

nm23-H1 Suppresses Invasion of Oral Squamous Cell Carcinoma-Derived Cell Lines without Modifying Matrix Metalloproteinase-2 and Matrix Metalloproteinase-9 Expression

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nm23-H1 is a candidate gene for the suppression of cancer metastasis. Several studies on human breast, hepatocellular, gastric, ovarian, and colon carcinomas and melanomas have shown that reduced nm23-H1 expression was closely related to metastatic progression with poor prognosis. However, the biochemical mechanism by which nm23-H1 suppresses the metastasis has yet to be elucidated. In this study, we analyzed the correlation between nm23 expression, cell motility, and the invasive abilities of six different oral squamous cell carcinoma cell lines (HSC2, HSC3, HSC4, KB, OSC19, and OSC20). Reduced mRNA/protein expression of the nm23-H1 was observed in three cell lines (HSC2, HSC3, and HSC4). These cell lines exhibited increased cell motility and an invasive character on organotypic raft culture. On the other hand, the cell lines (KB, OSC19, and OSC20) that showed a higher expression of nm23-H1 exhibited a threefold to fivefold reduced motility and also reflected fewer invasions compared to the former three cell lines. Because the HSC3 cells demonstrated the lowest nm23-H1 expression with the highest cell motility and invasive character, we established nm23-H1-transfected HSC3 cell lines to investigate whether exogenous nm23-H1 protein could inhibit cell migration and invasive activity. These transfectants showed a significant reduction in cell motility with exogenous nm23-H1 in a dose-dependent manner, and exhibited a noninvasive character. An immunofluorescence study demonstrated a distinct stress-fiber distribution at peripheral region of these transfectants. However, no significant difference of matrix metalloproteinase (MMP)-2 and MMP-9 expression was observed between mock transfectant and nm23-H1-transfected

cells. These findings suggest that nm23-H1 inhibits the invasive activity of oral squamous cell carcinoma by suppression of cell motility without altering the MMP-2 and MMP-9 status. (Am J Pathol 2001, 158:1785-1791)

One of the important features of a malignant tumor is its ability to invade its surrounding normal tissues. The invasive character is controlled by a group of proteinases that degrade the extracellular components.¹ For a metastatic process to be effective, a cell or a group of cells of a tumor must leave the primary site, invade the local host tissue, enter the circulation, arrest at a distant vascular bed, extravasate into the target organ interstitium and parenchyma, and proliferate as a secondary colony.¹

The nm23 gene was isolated as a metastatic suppressor gene.² Six human nm23³⁻⁸ genes have been reported; nm23-H1 encodes a protein of M_r 18,500 and nm23-H2 encodes a protein of M_r 17,000. Both nm23-H1 and nm23-H2 are localized on chromosome 17q21.3.⁹ They share 88% identity and are about 95 and 98% identical to the murine nm23-M1 and nm23-M2 proteins, respectively. The actual biochemical functions of nm23 is yet to be established, but significant homologies have been noted between nm23 and *Drosophila* abnormal wing disks (*awd*) developmental gene³ and nucleoside diphosphate (NDP) kinases from a variety of species.^{10,11}

In human infiltrating ductal breast carcinomas, with lymph node metastases contained quantitatively less nm23 mRNA than in tumors from patients without evidence of lymph node metastases.¹² Several experiments have shown that the exogenous expression of human nm23-H1 resulted in a significant reduction of metastatic potential *in vivo* and impairment of cell migration ability in response to several cytokines *in vitro*.¹³⁻¹⁵ However,

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other reports suggest, for example in neuroblastomas and colonic cancer that a high level of the nm23-H1 protein was associated with an advanced stage of the disease.^{16,17} It has become evident that the significance of nm23-H1 expression in human cancers differs from tissue to tissue and this may account for the discrepancies reported in the literature.

It is well established that the matrix metalloproteinases (MMPs) play a major role in tumor metastases. It is also known that distant metastases are frequent in invasive types of cancer. Many studies have indicated that the MMP-9 and MT-MMP activities of tumor cells strongly correlated to their metastatic potential. Our previous report demonstrated that MMP-9 expression has a potential role in helping oral squamous cell carcinoma (OSCC) cells to invade through the extracellular matrix.^{18,19} To investigate whether exogenous nm23-H1 could have an inhibitory effect on OSCC cell motility and as well as invasion, we established nm23-H1-transfected OSCC cell lines to determine their biological characteristics.

Materials and Methods

Cell Culture and Transfection

Six different established human OSCC cell lines (HSC2, HSC3, HSC4, KB, OSC19, and OSC20) were maintained for these experiments. All cell lines were cultured in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 IU/ml streptomycin. Cell lines were incubated at 37°C in an atmosphere of 5% CO₂. The full-length cDNA of nm23-H1 tagged the hemagglutinin (HA) was subcloned into the *Bam*HI and *Xba*I sites of the pcDNA3 (Invitrogen, Carlsbad, CA) plasmid. HSC3 cells were transfected with pcDNA3-HA-nm23-H1 and pcDNA3 vector alone using FuGENE 6 transfection reagent (Roche, Indianapolis, IN) according to the manufacturer's instructions. Three HSC3 clones (H1-4, H1-5, and H1-10) expressing HA-nm23-H1 protein were isolated after selection of cells in medium containing Geneticin (G418, 500 µg/ml; Life Technologies, Inc., Gaithersburg, MD) for further analysis. MRC5 cells (purchased from American Type Culture Collection, Rockville, MD) were maintained for collecting the conditioned medium as the chemoattractants for the motility assay.

Cell Migration Assay

To estimate the motility of each OSCC and nm23-H1 transfectant, the method of Boyden was used. Briefly, fibroblast-derived conditioned medium was added to the bottom wells of the chemotaxis chambers. A polycarbonate membrane of 8-µm porosity was placed onto the chemotaxis chamber. The upper chambers were then loaded with 5 × 10⁴ tumor cells/well, and incubated for 12 hours at 37°C in a 5% CO₂ incubator. Cells remaining on the upper surface of the membrane were removed and the cells that had passed through the filters were

stained by Giemsa's solution. All of the migrated cells were counted under a light microscope.

Western Blotting

Expression levels of both exogenous and endogenous nm23-H1 protein were determined by immunoblotting. Cells were lysed in a buffer containing 150 mmol/L NaCl, 50 mmol/L Tris, 5 mmol/L ethylenediaminetetraacetic acid, 0.2% Nonidet P-40, 20 µmol/L aprotinin, 20 µmol/L leupeptin, and 200 µmol/L phenylmethylsulfonyl fluoride. Equal amounts of lysate were electrophoresed with 12% sodium dodecyl sulfate-polyacrylamide gel and transferred onto nitrocellulose membranes. An anti-HA monoclonal antibody 12CA5, (Roche), and monoclonal anti-rat NDPKβ (reactive to human nm23-H1) were used for determining nm23-H1 protein expression. For detection, the ECL (Amersham Pharmacia Biotech, Little Chalfont Buckinghamshire, UK) system was used according to the manufacturer's instructions. For protein quantification analysis, we used image analyzer and KDS ID 3.0.1 software (digital science, IS 440CF; Eastman-Kodak, Rochester, NY).

Northern Hybridization

Total cell RNAs of six OSCC cell lines were prepared and analyzed as previously reported.¹⁸ In brief, 20 µg of each RNA sample was applied to a 1.2% agarose gel containing formaldehyde in MOPS buffer (3-[*N*-morpholino]propanesulfonic acid). After electrophoresis, samples were transferred onto nitrocellulose membranes; hybridized with random-primed ³²P-labeled nm23-H1, H2, and β-actin probes; and washed under highly stringent conditions. Finally, membranes were autoradiographed with Kodak X-OMAT X-ray film (Eastman-Kodak).

Gelatin Zymography

Conditioned media from three nm23-H1 transfectants and mock transfectant were collected after 24 hours of incubation. Equal amounts of protein were electrophoresed in a 7.5% sodium dodecyl sulfate-polyacrylamide gel containing 50 mg/ml gelatin. Gels were washed in 2.5% Triton X-100 for 1 hour at room temperature and subsequently transferred to a buffer containing 50 mmol/L Tris-HCl (pH -7.5), 10 mmol/L CaCl₂, 0.15 mol/L NaCl and incubated at 37°C for 16 hours. The gel was stained for 6 hours with 0.25% (w/v) Coomassie brilliant blue in 45% (v/v) methanol/1% (v/v) acetic acid and de-stained in 10% acetic acid (v/v)/25% methanol (v/v).

Immunofluorescence

nm23-H1 transfectants and mock transfected HSC3 cells were cultured on coverslips for 24 hours, then fixed with 100% methanol at room temperature for 5 minutes. These fixed cells were incubated with anti-HA monoclonal antibody (12CA5) for 1 hour at room temperature. Specimens

were visualized with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG and followed by rhodamine phalloidin (Molecular Probes, Eugene, OR) staining (3 U/ml). Each specimen was analyzed by using a confocal laser microscope (LSM 410 invert; Zeiss, Germany) and photographed.

In Vitro Raft Culture

A collagen matrix solution was made as described previously.^{19,20} Aliquots (3 ml) of the collagen fibroblast solution were poured into 35-mm plastic dishes and allowed to gel for 30 minutes at 37°C, and 2 ml of Dulbecco's modified Eagles medium/10% fetal bovine serum was added onto the collagen-fibroblast gels, which were then cultured in an incubator for 2 days. Cells from monolayer cultures were trypsinized and seeded onto the collagen-fibroblast matrix at 3×10^5 cells with 2 ml of Dulbecco's modified Eagles medium/10% fetal bovine serum added. At confluence, the collagen rafts were raised on stainless steel grids and then harvested after 10 days incubation. The Dulbecco's modified Eagles medium/10% fetal bovine serum was changed every 2 days. Each raft specimen was fixed with 2% paraformaldehyde for histological study. Specimens were embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histological evaluation.

Results

Expression of nm23-H1/H2 and Comparative Cell Motility/Invasion of Six OSCC Cell Lines

Six OSCC cell lines were analyzed for mRNA expression of the nm23-H1 and nm23-H2 genes. Higher mRNA expression of the nm23-H1 gene was observed in the KB, OSC19, and OSC20 cell lines and lower expression was observed in HSC2, HSC3, and HSC4 cells (Figure 1A). However, each cell line expressed about the same level of nm23-H2 mRNA. Then, we analyzed the protein expression of nm23-H1 by Western blotting (Figure 1B). The corresponding protein expression was also similar to mRNA expression of nm23-H1. The cell migration assay showed a contrasting relationship, ie, cell lines with higher expression of nm23-H1 had reduced cell motility, which was threefold to fivefold lower than for the lower nm23-H1-expressing cell lines (Figure 1C). Each cell line was then examined to determine the comparative behavior of the invasive patterns by organotypic raft culture. The HSC3 cells, which expressed the lowest amount of nm23-H1, exhibited an invasive morphology (Figure 2A), whereas the KB cells, with the highest nm23-H1 expression demonstrated a noninvasive and stratified growth pattern (Figure 2B). These results led us to speculate that the cell motility and invasive behavior of the OSCC cell lines could be related to alter expression of the nm23-H1.

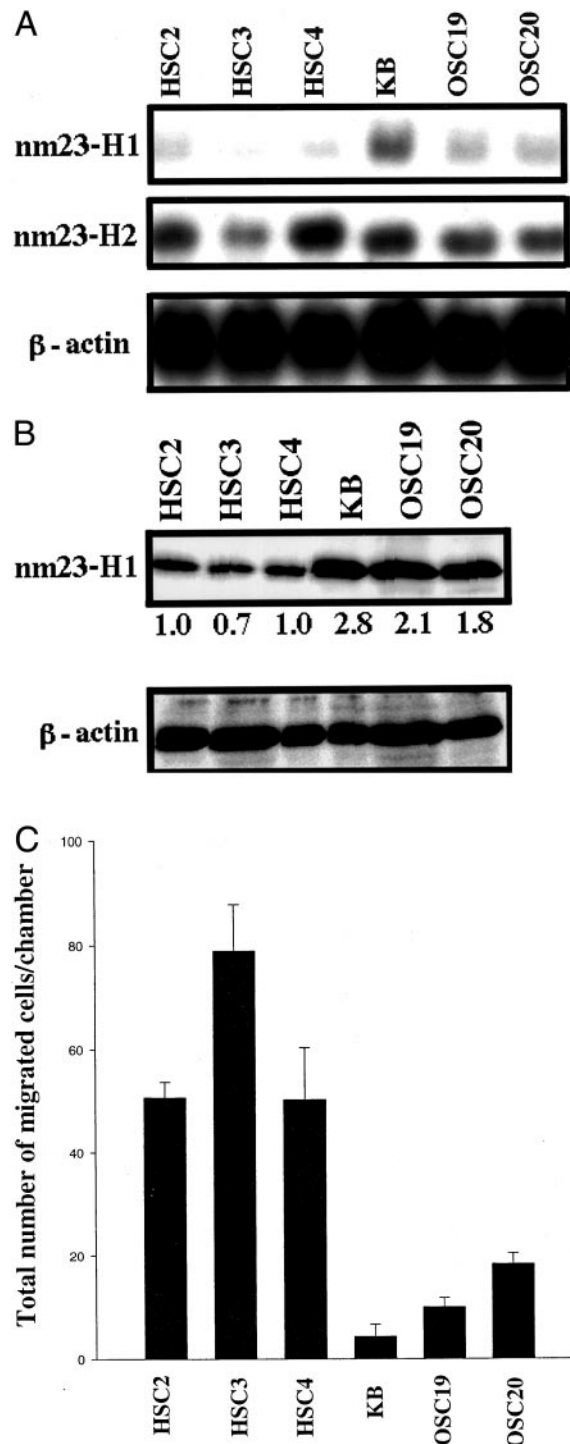


Figure 1. Expression of nm23-H1/H2 and comparative cell motility of six OSCC cell lines. **A:** Northern blot analysis of six different OSCC cell lines. High mRNA expression of the nm23-H1 gene was observed in three cell lines (KB, OSC19, and OSC20) and low expression was observed in the other three cell lines (HSC2, HSC3, and HSC4). No significant difference was observed in nm23-H2 expression. **B:** Total lysates from each cell line (25 μ g/lane) were analyzed for nm23-H1 protein expression. Detected nm23-H1 bands by immunoblotting were quantified using KDS 1D 3.0.1 software. The values indicated relative amount of nm23-H1 protein (adjusted with β -actin expression). High protein expression of the nm23-H1 was also observed in KB, OSC19, and OSC20 cell lines. **C:** Fibroblast-derived conditioned medium stimulated migration of those six cell lines. Data are expressed as means \pm SE ($n = 3$). Cells (KB, OSC19, and OSC20) with high mRNA/protein expression of nm23-H1 showed reduced cell motility, threefold to fivefold, compared to cell lines with reduced nm23-H1 expression.

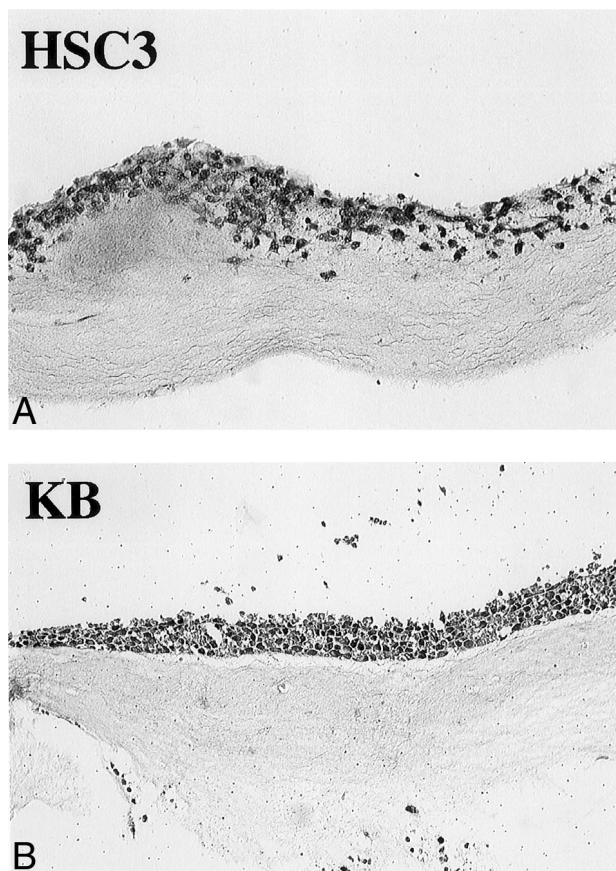


Figure 2. Histological findings of HSC3 and KB cells on raft culture. **A:** Cells tended to separate from each other and invaded deeply into the collagen gel, indicating a highly invasive phenotype. **B:** KB cells showed stratified epithelium on collagen gel, a noninvasive feature. Original magnifications, $\times 20$ (**A** and **B**).

Exogenous nm23-H1 Expression and Cell Motility/Invasion

Three clones (H1-4, H1-5, and H1-10) that stably expressed exogenous HA-tagged nm23-H1 were isolated (Figure 3A). As shown in Figure 3A, three clones expressed exogenous HA-tagged nm23-H1 protein at different levels. A quantitative analysis indicated that H1-10 clone expressed approximately fourfold to fivefold more amount of HA-nm23-H1 protein compared with H1-4 and H1-5. Those three transfectants and control cells were assayed for their ability to migrate (triplicate samples) in response to both fibroblast-derived conditioned medium and serum-free medium. In contrast to the control cells, all three transfectants (H1-4, H1-5, and H1-10) showed a significant reduction in cell motility with exogenous nm23-H1 in a dose-dependent manner (Figure 3B). Raft culture experiments demonstrated that one transfectant (H1-10), which had a significant reduction in cell motility, exhibited a stratified growth pattern without invading the collagen matrices, a noninvasive character (Figure 4B), whereas mock-transfected HSC3 cells demonstrated an invasive morphology with deep invasion and scattering into the collagen gel (Figure 4A).

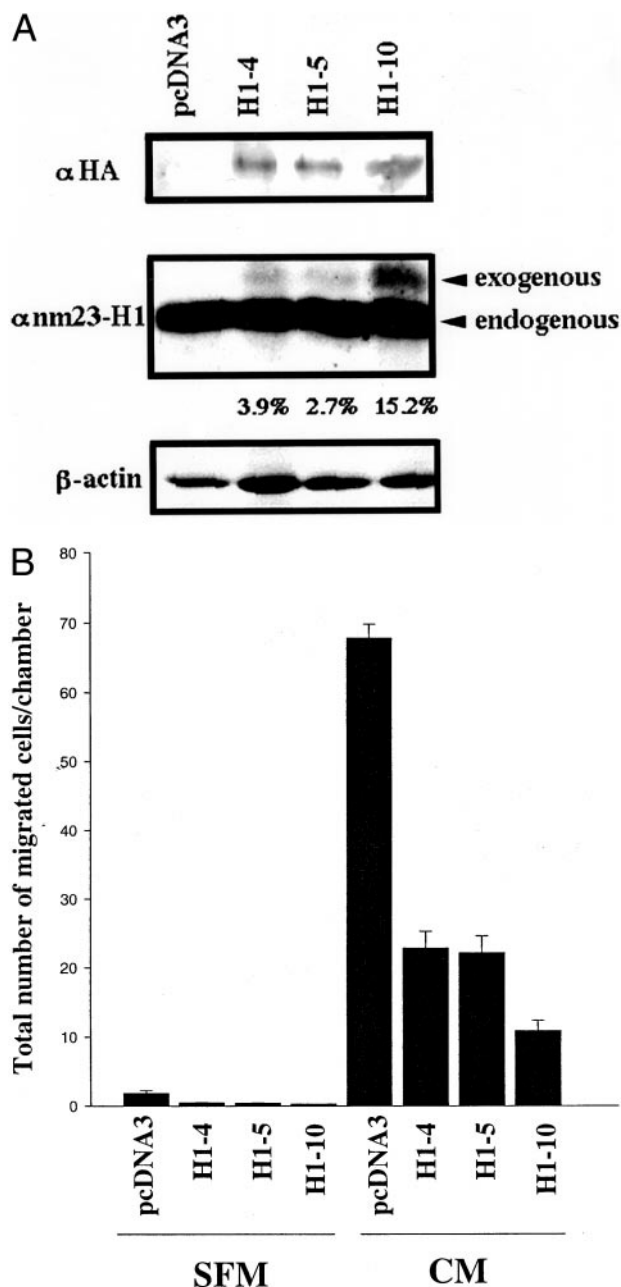


Figure 3. Exogenous nm23-H1 expression and cell motility assay. **A:** Exogenous protein level of nm23-H1-transfected HSC3 cells, as determined by immunoblotting using an anti-HA monoclonal antibody 12CA5. All three clones (H1-4, H1-5, and H1-10) expressed the HA-nm23-H1 protein whereas the mock transfectant did not. The monoclonal anti-rat NDPK β (reactive to human nm23-H1) detected both endogenous and exogenous nm23-H1 protein. Data showing the comparative exogenous nm23-H1 protein expression level (3.9% for H1-4, 2.7% for H1-5, and 15.2% for H1-10) to endogenous expression. **B:** Motility assay of mock transfectant and nm23-H1 transfectants (H1-4, H1-5, and H1-10). Data are expressed as means \pm SE ($n = 3$). The left four lanes were tested with serum-free medium (SFM) and the right four lanes with conditioned medium (CM). In contrast to the control, nm23-H1-transfected cells showed significantly decreased cell motility in a dose-dependent manner.

Gelatin Zymography for MMP-2 and MMP-9 Expression

Gelatinolytic (collagenase) activities of conditioned medium derived from those three nm23-H1 transfectants

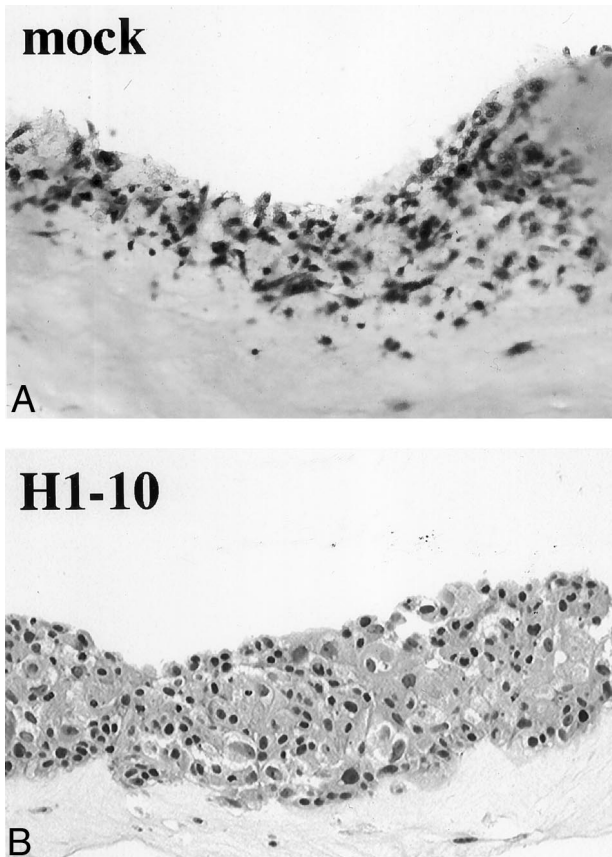


Figure 4. Morphological characteristics of mock transfectant and HA-nm23-H1 transfectant (H1-10) on raft culture. **A:** Control cells have a highly invasive phenotype, in which cells are scattered and invading deeply into the collagen gel. **B:** On the other hand, nm23-H1-transfected (H1-10) cells show a stratified growth pattern without invading the collagen matrices, indicating a noninvasive feature. Original magnifications, $\times 40$ (**A** and **B**).

and control cells were analyzed using gelatin zymography. Despite the reduced migration and noninvasiveness of the nm23-H1 transfectants, we did not observe a significant loss of metalloproteinase activity (MMP-2 and MMP-9) in any of the transfectant examined in comparison to mock-transfected cells (Figure 5).

Immunocytochemical Analysis of nm23-H1 Transfectants

An immunofluorescence study demonstrated a strong cytoplasmic signal of exogenous HA-tagged nm23-H1 in transfectant (Figure 6B). Rhodamine phalloidin (Molecular Probes) detected prominent actin bundles at the peripheral regions of cells (Figure 6D), whereas control cells exhibited few stress fibers, which were thin and attenuated (Figure 6C).

Discussion

The invasive potential of a carcinoma has an important prognostic significance. Invasive tumors often metastasize to local lymph nodes as well as to distant sites.²¹ The process of tumor invasion by OSCC involves degradation

of the demarcating basement membrane, which is primarily composed of type IV collagen.²² MMPs have been implicated for invasion and metastasis of tumor cells and therefore, expression of MMP-2 and MMP-9 are important phenotypic determinants of OSCC.^{19,23} Invasion of tumor cells into the basement membrane can be separated into three steps: attachment of cells to the basement membrane, matrix dissolution, and locomotion. In the third step of invasion, tumor cells propel across the basement membrane and stroma.¹ The precise mechanism by which nm23 protein interferes with the metastatic process has yet to be elucidated. Our results showed that the cell lines with higher levels of nm23-H1 expression had lower cell migration activities. Therefore the highest nm23-H1-expressing KB cells, and the lowest-expressing HSC3 cells were further investigated to compare their invasive potentials in a raft culture system. The HSC3 cells tended to separate from each other and invaded deeply into the collagen gel. On the other hand, KB cells showed stratified growth, which was a noninvasive feature. These results led us to speculate that the invasive phenotype of the OSCC cell lines might be related to different expression level of nm23-H1 gene, and we made a particular effort to examine the effects of exogenous nm23-H1 expression on HSC3 cell line. In the present study, we established three exogenous nm23-H1-expressing stable transfectants, which indicated a reduced cell migration activity, compared with mock transfectants. One clone (H1-10) exhibited almost the same motility and noninvasive character as shown by KB cells. We also demonstrated that exogenous nm23-H1 expression inhibited invasion of the HSC3 cells. However, gelatin zymography did not indicate loss of MMPs activities (MMP-2 and -9) in any of the transfected cells compared to control transfectants. It is likely that the amount of extracellular matrix-degrading enzymes secreted from human OSCC cells were not affected by the increased expression of nm23-H1. We therefore, concluded that the noninvasive behavior of exogenous nm23-H1-expressing clones were not because of reduced MMP activity. However, nm23-H1 protein may have helped the prevention of local invasion by interfering with cell motility.

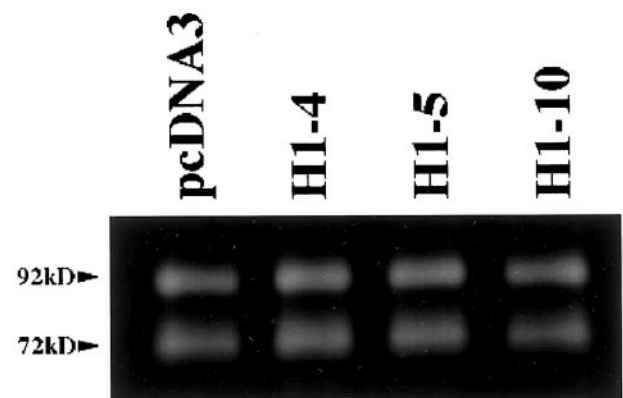


Figure 5. Gelatin zymography determined the expression levels of MMP-2 (72 kd) and MMP-9 (92 kd) of both control and nm23-H1 transfectants. Loss of metalloproteinases activity were not observed in any of the transfected cells in comparison to control.

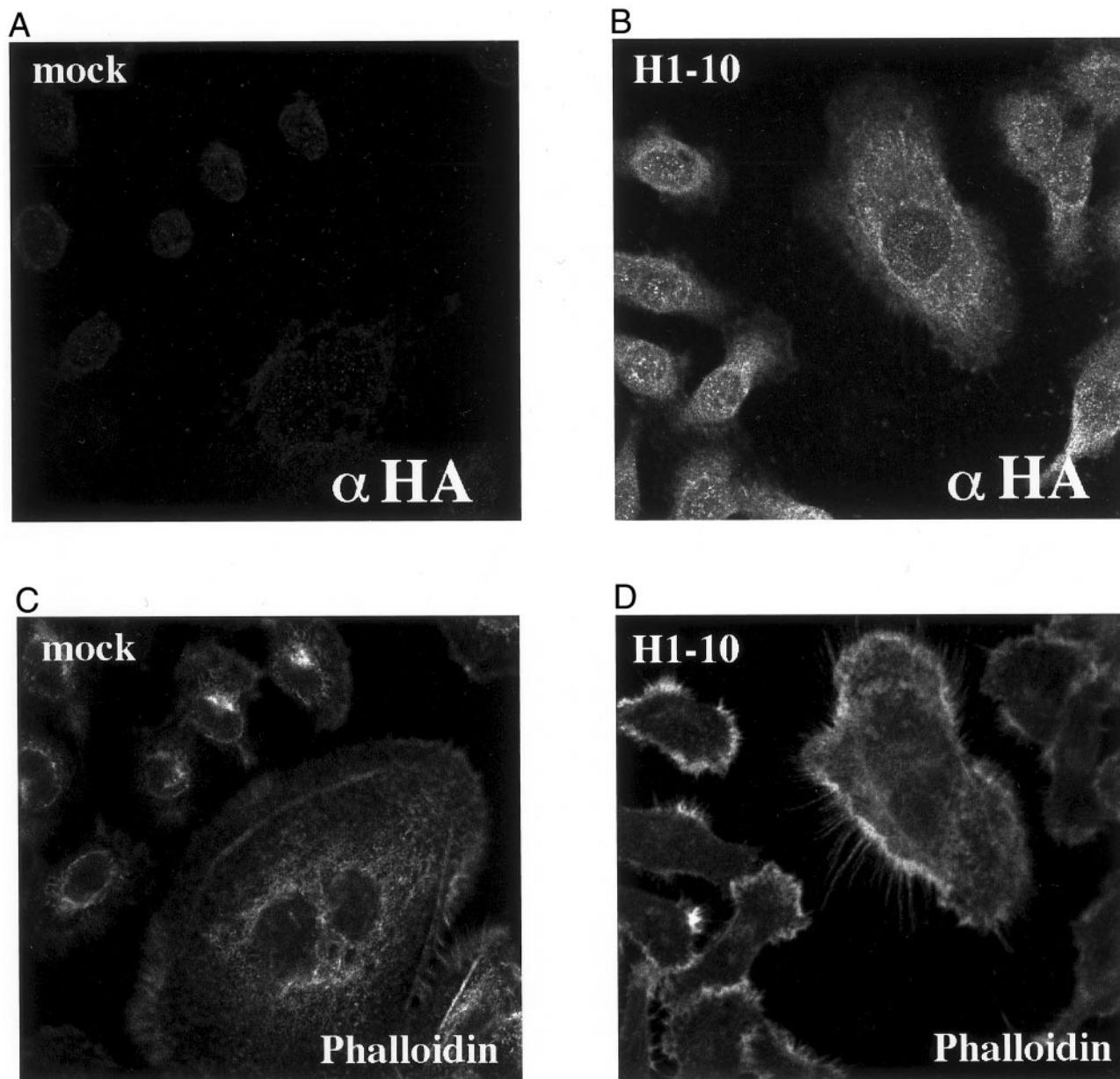


Figure 6. The subcellular localization of exogenous nm23-H1 and the distribution of stress fiber were analyzed by double staining with the anti-HA antibody and rhodamine phalloidin using confocal laser microscopy. Strong cytoplasmic signal of HA-nm23-H1 was observed in nm23-H1-transfected HSC3 cells (**B**), whereas no signal was detected in the control (**A**). nm23-H1-expressing cells showed distinct actin distribution at the peripheral regions of cell (**D**), whereas control showed faint attenuated signal (**C**). Same field of cells was shown in **A** and **C** (control) and **B** and **D** (nm23-H1).

Immunofluorescence experiments revealed that exogenous expression of nm23-H1 influenced the cytoskeletal status. The H1-10 clone had intense actin stress fiber at the peripheral region, whereas mock-transfected cells showed faint attenuated actin fibers. This alteration of actin stress fiber reconstruction might suggest that nm23-H1 is involved in certain signal transduction cascades. However, the amount of exogenous nm23-H1 of H1-10 was $\sim 15\%$ of total endogenous nm23-H1 protein of the HSC3 cell. The total amount of exogenous and endogenous nm23-H1 protein of H1-10 was $<50\%$ of the KB cell. The threshold protein expression level, which is optimum for inhibition of tumor invasion, should be assessed in future experiment.

It has been reported that transfection of nm23-H2 into the metastatic OSCC cell line caused reduction in the lung metastasis in an experimental metastasis assay but not by nm23-H1.²⁴ However, the authors have suggested that their nm23-H1 transfectant did not express high levels of nm23-H1 protein. There have been no clear data demonstrating metastatic suppression activity of nm23-H1 transfected with OSCC cell lines, although nm23-H1/NDP kinase A protein has been observed as a positive correlate to the lack of metastasis in human OSCC by immunohistochemical analysis.²⁵ Transfection of nm23-H1 into human breast carcinoma cells proved that there was a significant reduction of cell motility in response to a variety of chemoattractants that act through different receptors and it seemed

that the blockage of cell migration occurred in the downstream of the chemoattractant stimulation cascade.²⁶ In the nm23-H1 mutation study, S120G and P96S reversed the wild-type effect, whereas S44G closely resembled that of the wild type.¹⁴ Thus, these authors concluded that only two known sites of nm23-H1 protein, P96 and S120, were essential for its motility suppressor effect, but the mechanism underlying the inhibition of stimulated cell motility in nm23-transfected cells remains unclear.

In conclusion, our data indicate that nm23-H1 is a gene that can reduce local invasiveness of OSCC cells via suppression of cell motility. The proteolytic phenotype, at least MMP-2 and MMP-9 of OSCC are not affected by exogenous expression of nm23-H1, however other proteolytic enzymes or protease inhibitors that may influence this process are yet to be determined. Motility properties and proteolytic enzyme activities of tumor cells play a crucial role in metastasis and it seems that these phenotypic determinants are regulated by a diverse array of mechanisms.

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References

- Liotta LA, Steeg PS, Stetler-Stevenson WG: Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* 1991, 64:327–336
- Steeg PS, Bevilacqua G, Kopper L, Thorgeirsson UP, Talmadge JE, Liotta LA, Sobel ME: Evidence for a novel gene associated with low tumor metastatic potential. *J Natl Cancer Inst* 1988, 80:200–204
- Rosengard AM, Krutzsch HC, Shearn A, Biggs JR, Barker E, Margulies IM, King CR, Liotta LA, Steeg PS: Reduced Nm23/Awd protein in tumor metastasis and aberrant *Drosophila* development. *Nature* 1989, 342:177–180
- Stahl JA, Leone A, Rosengard AM, Porter L, King CR, Steeg PS: Identification of a second human nm23 gene, nm23-H2. *Cancer Res* 1991, 51:445–449
- Venturelli D, Martinez R, Melotti P, Casella I, Peschle C, Cucco C, Spampinato G, Darzynkiewicz Z, Calabretta B: Overexpression of DR-nm23, a protein encoded by a member of the nm23 gene family, inhibits granulocyte differentiation and induces apoptosis in 32Dc13 myeloid cells. *Proc Natl Acad Sci USA* 1995, 92:7435–7439
- Milon L, Rousseau-Merck MF, Munier A, Erent M, Lascu I, Capeau J, Lacombe ML: nm23-H4, a new member of the family of human nm23/nucleoside diphosphate kinase genes localized on chromosome 16p13. *Hum Genet* 1997, 99:550–557
- Munier A, Johansson M, Karlsson A, Lascu I, Capeau J, Janin J, Lacombe ML: The human nm23-H4 gene product is a mitochondrial nucleoside diphosphate kinase. *FEBS Lett* 1998, 434:289–294
- Tsuiki H, Nitta M, Furuya A, Hanai N, Fujiwara T, Inagaki M, Kochi M, Ushio Y, Saya H, Nakamura H: A novel human nucleoside diphosphate (NDP) kinase, Nm23-H6, localizes in mitochondria and affects cytokinesis. *J Cell Biochem* 1999, 76:254–269
- Backer JM, Mendola CE, Kovessi I, Fairhurst JL, O'Hara B, Eddy Jr RL, Shows TB, Mathew S, Murty VV, Chaganti RS: Chromosomal localization and nucleoside diphosphate kinase activity of human metastasis-suppressor genes NM23-1 and NM23-2. *Oncogene* 1993, 8:497–502
- Isikawa N, Shimada N, Munakata Y, Watanabe K, Kimura N: Isolation and characterization of a gene encoding rat nucleoside diphosphate kinase. *J Biol Chem* 1992, 267:14366–14372
- Munoz-Dorado J, Inouye M, Inouye S: Nucleoside diphosphate kinase from *Myxococcus xanthus*. *J Biol Chem* 1990, 265:2702–2706
- Bevilacqua G, Sobel ME, Liotta LA, Steeg PS: Association of low nm23 RNA levels in human primary infiltrating ductal breast carcinomas with lymph node involvement and other histopathological indicators of high metastatic potential. *Cancer Res* 1989, 49:5185–5190
- Hsu S, Huang F, Ossowski L, Friedman E: Colon carcinoma cells with inactive nm23 show increased motility and response to motility factors. *Carcinogenesis* 1995, 16:2259–2262
- MacDonald NJ, Freije JMP, Stracke ML, Manrow RE, Steeg PS: Site-directed mutagenesis of nm23-H1. Mutation of proline 96 or serine 120 abrogates its motility inhibitory activity upon transfection into human breast carcinoma cells. *J Biol Chem* 1996, 271:25107–25116
- Russell RL, Pedersen AN, Kantor J, Geisinger K, Long R, Zbieranski N, Townsend A, Shelton B, Brunner N, Kute TE: Relationship of nm23 to proteolytic factors, proliferation and motility in breast cancer tissues and cell lines. *Br J Cancer* 1998, 78:710–717
- Hailat N, Keim DR, Melhem RF, Zhu XX, Eckerskorn C, Brodeur GM, Reynolds CP, Seeger RC, Lottspeich F, Strahler JR, Hanash SM: High levels of p19/nm23 protein in neuroblastoma are associated with advanced stage disease and with N-myc gene amplification. *J Clin Invest* 1991, 88:341–345
- Haut M, Steeg PS, Willson JK, Markowitz SD: Induction of nm23 gene expression in human colonic neoplasms and equal expression in colon tumors of high and low metastatic potential. *J Natl Cancer Inst* 1991, 83:712–716
- Hida K, Shindoh M, Yasuda M, Hanzawa M, Funaoka K, Kohgo T, Amemiya A, Totsuka Y, Yoshida K, Fujinaga K: Antisense E1AF transfection restrains oral cancer invasion by reducing matrix metalloproteinase activities. *Am J Pathol* 1997, 150:2125–2131
- Shindoh M, Higashino F, Kaya M, Yasuda M, Funaoka K, Hanzawa M, Hida K, Kohgo T, Amemiya A, Yoshida K, Fujinaga K: Correlated expression of matrix metalloproteinases and ets family transcription factor E1A-F in invasive oral squamous-cell-carcinoma-derived cell lines. *Am J Pathol* 1996, 148:693–700
- Shindoh M, Sun Q, Pater A, Pater MM: Prevention of carcinoma in situ of human papillomavirus type 16-immortalized human endocervical cells by retinoic acid in organotypic raft culture. *Obstet Gynecol* 1995, 85:721–728
- Jakobsson PA, Eneroth CM, Killander D, Moberger G, Martensson B: Histological classification and grading of malignancy in carcinoma of the larynx. *Acta Radiol Ther Phys Biol* 1973, 12:1–8
- Hirota J, Yoneda K, Osaki T: Basement membrane type IV collagen in oral squamous cell carcinoma. *Head Neck* 1990, 12:400–405
- Juarez J, Clayman G, Nakajima M, Tanabe KK, Saya H, Nicolson GL, Boyd D: Role and regulation of expression of 92-kDa type-IV collagenase (MMP-9) in 2 invasive squamous-cell-carcinoma cell lines of the oral cavity. *Int J Cancer* 1993, 55:10–18
- Miyazaki H, Fukuda M, Ishijima Y, Takagi Y, Limura T, Negishi A, Hirayama R, Ishikawa N, Amagasa T, Kimura N: Overexpression of nm23-H2/NDP kinase B in a human oral squamous cell carcinoma cell line results in reduced metastasis, differentiated phenotype in the metastatic site, and growth factor-independent proliferative activity in culture. *Clin Cancer Res* 1999, 5:4301–4307
- Ohtsuki K, Shintani S, Kimura N, Matsumura T: Immunohistochemical study on the nm23 gene product (NDP kinase) in oral squamous cell carcinoma. *Oral Oncol* 1997, 33:237–239
- Kantor JD, McCormick B, Steeg PS, Zetter BR: Inhibition of cell motility after nm23 transfection of human and murine tumor cells. *Cancer Res* 1993, 53:1971–1973