Sentinel Lymph Node–Targeted Therapy by Oncolytic Sendai Virus Suppresses Micrometastasis of Head and Neck Squamous Cell Carcinoma in an Orthotopic Nude Mouse Model

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Abstract

In clinical N0 (cN0) cases with head and neck squamous cell carcinoma (HNSCC), a treatment selection is still controversial: elective neck dissection or watchful waiting. We focused on sentinel lymph node (SLN)-targeted therapy using the urokinase-type plasminogen activator (uPA)-dependent oncolytic Sendai virus "BioKnife." The objectives of this study were to investigate BioKnife migration into SLNs and elucidate its antitumor effect on lymph node metastases (LNM). We established an orthotopic nude mouse model of HNSCC, with LNM being frequently induced. We inoculated HSC-3-M3, human highly metastatic tongue squamous cell carcinoma cells, in the tongue of the nude mice, and after 2 weeks, we injected BioKnife into the primary tumor. We tracked BioKnife migration into the SLNs by immunostaining, RT-PCR, and an in vivo imaging system. We also examined its antitumor effects and mechanisms through serial section analysis of lymph nodes. GFP reporter expression was clearly visible in the lymph nodes of virus groups, which corresponded to SLNs. Relative GFP mRNA was significantly increased in both the tongues and lymph nodes in the virus groups compared with that in the control group (P < 0.05). Serial section analysis showed that BioKnife infected cancer cells and exhibited significant antitumor effect against LNM compared with the control groups (P < 0.05). We detected apoptosis in LNM infected by BioKnife. BioKnife migrated into SLNs after its injection into the primary tumor and effectively suppressed LNM, suggesting that SLN-targeted therapy using BioKnife has great potential to provide a novel and promising alternative to elective neck dissection in cN0 patients with HNSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the seventh most frequent cancer in the world, affecting over 680,000 patients annually (1). The standard treatment options for HNSCC are surgery and/or radiotherapy, with or without chemotherapy. However, despite advances in the cancer treatment, the overall survival rate of patients with HNSCC has not significantly improved over the past several decades (2).

HNSCC has a high rate of lymph node metastasis (LNM) through lymphatic system, even in early T stages. Because of this, the control of LNM is one of the most important prognostic factors in HNSCC treatment (3). In clinical N0 (cN0) cases, however, it is difficult to decide whether elective neck dissection or watchful waiting would be more appropriate treatment, and current imaging modalities, including CT, MRI, ultrasonography, and PET, are not reliable enough for detecting occult cervical LNM (4).

Sentinel lymph nodes (SLN), which are the first lymph nodes reached by cancer cells from the primary tumor, have received significant attention recently. SLN-targeted therapy has been anticipated by therapeutic agents such as proteins, cells, chemotherapeutic agents, and viral vectors (5). In addition, transoral surgery (6–12) and sentinel node navigation surgery (13–16) for patients with HNSCC have recently been reported, which are minimally invasive, organ-preserving surgeries that reduce the incidence of postoperative morbidities and avoid unnecessary neck dissection in early T and cN0 stages of HNSCC. The combination of these less invasive surgeries and SLN-targeted therapy is promising, offering head and neck surgeons minimally invasive treatment options to reduce the incidence of postoperative morbidities and improve the rate of survival of patients with HNSCC.

Oncolytic virotherapy (OV), which has recently been developed and applied in clinical settings, has the ability to selectively infect and kill cancer cells and avoid cross-resistance to standard anticancer therapies owing to different anticancer mechanism (17). We recently presented OV against anaplastic thyroid cancer and HNSCC using the recombinant Sendai virus (rSeV) "BioKnife," which shows urokinase-type plasminogen activator (uPA)-specific cell killing activity via cell–cell fusion (18, 19). If
BioKnife injected into the primary tumor migrates and infects LNM in the SLNs and acts to suppress tumor growth, SLN-targeted therapy with BioKnife could be used to prevent and/or suppress LNM in cN0 cases, enhancing the possibility of its application in clinical settings. We investigated BioKnife in an orthotopic tongue squamous cell carcinoma (SCC) xenograft mouse model to clarify the hypothesis that BioKnife injected into the primary tumor migrates into SLN and shows its antitumor effect on LNM.

Materials and Methods

Cell line and cell culture

HSC-3-M3, a highly metastatic human tongue SCC cell line, was kindly provided by Dr. Ota (Kanazawa Medical University, Uchinada, Japan; July 2011), who established the cell line (20). The authentication of the cell line was conducted by the comparison with the database of Japanese Collection of Research Bioresources Cell Bank on July 23, 2015. These cell lines were grown in DMEM (Life Technologies) supplemented with 10% FBS (HyClone Laboratories) and penicillin (50 units/mL)/streptomycin (50 µg/mL; Invitrogen) in a humidified 5% CO2 atmosphere at 37°C.

Preparation of nontransmissible rSeV

Sendai virus (SeV) has a negative sense, nonsegmented, single-strand RNA genome. The M (matrix protein) gene and F (fusion) gene of SeV are unique in that the F gene is involved in forming mature viral particles, and the F gene causes cell fusion in the presence of activated F glycoprotein. The control virus, mature viral particles, and the gene of SeV are unique in that the deleted nontransmissible rSeV-encoding GFP (BioKnife; 2.4 × 10^9 cell infectious units (CIU)/mL), and rSeV/dMFct14 (uPA2)-GFP (BioKnife; 2.4 × 10^9 CIU/mL) were prepared as described previously (18). Gene maps of wild-type SeV, rSeV/dM, and BioKnife are shown in Supplementary Fig. S1. rSeV/dM and BioKnife were manufactured and provided by ID Pharma and stored at −80°C until use.

In vitro cytotoxicity assay

To assess temporal dynamics of rSeV/dM (control virus) and BioKnife cytotoxicity against HSC-3-M3, fluorescence microscopy images were taken, as depicted in Fig. 1A. HSC-3-M3 cells (1 × 10^5 cells) were seeded onto a 35-mm dish, incubated for 24 hours at 37°C, and then infected with 10 multiplicities of infection (MOI) of rSeV/dM or BioKnife at 0 hour. Images of HSC-3-M3 cultured at 37°C in a humidified environment were taken at 0, 24, 48, 72, and 96 hours using a BZ8000 Fluorescence Microscope (Keyence). To assess the cytotoxicity of BioKnife against HSC-3-M3, a cytotoxicity assay was performed. HSC-3-M3 cells (4,000 cells/well) were seeded in 96-well plates. Twenty-four hours after preincubation, various titers of rSeV/dM or BioKnife were seeded in 96-well plates, incubated for 24 hours at 37°C, and then infected with 10 multiplicities of infection (MOI) of rSeV/dM or BioKnife at 0 hour. Images of HSC-3-M3 cultured at 37°C in a humidified environment were taken at 0, 24, 48, 72, and 96 hours using a BZ8000 Fluorescence Microscope (Keyence). To assess the cytotoxicity of BioKnife against HSC-3-M3, a cytotoxicity assay was performed. HSC-3-M3 cells (4,000 cells/well) were seeded in 96-well plates. Twenty-four hours after preincubation, various titers of rSeV/dM or BioKnife at 0.1–10 MOI were added and incubated before measurement. A WST-8 Assay (Cell Counting Kit-8; Dojindo) was performed at 100 hours after the addition of the viruses according to the manufacturer’s instructions. The absorbance at 450 nm was measured using a multilabel plate reader (PerkinElmer Japan). Relative cytotoxicity was calculated as reported previously (18, 19).

Animal model

All animal experiments were approved by the Committee on the Ethics of Animal Experiments and by the Safety Board on Animal model (permit no.: 16053 and 2012-24). Animal experiments were conducted using 5- to 8-week-old athymic BALB/c nude mice weighing 20–25 g. All animal procedures were performed under general anesthesia by the intraperitoneal injection of medetomidine (1.0 mg/kg, i.p.) and ketamine hydrochloride (75 mg/kg, i.p.), or inhalation anesthesia with isoflurane. The depth of anesthesia was determined by toe pinch.

To assess migration and antitumor effect of rSeV/dM and BioKnife in cervical lymph nodes, we established an orthotopic tongue SCC xenograft mouse model with LNM by using the highly metastatic human SCC cell line, HSC-3-M3. HSC-3-M3 (1 × 10^5 cells) was suspended in 20 µL of PBS and injected into the left flank of the tongue at day 0.

Migration of recombinant SeV into cervical lymph nodes

A schematic protocol of an orthotopic tongue SCC xenograft mouse model is shown in Fig. 2A. Mice were assigned into two groups: control (n = 5) and rSeV/dM (n = 5). rSeV/dM (1.0 × 10^8 CIU) was administered intratumorally in the rSeV/dM group at day 14; 20 µL of PBS was administered intratumorally in the control group at day 14. At day 21, the animals were sacrificed, and the tongues and all cervical lymph nodes were dissected and assessed for the following experiments.

Immuno-fluorescence staining

To confirm migration of rSeV into cervical lymph nodes and expression of its GFP reporter gene in them, fresh specimens of dissected tongue and cervical lymph nodes were embedded in optimal cutting temperature compound and snap frozen in liquid nitrogen. Ten-micrometer cryosections were prepared for immuno-fluorescence staining. The staining procedure was the same as described in our previous report (21). Rabbit monoclonal anti-GFP antibody was used as the primary antibody (1:100; Life Technologies). Alexa Fluor 488 IgG as the secondary antibody (1:200; Invitrogen). An antifade medium was used to mount the sample (VECTASHIELD with DAPI; Vector Laboratories). The tissues were observed by fluorescence microscopy using the Nikon C1 System (Nikon). A relative GFP intensity was calculated by using ImageJ as per a previous report (5).

qRT-PCR

To confirm migration of rSeV into cervical lymph nodes and expression of its GFP reporter gene in them, excised tongue and cervical lymph nodes were prepared as mentioned above. The preparation and protocol for qRT-PCR were as described in our previous reports (21, 22). Primer sequences were: GFP forward 5'-GGTCTTGCTCAGGGCGGGACT-3' and reverse 5'-GTTGTCGATCGTGCGGG-3'; and GAPDH forward 5'-TACGA-CAGTCCTCAAGATG-3' and reverse 5'-TCTCGGTCGAGTCG-3'. Results are shown as the means ± SEM.

In vivo imaging system

To confirm migration of rSeV into SLNs and expression of its GFP reporter gene in them, mice were assigned into three groups: control, rSeV/dM, and BioKnife. PBS (20 µL), rSeV/dM (2.5 × 10^6 CIU), or BioKnife (2.5 × 10^6 CIU) was administered intratumorally in each group at day 14. At day 21, macroscopic in vivo and ex vivo fluorescence was assessed via the IVIS system (PerkinElmer). Indocyanine green (ICG) stock solution was prepared in the same way as in our previous report (23). For biofluorescence imaging, mice were anesthetized and the working
solution of ICG was administered intratumorally. One hour after the administration of ICG, \textit{in vivo} and \textit{ex vivo} images of SLNs were acquired. Images of ICG were illuminated with a 780-nm excitation light and the fluorescence was determined using an 845-nm filter. Images of GFP were illuminated with a 480-nm excitation light and the fluorescence was determined using a 520-nm filter.

**Antitumor effect of BioKnife on LNM**

A schematic protocol for assessment of antitumor effect of BioKnife on LNM is shown in Fig. 4A. The mice were assigned into three groups: control (\(n = 10\)), rSeV/dM (\(n = 10\)) and BioKnife (\(n = 10\)). In this model, PBS (20 \(\mu\)L), rSeV/dM (1.0 \(\times\) 10\(^6\) CIU), or BioKnife (1.0 \(\times\) 10\(^6\) CIU) was administered intratumorally in each group at days 14, 16, and 18 respectively. Tumor size was measured using a digital caliper, as in our previous report (19). To confirm LNM, the mice were sacrificed, and the tongues and all cervical lymph nodes were dissected at day 25. Mice that lost over 20% of body weight or were not able to eat because of a ruptured tongue tumor were excluded, according to the guidelines for animal experiments. The metastasis rate was assessed by counting the number of animals with positive metastasis (at least one positive LNM per animal) and the number of metastasis-positive lymph nodes in each group as per our previous report (24).

**IHC**

To confirm that rSeV successfully migrated to and infected micrometastasized cervical lymph nodes, a similar staining procedure as in our previous report was used (25). Anti-SeV polyclonal antibody was used as a primary antibody (1:100; MBL International Corporation). After the primary antibody incubation, slides were incubated for 10 minutes in buffer to avoid
nonspecific binding of the primary antibody (29.22 g/L NaCl, 0.172 g/L NaH2PO4·H2O, 3.19 g/L NaHPO4·12H2O, 1 mL Tween20 in 1 L distilled water). SeV expression was observed under high magnification microscopy and images were captured using a CCD camera (Olympus).

qRT-PCR
To confirm migration of rSeV into cervical lymph nodes and expression of its GFP reporter gene in them, excised tongue and cervical lymph nodes were prepared, and qRT-PCR was conducted using the forementioned method.

Tumor apoptosis assay
To detect apoptosis within metastases in cervical lymph nodes, an ApopTag Peroxidase In Situ Apoptosis Detection Kit (Merck Millipore) was used according to the manufacturer’s instructions. The signal was visualized with 3,3′-diaminobenzidine and counterstaining was performed with 0.5% w/v methyl green (Sigma-Aldrich).

Statistical analysis
Statistical analyses were performed using Prism 5 (GraphPad Software, Inc.) and the Microsoft Excel add-in software Statcel (Ver. 3, OMS Publishing). Results are shown as the mean ± SEM. To evaluate differences between two groups, the Mann–Whitney U test was performed. Fisher exact probability test was performed to measure the LNM rate. Comparisons between the three groups were performed by combining the Kruskal–Wallis test with the Steel–Dwass test. Statistical significance was assumed at $P < 0.05$.

Results

In vitro cytotoxicity of BioKnife toward human highly metastatic tongue SCC cell line
BioKnife is a type of rSeV that selectively shows uPA-specific cell killing activity via cell–cell fusion. Fluorescent microscopic observation revealed the temporal dynamics of BioKnife, including syncytium formation and cell death in the highly metastatic tongue SCC cell line, HSC-3-M3. However, rSeV/dM group showed neither of these attributes, and therefore behaves as a control virus group of BioKnife (Fig. 1B). Time-lapse imaging of the movement of BioKnife toward HSC-3-M3 cells is shown in Supplementary Video S1, in which cell–cell fusion, syncytium formation, and cell death can clearly be observed. A cytotoxicity assay 100 hours after administration of the viruses showed that,
although cytotoxicity of rSeV/dM was 10%–16% regardless of the titer of rSeV/dM (0.1–1.0 MOI), BioKnife significantly induced cell death across all titers compared with rSeV/dM (cytotoxicity of BioKnife, 48%–87%; range of titers, 0.1–1.0 MOI, P < 0.05; Fig. 1C). These in vitro experiments revealed that BioKnife has significant cytotoxicity against HSC-3-M3 by inducing clearly visible syncytium formation.

**Migration of rSeV/dM and BioKnife into SLNs in an orthotopic tongue SCC xenograft mouse model**

To verify that rSeV/dM migrates into cervical lymph nodes and expresses its GFP reporter gene, tongues and cervical lymph nodes were dissected and processed for frozen sections at day 21, as described in Fig. 2A. Immunofluorescence staining of an anti-GFP antibody showed that rSeV/dM migration into cervical lymph nodes was clearly visible by GFP signals in the rSeV/dM group compared with the control group (Fig. 2B). Quantitative GFP expression analyzed using ImageJ also revealed that the fluorescence intensity of the rSeV/dM group was significantly higher than that of the control group in both tongues and cervical lymph nodes [tongues: 1.0 arbitrary units (a.u.) in the control group vs. 20.9 a.u. in the rSeV/dM group, P < 0.01; cervical lymph nodes: 0.4 a.u. in the control group vs. 28.2 a.u. in the rSeV/dM group, P < 0.01; Fig. 2C]. In RT-PCR, for dissected tongues and cervical lymph nodes, the relative mRNA expression of GFP was significantly increased in both tongues and cervical lymph nodes of the rSeV/dM group when compared with those of the control group (tongues: 1.5 a.u. in the control group vs. 139.2 a.u. in the rSeV/dM group, P < 0.05; cervical lymph nodes: 3.24 a.u. in the control group vs. 64.9 a.u. in the rSeV/dM group, P < 0.05; Fig. 2D).

Because rSeV injected into the primary tumor migrated into cervical lymph nodes, in vivo and ex vivo imaging analyses were performed to clarify that GFP reporter signals of rSeV/dM and BioKnife exist within SLNs. ICG was used to identify the SLNs. Figure 3 shows that SLNs with infrared signals of ICG simultaneously expressed GFP reporter signals in both the rSeV/dM and BioKnife groups.

We found that, by injecting rSeV into the primary tumor, rSeV migrated into cervical lymph nodes and expressed its GFP reporter gene. GFP-expressing lymph nodes were identical to lymph nodes into which ICG flowed, which mean SLNs.

**Antitumor effect of BioKnife in metastatic cervical lymph nodes of an orthotopic tongue SCC xenograft mouse model**

Next, we assessed whether intratumoral injection of BioKnife could suppress cervical LNM. Four mice with more than 20% weight loss or primary tumor rupture were excluded from the 10 mice in the control and rSeV/dM groups. Three administrations of viruses were selected in this model based on preliminary experiments, which did not significantly show LNM suppression by single BioKnife administration. A schematic protocol is shown in Fig. 4A.

During the time course, primary tongue tumor size was significantly different between the control group and the BioKnife group (36.4 mm\(^2\) in the control group vs. 7.3 mm\(^2\) in the BioKnife group, P < 0.01; Fig. 4B). Serial hematoxylin and eosin (H&E) sections of cervical lymph nodes demonstrated that the rate of positive metastasis per animal (at least one positive LNM per animal) was significantly lower in the BioKnife group than that in either the control or rSeV/dM groups (83.3% in the control group, P < 0.05; 30.0% in the BioKnife group, P < 0.05; Fig. 4C). The rate of positive metastasis per lymph node was significantly lower in the BioKnife group than in either the control or rSeV/dM groups (42.9% in the control group vs. 11.6% in the BioKnife group, P < 0.05; 27.8% in the rSeV/dM group vs. 11.6% in the BioKnife group, P < 0.05; Fig. 4D). BioKnife exhibited significant antitumor effects not only on primary tumors but also on LNM.

IHC of the anti-SeV antibody demonstrated that migration and infection of rSeV/dM and BioKnife in cervical lymph nodes were clearly visible by light microscopic imaging (brown in Fig. 5A). In RT-PCR for dissected tongues and cervical lymph nodes, the relative mRNA expressions of GFP were significantly increased in both the rSeV/dM group and the BioKnife group when compared with the control group in both tongues and cervical lymph nodes (tongues: 2.2 a.u. in the control group vs. 251.8 a.u. in the rSeV/dM group, P < 0.05; 2.2 a.u. in the control group vs. 238.2 a.u. in the BioKnife group, P < 0.05; cervical lymph nodes: 3.4 a.u. in the control group vs. 64.9 a.u. in the rSeV/dM group, P < 0.05; 3.4 a.u. in the control group vs. 47.5 a.u. in the BioKnife group, P < 0.05; Fig. 5B). These results indicate that both rSeV/dM and BioKnife can migrate through the lymphatic system to cervical lymph nodes and selectively infect LNM after the intratumoral injection of rSeV/dM or BioKnife.

![Figure 3](image-url)

**Figure 3.**

GFP reporter gene expression of rSeV/dM and BioKnife in SLN. Mice were assigned to three groups: control, rSeV/dM, and BioKnife. HSC-3-M3 was inoculated into the tongue at day 0, and each virus (2.5 × 10^6 CIU) was administered intratumorally to each group at day 14. The animals were analyzed at day 21. ICG was used to identify SLNs. In an *in vivo* imaging analysis, SLN with an infrared signal of ICG simultaneously expressed a GFP reporter signal in the BioKnife group (left images of each group). In an *ex vivo* imaging analysis, SLNs with infrared signals of ICG expressed GFP reporter signals in both the rSeV/dM and BioKnife groups (right images of each group).
Figure 5C showed that apoptosis was clearly detected in the BioKnife group, although there was no apoptosis in the control group and little apoptosis in the rSeV/dM group. We found that, by multiple injections of BioKnife into the primary tumor, BioKnife migrated into cervical lymph nodes, infected metastasized cells, and suppressed LNM in this model. Apoptosis is considered one of the mechanisms by which BioKnife suppressed LNM.

Discussion
Treatment options for cN0 cases of HNSCC are controversial despite the development of treatment paradigms, and an optimal treatment is yet to be established. Either elective neck dissection or the "watchful waiting policy" is generally employed in the cN0 cases as methods of treatment. Weiss and colleagues recommended that when the probability of occult cervical metastases is more than 20% (with a positive predictive rate above 20%), the neck should be electively treated (26). In these cases of micrometastasis of the SLN, minimally invasive techniques are desired over elective neck dissection. Furthermore, because minimally invasive, organ-preserving surgery for the primary lesions of HNSCC has become popular following the development of trans-oral resection procedures such as trans-oral laser microsurgery (6), transoral robotic surgery (7), or trans-oral video-laryngoscopic surgery (8–12), combining these trans-oral surgical procedures and a novel, less invasive treatment targeting micrometastases in the SLNs can provide head and neck surgeons with minimally invasive treatment options to reduce the incidence of postoperative morbidities and improve the survival of patients with HNSCC.

Here, we investigated the potential of our recently developed uPA-dependent oncolytic SeV, BioKnife, to treat SLN micrometastasis in an orthotopic tongue SCC xenograft mouse model. The key observations are as follows: (i) rSeV/dM or BioKnife, which were injected into the primary tumor, migrated into SLNs, and expressed their GFP reporter genes; (ii) BioKnife specifically infected and suppressed LNM; and (iii) BioKnife induced apoptosis in the affected LNM. In terms of (ii), to assess the treatment effect of OV, we first assessed how frequently HSC-3-M3 metastasized to cervical lymph nodes 2 or 3 weeks after orthotopically inoculation of HSC-3-M3 in this model. Two weeks after inoculation, 60% of mice were found to have metastases in the cervical lymph nodes, and after 3 weeks, nearly 90% similarly had metastases (Supplementary Fig. S2). This result is consistent with data showing that 3 weeks after HSC-3-M3 inoculation, 90% of mice had metastases into the cervical lymph nodes (20). On the basis of these data, it was obvious that LNM were suppressed (only 30% of mice were found with metastases 25 days after tumor inoculation, as shown in Fig. 4C), although the primary tumor was also suppressed significantly in the BioKnife group. From a clinical viewpoint, this novel and less-invasive OV is a very realistic and promising treatment option compared with prophylactic surgeries including elective neck dissection for occult neck LNM at early stages of HNSCC cases.

Figure 4.
Antitumor effect of BioKnife on metastases in cervical lymph nodes. A, Mice were assigned to three groups: control, rSeV/dM, and BioKnife. HSC-3-M3 was inoculated into the tongue at day 0, and each virus (1.0 × 10^5 CIU) was administered intratumorally in each group at days 14, 16, and 18. The animals were sacrificed and analyzed at day 25. B, During the time course, primary tongue tumor size was significantly different between control group and BioKnife group. Data represent means ± SEM. C, Serial H & E sections of cervical lymph nodes demonstrated that the rate of positive metastasis per animal (at least one positive LNM per animal) was significantly lower in the BioKnife group than that in the control or rSeV/dM groups. D, The rate of positive metastasis per lymph node was significantly lower in the BioKnife group than in the control or rSeV/dM groups. *, P < 0.05.
Figure 5. Histopathologic examination and qRT-PCR to track the migration of rSeV/dM and BioKnife into metastatic cervical lymph nodes (LN). Tongue and cervical lymph node specimens were prepared as per the same protocol in Fig. 4. A, IHC with anti-SeV antibody demonstrated that migration and infection of rSeV/dM and BioKnife in cervical LNM were clearly visible (brown). B, In RT-PCR of dissected tongues and cervical lymph nodes, the relative mRNA expressions of GFP were significantly increased in the rSeV/dM group and BioKnife groups compared with the control group in both tongues and cervical lymph nodes. Data represent means ± SEM. *, P < 0.05. C, Apoptosis was clearly detected in the BioKnife group (brown), although no apoptosis was observed in the control group and little apoptosis occurred in the rSeV/dM group.
It has been reported that BioKnife suppressed anaplastic thyroid carcinoma, glioblastoma multiforme, and malignant pleural mesothelioma in vivo (19, 27, 28). These malignant tumors frequently express uPA, and their invasiveness is closely related to uPA activity (29–32). BioKnife, which targets uPA, has the potential to kill aggressive malignant tumors, which show heightened uPA expression (31, 32). Supplementary Figure S3 shows that the HSC-3-M3 cell line, which frequently metastasizes to cervical lymph nodes, had higher uPA activity in vitro compared with the other SCC cell lines, based on uPA assay results. These results together indicated that BioKnife is a very promising oncolytic virus that not only suppresses uPA-expressing malignant primary tumors, but also suppresses their LNM.

The lymphatic system can be utilized as a route for the delivery of therapeutic agents. Although there has been little literature regarding SLN-targeted OV, Kikuchi and colleagues reported that the telomerase-specific oncolytic adenovirus selectively infected and eradicated regional LNM and improved the survival in an orthotopic early rectal cancer xenograft mouse model (5). Our study showed that BioKnife migrates into SLNs and suppresses metastatic outgrowth in draining SLNs. Advantages of our study are that (i) migration of BioKnife follows SLN theory by using ICG in an in vivo imaging system, and (ii) administration of BioKnife into the primary tumor suppresses cervical LNM by detecting BioKnife infection and induction of apoptosis in LNM. Moreover, it is well known that the therapeutic use of rSeV is considered safer than that of DNA viruses, because its RNA genome does not go through a DNA phase. rSeV is also nonpathogenic to humans and is well known that the therapeutic use of rSeV is considered safer than that of DNA viruses, because its RNA genome does not go through a DNA phase. rSeV is also nonpathogenic to humans. Recently, in several clinical studies, intranasal or intramuscular inoculation of SeV in humans produced no harmful outcomes (33). BioKnife injected into the primary tumor induces tumor immunoresponses (34). The antimetastatic effect of BioKnife is promising because the approach has the potential to prevent and suppress metastasis in HNSCC cases, which enhances the possibility of its application in clinical settings.

In conclusion, we have demonstrated that the uPA-dependent oncolytic SeV “BioKnife” migrated into metastatic foci in SLNs after its injection into the primary tumor, and effectively suppressed lymph node micrometastases in an orthotopic tongue SCC xenograft mouse model. These results suggest that BioKnife targeting of OVs to the SLNs has potential to provide a novel and promising alternative to elective neck dissection for cN0 patients with HNSCC.

Disclosure of Potential Conflicts of Interest
Y. Ueda is manager at ID Pharma Co., Ltd. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: Y. Tanaka, K. Araki, Y. Ueda, A. Shiotani
Development of methodology: Y. Tanaka, K. Araki, D. Kamide, Y. Ueda
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Tanaka, K. Araki
Writing, review, and/or revision of the manuscript: Y. Tanaka, K. Araki, A. Shiotani
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Araki, D. Kamide, Y. Ueda, A. Shiotani
Study supervision: K. Araki, A. Shiotani
Other (producing BioKnife and providing it to the executing team): Y. Ueda

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References

# Molecular Cancer Therapeutics

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