated with 200 ng/ml TNF-α for 4 h. Then 100 μl of the cultured cells (1×10^5/well) were added to the wells of the plate and further incubated for 30 min at 4 °C. After washing with PBS five times, the cells were fixed with glutaradehyde and stained with crystal violet. The numbers of cells adhered to HUVEC were counted in 8 HPFs (×200). The data were expressed by the mean value of adhered cells per HPF in triplicate with two independent experiments.

Determination of chemotaxic cell migration and invasion

The chemotaxic cell migration assay was performed using 24-well transwell units with polycarbonate filters of 8 μm pore size according to the method of Yu et al. (Yu et al. 1994) as described by Liu et al. (Liu et al. 2000). Each lower compartment of the transwell contained 600 μl of 0.5% FCS in DMEM as chemoattractant, or 0.5% BSA as negative control. 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 the cells that had migrated to the lower side of the polycarbonate filter were counted in 8 HPFs (×200). The data were expressed by the mean value of cells per HPF in triplicate with two independent experiments.

To assay chemotaxic cell invasion, the method was the same as in the chemotaxic cell migration assay, except that the upper side of polycarbonate filter was coated with 0.1 ml (20 μg/filter) of matrigel in cold DMEM to form a continuous thin layer (Yu et al. 1994). Cells (1×10^5) in 0.1 ml were added, and the incubation time was prolonged to 36 h. Cells were stained and counted as described above, and the number of cells that invaded the lower side of the filter was a measure of the invasive activity of the cells.

Inhibition of cell adhesion, migration, and invasion with monoclonal antibody

For inhibition of cell adhesion, migration, and invasion, the 7721 cells were pre-incubated with 10 ng/ml different monoclonal antibodies (CD15, KM93, FH6 or CA19–9) for 30 min at 4 °C. Subsequently, the cells were added to the coated wells of the culture plate for adhesion assay, or to the transwells for migration or invasion assay.

Determination of cell growth

The harvested cells were washed with PBS, and the viable cells were counted every day in triplicate after staining with trypan blue.

Assay of colony formation on soft agar

The wells of culture plate were coated with 0.8% agar in RPMI1640 to form a layer of gel, and 5×10^3 cells suspended in 0.3% agar gel/RPMI1640 were added onto the wells. The plates were incubated at 37 °C under 5% CO_2 for 2 weeks, then the cell colonies (> 50 cells) were counted.

Results

Expression of Lewis antigens on the surface of 7721 cell mock-transfected with pcDNA3 vector

Using monoclonal antibodies, FITC-labeled second antibodies, and flow cytometric methods, the expressions of four Lewis antigens on the surface of 7721 cells mock-transfected with pcDNA3 vector were determined; the fluorescence-activated cell spectra (FACS) are shown in Fig. 1. It was found that only SLe^x was significantly expressed on the mock cells (the peak of its FACS was obviously shifted to the right), when compared with the "(–) control" sample without the addition of the first antibody, while the other three Lewis antigens, Le^x, $SDLe^x$, and SLe^a expressed very little. These results were in accordance with the findings on the parental (quiescent) 7721 cells reported in our previous paper (Liu et al. 2001a).

Decrease of SLe^x expression on the cell surface after transfection with *nm23*-H1 cDNA

As shown in Fig. 2a and b, the expression of SLe^x on the cell surface was down-regulated after the transfection of *nm23*-H1 cDNA, as compared with the mock cells. Among the three selected *nm23*-H1 positive clones, SLe^x on clone 3 and clone 2 decreased significantly ($P < 0.05$), but that on clone 4 decreased slightly with no statistical significance. The down-regulation of the expression of the other three Lewis antigens could not be detected owing to their low level of expression.

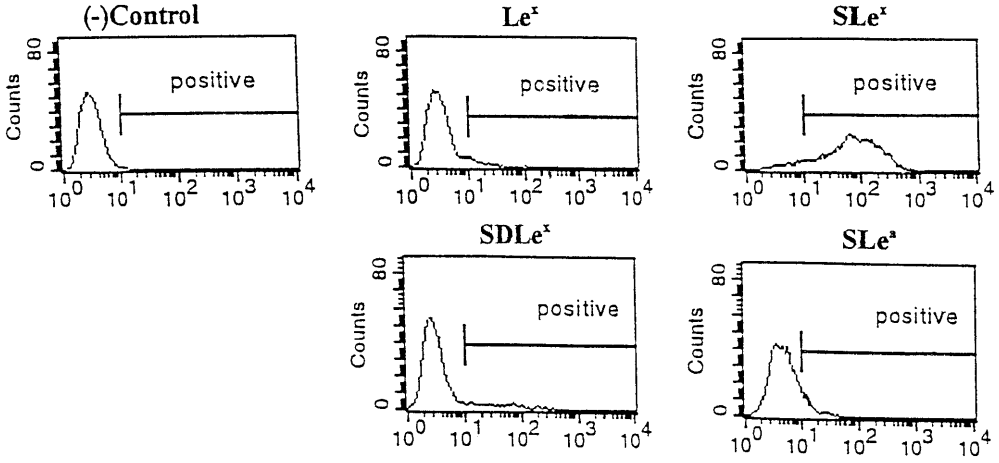


Fig. 1. Flow-cytometric analysis of the expressions of Lewis antigens on the surface of mock-transfected 7721 cells. (–) Control: background of fluorescence, the first antibody (mAb) was omitted in the assay; Le^x: Lewis X, detected with the monoclonal antibody CD15; SLe^x: sialyl Lewis X, detected with the monoclonal antibody KM93; $SDLe^x$: sialyl dimeric Lewis X, detected with the monoclonal antibody KH6; SLe^a: sialyl Lewis A, detected with the monoclonal antibody CA19–9. The procedure of the experiment is described in "Methods"

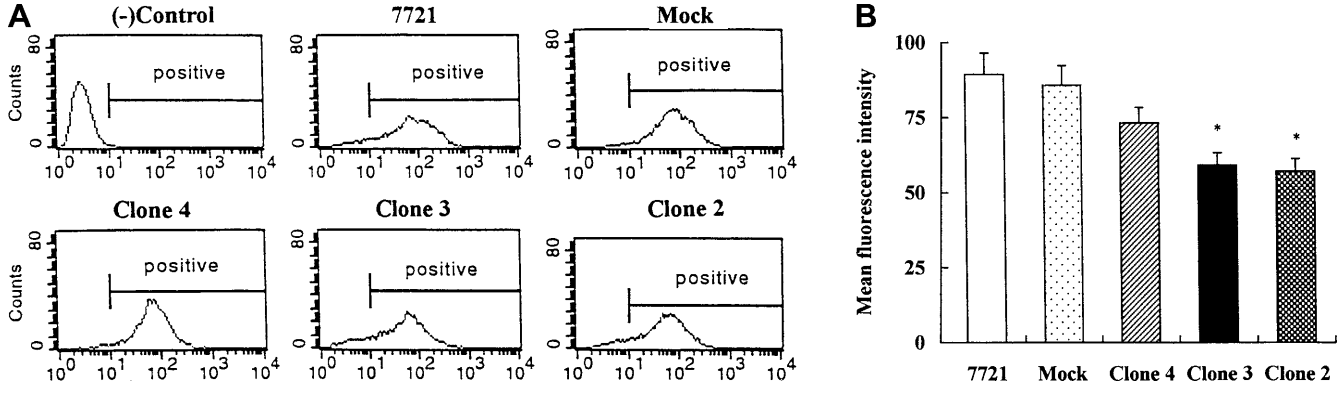
Decrease of the mRNA of α1,3 FucT-VII after transfection with *nm23*-H1 cDNA

SLe^x can be synthesized by αFucT-III, V, VI, and VII (Narimatsu 1998). We have found with Northern blot that the expression of α1,3 FucT-III and VI was very low in 7721 cells (Liu et al. 2001a; 2001b); the gene of α1,3 FucT- was reported to be silent in many tissues (Narimatsu 1998). We also found that the expression of α1,3 FucT-VII, but not α1,3/1,4 FucT-III and α1,3 FucT-VI, was increased after the transfection of the oncogene, *c-erb*B2/*neu* (Liu et al. 2001a). Therefore, α1,3 FucT-VII may be the main enzyme responsible for the synthesis of SLe^x in 7721 cells. α1,3 FucT-VII only shares 42–47% identity in amino acid sequences with αFucT-III/V/VI and α1,3 FucT-IV (Sasaki 1994), therefore, its cDNA probe is specific, and does not cross-hybridize with the mRNA of other α FuTs. As indicated in Fig. 3a, parental and mock-transfected cells expressed a moderate amount of α1,3 FucT-VII. The fragment of α1,3 FucT-VII mRNA transcript was about 2.3 kb. Its expression was decreased after the transfection of *nm23*-H1, and the magnitude of reduction was clone 3 > clone 2 > clone 4 (Fig. 3b), which was, in general, compatible with the expressions of SLe^x on these three clones. The decrease in clone 3 and 2 were statistically significant ($P < 0.05$) when compared with the mock cells. Using reverse transcription-polymerase chain reaction (RT-PCR), our laboratory also found that the transcript of α1,3 FucT-VII was higher than that of α1,3 FucT-VI (unpublished data).

Fig. 2a,b. Flow-cytometric analysis of SLe^x expression on the surface of parental, mock, and *nm23*-H1-transfected 7721 cell. **a** Fluorescence-activated cell spectra (FACS) for SLe^x; **b** Relative expression of SLe^x in different clones of cells. (–) Control: background of fluorescence, the first antibody (mAb) was omitted in the assay; 7721: parental cells without any transfection; mock: 7721 cells transfected with pcDNA3 vectors; clones 4, 3, 2: three clones of 7721 cells transfected with *nm23*-H1 cDNA. * $P < 0.05$; compared with the mock cells ($n = 3$). The procedure of the experiment is described in "Methods"

Decrease of cell adhesion to HUVEC after transfection with *nm23*-H1 cDNA

To mimic the sialyl Lewis antigen/selectin interaction in vivo, TNF-α was used for stimulating the expression of E/P-selectin on the surface of HUVEC before the determination of cell adhesion to coated HUVEC. It was



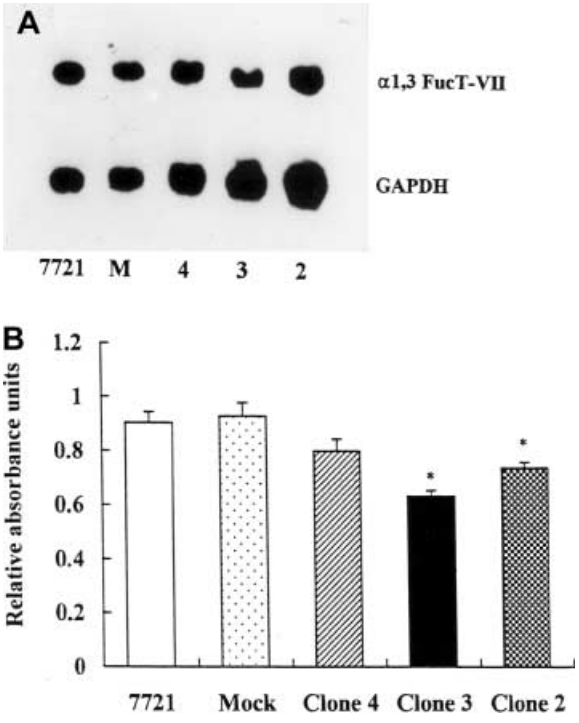


Fig. 3a,b. Expression of α1,3 FucT-VII mRNA in paretal, mock, and *nm23*-H1-transfected 7721 cells. **a** Northern blot profiles of α1,3 FucT-VII mRNA with α1,3 FucT-VII full-length cDNA as probe (an example of three experiments); **b** Densitometric quantification of αFuT-VII mRNA. 7721: parental 7721 cells without any transfection; M or mock: 7721 cells transfected with pcDNA3 vectors; clones 4, 3, 2: three clones of 7721 cells transfected with *nm23*-H1 cDNA. * $P<0.05$; compared with the mock cells ($n=3$). The procedure of the experiment is described in the "Methods"

observed that the adhesion of *nm23*-H1 transfected 7721 cells to HUVEC was reduced when compared with the parental or mock cells (Fig. 4). The degree of the reduction was most obvious in clone 3 ($P<0.01$), moderate in clone 2 ($P<0.05$), and not significant in clone 4, which was compatible with the order of the reduction of α1,3 FucT-VII expression.

Decrease of cell migration and invasion after transfection with *nm23*-H1 cDNA

The abilities of chemotaxic cell migration and invasion are important in the metastasis of malignant cells. Therefore, these abilities were examined in the *nm23*-H1transfected 7721 cells. Transfection of *nm23*-H1 into 7721 cells markedly decreased cell migration (Fig. 5a) and invasion (Fig. 5b). The number of migrated and invaded cells for clone 3 was the least ($P<0.01$), clone 2 was moderate ($P<0.05$), and clone 4 was the most ($P<0.05$). Therefore, the abilities of chemotaxic cell migration and invasion were correlated to the expressions of SLe^x and α1,3 FucT-VII, as well as the cell adhesion to HUVEC.

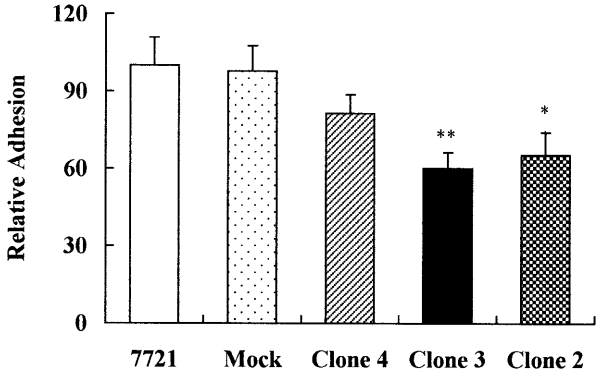


Fig. 4. Adhesion to HUVEC of parental, mock, and *nm23*-H1-transfected 7721 cells. 7721: parental cells without any transfection; mock: 7721 cells transfected with pcDNA3 vectors; clones 4, 3, 2: three clones of 7721 cells transfected with *nm23*-H1 cDNA. ** $P<0.01$,* $P<0.05$ compared with the mock cells ($n=6$). The procedure of the experiment in described is "Methods". HUVEC were pre-stimulated with 200 ng/ml TNF-α

Abolition of cell adhesion to HUVEC, cell migration, and invasion by antibodies of Lewis antigens

When different monoclonal antibodies against Lewis antigens were added to block the corresponding Lewis antigens on the surface of parental 7721 cells, it was found that only KM93 (anti-SLe^x mAb) showed significant inhibition on the cell adhesion to HUVEC ($P<0.01$). FH6 (anti-$SDLe^x$ mAb) slightly, but not significantly ($P>0.05$), suppressed the adhesion. In contrast, other antibodies (CD15 and CA-19–9) did not show any obvious blocking effects (Fig. 6a). Therefore, only KM93 and FH6 were chosen to further test their effects on cell migration and invasion. As shown in Fig. 6b, KM93 also obviously ($P<0.01$) and FH6 slightly ($P>0.05$) abolished these two biological activities of the cells. These findings indicate that sialyl Lewis antigens, especially SLe^x, plays a critical role in the cell adhesion to HUVEC, cell migration and invasion.

Cell growth and colony formation before and after the transfection of *nm23*-H1

After the 7721 cells were transfected with the cDNA of *nm23*-H1, cell growth was not altered (Fig. 7a). In addition, the ability of colonies to form on soft agar changed a little. Only the decrease of clone 3 was statistically significant ($P<0.05$) (Fig. 7b).

Discussion

The metastatic potential of malignant cells is better assayed in vivo by inoculation of the malignant cells into nude mice, followed by counting the number of metastatic foci in the lung. However, metastatic potential can be also assayed by means of a variety of cell-level techniques, such as cell adhesion, migration, invasion, and

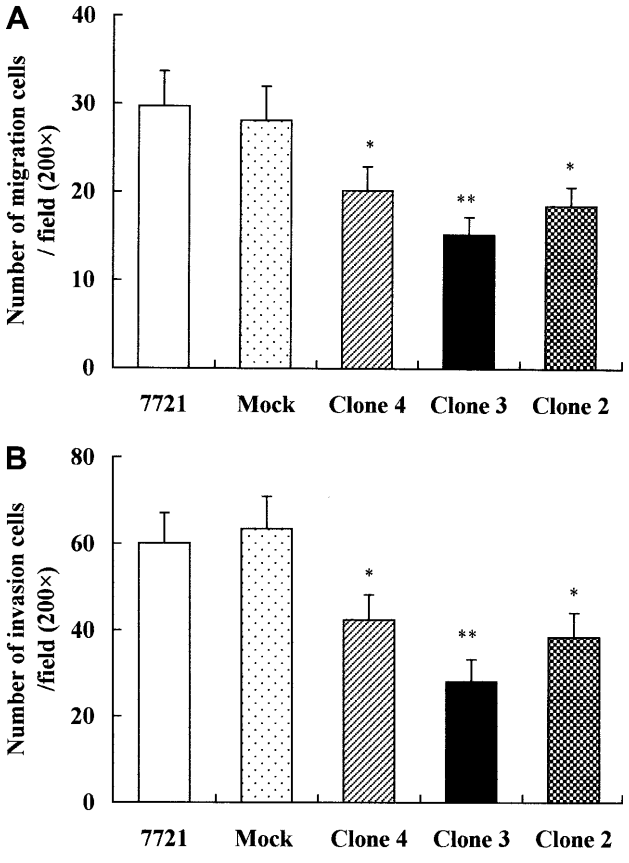


Fig. 5a,b. Migration and invasion of parental, mock, and *nm23*-H1-transfected 7721 cells. **a** Chemotaxic cell migration; **b** Chemotaxic cell invasion. 7721: parental cells without any transfection; mock: 7721 cells transfected with pcDNA3 vectors; clones 4, 3, 2: three clones of 7721 cells transfected with *nm23*-H1 cDNA. ** $P<0.01$,* $P<0.05$ compared with the mock cells ($n=6$). The procedure of the experiment is described in "Methods"

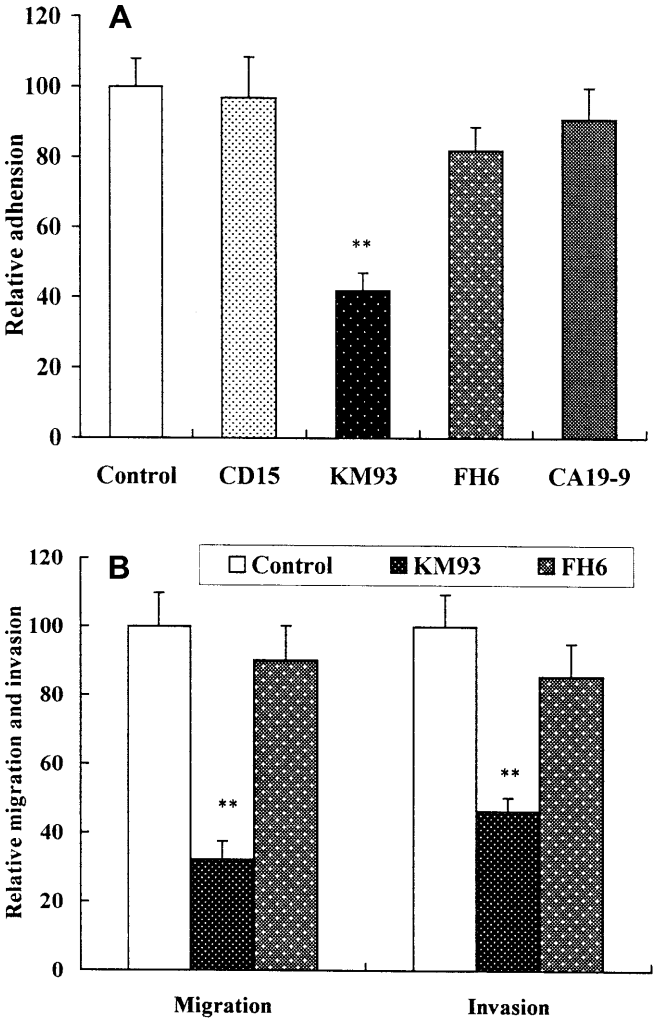


Fig. 6a,b. Effect of monoclonal antibodies on cell adhesion to HUVEC, cell migration, and invasion. **a** Inhibition of monoclonal antibodies on cell adhesion to HUVEC; **b** Inhibition of monoclonal antibodies on chemotaxic cell migration and invasion. Control: cells not treated with any antibodies; CD15: mAb to Le^x; KM93: mAb to SLe^x; FH6: mAb to $SDLe^x$; CA-19–9: mAb to SLe^a. The procedures of the experiments were the same as described under Fig. 4 and Fig. 5, except that the cells were pre-treated with different monoclonal antibodies against different Lewis antigens (10 ng/ml) for 30 min at 4 °C. ** $P<0.01$ compared with the "control" cells ($n=3$)

angiogenesis (Welch 1997), and these assays have been widely used in many laboratories. Our group has observed that cell adhesion to immobilized laminin was decreased in *nm23*-H1-transfected 7721 cells as compared with the mock-transfected cells (Guo et al. 2000a). In this study, we found that cell adhesion to TNF-α stimulated HUVEC, chemotaxic cell migration, and invasion were also reduced in *nm23*-H1 transfected 7721 cells. These findings indicate that the reduction of cell adhesion to laminin and HUVEC, as well as the cell migration and invasion are good indices for the assessment of metastatic potential at the cell level.

The results of this investigation indicate that the inhibition of *nm23*-H1 on the metastasis-related phenotypes of 7721 cells is at least partially mediated by the down-regulation of α1,3 FucT-VII and it product, SLe^x. This is evidenced by the following findings: (1) the decrease of cell adhesion to HUVEC, cell migration, and invasion were inversely correlated to the expression of *nm23*-H1 mRNA and protein in clones 3, 4, and 2. For example, clone 3 expressed the highest mRNA and protein of *nm23*-H1 (about two and four times as much, respectively, as those in the mock cells [Guo et al. 2000a]) and obtained the lowest metastatic-related phenotypes (this study), while clone 4 expressed the lowest *nm23*-H1 (only 30% and 50%, respectively, higher than those in the mock cells), but showed the highest metastatic potential; (2) in different clones of *nm23*-H1 transfected 7721 cells, the reduction of cell adhesion to HUVEC, cell migration, and invasion (Fig. 4 and Fig. 5) were generally proportional to the decrease of SLe^x and α1,3 FucT-VII expression (Fig. 2 and Fig. 3); (3) cell adhesion to HUVEC, as well as cell migration and invasion were significantly abolished only by the specific monoclonal antibody of SLe^x, KM93, suggesting that SLe^x is the most important Lewis antigen responsible for the above metastasis-related phenotypes in

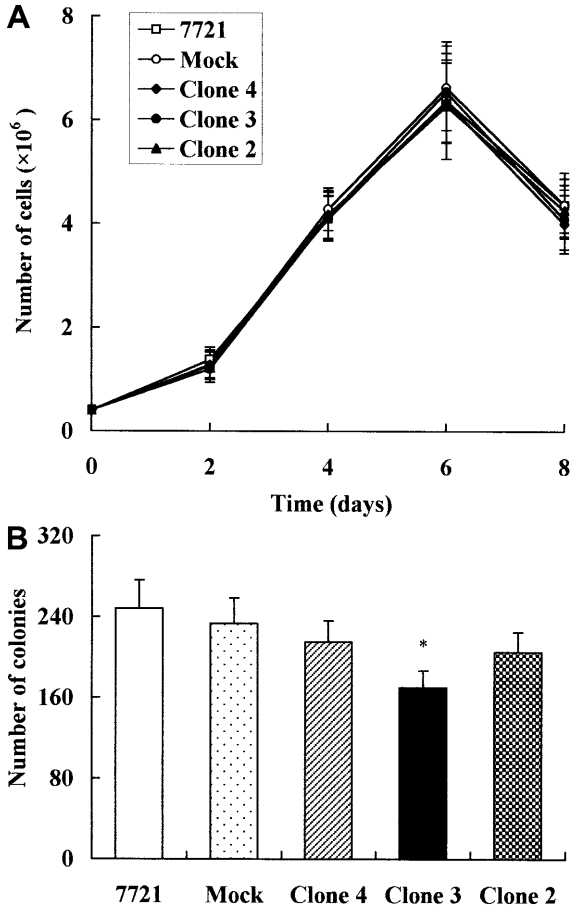


Fig. 7a,b. Cell growth and colony formation of parental, mock, and *nm23*-H1 transfected 7721 cells. **a** Growth curve of different cells; **b** Colony formation of different cells on soft agar 7721: parental cells without any transfection; mock: 7721 cells transfected with pcDNA3 vectors; clones 4, 3, 2: three clones of 7721-cells transfected with *nm23*-H1 cDNA. The procedure of the experiment is described in "Methods"

7721 cells: (4) the expression of α1,3/1,4 FucT-III, α1,3 FucT-VI, and their corresponding products, SLe^a and $SDLe^x$, were rather low in 7721 cells (Liu et al. 2001a, 2001b), but that of α1,3 FucT-VII and its product, SLe^x, were more expressed, indicating that α1,3 FucT-VII is the most likely enzyme in the synthesis of SLe^x in 7721 cells. The expression of α1,3 FucT-VII was down-regulated after transfection of *nm23*-H1 and was not very obvious (maybe due to the sufficient expression of *nm23*-H1 in the parental 7721 cells), but this result was repeatable and statistically significant in clones 3 and 2. However, the contribution of α1,3/1,4 FucT-III, and α1,3 FucT-VI – especially the latter – in the synthesis of SLe^x cannot be ruled out, as it was reported that α1,3 FucT-VI exhibited the strongest relative activity for SLe^x synthesis, almost 6.4-fold that of α1,3/1,4 FucT-III, and 1.5-fold that of α1,3 FucT-VII (Togayachi et al. 1999).

We have found that the transfection of oncogene, *H-ras*, and *v-sis*, up-regulated the expression of GnT-V and its product, GlcNAcβ1,6 branch in N-glycans, as well as metastasis-related phenotypes of 7721 cells (Guo et al. 2000b). Moreover, transfection of *c-erb*B2/*neu* augmented the expression of α1,3 FucT-VII, its product, SLe^x, and metastatic potential (Liu et al. 2001a). On the other hand, transfection of *nm23*-H1 simultaneously down-regulated the expressions of both GnT-V/GlcNAcβ1,6 branch of N-glycans and α1,3 FucT-VII/SLe^x, as well as the metastatic phenotypes (Guo et al. 2000a and this paper). In addition, transfection of the cDNA of GnT-V or α1,3 FucT-VII into 7721 cells resulted in the enhancement of the metastatic phenotypes (Guo et al. 2001; Liu et al. 2001a). These findings prove that not only GnT-V/GlcNAcβ1,6 branch of N-glycans, but also α1,3 FucT-VII/SLe^x are the metastasis-associated molecules for human hepatocarcinoma cells.

The rate of cell growth and the ability of colony formation on soft agar are two well-known indices for the assessment of cell malignancy. The transfection of *nm23*-H1 did not alter the cell growth, and slightly decreased the ability of colony formation on soft agar. However, among three *nm23*-H1 transfected clones, only clone 3 was decreased with statistically significant. These results provide the evidence that *nm23*-H1 is rather a metastasis-suppressive gene than a growth-inhibiting anti-oncogene.

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