The oncolytic herpes simplex virus vector G47Δ effectively targets breast cancer stem cells

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Abstract. Accumulating evidence suggests that breast cancer originates from cancer stem cells (CSCs), which comprise a small percentage of the overall tumor but are highly tumorigenic and pluripotent with unlimited proliferation potential. Furthermore, CSCs are highly resistant to conventional treatment, which may explain certain difficulties in treating cancer with current therapy options. In this study, the third generation oncolytic herpes simplex virus (oHSV) vector G47Δ effectively killed different subtypes of breast cancer cells, with more than 98% of the tumor cells killed by Day 5. Moreover, G47Δ targeted equally non-cancer stem cells (NCSCs) and CSCs which showed resistance to paclitaxel. We demonstrated that G47Δ effectively replicated and spread among CSCs. G47Δ also impaired the self-renewal ability of CSCs, as the viable cells were unable to form secondary tumor spheres. We also showed that G47Δ was able to induce the regression of tumor xenografts in BALB/c nude mice and demonstrated the ability of G47Δ to synergize with paclitaxel by killing both NCSCs and CSCs, suggesting that oHSV may be an effective treatment modality for patients with breast cancer.

Introduction

The cancer stem cell (CSC) theory proposes that tumor cells are composed of a heterotypic population of cells and the CSCs, which comprise a small percentage of the total tumor, remain at the top of the cellular hierarchy with unlimited proliferation potential and pluripotency (1,2). Recent evidence indicates that breast CSCs are relatively resistant to both radiation and chemotherapy (3,4). Therefore, new treatment strategies that effectively target both NCSCs and CSCs show promise as an effective treatment for breast cancer.

Materials and methods

Cells and viruses. Breast cancer cell lines MCF-7, SK-BR-3, MDA-MB-435, MDA-MB-436 and MDA-MB-468 were obtained from Dr Xie Xiao-ming (Sun Yat-sen University Cancer Center, China). G47Δ was constructed as previously described (7) and was obtained from Dr Samuel D. Rabkin, Molecular Neurosurgery Laboratory, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA. Viruses were grown and titered using Vero cells (African green monkey kidney cells; ATCC, Manassas, VA, USA).

In vitro cytotoxicity of the parental cell lines. Cells were infected with mock or viruses at multiplicity of infection (MOI) of 0.1 and 0.01, then incubated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, USA) supplemented with 1% heat-inactivated fetal calf serum. Cells were stained with trypan blue (Sigma, USA) and counted on a hemocytometer.

For combination therapy, cells were seeded into a 10-cm culture dish and infected with viruses at a MOI of 0.5, 1 nM paclitaxel (Sigma), G47Δ+ paclitaxel or a mock treatment. Cells were incubated at 37°C for 4 days in DMEM supplemented with 10% heat-inactivated fetal calf serum. Cells were harvested and...
counted on Day 4 and stained with fluorescein isothiocyanate-labeled (FITC) anti-CD44 and phycoerythrin-labeled (PE) anti-CD24 antibodies (both from Beckman Coulter, Brea, CA, USA). The stained cells were assayed by flow cytometry (Becton-Dickinson), and the percent of CD44<sup>+</sup>CD24<sup>-</sup> cells was calculated.

**Isolation and identification of CSCs.** As previously described (8), cells were cultured in DMEM/F12 medium supplemented with 10 µg/l basic fibroblast growth factor, 20 µg/l epidermal growth factor, 5 mg/l insulin and B27 (all from Invitrogen). Cells generally formed mammospheres within 10 days. The mammospheres and parental cell lines were trypsinized and stained with anti-CD44 and anti-CD24 antibodies as described above. The Aldefluor assay was performed according to the manufacturer's protocol (Stemcell Technologies, Canada).

**In vitro cytotoxicity of G47∆ on CSCs.** Mammospheres were dissociated, the resulting single cells were resuspended at 1x10<sup>7</sup> cells/ml and infected at an MOI of 0.1 or mock-infected for 90 min at 37°C. The cells were then centrifuged to remove unadsorbed viruses and seeded in 6-well plates at 1x10<sup>5</sup> cells per well. Cells were counted on Days 3 and 7 using a hemocytometer. Cells were harvested along with the associated supernatant at 12, 36 and 60 h post-infection. After three freeze/thaw cycles, the titers of infectious virus were tested using a plaque assay with Vero cells.

Seven days following virus infection, the viable cells were collected, resuspended in fresh medium, and seeded into 96-well plates at a density of 1 or 10 cells per well. Two weeks later, the number of wells containing mammospheres (diameter >40 µm) was recorded.

**In vivo treatment studies.** MDA-MB-468 mammosphere cells (1x10<sup>6</sup>) were implanted into the left flank of 6-week-old female BALB/c nude mice (Vital River Laboratory Animal Technology Co., Ltd., China). When tumors reached a maximal diameter of ~5 mm, mice were randomized into 4 groups of 7 mice per group. The groups consisted of a G47∆ treatment group that was given a 50-µl intratumor (i.t.) injection of G47∆ virus (2x10<sup>7</sup> plaque-forming units, pfu) on Days 0 and 3, a paclitaxel treatment group treated with an intraperitoneal (i.p.) injection of 3 mg/kg paclitaxel on Days 0 and 7, a combination treatment group that was treated with G47∆ and paclitaxel on the same dosing schedule, and a mock treatment group. Tumor volume was calculated using the formula: width (mm)<sup>2</sup> x length (mm) x 0.5.

Tumor tissue was minced and digested for ~6 h at 37°C using a solution of 100 U/ml collagenase I, 150 U/ml hyaluronidase, 10% calf serum, and 5 mg/l bovine insulin in DMEM (all from Invitrogen). Digested tissue was strained using a 40-µm strainer, the cell suspension was washed with PBS and the red blood cells were lysed for 5 min (Bomeike Biotechnology, China). The samples were analyzed for the percentage of CD44<sup>+</sup>CD24<sup>-</sup> cells using flow cytometry. All
animal procedures were approved by the Animal Care and Use Committee of Sun Yat-sen University.

**X-gal histochemistry.** X-gal staining was performed according to the manufacturer’s protocol (Beyotime Institute of Biotechnology, China).

**Statistical analysis.** Data are presented as the means ± standard deviation (SD). Each experiment was repeated in at least three independent trials. A Student's t-test was used to analyze the significance of differences between two treatment groups. SPSS version 13.0 software was used, and P<0.05 was considered to indicate a statistically significant difference.

**Results**

G47Δ effectively kills different types of breast cancer cell lines in vitro. Neve et al (9) reported that breast cancer cell lines effectively model primary breast tumors. MCF-7, SK-BR-3 and three other cell lines represent luminal subtype tumors, HER-2 overexpression subtype and basal-like subtype, respectively. We showed that G47Δ effectively killed different subtypes of tumors (Fig. 1A). MCF-7, MDA-MB-435 and MDA-MB-468 were highly sensitive to G47Δ-mediated killing with >98% of these tumor cells dying by Day 4 with an MOI of 0.01. By Day 3, almost all the MDA-MB-468 cells were infected by G47Δ (Fig. 1B). SK-BR-3 and MDA-MB-436 cells displayed a moderate sensitivity to G47Δ-mediated killing with >98% of cells dying by Day 5. These findings demonstrate that G47Δ can effectively kill different subtypes of breast cancer cells.

**Isolation and identification of CSCs.** CSCs typically form mammospheres within 10 days of anchorage-independent culture. Our mammosphere cultures could be passaged for >3 months. Moreover, single dissociated mammosphere cells were able to regenerate spheres, thereby indicating their clonogenicity (Fig. 2A and Table I). Using the cultured CSCs, we investigated the percentage of mammosphere cells...
that displayed the stem cell-like phenotype CD44+CD24- and aldehyde dehydrogenase (ALDH). In contrast to their respective parental cell lines, cells derived from mammosphere cells were enriched for CD44+CD24- and ALDH+ cells. CD44+CD24- cells accounted for 0.23±0.08% of parental SK-BR-3 cells, whereas CD44+CD24- cells accounted for 91.69±2.27% of mammosphere cells derived from the SK-BR-3 cell line (Fig. 2B). The percentage of ALDH+ cells in the MDA-MB-468 parental cell line was 2.31±0.27%, whereas ALDH+ cells accounted for 18.14±1.21% of mammosphere cells derived from the MDA-MB-468 cell line (P<0.001) (Fig. 2C). Mammosphere cells from the other breast cancer cell lines displayed a similar enrichment for CD44+CD24- or ALDH+ cells relative to their respective parental cell line (Fig. 2D).

These results show that anchorage-independent cell culture results in the production of mammosphere cells that are enriched with cells expressing breast CSC markers, and these cells display undifferentiated stem-like characteristics.

G47Δ effectively targets breast CSCs in vitro. CSCs were cultured under anchorage-independent conditions and infected with G47Δ at a MOI of 0.1. Our results showed that CSCs were sensitive to G47Δ (Fig. 3A). Similar to the parental cell lines, MCF-7, SK-BR-3 and MDA-MB-468 grown under anchorage-independent conditions were also effectively killed by G47Δ with >98% of CSCs killed by Day 7. To determine whether G47Δ could replicate and spread among CSCs, we quantified viral replication using the plaque-forming unit (PFU) assay. The CSCs and their associated supernatants were collected at indicated time points after infection, and virus titers were determined after three freeze/thaw cycles by the PFU assay using Vero cells. We found that G47Δ replicated considerably in MCF-7, SK-BR-3 and MDA-MB-468 cells (Fig. 3B). Collectively, these results demonstrate that G47Δ effectively targets breast CSCs.

G47Δ kills breast NCSCs and CSCs equally and synergizes with paclitaxel by killing both NCSCs and CSCs in vitro. A previous study demonstrated that CSCs are relatively resistant to chemotherapy (4). This study showed that treatment with the standard breast cancer drug paclitaxel resulted in an increase in the proportion of CD44+CD24- cells (4). Therefore, we determined whether NCSCs were more sensitive to G47Δ than CSCs. To this end, we tested the proportion of CD44+CD24- cells before and after treatment with G47Δ or paclitaxel. Similar to previous research, our results showed that CSCs were generally resistant to paclitaxel-mediated cell death, and, following treatment with paclitaxel, the proportion of CD44+CD24- cells increased (13.13±0.43%) when compared with the control group (5.41±0.42%, P<0.001) (Fig. 4). By contrast, the proportion of CD44+CD24- cells did not change relative to the control cells after treatment with G47Δ (5.44±0.39%, P>0.05). Moreover, when G47Δ was used in combination with paclitaxel...
taxel, we observed a synergistic effect resulting in the death of both NCSCs and CSCs, the percentage of stem cells remained unchanged (5.55±0.32%, P>0.05). Collectively, these results demonstrate that G47A is equally effective in killing both breast NCSCs and CSCs. Furthermore, G47A can synergize with paclitaxel to kill both NCSCs and CSCs in vitro.
The self-renewal of CSCs is impaired after G47Δ infection. Self-renewal is one of the defining properties of CSCs, and the ability to form spheres in vitro is considered an indicator of self-renewal ability. Wakimoto et al. (10) found that G47Δ infection impairs the self-renewal ability of glioblastoma stem cells. Therefore, we used single-cell-derived mammosphere cells to determine whether G47A could impair the self-renewal ability of breast CSCs. Although G47Δ killed almost all mammosphere cells by Day 7, we observed a minor population of cells that remained alive after 7 days of infection with G47Δ. Live cells were collected and subjected to a limiting dilution assay to determine whether these cells maintained the ability to generate secondary mammospheres. Our results showed that none of the cells that survived following G47Δ infection were able to generate secondary mammospheres when seeded at up to 10 cells per well (Table 1). By contrast, as few as one live cell from the control treatment group was able to form secondary mammospheres. Collectively, these results suggest that G47A may inhibit the self-renewal of breast CSCs regardless of whether they undergo oncology. However, the underlying mechanism remains to be determined and warrants further investigation.

G47Δ-induced regression of tumor xenografts in BALB/c mice. We chose MDA-MB-468 mammosphere cells to form tumors in BALB/c mice, as MDA-MB-468 is a basal-like cell line that is very aggressive and forms large tumors that are resistant to tamoxifen or trastuzumab (11). Mice were injected with MDA-MB-468 mammosphere cells, and after a tumor developed, the mice were divided into four treatment groups. One group was treated with a mock treatment, another group received an i.t. G47Δ injection, one group received an i.p. paclitaxel injection, and one group received G47Δ + paclitaxel. Our results showed that G47Δ effectively killed tumor cells in vivo since the mice that received G47Δ alone showed a significant reduction in their mean tumor volume (101.6±46.7 mm³) when compared with the mock treatment group (435.3±50.25 mm³) by Day 28 (P<0.001) (Fig. 5A and B). However, we also observed that the group of mice that received G47Δ + paclitaxel displayed a synergistic effect in vivo and a significant reduction in the mean tumor volume (45.9±35.4 mm³) when compared with G47Δ alone (101.6±46.7 mm³), paclitaxel alone (288.9±44.4 mm³), or mock treatment groups (435.3±50.25 mm³) by Day 28 (P<0.05 for all three comparisons) (Fig. 5A and B). Furthermore, the combined treatment showed no signs of toxicity as the body weights of the mice were similar among the four groups (mean body weight, 22.3 g), and body weight did not significantly change over the course of the study.

We also found that G47Δ effectively replicated and spread among tumor cells in vivo, and the most extensive X-gal staining was apparent 14 days post-inoculation (Fig. 5C). By Day 28, we found that G47Δ remained capable of in vivo replication.

We found that mammosphere cells derived from MDA-MB-468 could differentiate into different types of cancer cells in vivo. The proportion of CD44+CD24- cells of the control treatment group (7.23±0.45%) was similar to the MDA-MB-468 cell line in vitro (5.35±0.42%) (Fig. 5D). G47Δ effectively killed both CSCs and NCSCs in vivo, and the proportion of the CD44+CD24- cells (7.48±0.37%, P>0.05, compared with the control group) did not increase after treatment with G47Δ, which was similar to the in vitro results. However, the proportion of CD44+CD24- cells (23.6±2.54%, P<0.001, compared to the control group) increased 2.2-fold after treatment with paclitaxel. Notably, we found G47Δ and paclitaxel showed a synergistic effect by killing both NCSCs and CSCs in vivo. The proportion of CD44+CD24- cells in the combination treatment group was 7.51±0.63%, which was similar to the control treatment group (P>0.05, compared to the control group).

Collectively, these results suggest that G47A effectively kills breast NCSCs and CSCs in vivo and has a synergistic effect with paclitaxel by killing both CSCs and NCSCs.

Discussion

Different breast cancer subtypes categorized based on molecular characteristics have been shown to be highly associated with patient prognosis; these characteristics also serve as predictive tools for the efficacy of a particular therapy in a given patient. Luminal subtype of breast tumors usually have an excellent prognosis and do not benefit significantly from chemotherapy (12). For patients whose tumors highly express the HER-2 gene, trastuzumab significantly improves the disease-free and overall survival (13,14). However, although triple-negative breast cancers (TNBCs) or basal-like tumors tend to be highly sensitive to chemotherapy, they tend to have the worst prognosis and highest recurrence rates (15-17). Moreover, there are no approved drugs that specifically target TNBCs. There is a substantial ongoing effort to develop therapeutics that effectively target all subtypes of breast cancer and TNBC in particular. In this study, we have shown that G47Δ effectively kills all types of breast cancer cells regardless of the ER, PR and HER-2 status. G47Δ may represent a promising therapeutic agent for the treatment of all subtypes of breast cancer.

Breast CSCs were isolated based on the expression of the two cell surface markers CD44 and CD24 or expression of aldehyde dehydrogenase 1 (ALDH1) (18,19). Previous studies have demonstrated that CD44+CD24- cells are highly tumorigenic and can give rise to a large and diverse population of tumor cell types (18). Ginestier et al. (19) showed ALDH1+ cells were highly tumorigenic, and patients with a higher percentage of ALDH1+ cells had the worst clinical outcomes. Recent studies reveal that stem/progenitor tumor cells can grow in non-adherent conditions based on the unique properties of stem/progenitor cells that allow them to survive and grow in a serum-free suspension culture (8). Thus, the tumor-sphere assay allows researchers to culture breast tumor cells with stem/progenitor cell properties for a long period of time, which represents a suitable in vitro model for the study of breast cancer-initiating cells. We used anchorage-independent cell culture conditions to enrich for CSCs and found that mammosphere cells were enriched with cells expressing breast CSC markers, and these cells display undifferentiated stem-like characteristics.

Despite the improvements in treatment regimens, approximately 30% of patients with an early-stage disease eventually develop recurrent and metastatic lesions (20). CSCs are considered the main source of recurrence and metastasis (1,2). CSCs are relatively resistant to chemotherapy and radiotherapy;
moreover, breast CSCs appear to be ER negative and may play an important role in resistance to endocrine therapy (21).

oHSV viral vectors selectively replicate within tumors and directly destroy tumor cells by virus-induced cell lysis. This mechanism is distinctly different from routine cancer therapies, such as chemotherapy and erodincine therapy. We demonstrated that G47Aequally kills CSCs and NCSCs; G47A
displayed a synergistic effect with paclitaxel by killing both CSCs and NCSCs, and G47A showed no obvious signs of toxicity in mice. G47A might be an effective new strategy to target CSCs and to use in conjunction with conventional chemotherapy to achieve the greatest effect. Ahtaiainen et al (22) found that breast CSCs had innate immunity defects which rendered breast CSCs permissive to oncolytic adenovirus. Next, we may focus on the immune response of breast CSCs to G47A infection both in vitro and in vivo.

CSCs have the ability of self-renewal, and only CSCs are believed to have the ability to proliferate indefinitely to generate new tumors. Through asymmetric division, CSCs divide to produce one daughter stem cell and one daughter cell that may differentiate into a nontumorigenic cell. This theory suggests that agents that target the self-renewal pathways in CSCs could represent effective routes of therapy. Our results show that CSCs that survive G47A infection are unable to form secondary mammospheres, thereby indicating that the self-renewal ability of CSCs remains impaired by G47A even if lysis does not occur. Similarly, Wakimoto et al (10) reported that the inhibition of self-renewal by G47A may be due to factors secreted by G47A or due to G47A directly inhibiting the self-renewal pathways. The mechanism whereby G47A inhibits the self-renewal capability of stem cells remains unknown and warrants further investigation.

In conclusion, we have shown that G47A effectively and equally targets NCSCs and CSCs derived from different breast cancer subtypes. Furthermore, G47A appeared to impair the self-renewal ability of CSCs. G47A also demonstrated a synergistic effect with paclitaxel to amplify the killing of both NCSCs and CSCs in vitro and in vivo. To our knowledge, this is the first report on the targeted treatment of breast CSCs with oHSV.

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References