Transfection of nm23-H1 increased expression of β-Catenin, E-Cadherin and TIMP-1 and decreased the expression of MMP-2, CD44v6 and VEGF and inhibited the metastatic potential of human non-small cell lung cancer cell line L9981*

G. CHE[1], J. CHEN[1,2], L. LIU[1], Y. WANG[1], L. LI[1], Y. QIN[1], Q. ZHOU[1,3]**

[1]The Key Laboratory of Lung Cancer Molecular Biology in Sichuan Province, e-mail: Zhouqh1106@yahoo.com.cn, West China Hospital, Sichuan University, Chengdu 610041, China; [2]The Sylvester Comprehensive Cancer Center, University of Miami, 1580 NW, Miami, FL 33136, USA; [3]Division of Thoracic Surgery, Department of Surgery, Tianjin Medical University General Hospital, Tianjin 30052, China

Received March 6, 2006

Nm23 is a metastasis suppressor gene. In this report, we transfected nm23-H1 cDNA into L9981, a human large cell lung cancer cell line with nm23 negative expression, and made a stable transfectant. L9981-nm23-H1 cells exhibited lower cells proliferation rate, more G0/G1 phase growth and an increase in apoptosis with a dramatic decreased in the tumor cells ability to metastasize. L9981-nm23-H1 cells also demonstrated a significantly reduced lymph node and pulmonary metastatic capacity *in vivo* when injected into the nude mice. Furthermore, we used DNA microarray analysis to explore the change in expression of the metastasis-related genes in L9981-nm23-H1 cells. We found that the expression of β-Catenin, E-Cadherin and TIMP-1 were significantly increased while expression MMP-2, CD44v6, and VEGF was dramatically decreased in L9981-nm23-H1, as confirmed by RT-PCR and western blot. These results demonstrated that nm23-H1 can suppress the mobility and metastatic capacity of cancer cells and the molecular mechanism by which nm23-H1 suppresses tumor metastasis may be via increasing the expression of metastasis-related genes such as β-Catenin, E-Cadherin and TIMP-1 and decreasing the expression of MMP-2, CD44V6 and VEGF.

Key words: nm23-H1, metastasis suppressor gene, lung cancer, L9981 cell line

Metastasis suppressor genes are identified by their ability to suppress metastatic potential *in vivo* without a concomitant reduction in tumor growth or primary tumor size. Nm23 was identified by its reduced expression in highly metastatic melanoma cell lines, as compared with tumorigenesis in less metastatic cell lines [1]. Of the eight members of nm23 gene family, nm23-H1 is the most studied. Although most studies showed that the decreased expression of nm23 in primary breast carcinomas was significantly correlated with an aggressive clinical course, such as more lymph node metastasis and shortened patient survival, controversy still exists regarding the prognostic value of nm23 in breast cancer [2, 3]. Several studies showed that metastatic tumor cell lines transfected with nm23 dramatically decreased the metastatic ability *in vivo* [2–5]. Furthermore, over-expression of nm23-H1 in human MDA-MB-435 breast cancer cells reduced tumor cell colonization in soft agar and invasion [4]. Similar findings are observed in neural cells [6]. The role of nm23-H1 in lung cancer pathogenesis and tumor progression is less well defined.

Lung cancer is the leading cause of cancer death in both men and women. Tumor metastasis is an essential aspect of lung cancer progression. Our previous studies have revealed a significant relationship between the low-level expression nm23-H1 in primary non-small cell lung cancer (NSCLCs) with increased metastasis and a poor prognosis [7, 8]. The

*This work was supported by the grants from the Key Project of National Natural Science Foundation of China (No. 30430300, to Qinghua Zhou). The National Natural Science Foundation of China (No. 30070333, to Qinghua Zhou), the Research Fund for the Doctoral Program of Higher Education of China (No. 200040610060, to Qinghua Zhou) and The grant from China Medical Board of New York (No. 199506122, to Qinghua Zhou).

**Corresponding author

molecular mechanism by which nm23-H1 suppresses the metastasis is still unclear. Nm23-H1 was reported to bind to many proteins, such as small and heterotrimeric G-proteins and their exchange factors [9, 10]. These studies suggest that nm23-H1 protein may function as an upstream regulator that modulates downstream metastasis-related genes to inhibit the tumor metastasis.

Our lab established a primary human large cell lung cancer cell line L9981 that has a high metastatic potential with nm23-H1 negative expression [11]. In this study, we presented evidence that forced expression of nm23-H1 in L9981 cell line dramatically changed the expression of metastasis-related genes including increased expression of β-Catenin, E-Cadherin and TIMP-1 and decreased expression of MMP-2, CD44V6 and VEGF. Furthermore, the changes in the expression of these metastasis-related genes were accompanied by dramatically decreased tumor cell proliferation, invasion and metastatic ability *in vitro* and *in vivo*. Our studies indicate that nm23-H1 may decrease the metastatic ability of the lung cancer cell L9981 by controlling the expression of metastasis-related genes.

Material and methods

Construction of the recombinant plasmids carrying the nm23-H1 full cDNA. The full encoding nm23-H1 cDNA was amplified from plasmids provided by Dr. Steeg using PCR and the following primers: nm23-H1 forward: 5'-ATGGCCAACTGTGAGCGTACC-3', reverse: 5'-TCATTCATAGATCCAGTTCTG-3'. Then, nm23-H1 cDNA was cloned into pLXSN vector between EcoRI and XhoI sites. The PCR program was as follows: 94 °C for 5 min for the first cycle; 94 °C for 30 s, 60 °C for 45 s, 72 °C for 1 min for a total of 30 cycles; 7 minutes at 72 °C for the last cycle; and 4 °C on hold. The recombinant plasmid was confirmed by DNA sequencing.

Cell culture and transfection. Human lung cancer cell line L9981 was cultured in RMPI 1640 medium containing 10% fetal bovine serum at 37 °C with 5% CO_2. PLXSN-nm23-H1 and pLXSN vectors were first transfected into the packaging cells PT67 using the LipofectAMINE kit (Invitrogen). After 48 hours, the supernatant was used to infect L9981 cells. Stable transfectants were selected with 0.6 mg/ml G418 (GIBCO-BRL) and multiple colonies were maintained in the same medium for further use.

Western blot analysis. Cellular protein extraction and Western blot analysis were performed as described previously [12]. Monoclonal antibodies against nm23-H1, β-Catenin, VEGF, CD44S, CD44V6, E-Cadherin, MMP-2, TIMP-1 and β-actin were purchased from Santa Cruz. Images were quantified by using image analyzer and KDS ID 3.0.1 software (digital science, IS 440CF; Eastman-Kodak, Rochester, NY). ß-actin was used as an internal control.

Cell proliferation assay. $2x10^4$ cells/well were seeded into 96-well plates and incubated for 6 days. Cell proliferation was determined by MTT assay (Roche). Data are representative of three independent experiments performed in triplicate.

Flow cytometry analysis of apoptotic cells and cell cycle. To determine the effect of nm23-H1 protein on apoptosis, $1x10^5$ cells/well were seeded into 6-well plates and incubated for 48 hours. Cells were stained using Annexin V-FITC and Propidium iodine (PI) double staining method (Apoptosis Analysis Kit, MBL) and then subjected to a FACStar plus flow cytometry (Becton-Dickenson) to sort out the Annexin V-FITC stained apoptotic cells. Cell cycle analysis was evaluated with FACS (fluorescence-activated cell sorter) using PI and a cell cycle analysis kit (BD Biosciences).

Cloning in soft agar. Cells were plated in duplicates at $3x10^2$ cells/35-mm-dish with two agar layers: a 0.3% of soft agar (Seaplaque agar; FML Biozyme) upper layer and a 0.6% of agar lower layer in 2 x RMPI 1640 with 10% FBS. The plates were put at 37 °C in humidified incubator for 14 days. Then we used 0.5 ml of 0.005% crystal violet to stain the plates. Colonies were counted using a dissecting microscope.

Matrigel invasion assays. Matrigel invasion assay was performed as described previously (Zhou et al, 2003). In brief, 100 µl of the matrigel (1mg/ml) was put into the upper chamber of 24-well transwell and incubated for at least 2 hours at 37 °C. Cells in serum-free RPMI1640 medium (200 µl containing $1x10^5$ cells) were loaded into the upper compartment of the chamber. After incubating at 37 °C for additional 16–18 h, they were removed from the matrigel, washed, fixed and stained in Giemsa solution. The non-invading cells on the top of the transwell were scraped off with a cotton swab and the invaded cells were counted under a light microscope (x 200).

In vivo assay. Male athymic nude mice (BALB/c background, 6–7 weeks old) were used for tumor cell injections. Seven mice were used for each of the parent, nm23-H1 transfected and control cells. Briefly, cells from mixture clones were seeded into the 10 cm dish for 24 h and then harvested. Subcutaneous tumors were produced by injection of $2x10^6$ tumor cells in 0.2 ml of Ca^{2+} and Mg^{2+} free PBS over the right scapular region. Mice were checked everyday and the tumor size was measured every10 days with calipers. After 60 days, the mice were sacrificed. The subcutaneous tumors and the lungs were removed, washed and fixed in Bouin's solution. Tumor nodules on the surface of the lung were counted under a dissecting microscope. Sections of subcutaneous tumor and the nodules in the lung were further stained with HE (hematoxylin&eosin, x200) and examined by immunohistochemistry with polyclonal anti-nm23-H1 antibody (1:100 dilution).

Microassay. cDNA microarray slide with 14,000 spots was purchased from Shanghai Biomicroarray, Ltd (Shanghai, China). Total RNA was extracted from nm23-H1 and the control transfectants using Oligotex mRNA Midi Kit (Qiagen). The nm23-H1 transfectants mRNA was labeled with Cy5-dUTP while the mRNA from control was labeled with Cy3-dUTP. Then equal amounts of each of the labeled

mRNA were mixed together and hybridized with the slides in Hybchamber buffer at 60 °C for 15 to 17 hours. After being washed, the slides were read by Agilent laser scanner (Agilent: G2655AA) for both dyes (Cy3 and Cy5). The determination of differentially expressed genes was set the absolute value of natural logarithm of the ratio of Cy5 to Cy3 at greater than 6 and less than 0.16 (please see the results for the value stet detail). The ratios of Cy5 to Cy3 were calculated using Agilent 2100 Bioanalyzer software (Agilent: G2938B). Two independent microarrays were conducted in these experiments.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). RT-PCR was performed by using One-Step RT-PCR kit (Invitrogen) according to the manufacturer's instructions. PCR products were electrophoresed on a 2% agarose and quantitated by densitometry. β-actin was used as an internal control. The following are the primers used for each gene: β-Catenin forward:
5'-TTCTAGCTCATCATACTGGC-3', reverse:
5'-CAGGTACTCTGAATGTAAATC-3'; E-Cadherin forward: 5'-CTGAAGTGACTCGTAACGAC-3', reverse:
5'-CATGTCAGCCAGCTTCTTGAAG-3'; TIMP-1 forward:
5'-AGACATGTGATCAAGATTCC-3', reverse:
5'-CACGAGCAAAGGCATCATCC-3'; CD44S forward:
5'-CCAACTCCATCTGTGCAG-3', reverse:
5'-AACCTCCTGAAGTGCTGC-3'; VEGF forward:
5'-TCTACCTCCACCATGCCAAG-3', reverse:
5'-TCCTCAGTGGGCACAACTC-3'; MMP-2 forward:
5'-AGCTGCAACCTGTTTGTG-3', reverse:
5'-CCAATGATCCTGTATGTGATC-3'; CD44V6 forward:
5'-TTCTAGCTCATCATACTGGC-3', reverse:
5'-CAGGTACTCTGAATGTAAATC-3'; nm23-H1 forward:
5'-ATGGCCAACTGTGAGCGTACC-3', reverse:
5'-TCATTCATAGATCCAGTTCTG-3' and β-actin forward:
5'-GACTACCTCATGAAGATC-3', reverse:
5'-GATCCACATCTGCTGGAA-3'.

Statistical analysis. For cell proliferation assay and Matrigel invasion assays, each experiment was performed in triplicate and repeated at least three times. Data was analyzed using Students' t-test or F-test. A p≤0.05 was considered statistically significant.

Results

Forced expression of nm23-H1 in human lung cancer cell line L9981. To force-express nm23-H1 in the L9981 human lung cancer cell line, we constructed the recombinant plasmid carrying full encoding nm23-H1 cDNA. After stable transfection and colony selection, total RNA and cellular protein were extracted to verify the expression of nm23-H1 in the transfectants by RT-PCR and western blots as in Figure 1.

Nm23-H1 inhibited L9981 cell proliferation and invasion in vitro. To examine the role of nm-23-H1 in cell proliferation and apoptosis, the viability of L9881 cells transfected with nm23-H1 was assessed by the MTT assay (Roche). As illustrated in Figure 2a, the L9881 cells transfected with nm23-H1 exhibited a significant reduction in cell viability (80.5±2.9%) compared to the NEO-transfected control cells and parent cells (p<0.01). We further examined the apoptotic change by Annexin-V FITC cell flow cytometry. The cell cycle analysis showed that L9881 cells transfected with nm23-H1 had 82.13% in G0/G1 phase, while the control cells and parent cells exhibited only 71.80% and 65.26%, respectively in the same cell cycle phase (p=0.001). Consistent with the cell viability study, our experiments demonstrated that, compared with the control-transfected cells and the parent cells, the L9881 cells transfected with nm23-H1 had a higher percentage of apoptotic cells compared with the control-transfected cells and the parent cells (13.7% apoptotic cells *vs* 5.1% and 4.0%, respectively, p=0.02) (Tab. 1).

Table 1. The effect of nm23-H1 on cell cycle and apoptosis in L9981 cells

Groups	AI (%)	Cell cycle (%)		
		G0/G1	S	G2/M
L9981	4.0	65.26	15.26	19.46
L9981-pLXSN	5.1	71.80	12.71	15.48
L9981-pLXSN-nm23-H1	13.7	82.13	6.58	11.28
p value	0.02	0.001	0.002	0.071

FACS analysis of L9981 cells transfected with nm23-H1 x 10^5 cells/well were seeded into 6-well plates and incubated for 48 hours. Cells were stained using Annexin V-FITC and PI double staining method. Cell cycle analysis was evaluated with FACS using PI and a cell cycle analysis kit. Each experiment was repeated three times, and data shown are representative of three independent experiments.

Next, we studied the role of nm23-H1 in cell colony formation and invasion. Compared to the control cells and parent cells, nm23-H1 transfected L9981 cells had considerably decreased colony formations capacity in agar (10.33±0.67 vs 20.67±1.00, 21.67±1.33, p<0.001) (Fig. 2b). Matrigel invasion assay was used to detect the cell invasion ability in L9981 cell line. The invasion ability of the L9981 cells with stably transfected nm23-H1 (31±3 cells/0.723 mm^2, p<0.01) was dramatically decreased compared to that in the control and parent cells (122.33±5.33 cells/0.723 mm^2 and 157.00±6.33 cells/0.723 mm^2, respectively) (p<0.01) (Fig. 2c).

Nm23-H1 inhibited the metastatic ability of L9981 cell in vivo. We also examined the *in vivo* role of nm23-H1 in cell invasion by injection of L9881 cells transfected with nm23-H1 or the control plasmid into the subcutaneous layer of the right scapular region of nude mice (7 mice per group). After 60 days, the mice were sacrificed and the primary tumor weight and the metastatic lesions in lung were measured and counted. Contrary to previous studies that tumor suppressor genes do not affect primary tumor size, our results showed that the mean weight of the primary tumors injected with nm23-H1 transfectants was 0.6571±0.321 g, which was significant less than that in parent and control transfectants (3.767±0.104 and 3.239±0.657 g, respectively) (p<0.0001)

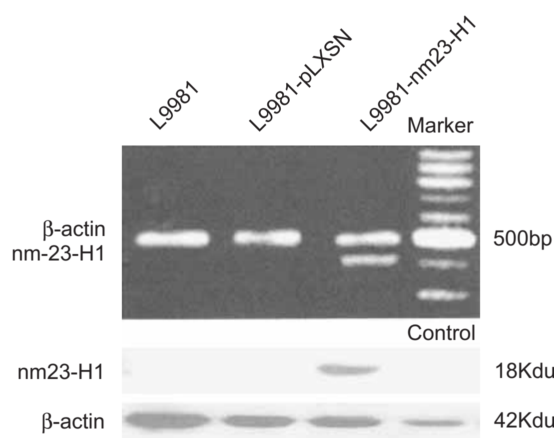

Figure 1. Expression of nm23-H1 in L9981 cells. The stable transfectants were made by taking the supernatant of PT67 cells packaged with the pLXSN-nm23-H1 to transduce L9981 cells, and selected by 600 mg/ml G418. Upper panel: RT-PCR assay for nm23-H1. Lower panel: Western blot assay with anti-nm23 antibody.

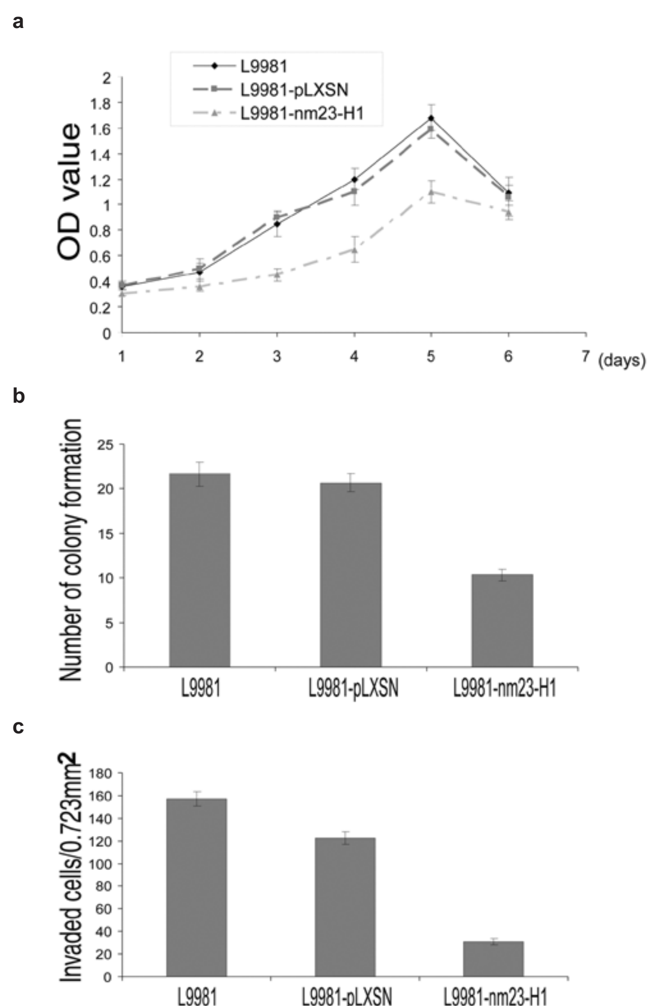

Figure 2. Characterization of L9981 cells transfected with nm23-H1 *in vitro*. (a) Cell proliferation assay: $2x10^4$ cells/well were seeded into 96-well plates, incubated for 6 days and analyzed by MTT. (b) Soft agar colonization. (c) Invasion assay: $1x10^5$ cells were placed on the upper compartment of the chamber, incubated for 16–18 h, and then stained with Giemsa. The invading cells were counted under a light microscope (x 200/0.723 mm^2).

(Fig. 3b). Interestingly, 3 of 7 mice with injection of nm23-H1 transfectants did not exhibit primary tumor in the injected sites (Fig. 3a, arrows). We used anti-nm23 antibody to detect nm23 expression in primary tumors by immunohistochemical staining in order to answer whether nm23-H1 was still expressed in the primary tumor transfected with nm23-H1. As showed in Figure 3c, nm23 protein expression was observed only in the nm23-H1 transfected-tumor. Furthermore, as shown in Figure 3d (upper panel), the autopsies revealed up to 9 small protruding lesions in the lung and 4–5 lesions in the mediastinum in the parent group (7/7 mice) and that in the control group (7/7 mice). The lesions on the surface of lung in both groups of L9981 and L9981-pLXSN were confirmed to be metastatic tumors by H&E staining (Fig. 3d lower panel). We did not find any metastatic lesions in nm23-H1 transfection group (0/7 mice).

The expression analysis of metastasis-related genes in L9881 cells transfected with nm23-H1. Furthermore, we used Microassay to investigate the change in the expression of metastasis-related genes in the L9981-nm23-H1 cells as compared with vector transfected control cells. The standard of determination for differentially expressed genes was initially set the absolute value of natural logarithm of the ratio of Cy5 to Cy3 at greater than 2 and less than 0.5. The change of gene expression was above 2 times, and the signal value of either Cy3 or Cy5 needed to be greater than 200. We obtained 1156 (8.26%, 1156/14000) upregulated genes and 642 (4.59%, 642/14000) downregulated genes in nm23 transfected cell line. Further analysis indicated there were 89.53% (1035/1156) unknown genes in upregulated group, and 84.27% (541/642) unknown genes in downregulated group. We therefore increased the absolute value to 6 times at greater than 6 and less than 0.16. There were 125 upregulated genes and 54 downregulated genes in nm23-H1 transfected cell line, as showed in supplementary Table 1 and 2. Further analysis revealed that many of the differentially expressed genes were involved in tumor mobility and metastasis including adhesion molecules (E-Cadherin and CD44), matrix metalloproteinases (MMP-1, -9, -21), tissue inhibitor of metalloproteinases family (TIMP-1, -2) and vascular endothelial growth factor family (VEGF-A, -C). As compared with the other study of microarray result in breast cancer cell lines [13], there were more downregulated genes found in nm23 transfected breast cancer cell line vs more upregulated genes found in lung cancer cell lines. Some genes such as

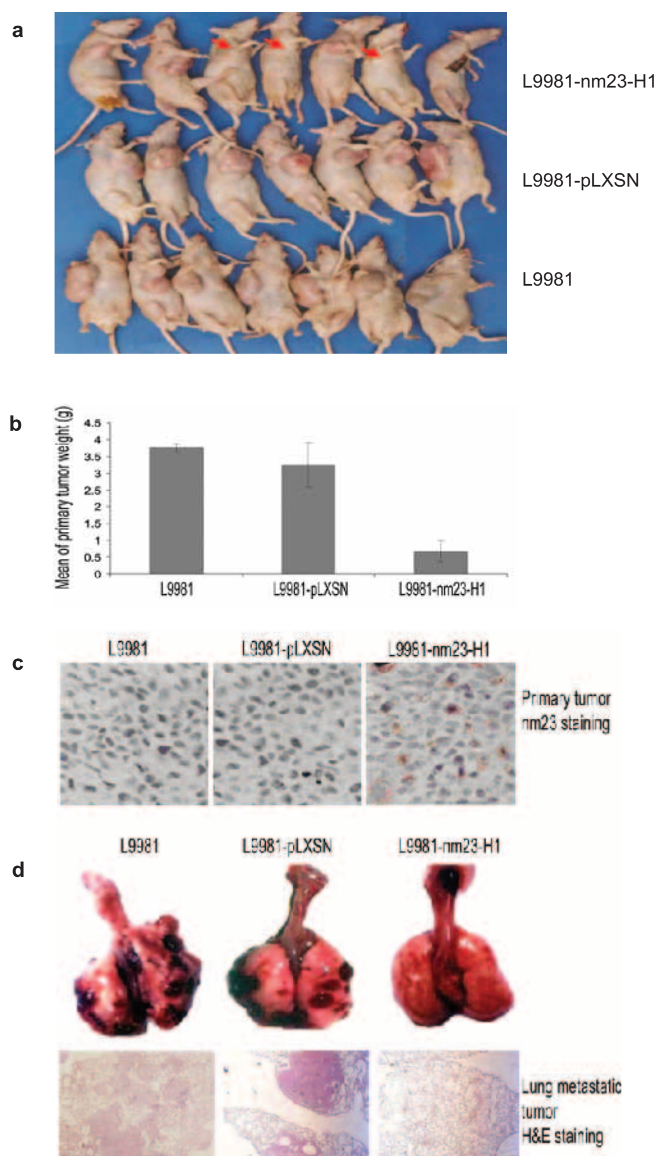

Figure 3. The effects of nm23-H1 on L9981 cells *in vivo*. (a) Sacrificed mice and the corresponding primary tumor. 3 of 7 mice in nm23-H1 transfection group did not exhibit the solid primary tumor (arrows). (b) The mean of primary tumor weight. (c) IHC staining with anti-nm23 antibody on the primary tumor. Nm23 was only expressed in the nm23-H1 transfection group (yellow staining, x200). (d) Lung metastasis. The protruding lesions were observed on the surface of the lung in both parent and control groups (upper panel), and were proved to be metastatic tumors by H&E staining (x100, lower panel). None of metastatic lesion was found in nm23-H1 transfected group.

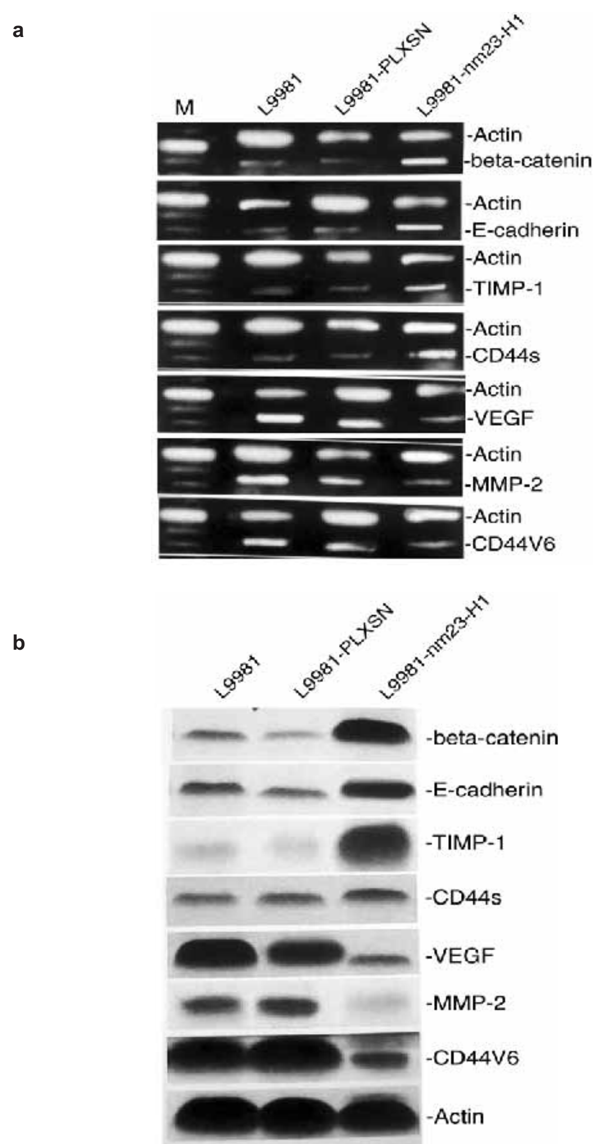

Figure 4. Analysis of the expression of the metastasis-related genes in L9881 cells with transfection of nm23-H1. (a) RT-PCR: 1 µg of total RNA was used as template to perform reverse transcription with proper primers using One-step RT-PCR kit. (b) Western blotting: 20 µg of protein was loaded on 10% SDS-PAGE and the procedure was done as previously described; β-actin was used as the internal control.

MMP-1, VEGF, RAS and RAS related GTP-binding protein were found to have a change in both experiments, but most changed genes were different in between these two experiments. One of the major reasons for the difference is due to the cell lines from different tissues. The other feasible explanation is that the technique skills used in both experiments were different.

Next, we tested the expression of these metastasis-related genes by RT-PCR in L9981 cell lines with or without transfection of nm23-H1. As shown in Figure 2a, the transcriptional expression of β-Catenin, E-Cadherin, TIMP-1 and CD44s in L9881-nm23-H1 cell line was dramatically increased, as compared with the parent and control cell lines, while the expression of MMP-2, CD44V6 and VEGF clearly decreased. Furthermore, similar change in protein expression of these molecules was observed by western blotting. The protein expression of β-Catenin, E-Cadherin and TIMP-1

was clearly increased, while that of MMP-2, CD44V6 and VEGF expression was decreased in L9881-nm23-H1 cell line. Unexpectedly, we did not find a significant change of the CD44s protein expression (Fig. 2b).

Discussion

nm23-H1 gene is the human homologue of *Drosophila awd.* To date, eight members of this human gene family have been identified. Increased expression of these genes has been correlated with the decreased tumor proliferation, increased cell differentiation and apoptosis; low level expression of nm23-H1 is correlated with the high metastatic potentials in a variety of tumors and tumor cell lines [2]. However, the prognostic value of nm23-H1 in these tumors is still controversial [14]. To date, few studies were conducted to investigate the role of nm23-H1 in the pathogenesis and disease progression in lung cancer. We previously showed that the low level of nm23-H1 expression was correlated with the advanced stages of non-small cell lung cancer (NSCLC) [7, 8]. Furthermore, there was a 26.67% (12/45) allelic deletion of nm23-H1 in NSCLC which was correlated with worse prognosis [15]. It is likely that abnormal expression of nm23-H1 has an important biological role in the invasion and metastasis of lung cancer.

The molecular mechanism by which nm23-H1 suppresses the metastatic potential of cancer cells is unclear. Nm23 gene encodes a nucleoside diphosphate kinase, which may have multiple biochemical activities. The metastasis-suppressive function of nm23 was previously linked to its histidine protein kinase activity by site-directed mutagenesis experiments [16]. To date, nm23-H1 protein has been observed to be bind to proteins from different subcellular compartments: heterotrimeric G-protein [9] and their exchange factors [10] on inner cell membranes, cytosolic heat shock proteins [17], centrosomes [18], glyceradehydephosphate dehydrogenase [19], cytoskeletal proteins [20], Prune [21] and nuclear receptors and proteins such as menin [22], muscarinic receptors [23] and ROR/RZR receptors [24]. The functional significance of these interaction remains unclear. Recently, HARTSOUGH et al observed that nm23-H1 phosphorylated the kinase suppressor of Ras (KSR) serine 392, a 14-3-3- binding site and Ser434 [21]. KSR is a scaffold protein for ERK-MAPK pathway, which is involved in the signal transduction pathway in tumor mobility and metastasis.

In our present study, we provided the first report of microassay which shows the change in the gene expression profile in L9981 cells transfected with nm23-H1, as compared to the control transfectants. Our experiments showed that the expression pattern of 179 genes changed after transfection with nm23-H1, including 125 genes that were up-regulated and 54 genes that were down-regulated. We further analyzed the expression of metastasis-related genes by RT-PCR and western blotting. While the expression of β-Catenin, E-Cadherin and TIMP-1 genes were significantly increased; the expression of MMP-2, CD44V6 and VEGF were dramatically decreased. Interestingly, we observed an increased expression of mRNA transcription but not protein translation of CD44s.

Our microassay, RT-PCR and western blot experiment results in L9981 lung cancer cells are consistent with the known function of metastasis-related genes and the postulated function of nm23-H1 to inhibit mobility/metastasis in other tumor models. E-cadherin is a cell surface adhesion molecule while β-catenin is an intracytoplasmic E-cadherin binding protein. They function to control cell-cell adhesion and cell migration. Reduced expression of E-cadherin and β-catenin has been associated with increased metastatic potential in cancer cells [25]. TIMP-1, the most studied member of TIMPs (Tissue inhibitors of Metalloproteinase), plays an important role to inhibiting matrix metalloproteinases, which in turn enables tumor cells to migrate through tissue matrix. Decreased expression of TIMP-1 has been associated with increased activity of MMPs in human hepatitis B-viral hepatoma cells [26]. Transfection of nm23-H1 in L9981 cells increased expression of E-cadherin, β-catenin and TIMP-1. An increased expression of β-Catenin, E-Cadherin and TIMP-1 therefore should result in decreased metastatic potential of the L9981-nm23-H1 cells. On the other hand, MMPs (Matrix metalloproteinases) and the plasminogen activation system (PA) play a central role in the process of cancer invasion and metastasis. Overexpression of MMP-2 and MMP-9 was found to correlate with the increased recurrence and poor survival in hepatocellular carcinoma (HCC) [27, 28], while a decreased expression of CD44s was contributed to the increased recurrence of human non-small cell lung carcinomas [29]. Mounting evidence has demonstrated that increased expression of VEGF (vascular endothelial growth factor) is associated with the increased tumor growth, metastatic potential, advanced disease stages and poor prognosis in various cancers. Transfection of nm23-H1 in L9981 cells decreased the expression of MMP-2, CD44v6 and VEGF, therefore leading to decreased tumor cell mobility and metastatic potential. CD44s, a standard isoform of CD44, binds to hyaluronate (HA) to regulate mobility and cell-matrix interaction [30]. We observed only an increased expression of mRNA transcription but not protein translation of CD44s in our studies. Our experiments suggested that the expression of CD44s is controlled mostly at the post-transcriptional level in L9981 cells.

Our experiments further showed that L9981-nm23-H1 cell lines exhibited significantly lower cell proliferation rate, more G0/G1 growth phase and more apoptotic cells than the controlled transfectants. More importantly the invasion/mobility of L9981 cells transfected with nm23-H1 was dramatically decreased compared with the control transfectants *in vitro* and *in vivo*. We did not detect any significant change in expression of the common genes involved in the control of cell cycle and apoptosis, such as p53, cyclins and Bcl-2 family proteins by microassay. Our experiments showed that transfection of nm23-H1 modulated gene expressions other

than p53, cyclins and Bcl-2 family proteins to regulate cell cycle and apoptosis. Alternatively, the changes in the expression of those genes were too moderate to be detected by microassay. Contrary to other metastasis-suppressive genes, which do not affect cell growth, transfection of nm23-H1 not only affected the expression of metastasis-related genes and decreased the mobility and metastatic potentials but also decreased the growth rate and increased the apoptosis of the L9981 cells. It would be interesting to investigate if nm23-H1 does so by phosphorylating these downstream molecules or binding to and modulating the expression of the promoters of the important genes.

In summary, our experiments strongly suggested that nm23-H1 functions as an upstream molecule that controls the expression of many important genes involved in lung cancer mobility/metastasis, and modulates the expression of downstream metastasis-related genes such as β-Catenin, E-Cadherin, TIMP-1, MMP-2, CD44V6 and VEGF. Nm23-H1 may serve as important molecule in developing targeted therapy to inhibit tumor growth and metastasis in the clinical management of lung cancer.

The authors would like to thank Dr. P.S. STEEG for kindly providing nm23-H1 cDNA; Professor ZHILIN SUN and Miss ZEFANG SUN for their technical assistance; Professor ZHILIN SUN and Dr. SHOU-CHING TANG for helpful comments on the manuscript.

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