Aberrant Myosin 1b Expression Promotes Cell Migration and Lymph Node Metastasis of HNSCC

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Abstract

Lymph node metastasis is the major clinicopathologic feature associated with poor prognosis in patients with head and neck squamous cell carcinoma (HNSCC). Here, web-based bioinformatics meta-analysis was performed to elucidate the molecular mechanism of lymph node metastasis of human HNSCC. Preferential upregulation of Myosin 1b (MYO1B) transcript in HNSCC datasets was identified. Myo1b mRNA was highly expressed in human HNSCC cells and patient tissue specimens compared with their normal counterparts as shown by quantitative PCR (qPCR) analyses. Immunohistochemistry (IHC)-detected Myo1b expression was significantly correlated with lymph node metastases in patients with oral cancer of the tongue. HNSCC with high expression of Myo1b and chemokine receptor 4 (CCR4), another metastasis-associated molecule, was strongly associated with lymph node metastasis. RNA interference (RNAi) of Myo1b in HNSCC cells, SAS and HSC4, significantly inhibited migratory and invasive abilities through decreased large protrusion formation of cell membranes. Finally, Myo1b knockdown in SAS cells significantly inhibited in vivo cervical lymph node metastases in a cervical lymph node metastatic mouse model system.

Implications: Myo1b is functionally involved in lymph node metastasis of human HNSCC through enhanced cancer cell motility and is an attractive target for new diagnostic and therapeutic strategies for patients with HNSCC. Mol Cancer Res. 13(4); 721–31. ©2014 AACR.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy worldwide (1). Despite improved locoregional control of HNSCC (2), the 5-year survival rate for HNSCC remains relatively unchanged, at under 50% for the past three decades (3). HNSCC tends to metastasize to lymph nodes before distant metastasis (4–6). Because lymph node metastasis is strongly associated with poor prognosis in patients with HNSCC (4, 7–9), understanding the mechanisms that underlie lymph node metastasis of HNSCC is important for improving diagnostic and therapeutic strategies.

Cancer cells use their intrinsic migratory ability to invade adjacent tissues and ultimately to metastasize to different organs. Cell migration is a highly integrated multistep process, initiated by protrusion of the cell membrane. Aberrant regulation of cell migration drives cancer invasion and metastasis. The protrusive structures formed by migrating and invading cancer cells are filopodia, lamellipodia, and invadopodia/podosomes, depending on their morphologic, structural, and functional characters (10). However, the mechanisms of human HNSCC cell migration and metastasis remain poorly understood.

Here, we investigated the mechanisms of lymph node metastasis of human HNSCC. Myosin 1b (Myo1b, also named myosin 1 alpha or Myr1), a member of the myosin family, was found to be upregulated in HNSCC through a web-based bioinformatics meta-analysis. Myo1b, a class 1 myosin, is a widely expressed, single-headed, actin-associated molecular motor, associated with cell migration of zebrafish embryo cells (11) and intracellular transport regulated by the formation of post-Golgi carriers (12, 13). However, its role in cancer biology has never been reported. Aberrant expression of Myo1b immunohistochemically (IHC) detected was correlated with lymph node metastases in patients with HNSCC (n = 31, P = 0.0320). Myo1b knockdown in human HNSCC cell lines by RNA interference inhibited in vitro migration and invasion abilities of the HNSCC cells accompanied by decreased large protrusions formation in cell membrane. Downregulation of Myo1b in human HNSCC cell lines resulted in inhibition of metastasis to cervical lymph nodes in cervical lymph node metastatic model using nude mice implanted with human HNSCC. These results demonstrate that Myo1b is involved in cancer cell motility and lymph node metastasis of human HNSCC, implying that Myo1b could be a novel diagnostic and therapeutic target for patients with HNSCC.
Materials and Methods
Patients and clinical samples
A total of 31 patients with tongue cancer for IHC and 7 patients with HNSCC for quantitative PCR assays, who underwent surgery at Kyoto Prefectural University of Medicine (Kyoto, Japan) between April 2008 and April 2012, were enrolled in a retrospective study. Data were collected from clinical and pathologic records with the written informed consent of individual patients after approval by the Ethics Committee of the institutes.

In silico gene expression studies
We used the Oncomine database (Compendia Bioscience; http://www.oncomine.org) to identify upregulated genes in HNSCC on May 26, 2013 and performed a microarray meta-analysis to compare all genes across 13 different datasets (14–22) that were identified by the following parameters: “mRNA,” “cancer vs. normal analysis,” and “head and neck cancer” (excluding nasopharyngeal cancer, salivary gland cancer, and thyroid cancer). To compare Myo1b expression, Myo1b expression fold changes were limited to P < 0.05.

Cell culture
Human oral tongue cancer cell lines, SAS and HSC-4, were purchased from RIKEN Bioresource Center Cell Bank (Tsukuba, Japan). Cells were cultured in RPMI-1640 supplemented with 10% FBS, penicillin (50 units/mL), and streptomycin (50 mg/mL) and were incubated at 37°C in a humidified chamber supplemented with 5% CO2. Human hypopharyngeal cancer, FaDu and Detroit562, human oral tongue cancer, HSC-3, human Hodgkin lymphoma, L-428, human melanoma, Skmel23, 92smel, and A375smel, human brain tumor, U87MG, human esophageal cancer, TE4 and TE9, lung cancer, LK2 and EBC1, pancreatic ductal adenocarcinoma, PK59, prostate cancer, PC3, breast cancer, Hs578 and MDA-MB-231, chronic myeloid leukemia, K562, acute myeloid leukemia, HL60, was obtained and cultured as previously reported (23–25). Human brain tumor, T98G, human cervical cancer, C33a and HeLa, were purchased from the ATCC and cultured in DMEM as well as in RPMI-1640. Human cervical cancer, SKG1, was a kind gift from Dr. Daisuke Aoki (Keio University, Tokyo, Japan) and cultured in DMEM as well as in RPMI-1640. SAS, HSC4, HSC3, Detroit526, FaDu, L-428, A375smel, U87MG, T98G, TE4, TE9, C33a, SKG1, HeLa, PK59, Hs578, and MDA-MB-231 were authenticated by short tandem repeat profiling.

Evaluation of mRNA and protein expression
Total RNA was isolated from cells using an RNeasy Mini Kit (Qiagen) with on-column DNase treatment (Qiagen), and TissueRuptor (Qiagen) was used in the total RNA isolation from patient tissue samples. cDNA was synthesized using Superscript III Reverse Transcriptase and Oligo (dT) 12–18 primers (Invitrogen). Quantitative real-time PCR was performed using a TaqMan probe (Applied Biosystems). Myo1b (Hs00362654_m1), Snail1 (Hs00195591_m1), Twist1 (Hs01675818_s1), CDH1 (Hs01023895_m1), and GAPDH (Hs03929097_g1), on ABI PRISM 7900 HT Sequence Detection System with TaqMan Gene Expression Master Mix (Applied Biosystems). Target gene expression was standardized to GAPDH levels using SDS Software v2.2 (Applied Biosystems). Relative expression of Myo1b mRNA was shown after normalization to the Myo1b expression level in HSC3 as the baseline value (1). Western blotting was performed by general procedure with rabbit anti-hMyo1b pAb (Sigma-Aldrich) and rabbit anti-GAPDH pAb (Santa Cruz Biotechnology).

Myo1b knockdown
siRNAs specific for human Myo1b (si-Myo1b-1; HSS106714 and si-Myo1b-2; HSS106715, Stealth Select RNAi), as well as scrambled siRNA (Stealth RNAi Negative Control Kit, medium GC), were purchased from Invitrogen. These siRNAs (final concentration during transfection, 100 nmol/L) were transfected into cells using Lipofectamine RNAi MAX (Invitrogen) according to the manufacturer’s recommendations. Assays were carried out 72 hours after transfection. Lentiviral pGIPZ shRNA vectors targeting human Myo1b (sh-Myo1b-1; V3LHS_356101, sh-Myo1b-2; V3LHS_356104) and nonsilencing pGIPZ control vector (sh-NC) were purchased from Open Biosystems (Thermo Fisher Scientific, Inc.), and they were integrated with turboGFP and puromycin resistance sequences. Lentivirus particles were produced in 293T cells according to the manufacturer’s instructions. The viral titer was calculated by evaluating GFP expression in infected 293T cells. SAS cells were transduced by the lentiviral particles in the same viral titer. The cells stably expressing shRNA were selected, pooled, and maintained with 2 μg/mL puromycin (Wako).

In vitro migration, invasion, and cell proliferation assays
Cell migration, invasion, or proliferation was evaluated by real-time monitoring using the xCELLigence RTCA DP Instrument (Roche Applied Science), as described before (26, 27). Briefly, for Transwell migration and invasion assays, 4 × 105 cells suspended in medium were seeded with plain medium in each upper chamber of a CIM-Plate 16 with 8-μm pores (Roche Applied Science). The plate was then monitored for 48 hours with 10% FBS-containing medium in the lower chambers. In addition, for invasion assays, 2.5% BD Matrigel matrix (BD Biosciences) was placed on upper chamber. For in vitro cell proliferation assays, 2 × 104 cells suspended in medium containing 10% FBS were seeded per well in an E-plate 16 (Roche Applied Science) and monitored for 48 hours. We acquired and analyzed data at some time points with RTCA software (version 1.2, Roche Applied Science). The correlation between cell index and manually counted cell number was previously confirmed (24).

Wound-healing assays
Cells were grown to confluence in 24-well dishes (BD Falcon). Scratch wounds were then made with sterile plastic 200-μl pipette tips. After washing and a change of medium, microscopic images of healing areas were photographically recorded at 0 and 12 hours after scratching. Healing area was calculated using Axio Vision LE software (version 4.7.2.0, Carl Zeiss Imaging Solutions GmbH).

Epithelial-to-mesenchymal transition induction by TGF-β1
Cells were prepared with recombinant human TGF-β1 (R&D Systems; 240-B, 2 ng/mL) for 24 hours. Their total RNA was then isolated.

Immunohistochemistry
Human HNSCC clinical samples embedded in paraffin blocks were cut into 4-μm-thick sections. After deparaffinization, sections were then pretreated with an antigen retrieval solution, HistoVTone (Nacalai Tesque), for 20 minutes at 90°C.
Endogenous peroxidase activity was then blocked by incubating with 1% hydrogen peroxide for 30 minutes at room temperature. After blocking in 1% normal goat serum, the specimens were incubated with primary antibody (rabbit anti-hMyo1b pAb, at 1:75; Sigma-Aldrich) at 4°C overnight. This pAb was also used in Western blotting. Negative controls were incubated with rabbit IgG in place of the primary antibody. The streptavidin-biotin method was performed with a Histofine Simple Stain (R) kit (Nichirei) according to the manufacturer's instructions. The sections were developed with 3,3’-diaminobenzidine (DAB) in 0.003% hydrogen peroxide (Muto Pure Chemicals) and counterstained lightly with hematoxylin. IHC staining for human CCR4, CCR7, and CXCR4 was performed and the results were interpreted as previously reported (24). Anti-hCCR4 mAb (Yowa Hakko Kōrin), anti-hCCRF mAb (BD Biosciences), and anti-hCCXCR4 mAb (R&D Systems) were used. IHC staining to detect lymphatic organs were collected and dissociated individually by mechanical disruption and incubation with collagenase for 30 minutes at 37°C. Collagenase activity was stopped, and samples were processed with Texas Red-X phalloidin (Molecular Probes; diluted 1:100) in PBS. Images were taken with a Zeiss LSM 700 Laser Scanning Microscope and analyzed using the LSM Software ZEN 2009 (Carl Zeiss). Cells having large protrusions of cell membrane were evaluated that by quantitative PCR assays on patient samples and confirmed to be aberrantly overexpressed in HNSCC datasets among various cancer types by the in silico gene expression analysis (Fig. 1B). Although SERPINC1 (also known as SERPINH1) was found to be aberrantly overexpressed in 3 of 5 human HNSCC cell lines, SAS, HSC4, and Detroit562, especially tongue cancer cell lines SAS and HSC4 (Fig. 1D). Analysis on DNA methylation status of Myo1b mRNA found to be aberrantly overexpressed in HNSCC tissues compared with adjacent normal tissues from healthy donor (Oncomine; see Materials and Methods). The overexpressing genes were ranked by median-rank analyses across each of 13 analyses. Myo1b was identified as one of the mostly upregulated genes in HNSCC tissues (Fig. 1A and Supplementary Fig. S1). Interestingly, Myo1b was preferentially overexpressed in the HNSCC datasets among various cancer types by the in silico gene expression analysis (Fig. 1B). Although SERPINC1 (also known as SERPINH1) was the highest ranked gene, it was previously shown to be involved in cancer progression of cervical squamous cancer (33). Therefore, in this study, we further evaluated the roles of Myo1b in the HNSCC progression. We have confirmed that by quantitative PCR assays on patient samples and cancer cell lines. Myo1b mRNA found to be aberrantly overexpressed in HNSCC tissues compared with adjacent normal tissues obtained from the same individuals (Fig. 1C and Supplementary Fig. S2; n = 7, P = 0.0253). Myo1b mRNA was highly expressed in 3 of 5 human HNSCC cell lines, SAS, HSC4, and Detroit562, especially tongue cancer cell lines SAS and HSC4 (Fig. 1D). Analysis on DNA methylation status of Myo1b promoter region using The Cancer Genome Atlas (TCGA) did not show significant alterations of DNA methylations in HNSCC (data not shown). Because the expression of EGFR which is expressed in HNSCC cells was previously reported to be associated with one of the myosin family, myosin6, in lung cancer cell line (34), the relationship...
between EGFR and Myo1b was examined, and it was found that Myo1b knockdown did not affect cell surface protein expression of EGFR in SAS and HSC4 cell lines, and stimulation of EGFR did not increase the expression of Myo1b mRNA and protein either in SAS and HSC4 cell lines (data not shown). Therefore, Myo1b is not involved in the expression and function of EGFR in HNCC cell lines. High Myo1b expression may be involved in the pathogenesis of HNCC through aberrant expression, although the mechanisms remain to be investigated.

Correlation of Myo1b expression with lymph node metastases of human HNSCC

Expression of Myo1b protein in human HNSCC was evaluated by IHC studies, and Myo1b was detected in all 31 human tongue cancer tissues evaluated but was not expressed in adjacent normal tissues (Fig. 2 and Supplementary Fig. S4A and S4B), which is consistent with results of the gene expression analysis. Three representative samples with different staining patterns as described in the Materials and Methods are shown in Fig. 2.

**Figure 1.** Myo1b is highly expressed preferentially in human HNSCC. Myo1b mRNA is significantly upregulated in HNSCC. A, a microarray meta-analysis was performed using Oncomine to compare all genes across 13 different datasets, which included 222 patients with HNSCC and 135 healthy individuals (see Materials and Methods). Myo1b was identified as one of the most upregulated genes in HNSCC tissues. The top 20 overexpressed genes identified by meta-analysis are shown. A gene's rank is its median rank across each of the analyses. P value: gene's P value for the median-ranked analysis. Black and white indicates expression level in each analysis, and the diagonal line indicates no analysis. B, Myo1b is preferentially overexpressed in HNSCC. Fold changes of Myo1b mRNA expression in various cancer types are plotted for comparison. C, Myo1b mRNA expression was significantly upregulated in cancer tissues compared with normal tissues from the same individuals by quantitative PCR analysis (n = 7, *P = 0.0253, Mann-Whitney U test). Relative expression of Myo1b mRNA was standardized by using GAPDH gene expression as an internal reference, and each value was shown after normalization to the Myo1b expression level in HSC3 as the baseline value (= 1). D, Myo1b mRNA was highly expressed in 3 of 5 human HNSCC cell lines, particularly tongue cancer cell lines, SAS and HSC4 among various cancer cell lines. Relative expression of Myo1b mRNA was standardized by using GAPDH gene expression as an internal reference, and each value was shown after normalization to the Myo1b expression level in HSC3 as the baseline value (= 1): representative of 3 separate experiments.
Correlation between Myo1b and clinicopathologic features

**Table 1.**

<table>
<thead>
<tr>
<th>Clinicopathologic factors</th>
<th>Myo1b IHC score</th>
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<td>Sex</td>
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<td></td>
</tr>
<tr>
<td>Female</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Male</td>
<td>11</td>
<td>11</td>
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<tr>
<td>Age (y)</td>
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<td></td>
</tr>
<tr>
<td>&lt;70</td>
<td>10</td>
<td>7</td>
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<td>≥70</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>T stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤2</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>≥3</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Histologic type/differentiation</td>
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<td></td>
</tr>
<tr>
<td>Well</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Moderate/poorly</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Lymphatic invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Present</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Venous invasion</td>
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<tr>
<td>Absent</td>
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<td>12</td>
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<tr>
<td>Present</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Preoperative chemotherapy</td>
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<td></td>
</tr>
<tr>
<td>Not performed</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Performed</td>
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<td>7</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>Positive</td>
<td>4</td>
<td>11</td>
</tr>
</tbody>
</table>

**NOTE:** Myo1b expression was significantly correlated with lymphatic invasion and cervical lymph node metastases.

**Table 2.**

<table>
<thead>
<tr>
<th>Other metastasis-related molecules</th>
<th>Myo1b IHC score</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Negative</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>CCR7</td>
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<td></td>
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<tr>
<td>Positive</td>
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<td>7</td>
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<td>CXCR4</td>
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<tr>
<td>Positive</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Negative</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>

**NOTE:** No correlation was observed between Myo1b expression and other molecules associated with lymph node metastasis of human HNSCC, chemokine receptors CCR4, CXCR4, and CCR7. P value by Fisher exact test.

Promotion of Lymph Node Metastasis of HNSCC by Myosin Ib

**Table 3.**

<table>
<thead>
<tr>
<th>Lymph node metastasis</th>
<th>Myo1b high and CCR4 positive</th>
<th>The others</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>10</td>
<td>5</td>
<td>0.0006**</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** Human HNSCC with high expression of both Myo1b and CCR4 are strongly associated with lymph node metastasis. ***, P < 0.01 by the Fisher exact test.

2A. Sample 1 (T2N2bM0): staining intensity 3, 95% Myo1b⁺ cancer cells, Myo1b IHC score 285; Fig. 2C, Sample 2 (T1N0M0): staining intensity 2, 75% Myo1b⁺ cancer cells, Myo1b IHC score 150; Fig. 2E, sample 3 (T1N0M0): staining intensity 1, 75% Myo1b⁺ cancer cells, Myo1b IHC score 75; Fig 2B, D, and F show control staining with rabbit IgG. Myo1b protein was expressed in cytoplasm of cancer cells and tended to increase in tumor borders. Myo1b protein expression in cervical lymph node metastasis appeared similar to that in primary lesions (data not shown).

To evaluate the clinicopathologic roles of Myo1b in human HNSCC, correlations between Myo1b expression (Myo1b IHC score) and various clinicopathologic features were examined. Myo1b expression levels (high and low) were classified by the median value of the Myo1b IHC score. In addition, we also evaluated the DAB intensity of Myo1b IHC using ImageJ (see Materials and Methods) and confirmed the significant correlation between the results by the Myo1b IHC score and the ImageJ analyses (Supplementary Fig. S3). High Myo1b expression was significantly correlated with lymphatic invasion and lymph node metastasis (Table 1, Supplementary Fig. S4C and S4D, n = 31, P = 0.032), although other clinicopathologic factors, including T-stage and histologic type, showed no significant correlation. High Myo1b expression was not correlated with expression of CCR4, CXCR4, or CCR7, which are known to correlate with lymph node metastasis in HNSCC (24, 35–37), suggesting that Myo1b and these chemokine receptors were independent factors for lymph node metastasis (Table 2). Interestingly, cancer cells with both high Myo1b and CCR4⁺ status was strongly correlated with lymph node metastasis (Table 3, P = 0.0006). Further multivariate analysis on their relationships in lymph node metastasis, confirmation of diagnostic power of the Myo1b and CCR4 combination, using increased number of HNSCC samples is required. These results, together with previous reports indicating the Myo1b involvement in motility of non-cancer cells (11), demonstrate that Myo1b is involved in migration, lymphatic invasion, and lymph node metastasis.

Knockdown of Myo1b inhibited migration and invasion of human HNSCC cells through reduced formation of large protrusions in cell membrane

We evaluated the functional role of Myo1b in cell motility of human HNSCC cell lines. Myo1b expression was downregulated by 2 different Myo1b-specific siRNAs (si1 and si2) and shRNAs (sh1 and sh2; Fig. 3A–C, Western blotting). These siRNAs or shRNAs significantly inhibited cell migration and invasion of SAS and HSC4 when evaluated by the in vitro Transwell migration and invasion assays using the xCELLigence RTCA DP Instrument (Fig. 3D, E, G, and H; Supplementary Fig. S5A and S5B) without affecting cell proliferation (Fig. 3F and I; Supplementary Fig. S5C). These observations were also confirmed by wound-healing assays with the Myo1b-knockdown SAS and HSC4 (Fig. 3J and K). These results indicate that Myo1b is functionally involved in cell migration and invasion of human HNSCC.

Because epithelial-to-mesenchymal transition (EMT) is one of the mechanisms of enhanced cancer cell migration and invasion, relationships between Myo1b expression and some EMT-related molecules were evaluated. However, Myo1b expression did not affect expression of EMT-governing transcription factors such as Snail1 and Twist1, or EMT-related molecules such as CDH1 (E-cadherin), as shown using quantitative PCR analysis (Supplementary Fig. S6A–S6F). In addition, TGF-β1–induced EMT in SAS and HSC4 did not result in Myo1b induction (Supplementary Fig. S6G–S6J). Therefore, Myo1b regulates cell motility of human HNSCC not depending on the major EMT transcription factors.

To investigate the mechanism of enhanced HNSCC motility via Myo1b expression, possible Myo1b regulation of membrane protrusion formation, including filopodia, lamellipodia, and invadopodia/podosomes, was evaluated because they are known to be involved in cancer cell migration, and Myo1b was reported to localize at membranes of these protrusions (38, 39). Large...
protrusions formation in cell membrane of SAS with or without Myo1b shRNA transfection was evaluated in scratch wound assays. SAS treated with control shRNA showed large protrusions formation of cell membrane that were larger than their nuclei 1 hour after scratching (Fig. 4A and C), whereas large protrusions formation was reduced in the Myo1b knockdown SAS (Fig. 4B and D). Similar results were shown in HSC4 with or without Myo1b siRNA transfection (Fig. 4F and G). Cells having large protrusion of cell membrane were counted by using confocal microscopy, and significant reduction in the number of cells with large protrusion was observed in both SAS and HSC4 transduced with Myo1b-specific siRNAs and shRNAs (Fig. 4E and H). Lamellipodia was observed in some large protrusions of cell membrane. Filopodia formation in Myo1b knockdown SAS was not changed (data not shown). Therefore, Myo1b augments cancer cell motility possibly through formation of large protrusion in cell membrane.

**Myo1b knockdown inhibits cervical lymph node metastases of human HNSCC in cervical lymph node metastatic model using nude mice implanted with human HNSCC cells**

To investigate the roles of Myo1b in lymph node metastasis, we developed a cervical lymph node metastatic model by using nude mice implanted with Myo1b-expressing human tongue cancer cell line, SAS. SAS-shRNA-Myo1b GFP+ and SAS-shRNA-NC GFP+ cell lines were constructed by lentiviral transduction into SAS with GFP gene and either Myo1b-specific shRNA or control shRNA. More than 97% of each cell line expressed GFP (data not shown). These cell lines were injected into right masseter muscle of nude mice as cervical lymph node metastatic model. There was no difference in *in vivo* tumor growth between the Myo1b-specific shRNA-transduced and control shRNA-transduced SAS (Fig. 5A), consistent with no difference of their *in vitro* cell proliferation (Supplementary Fig. S5D). By flow cytometric analysis, significant decrease of GFP+ cancer cells was detected in the draining lymph nodes of mice implanted with SAS transduced with Myo1b-specific shRNA-transduced and control shRNA-transduced SAS (Fig. 5A), with no difference of their *in vitro* cell proliferation (Supplementary Fig. S5D). By flow cytometric analysis, significant decrease of GFP+ cancer cells was detected in the draining lymph nodes of mice implanted with SAS transduced with Myo1b-specific shRNA-transduced and control shRNA-transduced SAS (Fig. 5A).

**Discussion**

The roles of myosin family motor proteins in cancer cell characteristics have not yet been well-investigated (34, 40, 41),
although the role of myosin family motor proteins—particularly myosin 2—in cancer motility through cross-linking actin filaments has previously been reported (42, 43). In this article, we have revealed that aberrant overexpression of Myo1b in human HNSCC enhanced lymph node metastasis, possibly through increased cell motility and that Myo1b may be an attractive molecule for the development of new diagnostic and therapeutic strategies for patients with HNSCC. Although we attempted to identify the mechanisms of Myo1b overexpression in human HNSCC, it remains to be investigated. No altered DNA methylation in the Myo1b promoter region was found, and stimulation of EGFR, which is known to be amplified.
in HNSCC, did not change Myo1b expression (data not shown). Interestingly, our in silico gene expression analysis and quantitative PCR analysis demonstrated preferential overexpression of Myo1b in HNSCC, and some of the other squamous cell carcinomas, including esophageal cancer and cervical cancer, which suggest that squamous cell type–related mechanisms may contribute to Myo1b overexpression. In contrast to Myo1b overexpression in HNSCC, downregulation of Myo1b expression was observed in certain types of cancers (Fig. 1B), suggesting that the role of Myo1b in metastasis may be different among cancer types.

Further investigation is needed to evaluate the function of Myo1b in other cancers.

The functional roles of Myo1b in cancer biology have not previously been known, although its association with cell migration of zebrafish embryo cells (11) and intracellular transport regulated by the formation of post-Golgi carriers were reported (12, 13). Myo1b was reported to localize at plasma membrane structures, including filopodia, ruffles, and lamellipodia (38, 39, 44). Our in vitro study indicated that knockdown of Myo1b inhibited HNSCC cell migration and invasion through decreased cell membrane protrusion. The functional roles of Myo1b in cancer biology have not previously been known, although its association with cell migration of zebrafish embryo cells (11) and intracellular transport regulated by the formation of post-Golgi carriers were reported (12, 13). Myo1b was reported to localize at plasma membrane structures, including filopodia, ruffles, and lamellipodia (38, 39, 44). Our in vitro study indicated that knockdown of Myo1b inhibited HNSCC cell migration and invasion through decreased cell membrane protrusion.
large protrusion formation of cell membrane. Recently, some myosin family proteins such as myosin 2 were reported to be highly expressed in cancer cells and to be correlated with cancer cell motility and metastasis partly through cross-linking actin filaments (34, 41, 42). Although myosin 6 was reported to be involved in polarized delivery of vesicles containing EGFR into leading edges of cancer cells (34), $\text{Myo1b}$ knockdown did not affect cell surface protein expression of EGFR (data not shown). We also evaluated the relationship between $\text{Myo1b}$ and intracellular transport of various molecules other than EGFR (e.g., HLA, IL6, IL8, and CCL2) but no difference was observed (data not shown). Further study on possible $\text{Myo1b}$-associated trafficking changes of molecules involved in cell migration may be required (12, 13).

Our clinicopathologic analyses indicate that $\text{Myo1b}$ expression in primary HNSCC tissues is a potential diagnostic marker to predict lymph node metastases and possibly subsequent prognosis of patients with HNSCC, as lymph node metastases is the major factor in overall survival of patients with HNSCC. This may enable better clinical management, including decisions for prophylactic neck dissection or postsurgical chemoradiotherapy. Moreover, combinatorial uses of other predictive markers such as chemokine receptors may further improve the accuracy of predicting power. In fact, cancer cells with both high-$\text{Myo1b}$ expression and CCR4 status are strongly associated with high occurrence of lymph node metastasis in this study (Table 3, $P = 0.0006$). Further analyses including multivariate analysis with increased numbers of patients are warranted for confirmation of

Figure 5. $\text{Myo1b}$ knockdown inhibits cervical lymph node metastasis of human HNSCC cell line implanted in nude mice. $\text{Myo1b}$ knockdown reduced cervical lymph node metastasis in cervical lymph node metastatic model using nude mice implanted with SAS cell line. SAS-shRNA-Myo1b-GFP$^+$ (SAS-sh-Myo1b) and SAS-shRNA-NC GFP$^+$ (SAS-sh-NC) cell lines were constructed by lentiviral transfection into SAS with GFP gene and either human Myo1b-specific shRNA (sh-Myo1b-2) or control shRNA. Each cell line expressed GFP in more than 97% cells (data not shown). These cell lines were implanted into masseter muscle of nude mice. A, there was no difference in in vivo tumor growth between the $\text{Myo1b}$-specific shRNA-transduced and control shRNA-transduced SAS. Tumor volume (mean ± SD) of transplanted SAS-sh-Myo1b and SAS-sh-NC in vivo (n = 7, 6; n.s., not significant; Student t test), representative of 3 separate experiments. The average tumor volume of SAS-sh-Myo1b was 608 mm$^3$ and SAS-sh-NC was 603 mm$^3$. B and C, by flow cytometric analysis, a significant decrease of GFP$^+$ cancer cells was detected in the draining lymph nodes of mice implanted with SAS transduced with $\text{Myo1b}$-specific shRNA cells compared with SAS transduced with control shRNA cells; however, the GFP$^+$ cells were not detected in lung tissues. B, numbers of GFP$^+$ cells per $1 \times 10^5$ cells derived from tumor-draining cervical lymph node were plotted (n = 7, 6; *, $P = 0.0455$; Mann–Whitney U test), representative of 3 separate experiments. C, GFP$^+$ cells were detected by flow cytometry in cervical lymph node (top) and lung (bottom) samples. Each depicted number shows the percentage of GFP$^+$ cells among total cells. Left, non–tumor-bearing mouse; middle, SAS-sh-NC-bearing mouse; right, SAS-sh-Myo1b-bearing mouse. Representative dot plots are shown.
the diagnostic powers of Myo1b expression along with other markers such as chemokine receptor for lymph node metastasis and subsequent survival of patients with HNSCC.

Because Myo1b knockdown inhibited lymph node metastases, possibly due to decreased cancer cell motility, Myo1b could also be a therapeutic target for prevention of lymph node metastasis and subsequent improvement of overall survival. Recently, pentachloropseudillalin, a low-molecular-weight chemical, was reported to be a reversible and allosteric inhibitor of class 1 myosin motor activity (45). As Myo1b is expressed in various normal tissues, systemic administration of Myo1b inhibitors such as low-molecular-weight chemicals and siRNAs may cause severe adverse effects. Therefore, a preferential delivery method to cancer cells may be required. Otherwise, upstream or downstream molecules in the Myo1b-related cell mobilization axis may also be targets for HNSCC. Further studies are required to develop therapeutic strategies that target Myo1b-related malignant features.

In summary, we have shown that aberrant overexpression of Myo1b in human HNSCC augments cancer cell motility via enhanced large protrusion formation of cell membrane and promotes lymph node metastasis. Therefore, Myo1b is an attractive target for the development of new diagnostic and therapeutic strategies for patients with HNSCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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