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, 928mel, and A375mel, human brain tumor, U87MG, human esophageal cancer, Te4 and Te9, lung cancer, LC2 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. Daijiike Aoki (Keio University, Tokyo, Japan) and cultured in DMEM as well as in RPMI-1640. SAS, HSC4, HSC3, Detroit526, FaDu, L-428, A375mel, U87MG, T98G, Te4, Te9, lung cancer, LC2 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. Daijiike Aoki (Keio University, Tokyo, Japan) and cultured in DMEM as well as in RPMI-1640.

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_s1), 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 (ΔΔCt). 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 × 104 cells suspended in 100 μL of medium were seeded 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 then 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 (Kyowa Hakko Kirin), anti-hCCR7 mAb (BD Biosciences), and anti-hCXCR4 mAb (R&D Systems) were used. IHC staining to detect lymphatic invasion and lymph node metastasis was performed by similar protocol. Anti-human D2-40 mAb (Dako) and anti-TurboGFP pAb (Evrogen) were used.

Immunostaining was examined by an experienced pathologist and an experienced oncologist who were blinded to patients' clinical outcomes. Immunostaining was classified by staining intensity and percentage of stained cancer cells, as previously reported (28). Briefly, staining intensity was determined as 3 (strong), 2 (moderate), 1 (weak), or 0 (absent; Fig. 2A, C and E). Expression levels of Myo1b were semiquantified using an IHC score (Myo1b IHC score, range: 0–300) calculated by multiplying the staining intensity by the percentage of positive cancer cells. The median value of Myo1b IHC score was used as a cutoff point to classify Myo1b expression (high and low). The DAB densities were evaluated by the ImageJ color deconvolution method according to the reported protocol (29, 30). The Myo1b ImageJ score was calculated by using the average DAB density of 5 randomly selected fields in each sample (range, 68.8626–150.0392; median, 113.9958).

Scratch wound assays

For scratch wound assays, siRNA-treated HSC4, SAS-sh-NC, SAS-sh-Myo1b-1, or SAS-sh-Myo1b-2 were plated on coverslips at 1 × 10^5 cells suspended in 500 μL of RPMI and 24 hours later, when the cells were confluent, scratched with a pipette tip. SAS-sh-NC, SAS-sh-Myo1b-1, and SAS-sh-Myo1b-2 were incubated in RPMI for 60 minutes and siRNA-treated HSC4 were incubated for 120 minutes. They were then fixed with 4% paraformaldehyde for 10 minutes at room temperature, permeabilized with 0.3% Triton X-100, blocked with 1% BSA in PBS for 10 minutes at room temperature and incubated at room temperature for 5 minutes with 4′,6-diamidino-2-phenylindole (DAPI; Dojindo Laboratories; 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 (i.e., larger than their nuclei) into the open area were counted in 4 randomly selected lesions.

Animal model

SAS/nude mouse model was used to establish a cervical lymph node metastatic model, which was modified from our previously reported model (24). Briefly, female nude mice (BalbC nu/nu) ages 5 to 6 weeks (from CLEA Japan, Tokyo, Japan) were intramuscularly injected with 1 × 10^6 SAS cells suspended in 100 μL of RPMI into the right masseter muscle, as described (31). Tumor diameters were measured using a digital caliper twice a week. Tumor volume (V) was calculated according to the formula: V = (A × B^2)/2, where A and B were the long and short axes, respectively. After sacrifice at day 14, cells were extracted from tumor tissues, right cervical lymph nodes, and lung. Tissue dissociation, sample preparation, and flow cytometric analysis for detecting GFP + metastatic cells were performed as described (32). Briefly, upon sacrifice, tumor and 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 passed through a 70-μm cell strainer (BD biosciences). Cell suspensions were centrifuged and incubated in ice-cold 0.17 mol/L NH4Cl for 10 minutes. Finally, cells were washed and resuspended in 1% FBS/PBS and fixed by 2% paraformaldehyde/PBS. After wash and fixation, cells were analyzed using the Gallios and the Kaluza software (Beckman Coulter). Prior approval from the Institutional Animal Care and Use Committee was obtained in all animal experiments.

Statistical analysis

All analyses were carried out on Microsoft Excel, version 2010 (Microsoft Corp.), Ekuseru-Toukei version 2010 (SSRI). Data were analyzed using one of the following tests for significance: Student t test, Fisher exact test, or Mann–Whitney U test. P < 0.05 was considered statistically significant.

Results

Aberrant overexpression of Myo1b in human HNSCC

To identify molecules involved in lymph node metastasis of human HNSCC, a microarray meta-analysis was performed to compare all genes across 13 different datasets including 222 cancer tissues from HNSCC patient and 135 head and neck normal tissues from healthy donor (Oncomine; see Materials and Methods). The overexpressing genes were ranked by median-ranked 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 SERPINH1 (also known as HSP47) 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 methylation 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.
Correlation between Myo1b and clinicopathologic features

Table 1. Correlation between Myo1b and clinicopathologic features

<table>
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<tr>
<th>Clinicopathologic factors</th>
<th>Myo1b IHC score</th>
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<th>High (≥140)</th>
<th>P</th>
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<td>4</td>
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<td>≥3</td>
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<tr>
<td>Histologic type/differentiation</td>
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<tr>
<td>Well</td>
<td></td>
<td>12</td>
<td>13</td>
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<tr>
<td>Moderate/poorly</td>
<td></td>
<td>3</td>
<td>3</td>
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<td>Lymphatic invasion</td>
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<tr>
<td>Absent</td>
<td></td>
<td>10</td>
<td>4</td>
<td>0.0320</td>
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<tr>
<td>Present</td>
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<td>Preoperative chemotherapy</td>
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<tr>
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<td>4</td>
<td>11</td>
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NOTE: Myo1b expression was significantly correlated with lymphatic invasion and cervical lymph node metastases. *P < 0.05 by the Fisher exact test.

Correlation between Myo1b and other metastasis-related molecules

Table 2. Correlation between Myo1b and other metastasis-related molecules

<table>
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<th>Other metastasis-related molecules</th>
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<tr>
<td>Negative</td>
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<td>12</td>
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</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.

Correlation between lymph node metastasis and cancer cell expression of Myo1b and CCR4

Table 3. Correlation between lymph node metastasis and cancer cell expression of Myo1b and CCR4

<table>
<thead>
<tr>
<th>Lymph node metastasis</th>
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<th>The others</th>
<th>P</th>
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<tbody>
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<td>Positive</td>
<td>10</td>
<td>5</td>
<td>0.0006 **</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>15</td>
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</tr>
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</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.

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 to 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.

**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

Figure 3.
Myo1b knockdown inhibits in vitro migration and invasion of human HNSCC cell lines. Myo1b knockdown using siRNA and shRNA inhibits in vitro cell migration and invasion of human HNSCC. A–C, decrease of Myo1b protein by Myo1b-specific siRNAs and shRNAs in human HNSCC cell lines, SAS and HSC4, was confirmed by Western blotting. GAPDH is an internal control. D–I, these siRNAs significantly inhibited cell migration and invasion of SAS and HSC4. SAS and HSC4 transfected with siRNAs were used for Transwell migration, invasion, and proliferation assays. Migration, invasion, and proliferation were evaluated by real-time monitoring for 48 hours using the xCELLigence RTCA DP instrument as described in Materials and Methods. Transfections were as follows: si negative control (siNC): scrambled siRNA; si1 or si2: siRNAs specific for human Myo1b (si-Myo1b-1 and si-Myo1b-2, respectively). Results show cell index at representative time points (28 hours for SAS migration and invasion, 38 hours for SAS proliferation, 14 hours for HSC4 migration, invasion, and proliferation) compared with each cell index of siNC (1), shown as mean ± SD (n = 4; n.s., not significant; * , P < 0.05; ** , P < 0.01; Student t test), representative of 3 separate experiments. J and K, these observations were also confirmed by wound-healing assays with Myo1b knockdown SAS and HSC4. SAS and HSC4 were transfected with shRNAs and siRNAs, respectively, and used for wound-healing assays. Transfections were as follows: shNC, control shRNA; sh1 or sh2, shRNAs specific for human Myo1b (sh-Myo1b-1 or sh-Myo1b-2, respectively); siNC, scrambled siRNA; si1 or si2, siRNAs specific for human Myo1b (si-Myo1b-1 and si-Myo1b-2, respectively). Results show wound-healing area after 12 hours incubation as mean ± SD (n = 9 or 12; * , P < 0.05; ** , P < 0.01; Student t test), about SAS: representative of 3 separate experiments and HSC4: 1 experiment.
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...
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), Myo1b knockdown did not affect cell surface protein expression of EGFR (data not shown). We also evaluated the relationship between 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 Myo1b-associated trafficking changes of molecules involved in cell migration may be required (12, 13).

Our clinicopathologic analyses indicate that 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-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
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, pentachlorophenol, 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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Aberrant Myosin 1b Expression Promotes Cell Migration and Lymph Node Metastasis of HNSCC

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