Sensitivity of Squamous Cell Carcinoma Lymph Node Metastases to Herpes Oncolytic Therapy

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Abstract

Purpose: Cancer metastases may have phenotypic and genetic differences from their primary cancers of origin. Engineered, replication-competent, attenuated viruses based on herpes simplex virus-1 (HSV-1) have shown potent oncolytic effects in treating primary tumors in animal models, but their efficacy in treating lymph node metastases is poorly understood. We compared the efficacy of an attenuated oncolytic HSV-1 (NV1023) in treating a series of murine squamous carcinoma cell lines derived from serial implantation and harvest from metastatic lymph nodes.

Experimental Design and Results: The auricles of C3H/HeJ mice were implanted with SCCVII. Cervical nodal metastases were isolated, expanded in vitro, and reimplanted into new mice. A series of cell lines (LN1-LN7) were generated through seven serial passages. Cells from higher LN passages showed consistent trends toward increased migratory and invasive ability, increased cell surface nectin-1 (an HSV-1 receptor) expression, and increased glycoprotein D binding. Exposure to NV1023 showed increased viral entry, replication, and cytotoxicity with higher LN passages. Intratumoral injection of NV1023 in a murine flank tumor model caused significantly greater tumor regression and increased viral infection of LN7 compared with SCCVII.

Conclusions: These results show that lymph node metastases may undergo selection for characteristics, including increased nectin-1 expression, that make them more sensitive targets for herpes oncolytic therapy. These findings support the clinical application of these agents for the treatment of lymph node metastases.

Smoking and alcohol usage are major causative factors for squamous cell carcinoma of the upper aerodigestive tract. Interestingly, squamous cell carcinoma lymph node metastases may have phenotypic and genetic differences from their primary cancers of origin (1). In some cases, the lymph node metastases may exhibit more aggressive behavior than the primary tumor, and the treatment of progressive nodal disease can present the greater clinical challenge. The response of nodal disease to a treatment, such as radiation or chemotherapy, may also be different from the response of the primary tumor. Novel therapies for the treatment of nodal metastases would be of significant clinical value.

Genetically engineered, replication-competent, attenuated herpes viruses have shown significant oncolytic effects against a variety of human malignancies in animal models (2–7). Recent early clinical trials have also suggested excellent safety profiles in the treatment of brain malignancies and colon cancer liver metastases (8–10). Our group described the construction of NV1023, a single γ134.5-deleted, attenuated, oncolytic herpes simplex virus-1 (HSV-1) that expresses β-galactosidase (lacZ; ref. 11). The NV1023 series of viruses have exhibited potent oncolytic activity in treating a variety of human malignancies and have therapeutic efficacy in animal models when delivered through intratumoral, intracavitary, and intravascular routes (6, 7, 11–13).

Our group showed that the administration of NV1023 to a primary tumor site can lead to successful lymphatic trafficking of virus to regional lymph nodes, which may infect and treat metastatic tumor deposits in these lymph nodes (14, 15). However, there is little known about the relative susceptibility of lymph node metastases to herpes oncolytic therapy in relation to the primary tumor of origin. Cancer cell surface nectin-1, an HSV-1 glycoprotein D receptor, is expressed on invasive carcinomas and is an important determinant of NV1023 viral entry and oncolysis in squamous cell carcinoma (16). We showed that squamous carcinoma cell lines with increasing migratory and invasive abilities have increasing cell surface expression of nectin-1 and NV1023 sensitivity (17). In this study, we hypothesize that lymph node metastases may exhibit alterations in cell surface nectin-1 compared with the primary carcinoma of origin. We generated a series of cell lines from an animal...
model of squamous cell carcinoma lymph node metastases and compared their relative susceptibilities to NV1023 viral entry, replication, and oncolysis.

**Materials and Methods**

**Cell lines.** The murine SCCVII cell line is a cutaneous squamous cell carcinoma that arose from the C3H/HeJ mouse. SCCVII cells were grown in vitro in MEM with 10% FCS. The CHO.K1 cell line was grown in F12 medium containing 10% FCS and 1% P&G and was used for the generation of the soluble gD:Fc protein. A series of cell lines based on SCCVII were generated by serial selection of metastatic cells using an in vivo murine model. SCCVII cells were implanted into the auricles of C3H/HeJ mice. After reaching 1.5 cm in size, tumors were excised (Fig. 1A). A cervical lymph node metastasis that subsequently developed over the next several weeks was excised, minced, digested with collagenase, and expanded in cell culture (Fig. 1B). Cells were injected into the auricles of new mice, and additional aliquots were frozen. Seven serial passages were done, and resulting cell lines were termed LN1 to LN7.

**Virus.** We previously described the construction of NV1023, an attenuated, replication-competent, oncolytic herpes virus (11). NV1023 was derived from R7020, an HSV-1 vector originally designed as an HSV-1/HSV-2 vaccine candidate (18). NV1023 carries a 5.2-kb fragment of HSV-2 DNA (containing US2-2 to US2-5) inserted in the UL/S junction. NV1023 is attenuated by a 15-kb deletion in the inverted repeat region that extends from the 3’ end of UL55 to the promoter for ICP4, thus deleting UL56 and one copy of the diploid genes ICP0 and ICP4 and the γ134.5 neurovirulence gene. NV1023 also contains the *Escherichia coli* β-galactosidase gene (lacZ) inserted at the US10-12 locus to serve as a marker of infection. NV1023 was provided by MediGene, Inc.

**Cell line proliferation and morphology.** Cell proliferation was measured for SCCVII and selected LN lines to determine if the selection process resulted in alteration of replication rate. SCC7, LN2, LN4, and LN7 were seeded onto six-well plates at 6 × 10⁴ cells per well in 3 mL of MEM with 10% FCS. Wells were trypsinized and stained with trypan blue, and cells were counted at daily intervals to determine the number of viable cells. At day 4, wells underwent replacement with fresh medium. All samples were assessed in triplicate.

To assess for differences in cell morphology, cell lines grown in T-175 flasks at both sparse and confluent conditions were photographed with phase contrast light microscopy at 200× magnification.

**Migration and invasion assays.** Migration and invasion assays were done on SCC7, LN2, LN4, and LN7 to validate phenotypic differences between these cell lines. For migration assays, polycarbonate filters (8-μm pore size, transwell inserts, BD Biosciences) were used in 24-well plates. For migration assays, Matrigel invasion chambers (BD Biosciences) were used in 24-well plates. Cells...
confluent Vero cells for 4 h. Wells were washed with medium and covered with 1% agarose in medium. After 48 h, 2 mL of neutral red solution (2% by volume) was added, and viral plaques were counted 24 h later. Samples were done in triplicate.

**Cytotoxicity assays.** SCCVII, LN3, and LN7 cells were plated at 1 × 10^4 per well in 96-well plates in 100 μL medium. After 6 h, NV1023 (50 μL) was added to each well at an MOI of 0 or 1. Viral cytotoxicity was tested at daily intervals. CellTiter 96 AQ One Solution Reagent (Promega) was added (30 μL) to each culture well. Plates were incubated for 1 h at 37°C. The amount of soluble formazan produced by cellular reduction was measured at 490 nm on a spectrophotometer (SpectraMax 190, Molecular Devices Corporation). Results were expressed as the surviving percentage of cells, determined by comparing test samples to control cell samples considered 100% viable. Samples were done in quadruplicate.

**Murine flank tumor therapy.** Animal procedures were done with the approval of the Memorial Sloan-Kettering Institutional Animal Care and Use Committee. Eight-week-old C3H/HeJ mice (Jackson Laboratory) were anesthetized with inhalational methoxyflurane for all procedures and were sacrificed by CO₂ inhalation. Flank tumors were established for the SCCVII and LN7 cell lines by injecting 1 × 10^6 tumor cells in 50 μL of PBS into the subcutaneous flanks of mice. When tumors had reached ~75 mm³ in largest dimension, animals were distributed into experimental groups (n = 6 per group) with equitable starting tumor volumes. Flank tumors were treated by intratumoral injections of a single dose of NV1023 at 1 × 10^5 plaque-forming units (pfu) or PBS as a control. Tumor dimensions were serially measured, and tumor volumes were calculated by the formula for the volume of an ellipsoid: volume = (4/3) × π × (length / 2) × (width / 2)². Photographs were taken of representative animals at day 10.

**5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside histochemistry.** Established flank tumors of SCCVII and LN7 were injected with NV1023 (1 × 10^5 pfu). After 24 h, animals were sacrificed and flank tumors were excised, frozen in Tissue Tek solution, and sectioned. Slides were fixed with 1% glutaraldehyde for 5 min, washed with PBS, and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (1 mg/mL) in an iron solution of 5 mmol/L K₄Fe(CN)₆ and 2 mmol/L MgCl₂ at 37°C for 2 h. Slides were counterstained with nuclear fast red.

**Results**

**Higher LN cell lines exhibit altered morphology and more invasive behavior.** We assessed if differences in cell shape morphology and growth patterns could be appreciated with higher LN passage cell lines. SCCVII cells grew densely confluent together, with complete cell-cell contact. LN3 cells showed small gaps between mostly confluent cells, whereas LN5 showed slightly larger, circular-shaped gaps. LN7 showed large circular gaps even at maximal confluence. Higher LN passages, therefore, exhibited gradually decreasing degrees of cell-cell contact and increasing spindle morphology (Fig. 1C), suggestive of epithelial mesenchymal transition. There were no differences in cell proliferation rates in vitro between SCCVII, LN2, LN4, and LN7 (Fig. 1D).

Significant alterations in migratory and invasive ability by higher LN passage cell lines were identified. There were highly significant correlations between increased migratory ability and higher LN passage (P < 0.001 by Pearson's correlation, R = 0.80). The number of successfully migratory cells counted increased from 8.6 ± 1.3 (SCCVII) to 16.7 ± 1.6 (LN2), 16.5 ± 1.0 (LN4), and 52.0 ± 2.7 (LN7; Fig. 2A). Similarly, there was also a strong correlation between...
increased invasive ability and higher LN passage ($P < 0.001$, $R = 0.89$ by Pearson’s correlation). The number of successfully invasive cells counted increased from 99.0 ± 9.0 (SCCVII) to 237.7 ± 18.5 (LN2), 359.8 ± 26.8 (LN4), and 494.4 ± 43.5 (LN7; Fig. 2C). Representative photographs are shown of successfully migrating (Fig. 2B) and invading (Fig. 2D) cells. These results show that the process of serial selection of the LN cell lines led to increasing degrees of cell migratory and invasive ability.

**Nectin-1 expression and glycoprotein D binding are enhanced on higher LN cell lines.** Nectin-1 is a cell surface HSV-1 glycoprotein D receptor that also functions as an intercellular adherens junction protein (19). Nectin-1 may have increased exposure at conditions with decreased cell-cell junction integrity (20). Immunofluorescence microscopy showed gradually increasing cell surface expression of nectin-1 for the higher LN passages compared with the original SCCVII cell line (Fig. 3A). Quantitation of surface nectin-1 with cellular ELISA showed a strong correlation between increasing levels of measured nectin-1 with higher LN passage number ($P < 0.001$, $R = 0.91$; Fig. 3B).

The interactions between HSV-1 envelope glycoprotein D and nectin-1 or other glycoprotein D receptors (HVEM or HS-3OST) are essential for successful HSV-1 entry into a target cell. We used a soluble gD:Fc fusion protein to quantitatively measure total glycoprotein D receptor expression on our cell lines using cellular ELISA. There was a strong correlation between increasing gD:Fc protein binding, as measured by absorbance and increasing LN passage ($P < 0.001$, $R = 0.88$; Fig. 4A).

**Higher LN cell lines are more susceptible to NV1023 entry, replication, and oncolysis in vitro.** The ability of NV1023, an oncolytic HSV-1, to enter into the LN cell lines was assessed to determine if alterations in nectin-1 expression would lead to increased sensitivity to herpes viral infection. Measured levels of NV1023 viral entry into cells 6 hours after viral exposure were strongly correlated with increasing LN passage number ($P < 0.001$, $R = 0.73$; Fig. 4B). The ability of NV1023 to replicate within these cell lines was also assessed using viral plaque assays. Strong correlations were found between viral titers measured 4 days after viral exposure and increasing LN passage number ($P < 0.01$, $R = 0.79$; Fig. 4C). Titers varied from $1.3 \times 10^6$ pfu (SCCVII) to $5.2 \times 10^6$ pfu (LN5).

The ability of NV1023 to lyse the LN cell lines was compared with determining if the measured differences in viral entry translate to oncolysis, as well. At MOI of 1, NV1023 showed increasing cytotoxic effects with higher LN passage at day 3 (Fig. 4D). Mean cell survival percentage was 68±9% for...
SCCVII, 30±6% for LN3, and 12±2% for LN7 (P < 0.05 for all comparisons, t test).

**Higher LN cell lines are more susceptible to NV1023 infection and oncolysis in vivo.** Flank tumors of SCCVII and LN7 were established and allowed to reach ~75 mm³ in size. A single intratumoral injection of 1 × 10⁷ pfu of NV1023 gave only minimal tumor growth inhibition of SCCVII (P = not significant, t test; Fig. 5A and C). In contrast, the same dose of NV1023 caused a more significant tumor growth inhibition of LN7 (P < 0.05, t test, days 8 and 10; Fig. 5B and D). Despite equivalent growth rates in vitro between SCCVII and LN7, we noted a more rapid flank tumor growth in vivo by the LN7 cell line, with the mean LN7 tumor volume exceeding 1,500 mm³ by day 10, in contrast to just under 900 mm³ for SCCVII. LN7 control animals were sacrificed after day 10 due to tumor ulceration.

Established SCCVII and LN7 flank tumors were injected with NV1023 and then excised 24 hours later for 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside histochemistry to track NV1023 infection. Histology shows modest staining for SCCVII tumors (Fig. 5E) but much more robust staining for LN7 tumors (Fig. 5F), reflecting more effective early tumor infection and lacZ expression by NV1023.

**Discussion**

The presence of lymph node metastases for most solid tumor malignancies is a significant adverse prognostic factor. For head and neck squamous carcinomas, the presence of cervical lymph node metastases decreases patient survival rates by ~ 50% (21). The identification and management of lymph node metastases is essential to achieving optimal outcomes. In some cases, the clinical behavior of lymph node metastases may be more aggressive than the primary tumor of origin. Cancer cells that are able to metastasize frequently carry specific genetic and phenotypic traits that have been selected from a larger, diverse population of primary tumor cells (1). The clinical behavior and therapeutic response of nodal metastases may therefore differ from their primary carcinomas of origin.

The application of gene therapy approaches to head and neck cancer has been studied with adenoviruses in early clinical trials. An adenovirus engineered to express wild type p53 has been studied in a phase I clinical trial in patients with advanced head and neck cancers and showed significant tumor regression in 12% of patients (22). Replication-competent adenoviruses designed to be selective for malignant tumors (ONYX-015) have shown an ability to be oncolytic to tumor targets. In a phase II clinical trial, patients with recurrent head and neck cancers showed a 14% partial to complete tumor regression and 41% with stable disease. Fever, and injection site pain were the main treatment-related toxicities (23). Other oncolytic agents, such as replication-competent, attenuated herpes simplex viruses have shown promising preclinical activity against human head and neck cancer cell lines (6).

In this study, we sought to compare the efficacy of applying an oncolytic herpes virus for treating cancer cell nodal metastases of squamous carcinoma compared with the original primary tumor. We sought to determine if the process of lymph node metastasis may naturally select for cancer cell characteristics that alter sensitivity to oncolytic herpes viruses. We did serial auricular injections of a murine squamous carcinoma cell line (SCCVII), subsequent cervical lymph node harvesting, expansion of metastatic nodal cancer cells in vitro, and reinjection into the auricles of new mice. The generation of this series of cell lines allows for (a) the study of an enhanced metastatic phenotype in the high LN passages and (b) the examination of stepwise trends across a range of cell lines for...
alterations in cell characteristics and HSV sensitivity. Migration and invasion assays of the LN series of cell lines were done showing strong, significant correlations between increasing migratory or invasive ability and higher passage number.

A comparison of cell growth patterns at confluence showed that the higher LN passages exhibit decreased cell-cell contact, with increasing gaps remaining between cells at higher passage. This suggests that intercellular adhesion may be decreased in the higher LN passages. Alterations in intercellular adhesion may reflect changes in cell surface adhesion molecules, which can be linked to herpes viral receptor expression (20). Interellular adherens junctions are an important structure governing cell-cell contact and organization and are formed by bridges of E-cadherin and nectin-1. Adherens junction integrity is frequently impaired in highly invasive and metastatic cancers through the dysfunction of E-cadherin (24). Nectin-1 also functions as an important receptor to HSV-1 envelope glycoprotein-D (19). We previously showed that nectin-1 expression is a determinant of oncolytic susceptibility, seems inversely linked to E-cadherin expression, and may be more prevalent on highly invasive and migratory cells (16, 17). We therefore hypothesized that cancer cells in nodal metastases might have altered nectin-1 expression and differential sensitivity to oncolytic herpes viral therapy compared with their primary cancer cells of origin.

We examined the SCCVII and LN cell lines for nectin-1 receptor expression and their abilities to functionally support herpes viral entry and other downstream events. Multiple factors correlating with higher LN passage were identified, including (a) increased migratory and invasive function, (b) increases in cell surface nectin-1 expression, (c) increases in glycoprotein D protein binding, corroborating nectin-1 functional activity as a glycoprotein D receptor, (d) increases in NV1023 viral entry, (e) increases in NV1023 viral replication, a downstream event after viral entry, and (f) increases in NV1023 cytotoxicity. Pearson’s coefficients showed highly significant correlations between each of these characteristics and increasing LN passage number.

These observations in vitro translated into clinically relevant differences in vivo in a murine flank tumor model. Significantly enhanced sensitivity of LN7 over SCCVII to tumor volume reduction in vivo was observed after a single intratumoral injection of NV1023. LacZ staining at 24 hours showed more extensive viral spreading in LN7 tissues over SCCVII, suggesting more effective NV1023 infection in vivo in LN7. Interestingly, the LN7 cell line grew more rapidly in the murine flank than the parent SCCVII cell line. Despite equivalent growth rates in vitro, it is likely that LN7 acquired other genetic changes that gave it a growth advantage in vivo. These observations suggest that events promoting nodal metastases of squamous carcinoma may lead to increased nectin-1 expression, thereby making metastatic deposits more susceptible to herpes oncolytic therapy.

We previously showed that an oncolytic herpes virus applied to a primary tumor site may transit through lymphatics to deposit in regional lymph nodes (14, 15). The natural transit of oncolytic HSV through lymphatics may have both diagnostic and therapeutic application and represents a simple method for potentially treating microscopic nodal metastases. For gross nodal metastases, however, lymphatic drainage is often obstructed by tumor, which would impede viral transit and delivery. In this scenario, the direct injection of oncolytic

![Fig. 4.](https://example.com/fig4.png) Measures of activity by an oncolytic HSV-1 (NV1023) are increased in vitro with higher LN passage. A, the binding of glycoprotein D, the HSV-1 envelope ligand, to cell surface nectin-1 increases with higher LN passage as measured by cellular ELISA ($R = 0.88$, $P < 0.001$). B, viral entry of NV1023 (MOI, 5), an oncolytic HSV-1, shows a trend toward increasing efficacy with higher LN passage as measured by lacZ assays 6 h after viral exposure ($R = 0.73$, $P < 0.01$). C, viral replication by NV1023 (MOI, 1) showed a trend toward increasing viral titers in higher LN passage LN cell lines after 4 d of exposure ($R = 0.79$, $P < 0.01$). D, viral cytotoxicity after exposure to NV1023 (MOI, 1) was measured by lactate dehydrogenase assays and shows significantly increased cytotoxicity in LN7 cells compared with LN3 and with SCCVII ($P < 0.05$ for both comparisons, t test).
herpes viruses into nodal disease may allow for effective viral
delivery of much higher viral doses and potentially more
effective overall therapy.

In summary, we show that the natural selection process of
primary cancer cells for traits favoring lymph node metastasis
may also lead to the selection of enhanced cancer cell nectin-1
expression, HSV-1 glycoprotein D binding, HSV-1 entry, repli-
cation, and sensitivity to HSV-1 oncolysis. These findings
support the further study and potential clinical application of
oncolytic HSV for (a) cancers with a proclivity for nodal meta-
stases and (b) existing nodal metastases in upcoming clinical
trials using these promising agents.

Fig. 5. NV1023 infection and tumor volume reduction
in vivo are enhanced in LN7. A-D, murine flank tumors of
SCC VII or LN7 were treated with NV1023 (1 × 10^7 pfu)
by a single intratumoral injection. Differential response
rates between the SCC VII tumors (A) and the LN7
tumors (B) were noted, with a significant reduction
of tumor volume noted for LN7 but not for SCC VII.
Representative SCC VII tumors (C) and LN7 tumors (D)
are shown at day 10. E-F, established SCC VII (E) and
LN7 (F) tumors treated with a single intratumoral
injection of NV1023 (1 × 10^7 pfu) were excised after
24 h for lacZ histochemical (blue) and background
nuclear fast red staining. LN7 tumors supported more
intense early infection and lacZ gene expression by
NV1023 compared with SCC VII tumors.
References


