

Therapeutic Efficacy of Prodrug Activator Gene Therapy Using Retroviral Replicating Vectors for Human Ovarian Cancer

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Abstract. *Background/Aim:* Retroviral replicating vectors (RRV) have exhibited efficient tumor transduction and improved therapeutic benefits in a variety of cancer models. In this study, we validated two RRV created from amphotropic murine leukemia virus (AMLV) and gibbon ape leukemia virus (GALV), which use different cell receptors for virus entry, in human ovarian cancer (OC) cells. *Materials and Methods:* Expression levels of the receptors for AMLV (PiT-2) and GALV (PiT-1) in human OC cell lines (A2780, Caov3, RMG-1, SKOV-3), fibroblasts and HEK293 cells were evaluated using quantitative RT-PCR. *In vitro* RRV-GFP replication was monitored using flow cytometry, and cytotoxicity quantitated using AlamarBlue assay after 5-fluorocytosine treatment of OC cells transduced with RRV expressing the yeast cytosine deaminase prodrug activator gene. *In vivo* antitumor effect of RRV-mediated prodrug activator gene therapy was investigated in a SKOV-3 subcutaneous tumor model. *Results:* Quantitative RT-PCR analysis revealed high expression levels of PiT-2 (AMLV receptor) and PiT-1 (GALV receptor) in the RMG-1 and SKOV3 OC cell lines, compared with their levels in non-malignant cells. In RMG-1 and SKOV3 cells, both RRV showed highly efficient RRV replication and spread leading to over 90% transduction

by Days 10-13. Additionally, both RRV that express the yeast cytosine deaminase gene demonstrated effective cell killing of RMG-1 and SKOV-3 cells upon treatment with the prodrug 5-fluorocytosine. Notably, RRV-mediated prodrug activator gene therapy showed significant inhibition of subcutaneous SKOV-3 tumor growth in nude mice. *Conclusion:* RRV-mediated prodrug activator gene therapy may be used for treating PiT-expressing human OC.

Ovarian cancer (OC) is a major cause of female cancer-related deaths worldwide, with 295,000 new cases and 185,000 deaths annually (1). OC has few early symptoms and is often detected late, leading to peritoneal dissemination at diagnosis. The fatality rate is high, with a 5-year survival rate below 45%, given that most OCs are discovered at advanced stages (2). Thus, the development of novel therapeutic paradigms is crucial.

As a new therapeutic paradigm for OC, oncolytic viruses, which selectively replicate in tumors, are gaining attention (3). Various viruses, including adenovirus (4, 5), measles virus (6), reovirus (7), herpes simplex virus type 1 (8, 9), and vesicular stomatitis virus (10), are being developed and evaluated in clinical studies, but further research is needed before implementing this strategy.

Retroviral replicating vectors (RRV) can only effectively transduce and replicate in cancer cells due to their inability to infect post-mitotic cells, defective anti-retroviral immunity in normal cells, and suppression of acquired immunity in the tumor microenvironment (11-13). Unlike other oncolytic viruses, RRV, which were developed based on the amphotropic murine leukemia virus (AMLV), are not inherently cytolytic. However, prodrug activator genes can be engineered into RRV to induce synchronized cell killing of infected tumor cells upon prodrug administration. Using RRV expressing the yeast cytosine deaminase (CD) prodrug activator gene, we demonstrated highly effective killing of various cancer cells *in*

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Key Words: Ovarian cancer, retroviral replicating vectors, prodrug activator gene therapy, cancer virotherapy, amphotropic murine leukemia virus, gibbon ape leukemia virus.



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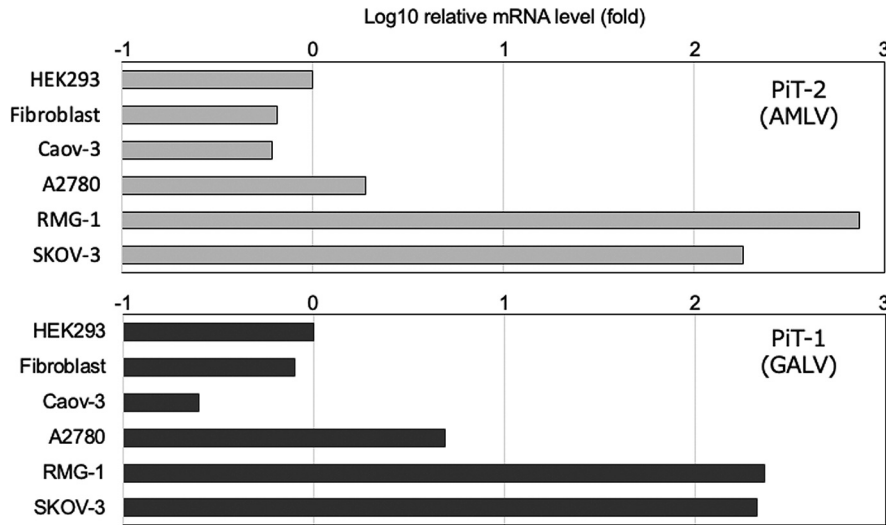


Figure 1. Expression of the cellular receptor of AMLV and GALV in OC cells. Total RNA was extracted from non-malignant human cell lines (HEK293, fibroblasts) and human OC cell lines (Caov-3, A2780, RMG-1, and SKOV3). The RNA samples were prepared in triplicate, reverse-transcribed, and amplified using PCR with RRV-specific primers for PiT-2 (AMLV receptor), PiT-1 (GALV receptor), and GAPDH. GAPDH was used as an internal control to normalize for different amounts of total RNA.

vitro and *in vivo* (11-18). Clinical trials for RRV-mediated prodrug activator gene therapy have started, showing therapeutic benefit in recurrent high-grade glioma (11, 19-21).

We have also recently developed another RRV derived from the gibbon ape leukemia virus (GALV) (17). Although both GALV and AMLV are members of the gamma retrovirus genus, they infect cells through different receptors, with GALV entering the host cell *via* the PiT-1 phosphate transporter and AMLV *via* the PiT-2 phosphate transporter (22). PiT-1 and PiT-2 proteins regulate intracellular inorganic phosphate balance in normal cells, but we previously found that their expression varies in various cancer cell lines (16, 17, 23, 24). Low expression of these receptors restricts the proliferative propagation of RRV and reduces tumor cell-killing effects (16, 17, 23). Therefore, evaluating PiT-1 and PiT-2 levels in cancerous tumors could be an effective biomarker for therapy selection.

In the present study, we assessed the tumor-selective, highly efficient gene delivery and the therapeutic efficacy of AMLV and GALV in human OC cells.

Materials and Methods

Cell lines. Human dermal fibroblasts were purchased from Cell Systems Corporation (Kirkland, WA, USA) and cultivated in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO, USA). The human embryonic kidney 293 cell line (HEK293; Microbix, Toronto, Canada) (25) and transformed human embryonic kidney 293T cell lines (26) were cultivated in DMEM (Nacalai Tesque) supplemented with 10% FBS. Among human OC cell lines,

Caov-3 and SKOV3 were obtained from the American Type Culture Collection (Manassas, VA, USA). A2780 and RMG-1 cell lines were obtained from the National Institutes of Biomedical Innovation, Health, and Nutrition (Ibaraki, Japan). Caov-3 and RMG-1 cells were grown in DMEM supplemented with 10% FBS. A2780 and SKOV3 were grown in RPMI supplemented with 10% FBS. All cells were cultured at 37°C with 5% CO₂.

Quantitative reverse-transcription PCR to assess PiT-1 and PiT-2 expression in cell lines. Total RNA was extracted from semi-confluent cell cultures that had been grown on 10-cm plates using RNA extraction solution (Sepasol-RNA I Super G, Nacalai Tesque). After treatment with DNase I (Takara Bio, Kusatsu, Japan), the TaqMan One-Step RT-PCR Master Mix Reagents kit (Applied Biosystems Japan, Tokyo, Japan) was used to perform quantitative (q)PCR to evaluate the expression of PiT-1, PiT-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (17). In brief, 20 ng of total RNA was added to a reaction mixture containing 18 pmol of each primer and 5 pmol of the probe, and the reaction was run on the ABI 7500 Fast Real-Time PCR System (Applied Biosystems Japan) for one cycle of 48°C for 30 min and 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min.

Viral vector plasmid and virus production. The RRV vector plasmids pAMLV-GFP, pGALV-GFP, pAMLV-CD, and pGALV-CD have all been previously described (17); each contains a full-length amphotropic AMLV or GALV with an additional internal ribosome entry site (IRES)-GFP or IRES-CD cassette, respectively. To produce each RRV, 293T cells were transfected with the vector plasmid using Lipofectamine 2000 (Life Technologies Japan, Tokyo, Japan). Forty-eight hours later, the supernatant was collected, filtered, and stored at -80°C. Using a FACS Calibur flow cytometer (Becton Dickinson Japan, Tokyo, Japan), RRV titers were calculated

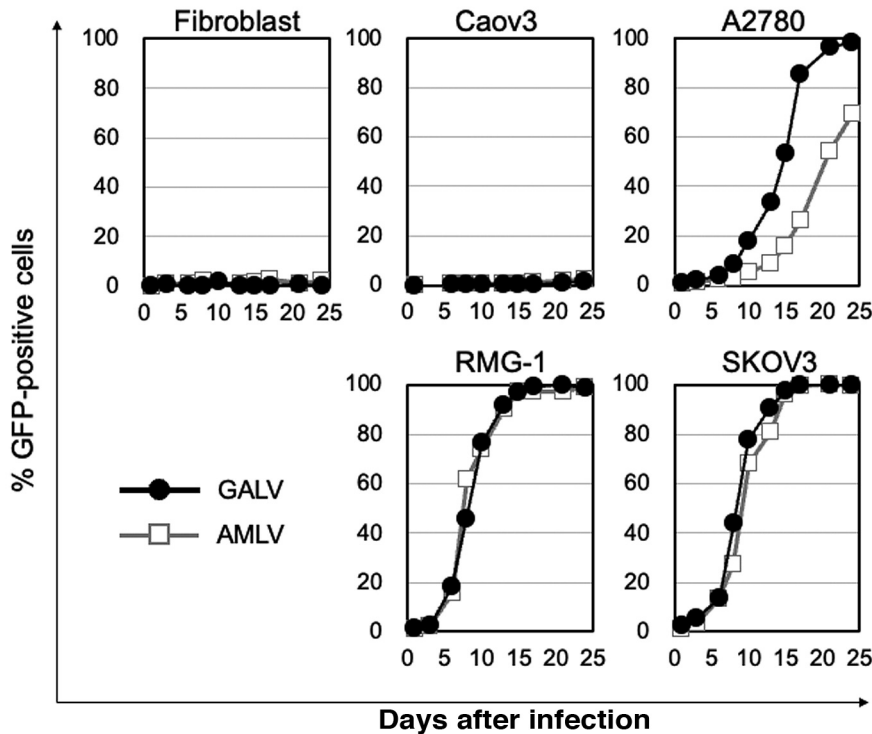


Figure 2. Replication kinetics of AMLV and GALV vectors in human OC cells. Human fibroblasts and OC cells (A2780, Caov-3, RMG-1, and SKOV3) were inoculated with AMLV-GFP or GALV-GFP vectors at an MOI of 0.01. On the days of passage, cells were analyzed for GFP expression using flow cytometry. Representative data of three independent experiments are shown.

based on the expression of fluorescent protein and expressed as transducing units per ml.

In vitro replication kinetics of RRV. RRV (AMLV-GFP and GALV-GFP) were used to infect different human cell lines at 20% confluence with a multiplicity of infection (MOI) of 0.01. At the indicated time following infection, the cells were trypsinized, one-quarter of the cells were replated, and the remaining cells were analyzed for GFP expression using flow cytometry as described above.

In vitro cytotoxicity assay. Human OC cells (A2780, Caov-3, RMG-1, and SKOV3, 1×10^4 cells/well) were infected with AMLV-CD or GALV-CD at an MOI of 0.01 and maintained for 15 days. The pretransduced cells were then replated and cultured in triplicate wells in 96-well tissue culture plates with different concentrations of 5-fluorocytosine (5-FC) (Wako Pure Chemical Industries, Osaka, Japan). After four days, the viable cell counts of triplicate cultures were determined using the AlamarBlue method (Alamar Biosciences, Sacramento, CA, USA), as shown previously (16, 24). Fluorescence was measured using an ARVO X4 multilabel plate reader (PerkinElmer Japan, Tokyo, Japan), and the percentage of viable cells was determined by calculating the fluorescence of viable cells relative to those of wells lacking 5-FC.

Subcutaneous xenograft model of human OC. BALB/c-nu/nu (nude) mice (Charles River Japan, Yokohama, Japan) were maintained under specific-pathogen-free conditions in the Center for Comparative

Medicine, Hyogo Medical University. All studies followed protocols approved by Hyogo Medical University Animal Research Committee. Human OC xenografts were established in 5-week-old female nude mice by the subcutaneous inoculation of 1×10^6 SKOV3 cells into the right dorsal flank. On day 0, the mice with tumor diameters greater than 5 mm were randomly divided into three groups and injected intratumorally with 50 μ l of PBS (n=12 per group), AMLV-CD (n=13), or GALV-CD (n=12), followed by the intraperitoneal administration of 5-FC (500 mg/kg/day) three times a week from Day 11 to Day 42. Throughout the experiment, the mice were monitored for overall health status daily, and the tumors were measured twice a week using a digital caliper. The tumor volume values were calculated using the formula: Length \times Width² \times 0.5.

Statistical analysis. Data are presented as the mean \pm standard deviation (SD) or standard error (SE). All data were analyzed using Student's *t*-test. A *p*-value of 0.05 or less was considered statistically significant.

Results

First, we analyzed the expression levels of RRV receptors in human OC cell lines using qPCR (Figure 1). When HEK293 cells were employed as a positive control, PiT-1 and PiT-2 mRNA levels were low in fibroblast and Caov-3 cells.

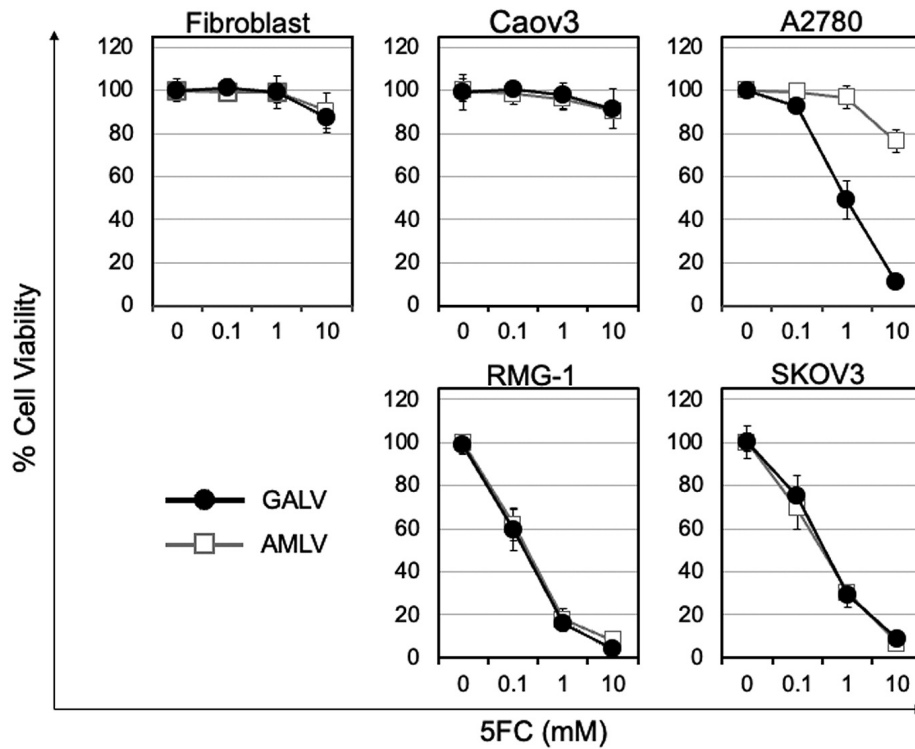


Figure 3. Prodrug activator gene-mediated cell killing effect after AMLV and GALV infection in vitro. Caov-3, A2780, RMG-1, and SKOV3 cells were inoculated with GALV-CD or AMLV-CD at an MOI of 0.01 on Day 1, and exposed to different concentrations of the 5-FC prodrug from Day 16 for four days. On Day 19, cell viability was examined using the AlamarBlue assay. The data are means and SDs from experiments performed in triplicate.

Comparing A2780 cells to HEK293, both receptor expression levels were higher, with that of PiT-1 being higher than that of PiT-2. RMG-1 and SKOV3 cells had higher mRNA levels for both PiT-1 and PiT-2. Therefore, OC cell lines other than Caov-3 expressed the cellular receptor for RRV at higher levels than fibroblasts and HEK293 cells.

Next, we compared the replication efficiency of RRV in human OC cells (Figure 2). In negative control fibroblasts and Caov-3 cells (low PiT-2 and low PiT-1 expression), the percentage of GFP-positive cells was still less than 3% at 24 days after RRV inoculation. In A2780 cells (PiT-2 < PiT-1), AMLV and GALV displayed distinct growth propagation patterns, with GALV replicating more efficiently than AMLV. Meanwhile, in RMG-1 and SKOV3 cells (high PiT-2, high PiT-1), the two RRV had similar replicative kinetics with highly efficient propagation leading to over 90% transduction by Days 10-13. These findings suggest that PiT-2 and PiT-1 expression levels are crucial in determining the replication efficiency of AMLV and GALV (Figure 1 and Figure 2).

We then tested the efficacy of RRV-mediated prodrug activator gene therapy in human OC cells (Figure 3). In fibroblasts and Caov-3 cells infected with either RRV, cell

viability remained above 80% even at the highest concentration of 5-FC (10 mM). In contrast, for RMG-1 and SKOV3 cells (high PiT-2 and high PiT-1), significant reductions in cell viability were noted upon infection with either RRV. This reduction was dose-dependent for 5-FC and fell below 5% at 10 mM 5-FC. In A2780 cells (PiT-2 < PiT-1), different patterns of cell viability were observed between AMLV (76.2±5.2%) and GALV (11.7±2.7%) at 10 mM 5-FC. These results matched the levels of RRV receptor expression in these cells (Figure 1) and the ability of RRV to replicate in these cells (Figure 2).

To analyze the antitumor effects of RRV-mediated CD/5-FC prodrug activator gene therapy, we administered AMLV-CD, GALV-CD, or PBS intratumorally to SKOV-3 subcutaneous tumors in nude mice (Figure 4). In the PBS group, tumor growth persisted even after 5-FC administration began. Meanwhile, both AMLV and GALV groups showed significant tumor growth inhibition compared with the PBS group after Day 10 of 5-FC administration. No significant difference in antitumor activities was found between the AMLV and GALV groups. These results indicate that RRV-mediated prodrug activator gene therapy has a significant antitumor effect in a human SKOV-3 OC model.

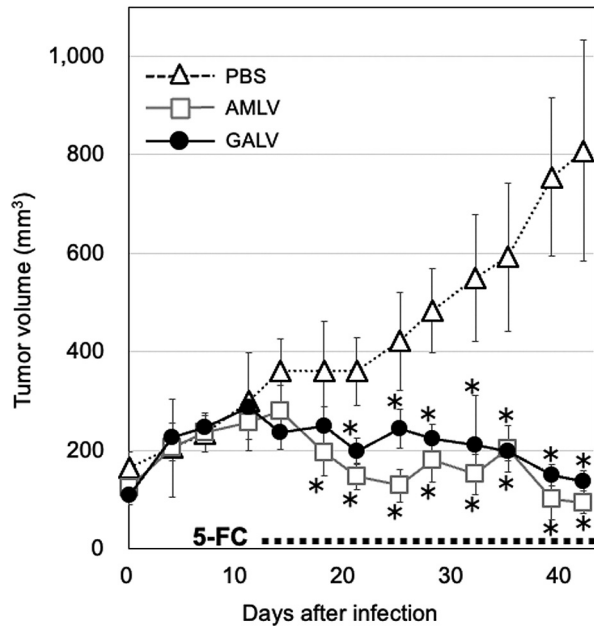


Figure 4. Antitumor effect of RRV-mediated prodrug activator gene therapy in an OC subcutaneous tumor model. Human OC xenografts were established in nude mice by subcutaneous inoculation of 1×10^6 SKOV3 cells into the right dorsal flank. When the tumor diameter reached 5 mm, tumors were injected with 2×10^4 TU (100 μ l) of either AMLV-CD ($n=13$ per group) or GALV-CD ($n=12$) vector, or PBS (100 μ l) ($n=12$). From Day 11 onwards, the mice received 5-FU three times a week. The data are presented as the means and SEs. * $p < 0.05$ (each RRV-treated group vs. PBS group).

Discussion

In this study, we showed that RRV can rapidly spread in susceptible OC cells, causing cancer cell-selective cytotoxicity *in vitro* and antitumor efficacy *in vivo* upon administration of the prodrug, demonstrating the potential of RRV-mediated prodrug activator gene therapy for OC treatment.

We have previously demonstrated the efficacy of RRV-mediated prodrug activator gene therapy in the treatment of a variety of tumors, including mesothelioma (12, 24), lung cancer (18), pancreatic cancer (15), gastric cancer (23), and osteosarcoma (16). Common to the results of previous studies and the present study is that the transduction efficiency and cytotoxic/antitumor effects of RRV depends on viral receptor expression levels (16, 17, 23) (Figure 1 and Figure 2). These findings demonstrated the feasibility of using viral receptor expression levels in cancer cell lines as a potential biomarker of RRV efficacy. In this regard, clinical applications are anticipated to enable the selection and administration of efficient RRV before the start of treatment by assessing the expression levels of PiT-1 and PiT-2 receptors in patient tumor biopsies as markers for personalized cancer virotherapy.

Chemotherapy combined with immunostimulatory gene therapy, utilizing oncolytic viruses as carriers, is a promising therapeutic strategy targeting various pathways to cause tumor cell death, regulate growth, and prevent metastasis (3). Similarly to other oncolytic viruses, combinations of chemotherapy with RRV-mediated prodrug activator gene therapy showed promising results. For example, combinations of RRV-CD/5-FU with temozolomide achieved a synergistic long-term survival benefit in mice bearing orthotopic temozolomide-sensitive glioma (27). Additionally, checkpoint inhibitors, such as anti-CTLA4 or anti-PD-L1, have shown additive effects in animal models (28, 29). Moreover, combinations with intracellular 5-FU produced during RRV-CD/5-FU therapy have been reported to exert radiosensitizing effects on human glioma cell lines, supporting the rationale for combining radiation with RRV-CD/5-FU treatment for the treatment of patients with brain tumors (30). We previously reported that dual-vector prodrug activator gene therapy using two distinct RRV carrying different prodrug activator genes (CD/5FC and herpes simplex virus thymidine kinase/ganciclovir) achieved synergistic cytotoxic effect compared to single-vector gene therapy (24). Against this background, a wide range of combination therapies with RRV-mediated prodrug activator gene therapy may be possible.

Conclusion

Our results indicate that RRV-mediated prodrug activator gene therapy could be a new and effective treatment modality for PiT-expressing human OC.

Conflicts of Interest

All Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

SK conceived the study; LI, ESF, and HF performed experiments; LI, ESF, and TT collected samples and data; SK, ESF, TT, and KH analyzed and interpreted data; LI, TT, KH, NK, and SK wrote the manuscript; all Authors reviewed the manuscript.

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