SPANDIDOS PUBLICATIONS

p53 enhances the Δ-24 conditionally replicative adenovirus anti-glioma effect

P.G. MITLIANGA[1], C. SIOKA[1], G. VARTHOLOMATOS[4], A. GOUSSIA[2], K. POLYZOIDIS[1], J.S. RAO[5] and A.P. KYRITSIS[3]

[1]Neurosurgical Institute, [2]Department of Histopathology, [3]Department of Neurology, University of Ioannina Medical School; [4]Molecular Biology Unit, Hematology Laboratory, University Hospital of Ioannina, Ioannina, GR-451 10, Ioannina, Greece; [5]Division of Cancer Biology, Department of Biomedical and Therapeutic Sciences, University of Illinois College of Medicine at Peoria, Peoria, IL, USA

Received July 1, 2005; Accepted August 19, 2005

Abstract. Previous studies have demonstrated that the conditionally replicative adenovirus Ad5Δ24 is a powerful cytolytic agent against glioma selectively affecting cells with a defective p16/Rb/E2F pathway. The p53 protein is also known to be an apoptotic factor for glioma cells. In this study, we examined the simultaneous delivery of the combination of exogenous p53 and Ad5Δ24 adenovirus in glioma cells. Infecting cells with low doses of adenovirus *p53* and Ad5Δ24 resulted in an additive effect on cell death. The cell death induced by both agents was independent of the p53 status of cells. Flow cytometry revealed that the potent anti-tumor effect induced by the mixture of Ad5CMV-*p53* and Ad5Δ24 adenoviruses was due to a combination of apoptosis and cell lysis. Our results indicate that Ad5CMV-p53 enhances the oncolytic effect of the Ad5Δ24 adenovirus, and the combination of adenovirus Ad5Δ24 and Ad5CMV-*p53* may thus be a potential therapeutic tool for gliomas.

Introduction

Malignant gliomas are the most common primary brain tumors in humans, and highly resistant to all current therapies, including widely investigated approaches to gene therapy. At the molecular level, glioblastomas are highly heterogeneous tumors, as several populations of cells with different gene abnormalities co-exist within a given tumor (1). Secondary glioblastomas that develop from pre-existing low-grade astrocytomas frequently contain *p53* mutations (1), and abnormalities of the p16/Rb/E2F pathway are present in most gliomas (2,3).

Correspondence to: Dr Paraskevi Mitlianga, NeuroSurgical Institute, University of Ioannina, Ioannina, GR-451 10, Greece
E-mail: pmitliag@cc.uoi.gr

Key words: glioma, apoptosis, conditionally replicative adenovirus, gene therapy

Previous studies have demonstrated that the transfer of exogenous wild-type *p53* cDNA causes apoptosis in glioma cells expressing endogenous mutant p53, but not in cells expressing endogenous wild-type *p53* (4,5). However, only 50% of gliomas exhibit mutant *p53*, and the mutant cell population usually constitutes <30% of tumor mass (6).

More than 90% of malignant gliomas exhibit abnormalities in the p16/Rb/E2F pathway (7). The adenovirus Ad5Δ24 carries a 24-bp deletion in the E1 region corresponding to amino acids 122-129 specific for binding the Rb protein. This virus produces an anti-glioma effect *in vitro* and *in vivo* in tumors with altered p16/Rb/E2F pathway (8). The conditionally replicative adenoviruses (e.g. Ad5Δ24 and ONYX-15) kill tumor cells selectively, and their replication leads to amplification of their oncolytic potential. Considerable preclinical data support this notion (8-11). In this study, we tested if the combination of an apoptotic molecule (p53) with an oncolytic adenovirus (Ad5Δ24) could have an additive effect on cell death in malignant glioma cells.

Materials and methods

Cell lines, adenoviral vectors, and infection conditions. The human glioma cell line U-251 MG was obtained from Dr W.K. Alfred Yung (Department of Neuro-Oncology, M.D. Anderson Cancer Center, Houston, TX). U-87 MG was obtained from ATCC (Manassas, VA) and HFK (human kidney fibroblasts) from Dr T. Fotsis (Laboratory of Biological Chemistry, University of Ioannina Medical School, Ioannina, Greece). The conditionally replicative adenovirus Δ24 (8), recombinant replication-deficient adenovirus vector carrying the p53 cDNA (Ad5CMV-p53) (9) and control vector Ad5CMV-pA (7) were generated and characterized, as described elsewhere. The cell lines were cultured and infected as reported previously (4). The human kidney fibroblasts were cultured according to manufacturer's instructions. For these experiments, we used a multiplicity of infection (MOI; the ratio of the number of infectious virions to the number of susceptible cells) of 1, 2, 5, 10 and 20 for the Δ24 adenovirus, and 20 for p53 adenovirus.

Immunoblot analysis. Western blot analysis was performed as described previously (4). Briefly, U-87 MG cells were infected with 20 MOI of Ad5CMV-pA, Ad5Δ24, Ad5CMV-*p53*, Ad5Δ24+Ad5CMV-*p53* or mock infected. After 72 h, cells were collected and lysed with RIPA-A buffer in the presence of protease inhibitors. Protein (20 μg) from each sample was fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with p53 antibody (diluted 1:2000) or anti-human ß-actin (diluted 1:500). The secondary antibodies were horseradish appropriate conjugates. The membranes were developed according to ECL protocol (Amersham).

Viability assay. Cell viability was assessed by both crystal violet and trypan blue exclusion test.

a) Crystal violet: Monolayers of human glioma cells or normal human fibroblasts were infected at the indicated MOI with Ad5Δ24 (1, 2, 5, 10 and 20 MOI) AdCMV-*p53* (20 MOI) and their combination or UV-inactivated Ad5Δ24 (at different MOIs mentioned above). Viable cells were stained with crystal violet when 5 MOI of Ad5Δ24 produced >75% of the cytopathic effect.

b) Trypan blue exclusion test: Cell viability assessment of human glioma cells or normal human fibroblasts with trypan blue exclusion test. Cells were infected at different MOI of 5 and 10 plaque-forming units (pfu) per cell of Ad5Δ24, 20 pfu/cell of Ad5CMV-*p53*, 5 and 10 pfu/cell of UV-inactivated Ad5Δ24, or mock infected, and cell viability was measured by trypan blue exclusion test. Each assay was carried out at least 3 times and is represented as cell viability relative to mock-treated cells (equal to 100%). Note that for U-251 MG, both the crystal violet and trypan blue exclusion tests were performed at day 5 after infection, while for U-87 MG the assays were performed at day 8 after infection. As expected, no cytotoxicity was observed for the human fibroblast cells (HFK-2 cells; assays were performed 8 days after infection).

Replication assay. U-87 MG cells were infected with 10 MOI of Ad5Δ24 adenovirus. Cells were collected 3 days after infection and freeze-thawed 3 times. Supernatant was collected, and the $TCID_{50}$ method was used to determine viral replication. Briefly, 293 cells were seeded to 96-well plates and infected with the viral supernatant at different dilutions after 24 h. Ten days after infection, cells were observed under a light microscope to assess the cytopathic effect.

Flow cytometric analysis of DNA content. Cells were infected at MOI of 10 pfu/cell of Ad5Δ24, 20 pfu/cell of Ad5CMV-*p53* or mock infected. When cytotoxicity was observed (5 days for U-251 MG or 8 days for U-87 MG), they were trypsinized, fixed in 70% cold ethanol, and incubated with propidium iodide (50 mg/ml) and ribonuclease A (20 mg/ml) for 20 min at 37˚C and processed through a FACS. At least 10,000 events per sample were analyzed, and fluorescein isothiocyanate fluorescence was collected with a 525-nm bandpass filter. Coulter's cytologic program was used to analyze the data, and the mean peak fluorescence was determined for each histogram.

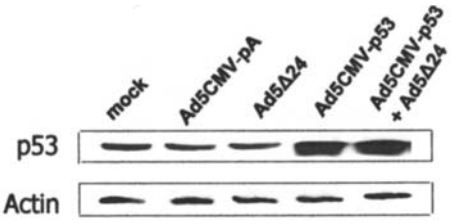

Figure 1. p53 protein expression in U-87 MG glioma cells. Cells were infected with the adenovirus carrying p53 cDNA. Total cell lysates were prepared 3 days after infection, and the expression of the protein was assessed by Western blot analysis. The level of ß-actin expression was used to verify equal protein loading.

Results

Transfer of exogenous p53 to human glioma cells. We used the Ad5CMV vector to transfer *p53* to glioma cell lines (U-87 MG and U-251 MG) and assessed the expression of exogenous proteins by the cells with Western blot analysis (Fig. 1; data shown for U-87 MG). The amount of exogenous p53 protein expressed was at least 3-fold higher than the amount of the endogenous counterpart. Infection of glioma cells with both adenoviruses (oncolytic Ad5Δ24 and Ad5CMV-*p53*) resulted in over-expression of the p53 protein at the same levels as Ad5CMV-*p53* alone.

Cell viability. For the cell viability experiments, two human glioma cell lines (U-87 MG and U-251 MG) were infected with Ad5Δ24, Ad5CMV-*p53* or ultraviolet (UV)-inactivated Ad5Δ24 adenoviruses and their combination at doses of 1, 2, 5, 10 and 20 MOI for Ad5Δ24 and 20 MOI for Ad5CMV-*p53*. Cell viability was first assessed by crystal violet assay, then quantified with the trypan blue exclusion test. Both techniques showed a consistent dose-response effect of Ad5Δ24 alone or in combination with Ad5CMV-*p53* on the two glioma cell lines (Fig. 2). The cytotoxic effect was more pronounced and exhibited earlier when the combination of the two viruses was used. Cultures were monitored every day by light microscopy, and a weak cytopathic effect was evident at day 3 post-infection. U-251 MG demonstrated a strong cytopathic effect at 5 days post-infection and U-87 MG at 8-9 days post-infection. The cytopathic effect was evidenced by the detachment of the cultures and formation of rounded unhealthy cells (data not shown). Furthermore, the transfer of Ad5Δ24 and Ad5CMV-*p53* resulted in a high percentage of cell death in U-87 MG, a p53-resistant glioma cell line (Fig. 2a), as monitored by staining with crystal violet. In addition, the transfer of Ad5Δ24 did not interfere with the apoptotic properties of exogenous wild-type p53 in U-251 MG cells, but resulted in an additive cytotoxic effect (Fig. 2a). The cytopathic effect was noticeable with the combination of 5 MOI of Ad5Δ24 and 20 MOI of Ad5CMV-*p53* on the third day for U-251 MG and on the sixth day for U-87 MG. This effect was >75% at 5 and 8 days post-infection for U-251 MG and U-87 MG, respectively. Trypan blue exclusion tests further showed that the decreased viability observed in the crystal violet assays was highly reproducible and dose-dependent in the cell lines tested (Fig. 2b). Infection of U-87 MG for 8 days with 20 MOI of Ad5CMV-*p53* did not result

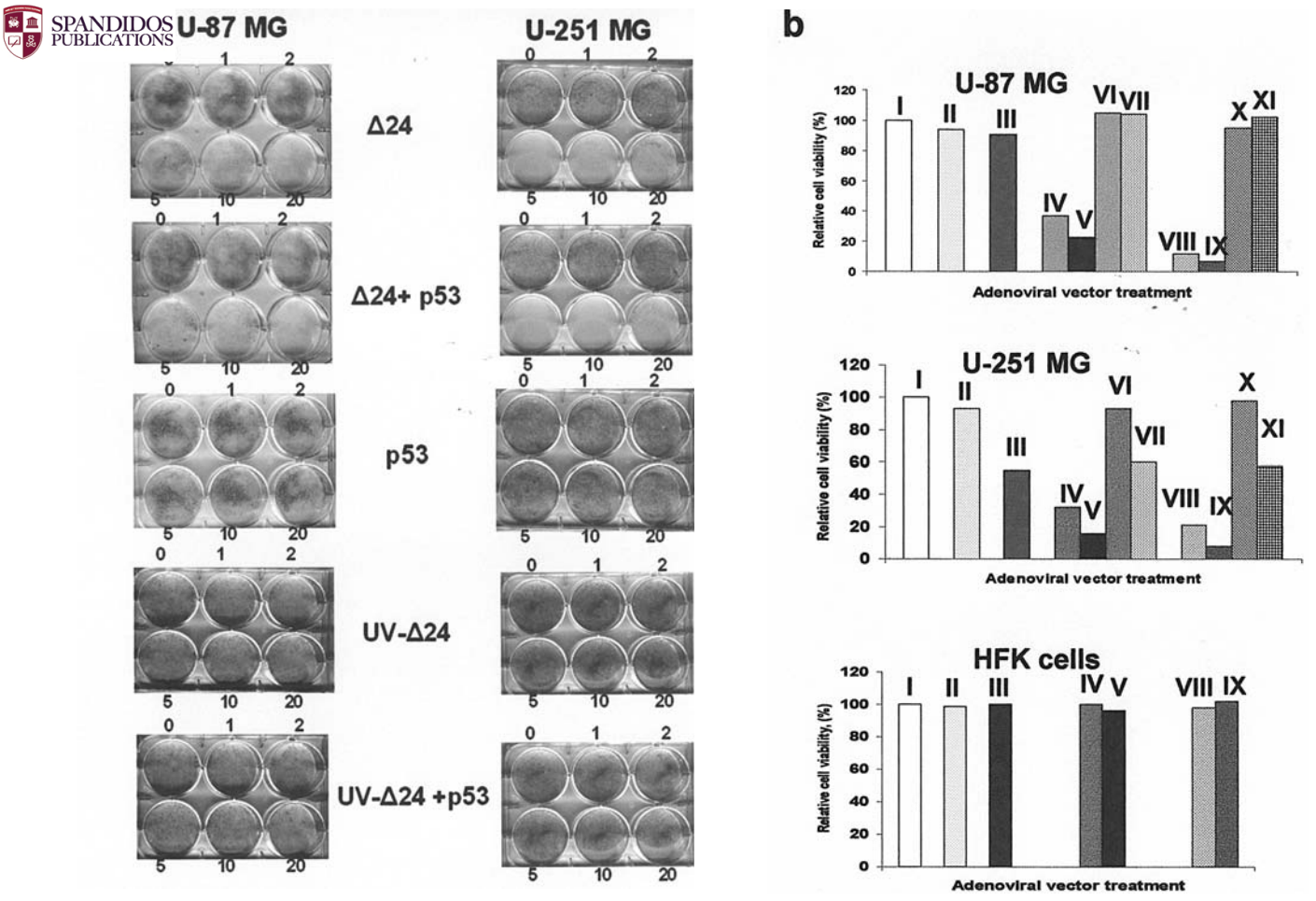

Figure 2. Anticancer effect of the combination of Ad5Δ24 and AdCMV-*p53 in vitro*. (a) Crystal violet test was used to measure viable cells (Δ24, Ad5Δ24+*p53*, Ad5CMV-*p53*, UV-Δ24 and UV-inactivated Ad5Δ24). (b) Cell viability assessment of human glioma cells or normal human fibroblasts with trypan blue exclusion tests: I, mock infection; II, 20 MOI Ad5CMV-pA; III, 20 MOI Ad5CMV-*p53*; IV, 5 MOI Ad5Δ24; V, 5 MOI Ad5Δ24+20 MOI Ad5CMV-*p53*; VI, 5 MOI UV-inactivated AdΔ24; VII, 5 MOI UV-inactivated AdΔ24+20 MOI AdCMV-*p53*; VIII, 10 MOI Ad5Δ24; IX, 10 MOI Ad5Δ24+20 MOI Ad5CMV-*p53*; X, 10 MOI UV-inactivated Ad5Δ24; and XI, 10 MOI UV-inactivated Ad5Δ24+20 MOI Ad5CMV-*p53*. Values shown are the means and standard deviations from 3 separate experiments. Values are normalized to mock-infected cells.

in substantial growth inhibition since 95% of cells remained viable relative to mock-infected cells (100%) (Fig. 2b). However, infection of the above cell line with 5 or 10 MOI of Ad5Δ24 resulted in 60% and 88% of growth inhibition, respectively, 8 days post-infection. Cell viability of U-87 MG infected with both Ad5Δ24 (5 and 10 MOI) and Ad5CMV-*p53* (20 MOI) 8 days post-infection was demonstrated to be 20% and 5%, respectively. Growth inhibition of U-87 MG by both viruses was not observed when Ad5Δ24 was first UV-inactivated, then added to the cell cultures. This experiment showed that the replication of Ad5Δ24 was the initial event that triggered cell death, and became more pronounced when Ad5CMV-*p53* was added to the cell cultures simultaneously. Infection of U-251 MG for 5 days with 20 MOI of Ad5CMV-*p53* resulted in 40% growth inhibition, an expected result since the U-251 MG cell line was a p53-mutated cell line and the exogenous p53 provoked apoptosis (Fig. 2b). Infection of the above cell line with 5 or 10 MOI of Ad5Δ24 resulted in 65% and 85% growth inhibition, respectively, 5 days post-infection. Cell viability of U-251 MG infected with both Ad5Δ24 (5 and 10 MOI) and Ad5CMV-*p53* (20 MOI) 5 days post-infection was demonstrated to be 15% and 5%, respectively. Growth inhibition of U-251 MG by both viruses was not observed when Ad5Δ24 was first UV inactivated, then added to the cell cultures. This experiment showed that the transfer of Ad5Δ24 did not interfere with the apoptotic properties of exogenous wild-type p53 in U-251 MG cells, but resulted in an additive cytotoxic effect (Fig. 2b). It is well established that Ad5Δ24 exhibits this potent oncolytic effect due to its ability to replicate in cancer cells with alterations in the p16/Rb/E2F pathway (8,11). In order to examine if Ad5Δ24 replication and p53 over-expression affected normal cells, normal human kidney fibroblasts (HFK) were grown as a low confluency monolayer and arrested by serum starvation. The arrested cells were infected with Ad5CMV-*p53*, Ad5Δ24 (5 and 10 MOI) and their combination and monitored every day for 8 days. Most cells retained their morphology and were attached to the culture dishes by the end of the experiment. These results were quantified by trypan blue viability assay and are shown in Fig. 2b. There were no significant differences in the viability of cells infected with Ad5CMV-*p53*, Ad5Δ24 (5 and 10 MOI) and their combination. These results show that the oncolytic Ad5Δ24 cannot replicate in normal quiescent cells, and p53 over-expression did not affect the growth status of such cultures.

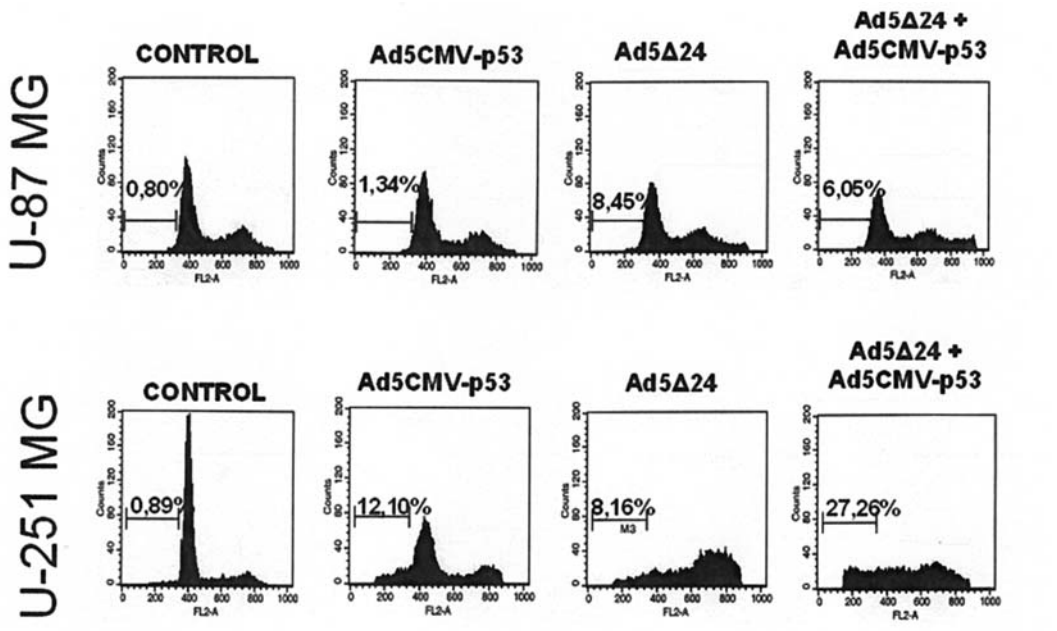

Figure 3. Fluorescence-activated cell sorting analysis of U-87 MG and U-251 MG cells. Cells were mock-infected or infected with adenoviruses Adp53, AdΔ24, and the combination of Adp53 plus AdΔ24. The x-axis represents DNA content, and the y-axis represents cell number. The cursor (sub-G1) marks the position of apoptotic cells (cells with DNA content <2n). The percentage of cells in the sub-G1 peak is indicated.

This observation is important when considering this combination gene effect as a potential treatment for gliomas with or without minimal toxicity.

Replication assay. We used $TCID_{50}$ assays to quantify the cytopathic effect in terms of viral titers. Virus yields were determined by titer in 293 cells at 10 days after the infection of U-87 MG with 10 MOI of the Ad5Δ24 adenovirus. The Ad5Δ24 titer was a million times higher than the initial dose with the U-87 MG lysates in two independent experiments.

Flow cytometry. Flow cytometric analysis of DNA content of the two human glioma cell lines after adenoviral infection showed that the transfer of Ad5Δ24 resulted in ≤8.5% of cells in sub-G1, 8 days and 5 days post-infection (Fig. 3; representative experiment on U-87 MG and U-251 MG cells, respectively). At that point, the cytopathic effect was widespread. The majority of the cells detached from the dish and cellular debris was substantial. However, there was only a small cell population in the sub-G1 area. The discrepancy between the morphological and flow cytometric data suggests that the virus-mediated cell death was probably due to both cell lysis and apoptosis. Additionally, expression of the p53 protein induced apoptosis in about 12.1% of U-251 MG cells and <1.5% of U-87 MG cells (Fig. 3). Our results on apoptosis in U-251 MG and U-87 MG cells after the adenoviral-mediated transfer of p53 agree with previous findings from our laboratory (4); the relatively small percentage of apoptotic cells in this study probably reflects the small adenoviral dose (20 MOI). U-251 MG cells over-expressing p53 and carrying the oncolytic Ad5Δ24 at 10 MOI were apoptotic at a degree of 27.26%, but no apoptotic cell death was detected under the same conditions in U-87 MG cells according to flow cytometry data (Fig. 3). The results obtained when U-251 MG cells were infected with both viruses (Ad5Δ24 and Ad5CMV-p53) agree with previous reports that p53 can cooperate with other viral and cell proteins to enhance adenovirally mediated cell death (12).

Discussion

Transferring a single gene for gene therapy for cancer may not be successful for several highly heterogeneous tumors e.g. glioblastomas (1). Several approaches using the transfer of more than one gene have been published, and others are under investigation (13-15). Conditionally replicative oncolytic viruses were also engineered as a second-step tool to adenoviral gene transfer therapy for cancer. Many reports suggest that this approach could be successful in the treatment of gliomas and other tumors (16-18).

In the present study, we examined the effect of the combined oncolytic (Ad5Δ24) adenovirus and apoptotic gene (*p53*) transfer in cultured glioma cells. Our results showed that co-infection of glioma cells with Ad5Δ24 adenovirus and Ad5CMV-*p53* adenovirus led to cell death by both apoptosis and cell lysis. The simultaneous use of both agents was effective, but each one alone did not inhibit the cytolytic or apoptotic action of the other. Thus, it appeared that the simultaneous presence of the two molecules tested here exerted an additive effect, even though each molecule exhibited its own death pathway. This finding suggests that p53 does not interfere with the capacity of Ad5Δ24 to conditionally proliferate, a fact that was also observed in previous studies (12,20).

In summary, our results indicate that the combination of an adenovirus carrying the apoptosis-inducing *p53* gene and a cytolytic adenovirus specific to cells with a defective p16/Rb/E2F pathway have an additive effect on cell death of gliomas *in vitro*. Even though *in vivo* experiments were not

d in this study, our data suggest that this gene ion approach may be a potential therapeutic tool for gliomas.

References

1. Lang FF, Miller DC, Koslow M and Newcomb EW: Pathways leading to glioblastoma multiforme: a molecular analysis of genetic alterations in 65 astrocytic tumors. J Neurosurg 81: 427-436, 1994.
2. Fueyo J, Gomez-Manzano C, Yung WKA, and Kyritsis AP: The functional role of tumor suppressor genes in gliomas: clues for future therapeutic strategies. Neurology 51: 1250-1255, 1998.
3. Fueyo J, Gomez-Manzano C, Yung WK, Liu TJ, Alemany R, Bruner JM, Chintala SK, Rao JS, Levin VA and Kyritsis AP: Suppression of human glioma growth by adenovirus-mediated Rb gene transfer. Neurology 50: 1307-1315, 1998.
4. Gomez-Manzano C, Fueyo J, Kyritsis AP, McDonnell TJ, Steck PA, Roth JA, Steck KD, Levin VA, and Yung WKA: Adenovirus-mediated transfer of the p53 gene produces rapid and generalized death of human glioma via apoptosis. Cancer Res 56: 694-699, 1996.
5. Gomez-Manzano C, Fueyo J, Kyritsis AP, McDonnell TJ, Steck PA, Levin VA and Yung WKA: Characterization of p53 and p21 functional interactions in glioma cells via apoptosis. J Natl Cancer Inst 89: 1036-1044, 1997.
6. Kyritsis AP, Zhang B, Zhang W, Xiao M, Takeshima H, Bondy ML, Cunningham JE, Levin VA and Bruner J: Mutations of the p16 gene in gliomas. Oncogene 12: 63-67, 1996.
7. Alemany R, Gomez-Manzano C, Balague C, Yung WKA, Curiel DT, Kyritsis AP and Fueyo J: Gene therapy for gliomas: molecular targets, adenoviral vectors, and oncolytic adenoviruses. Exp Cell Res 252: 1-12, 1999.
8. Fueyo J, Gomez-Manzano C, Alemany R, Lee PS, McDonnell TJ, Mitlianga P, Shi YX, Levin VA, Yung WKA and Kyritsis AP: A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect *in vivo*. Oncogene 19: 2-12, 2000.
9. Bischoff JR, Kirn DH, Williams A, Heise C, Horn S, Muna M, Ng L, Nye JA, Sampson-Johannes A, Fattaey A and McCormick F: An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. Science 274: 373-376, 1996.
10. Heise C, Herminston T, Johnson L, Brooks G, Williams A, Sampson-Johannes A, Hawkins L and Kirn D: An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. Nat Med 6: 1134-1139, 2000.
11. Gomez-Manzano C, Balague C, Alemany R, Lemoine MG, Mitlianga P, Jiang H, Khan A, Alonso M, Lang FF, Conrad CA, Liu T-J, Bekele NB, Yung WKA and Fueyo J: A novel E1A-E1B mutant adenovirus induces glioma regression *in vivo*. Oncogene 23: 1821-1828, 2004.
12. Ridgway P, Hill AR, Myers CJ and Braithwaite AW: p53/E1b 58 kDa complex regulates adenovirus replication. Virology 237: 404-413, 1997.
13. Mitlianga PG, Kyritsis AP, Gomez-Manzano C, Lemoine M, Hu M, Liu TJ, Yung WKA and Fueyo J: Co-expression of E2F-2 enhances the p53 anti-cancer effect in human glioma cells. Int J Oncol 18: 343-347, 2001.
14. Mitlianga PG, Gomez-Manzano C, Kyritsis AP and Fueyo J: Overexpression of E2F-1 leads to bax-independent cell death in human glioma cells. Int J Oncol 21: 1015-1020, 2002.
15. Rubinchik S, Yu H, Woraratanadharm J, Voelkel-Johnson C, Norris JS and Dong JY: Enhanced apoptosis of glioma cell lines is achieved by co-delivering FasL-GFP and TRAIL with a complex Ad5 vector. Cancer Gene Ther 10: 814-822, 2003.
16. Kirn D, Martuza RL and Zwiebel J: Replication-selective virotherapy for cancer: biological principles, risk management and future directions. Nature Med 7: 781-787, 2001.
17. Hawkins LK, Lemoine NR and Kirn D: Oncolytic virotherapy: a novel therapeutic platform. Lancet Oncol 3: 17-26, 2002.
18. Gomez-Manzano C, Yung WK, Alemany R and Fueyo J: Genetically modified adenoviruses against gliomas: from bench to bedside. Neurology 63: 418-426, 2004.
19. Fueyo J, Alemany R, Gomez-Manzano C, Fuller GN, Khan A, Conrad CA, Liu TJ, Jiang H, Lemoine MG, Suzuki K, Sawaya R, Curiel DT, Yung WK and Lang FF: Preclinical characterization of the antiglioma activity of a tropism-enhanced adenovirus targeted to the retinoblastoma pathway. J Natl Cancer Inst 95: 652-660, 2003.
20. Van Beusechem VW, van den Doel PB, Grill J, Pinedo HM and Gerritsen WR: Conditionally replicative adenovirus expressing p53 exhibits enhanced oncolytic potency. Cancer Res 62: 6165-6171, 2002.