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

Metastasis of HNSCC

Gaku Ohmura1,2*, Takahiro Tsujikawa1,2*, Tomonori Yaguchi1, Naoshi Kawamura1, Shuji Mikami3, Juri Sugiyama1, Kenta Nakamura1, Asuka Kobayashi1, Takashi Iwata1, Hiroshi Nakano2, Taketoshi Shimada2, Yasuo Hisa2, Yutaka Kawakami1

*These authors contributed equally to this work.

1 Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

2 Department of Otolaryngology-Head and Neck Surgery, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kamigyo-ku, Kyoto City, Kyoto 602-8566, Japan

3 Division of Diagnostic Pathology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

Running title: Promotion of lymph node metastasis of HNSCC by Myosin 1b

Corresponding author: Yutaka Kawakami, Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Tel: +81-3-5363-3778; Fax: +81-3-5362-9259; E-mail: yutakawa@z5.keio.jp
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**Abbreviations used:** EMT, epithelial to mesenchymal transition; FBS, fetal bovine serum; GFP, green fluorescent protein; HNSCC, head and neck squamous cell carcinoma; IHC, immunohistochemistry; mAb, monoclonal antibody; Myo1b, myosin 1b; NC, negative control; pAb, polyclonal antibody; PCR, polymerase chain reaction; SD, standard deviations; shRNA, short-hairpin RNA; siRNA, short-interfering RNA.

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Abstract

Lymph node metastasis is the major clinicopathological 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 to their normal counterparts as shown by quantitative-PCR (qPCR) analyses. Immunohistochemical (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 migration and invasion ability 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.
Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy worldwide (1). Despite improved loco-regional control of HNSCC (2), the 5-year survival rate for HNSCC remains relatively unchanged, at ~50% for the past 3 decades (3). HNSCC tends to metastasize to lymph nodes prior to 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 morphological, 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 I 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 detected was correlated with lymph node metastases in HNSCC patients (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 patients for immunohistochemistry (IHC) and 7 patients with HNSCC for quantitative PCR assays, who underwent surgery at Kyoto Prefectural University of Medicine 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, Ann Arbor, MI, http://www.oncomine.org) to identify upregulated genes in HNSCC on 26 May 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% fetal bovine serum (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’s lymphoma, L-428, human melanoma, Skmel23, 928mel and A375mel, 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 American Type Culture Collection (Rockville, MD) and cultured in DMEM as same as in RPMI-1640. Human cervical cancer, SKG1, was a kind gift from Dr. Daisuke Aoki (Keio University) and cultured in DMEM as same as in RPMI-1640. SAS, HSC4, HSC3, Detroit526, FaDu, L-428, A375mel, 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 a RNeasy Mini Kit (Qiagen, Chatsworth, CA) 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, San Diego, CA). Quantitative real-time PCR was performed using a TaqMan probe (Applied Biosystems, Foster City, CA), *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**

Short-interfering RNA (siRNA) 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 nM) were transfected into cells using Lipofectamine RNAi MAX (Invitrogen) according to the manufacturer’s recommendations. Assays were carried out 72 h after transfection. Lentiviral pGIPZ short hairpin RNA (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, Osaka, Japan).

**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, Indianapolis, IN), as described before (26, 27). Briefly, for Transwell migration and invasion assays, 4×10^4 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 h with 10% FBS-containing medium in the lower chambers. Additionally, for invasion assays, 2.5% BD Matrigel matrix (BD Biosciences, San Jose, CA) was placed on upper chamber. For *in vitro* cell proliferation assays, $2 \times 10^4$ cells suspended in medium containing 10% FBS were seeded per well in an E-plate 16 (Roche Applied Science) and monitored for 48 h. 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 h after scratching. Healing area was calculated using Axio Vision LE software (version 4.7.2.0, Carl Zeiss Imaging Solutions GmbH, Hallbergmoos, Germany).

**EMT induction by TGF-β1**

Cells were prepared with recombinant human TGF-β1 (R&D Systems, Minneapolis, MN, 240-B, 2ng/ml) for 24 h. 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, Kyoto, Japan), for 20 min at 90°C. Endogenous peroxidase activity was then blocked by incubating with 1% hydrogen peroxide for 30 min 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, St. Louis, MO) 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, Tokyo, Japan) according to the manufacturer’s instructions. The sections were developed with DAB in 0.003% hydrogen peroxide (Muto Pure Chemicals, Tokyo, Japan) and counterstained lightly with hematoxylin. Immunohistochemical staining for human CCR4, CCR7 and CXCR4 were performed and the results were interpreted as previously reported (24). Anti-hCCR4 mAb (Kyowa Hakko Kirin, Tokyo, Japan), anti-hCCR7 mAb (BD Biosciences, San Jose, CA) and anti-hCXCR4 mAb (R&D Systems, Minneapolis, MN) were used. Immunohistochemical staining to detect lymphatic invasion and lymph node metastasis was performed by similar protocol. Anti-human
D2-40 mAb (Dako, Carpinteria, CA) and anti-TurboGFP pAb (Evrogen, Moscow, Russia) 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, 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 five-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 $1 \times 10^5$ cells suspended in 500 μl of RPMI and 24 h 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 min and siRNA-treated HSC4 were incubated for 120 min. They were then fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.3% Triton-X-100, blocked with 1% BSA in PBS for 30 min at 37°C, processed with Texas Red-X phalloidin (Molecular Probes, Eugene, OR, diluted 1:40) with 1% BSA in PBS for 10 min at room temperature and incubated at room temperature for 5 min with DAPI (Douseind Laboratories, Kumamoto, Japan, 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, Oberkochen, Germany). Cells having large protrusions of cell membrane (i.e., larger than their nuclei) into the open area were counted in four 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) aged 5–6 weeks (from CLEA Japan, Tokyo, Japan) were intramuscularly injected with $1 \times 10^6$ SAS cells suspended in 100 μl of RPMI into the right masseter region, as described (31). Tumor diameters were measured using a digital caliper twice a week. Tumor volume ($V$) was calculated according to the formula: $V =$
\[
\frac{(A \times B^2)}{2}, \text{ where } A \text{ and } B \text{ were the long and short axes, respectively. After sacrifice at day 14, cells were extracted from tumor tissues, right cervical lymph nodes, and lung.}
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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 min 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 min. Finally cells were washed and resuspended in 1% fetal bovine serum/PBS and fixed by 2% paraformaldehyde/PBS. After wash and fixation, cells were analyzed employing the Gallios and the Kaluza software (Beckman Coulter, Brea, CA). 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., Redmond, Wash), Ekuseru-Toukei version 2010 (SSRI, Tokyo, Japan). Data were analyzed using one of the following tests for significance: Student’s t-test, Fisher’s 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, Supplementary Fig. 1). 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 heat shock protein 47) 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, Supplementary Fig. 2, 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). Since 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. Therefore, high *Myo1b* expression may be involved in the pathogenesis of HNSCC 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 immunohistochemical (IHC) studies, and Myo1b was detected in all 31 human tongue cancer tissues evaluated, but was not expressed in adjacent normal tissues (Fig. 2, Supplementary Fig. 4 A ,B), 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 (Fig 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 2 B, 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 clinicopathological roles of Myo1b in human HNSCC, correlations between Myo1b expression (Myo1b-IHC score) and various clinicopathological 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. 3). High Myo1b expression was significantly correlated with lymphatic invasion and lymph node metastasis (Table 1, Supplementary Fig.4 C, D, n = 31, P = 0.032), although other clinicopathological factors, including T-stage and
histological 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 2A). Interestingly cancer cells with both high Myo1b and CCR4+ status was strongly correlated with lymph node metastasis (Table 2B, \( P = 0.0006 \)). 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.

**Knock down 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 down-regulated by two different Myo1b-specific siRNAs (si1 and si2) and shRNAs (sh1 and sh2) (Fig 3A, B and 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 assay using the
xCELLigence RTCA DP Instrument (Fig. 3D, E, G, H, Supplementary Fig. 5A, B) without affecting cell proliferation (Fig. 3F, I, Supplementary Fig. 5C). These observations were also confirmed by wound healing assays with the Myo1b knockdown-SAS and HSC4 (Fig. 3J, K). These results indicate that Myo1b is functionally involved in cell migration and invasion of human HNSCC.

Since 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. 6A-F). Additionally, TGF-β1-induced EMT in SAS and HSC4 did not result in Myo1b induction (Supplementary Fig. 6G-J). 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 one hour after scratching (Fig. 4A, C), whereas large protrusions formation was reduced in the Myo1b knockdown SAS (Fig. 4B, D). Similar results were shown in HSC4 with or without Myo1b siRNA transfection (Fig. 4F, 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, 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. Over 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. 5D). 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 cells compared with SAS transduced with control shRNA cells (Fig. 5B, C). Moreover, presence of focal metastatic lesions in cervical lymph nodes was also confirmed by immunohistochemical method (Supplementary Fig. 7). However, the GFP+ cells were not detected in lungs. These results indicate that Myo1b knockdown in human HNSCC cells reduces lymph node metastasis.

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 paper, 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 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 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, IL-6, IL-8, 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 clinicopathological analyses indicate that Myo1b expression in primary HNSCC tissues is a potential diagnostic marker to predict lymph node metastases and possibly
subsequent prognosis of HNSCC patients, as lymph node metastases is the major factor in overall survival of HNSCC patients. 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 2B, $P = 0.0006$). 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 HNSCC patients.

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, pentachloropseudulolin, 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
development of new diagnostic and therapeutic strategies for patients with HNSCC.

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References


Figure legends

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-change 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 three separate experiments.

Figure 2. Myo1b is expressed in tongue cancer tissues. Expression of Myo1b protein in human HNSCC was evaluated by immunohistochemical (IHC) studies, and Myo1b was detected in all 31 human tongue cancer tissues evaluated, but was not expressed in adjacent normal tissues. (A, C, E) Three representative immunostaining results of tongue cancer tissues expressing various Myo1b level with rabbit anti-Myo1b pAb are shown among 31 patients evaluated. The Myo1b IHC score was calculated as described in the Materials and methods. Dotted lines indicate tumor border. (A) Sample 1 (T2N2bM0) has intensity of 3 and is 95% Myo1b+ cancer cells, so its Myo1b IHC score is 285. (C) Sample 2 (T1N0M0) has intensity of 2 and is 75% Myo1b+ cancer cells, so its Myo1b IHC score is 150. (E) Sample 3 (T1N0M0) has intensity of 1 and is 75% Myo1b+ cancer cells, so its Myo1b IHC score is 75. (B, D, F) The same sections were stained by control rabbit IgG. Bar: 100μm.
**Figure 3.** *Myo1b* knockdown inhibits *in vitro* migration and invasion of human HNSCC cell lines. 

**HNSCC cell lines.** *Myo1b* knockdown using siRNA and shRNA inhibits *in vitro* cell migration and invasion of human HNSCC. (A, B, 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 h using the xCELLigence RTCA DP instrument as described in the Materials and methods. Transfections were as follows. si negative control (siNC): scrambled siRNA; si1 or si2: siRNA specific for human *Myo1b* (si-Myo1b-1 and si-Myo1b-2 respectively). Results show cell index at representative time-points (28 h for SAS migration and invasion, 38 h for SAS proliferation, 14 h 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’s *t*-test), representative of three separate experiments. (J, 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: shRNA specific for human *Myo1b* (sh-Myo1b-1 or sh-Myo1b-2 respectively); siNC: scrambled siRNA; si1 or si2: siRNA specific for human *Myo1b* (si-Myo1b-1 and si-Myo1b-2 respectively). Results show wound healing area after 12 h incubation as mean ± SD (n = 9 or 12; *P* < 0.05; **P** < 0.01, Student’s *t*-test), about SAS: representative of three separate experiments and HSC4: one experiment.

**Figure 4.** *Myo1b* knockdown inhibits formation of large protrusion in cell membrane in both SAS and HSC4 cell lines. *Myo1b* knockdown reduces large protrusion of cell membrane in HNSCC cell lines in scratch wound assays. (A-D) Large protrusions formation in cell membrane of SAS cell line with or without *Myo1b* shRNA transfection was evaluated in scratch wound assays (see Materials and methods). SAS treated with control shRNA showed large protrusions of cell membrane that were larger than their nuclei one hour after scratching (A, C), whereas large protrusions formation in cell membrane was reduced in the *Myo1b* knockdown SAS (B, D). Transfections were as follows. shNC: control shRNA; sh1 or sh2: shRNA specific for human *Myo1b* (sh-Myo1b-1 or sh-Myo1b-2 respectively). Representative large protrusion is larger than cell nucleus in SAS-shNC (C); small protrusion is smaller than cell nucleus in SAS-sh2 (D). (E) Cells having large protrusion of cell membrane were counted by using...
confocal microscopy, and a significant reduction in the number of cells with large protrusion was observed in SAS transduced with Myo1b-specific shRNA. (F, G) The similar results were shown in HSC4. Transfections were as follows. siNC: scrambled siRNA; si1 or si2: siRNA specific for human Myo1b (si-Myo1b-1 and si-Myo1b-2 respectively). HSC4-siNC formed large protrusion of cell membrane (F), whereas HSC4-si2 formed few large protrusions (G). (H) Significant reduction of number of cells with large protrusion was observed in HSC4 transduced with Myo1b-specific siRNA. White: Phalloidin, Red: DAPI. White arrows indicate large protrusion of cell membrane. Bar: 200 μm (A, B, F, G). Bar: 20μm (C, D). Data are shown as means ± SD from 4 optical areas (n = 4; *P < 0.05, **P < 0.01, Student’s t-test), about SAS: representative of three separate experiments and HSC4: representative of two separate experiments.

**Figure 5.** *Myo1b* knockdown inhibits cervical lymph node metastasis of human HNSCC cell line implanted in nude mice. *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 over 97% cells (data not shown). These cell lines were implanted into masseter lesions of nude mice. (A) There was no difference in in vivo tumor growth between the 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’s t-test), representative of three separate experiments. The average tumor volume of SAS-sh-Myo1b was 608 mm³ and SAS-sh-NC was 603 mm³. (B, 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 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 × 10⁵ live cells derived from tumor-draining cervical lymph node were plotted (n = 7, 6; *P = 0.0455; Mann–Whitney U-test), representative of three separate experiments. (C) GFP⁺ cells were detected by flow cytometry in cervical lymph node (upper) and lung (lower) samples. Depicted numbers each show the percentage of GFP⁺ cells among total live cells. Left: non-tumor bearing mouse; middle: SAS-sh-NC bearing mouse; right: SAS-sh-Myo1b bearing mouse.

Representative dot plots are shown.
Table 1. Correlation between Myo1b expression and clinicopathological features in human HNSCC patients. Myo1b expression was significantly correlated with lymphatic invasion and cervical lymph node metastases.

Table 2. Relationship between Myo1b expression and other molecules associated with lymph node metastasis of human HNSCC. (A) No correlation was observed between Myo1b expression and other molecules associated with lymph node metastasis of human HNSCC, chemokine receptors CCR4, CXCR4 and CCR7. (B) Human HNSCC with high expression of both Myo1b and CCR4 are strongly associated with lymph node metastasis.
### Table: Gene Expression Analysis

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### Figure 1: Gene Expression Patterns

- **Upregulated**
  - Prostate
  - Pancreatic
  - Ovarian
  - Melanoma
  - Lung
  - Liver
  - Kidney
- **Downregulated**
  - Head & Neck
  - Gastric
  - Esophageal
  - Colorectal
  - Cervical
  - Breast
  - Brain
  - Bladder

**Relative expression of Myo1b/mRNA (HSC3 = 1)**

- **HNSCC**
- **Other SCC**
- **Other cancers**

---

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Figure 2

A) Anti hMyo1b

Intensity 3, Myo1b+ 95%, IHC score 285

B) Negative control

Intensity 2, Myo1b+ 75%, IHC score 150

C) Intensity 1, Myo1b+ 75%, IHC score 75
Figure 3
Figure 5

(A) Tumor volume (mm$^3$) vs. Days after implantation for sh-Myo1b and sh-NC groups. The graph shows a significant increase in tumor volume over time, with a non-significant difference (n.s.) between the two groups.

(B) GFP+ cells / 100000 cells for sh-NC and sh-Myo1b groups. The data points indicate a trend with a * symbol indicating a statistically significant difference.

(C) Flow cytometry analysis of lymph node and lung tissue from non-tumor bearing, sh-NC, and sh-Myo1b groups. The percentage of GFP+ cells is shown for each group:
- Lymph node: non-tumor bearing 0.00%, sh-NC 0.05%, sh-Myo1b 0.01%
- Lung: non-tumor bearing 0.00%, sh-NC 0.00%, sh-Myo1b 0.00%
Table 1. Correlation between Myo1b and clinicopathological features

| Clinicopathological factors                  | Myo1b IHC score |   |   |   |   |
|---------------------------------------------|-----------------|--|--|--|--|--
|                                             | Low (<140)      | High (≥140) | P value |   |   |
| Sex                                         |                 |             |         |   |   |
| Female                                      | 4               | 5           | 1.0000  |   |   |
| Male                                        | 11              | 11          |         |   |   |
| Age                                         |                 |             |         |   |   |
| <70                                         | 10              | 7           | 0.2852  |   |   |
| ≥70                                         | 5               | 9           |         |   |   |
| T stage                                      |                 |             |         |   |   |
| ≤2                                          | 13              | 12          | 0.6539  |   |   |
| ≥3                                          | 2               | 4           |         |   |   |
| Histological type/differentiation           |                 |             |         |   |   |
| Well                                        | 12              | 13          | 1.0000  |   |   |
| Moderate/poorly                             | 3               | 3           |         |   |   |
| Lymphatic invasion                          |                 |             |         |   |   |
| Absent                                      | 10              | 4           | 0.0320  | *|   |
| Present                                     | 5               | 12          |         |   |   |
| Venous invasion                             |                 |             |         |   |   |
| Absent                                      | 15              | 12          | 0.1012  |   |   |
| Present                                     | 0               | 4           |         |   |   |
| Preoperative chemotherapy                   |                 |             |         |   |   |
| Not performed                               | 8               | 9           | 1.0000  |   |   |
| Performed                                   | 7               | 7           |         |   |   |
| Lymph node metastasis                       |                 |             |         |   |   |
| Negative                                    | 11              | 5           | 0.0320  | *|   |
| Positive                                    | 4               | 11          |         |   |   |

*P < 0.05 by Fisher’s exact test.
Table 2A. Correlation between Myo1b and other metastasis related molecules

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P value by Fisher’s exact test.

Table 2B. Correlation between Lymph node metastasis and cancer cell expression of Myo1b and CCR4

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<td>Negative</td>
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**P < 0.01 by Fisher’s exact test.
Molecular Cancer Research

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

Gaku Ohmura, Takahiro Tsujikawa, Tomonori Yaguchi, et al.

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