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Upregulation of sialidase NEU3 in head and neck squamous cell carcinoma associated with lymph node metastasis

Kiyoto Shiga,[1,2] Kohta Takahashi,[3,4] Ikuro Sato,[5] Kengo Kato,[2] Shigeru Saijo,[2] Setsuko Moriya,[3] Masahiro Hosono[4] and Taeko Miyagi[3]

[1]Department of Otolaryngology–Head and Neck Surgery, Iwate Medical University, Morioka; [2]Department of Head and Neck Surgery, Miyagi Cancer Center, Natori; Divisions of [3]Cancer Glycosylation Research; [4]Cell Recognition Study, Institute of Molecular Biomembrane and Glycobiology, Tohoku Pharmaceutical University, Sendai; [5]Department of Pathology, Miyagi Cancer Center, Natori, Japan

Key words

Epidermal growth factor receptor, head and neck squamous cell carcinoma, lymph node metastasis, MMP9, sialidase

Correspondence

Kiyoto Shiga, Department of Otolaryngology–Head and Neck Surgery, Iwate Medical University, 19-1 Uchimaru, Morioka, Iwate 020-8505, Japan.
Tel: +81-19-651-5111; Fax: +81-19-652-8642;
E-mail: kshiga@iwate-med.ac.jp
and
Taeko Miyagi, Division of Cancer Glycosylation Research, Institute of Molecular Biomembrane and Glycobiology, Tohoku Pharmaceutical University, 4-4-1, Komatsushima, Aoba-ku, Sendai 981-8558, Japan.Tel: +81-22-727-0157;
Fax: +81-22-275-2013;
E-mail: tmiyagi@tohoku-pharm.ac.jp

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Regional lymph node metastasis in head and neck squamous cell carcinoma (HNSCC) is a crucial event for its progression, associated with a high rate of mortality. Sialidase, a key enzyme for the regulation of cellular sialic acids through catalyzing the initial step of degradation of glycoproteins and glycolipids, has been implicated in cancer progression. To facilitate the development of novel treatments for HNSCC, we have investigated whether sialidase is involved in the progression of this cancer. We found plasma membrane-associated sialidase (NEU3) to be significantly upregulated in tumor compared to non-tumor tissues; particularly, an increase in its mRNA levels was significantly associated with lymph node metastasis. To understand the mechanisms, we analyzed the NEU3-mediated effects on the malignant phenotype using squamous carcinoma HSC-2 and SAS cells. NEU3 promoted cell motility and invasion, accompanied by the increased expression of MMP-9, whereas NEU3 silencing or the activity-null mutant did not. NEU3 enhanced phosphorylation of epidermal growth factor receptor (EGFR), and an EGFR inhibitor, AG1478, abrogated the NEU3-induced MMP9 augmentation. These findings identify NEU3 as a participant in HNSCC progression through the regulation of EGFR signaling and thus as a potential target for inhibiting EGFR-mediated tumor progression.

Head and neck squamous cell carcinoma (HNSCC) includes epithelial malignancies of the oral cavity, pharynx, and larynx, and is the sixth most common cancer worldwide.[1,2] Despite extensive advances in research and treatment, the 5-year overall survival rates has not improved significantly in the last 50 years. Head and neck squamous cell carcinoma is characterized by a highly invasive and metastatic malignancy, and the high mortality rate is attributed to metastatic diseases at regional and distant sites. A more detailed analysis of the molecular mechanisms underlying its invasiveness is required for the development of novel treatment strategies. To improve HNSCC treatment, we have attempted to identify the molecules involved in its progression.

Alteration in glycosylation is a characteristic feature of tumorigenesis, and aberrant sialylation, in particular, has been implicated in the malignant phenotype with reference to metastatic potential and invasiveness.[3,4] Sialidases are key enzymes for the control of cellular sialic acid contents, by catalyzing the removal of sialic acid residues from their carbohydrate portions. Among the four types of human sialidases so far identified (NEU1–NEU4), which differ in major subcellular localization and enzymatic properties, as well as in positioning on the chromosomes,[5] the plasma membrane-associated sialidase NEU3 appears to play particular roles in controlling transmembrane signaling by the modulation of gangliosides,[6] and its aberrant expression is closely related to the pathogenesis of cancer.[7] We previously shown that NEU3 is upregulated in tumor compared to adjacent non-tumor tissues in colon, renal, prostate, and ovarian cancers.[8–11] NEU3 enhances cancer cell survival,[8,12] cell migration and attachment,[13] and actually enhances the epidermal growth factor (EGF)-stimulated tyrosine phosphorylation of EGF receptor (EGFR).[12]

In the present study, we investigated whether any form of human sialidase is involved in the progression of HNSCC by

determining the sialidase expression in surgical specimens. We found that plasma membrane-associated sialidase NEU3 was markedly upregulated in cancer tissues, especially in association with lymph node metastasis, so it is probably a molecule that promotes metastasis through EGFR signaling activation. Targeting the sialidase may thus be a therapeutic approach for this cancer.

Materials and Methods

Patient samples. Surgical specimens were obtained from 30 consecutive HNSCC patients from April 1999 to October 2000 who underwent radical therapy at Miyagi Cancer Center Hospital. Informed consent was obtained from each patient to allow the use of portions of tissue for research purposes, and the study was approved by the Committee on Human Rights in Research at Miyagi Cancer Center (Natori, Japan). The clinical characteristics of these patients are listed in Table 1. The stage grouping and the TNM system of the patients were defined according to the International Union Against Cancer (UICC; 1997).

Cell culture. Oral squamous cell carcinoma HSC-2 and SAS cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Cells were cultured in DMEM supplemented with 10% FBS (Invitrogen, Grand Island, NY, USA) at 37°C in a 5% CO_2 atmosphere.

Antibodies. Antibodies for phospho-EGFR (Y-845), phospho-ERK, and ERK, from Cell Signaling Technology (Danvers, MA, USA), EGFR from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and a monoclonal anti-NEU3, prepared as described previously,[14] were used in immunoblotting analysis.

Table 1. Clinical characterization of head and neck squamous cell carcinoma patients (n = 30)

	Age, years/gender	Tumor site	Stage	TNM classification
1	74/M	Hypopharynx	IV	T4N2cMO
2	60/F	Tongue	III	T2N1M0
3	61/M	Tongue	IV	T3N2bM0
4	61/M	Hypopharynx	IV	T3N2bM0
5	50/M	Gingiva	IV	T4N2bM0
6	27/M	Mesopharynx	IV	T3N2bM0
7	68/M	Tongue	IV	rT4N0M0
8	67/F	Tongue	II	T2N0M0
9	80/M	Larynx	IV	T4N0M0
10	46/M	Tongue	IV	rN3M0
11	61/M	Mesopharynx	II	T2N0M0
12	48/M	Mesopharynx	IV	T3N2bM0
13	67/M	Hypopharynx	IV	T4N2M0
14	55/M	Mesopharynx	IV	T2N2bM0
15	70/F	Hypopharynx	IV	T2N2bM0
16	56/M	Maxilla	III	T3N0M0
17	85/M	Lip	IV	T3N2cM0
18	48/M	Larynx	I	rT1bNOM0
19	65/M	Tongue	III	T2N1M0
20	58/M	Gingiva	IV	T4N2bM0
21	63/M	Tongue	II	T2N0M0
22	77/M	Tongue	III	T3N1M0
23	76/M	Larynx	IV	T4N2cM0
24	52/F	Tongue	I	T1N0M0
25	48/M	Maxilla	IV	T4N2cM0
26	56/F	Tongue	I	T1N0M0
27	64/M	Mesopharynx	IV	T3N2bM0
28	62/M	Larynx	IV	T4N0M0
29	64/M	Mesopharynx	IV	rT2N0M0
30	72/M	Mesopharynx	IV	T2N2bM0

Quantitative RT-PCR analysis. Real-time PCR was carried out according to the methods described previously.[12] The sequence primers are listed in Table S1. The expression of glyceraldehyde-3-phosphate dehydrogenase was determined as an internal control.

Plasmids, siRNA, and transfection. Sialidase expression vectors were constructed by subcloning *NEU3* cDNA into an expression vector pCAGGS vector. Transient cDNA transfection was accomplished using FuGENE (Promega, Madison, WI, USA) for HSC-2 and SAS cells. For the NEU3 silencing, specific siRNA synthesized by Dharmacon (Lafayette, CO, USA) as described[12] was transfected using RNAiMAX (Invitrogen), and its efficiency was evaluated by RT-PCR.

Sialidase activity assay. Cell homogenates and the particulate fractions of tissue homogenates were prepared and assayed for sialidases NEU1 and NEU3 as described previously.[8] Briefly, for the assays, NEU1 sialidase activity was evaluated with synthetic substrate 4-methylumbelliferyl-neuraminic acid (4MU-NeuAc) at pH 4.6 at 37°C for 30–60 min, and the 4-methylumbelliferone released was determined fluorometrically. NEU3 activity was assayed with GM3 gangliosides as a substrate in the presence of 0.1% Triton X-100. The assays with the tissue particulates as the enzyme source were determined by the thiobarbituric acid method after passing through an AG1X-2 minicolumn. One unit was defined as the amount of enzyme that cleaved 1 nmol sialic acid/h. Protein concentrations were determined by dye-binding assay (Bio-Rad Laboratories, Hercules, CA, USA).

Immunoblotting. Cells were treated with or without EGF (100 ng/mL), washed with PBS and lysed in cold lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1% Nonidet P40, 2 mM EDTA, 7.5 μg/mL aprotinin, 10 μg/mL leupeptin, 10 mM NaF, 2 mM orthovanadate, and 2 mM PMSF). After centrifugation (12 000 *g* for 15 min), cellular lysates were subjected to SDS-PAGE and immunoblotting. For EGFR inhibition, the cells were treated with 10 μM of specific inhibitor AG1478 (Calbiochem, La Jolla, CA, USA).

Immunohistochemistry. Removed tissues were fixed in 10% neutral buffered formaldehyde for 3 days, routinely processed for embedding in paraffin, and sectioned at a thickness of 2.5 mm. The sections were incubated with anti-monoclonal NEU3 antibody.

Gelatin zymographic assay. The levels of gelatinases, MMP2 and MMP9, were measured by zymographic assay. Cells were cultured with serum-free medium for 16 h, and the conditioned medium collected was mixed with SDS buffer without reducing reagent. After SDS-PAGE on gels containing 0.1% gelatin (Sigma-Aldrich, St. Louis, MO, USA), the gels were washed with 2.5% Triton X-100 in Tris-HCl (pH 8.0), incubated with Tris-HCl (pH 8.0) containing 0.5 mM $CaCl_2$ and 1 mM $ZnCl_2$ at 37°C for 16 h, and then stained with 0.1% Coomassie R-250 (Bio-Rad Laboratories). Proteins with gelatinolytic activity were visualized as clear zones in an otherwise blue gel.

Cell motility and invasion assays. The assays for cell motility and invasion were carried out as previously described.[15] Cell motility assays were carried out with cell culture inserts (Corning, Tewksbury, MA, USA). At 24 h after transfection, cells were seeded at 2.5×10^5/well onto their upper surface membranes and the lower chambers were filled with medium containing 10% FBS. After 24 h the cells were fixed and stained with Wright–Giemsa solution and all those present on the

lower surfaces of the membranes were counted under a microscope. For the assay of invasive potential, 1×10^6 cells were incubated for 24 h with Biocoat Matrigel Invasion Chambers (Corning).

Thin-layer chromatography. Glycolipids were extracted from cells as described elsewhere,[9] fractionated by thin-layer chromatography on high-performance thin-layer chromatography plates (Merck, Darmstadt, Germany) and visualized with orcinol–H_2SO_4.

Statistical analysis. Results are expressed as mean ± SD. All values were compared using Student's *t*-test.

Results

Increased expression of sialidase NEU3 is associated with lymph node involvement. Among the four human sialidases, NEU1, NEU3, and NEU4 generally show evident expression,[5] whereas the level of NEU2 is extremely low and hardly detectable.[16] NEU4 expression is especially strong in liver, colon, and brain, but weak in other tissues,[17,18] as assessed by quantitative real-time RT-PCR. On the basis of these previous studies, we determined the expression levels of major sialidases, NEU3 and NEU1, by RT-PCR. Figure 1(a,b) shows the results of NEU3 and NEU1 expression in matched tumor (T) and adjacent non-tumor (N) mucosa as the T/N ratio. Increases in NEU3 and NEU1 mRNA levels in tumor compared with non-tumor tissue were detected in 22 (73%) and 18 (60%) in 30 cases, with T/N ratios of 6.67 ± 6.79 and 3.11 ± 3.94, respectively. According to the clinical features of the HNSCC patients, T/N ratios for NEU3 and NEU1 were analyzed, as shown in Table 2. Interestingly, a statistically significant difference in NEU3 mRNA level was observed

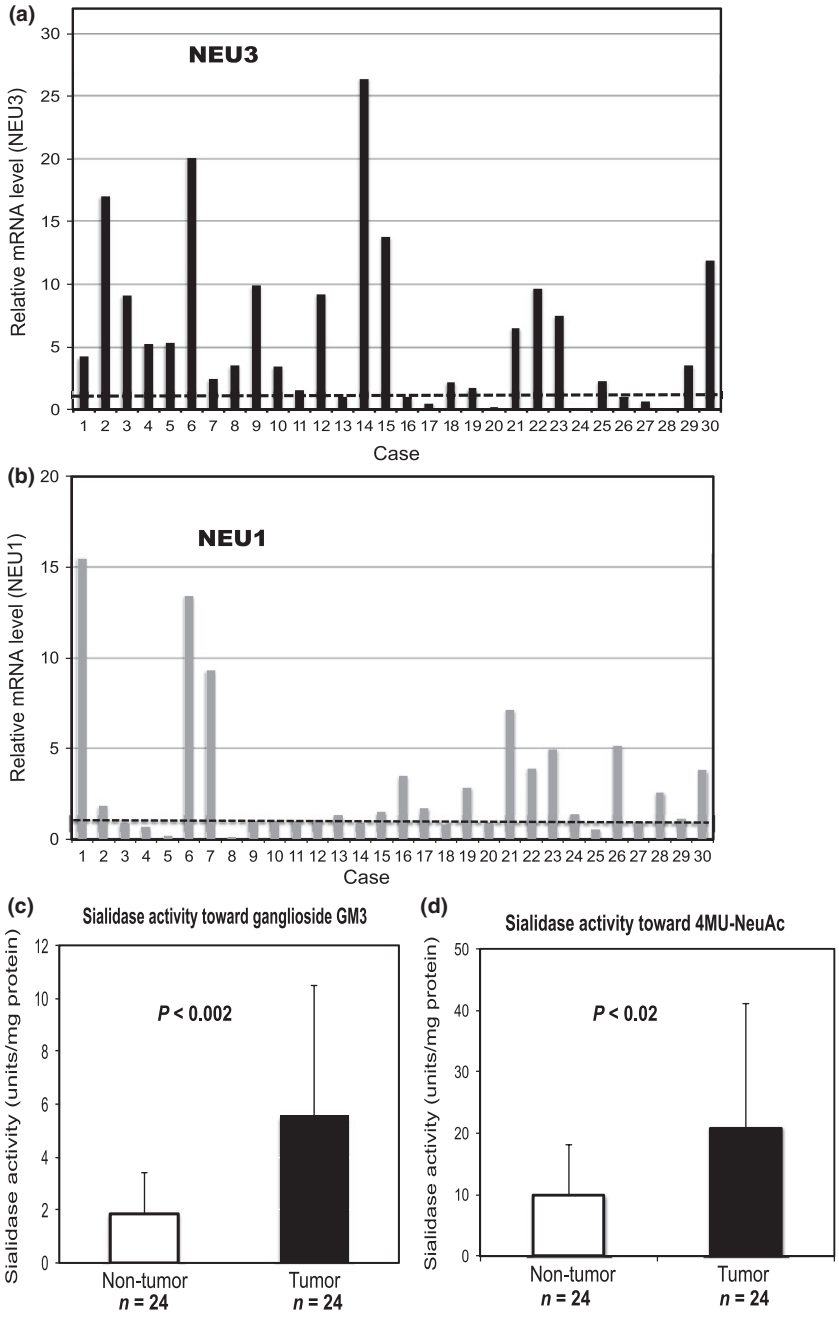


Fig. 1. Sialidase expression in matched tumor and adjacent non-tumor mucosa samples in head and neck squamous cell carcinoma. (a,b) Increased sialidase NEU3 (a) and NEU1 (b) mRNA levels in tumor compared with non-tumor mucosa. The endogenous levels were determined by quantitative RT-PCR. Dotted lines indicate a tumor/non-tumor ratio of one in each graph. (c) Increased sialidase activity toward ganglioside GM3 as the substrate in tumor compared with non-tumor mucosa (5.36 ± 5.12 and 1.84 ± 1.57 units/mg protein, respectively; $P = 0.0028$). (d) Increased sialidase activity toward 4MU-NeuAc as the substrate in the tumor compared to those in non-tumor mucosa (21.08 ± 20.79 and 10.15 ± 8.33 units/mg protein, respectively; $P = 0.024$). For evaluation of the apparent NEU3 and NEU1 activities, sialidase assays were carried out using ganglioside GM3 and 4MU-NeuAc, respectively.

Table 2. Clinical features of head and neck squamous cell carcinoma patients and association to sialidase NEU3 mRNA level in lymph node metastasis

TNM category		No. of patients	T/N ratio	*P*-value
NEU3				
Total		30	6.67 ± 6.79	
Stage	I–II	6	2.96 ± 4.04	0.055
	III–IV	24	6.88 ± 6.82	
Tumor size	T1–T2	12	7.36 ± 7.88	0.580
	T3–T4	17	6.06 ± 6.53	
Lymph node metastasis	N0	11	2.81 ± 2.89	0.019*
	N1–N3	19	7.79 ± 7.17	
NEU1				
Total		30	3.11 ± 3.94	
Stage	I–II	6	2.61 ± 2.59	0.470
	III–IV	24	3.23 ± 4.20	
Tumor size	T1–T2	12	2.17 ± 2.03	0.190
	T3–T4	17	3.16 ± 4.05	
Lymph node metastasis	N0	11	3.01 ± 2.84	0.490
	N1–N3	19	3.16 ± 4.45	

*Significant difference was observed between lymph node metastasis N0 and N1–N3 in sialidase NEU3 mRNA level.

between cases of lymph node metastasis N0 and N1–N3 ($P = 0.019$), indicating a close association between NEU3 expression and lymph node metastasis. To confirm whether the sialidase activity level changes in association with the metastasis, the activity assays were carried out using ganglioside GM3 for NEU3, and 4MU-NeuAc for NEU1 as the substrate, although the values do not exactly represent the activity of each sialidase type because of substrate redundancy among the sialidases. As shown in Figure 1(c,d), the activity levels were compared between tumor and adjacent non-tumor mucosa in 23 of the above 30 cases. The activities of putative NEU3 and NEU1 were significantly higher in the tumor tissues than in mucosa (T/N ratios, 4.01 ± 3.92 and 2.26 ± 1.86, respectively), although there was no statistically significant correlation with the clinical features, including lymph node involvement, for the activity levels of NEU3 and NEU1. This may be attributable to the involvement of some NEU4-dependent activity that could have been included, despite such activity being expected to be low, because NEU4 can act on both GM3 and 4MU-NeuAc.

To verify the marked expression of NEU3, immunohistochemical staining was then carried out with anti-NEU3 mAb. The tumor tissues of 10 cases having strong NEU3 mRNA expression were chosen for the analysis. As shown in Figure 2, the representative results of immunohistochemical analyses indicated marked expression of NEU3, with strong staining in tumor tissues, but almost no expression in non-tumor tissues. All of the 10 cases examined gave similar patterns. These results together show that sialidase NEU3 was significantly increased in terms of its mRNA level as well as its activity and protein levels in tumor tissues compared with those in the mucosa; in particular, it had strong association with lymph node involvement. We then focused on NEU3 in terms of its relationship to lymph node metastasis.

Upregulation of NEU3 enhances cell migration and invasion in squamous cell carcinoma. To elucidate the role of NEU3 upregulation in HNSCC, oral squamous cell carcinoma HSC-2 and SAS cells were used to overexpress or silence the *NEU3* gene. Endogenous expression of the four sialidases in the cells was first evaluated by quantitative RT-PCR (Fig. 3a). To compare the levels among these forms, a standard curve for each cDNA was generated by serial dilution of the pBluescript vector containing the gene encoding the entire ORF, as described previously.[18] As expected, NEU2 was not detectable and the level of NEU4 was extremely low, being at most only one-thousandth of the levels of NEU1 and NEU3 in these cells, suggesting little involvement of NEU2 and NEU4 in the increased sialidase expression in the carcinomas. It should be noted here that the carcinoma cells showed an NEU3 level of approximately one-quarter that of NEU1, probably due to its upregulation in carcinogenesis, because the NEU3 level is generally one-tenth to one-twentieth that of NEU1 in normal tissues.

To determine the relationship between NEU3 upregulation and the invasive phenotype of the two squamous cell carcinoma cells, cell migration and invasiveness were determined (Fig. 3b). NEU3 transient transfection yielded an increase of GM3 hydrolyzing activity by approximately 60- and 25-fold in HSC-2 and SAS cells, respectively, and apparently enhanced the cell motility compared with the vector control in both these cells. In contrast, its silencing resulted in a significant decrease of cell motility in HSC-2 and SAS cells (78% and 85% reduction in mRNA levels, respectively). For the invasion assay, both HSC-2 and SAS cells showed decreased invasiveness after NEU3 silencing. Cell proliferation ability was hardly changed by NEU3 alteration in these cells (data not shown).

NEU3 alters MMP expression through EGFR signaling. To understand the mechanism by which NEU3 affects cell motility and invasion, we then studied the expression of several genes associated with these cellular phenotypes, including epithelial–mesenchymal transition and MMP genes in HSC-2 and SAS cells, by RT-PCR. Despite lacking statistical significance, E-cadherin expression levels showed a tendency to decrease with NEU3 overexpression and, in contrast, a tendency to increase with NEU3 silencing in HSC-2 cells. N-cadherin expression was clearly reduced in NEU3-silenced cells (Fig. 4a, upper). In SAS cells, NEU3 significantly increased, and its silencing decreased N-cadherin levels (Fig. 4a lower). However, the alteration of NEU3 expression did not show any particular changes in cell morphology, nor constant influence on the gene expression of *Snail*, *Twist*, or *Vimentin* in these cells. As a number of reports have described that MMP is essential to the invasiveness of HNSCC,[19,20] the expression of membrane-type 1 (MT1)-MMP, MMP2, and MMP9 was also examined in these cells (Fig. 4b). Although NEU3 overexpression enhanced only MMP9 expression, silencing of NEU3 caused significant decreases in both MMP2 and MMP9, and no change in MT1-MMP, in both HSC-2 and SAS cells, indicating that the alteration of NEU3 expression profoundly influences MMP expression. To define the mechanism behind the NEU3-mediated effect on MMP in more detail, we examined the impact of EGF on MMP expression, because EGFR activation has been found to occur with NEU3 upregulation[12] and also to be the most common genetic event in oral squamous cell carcinoma[1,21,22] causing MMP stimulation.[23,24] Figure 5(a) shows that NEU3 silencing downregulated the expression of MMP9 and, in particular, caused a marked decline in its

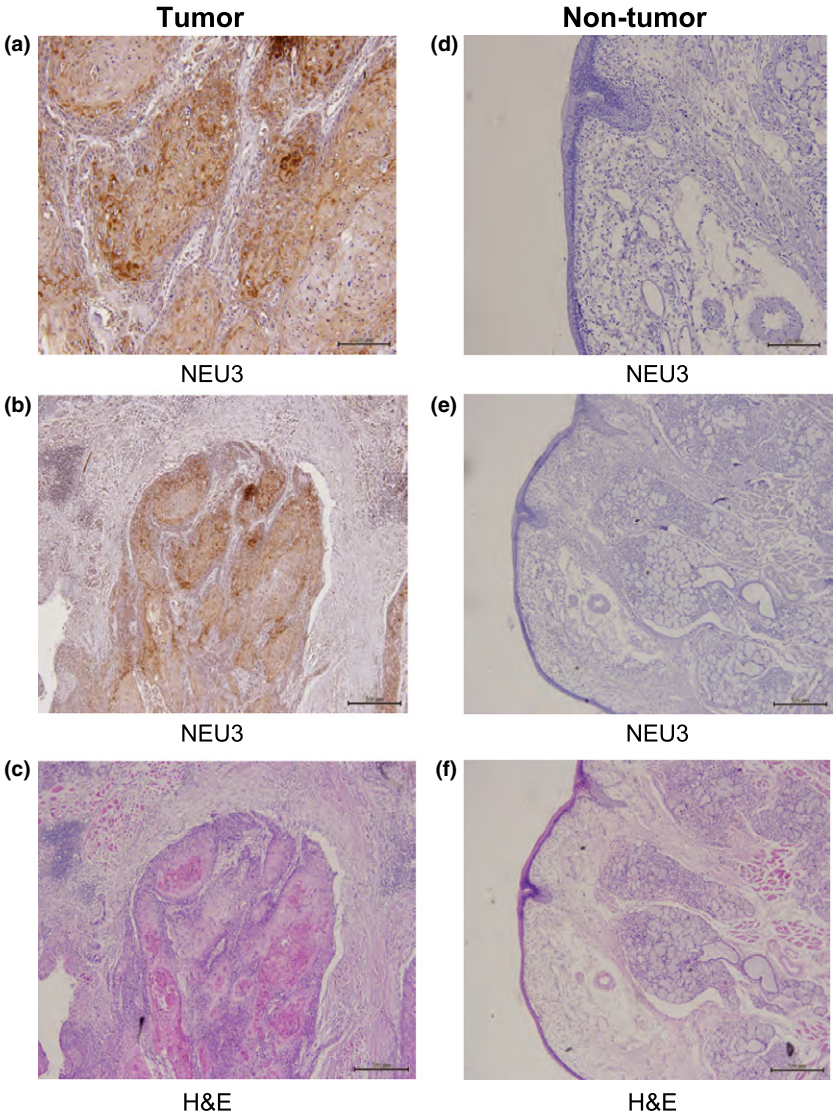


Fig. 2. Immunohistochemistry of tumor and non-tumor tissues from head and neck squamous cell carcinoma patients with anti-NEU3 mAb. Sections from tumor and non-tumor tissues were stained with anti-NEU3 mAb (a,b,d,e) and H&E (c,f). Bar = 200 μm (a,d), and 500 μm (b,c,e,f).

EGF-induced upregulation in both carcinoma cell lines, whereas MMP2 expression was hardly affected by EGF addition (data not shown). On the basis of these results, MMP9 expression was evaluated in terms of whether it is influenced by EGFR activation. Consistent with these findings on mRNA levels, gelatin zymography analyses confirmed that MMP9 secretion was increased in a manner dependent on EGF and was decreased by NEU3 silencing (Fig. 5b). Next, EGFR signaling was investigated in these cells. Figure 5(c) shows that NEU3 enhanced the phosphorylation of EGFR and subsequent activation of ERK and Akt in HSC-2 cells, whereas NEU3 silencing in SAS cells resulted in the opposite effect. Furthermore, NEU3-mediated elevation of MMP9 was abrogated by EGFR inhibitor AG1478 in HSC-2 and SAS cells, indicating the involvement of NEU3 in the regulation of MMP expression, probably through EGFR signaling (Fig. 5d). It should be noted here that, even without EGF, NEU3 slightly but significantly elevated MMP9 expression in both cells, which is likely to be attributed to NEU3-mediated activation of the EGFR pathway under the same conditions, as shown in Figure 5(c).

NEU3-mediated enhancement of cell migration requires its catalytic activity. Human NEU3 is a ganglioside hydrolyzing sialidase[5] and has recently been proved to activate EGFR signaling through its catalytic activity.[25] Alterations of the cellular ganglioside pattern in NEU3-overexpressing HSC-2 cells were analyzed by thin-layer chromatography. As shown in Figure 6(a), NEU3 overexpression resulted in a decrease in the glycolipid with similar mobility to GM3, a good substrate of NEU3, together with a slight increase in that with similar mobility to lactosylceramide, a product of NEU3, in HSC-2 cells, as confirmed statistically by three independent experiments (Fig. 6a, lower). To verify whether the cell motility is altered by glycolipid changes, GM3 was then added exogenously to the chamber. Figure 6(b) shows the GM3 significantly reduced the cell motility, as expected. Although these glycolipid changes are generally observed in most cells by NEU3 overexpression,[25] the activity-null mutants did not show any change in glycolipids. In this context, we used one of these NEU3 mutants (Y370C) to assess the requirement for this molecule's activity for NEU3-mediated increased cell migration. Compared with the cells transfected with wild-type NEU3, the mutant did not enhance cell migration, similar to

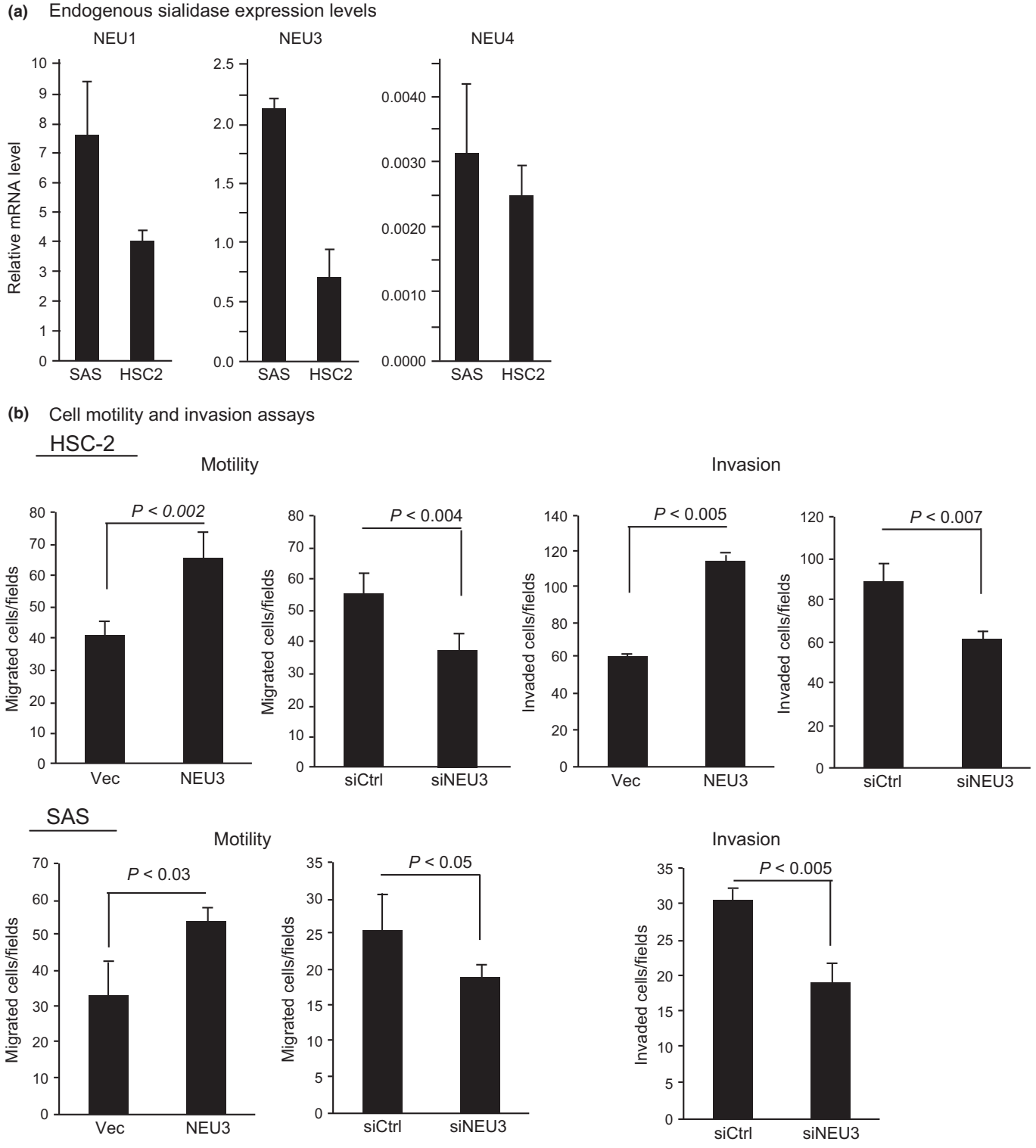


Fig. 3. Regulation of cell motility and invasion by sialidase NEU3 in squamous cell carcinoma HSC-2 and SAS cells. (a) Evaluation of endogenous sialidase mRNA levels in the two carcinoma cell lines by quantitative RT-PCR. (b) Enhanced cell motility and invasion in NEU3 transiently transfected cells (NEU3) and its suppression in NEU3 silenced cells (siNEU3). The sialidase activities with GM3 substrate were 158.7 ± 49.6 and 2.5 ± 1.1 units/mg protein for HSC-2 cells and 138 ± 37 and 5.5 ± 2.1 units/mg protein for SAS cells in NEU3-transfected cells (30–40% transfection efficiency assessed by simultaneous transfection with GFP-tagged cDNA) and vector control cells (Vec), respectively. The efficiency levels of NEU3 silencing were 78% and 85% in terms of mRNA in HSC-2 and SAS cells, respectively. siCtrl, control siRNA.

the control cells. These results suggest that NEU3-dependent glycolipid changes induced by its catalytic action may be necessary for its enhancement of cell migration (Fig. 6c), although other mechanisms including the molecular interaction of NEU3 with EGFR[25] or phosphatidic acid[26] cannot be excluded in the cellular event.

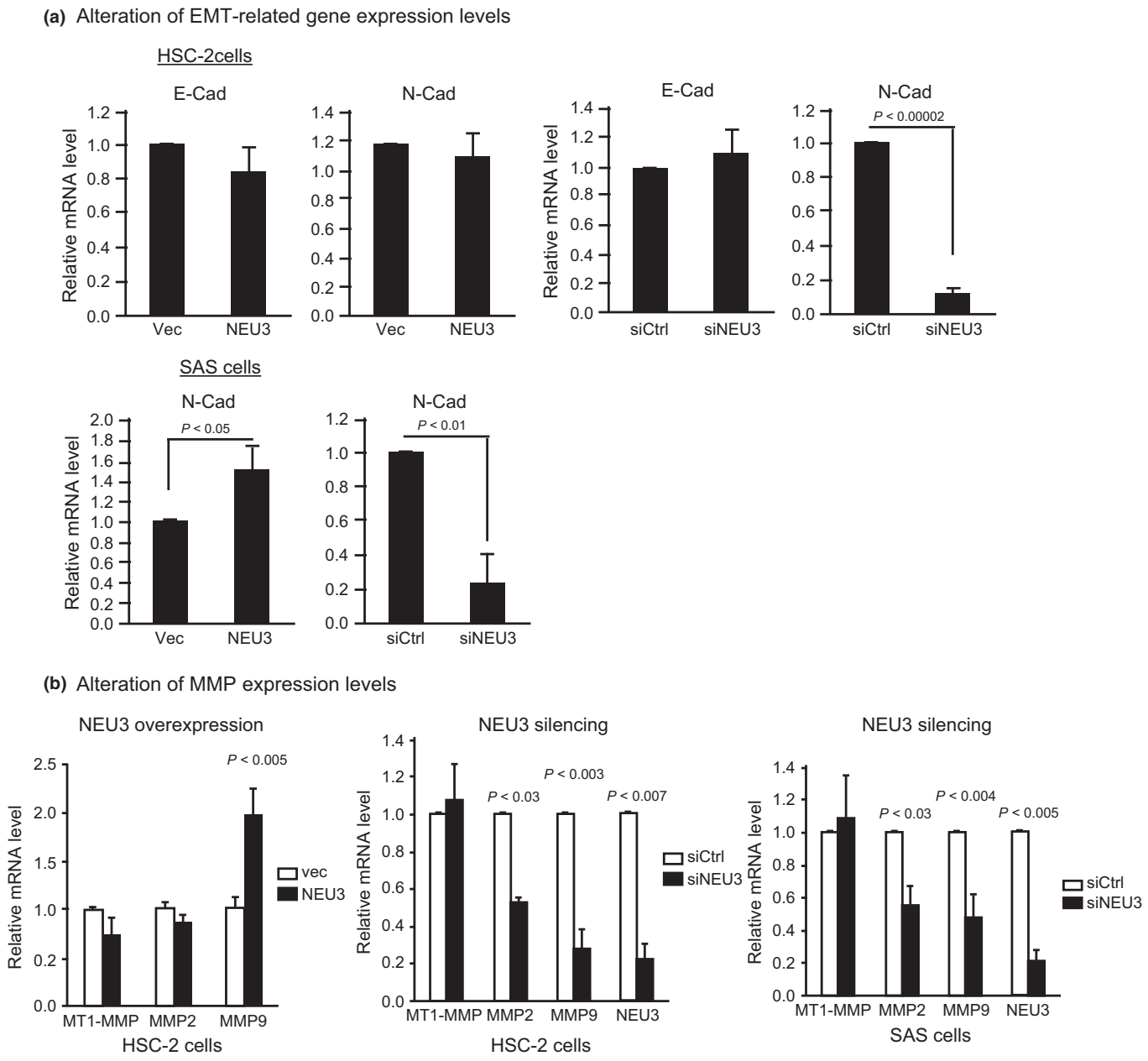
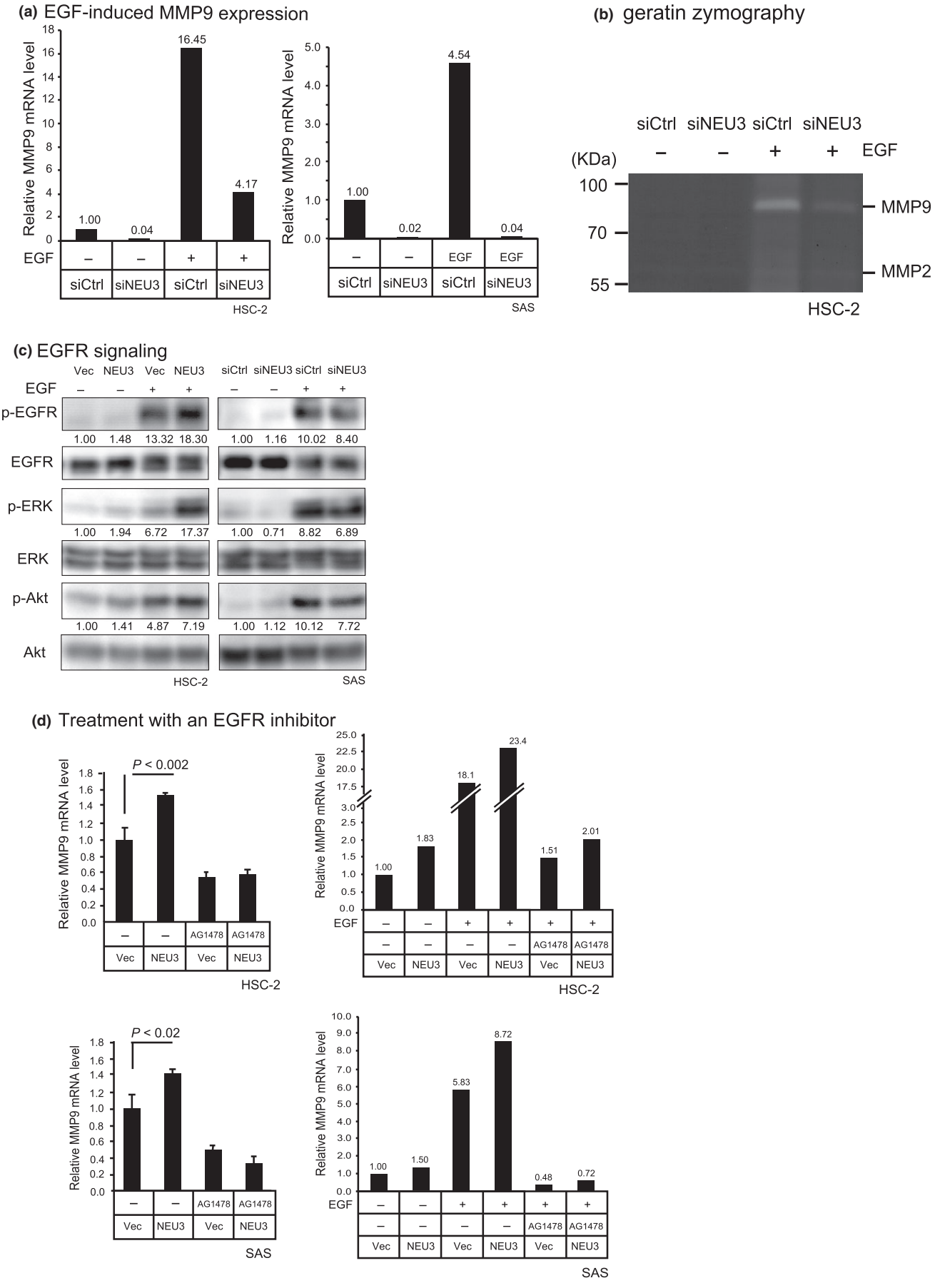


Fig. 4. Regulation of epithelial–mesenchymal transition (EMT)-related and MMP gene expression by NEU3 in squamous cell carcinoma HSC-2 and SAS cells. (a) Alterations of E-cadherin (E-Cad) and N-cadherin (N-Cad) mRNA levels in NEU3-overexpressing or NEU3-silenced HSC-2 cells. (b) Alterations of membrane-type 1 (MT1)-MMP, MMP2, and MMP9 mRNA levels in NEU3-overexpressing HSC-2 cells and in NEU3-silenced HSC-2 and SAS cells. siCtrl, control; Vec, vector.

Discussion

Lymph node metastasis is a critical event in HNSCC progression. Identification of the molecules associated with metastasis would provide information to facilitate diagnosis and treatment of this cancer. Molecules involved in regulation of tumor metastasis have been focused on the ECM, such as fibronectin[27] and laminin,[28] and on their transmembrane matrix receptors, integrins.[29] Cell surface glycoproteins including cadherin[30] and neural cell adhesion molecule[31] have also

Fig. 5. Involvement of NEU3 in epidermal growth factor (EGF)-induced elevation of MMP9 expression in head and neck squamous cell carcinoma cells. (a) Attenuation of EGF-induced MMP9 elevation in NEU3-silenced HSC-2 and SAS cells in terms of mRNA levels. (b) Attenuation of EGF-induced MMP2 and MMP9 secretion and activity by NEU3 silencing in HSC-2 cells, as assessed by gelatin zymography. (c) Enhanced phosphorylation of EGF receptor (EGFR), ERK, and Akt by NEU3 overexpression and its reduction by NEU3 silencing, as assessed by Western blotting. Each value shown under the blot represents a value relative to that in the vector control (Vec) without EGF. (d) Abrogation of NEU3-mediated as well as EGF-induced augmentation of MMP9 expression by EGFR inhibitor AG1478.



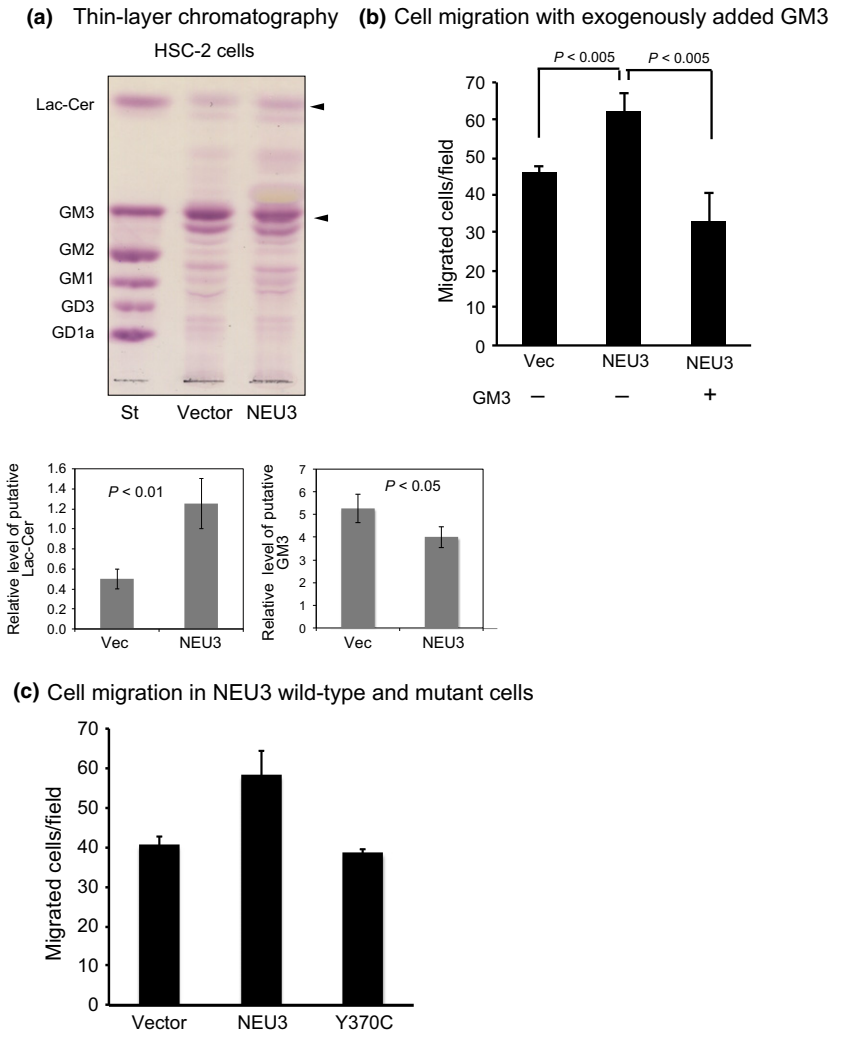


Fig. 6. Requirement of NEU3 catalytic activity for the enhanced motility of HSC-2 cells. (a) Analysis of altered glycolipids as a result of NEU3 catalytic reaction by thin-layer chromatography. The relative changes in putative lactosylceramide (Lac-Cer) and GM3 were quantified in independent experiments carried out in triplicate, as shown in the graphs below. St, standard gangliosides. (b) The effects of exogenously added GM3 on cell motility. The chambers were filled with medium containing GM3 at final concentrations of 20 μM, and the medium in lower chambers additionally contained 10% FBS. (c) Abrogation of NEU3-mediated increase in cell motility in NEU3 mutant (Y370)-transfected cells (sialidase activity, 1.9 ± 0.5 units/mg protein).

been thought to mediate the tissue-specific recognition process that is impaired in tumor progression and metastasis. In addition to these glycoproteins, gangliosides, components of cell-surface membranes, exert a wide variety of biological functions, including alterations associated with cell growth, differentiation, and tumorigenesis.[4] Sialidases are key enzymes to regulate degradation of glycoproteins and gangliosides by the removal of sialic acid residues. In the present study, we investigated sialidase expression and found ganglioside-specific sialidase NEU3 to be significantly upregulated in specimens of HNSCC patients with lymph node metastasis. NEU1 might also influence the malignant phenotype through degradation of glycoproteins, although there was no statistical significance in our findings. Our previous observations showed that NEU3 was upregulated in various human cancers and its involvement in the expression of malignant properties differed depending on the cancer types. However, the present study revealed a novel function of NEU3 in HNSCC, namely, participation in the regulation of MMP2 and MMP9 expression through activation of EGFR signaling, leading to enhanced cell invasion and migration.

Previous observations by others have identified the clinically correlated biomarkers, including EGFR and MMP9, as predictors of prognosis in HNSCC.[1,19–24] Our results here show that NEU3 is essentially involved in regulation of the expression and activation of these genes upstream of EGFR. Aberrant upregulation of NEU3 in this carcinoma probably elevates the phosphorylation of EGFR and subsequently leads to ERK activation, followed by MMP activation and secretion, ending with enhanced cell invasion. It is of particular interest that *NEU3*[32] as well as *EGFR*, *MMP2*, and *MMP9*[33] are all target genes of transcription factors Sp1 and Sp3, which were initially considered as constitutive activators of housekeeping genes and other TATA-less genes, but are now known to play crucial roles in regulating the transcription of genes involved in cell growth control and tumorigenesis.[33] Therefore, the activation of EGFR signaling and consequent increased expression of MMP caused by NEU3 upregulation seem to influence each other in a positive feedback manner, because Sp1 and Sp3 can also be activated through Ras/ERK signals.

Consistent with our recent data,[25] we showed that NEU3-mediated activation of EGF signaling occurs in a manner dependent on the sialidase catalytic reaction, with the putative product lactosylceramide possibly affecting EGFR phosphorylation. Figure 6 reveals that abrogation of NEU3-mediated enhancement of cell motility occurred in an NEU3 activity-null mutant. In addition to the deletion of NEU3 activity, the present data provided evidence that NEU3 silencing reversed the malignant phenotype, probably due to accumulation of

GM3, as evidenced by results of the experiment with exogenously added GM3. Chemoprevention trials with erlotinib or cetuximab aimed at EGFR inhibition have been encouraging but remain unclear.[2] The present findings identify NEU3 as a pivotal molecule regulating the EGFR signaling pathway and MMP expression upstream of EGFR through ganglioside modulation, which is a signaling event closely associated with lymph node metastasis of HNSCC. Thus, NEU3 is a potential target for the development of cancer treatment.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Primer sequences used in this study.