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, 18,500 and nm23-H2 encodes a protein of M, 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 gene9 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,
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.\textsuperscript{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 correlate 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.\textsuperscript{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 determined 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% CO\textsubscript{2}. The full-length cDNA of nm23-H1 tagged the hemagglutinin (HA) was subcloned into the BamH\textsubscript{I} and Xba\textsubscript{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 \textmu g/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-\mu m porosity was placed onto the chemotaxis chamber. The upper chambers were then loaded with 5 x 10\textsuperscript{4} tumor cells/well, and incubated for 12 hours at 37°C in a 5% CO\textsubscript{2} 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 \mu g/ml aprotinin, 20 \mu g/ml leupeptin, and 200 \mu g/ml 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 NDPK\beta (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.\textsuperscript{18} In brief, 20 \mu g 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 \textsuperscript{32}P-labeled nm23-H1, H2, and \beta-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 CaCl\textsubscript{2}, 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 pre-
viously.19,20 Aliquots (3 ml) of the collagen fibroblast
solution were poured into 35-mm plastic dishes and al-
lowed to gel for 30 minutes at 37°C, and 2 ml of Dulbe-
cco’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 monol-
ayer cultures were trypsinized and seeded onto the col-
lagen-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 me-
dium/10% fetal bovine serum was changed every 2 days.
Each raft specimen was fixed with 2% paraformaldehyde
for histological study. Specimens were embedded in par-
affin, 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 ex-
pression 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 ex-
pression 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, i.e., 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 behav-
ior 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 expres-
dion 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 alteration of 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
μg/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 ±
SE (n = 3). Cells (KB, OSC19, and OSC20) with high mRNA/protein expres-
sion of nm23-H1 showed reduced cell motility, threelfold to fivefold, com-
pared to cell lines with reduced nm23-H1 expression.
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).

Gelatin Zymography for MMP-2 and MMP-9 Expression

Gelatinolytic (collagenase) activities of conditioned medium derived from those three nm23-H1 transfectants...
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. The process of tumor invasion by OSCC involves degradation of the demarcating basement membrane, which is primarily composed of type IV collagen. 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. 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. 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.
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. 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. 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 chemotactrant stimulation cascade. In the nm23-H1 mutation study, S120G and P96S reversed the wild-type effect, whereas S44G closely resembled that of the wild type. 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.

Acknowledgments

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