The oncolytic herpes simplex virus vector, G47Δ, effectively targets tamoxifen-resistant breast cancer cells

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Abstract. The aim of the present study was to establish a tamoxifen-resistant cell line (MCF-7/TAM-R) and to investigate the therapeutic effect of G47Δ on this cell line both in vitro and in vivo. In the present study, the MCF-7/TAM-R monoclonal subline was established after exposing MCF-7 cells to tamoxifen for 21 days. Then, it was compared with a wild-type MCF-7 subline (MCF-7/Δ), which was not treated with tamoxifen. Cell proliferation, viability, cell cycle and apoptosis analyses were carried out to examine the characteristics of the MCF-7/TAM-R cells. Both in vitro and in vivo toxicity studies were conducted to investigate the therapeutic effect of G47Δ on the MCF-7/TAM-R cells. Compared to the MCF-7W cells, we found that the MCF-7/TAM-R cells exhibited a higher proliferation ability (P<0.05) and a stronger resistance to the cytotoxic effects induced by 4-hydroxytamoxifen (4-OHT) (P<0.05). G47Δ demonstrated a high cytotoxic effect on both the MCF-7/TAM-R and MCF-7W cell lines. After being infected with G47Δ at an MOI of 0.01, >90% of the MCF-7/TAM-R and MCF-7W cells died on day 5. G47Δ induced cell cycle arrest in the G2/M phase. Furthermore, G47Δ inhibited tumor growth in subcutaneous tumor models of both MCF-7/TAM-R and MCF-7W. Thus, we conclude that G47Δ, a third generation oncolytic herpes simplex virus, is highly sensitive and safe in targeting tamoxifen-resistant breast cancer cells both in vitro and in vivo.

Introduction

Breast cancer is the most common malignant neoplasm in women, and more than 1.6 million newly diagnosed cases were reported in 2012 worldwide (1). The National Cancer Institute has reported that over 70% of breast cancers express the estrogen receptor (ER) (2), providing biological evidence to administer endocrine therapy as referenced management for patients with ER-positive diseases. This strategy has been widely used in breast cancer prevention and treatment (3-8). Unfortunately, recent studies have revealed that ~50% of cases of ER-positive breast cancer (9) do not respond or partially respond to hormonal therapy due to either intrinsic or acquired therapeutic resistance. Among all antiestrogenic compounds, tamoxifen, which blocks ER signaling by binding to the corresponding receptor, is the most frequently used medication in the past decades (7,10,11). However, nearly one-third of ER-positive breast cancer patients are not sensitive to tamoxifen (12). In such cases, aromatase inhibitors (AIs) alone or in combination with ovarian suppression are used to treat breast cancer. Nonetheless, AIs and ovarian suppression are associated with several adverse events, which severely hamper the quality of life of such patients. Ovarian ablation (surgical or radiation) completely blocks the source of ovarian estrogen, leading to an irreversible menopause at a young age. Moreover, bone density loss (13) and cardiovascular events (8,14) usually occur in patients treated with AIs or ovarian suppression. At present, only few therapeutic strategies with significant side-effects are available for tamoxifen-resistant patients. Therefore, safer and more effective treatments for these patients are urgently needed.

Oncolytic herpes simplex virus (oHSV) is a promising agent for various types of cancers. It has been widely used in cancer research studies (15). Furthermore, several oncolytic viruses have been tested in clinical trials, such as NV1020, G207, HF10 and OncoVEX (GM-CSF). These viruses can safely and effectively target cancer cells (16-18). G47Δ is a third generation vector; it is generated from G207 by deleting the non-essential α-47 gene. A previous study reported that, compared to G207, G47Δ replicates and spreads better in infected cells. Furthermore, G47Δ was found to be more efficient in inhibiting the growth of tumors (19). G47Δ has also been employed in a phase I clinical trial of progressive glioblastoma (20).

In the present study, we established the MCF-7 tamoxifen-resistant monoclonal subline and the MCF-7 wild-type monoclonal subline (21), and investigated the cytotoxic effects of the G47Δ oHSV vector on the tamoxifen-resistant cell line in vitro and in vivo.
Materials and methods

Cell culture and virus. MCF-7 and Vero cells were provided by Dr Mu Sheng Zeng (Sun Yat-Sen University Cancer Center, Guangzhou, China). We cultured both types of cells in Dulbecco's modified Eagle's medium (DMEM; Gibco, Shanghai, China), which was supplemented with glucose (4.5 g/l) and 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) at 37°C in an atmosphere containing 5% CO₂.

Oncolytic HSV G47Δ was purchased from MediGene Inc. (San Diego, CA, USA), and it was reproduced in Vero cells. After achieving approximately 80% confluence, the Vero cells were infected with G47Δ at a multiplicity of infection (MOI) of 0.03 and incubated at 37°C in 5% CO₂. All infected cells were harvested and centrifuged at 1,000 x g for 6 min, and then the liquid supernatant was discarded and 1 ml virus buffer (150 mM NaCl/20 mM Tris, pH 7.5) was added to the system. Thereafter, we subjected the cells to three freeze-thaw cycles; the resultant liquid supernatant was stored at -80°C for future analyses.

Virus titers were determined by plaque assays on the Vero cells. In brief, the Vero cells were seeded into 6-well plates with DMEM and 10% FBS at 37°C in 5% CO₂ and infected with 700 µl virus dilute (10⁻¹/ml, 10⁻²/ml, 10⁻³/ml or 10⁻⁴/ml) when cells achieved 100% confluency. The infected cells were cultured at 37°C in 5% CO₂ for 48 h before subsequent X-gal staining for plaque counting. The following equation was applied to calculate virus titers: Virus titers = plaque numbers/0.7 x dilution ratio (19).

Establishment of the tamoxifen-resistant cell line. We derived MCF-7/tamoxifen-resistant (MCF-7/TAM-R) and wild-type MCF-7 (MCF-7W) cells from the tamoxifen-sensitive MCF-7 cell line (21). To establish the MCF-7/TAM-R monoclonal subline, MCF-7 cells were seeded in a 10-cm dish at a density of 1.0x10⁶ cells/dish. The cells were cultured in DMEM (5% FBS) containing 1 µM of 4-hydroxytamoxifen (4-OHT); this culture was maintained for 21 days at 37°C in 5% CO₂. Then, the survived monoclonal cells were cultured in complete medium without tamoxifen for 7 days. After that, each MCF-7/TAM-R monoclonal was separately picked and cultured in a 96-well plate. In order to maintain tamoxifen resistance in the MCF-7/TAM-R cells, we cultured them in complete medium containing 0.1 µM of 4-OHT. To obtain the MCF-7W monoclonal subline, MCF-7 cells were seeded in a 10-cm dish at a density of <100 cells/dish and cultured without 4-OHT for 30 days. Then, we selected the MCF-7W monocline and incubated the cells in a 96-well plate.

Cell proliferation assays. MCF-7/TAM-R and MCF-7W cells were separately seeded into a 96-well plate at a density of 5.0x10⁵ cells/well and cultured in DMEM (5% FBS) at 37°C in 5% CO₂. Cell proliferation was measured by Cell Counting Kit-8 assay (CCK-8; Dojindo, Japan) for 5 days. All experiments were triplicated.

Cell viability assays. MCF-7/TAM-R and MCF-7W cells were separately seeded overnight in a 96-well plate at a density of 5.0x10⁴ cells/well. The cells were then treated with 4-OHT or vehicle (ethanol, control). In the treatment group, the doses of tamoxifen were exponentially increased (10⁻⁸, 10⁻⁷, 10⁻⁶, 10⁻⁵ and 10⁻⁴ mol/l). After 48 h, cell viability was evaluated using the CCK-8 assay. Prior to this measurement, we added 100 µl DMEM, which contained 10 µl CCK-8, to each well and incubated them for 120 min at 37°C. Subsequently, we measured the absorption at 450 nm using an enzyme standard instrument (BioTek, Winooski, VT, USA). ED₅₀ was calculated using CompuSyn software (CompuSyn Inc., New York, NY, USA). Cell survival rate and resistance-index were computed using the following formulas:

\[ \text{Cell survival rate}=\left(\frac{\text{Average number of cells from virus-treated group}}{\text{Average number of cells from control group}}\right)\times 100\% \]

\[ \text{Resistance-index}=\frac{(\text{MCF-7/TAM-R ED}_{50})}{(\text{MCF-7W ED}_{50})} \]

All of the experiments were performed in triplicate.

Cell cycle analyses. MCF-7/TAM-R and MCF-7W cells were seeded separately in 6-well plates (5.0x10⁵ cells/well). Forty-eight hours after the intervention, we collected the virus-treated (MOI, 0.01), 4-OHT-treated (1 µmol/l) and control group, individually. Then, each group of cells was washed with phosphate-buffered saline (PBS) and centrifuged at 1,000 x g for 5 min. The sediments were resuspended into PBS at a concentration of 1.0x10⁶ cells/ml. Finally, they were fixed overnight in 70% ethyl alcohol at 4°C. A cell cycle detection kit (KeyGen, Nanjing, China) was used to analyze the phase distribution of the cell cycle. In brief, the samples were first centrifuged and resuspended in 0.1 ml of RNase A (KeyGen). After incubating the cells at 37°C for 30 min, we introduced 0.5 ml of propidium iodide (PI; KeyGen) staining solution to the system. The distribution of the cell cycle was evaluated by flow cytometry (BD LSR II; BD Biosciences, Franklin Lakes, NJ, USA).

Analysis of cell apoptosis. MCF-7/TAM-R and MCF-7W cells were separately seeded in a 6-well plate (5.0x10⁵ cells/well) and harvested after 48 h. Each group of cells was washed twice with PBS. Then, 5.0x10⁵ cells of each group were suspended in 500 µl binding buffer. Then, the cells were stained with Annexin V and PI [Annexin V-FITC apoptosis detection kit (KeyGen)] according to the manufacturer's instructions. All the data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Western blot analyses. MCF-7/TAM-R and MCF-7W cells were placed into 6-well plates. Then, the cells were treated with 4-OHT or vehicle (ethanol) for 48 h. After the intervention, the cells were washed twice with ice-cold PBS and lysed with a whole cell lysis kit (KeyGen). The protein density was measured using the BCA protein quantification kit (KeyGen). Then, all the samples were stored at -20°C before being subjected to electrophoresis. Briefly, the electrophoresis was carried out by separating 50 µg of total cellular protein on 10% or 15% SDS-polyacrylamide gel. Then, the cellular protein was electrotransferred onto a 0.22-µm thick nitrocellulose membrane (Millipore, Billerica, MA, USA). Thereafter, the membrane was blocked in 5% bovine serum albumin (Roche, Basel, Switzerland) in TBS-Tween buffer (TBS-T) for 1 h and
submitted to the primary and secondary antibodies. Briefly, the membrane was incubated overnight with the primary antibodies at 4°C. After washing the membrane thrice in TBS-T, it was incubated in the secondary antibody for 1 h at room temperature. The immunoreactive protein bands were detected using a chemiluminescence imaging system (Tanon-5200; Tanon, Guangzhou, China). The following antibodies were used: ER-α receptor (1:1,000 diluted), ER-β receptor (1:1,000 diluted) (both from Abcam, Cambridge, UK), Bcl-2-interacting killer (BIK; 1:1,000 diluted), caspase-7 (1:1,000 diluted) and β-actin (1:3,000 diluted) (all from Cell Signaling Technology, Boston, MA, USA). The secondary antibody was HRP-goat anti-rabbit IgG (1:2,000 diluted; Invitrogen, Shanghai, China).

In vitro cytotoxicity. MCF-7W and MCF-7/TAM-R cells were either infected with G47Δ at different MOIs (MOI, 0.01, 0.1 or 1) or mock infected and incubated in DMEM (1% FBS) at 37°C in 5% CO₂. After infection, the cells were stained with X-gal solution (Beyotime, Shanghai, China) and counted on a hemocytometer (Qiujing, Shanghai, China) for 5 days. The average number of cells from duplicate wells was recorded as the percentage of mock-infected cells.

In vivo experiment. All the protocols for the animal experiments were approved by the Ethics and Use Committee of the Research Institute at Sun Yat-Sen University, Guangdong, China. Female BALB/c nude mice (4 weeks of age, 14-16 g in weight) were purchased from Vital Rival Laboratories (Beijing, China). The mice were randomly divided into the following four groups (5 mice/group): MCF-7/TAM-R control group, MCF-7/TAM-R virus-treated group, MCF-7W control group, and MCF-7W virus-treated group. MCF-7/TAM-R and MCF-7W cells (1.0x10⁷) were suspended in 100 µl of DMEM containing 25% Matrigel (Corning, USA) and separately injected subcutaneously into the groin of each mouse. When the tumor attained a maximum diameter of 5 mm, we injected 50 µl of G47Δ (2.0x10⁷ pfu, repeated 4 times at an interval of 3 days) in the two virus-treated groups; the same volume of virus buffer (150 mM NaCl, 20 mM Tris, pH 7.5) was injected into the two control groups. The tumor size was evaluated using a Vernier caliper every 3 days, and the tumor volume = (width² x length)/2. On day 7 after infecting the virus, a mouse from each group was sacrificed to obtain subcutaneous tumors. Then, X-gal stain was applied to the frozen tumor sections to determine the efficacy of infection in each group (tissue showing blue staining was considered to be infected with G47Δ). The remaining animals were sacrificed on day 60, and their tumors were resected and fixed in 4% paraformaldehyde (Beijing Leagene Biotech Co., Beijing, China). The date of death was recorded for further survival analyses. H&E staining and immunohistochemical staining (Histostain-Plus kit; ZSGB-BIO, Beijing, China) were carried out to determine the tumor characteristics and to analyze the expression of ER-α and ER-β. For ER-α and ER-β analyses, five fields were randomly selected from each sample and examined under a microscope (magnification, x100).

Statistical analyses. The statistical analyses were carried out using SPSS 22.0 (IBM, Armonk, NY, USA). The reported values were presented as mean ± standard deviation (SD). The difference between the groups was evaluated using paired-sample t-test and one-way ANOVA. A P-value of <0.05 was considered to indicate a statistically significant result.

Results

Characteristics of the MCF/TAM-R and MCF-7W cells. As shown in Fig. 1, we successfully established MCF-7/TAM-R and MCF-7W cells. The cell proliferation analyses showed that compared to the MCF-7W cell line, the MCF-7/TAM-R cell line had a higher proliferation rate (P=0.018; Fig. 2A). Tamoxifen toxicity experiments confirmed that the ED₅₀ of 4-OHT in the MCF-7W cells was 2.40±0.08x10⁻⁶ mol/l, while the ED₅₀ of 4-OHT in the MCF-7/TAM-R cells was 2.98±0.51x10⁻⁵ mol/l (P=0.007; Fig. 2B). The resistance-index was 12.41±1.76.

After 48 h of treatment, 4-OHT (1 µM) increased cell cycle arrest in the G0/G1 phase (P=0.002) of the MCF-7W cells (Fig. 2D); however, 4-OHT did not show a significant impact on the MCF-7/TAM-R cell cycle (Fig. 2C).
Annexin V-FITC/PI staining indicated that 4-OHT induced apoptosis in the MCF-7W cells (mock vs. 4-OHT, 0.74±0.89 vs. 5.89±0.88%; P=0.001). However, no significant difference was detected between the 4-OHT-treated MCF-7/TAM-R and the mock control cells (0.94±0.19 vs. 0.64±0.94%; P=0.71) (Fig. 2E).

Caspase-7 and BIK were assessed to determine whether 4-OHT induced the expression of apoptotic proteins in the MCF-7W and MCF-7/TAM-R cells. Western blot analyses showed that tamoxifen increased the expression of caspase-7 and BIK in the MCF-7W cell line; however, no significant difference was detected in the MCF-7/TAM-R cell line (Fig. 2F). This indicates that the MCF-7/TAM-R cells showed significant drug resistance to 4-OHT. According to western blot analyses, the expression of ER-α and ER-β in the MCF-7/TAM-R cells was significantly lower than that in the MCF-7W cells (Fig. 2F).

G47Δ effectively targets MCF-7/TAM-R and MCF-7W cells. To determine the susceptibility of the tamoxifen-resistant MCF-7 cell line to G47Δ, MCF-7/TAM-R and MCF-7W cells were cultured in 6-well plates separately, and infected with G47Δ at MOI=0.01, 0.1 and 1. G47Δ exhibited a similar cytotoxic effect on the MCF-7/TAM-R and MCF-7W cell lines (Fig. 3A and B). At MOI=0.01, 91% of the MCF-7/TAM-R and 85% of the MCF-7W cells were killed on day 5. At MOI=0.1, 91% of the MCF-7/TAM-R and 80% of the MCF-7W cells were killed on day 4. At MOI=1, 90% of the MCF-7/TAM-R and 87% of the MCF-7W cells were killed on day 3 (Fig. 3C and D).
Δ induces cell cycle arrest and increases the levels of apoptosis. G47Δ significantly increased cell apoptosis in the MCF-7/TAM-R cells (control vs. virus: 2.43±0.97 vs. 14.88±0.85%; P=0.005) (Fig. 4) and in the MCF-7W cells.
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(mock vs. virus: 2.49±0.40 vs. 15.99±0.22%; P=0.001) (Fig. 4).

At a definite dose (MOI=0.01), G47Δ also significantly impacted the cell cycle in both the MCF-7W and MCF-7/TAM-R cells. Virus infection induced cell cycle arrest at the G2/M phase in both cell lines (MCF-7/TAM-R and MCF-7W; P=0.006 and P=0.004) (Fig. 5).

Antitumor efficacy of G47Δ in vivo. We successfully established subcutaneous tumor models of MCF-7/TAM-R and MCF-7W cells in 4-week-old female BALB/c nude mice. G47Δ significantly inhibited tumor growth in both models (for MCF-7/TAM-R: virus vs. control, 23.83±19.64 vs. 163.81±35.97 mm³; P=0.002; for MCF-7W: virus vs. control, 12.63±9.15 vs. 127.33±23.64 mm³; P=0.006). G47Δ was able to replicate in the MCF-7/TAM-R and MCF-7W models (Fig. 7). During the observation period, no mouse mortality was noted in both the control and virus-treated groups. Immunohistochemical staining revealed that the expression of ER-α and ER-β in MCF-7/TAM-R tumors was significantly lower than that in the MCF-7W tumors (Fig. 6).

Discussion

In the present study, we established MCF-7/TAM-R and MCF-7W monoclonal sublines to investigate the cytotoxic effect of G47Δ, a third generation oHSV, on tamoxifen-resistant
breast cancer cells. The results indicated that G47Δ effectively targeted tamoxifen-resistant cells as well as wild-type MCF-7 cells. We further confirmed that when G47Δ was injected inside the tumor, it not only induced a significant slowdown in tumor growth but also prolonged the survival of both MCF-7/TAM-R and MCF-7W subcutaneous models.

Traditionally, tamoxifen is the most frequently used medication in endocrine therapy, since it effectively inhibits tumor growth and improves the survival rate of ER-positive patients. Nonetheless, the intrinsic or acquired therapeutic resistance towards tamoxifen limits its long-term application in breast cancer treatment. In the past decades, several scientists have tried to employ a virus vector in breast cancer treatment. G47Δ, a third generation HSV vector generated from G207, lacks both copies of neurovirulence γ34.5 gene and is inserted in an E. coli LacZ gene into UL39 when compared to wild-type HSV to inactive the ICP6 gene (22). The γ34.5 gene is the major determinant of neurovirulence in HSV, and it also significantly enhances the replication ability of HSV-1. Furthermore, the HSV-1 ICP6 gene encodes ribonucleotide reductase and it can reduce ribonucleic acid to deoxyribonucleic, which is associated with DNA damage repair and cell division (23). The deletion of γ34.5 and the inactivation of ICP6 ensure that the vector selectively replicates...
in tumor cells but does not introduce any neurotoxicity in normal cells. Moreover, the non-essential ICP47, which inhibits antigen presentation, is deleted in G47Δ. This enhances MHC class I presentation, increases stimulation of lymphocytes, and decreases NK cytolysis. The promoter region of US11 that inhibits protein synthesis was also nulled, so as to increase virus replication and efficacy (19). In our previous research, G47Δ was found to be effective and safe in treating various tumors, including nasopharyngeal (20,22,24-28), thyroid (27) and hepatocellular carcinoma (29), breast (20,22,24-26,30) and prostate cancer (31), and malignant glioma (32,33). However, no previous study has confirmed that G47Δ can effectively target tamoxifen-resistant breast cancer.

Our data confirmed that tamoxifen arrested the cell cycle in the G0/G1 phase and reduced the proportion of MCF-7W cells in the S phase (34). However, it did not control the cell cycle distribution in the MCF-7/TAM-R cell line. MCF-7 cells are null for the CASP3 gene, therefore the apoptosis requires sequential activation of caspase-9, caspase-7 and caspase-6 (21). BIK is a pro-apoptotic factor of the Bcl-2 protein family and previous research has demonstrated that BIK inhibited Bcl-2 and Bcl-X factormediating cell proliferation in MCF-7 cells (35). In our study, the expression of caspase-7 and BIK in the MCF-7/TAM-R cells did not change after 4-OHT intervention, confirming that the MCF-7/TAM-R cells were resistant to tamoxifen and were not sensitive to the negative regulatory effects mediated by tamoxifen.

Tamoxifen inhibits breast cancer cell growth by blocking ER, therefore, the expression of ER-α is an important predictor in hormonal therapy. In ER-α-positive breast cancer cells, the expression of ER-β can increase the sensitivity towards tamoxifen as it downregulates HER2/HER3 signaling and increases the expression of PTEN (36). However, various other research studies have indicated that higher levels of ER-β indicate tamoxifen sensitivity (35,37). In cases showing acquired resistance towards tamoxifen, ER-α and ER-β exhibit altered structure and function (38). Our results indicated that MCF-7/TAM-R cells had decreased expression of ER-α and ER-β, demonstrating that the tamoxifen-resistant monoclonal subline was successfully established.

In order to evaluate the impact of G47Δ on tamoxifen-resistant cells, MCF-7/TAM-R and MCF-7W cells were infected with G47Δ at various MOIs (0.01, 0.1 and 1). In both the MCF-7/TAM-R and MCF-7W cell lines, we observed extremely strong cytotoxic effects even at a very low MOI (0.01). Similarly, G47Δ significantly influenced cell cycle distribution, inducing cell cycle arrest at the G2/M phase in both the MCF-7W and MCF-7/TAM-R cells. Apoptosis analysis also confirmed that G47Δ introduced cell apoptosis in the MCF-7W and MCF-7/TAM-R cells. The above results indicate that G47Δ has great potential in reducing the replication and division of cancer cells. To further verify the anti-tumor effect of G47Δ on tamoxifen-resistant breast cancer in vivo, we employed MCF-7/TAM-R and MCF-7W subcutaneous mouse models in the present study. An intra-tumor G47Δ injection significantly reduced tumor growth in both models. In addition, no mouse mortality occurred in the virus-treated group at the end of the study.

There are several limitations to our study. MCF-7 cells alone may not represent all the characteristics of ER-positive breast cancer cells. Additional ER-positive cell lines should be included in future studies. Furthermore, the underlying mechanism involved in the effective inhibition of MCF-7/TAM-R cell growth by G47Δ remains undetermined.

In conclusion, G47Δ, a third generation oHSV, is highly sensitive and safe for targeting tamoxifen-resistant breast cancer cells both in vitro and in vivo.

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References


