

RESEARCH ARTICLE

Treatment of Human Thyroid Carcinoma Cells with the G47delta Oncolytic Herpes Simplex Virus

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Abstract

Background: Thyroid carcinoma is the most common malignancy of the endocrine organs. Although the majority of thyroid cancer patients experience positive outcomes, anaplastic thyroid carcinoma is considered one of the most aggressive malignancies. Current therapeutic regimens do not confer a significant survival benefit, and new therapies are urgently needed. Oncolytic herpes simplex virus (oHSV) may represent a promising therapy for cancer. In the present study, we investigated the therapeutic effects of a third-generation HSV vector, G47 Δ , on various human thyroid carcinoma cell lines *in vitro*. Two subcutaneous (s.c.) models of anaplastic thyroid carcinoma were also established to evaluate the *in vivo* anti-tumor efficacy of G47 Δ . **Materials and Methods:** The human thyroid carcinoma cell line ARO, FRO, WRO, and KAT-5, were infected with G47 Δ at different multiplicities of infection (MOIs) *in vitro*. The survival rates of infected cells were calculated each day. Two s.c. tumor models were established using ARO and FRO cells in Balb/c nude mice, which were intratumorally (i.t.) treated with either G47 Δ or mock. Tumor volumes and mouse survival times were documented. **Results:** G47 Δ was highly cytotoxic to different types of thyroid carcinomas. For ARO, FRO, and KAT-5, greater than 30% and 80% of cells were killed at MOI=0.01 and MOI=0.1, respectively on day 5. WRO cells displayed modest sensitivity to G47 Δ , with only 21% and 38% of cells killed. In the s.c. tumor model, both of the anaplastic thyroid carcinoma cell lines (ARO and FRO) were highly sensitive to G47 Δ ; G47 Δ significantly inhibited tumor growth and prolonged the survival of mice bearing s.c. ARO and FRO tumors. **Conclusions:** The oHSV G47 Δ can effectively kill different types of human thyroid carcinomas *in vitro*. G47 Δ significantly inhibited growth of anaplastic thyroid carcinoma *in vivo* and prolonged animal survival. Therefore, G47 Δ may hold great promise for thyroid cancer patients.

Keywords: Thyroid carcinoma - oncolytic herpes simplex virus - cytotoxicity - subcutaneous models

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Introduction

Thyroid carcinoma, which consists of a group of diseases with different clinicopathologic features, is the most common malignancy of the endocrine organs (Vanderpump, 2011). Differentiated thyroid carcinoma (DTC) includes 2 types of malignancies: papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC) (Zaman et al., 2012). DTC is the most common thyroid malignancy and is often associated with an indolent clinical course and 5-year survival rates greater than 90% (Sipos and Mazzaferri, 2010). However, no treatment has been proven to be effective for radioiodine-resistant metastatic disease, and the 10-year survival rate is less than 15% for these patients (Sherman et al., 2008). Medullary thyroid carcinoma (MTC) originates from the

canteration of parafollicular C cells and presents a more aggressive clinical course than DTC. In addition, MTC is resistant to radioactive iodine therapy (Pacini et al., 2010; Vanderpump, 2011). Anaplastic thyroid carcinoma (ATC), or undifferentiated thyroid carcinoma, accounts for approximately 1-2% of thyroid malignancies (Lin et al., 2008; Smallridge et al., 2012). ATC is one of the most aggressive tumor types, with a median survival time of no more than 6 months (Are and Shaha, 2006; Lin et al., 2008; Smallridge et al., 2012). In addition, one-half of all ATC patients have distant metastases at diagnosis, and radiation and chemotherapy are of little sensitivity and do not result in improvements in survival (Are and Shaha, 2006). Therefore, new therapies are needed to target thyroid tumors, especially metastases and ATC.

Oncolytic viruses selectively infect tumor cells and

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replicate within them. The tumor cells are then destroyed, and the viruses are released from them, infecting other tumor cells (Russell et al., 2012). Herpes simplex virus is one of the most common viruses used for tumor therapy. Oncolytic herpes simplex virus (oHSV) can be genetically modified, thereby increasing tumor selectivity and safety (Varghese and Rabkin, 2002). Preclinical and clinical experiments have demonstrated that oHSV can effectively and safely treat various tumors (Varghese and Rabkin, 2002). G47 Δ is a third-generation oHSV vector, and our previous studies have indicated that G47 Δ can effectively treat breast and nasopharyngeal carcinomas (Wang et al., 2011a; Wang et al., 2011b; Li et al., 2012; Zeng et al., 2013a; Zeng et al., 2013b).

In the present study, we investigated the cytotoxic effects of the G47 Δ oHSV vector on various human thyroid cancer cell lines *in vitro*. Furthermore, we evaluated the therapeutic effects of G47 Δ on subcutaneous (s.c.) xenograft models of 2 human ATC cell lines.

Materials and Methods

Ethics statement

The animal work in the present study was conducted under the institutional guidelines of Guangdong Province and was approved by the Use Committee for Animal Care and the Sun Yat-sen University Institute Research Ethics Committee.

Cell lines and virus

The human papillary thyroid carcinoma cell line KAT-5, the human follicular thyroid carcinoma cell line WRO, and human undifferentiated thyroid carcinoma cell lines, ARO and FRO, were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, United Kingdom). The cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 Media containing glucose (4.5 g/l; Mediatech, Inc., Herndon, VA) and supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT) and were grown at 37°C in 5% CO₂. Vero cells (African green monkey kidney cells purchased from The Committee on Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing glucose (4.5 g/l; Mediatech, Inc., Herndon, VA) and supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT) and were grown at 37°C in 5% CO₂. The oHSV G47 Δ was provided by MediGene, Inc. (San Diego, CA). G47 Δ is a conditionally replicating HSV-1 vector that was constructed as previously described (Todo et al., 2001). Briefly, the a47 gene, the promoter region of the US11 gene, and both copies of neurovirulence gene γ 34.5 were deleted, and the ICP6 gene was inactivated by the insertion of a lacZ gene. As a result of these genetic mutations, G47 Δ selectively replicates in tumor cells but spares normal cells.

In vitro cytotoxicity

The cancer cells were seeded in 6 well plates at densities of 1×10⁵ cells per well. After 1 night of incubation, the cells were infected with the mock virus

or G47 Δ at multiplicities of infection (MOI) of 0.01 and 0.1. The cells were then cultured in RPMI 1640 media supplemented with 1% fetal calf serum (Hyclone Laboratories, Logan, UT) and grown at 37°C in 5% CO₂. The number of surviving cells was calculated each day using a hemocytometer. The average number of cells from triplicate wells are plotted as percentages of the mock wells. X-gal histochemistry was also performed each day post-infection according to the manufacturer's recommended protocol (Beyotime Institute of Biotechnology, China). The infected cells expressed Lac Z and were stained blue.

Animal studies

Four-week-old female Balb/c nude mice were purchased from the Shanghai Institutes for Biological Sciences, CAS, Shanghai, China. Human undifferentiated thyroid carcinoma cells, ARO (3×10⁶) and FRO (3×10⁶), were suspended in 0.1 mL of RPMI 1640 media with 25% Matrigel (BD Biosciences) and implanted s.c. into the left flanks of 4-week-old nude mice. When the maximal diameter of the tumors reached approximately 5 mm, the mice were randomly assigned to 1 of 2 groups (6 mice/group): intratumoral (i.t.) injection of 50 μ l of virus buffer [150 mM NaCl and 20 mM Tris, (pH 7.5)] or 2×10⁷ plaque forming units (pfu) of G47 Δ twice per week for 2 weeks. The lengths (a) and widths (b) of the tumors were recorded twice per week, and the tumor volumes were calculated ($V=ab^2/2$). The mice were sacrificed when the maximal diameter of the tumor exceeded 18 mm or when the mice seemed moribund, and this day was recorded as the date

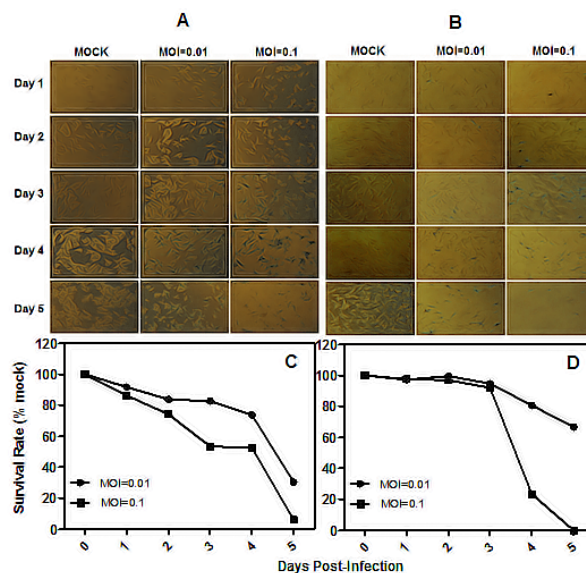


Figure 1. Cytotoxicity of 2 Thyroid Carcinoma Cell Lines *in vitro*. (A) X-gal staining of ARO cells infected with G47 Δ . Monolayers of ARO cells in 6-well dishes were infected with G47 Δ or mock virus and incubated with RPMI 1640 medium/1% heat-inactivated FBS at 37°C. X-gal staining was performed each day, and infected cells expressing Lac Z were stained blue. (B) X-gal staining of FRO cells infected with G47 Δ . (C and D) Monolayers of ARO and FRO cells in 6-well dishes were infected with G47 Δ at MOI=0.01 or MOI=0.1 and incubated in RPMI 1640 medium/1% heat-inactivated FBS at 37°C, and the number of viable cells was counted each day. The data represent the results of 3 independent trials

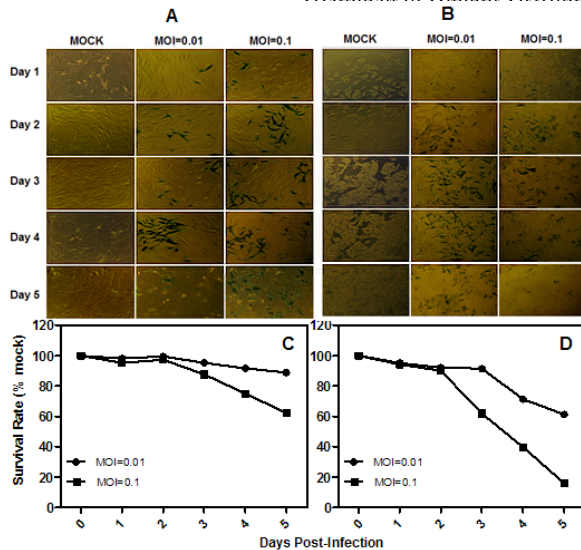


Figure 2. Cytotoxicity of 2 Thyroid Carcinoma Cell Lines *in vitro*. (A) X-gal staining of WRO cells infected with G47 Δ . Monolayers of WRO cells in 6-well dishes were infected with G47 Δ or mock virus and incubated with RPMI 1640 medium/1% heat-inactivated FBS at 37°C. X-gal staining was performed each day, and infected cells expressing Lac Z were stained blue. (B) X-gal staining of KAT-5 cells infected with G47 Δ . (C and D) Monolayers of WRO and KAT-5 cells in 6-well dishes were infected with G47 Δ at MOI=0.01 or MOI=0.1 and incubated in RPMI 1640 medium/1% heat-inactivated FBS at 37°C, and the number of viable cells was counted each day. The data represent the results of 3 independent trials

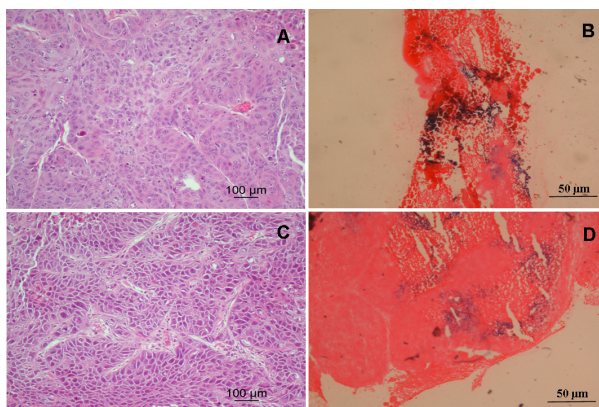


Figure 3. Established s.c. tumors derived from the ATC cell lines ARO (3×10^6) and FRO (3×10^6). ATC cells were suspended in 100 μ l of RPMI 1640 containing 25% Matrigel (BD Biosciences) and then s.c. implanted into the left flanks of Balb/c nude mice (4 weeks old). When the s.c. tumors were palpable, $2 \times 10^7/50 \mu$ l of G47 Δ or virus buffer [150 mM NaCl and 20 mM Tris, (pH 7.5)] was injected into the s.c. tumors twice per week. (A and B) Hematoxylin and eosin staining of ARO and FRO s.c. tumors ($\times 200$). (C and D) Coronal section through ARO and FRO s.c. tumors of Balb/c nude mice 3 days after G47 Δ inoculation to illustrate viral replication in the s.c. tumors. Coronal sections were stained with X-gal and counterstained with eosin to identify replicating G47 Δ (blue)

of death for the survival studies. The s.c. tumors were excised for hematoxylin-eosin staining. Another 2 nude mice were sacrificed 3 days after G47 Δ inoculation. The tumors were excised and frozen at 80°C, cryostat sections 10 μ m in thickness were prepared, and X-gal staining was

performed according to the manufacturer's recommended protocol (Beyotime Institute of Biotechnology, China). Sections were stained with X-gal, and counterstained with eosin to identify cells containing replicating G47 Δ (blue).

Results

In vitro cytotoxicity

To assess the susceptibility of human thyroid carcinoma cell lines to G47 Δ , various thyroid carcinoma cells were infected with G47 Δ at a low MOI (MOI 0.01 and 0.1). For ARO cells, greater than 70% and 90% of cells were killed at MOI=0.01 and MOI=0.1, respectively, on day 5 (Figure 1C). For FRO cells, greater than 35%

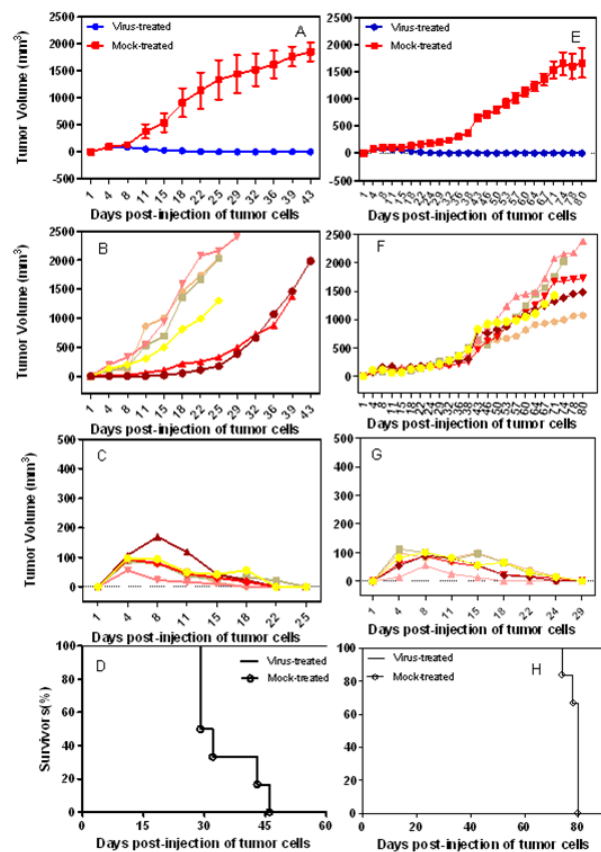


Figure 4. G47 Δ Significantly Inhibited ATC Growth *in vivo* and Prolonged Mouse Survival. Tumor volume was recorded twice per week. The mice were sacrificed when the maximal diameter of the tumor exceeded 18 mm or when the mice seemed moribund, and this day was recorded as the date of death for survival studies. G47 Δ significantly inhibited tumor growth compared to the control group, and all of the tumors in the treated group regressed completely. (A) The mean tumor volume is shown for the virus-treated and mock-treated ARO groups at different time points. (B and C) The growth of individual treated tumors in the mock- and virus-treated ARO groups, respectively. (D) The median survival time increased from 27 days for the control group to 60 days for the G47 Δ -treated s.c. ARO tumor group ($P < 0.05$, log-rank test). (E) The mean tumor volume is shown for the virus- and mock-treated FRO groups at different time points. (F and G) Growth of individual treated tumors in the mock- and virus-treated FRO groups, respectively. (H) The median survival time increased from 80 days for the mock-treated group to 90 days for the G47 Δ -treated s.c. FRO tumor group ($p < 0.05$, log-rank test)

of cells were killed at MOI=0.01 on day 5, while 100% of cells were killed at MOI=0.1 on day 5 (Figure 1D). For WRO cells, 21.1% and 37.8% cells were killed at MOI=0.01 and MOI=0.1, respectively, on day 5 (Figure 2C). For KAT-5 cells, greater than 30% and 80% cells were killed at MOI=0.01 and MOI=0.1, respectively, on day 5 (Figure 2D). G47 Δ contains the lacZ gene and can therefore be traced both *in vitro* and *in vivo*. Similar to the cytotoxicity results, X-gal staining revealed that G47 Δ effectively replicated and spread among ARO (Figures 1A), FRO (Figures 1B), WRO (Figures 2A), and KAT-5 cells (Figures 2B).

In vivo study

ATC is very aggressive and is considered a fatal disease. The majority of patients initially present with a rapidly enlarging tumor in the neck (Are and Shaha, 2006). To determine the therapeutic effects of G47 Δ on thyroid cancer *in vivo*, we selected 2 ATC cell lines, ARO and FRO, to form s.c. tumors in Balb/c nude mice, after which we injected G47 Δ or virus buffer i.t. To evaluate G47 Δ replication, the virus-treated nude mice were sacrificed 3 days after G47 Δ injection, and X-gal staining was performed on the sectioned ATC tumors. Blue-stained sections were evaluated for G47 Δ replication in ARO (Figures 3B) and FRO s.c. tumors (Figures 3D).

Similar to the results obtained *in vitro*, G47 Δ elicited prominent antitumor effects and demonstrated a significant inhibition of tumor growth (Figures 4A and E). In G47 Δ -treated mice bearing s.c. ARO and FRO tumors, all of the tumors completely regressed as a result of treatment (Figures 4C and G). In addition, none of the mice appeared to be moribund, so they were sacrificed on days 60 (ARO) or 90 (FRO). In contrast, the tumors grew rapidly in the control group (Figures 4B and F). Due to the rapid growth of ARO tumors, all of the mice in the control group were sacrificed by day 47, with a median survival time of 27 days (Figures 4D). For the FRO tumors, the median survival time was 80 days (Figures 4H). G47 Δ significantly prolonged the survival of mice bearing ARO or FRO tumors ($p < 0.05$, log-rank test).

Discussion

The use of an oncolytic virus is a relatively new therapeutic strategy for cancers. oHSV was first used for the treatment of glioblastoma in 1991 (Martuza et al., 1991). Since then, more than 20 different oHSVs have been used to treat various solid tumors in preclinical studies, including bladder, breast, colorectal, gastric, glioma, head and neck, liver, melanoma, neuroblastoma, pancreatic, and prostate, and ovarian (Reinblatt et al., 2007). Currently, six oncolytic HSV vectors, G207, G47 Δ , 1716, HF10, NV1020, and OncovexGM-CSF (talimogene laherparepvec) have progressed to clinical trials, with OncovexGM-CSF successfully reaching its primary endpoint in a randomized phase III trial for metastatic melanoma (Campadelli-Fiume et al., 2011).

The results of our previous studies indicated that oHSV G47 Δ was an effective and safe therapy for breast and nasopharyngeal carcinomas (Wang et al., 2011a;

Wang et al., 2011b; Li et al., 2012; Zeng et al., 2013a; Zeng et al., 2013b). G47 Δ effectively killed breast cancer cells both *in vitro* and *in vivo* but spared normal breast epithelial cells (Wang et al., 2011a). G47 Δ is a third-generation oHSV, which was constructed from G207, and contains 3 deletions/mutations that result in selective cytotoxicity to tumor cells (Todo et al., 2001). In addition, both copies of the γ 34.5 gene are deleted in this vector, which removes the major HSV neurovirulence gene and precludes the shut-off of protein synthesis in response to viral infection in host cells (Mohr et al., 2001); thus, this mutation attenuates neurovirulence, decreases the chance of reverting to wild-type virus, and causes the virus to preferentially replicate in tumor cells. The ICP6 gene encodes the large subunit of ribonucleotide reductase, which is the key enzyme involved in deoxyribonucleic acid synthesis and viral DNA synthesis in nondividing cells (but not in dividing cells) (Goldstein and Weller, 1988). The ICP6 gene is inactivated by insertion of the lacZ gene, which confers tumor cell selectivity to the virus. The α 47 gene and the promoter region of the US11 gene are also deleted, which places the US11 gene under the control of the immediate-early α 47 promoter. These mutations enhance the viral replication ability and increase the antitumor immune response by increasing MHC class I presentation (Todo et al., 2001).

Although differentiated thyroid cancer is the most common type of thyroid cancer and the majority of patients experience positive outcomes, the 10-year recurrence rate among elderly patients with tumors larger than 4 cm in diameter or those with tumors that have spread beyond the thyroid, such as those with lymph node metastases, ranges from 20% to 30% (Sherman et al., 2008). MTC represents 5% to 8% of all thyroid cancers and is more aggressive. One-half of all MTC patients initially develop distant metastases, and the 10-year survival rate is 10% for these patients. Moreover, no treatment has been proven to prolong the survival of these patients (Pacini et al., 2010). Although ATC represents less than 2% of all thyroid cancers, it accounts for approximately 40% of thyroid cancer mortality (Lai et al., 2005). Therefore, new therapies are needed to target thyroid tumors, especially metastases and ATC.

In the present study, we evaluated the cytopathic effects of the third-generation oHSV G47 Δ on different types of human thyroid carcinoma cells *in vitro*. G47 Δ effectively killed multiple types of thyroid tumor cells, with the ATC cell lines ARO and FRO displaying high sensitivity to G47 Δ . The ARO cell line was especially sensitive at low MOI, while the DTC cell lines KAT-5 and WRO showed modest sensitivity. ARO was previously found to be one of the more sensitive thyroid cancer lines to NV1023, another oHSV with different mutations (Yu et al., 2004). KAT-5 was reported to express high levels of nectin-1, one of the HSV receptors, while WRO did not to express nectin-1, which might explain its poor sensitivity here except we found reasonable X-gal staining, indicative of virus infection (Huang et al., 2007). Finally, we found that G47 Δ effectively inhibited ARO and FRO s.c. tumor growth *in vivo*. All of the tumors completely regressed in the G47 Δ treatment group, and survival in this group was

significantly prolonged. Moreover, no mouse displayed any side effects due to G47 Δ administration. Yu et al showed that ARO tumors were also sensitive to NV1023, although only 4 of 6 mice remained tumor-free long term after 3 i.t. injections (Yu et al., 2004).

In conclusion, the third-generation oHSV G47 Δ effectively killed different types of human thyroid carcinoma cells *in vitro*. Of particular interest, G47 Δ significantly inhibited ATC growth *in vivo* and prolonged animal survival in 2 different tumors, indicating its potential as an effective treatment for different types of thyroid tumors.

Acknowledgements

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