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ORIGINAL ARTICLE

Effects of tumor selective replication-competent herpes viruses in combination with gemcitabine on pancreatic cancer

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Abstract
Purpose Pancreatic cancer still has a poor prognosis, even if aggressive therapy is pursued. Currently, new modalities of oncolytic virus therapy are being tested against this cancer. The combination of one of two representative mutant herpes simplex viruses (R3616: $\gamma_1$34.5 inactivated, hrR3: UL39 inactivated) with a standard anti-pancreatic cancer chemotherapy drug (gemcitabine), was investigated in this study.
Experimental design The intracellular concentration of ribonucleotide reductase was estimated by Western blotting. The effect of gemcitabine on viral replication and the total cytotoxic effect of the combination therapy were investigated on pancreatic cancer cell lines. We compared the results of two oncolytic viruses, R3616 and hrR3. A mouse model of pancreatic cancer with peritoneal dissemination was used to evaluate the in vivo effect of the combination therapy.
Results Although the replication of both viruses was inhibited by gemcitabine, the combination caused more tumor cell cytotoxicity than did virus alone in vitro. The results with R3616 were more striking. Although the difference was not statistically significant, R3616 with gemcitabine had a greater effect than did R3616 alone, while hrR3 with gemcitabine had a weaker effect than did hrR3 alone in vivo experiments.
Conclusion The combination of oncolytic virus with gemcitabine is a promising new strategy against advanced pancreatic cancer. Each virus has different functional characteristics, and can affect the results of the combination of viruses and chemotherapy drugs. The results indicate that there is a complicated interaction among viruses, cells, and chemotherapy drugs and that the best combination of oncolytic virus and chemotherapeutic agents should be studied more extensively before embarking on a clinical trial.

Keywords Herpes oncolytic virus · Pancreatic cancer · Gemcitabine · Combination therapy

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Introduction

Pancreatic cancer is a disease with an extremely poor prognosis. Surgical therapy for pancreatic cancer is still insufficient to cure most patients [1]. Recently gemcitabine has shown a modest survival advantage over 5-fluorouracil (5-FU) in patients with this cancer [2]. Gemcitabine has become one of the standard chemotherapy drugs against pancreatic cancer but more effective therapies must be devised in order to significantly improve survival. Oncolytic virus therapy has been highly trusted as a new type of therapy for advanced incurable pancreatic cancer, and may provide some clinical benefit to those patients in the near future. Currently, clinical trials using oncolytic viruses have been started against many types of cancer in world-wide [3], such as brain cancer [4, 5], prostate cancer [6, 7], pancreatic cancer [8], breast cancer [9], and head and neck cancer [10, 11]. This study investigated the possibility of combination therapy using gemcitabine and two herpes mutant oncolytic viruses (R3616 and hrR3) against pancreatic cancer.

Gemcitabine (difluorodeoxycytidine; dFdC) is intracellularly phosphorylated to difluorodeoxycytidine diphosphate (dFdCDP) and difluorodeoxycytidine triphosphate

(dFdCTP). dFdCTP competes with deoxycytidine triphosphate (dCTP) for incorporation into DNA, and DNA synthesis is inhibited [2, 12, 13]. In addition, dFdCDP acts as an inhibitor of ribonucleotide reductase (RR) in cells, which in turn causes a major decrease in the dCTP pool. Therefore, gemcitabine reduces the activity of RR in cancer cell lines [14] (Fig. 1a). However, some cells have been known to acquire chemoresistance to gemcitabine due to over expression of RR [15–21].

R3616 and hrR3 are genetically engineered herpes simplex viruses [3]. R3616 lacks the $\gamma_1$34.5 gene that produces the ICP34.5 protein. Replication of R3616 is severely restricted in normal cells, because the expression of ICP34.5 in normal cells prevent a protein shutoff mechanism that is associated with eIF2α dephosphorylation through the protein kinase receptor (PKR). Most cancer cells lose this normal protein shutoff mechanism so that viral replication can proceed, which induces the virally infected cells to undergo apoptosis to protect the integrity of the cell's DNA and block viral replication [3, 22–24]. hrR3 lacks the UL39 gene that produces the ICP6 proteins (viral RR), a key enzyme in the biosynthesis of DNA in all prokaryotic and eukaryotic cells. The viral replication of hrR3 is severely restricted in cells that have high levels of holding proteins involved in nucleic acid synthesis such as cancer cells [25, 26] (Fig. 1b).

We investigated the effect of tumor-selective, replication-competent herpes viruses (R3616 and hrR3) against pancreatic cancer under the same conditions in which gemcitabine effects cancer cells. Our major concern was how gemcitabine may interrupt viral replication, and whether the combination of an oncolytic virus with gemcitabine can significantly improve anti-pancreatic cancer therapy.

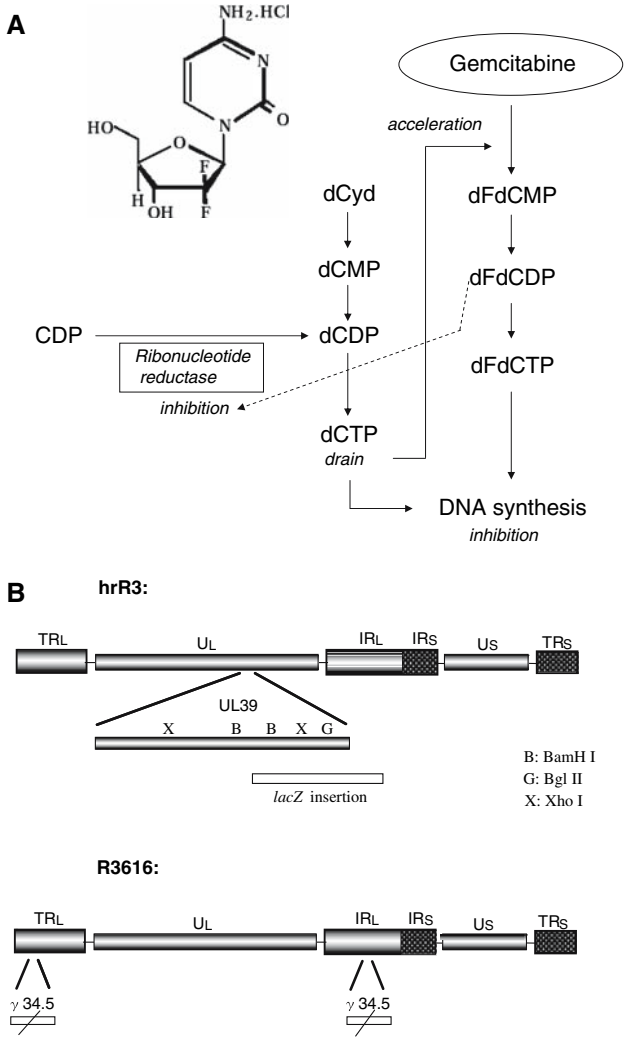

Fig. 1 **a** Gemcitabine structure and pathway. Gemcitabine HCl is a nucleoside analog that exhibits anti-tumor activity. Gemcitabine HCl is 2′-deoxy-2′, 2′-difluorocytidine monohydrochloride (β-isomer). The empirical formula for gemcitabine HCl is C9H11F2N3O4 × HCl. It has a molecular weight of 299.66. Gemcitabine is metabolized intracellularly by nucleoside kinases to the active diphosphate (dFdCDP) and triphosphate (dFdCTP) nucleosides. **b** Schematic illustration of hrR3 and R3616. hrR3 is a mutated herpes simplex virus (*HSV*) that has the LacZ gene inserted into the site of UL39 (ICP6), causing inactivation of ribonucleotide reductase activity that is associated with UL39. Ribonucleotide reductase is a key enzyme for viral DNA synthesis. R3616 is a mutated HSV that has a deletion of both $\gamma_1$34.5 genes. The $\gamma_1$34.5 gene produces ICP 34.5 that dephosphorylates eIF2α-phosphate to permit continued viral protein synthesis. Those mutated HSVs replicate and destroy only the cancer cells

Materials and methods

Viruses and cells

R3616 was kindly provided by Bernard Roizman Sc. D (University of Chicago, Chicago, IL, USA) and hrR3 was kindly provided by Sandra K. Weller Ph.D. (University of Connecticut, Storrs, CT, USA). SW1990, derived from a human pancreatic carcinoma, was kindly provided by Dr. T. Sawada (First Department of Surgery, Osaka City University, Osaka, Japan). CAPAN 1, also derived from a human pancreatic carcinoma, was obtained from the Japanese Cancer Research Resources Bank, Tokyo, Japan. PACA2, another cell line derived from a human pancreatic carcinoma, was obtained from the American Type Culture Collection, Manassas, VA, USA. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 1% penicillin/streptomycin at 37°C (Sigma, Tokyo, Japan).

Western blot assay

A total of 10^6 cells were harvested and rinsed twice with phosphate-buffered saline, pH 7.4. Cell extracts were prepared with lysis buffer (20 mM Tris, pH 7.5, 0.1% Triton-X, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and clarified by

centrifugation at 12,000*g*, for 15 min, at 4°C. Cell lysates containing equal amounts of protein as determined by a BCA assay kit were electrophoresed on a NuPAGE, Novex 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA), and the resolved proteins were transferred to PVDF membranes (Invitrogen).The membranes were blocked with 5% nonfat milk overnight at room temperature, and incubated with 0.2 μg/ml human anti-RRM1 antibody (CHEMICON International, Temecula, CA, USA) for 1 h. RRM1 was detected using an enhanced chemiluminescence (ECL) system following the manufacturer's instructions (Amersham Life Science, Uppsala, Sweden). β-actin also was detected on the same membrane to serve as a control for the amount of protein loaded.

Cytotoxic assay

Gemcitabine and viral-induced cytotoxicity assays were performed using the MTT assay as previously described [27, 28]. Briefly, 10^6 cells were plated in a 10-cm plate and 10 μg/ml of gemcitabine was added. After 24 h, a replication-competent virus (R3616 or hrR3) was added at multiplicity of infection (MOI) values ranging from 0.01 to 10 and incubated for an additional 48 h. The number of surviving cells was quantified by a colorimetric MTT assay. The results, expressed as mean ± SD of four samples, were compared with the results from the cytotoxicity assays of gemcitabine alone and the virus alone. Statistical significance was determined by the two-sided Student's *t*-test using SPSS (SPSS, Chicago, IL, USA).

Viral replication assay

Viral replication assays were performed as described [28, 29]. Briefly, 10^6 cells were plated in a 10-cm plate and 10 μg/ml of gemcitabine was added. After 24 h, replication competent viruses (R3616 or hrR3) were added at MOI of 2. Forty-eight hours after infection, the supernatant and cells were harvested, exposed to three freeze- thaw cycles to release the virions, and titered. The results were compared with the assays of viral replication without gemcitabine.

Animal studies

Mice (6-week-old females BALB/c nu/nu) were obtained from the Charles River Japan, Yokohama, Japan. Animal studies were performed in accordance with the guidelines issued by the Nagoya University Animal Center. The mice, used in a peritoneal-disseminated carcinoma model, were injected with 10^6 PACA2 cells into the intraperitoneal cavity. The condition of the animals was checked once or twice a day for the duration of the study. The mice were divided randomly into six groups (A–F). Group A (n = 10), group D (n = 10), and group E (n = 10) were injected with 1 mg of gemcitabine into the intraperitoneal cavity on day 14 after the injection of the PACA2 cells. The mice in groups A and B (n = 10) each were injected with 10^6 particles of R3616 on day 15 after the injection of the PACA2 cells. Group C (n = 10) and group D (n = 10) were injected with 10^6 particles of hrR3 on day 15 after the injection of the PACA2 cells. Group F (n = 10) was the control group, which was injected with only PACA2 cells into the intraperitoneal cavity.

Statistical differences between groups were determined by the log-rank test with the use of JMP 5.0 software (SAS Inc., Cary, NC, USA). $P < 0.05$ was considered statistically significant.

Results

Expression of RRM1 by Western blotting

As previously reported by many researchers on their papers, overexpression of ribonucleotide reductase subunit 1 (RRM1) is associated with chemoresistance to gemcitabine [15–17]. We examined the intensity of RRM1 protein expression in Capan1, PACA2, and SW1990 cells (Fig. 2). The intensity of RRM1 expression in the PACA2 cells was greater than in the other cell lines. The results from many previous related papers regarding chemoresistance to gemcitabine, indicated that PACA2 cells might have the highest potential of chemo resistance to gemcitabine among the three cell lines.

Comparison of cytotoxic assays between hrR3 and R3616, with or without gemcitabine

We compared the cytotoxicity of R3616 ($\gamma_1$34.5 deficiency) and hrR3 (ICP6: RR gene deficiency) viruses' combination with gemcitabine by the MTT assay (Fig. 3). With both R3616 and hrR3, the cytotoxicity was increased by their

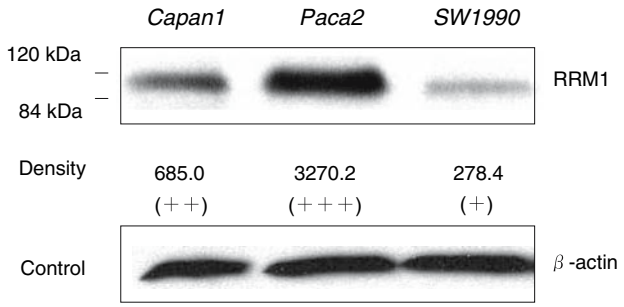

Fig. 2 Expression of ribonucleotide reductase M1 (*RRM1*) by Western blotting. PACA2 cells expressed the most ribonucleotide reductase M1 (*RRM1*) by Western blot assays among three pancreatic cancer cell lines tested. β-actin served as a control for the amount of protein loaded in each lane

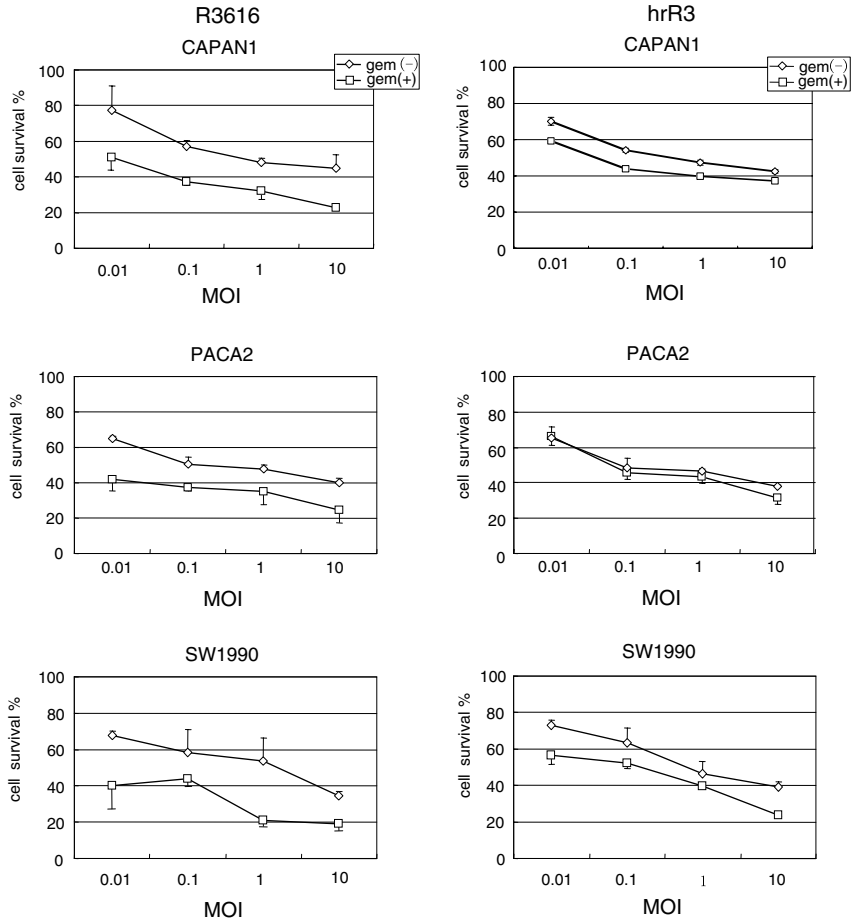

Fig. 3 Comparison of cytotoxic assays between hrR3 and R3616, with or without gemcitabine. For both R3616 ($\gamma_1$34.5 gene inactivated) and hrR3 (ICP6: ribonucleotide reductase gene inactivated), the cytotoxity was increased when combined with gemcitabine. However, there was a trend toward greater cytotoxity with R3616 than with hrR3 against all cell lines

combination with gemcitabine, but the more significant increase in cytotoxicity was observed with R3616 than hrR3. On the other hand, PACA2 cells, which expressed the most RRM1 by a Western blot assay, had the lowest increase in cytotoxicity with the combination of hrR3 and gemcitabine.

Comparison of cytotoxic assays between gemcitabine alone and gemcitabine with low titer virus

We also compared the cytotoxicity between gemcitabine alone and gemcitabine with low titer virus by the MTT assay (Fig. 4). Of all cell lines, the combination of gemcitabine and an MOI 0.01 of R3616 showed more cytotoxic tendency than did gemcitabine alone ($P = 0.04$ on PACA2 cell line), while the combination of gemcitabine with an MOI 0.01 of hrR3 tend to be less cytotoxic than gemcitabine alone. PACA2 cells.

Comparison of viral replication between hrR3 and R3616, with or without gemcitabine

We compared the viral replication between R3616 and hrR3 in the presence of gemcitabine by the plaque-forming assay (Fig. 5). The replication of both viruses was inhibited by gemcitabine. The titer of hrR3 declined more than did R3616 in combination with gemcitabine. The replication of hrR3 was inhibited by gemcitabine in all cell lines. PACA2 cells expressed the most RRM1 by Western blot assay, and hrR3 replicated more vigorously with gemcitabine in the PACA2 cells than in the other two cell lines, while R3616 was also inhibited by gemcitabine in all cell lines but with somewhat weaker inhibition comparing to hrR3.

Animal studies

Long-term survival (LTS: 100 days) was achieved in 60% of mice treated with an intraperitoneal injection of R3616 followed by gemcitabine (group A). Mice treated with an intraperitoneal injection of R3616 had only a 50% LTS (group B). Mice treated with hrR3 had a 30% LTS (group C). Mice treated with hrR3 followed by gemcitabine had a 20% LTS (group D). Mice treated with gemcitabine alone had only a 10% LTS (group E). All mice in the control group died within 60 days (group F) (Fig. 6). Statistical differences in the survival rates were determined by log-rank analyses (group A versus group F, $P = 0.0011$; group A versus group D, $P = 0.0078$; group E versus group F,

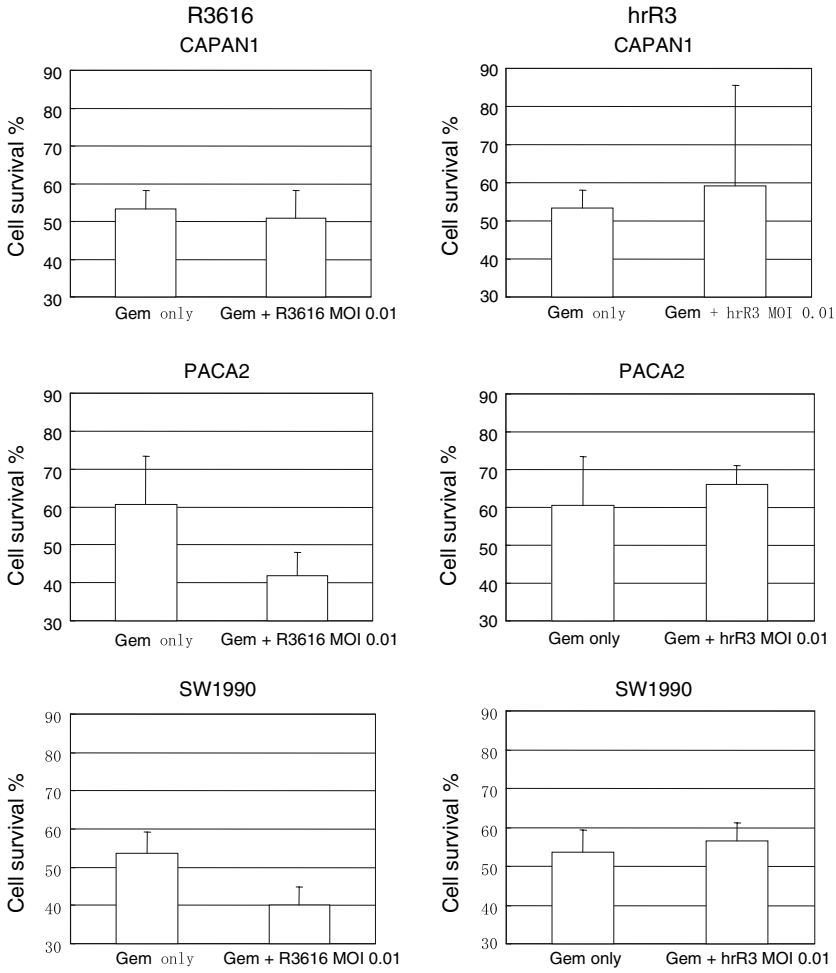

Fig. 4 Comparison of cytotoxic assays between gemcitabine alone and gemcitabine with a very low titer of virus. For all cell lines, the combination of gemcitabine and a very low titer (MOI 0.01) of R3616 tended to exhibit greater cytotoxicity than did gemcitabine alone ($P = 0.04$ on PACA2 cell line), on the other hand, the combination of gemcitabine and a very low titer (MOI 0.01) of hrR3 tended to be less cytotoxic than gemcitabine alone

$P = 0.006$; group B versus group D, $P = 0.0174$; group B versus group F, $P = 0.0049$). There were no other statistically significant differences between the other groups except for shown above. Although it was not significantly different, R3616 with gemcitabine tended to have a stronger effect than did R3616 alone, while hrR3 with gemcitabine tended to be weaker than hrR3 alone.

Discussion

In this study, we compared the efficacy of hrR3 or R3616 plus gemcitabine against pancreatic cancer. An in vitro cytotoxic assay indicated that R3616 plus gemcitabine caused a significant increase in the cell-killing effect in all three pancreatic cancer cell lines than did hrR3. We postulate that this result was due to the functional differences caused by each deleted viral gene. The viral replication of hrR3 might be more interrupted by the effect of gemcitabine than that of R3616, and this reduction might have been responsible for the slight decrease in the cell-killing effect of hrR3. Cellular RR is important for viral replication especially for hrR3 that has no RR [3, 25–27, 29, 30]. Gemcitabine is well known to reduce the activity of cellular RR in cancer cell lines [14]. Therefore, it is a possible that the effect of gemcitabine was greater in combination with hrR3 than with R3616 reducing the replication and cytotoxicity of the viruses.

Interestingly, infection with hrR3 at a very low concentration (MOI 0.01) in the presence of gemcitabine caused less cytotoxic than did gemcitabine alone. This may be the result of the virus protecting the cancer cells from the apoptosis caused by gemcitabine. The virus itself has some anti-apoptotic effects on cells in order to protect the host cells from bursting too early and until the virus particles have matured. Although gemcitabine reduced the replication of hrR3, some viral anti-apoptosis genes might still have worked in the infected cells without the burst-cell effect that is caused by an abundance of mature viruses. The apoptosis mechanism might malfunction as a result of this low virus concentration, causing an anti-apoptotic effect against gemcitabine. This effect might apply not only to HSV, a critical

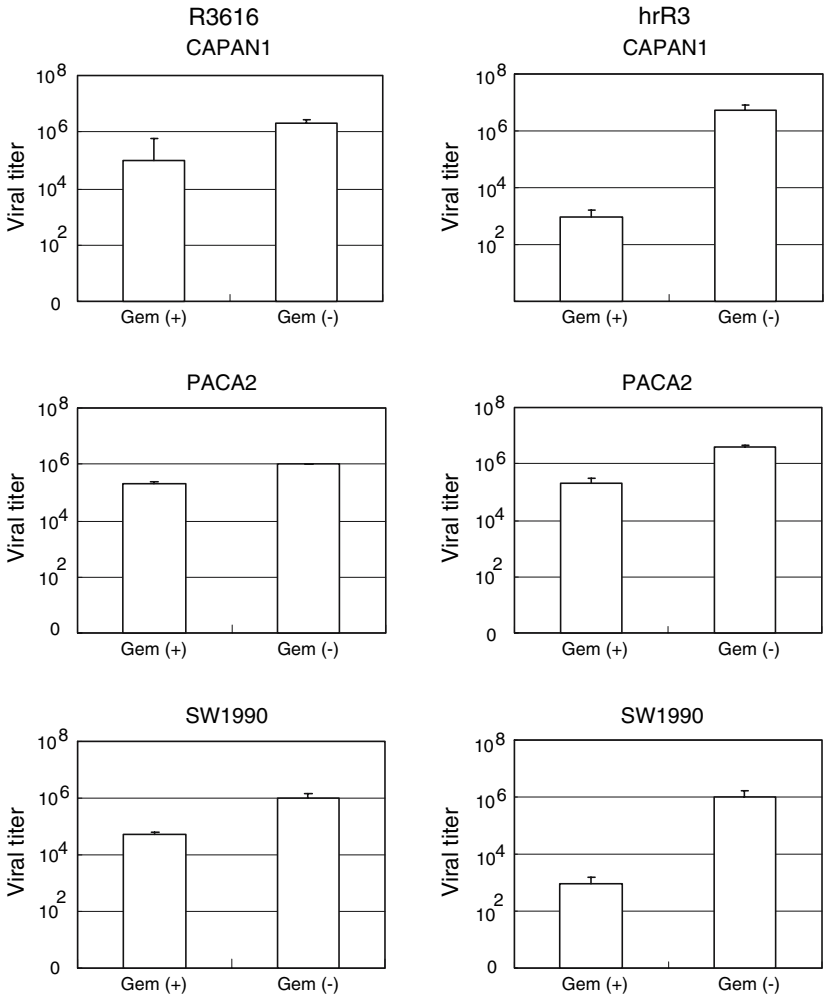

Fig. 5 Comparison of viral replication between hrR3 and R3616, with or without gemcitabine. The viral replication of both R3616 and hrR3 were inhibited by the presence of gemcitabine. This phenomenon was more prominent with hrR3 and gemcitabine than with R3616

consideration when using a viral vector or an oncolytic virus with chemotherapy drugs, because most viruses have such an anti-apoptosis gene. Examples include US3 and US5 in HSV [31, 32], and E1b 19 kDa in adenovirus [33]. Furthermore, several distinct viruses have been shown to develop mechanisms to block premature apoptosis of infected cells [34–36]. This phenomenon should be considered when using any viral vector for gene therapy or oncolytic virus therapy with chemotherapy drugs. In our opinion, the anti-apoptosis genes in a virus should be studied more intensively if future development of oncolytic virus therapy is to proceed.

PACA2 cells had the highest density of RR by Western assays and also the lowest cytotoxic effect from single agent gemcitabine among the three pancreatic cancer cell lines tested as 60% cell survival in Fig. 4, which indicates that PACA2 cells have some type of resistance against the cytotoxicity of gemcitabine comparing to other two cell lines. For the combination of R3616 with gemcitabine, increased efficacy was observed against all the pancreatic cancer cell lines even if the cells had some resistance to the chemotherapy alone. On the other hand, the combination of hrR3 with gemcitabine was of weak cytotoxity toward PACA2 cells, which expressed the most RRM1 by Western blot assay, and the effect was less pronounced than when R3616 was used. These results suggest that, the combination of R3616 and gemcitabine might be suitable for the cancer cell type that is expected to offer resistance to gemcitabine.

In the in vivo experiments, the combination of R3616 with gemcitabine yielded a 60% LTS rate (100 days) in the mice. This was higher than in mice treated with an intraperitoneal injection of R3616 alone that resulted in a 50% LTS rate, while mice treated with only hrR3 had a 30% LTS rate; however, there was no statistically significant difference in the LTS rate between R3616 and R3616 with gemcitabine. Thus, combination therapy with R3616 and gemcitabine had the same or slightly higher efficacy than the virus alone. However, mice treated with hrR3 followed by gemcitabine showed a lower LTS rate (20%) than those treated with hrR3 alone. And moreover, there was a statistically significant difference between group A (R3616 + GEM) and group D (hrR3 + GEM) ($P = 0.0078$). From the results of our in vivo and in vitro, we determined that the

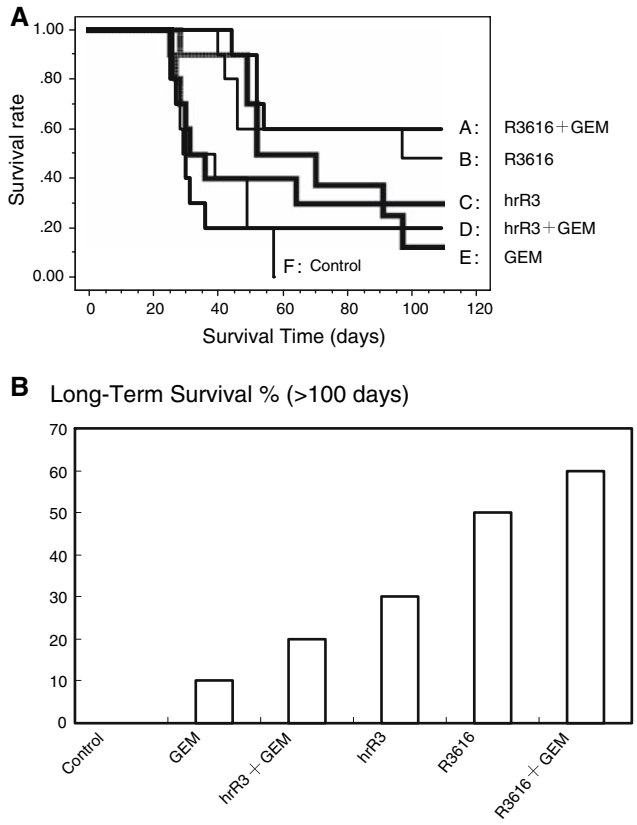

Fig. 6 **a** Cumulative survival curves of an in vivo mouse model. The PACA2 cells were injected into the peritoneal cavity. Each group was treated as shown below. Group A: R3616 + gemcitabine (GEM), group B: R3616 only, group C: hrR3 only, group D: hrR3 + GEM, group E: GEM only, and group F: no treatment. Differences in the survival rates were assessed by log-rank analysis (group A versus group F, $P = 0.0011$; group A versus group D, $P = 0.0078$; group E versus group F, $P = 0.006$; group B versus group D, $P = 0.0174$; and group B versus group F, $P = 0.0049$). There were no other statistic ally significant differences between the other groups except for shown above. There was a statistically significant difference between group A (R3616 + GEM) and group D (hrR3 + GEM) ($P = 0.0078$). **b** Long-term survival (*LTS*: over 100 days). LTS was achieved in 60% of mice treated with an intraperitoneal injection of R3616 followed by gemcitabine (group A). Mice treated with an intraperitoneal injection of R3616 only had a 50% LTS survival rate (group B). Mice treated with hrR3 only had a 30% LTS rate (group C). Mice treated with hrR3 followed by gemcitabine had a 20% LTS rate (group D). Mice treated with gemcitabine alone had only a 10% LTS rate (group E). The untreated group (group F) had a 0% LTS survival rate

combination of gemcitabine with R3616 ($\gamma_1$34.5 inactivated) might be more effective than the combination with hrR3 (RR inactivated).

Potentially, chemotherapy drugs connote to inhibit oncolytic virus replication to some degree, but this effect may be influenced by the differences in the characteristics of each virus caused by gene mutation. UL 39 (ICP6)-deleted HSVs, such as G207 [3, 37], and Myb34.5 [38, 39] also have some kind of potential likely to be inhibited by gemecitabine, as is hrR3, because of the genetic characteristics of RR. In other words, UL39-intact HSVs, such as HF10 [40, 41], RH105 [42], and DFγ34.5 [43] are likely to interact differently from a UL 39-deleted HSV (e.g., hrR3), in combination therapy with gemcitabine. Additional studies must be needed for further confirmation of the efficacy depending upon the functional characteristics among the chemotherapy drugs, viruses, and the cancer cells.

In the future, oncolytic virus therapy in combination with chemotherapy drugs may become more popular for use in clinical trials. Therefore, the characteristics of each virus must be considered carefully to determine if they are suitable for use with the chemotherapy drugs chosen.

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