Oncolytic Herpes Simplex Virus Prevents Premalignant Lesions from Progressing to Cancer

Yanghee Woo,1,2 Vincent Reid,1,3 Kaitlyn J. Kelly,1,4 Diane Carlson,5 Zhenkun Yu,1,6 and Yuman Fong1,2

Early detection and timely treatment of precancerous lesions are hallmarks of successful strategies to prevent deaths due to cancer. Oncolytic viruses are a group of promising anti-cancer agents with wide-ranging experimental and clinical efficacy against solid tumors. Previously, we have shown that NV1066, an oncolytic herpes simplex-1 virus encoding enhanced green fluorescent protein, selectively infects, replicates in, and kills various cancer types. In this study, we sought to determine whether this oncolytic agent can treat precancerous lesions to prevent cancer formation. Using an oral chemical carcinogenesis model in hamsters, we assessed the ability of NV1066 to infect precancerous and cancerous lesions. NV1066 consistently infected dysplastic cells, carcinoma in situ, and squamous cell carcinoma. Animals receiving an intramucosal injection of NV1066 for 7 weeks showed significantly fewer (3-fold) and smaller (4-fold) lesions compared to animals that did not receive viral treatment. Results indicate that infectivity might be dependent on the herpes simplex virus 1 receptor, nectin-1. This study demonstrates that not only can NV1066 treat oral squamous cell carcinoma, but it can also infect and treat premalignant lesions, thus delaying cancer progression. Overall, our study shows the potential of the oncolytic virus NV1066 as a cancer prevention tool.

INTRODUCTION

Preventing cancer initiation and progression could decrease associated mortality, but this relies on screening and early detection programs.1–4 Current screening methods include detection of serum molecular biomarkers, direct endoscopic visualization, and imaging techniques. Despite significant advances in the diagnostic capability of these approaches,5 most solid malignancies continue to be discovered at advanced stages that portend poor long-term outcomes.6–7 In 2018, an estimated 609,640 deaths due to cancer were reported.8 Moreover, current screening approaches do not confer therapeutic benefits.

Oncolytic viruses (OVs) have been shown to have tropism for cancerous lesions, selectively infecting, replicating in, and killing cancer cells without affecting normal cells. OVs thus hold great potential in the diagnosis and treatment of various cancers.9–11 In October 2015, the US Food and Drug Administration (FDA) approved T-VEC (talimogene laherparepvec, Amgen, Thousand Oaks, CA, USA), a herpes simplex virus 1 (HSV-1)-expressing granulocyte-macrophage colony-stimulating factor (GM-CSF), which represents the translational success of oncolytic HSV-1 for the treatment of advanced-stage melanoma.9 Another replication-competent HSV-1, NV1066, genetically engineered to express enhanced green fluorescent protein (EGFP), has been shown to assist in the detection and killing of primary malignancies in the pleural and peritoneal cavity, as well as in cancer harboring lymph nodes and distant organs.11–13 Importantly, these studies support NV1066 for both diagnostic and therapeutic applications.12,13,16–20 However, it is unclear whether OVs have tropism for cells early in the spectrum of malignant transformation. In this study, we investigated whether NV1066 can infect and kill premalignant cells and delay cancer progression using the hamster cheek pouch model of oral carcinogenesis.

RESULTS

Selective Detection of Dysplasia by EGFP-Expressing NV1066

We first tested whether NV1066 had tropism for cells at premalignant stages of transformation. Chemical carcinogenesis was induced in hamster cheek pouches, as previously described.21 Control animals (group A) were not exposed to the carcinogen, whereas the test animals (group B) received 0.5% 7,12-dimethylbenz[a]anthracene (DMBA) on cheek pouches twice a week for 8 weeks, followed by NV1066 treatment of both groups. Figure 1A shows the schema for infection experiments. As expected, no lesions developed in the cheek pouches of control animals (Figure 1B). Fluorescence stereoscopic examination of representative slides of control cheek pouches showed an absence of EGFP signal (Figure 1C), while hematoxylin and eosin (H&E) confirmed the absence of dysplasia (Figure 1D), and immunohistochemistry (IHC) staining confirmed HSV-1 infection (Figure 1E).

Eighteen of the group B 29 animals sacrificed (three per week) between weeks 9 and 14 did not show any EGFP-positive lesions.
Figure 1. Stereoscopic Fluorescence Microscopy of Oral Lesions Helps Identify Areas of EGFP Expression
(A) Schema representing a timeline of infection experiments. (B–E) Normal mucosa. (B) Bright-field stereoscope image of a normal cheek pouch that received NV1066. (C) The corresponding fluorescence stereoscope examination demonstrating absence of EGFP expression. (D) H&E staining of the tissue confirmed normal histology. (E) IHC for HSV-1 demonstrates absence of the virus in the lesion. (F and G) Lesion with dysplasia: a gross photograph of a polyp indicated by a black arrow (F), and area of corresponding EGFP expression under the fluorescent filter (G). (H and I) H&E staining for a section of the polyp showing marked dysplasia (H), which corresponds to positive (legend continued on next page)
>5 mm or any histological abnormality in the cheek pouch areas. Cheek pouches in the 11 remaining group B animals developed a total of 16 lesions >5 mm in diameter between weeks 15 and 20 (Figures 1F–1I). Ten of the 16 identified lesions were EGFP-negative, and histological analysis confirmed that all of these were normal or hyperplastic without evidence of dysplastic changes or cancerous cells.

Fluorescence stereomicroscopy showed that 6 of the 16 lesions were EGFP-positive (Figure 1G, representative image) and histologically heterogeneous, and all lesions harbored areas of premalignancy with morphology consistent with dysplasia (Figure 1H). In addition, these EGFP-positive dysplastic areas were confirmed to be infected with NV1066 by IHC staining for HSV-1 (Figure 1I). A specimen representative of a large lesion that had replaced a cheek pouch (Figure 1J) demonstrated heterogeneous EGFP expression with a focal area of grossly necrotic tumor (Figure 1K). EGFP-positive areas in the lesion corresponded to dysplasia or carcinoma (Figure 1L) and stained positive for HSV-1 (Figure 1M) as observed by IHC, thus confirming NV1066 infection of the lesion. Taken together, these findings demonstrate the ability of NV1066 to infect premalignant tissue, as indicated by the infected areas of dysplasia, in addition to carcinoma lesions.

**NV1066 Prevents Cancer Progression**

In agreement with previous results, we observed that the PBS control group cheek pouches developed obvious lesions by week 15. Animals in the NV1066 treatment group displayed significantly fewer tumors at 15 weeks (Figure 2). Figure 2B represents a gross photograph of a cheek pouch from a control animal after 7 weeks with numerous visible lesions. All lesions >5 mm were measured and counted, and a significant (3-fold) decrease in the number of tumor nodules was observed in the NV1066-treated cheek pouches compared to the controls (with an average of two lesions versus six lesions, respectively; p < 0.01) (Figure 2D). In addition, the lesions that were found in NV1066-treated pouches had a significantly smaller size as compared to controls (Figures 2C and 2E). Several large areas of tumor were present in cheek pouches of control animals (Figure 2B), while these were absent in the NV1066-treated cheek pouches (Figure 2C). The average tumor volume in the control group was 120 ± 15 mm³, while in the NV1066-treated group the volume was 33 ± 7 mm³, indicating a significant slowing of tumor growth with NV1066 treatment (Figure 2E, p < 0.05). These results show an almost 4-fold decrease in tumor volume in the NV1066-treated group by week 15 compared to the control group, suggesting that NV1066 delays the growth of tumors in these animals.

**Precancerous Lesions Are Characterized by Free Nectin-1**

HSV enters cancer cells via specific receptors such as nectin-1, nectin-2, herpes virus entry mediator, and modified heparan sulfate. Nectin-1 is an adherens junction protein that, when free, serves as an entry receptor for neurotropic HSV. To identify the potential role of nectin-1 in the premalignant tropism of NV1066, we performed IHC staining using representative sections from control and test groups. Staining of free nectin-1 was observed in areas of dysplasia (Figures 3A and 3B) but not in normal mucosal cells (Figures 3C and 3D). These data suggest that the presence of free nectin-1 on premalignant cells may facilitate the infection of these early lesions by NV1066.

**DISCUSSION**

Although tumor tropism by OVs has been studied previously, it is unclear whether OVs have tropism for cells early in the spectrum of malignant transformation and the mechanisms by which this may occur. Approval of T-VEC for the treatment of melanoma has established a platform for testing newer OV therapies. NV1066 shows great potential for viral gene therapy not only for diagnostic but also for therapeutic applications.

Nectin-1 is a component of intracellular adherens junctions, which in its free form serves as a cell surface receptor for the HSV envelope glycoprotein D (HSV-gD). HSV-gD binds to nectin-1 and can fuse with the cell membrane and subsequently enter the cell. The presence of free nectin-1 on the surface of cancer cells has been shown to be predictive of sensitivity to oncolysis by HSV. In addition, loss of E-cadherin expression has been shown to occur early in the preneoplastic steps of head and neck squamous cell carcinoma carcinogenesis. Interestingly, loss of E-cadherin expression leads to disruption of adherens junctions, which in turn frees nectin-1 to function as an HSV receptor.

In this proof-of-principle study, we demonstrate for the first time the ability of an OV to infect precancerous lesions and prevent the progression of such lesions to cancer. NV1066 was capable of infecting and ablating lesions at the dysplasia stage of cancer formation, as observed by EGFP expression in the infected premalignant cells. Treatment with NV1066 early in the progression of oral carcinogenesis significantly reduced both the number (~4-fold) and the size (~3-fold) of tumors in the oral mucosa within seven rounds of weekly treatment with the virus. NV1066 activity against these premalignant cells forestalls the development of cancer. Even more striking were the visual differences of the NV1066-treated buccal pouches, where lesions could barely be observed compared with large tumors found in control animals. IHC results showed the presence of free nectin-1 in areas of dysplasia but not in healthy tissue sections. Based on the role of nectin-1 as a cell surface receptor for HSV-gD, our results indicate that nectin-1 may play a role in NV1066 tropism to premalignant lesions.
CANCER PREVENTION EXPERIMENTS

| Week: | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|-------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|
| PBS Control group (n=10) | + carcinogen | + virus injections | 0.5% DMBA exposure | PBS weekly injections | Visual inspection imaging tissue collection and analysis |
| Treatment Group (n=18) | + carcinogen | + virus injections | 0.5% DMBA exposure | NV1066 virus weekly injections |

Figure 2. NV1066 Treatment Reduces the Number and Size of DMBA-Induced Lesions

(A) Schema representing the treatment strategy for prevention. (B) Multiple large lesions were visible by gross examination on cheek pouch of PBS-treated group (lesions >5 mm were counted, as indicated by black circles). (C) Significantly fewer lesions >5 mm were present in the NV1066-treated group; (D and E) bar graphs show significantly decreased average nodule count (D) and average volume (E) in the NV1066-treated group compared to the PBS-treated group. A total of 123 lesions in 20 cheek pouches of the PBS-treated group were present as compared to 82 lesions in 36 check pouches in the NV1066-treated group. DMBA, 7,12-dimethylbenz[a]anthracene. The number of lesions was counted, and tumor volume was measured in each cheek pouch, and a paired t test was used to compare the two groups. A p value of 0.05 or less was considered statistically significant.
the freeze-thaw method of cell lysis. NV1066 stocks were propagated on Vero cells and harvested using the freeze-thaw method of cell lysis. Under the control of a cytomegalovirus (CMV) promoter for constitutive expression, these deletions, the marker gene encoding EGFP has been inserted for the detection of infected cells by fluorescence microscopy. NV1066 expresses its early genes, including EGFP, which then allows for the detection of infected cells by fluorescence microscopy. NV1066 stocks were propagated on Vero cells and harvested using the freeze-thaw method of cell lysis.

In conclusion, the results suggest that NV1066 can infect and treat dysplastic precancerous lesions, deterring the progression of such lesions into squamous carcinoma. This novel finding—that the oncolytic efficacy of replication-competent HSV extends beyond the treatment of frankly malignant cells to delaying the progression of early dysplastic lesions—has potential to be a new and noninvasive therapeutic option. Such therapies might particularly benefit patients with dysplastic oral lesions and could significantly impact the prognosis of other solid tumor malignancies. Further studies investigating whether NV1066 or other OV can detect premalignant lesions of other tissue origins are currently underway, as are their molecular and immune mechanisms of action.

MATERIALS AND METHODS

OV: NV1066

NV1066 is a replication-competent, attenuated HSV-1 mutant virus that has single-copy deletions of the viral genes ICP-4, ICP-0, and γ3,4,5, as has been described extensively. In addition to these deletions, the marker gene encoding EGFP has been inserted under the control of a cytomegalovirus (CMV) promoter for constitutive expression. Four to six hours after infection of a host cell, NV1066 expresses its early genes, including EGFP, which then allows for the detection of infected cells by fluorescence microscopy. NV1066 stocks were propagated on Vero cells and harvested using the freeze-thaw method of cell lysis.

Animal Model of Carcinogenesis: Hamster Cheek Pouch Oral Carcinogenesis

All animal procedures were conducted with the approval of the Memorial Sloan Kettering Cancer Center Institutional Animal Care and Use Committee (IACUC) (New York, NY, USA) and in accordance with their guidelines. Golden Syrian hamsters 4–6 weeks old, initially 60–80 g in body weight, were purchased from Harlan Sprague-Dawley (San Diego, CA, USA) and provided with food and water ad libitum. Animals were subjected to a well-established standard carcinogenesis protocol using the topical application of 0.5% DMBA in dimethyl sulfoxide (DMSO) on bilateral buccal pouches with the modification of twice weekly for 8 weeks instead of three times weekly for 10–14 weeks.

Premalignant Lesion Infection Experiments

The animals were divided into two groups: the control group (A), no carcinogen exposure and virus infection (n = 2); and the experimental group (B), carcinogen exposure and virus infection (n = 29). Animals in both groups received a direct injection of 5e7 plaque-forming units (PFUs) of NV1066 virus into the cheek pouch mucosa and were euthanized 48–72 h after viral injection. From week 10 to week 20 after the start of carcinogen exposure, two or three hamsters from the experimental group were euthanized weekly. The two animals in the control group were treated and euthanized at week 20. Euthanasia was performed by CO2 inhalation in compliance with the Memorial Sloan Kettering Cancer Center IACUC guidelines. Immediately postmortem, we performed complete dissection of the buccal pouches and carefully removed all layers of the mucosa, submucosa, and part of the muscular layer. The fresh buccal pouches were mounted on a black backboard and were examined grossly by stereomicroscopy. Bright-field and fluorescence-filtered lenses were employed to assess for the presence of lesions and for EGFP expression, respectively, in the buccal pouches. The specimens were designated EGFP-positive or EGFP-negative on visual examination under the stereomicroscope.

Cancer Prevention Experiment

To assess for the ability of NV1066 to prevent progression of disease in the viral treatment experiments, animals were divided into two additional groups: the control group (D) (n = 10), and the treatment group (E) (n = 18). Animals in both groups were induced with DMBA to develop premalignant lesions for 8 weeks. At the end of week 8, animals in group D received weekly injections of PBS, while animals in group E received a weekly injection of NV1066 (5e7 PFUs) into the mucosa for 7 weeks. At week 15, hamster cheek pouches were visually inspected for the presence of lesions. The number and volume of the nodules in each cheek pouch were evaluated and compared between the groups.

Histologic Examination of Tissues

A portion of each cheek pouch specimen collected at the time of animal sacrifice was frozen in Tissue-Tek OCT embedding medium (Sakura, Finetek, Torrance, CA, USA) and sectioned by cryotome. Tissue-Tek-preserved specimens were cut into 5-mm consecutive sections and freshly mounted on glass slides. The sections were then stained with rabbit polyclonal HSV-1 antibody or mouse anti-nectin-1 monoclonal antibody, 5 μg/mL (Zymed Laboratories, San Francisco, CA, USA). An institutional pathologist reviewed all results.
and confirmed any histologic and immunohistochemical stain findings.

**Statistical Analysis**
The number of lesions was counted, tumor volume was measured in each cheek pouch, and a paired t test was used to compare the two groups. A p value of 0.05 or less was considered statistically significant.

**AUTHOR CONTRIBUTIONS**
Study concept and design: Y.W., Z.Y., and Y.F. Data collection, analysis, and interpretation: Y.W., V.R., K.J.K., D.C., and Y.F. Manuscript preparation and critical revision: Y.W., V.R., K.J.K., Z.Y., D.C., and Y.F. Final approval of manuscript: Y.W., V.R., K.J.K., Z.Y., D.C., and Y.F.

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