A novel Ad5/11 chimeric oncolytic adenovirus for improved glioma therapy

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Abstract. Effective therapies are needed for malignant glioma patients because of the poor prognosis. Gene therapy combined with virotherapy could be the strategy of choice. In this study, we constructed a modified conditionally replicating adenoviral vector CRAd5/11-Sp-eGFP. The novel vector has the following features: i) the transduction efficiency of CRAd5 was increased using a chimeric fiber 5/11 consisting of an Ad5 tail and an Ad11 shaft and knob; ii) the tumor-specific replication of the vector was improved by utilizing the human survivin promoter to control E1 expression and a poly-A signal inserted right after the inverted terminal repeat (ITR) to stop the non-specific transcriptional activity of the ITR; iii) an expression cassette was inserted into the region between the fiber and E4 region for expressing eGFP. *In vitro* assays demonstrated that the novel vector could efficiently replicate and kill human glioma cells. Furthermore, CRAd5/11-Sp-eGFP exhibited significantly increased antitumor effects compared with the control adenoviruses in a xenograft model of glioma. Our results indicate that CRAd5/11-Sp-eGFP represents a promising candidate drug in the treatment of malignant gliomas.

Introduction

Glioblastoma multiforme (GBM) is the most common type of brain tumor in adults, exhibiting relentless and malignant progression characterized by widespread invasion throughout the brain, destruction of normal brain tissue, and resistance to surgery, radiotherapy or chemotherapy (1). GBM usually recurs at its original location and within 12 months post-resection (2). Recurrent GBM tumors are usually comprised of tumor cells that are difficult for radiation and/or chemotherapy and do not

respond adequately to further treatments. Therefore, the developments of novel agents are urgently needed to enhance their therapeutic effects for GBM tumors.

Because GBM usually relapses around the original site and is localized in the central nervous system (CNS), direct delivery of an oncolytic adenovirus provides the potential to effectively target these tumors (3). Conditionally replicating adenovirus (CRAd) propagates in tumor cells but not in normal cells, which represents a novel approach for cancer treatment. Recent preclinical research suggested the potential efficacy of these viruses, and clinical studies confirmed the safety profile of these agents (4-7). However, the numbers of cancer gene therapy clinical trials performed to date had fallen short with respect to initial expectations of demonstrable therapeutic outcome. A major limitation of these vectors has been the poor replication and weak transduction in neighboring tumor cells. A better viral vector can be achieved by the application of tumor-specific promoters (TSP) to improve tumor-specific replication of the vector and by the modifications of viral capsids to enhance viral transduction.

One of the approaches to improve the replicative specificity of CRAd is based on the tissue- or tumor-specific transcriptional control of the essential early genes required for viral replication. For CRAd, the adenovirus genome is usually genetically modified to replace the E1A promoter region with the desired tissue- or tumor-specific expression profile. The ideal tumor specific promoter (TSP) would display an alternatively 'tumor on/normal tissue off' expression profiles. To develop TSP-based CRAd, one of the commonly used methods is to drive E1 gene expression with a selected promoter. Survivin, a member of the inhibitor of apoptosis protein (IAP) family, is highly expressed in most human tumors and fetal tissues, but rarely detectable in terminally differentiated cells (8). Eighty percent of GBM tumors demonstrate abundant survivin expression (9), and a clear correlation is seen between the histological grade of a glioma and the fraction of survivin-positive tumor cells (10). Previous studies showed that the adenoviral E1A driven by the human survivin promoter was responsible for the enhanced viral replication and improved oncolytic ability in malignant gliomas (10,11). Therefore, this promoter is a promising candidate to drive E1 expression in the development of a new CRAd agent.

The wild-type human adenovirus (Ad5) has demonstrated relatively poor transduction efficiency in malignant glioma cells

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because of the low-level expression of the Coxsackievirus and adenovirus receptor (CAR) on the tumor cells (12). To circumvent this issue, the adenovirus fiber protein could be modified by genetic method for improving the transduction of adenoviral vector in CAR-deficient cells. One of the approaches is the substitution of the knob domain of Ad5 with knobs from alternate Ad serotypes, for example, species B (13). Human species B adenoviruses can utilize ubiquitously expressed CD46 as a receptor for entry into host cells (14-16). Ad11, which belongs to adenovirus species B, was reported to possess strong ability to bind and infect human CD46 positive cells (17,18).

Up to now, modified oncolytic adenoviral vectors have used different methods including introduction of tumor/tissue-specific promoters, hexon or fiber modification. In this study, we hypothesized that transcriptional and transductional control of viral replication would enhance the oncolytic effect of virus against malignant gliomas. To test this hypothesis, we for the first time constructed chimeric oncolytic adenoviral vector CRAd5/11-Sp-eGFP by combinationally using a survivin-driven and chimeric 5/11 fiber and examined the targeting and oncolytic efficacy of CRAd5/11-Sp-eGFP *in vitro* and *in vivo*. This novel vector CRAd5/11-Sp-eGFP was found to exhibit enhanced antitumor activity compared with the wild-type fiber.

Materials and methods

Cells and culture conditions. Human malignant glioma cell lines U87, A172, U251 and human embryonic kidney cell line HEK-293 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and L-glutamine (200 μg/ml) and incubated in a humidified 37°C atmosphere of 95% air and 5% CO_2.

Construction of recombinant adenovirus. CRAd5/11-Sp-eGFP was constructed as follows: i) The generation of the shuttle vector pAd5-E1-Sp which the E1 gene was under the control of survivin promoter. The survivin promoter fragment corresponding to the region from nt2532 to nt2764 in the DNA sequence (GenBank Access no: U75285) was amplified from human genomic DNA by PCR using forward primer, 5'-AGGATCCGCACGCGTTCTTTGAAAGC-3', and reverse primer, 5'-TAAGCTTCCACCTCTGCCAACGGGTC-3' (19). The amplified survivin promoter was subcloned into the plasmid pshuttle-Ad5-E1 by *Bam*HI and *Hind*III restriction sites. A SV40 poly-A (PA) fragment was then cut with *Xba*I/ *Bam*HI from a pGL3B vector (Invitrogen, Carlsbad, CA, USA) and inserted into the pshuttle-Ad5-E1 by use of the same restriction sites. The resultant plasmid was named pAd5-E1-Sp. ii) The construction of pCRAd5-Sp, which was achieved by co-transforming *Sca*I linearized pAd5-E1-Sp and *Cla*I digested pTG3602/*Swa*I into *E. coli* BJ5183 (20). iii) The construction of shuttle plasmid pBS/Ad5/11-eGFP which includes the chimeric fiber Ad5/11 and eGFP. First, we synthesized a gene which includes Ad5 tail (522-608 bp), Ad11 shaft and knob sequence in pcDNA 3.1(+) (Sangon, Shanghai, China). The plasmid was named pcDNA 3.1(+)/Ad5/11 which included a unique *Nde*I and *Spe*I restriction introduced separately in its 5' and 3' end. The pcDNA 3.1(+)/Ad5/11 digested

by *Nde*I and *Spe*I was ligated with an *Nde*I/*Spe*I-linearized pBS shuttle vector at 4:1 (v/v) ratio (20). The positive clones were screened by enzyme digestion and DNA sequencing and named pBS/Ad5/11. Second, we constructed an expression cassette that the CMV promotes eGFP expression. This cassette digested by *Bam*HI and *Sfu*I was inserted into the region between E4 and the fiber located in the pBS/Ad5/11 to acquire the shuttle vector pBS/Ad5/11-eGFP. iv) For the generation of CRAd5/11-Sp-eGFP vector, the pCRAd5-Sp digested with *Swa*I and the pBS/Ad5/11-eGFP digested with *Xho*I was co-transformed into *E. coli* BJ5183 cells. The resulting plasmid was designated pCRAd5/11-Sp-eGFP. *Pac*I linearized pCRAd5/11-Sp-eGFP was transfected into HEK-293 cells. Then recombinant adenovirus CRAd5/11-Sp-eGFP was propagated in HEK-293 cells and purified by cesium chloride gradient methods. The titers were detected by spectrophotometry at an absorbance (A) of 260 nm. Using similar strategy, the recombinant adenoviruses CRAd5-Sp-eGFP was produced.

Viral oncolytic potency in human glioma cells. For determination of virus-mediated cell killing ability, $5x10^4$ glioma cells (U87, A172 and U251) were seeded in 24-well plates and infected with adenoviruses at various multiplicity of infection (MOI) or phosphate-buffered saline (PBS) for 4 h followed by replacement of infection media with growth media. Seven days after infection, in order to visualize cell killing, cells were fixed and stained with 2% crystal violet in 70% ethanol for 20 min followed by washing with distilled water to remove excess dye. The plates were dried and images were captured with an Alpha Innotech FluroChem (Santa Clara, CA, USA).

Cell viability assay. U87, A172 and U251 were cultured in 96-well plates at $5x10^3$ cells per well. Twenty-four hours later, viruses at 5 MOI were administered into cells. The plates were incubated at 37°C and supplemented with 5% CO_2. PBS was used as a control. The medium was removed every 24 h and fresh medium containing 20 μl of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 1 μg/ml) solution was added to each well. Four hours later, MTT was discarded and 150 μl of DMSO was loaded. The spectrophotometric absorbance of the samples was measured with a Multiskan MK3 (Thermo Electron Corporation, Waltham, MA, USA) at 570 nm. The percentage of cell viability was calculated using the following formula: cell viability = absorbance value of infected cells/absorbance value of uninfected control cells. Six duplicate wells were measured and the experiments were done at least three times.

Quantitative real-time PCR for the Ad5 E4 gene. U87, A172 and U251 cells were seeded in 6-well plates ($5x10^5$ cell/well) and infected with Ad5-eGFP, CRAd5-Sp-eGFP and CRAd5/11-Sp-eGFP at a MOI of 5. At each 24 h post-infection, the cells were washed with PBS and harvested. The DNA was then purified with a TIANamp blood DNA kit (Tiangen, China). Quantitative polymerase chain reaction (PCR) was performed with primers (Ad5 E4 for, 5'-CATGCGCCGCTGCCCTGATA-3'; and Ad5 E4 reverse, 5'-TTCCCGCTCCTCCCGTGTGT-3') against a 99 base pair region of the E4 gene. The amplified Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) based on specific primers (GAPDH for, 5'-TGGTGAAGACGCCAGTGGA-3'; and GAPDH reverse, 5'-GCACCGTCAAGGCTGAGAAC-3') was used for

an internal control. All reactions, performed in triplicate in a total reaction volume of 20 μl, using SYBR Premix Ex Taq™ II (Takara, Japan), were carried out in a Bio-Rad iQ5 instrument (Bio-Rad, Hercules, CA, USA). The parameters were used for amplification and melting curve analysis was described by Li *et al* (21). For quantitative analysis, viral genomes were purified from each group using the same quantity of viral particles (assessed by spectrophotometrically) and serially diluted to generate a standard curve.

Adenovirus transduction analysis. U87, A172 and U251 cells were plated at a density of 5x10^5/well in a 60-mm dish culture plate for 24 h prior to infection, then Ad5-eGFP, CRAd5-Sp-eGFP and CRAd5/11-Sp-eGFP were applied to the U87, A172 and U251 cells at the dosage of 500 viral particle/cell. For quantification of eGFP expression level, photographs were taken at 24 h post-infection with 10 x objective (3-sec exposure time). The infection efficiency was quantified by counting eGFP-positive cells.

Studies of xenograft tumors in nude mice. U87 tumor xenografts were established by subcutaneously inoculating 2x10^6 cells into the right flanks of 4- to 6-week-old female BALB/c nude mice (Animal Research Committee of the Institute of Biochemistry and Cell Biology, Shanghai, China). When tumors reached between 70 to 100 mm^3, 28 mice were randomly assigned to PBS treated group, Ad5-eGFP treated group, CRAd5-Sp-eGFP treated group and CRAd5/11-Sp-eGFP treated group, respectively. The established tumors were injected with 50 μl of PBS or 5x10^8 plaque-forming units (PFU) of virus. The injections were repeated four times, every other day. Tumor growth was monitored by periodic measurements with calipers and tumor volume was calculated using the following formula: tumor volume (mm^3) = length (mm) x width^2 (mm^2)/2. The tumor volumes of the 7 mice in each group were measured every 2 days for 1 month.

Statistical analysis. Statistical analysis for all experimental conditions was performed using the Statistical Package for the Social Sciences (SPSS) software (version 13, Chicago, IL, USA). The difference between each control and experimental groups was analyzed by using one-way analysis of variance between groups (ANOVA/LSD). In all cases, a p-value <0.05 was considered statistically significant.

Results

Chimeric fiber 5/11 modified oncolytic adenovirus CRAd5/11-Sp-eGFP for improved cell killing ability in glioma cells in vitro. In order to assay biological activity of the oncolytic adenoviruses (Fig. 1), glioma cell lines of U87, A172 and U251 were infected with CRAd5-Sp-eGFP, CRAd5/11-Sp-eGFP or replication-deficient Ad5-eGFP virus at the dose of 0, 0.1, 0.5, 1, 5 and 10 MOI, respectively. Cell killing effect was then assessed via crystal violet staining. Of the tested vectors, CRAd5/11-Sp-eGFP demonstrated a dose-dependent cell killing effect in all the human glioma cell lines (Fig. 2) with a dose as low as 0.1 MOI in A172 cells and at 1 MOI in U87 and U251 cells. Of note, the oncolytic effect of CRAd5/11-Sp-eGFP was about 100 times superior to that of CRAd5-Sp-eGFP in the A172 cell
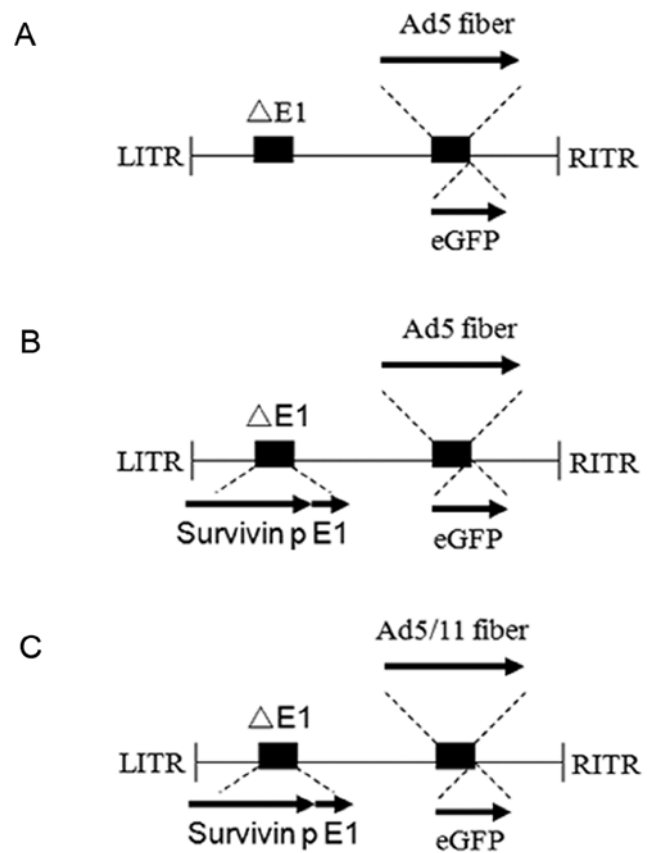

Figure 1. Schematic of adenoviral genomes. Maps of Ad5 genomes containing fiber modifications. (A) Ad5-eGFP, a replication-defective adenovirus with unmodified fiber. (B) CRAd5-Sp-eGFP and (C) CRAd5/11-Sp-eGFP are constructed by the insertion of a suvivin promoter-driven E1 expression cassette. CRAd5-Sp-eGFP carries the wild-type Ad5 fiber, whereas CRAd5/11-Sp-eGFP is an infectivity enhanced version of CRAd5-Sp-eGFP that carries the chimeric Ad5/11 fiber.

lines. Less or no cell killing effect was observed with the control, the replication-defective Ad5-eGFP vector. Cell viability was quantified by MTT assay. Tumor cell lines (U87, A172 and U251) were infected with CRAd5-Sp-eGFP, CRAd5/11-Sp-eGFP or replication-deficient adenovirus Ad5-eGFP at MOI of 5. As shown in Fig. 3, CRAd5-Sp-eGFP and CRAd5/11-Sp-eGFP was able to inhibit growth of glioma cells in a time-dependent manner. In addition, CRAd5/11-Sp-eGFP significantly inhibited the growth of all three tumor cell lines compared with CRAd5-Sp-eGFP. The results indicated that the fiber chimeric oncolytic virus constructed by us significantly enhanced cell killing effect against glioma cells compared with fiber unmodified oncolytic adenovirus.

Efficient replication of chimeric fiber 5/11 modified CRAd in glioma cells. Efficient viral replication can greatly contribute to the antitumor capacity of CRAd. Here, in order to investigate the replicative ability of the modified CRAds in glioma cell, U87, A172 and U251 cells were infected at 5 MOI of Ad5-eGFP, CRAd5-Sp-eGFP and CRAd5/11-Sp-eGFP and harvested in triplicate at 0, 1, 2 and 3 days after infection. Total DNA was isolated from the cells and viral replication was measured by qPCR using primers for the Ad5 E4 gene. As seen in Fig. 4, CRAd5-Sp-eGFP
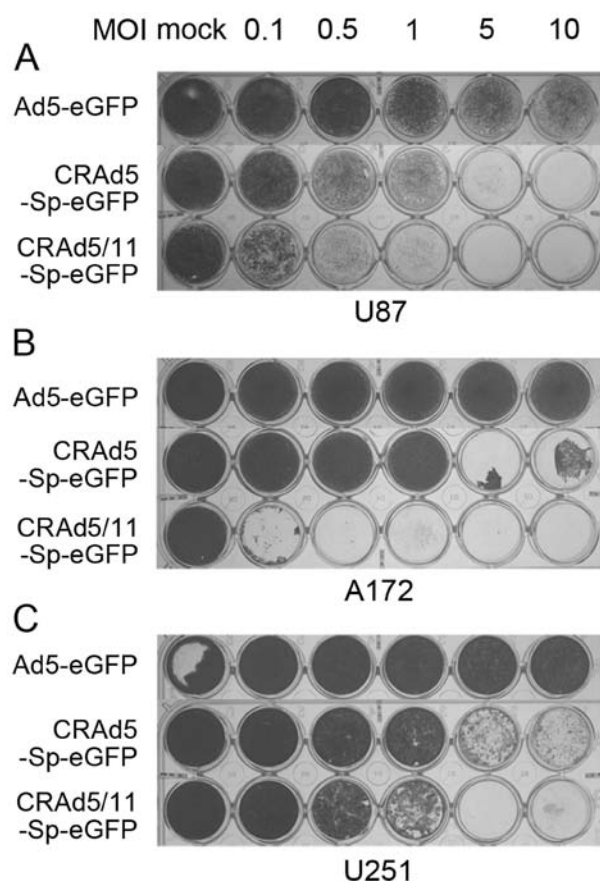
Figure 2. CRAd5/11-Sp-eGFP exhibits improved oncolytic activity in human glioma cells. CRAds at the indicated dosage were incubated with the following human glioma cell lines: (A) U87, (B) A172 and (C) U251. Seven days later, the cells were stained with 2% crystal violet and photographed. Experiments were repeated three times independently.

and CRAd5/11-Sp-eGFP all demonstrated significantly enhanced replication of viral genome compared with Ad5-eGFP (p<0.001). This result indicated that U87, A172 and U251 cells maintained high level of survivin promoter activity, which therefore resulted in specific replication of CRAd5-Sp-eGFP and CRAd5/11-Sp-eGFP in these three glioma cell lines. The result also suggested that the eGFP expression cassette located between the fiber and E4 region did not influence the replication capacity.

Enhanced transduction efficiency of fiber modified adenovirus in target cells. To confirm that the improved cytotoxic effect of Ad5/11 fiber chimeric CRAd in glioma cells, the transduction efficiency of the chimeric fiber adenovirus was evaluated. Ad5-eGFP, CRAd5-Sp-eGFP and CRAd5/11-Sp-eGFP were applied to U87, A172 and U251 cells that express a high level of CD46, the receptor of Ad11. The results showed that the percentage of eGFP positive cells in CRAd5/11-Sp-eGFP group was higher than that in the control groups Ad5-eGFP and CRAd5-Sp-eGFP in U87 and A172 cells (Fig. 5). This indicated that the tropism efficiently enhanced U87 and A172 target cells and it was mainly attributed to the modified fiber.

CRAd5/11-Sp-eGFP for improved antitumor activity in nude mice. In order to evaluate antitumor activity of CRAd5/11-Sp-eGFP *in vivo*, U87 human glioma xenograft model was established in nude mice. When the tumors reached 70-100 mm^3, 50 μl of Ad5-eGFP, CRAd5-Sp-eGFP, CRAd5/11-Sp-eGFP (5x10^8 PFU each) or PBS as the control was injected intratumorally every other day four times. The results indicated that animals treated with CRAd5/11-Sp-eGFP exhibited significant suppression of tumor growth compared with those treated with PBS (p<0.05) or Ad5-eGFP (p<0.05).
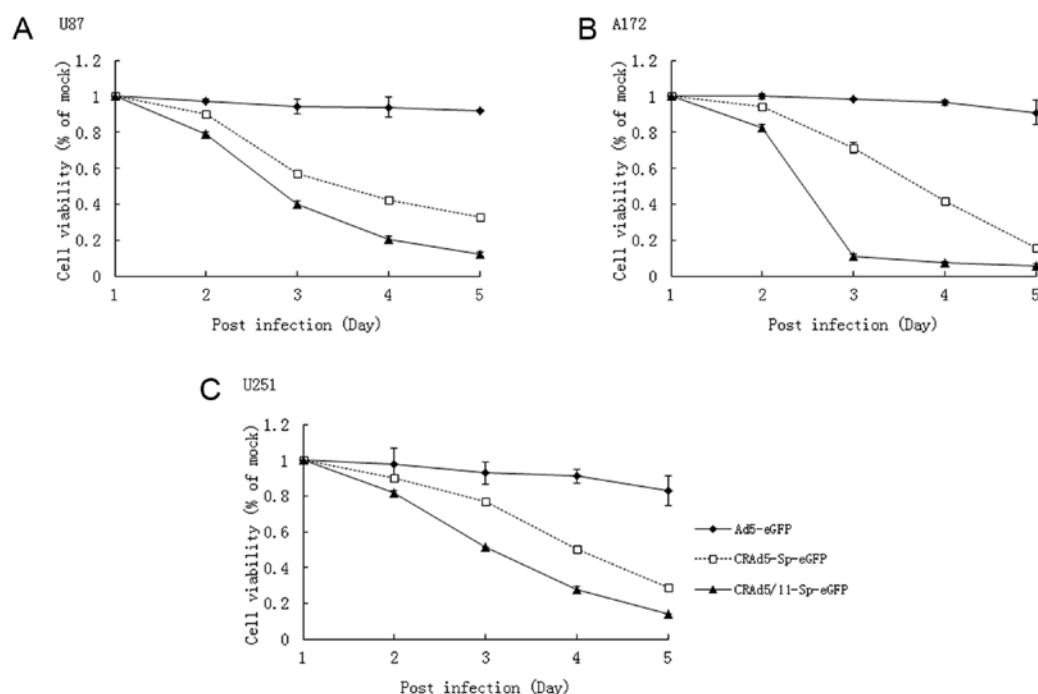

Figure 3. Cell viability was assayed by MTT test. Tumor cells (A) U87, (B) A172 and (C) U251 were infected with Ad5-eGFP, CRAd5-Sp-eGFP and CRAd5/11-Sp-eGFP at 5 MOI. At the indicated time, cell viability was determined by MTT assay. Data are presented as means ± SEM (standard error of the mean) of triplicate experiments and are expressed as a percentage of untreated control cells.
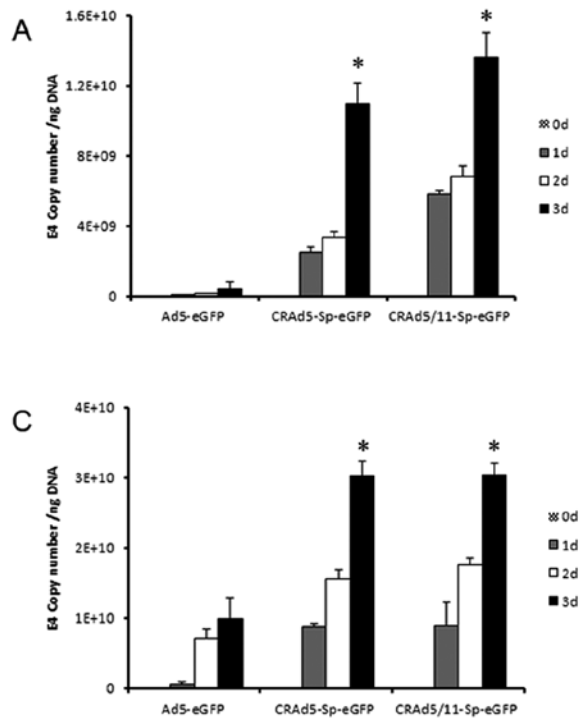
Figure 4. Replication ability of adenoviral vectors were assayed in (A) U87, (B) A172 and (C) U251 cells *in vitro*. These three cell lines were infected with 5 MOI of Ad5-eGFP, CRAd5-Sp-eGFP or CRAd5/11-Sp-eGFP, respectively, and the cells were harvested at days 0, 1, 2 and 3. Total DNA was isolated from these cells and viral replication was measured by qPCR using primers for the Ad 5 E4 gene. *p<0.05 (statistical significance in comparison with Ad5-eGFP at day 3).
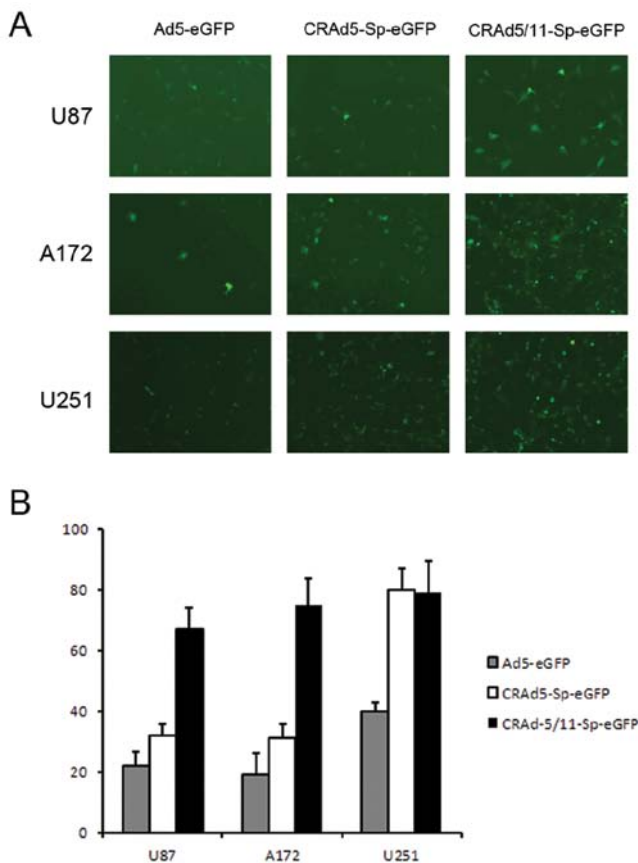

Figure 5. U87, A172 and U251 cells (5x10^5 cells/well) were infected with 500 viral particles/cell of Ad5-eGFP, CRAd5-Sp-eGFP or CRAd5/11-Sp-eGFP and analyzed for eGFP expression 24 h after infection. (A) The photographs were taken and the representative areas were imaged at x10 magnification. (B) Quantification of eGFP-positive cells under fluorescence microscope (x10 magnification).
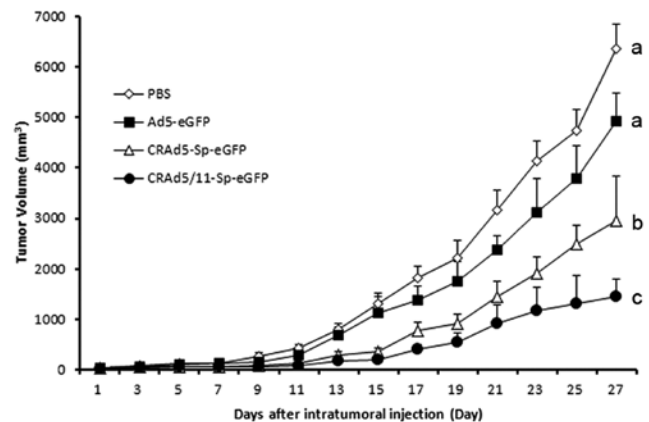

Figure 6. Therapeutic efficacy of CRAd5/11-Sp-eGFP was assayed in a model of glioblastoma. Tumor model were established by s.c. injection of U87 cells (2x10^6) into female nude mice. When tumors reached the size of 70-100 mm³, the mice were treated four times every other day by intratumoral injection of 50 μl PBS or Ad5-eGFP, CRAd5-Sp-eGFP, CRAd5/11-Sp-eGFP at 5x10^8 PFU of virus in a 50 μl volume. PBS was also used as a control. Tumor size was measured and tumor volume error bars represent the SEM. The bars that do not share a letter designation were determined to be significantly different by LSD one-way ANOVA comparison (p<0.05).

The average tumor volume of the CRAd5/11-Sp-eGFP group was about 1,400 mm³ at 29 days after virus treatment, whereas the tumor volume of PBS- or Ad5-eGFP-treated mice was 6,351±496.8 or 4,933.4±542.6 mm³, respectively. In addition, the CRAd5-Sp-eGFP-treated group showed notable antitumor efficacy compared with the PBS- or Ad5-eGFP-treated group (p<0.05) (Fig. 6). These data indicated that survivin-driven fiber chimeric oncolytic adenovirus, CRAd5/11-Sp-eGFP exerted strong antitumor activity.

Discussion

In the present study, we constructed a survivin-driven, chimeric 5/11 fiber-modified conditionally replicating adenovirus, CRAd5/11-Sp-eGFP. In previous studies, the exogenous gene expression cassettes usually were located in the E1 region or E3 region (22-26). In this study, the exogenous gene expression cassette was inserted into the region between E4 and the fiber by using a novel shuttle vector which contained the modified fiber and an expression cassette. This method provided us a new option for expressing an exogenous gene in oncolytic adenovirus except for conventional E1 or E3 region. In addition, the modification of the fiber protein and the insertion of an exogenous gene expression cassette into the region between the fiber and E4 could be conveniently done via one step homologous recombination by using the novel shuttle vector. The results showed that this novel oncolytic adenoviral vector exhibited enhanced tumor targeting and antiglioma activity both *in vitro* and *in vivo*.

The replicative specificity of CRAd is based on tumor-specific transcriptional control of the essential early genes required for replication. The ideal tumor specific promoter (TSP) would exhibit accurate expression profiles in the specific cancer tissue. Survivin is an important member of the IAP family that serves a dual role in the inhibition of apoptosis and regulation of cell division. One of the most important features of survivin is the differential expression of this protein in cancer versus normal tissue. Dramatic overexpression of survivin has been demonstrated in brain tumor, including malignant glioma (27,28). Zhu *et al* showed that the survivin promoter is upregulated in brain tumors (27). Moreover, a study from Van Houdt *et al* demonstrated that survivin promoter-based CRAd could be efficiently replicated within and kill a variety of established glioma tumor cells and significantly inhibit the growth of glioma xenografts *in vivo* (10). Similarly, in our study the CRAd that uses the survivin promoter revealed improved replication activity and oncolytic effect in glioma cells compared with the controls.

CRAd-mediated gene therapy in the context of malignant brain tumors is hindered by lack of a sufficient number of Ad receptors or CAR, on the glioma cell surface (29). Fiber chimeric adenovirus vectors from species B adenovirus have been reported (3,13,30,31), but 5/11 fiber with a replication-competent vector to improve virus transduction in gliomas has not been identified. There is a lack of 5/11 chimeric fiber adenovirus vector that have the high replication efficiency in gliomas and still carry a quantifiable marker gene. A precise evaluation of the oncolytic vectors with 5/11 chimeric fiber in different glioma cells remains to be addressed. In this study, the viral entry into glioma cells is significantly enhanced when the Ad5 fiber knob is replaced with the Ad11 knob, the latter of which recognizes the CD46 receptor that is widely expressed on tumor cells including gliomas (13,18). In addition, the Ad11 belongs to species B group 3 which preferentially interacts with CD46, but also uses receptor desmoglein 2 (32). The Ad3 shares the receptor desmoglein 2 with Ad11 and Ad35 nearly exclusively uses CD46 as its receptor. Thus, we hypothesis Ad11 have a more wide-range to target tumor cells and the results need further study. In the present study, we have shown that the virus of CRAd5/11-Sp-eGFP efficiently infected the U87 and A172 cell lines, as demonstrated by fluorescent microscopy. Therefore, infection of CRAd5/11-Sp-eGFP significantly inhibited U87

and A172 cell growth. Nevertheless, in U251 cell, the adenovirus of CRAd5-Sp-eGFP and CRAd5/11-Sp-eGFP had little difference in transduction efficiency. Allen *et al* reported U251 expresses the high level of CAR compared with other gliomas, such as U87, U118 and TE671 (33). Mäenpää *et al* found that U251 was moderately positive for CD46 expression (34). These results illustrated that CRAd5-Sp-eGFP and CRAd5/11-Sp-eGFP possessed the same level of eGFP-positive cells.

Compared with CRAd5-Sp-eGFP *in vitro*, CRAd5/11-Sp-eGFP exhibited higher infection efficiency and stronger oncolytic ability in glioma cells, and enhanced antitumor efficacy in human glioma U87 xenograft models. In summary, survivin-driven and chimeric 5/11 fiber-modified CRAds vector CRAd5/11-Sp-eGFP is a promising candidate in the treatment of malignant glioma.

Acknowledgements

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