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RESEARCH ARTICLE

Epidermal growth factor receptor targeting enhances adenoviral vector based suicide gene therapy of osteosarcoma

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Abstract

Background Despite improvements in the treatment of osteosarcoma (OS) there are still too many patients who cannot benefit from current treatment modalities. Therefore, new therapeutic approaches are warranted. Here we explore the efficacy of targeted adenoviral based suicide gene therapy.

Methods and results Immunohistochemistry and FACS analysis detected low or absent expression levels of the primary adenovirus receptor CAR on human primary OS and human OS cell lines. These results predict a low infection efficiency and thus a reduced therapeutic effect. Targeting the adenoviruses to another receptor highly expressed on OS could overcome this limitation. We found epidermal growth factor receptor (EGFR) to be widely expressed on primary OS. Immunohistochemistry on primary tumor samples and FACS analysis on primary short-term cultures and four OS cell lines showed that EGFR was consistently expressed. The recombinant bispecific single-chain antibody 425-s11 redirects adenoviral vectors towards the EGFR. Adenovirus transduction experiments in the presence or absence of 425-s11 showed significantly enhanced gene transfer with the targeted adenoviral vector compared with the native vector (OS cell lines 2.5 to 7.2 times enhanced gene transfer and OS primary short term cultures 1.7 to 10 times enhanced gene transfer). On this basis, targeted suicide gene therapy experiments with AdCMVHSV-TK in combination with ganciclovir were performed. These experiments demonstrated up to 3.5-fold enhanced kill of OS cell lines and primary short-term cultures by the EGFR targeted vector.

Conclusions Suicide gene therapy with adenovirus targeted towards EGFR may have favorable therapeutic characteristics for future gene therapy applications in OS. Copyright © 2002 John Wiley & Sons, Ltd.

Keywords EGFR; targeting; suicide gene therapy; osteosarcoma; primary short-term cultures

Introduction

Osteosarcoma (OS) is the second most common primary malignancy of the bone in children and young adults. OS can occur at any age but more than 75% occurs in patients between 12 and 25 years of age. A second peak occurs above the age of 50 years. Management of OS has dramatically changed over the past two decades. Amputation of the extremity was a standard treatment, with an overall survival of 5–20% at 2 years [1]. With current use of neoadjuvant chemotherapy, the 5-year survival rate for OS varies from 50–65% [2–8]. However, despite these recent improvements, there are still

too many patients who do not benefit from current treatment modalities. In addition, patients with metastatic OS have poor survival statistics (30% survival) [9,10]. Therefore, there is a need to develop novel therapeutic approaches. Gene therapy may offer new treatment options for this disease.

Adenoviral vectors are widely applied to transfer genes into tumor cells, since they can be produced in high titers and can infect many different cell types.

Adenoviral vector based suicide gene therapy [herpes simplex virus thymidine kinase (HSV-TK) in combination with ganciclovir] has been described as selective in blocking the growth of OS in experimental models. Furthermore, the combination of this therapy with metrotrexate showed to have better anti-tumor effects with reduced toxicity compared with the conventional chemotherapy protocols alone [11–13].

Adenoviral vectors have also shown efficacy in eradicating OS lung metastases. Nasal delivery of a vector encoding interleukin-12 resulted in the inhibition of pulmonary metastases in a nude mouse xenograft model [14]. The studies mentioned above suggest that adenoviral vector based gene therapy is feasible in OS. This has, however, not yet been confirmed on primary human OS tumor material.

Adenoviruses of human serotype 5 bind to the Coxsackie/adenovirus receptor (CAR) [15]. Following viral attachment, internalization is potentiated through cellular α_v-integrins via the RGD motif of the penton base of the virus [16]. Studies on other types of tumors have shown that application of adenoviral vectors for cancer gene therapy is hampered due to lack of CAR expression on primary tumor cells [17–19]. The lack of this receptor results in low infection efficiency, thus reducing the therapeutic efficacy of adenoviral vectors tremendously [20]. It is therefore important to provide adenoviral vectors with alternate and more effective pathways to enter into primary tumor cells. Retargeting of adenoviruses towards antigens present on tumor cells circumvents low CAR expression and increases the efficacy of gene transfer [21,22]. One such antigen is the human epidermal growth factor receptor (EGFR). EGFR is characteristically expressed on cells derived from all three germ layers and at particularly high levels on epithelia [23]. We have developed a bispecific single-chain antibody (scFv) that targets adenoviral vectors towards tumor cells via the EGFR [24]. This bispecific scFv mediated efficient adenoviral transduction of tumor cell lines from various tissue origin [24] and of primary human glioma cells [17,32].

In this study we investigated CAR and EGFR expression on primary human OS. Furthermore, we evaluated EGFR as a possible alternative target for adenoviral vector mediated gene transfer into OS. Finally, we carried out experiments to assess the efficacy of targeted adenoviral vector based suicide gene therapy for OS.

Materials and methods

Immunohistochemistry

Paraffin-embedded and frozen tumor tissue samples from 12 patients with OS (pre-chemotherapy) were obtained from the Departments of Pathology of the University of Munster (Germany) and the VU University Medical Center (Amsterdam, The Netherlands).

Tumor samples were sectioned at 4-μ-thick and stained immunohistochemically for EGFR and CAR. Mouse anti-CAR monoclonal antibody RmcB [15] was obtained from Dr. R. L. Crowell (Hahnemann University, Philadelphia, PA, USA). Supernatant of the 425-hybridoma culture, purchased from the ATCC (American Type Culture Collection, Manassas, USA), was used as a source for MoAb anti-EGFR. In addition, an EGFr antibody from Novocastra Laboratories (Newcastle upon Tyne, UK) was used [23].

Frozen or paraffin-embedded sections were pre-incubated with normal rabbit serum 1 : 50 (DAKO, Glostrup, Denmark) followed by overnight incubation with the primary antibody (425 non-diluted, EGFR-Novocastra 1 : 20, RmcB 1 : 100) at room temperature (EGFr) or 4 °C (CAR). Slides were rinsed with phosphate-buffered saline (PBS) and the biotinylated secondary antibody rabbit-anti-mouse IgG (DAKO) 1 : 500 was applied for 30 min. After rinsing, the slides were incubated with the avidin-biotin complex 1 : 200 (Strep ABComplex, DAKO) for 1 h. As substrate, chromogen 3′-diaminobenzidine tetrahydrochloride (DAB chromogen, DAKO) was applied for 5 min. The slides were counterstained with hematoxilin, dehydrated and cover slipped with entalan (Merck Eurolab B.V., Amsterdam, The Netherlands).

Tissue from normal skin was used as a positive control for EGFr staining, and normal brain tissue was used as a positive control for CAR staining. As a negative control, the primary antibody was replaced by PBS. Immunohistochemical results were evaluated by two independent observers and scored as follows: −, 0 positive cells; ±, ≤10% positive cells; +, 10–30% positive cells; ++, 30–60% positive cells; and +++, 60–100% positive cells.

Cells and culture conditions

The following human OS cell lines were used: MG-63 [25], courtesy of Dr. Clemens-Lowik, Leiden University Medical Center; MNNG-HOS [26]; SaOs-2 [27], courtesy of Dr. F. van Valen, Westfalische Wilhelms-Universitat Munster; and CAL-72 [28], courtesy of Dr. J. Gioanni, Laboratoire de Cancerologie, Faculte de Medicine, Nice, France.

All OS cell lines were maintained in DMEM supplemented with 10% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine (all from GibcoBRL, Life Technologies B.V., Breda, The Netherlands), at 37 °C in a 5% CO_2 humidified atmosphere.

Patient material

Tumor material (open biopsy procedure) was received directly after surgery and kept under sterile conditions at 4 °C. Biopsies were processed immediately. The biopsies were washed in sterile PBS, cut into small pieces and digested with liver digestion medium (GibcoBRL, Life Technologies) during four consecutive 30-min incubations in a water bath at 37 °C. After each round of digestion the cells were collected and washed. Finally, all cells were pooled and washed. Cells were directly used for adenoviral vector infection experiments and if possible for analysis by fluorescence-activated cell sorting (FACS) and brought into culture. OS tumor cell morphology of these short-term cultures (passage 0–5) was confirmed by a histopathologist (author: J.B).

Analysis by FACS

Human OS cell lines and cells of primary short-term cultures (OS-1 and LOS-5 cells used directly after digestion and OS-1a, OS-2 and OS-6 cells used after passage 1–5) were incubated with anti-EGFr 425 MoAb non-diluted or anti-CAR MoAb RmcB 1 : 100 diluted in PBS containing 0.1% BSA for 1 h on ice, followed by FITC-conjugated rabbit anti-mouse IgG antibody (DAKO, Glostrup, Denmark) for 1 h on ice. The samples were fixated in PBS/1% formaldehyde and analyzed on a FACscan (Becton Dickinson) using Cell Quest software within 24 h after fixation.

Adenoviral vectors and bispecific single-chain antibody

AdCMVluc [29], AdCMVHSV-TK [30] and AdCMVGFP are all E1/E3-deleted replication-deficient Ad5 vectors which express firefly luciferase, HSV-TK and green fluorescent protein, respectively, under the control of the immediate-early cytomegalovirus (CMV) promotor. The adenoviruses were propagated on the permissive line 293. All virus aliquots were stored at −80 °C until use. Adenovirus titers (pfu/ml) were determined by limiting dilution titration on 293 cells. All studies were performed with one batch of each adenoviral vector.

Anti-EGFR targeting bispecific single-chain antibody 425-s11 has been described previously [24]. It has dual specificity for the adenovirus fiber knob and human EGFR. Chinese hamster ovary (CHO) cells were stable transfected with this construct and cultured in an INTEGRA CL 350 cultivation system (INTEGRA Biosciences AG, Wallisellen, Switzerland) in CHO-S-SFM II medium (GibcoBRL, Life Technologies B.V.). Undiluted culture supernatant of the same batch was used for all targeting experiments.

Targeting experiments

OS cell lines were plated in a 24-well plate (2.5×10^4 cells/well); primary OS tumor cells were plated similarly or in a 96-well plate (10^4 cells/well). To allow adherence, cells were incubated overnight in DMEM 10% FCS medium. Viral particles, AdCMVLuc or AdCMVGFP (10^8), were diluted in 25 µl DMEM 2.5% FCS and incubated with 75 µl 425-s11 supernatant or 75 µl DMEM 2.5% FCS (control) for 30 min at room temperature. The optimal ratio of 425-s11 to AdCMVluc for targeted gene transfer into EGFr-positive cells was previously determined by titration. Subsequently, virus was diluted in DMEM 2.5% FCS and cells were infected in triplicate at a multiplicity of infection (MOI) of 25 pfu/cell for OS cell lines or MOIs ranging from 100 to 250 pfu/cell for primary OS tumor cells. One hour after infection the virus was removed from the cells and fresh DMEM 10% FCS medium was added. Twenty-four hours post-infection, cells were assayed for luciferase or GFP expression. Luciferase activity was measured using the luciferase assay system (Promega, Madison, WI, USA). Medium was removed from the cells and lysis buffer was added to the wells followed by a freeze-thaw step. Luminescence was measured during 10 s immediately after initiation of the light reaction in a Luminat LB9507 luminometer (EG&G Gerthold, Bad Wildbad, Germany). Values were protein normalized (Protein Assay, Bio-Rad Laboratories, Veenendaal, The Netherlands). Results were expressed as relative light units (RLUs) per mg protein. GFP expression was measured as percentage positive cells by FACS analysis.

Suicide gene therapy AdCMVHSV-TK experiments

Cells (5×10^3/well) were plated in a 96-well plate and allowed to attach overnight. After 24 h, cells were infected with AdCMVHSV-TK at an MOI of 100 pfu/cell in the presence or absence of 425-s11. Twenty-four hours post-infection, a range of ganciclovir (Cymevene®; Roche, Mijdrecht, The Netherlands) concentrations (0–243 µM) was added. Cell viability was determined by WST-1 cell proliferation reagent (Roche Molecular Biochemicals, Mannheim, Germany) conversion on day 7 after ganciclovir administration. We have observed inherent differences in sensitivity to chemotherapeutic reagents between OS cell lines and OS primaries; therefore, the correct concentration of GCV in combination with AdSMVHSV-tk was established in pilot experiments: 9 µM GCV for OS cell lines and 27 µM GCV for primary OS. Statistical analysis was performed by using student's t-test.

Results

CAR and EGFr expression on OS cell lines and primary OS

CAR and EGFr expression was analyzed on primary OS tumor samples by immunohistochemistry (Table 1). In all

tested cases, CAR expression was found to be low (10%) or absent. CAR staining was localized on the membrane and/or in the cytoplasm. EGFr expression was present in 10 of the 12 primary OS samples, varying from 30% to >90% of the cells being positive. Positive cells were scattered throughout the tumor. EGFr staining was most often localized on the membrane and in some cases there was some cytoplasmic staining as well.

Short-term cultures were established from the tumor material of seven patients. Patient characteristics are described in Table 3. From five patients, cell cultures were established from the primary tumor (OS). In two cases, cells were cultured from an OS lung metastasis (LOS). From patient no.1, cells were cultured from the primary tumor before (OS-1) and after (OS-1a) chemotherapy. FACS analysis on these short-term cultures (passage 0–5) was performed to assess CAR and EGFr expression. All samples were negative for CAR and positive for EGFr (Figure 1A).

The OS cell lines (MG-63, MNNG-HOS, CAL-72 and SaOs-2) were negative (MG-63, CAL-72), low (MNNG-HOS) or moderately (SaOs-2) positive for CAR (Figure 1B). EGFr was present on all cell lines, ranging from highly (MG-63, MNNG-HOS) to moderately (CAL-72, SaOs-2) positive.

Targeting adenoviral vectors towards EGFr on OS cell lines

To determine whether redirecting adenoviral vectors towards EGFr enhances gene transfer into OS cell lines, transduction experiments were performed with AdCMVLuc. Luciferase gene transfer was measured 24 h post-infection (Figure 2). In two independent experiments, enhanced gene transfer by EGFr targeting was observed on all OS cell lines, i.e., average 7.2 times for MG-63, 4.8 times for MNNG-HOS, 5.4 times for CAL-72 and 2.5 times for SaOs-2. Augmentation correlated with the relative CAR and EGFr expression levels (see Figure 1B).

Table 1. CAR and EGFR expression on primary OS tumor samples

Code	Age	M/F	Diagnosis	CAR*	EGFR
I	10	M	High grade OS, lung metastasis	±	+
II	16	M	High grade OS, lung metastasis	−	+
III	18	M	High grade OS	−	+++
IV	65	F	High grade OS, chondroblastic component	±	++
V	23	M	High grade OS, chondroblastic component	−	++
VI	45	M	High grade OS	±	++
VII	27	M	Low grade OS, Juxtacortical OS	−	++
VIII	10	F	High grade OS, chondroblastic component	ND**	+
IX	35	M	High grade OS	ND	−
X	9	M	High grade OS, chondroblastair component	ND	−
XI	19	M	Small cell OS	ND	+
XII	15	F	High grade OS	ND	+

*Expression levels were scored as follows: – accounts for 0 cells positive, ± ≤10% positive cells, + 10–30% positive cells, ++ 30–60% positive cells and +++ 60–100% positive cells.
ND**Samples 8–12 were not eligible for CAR staining; no frozen samples were available.

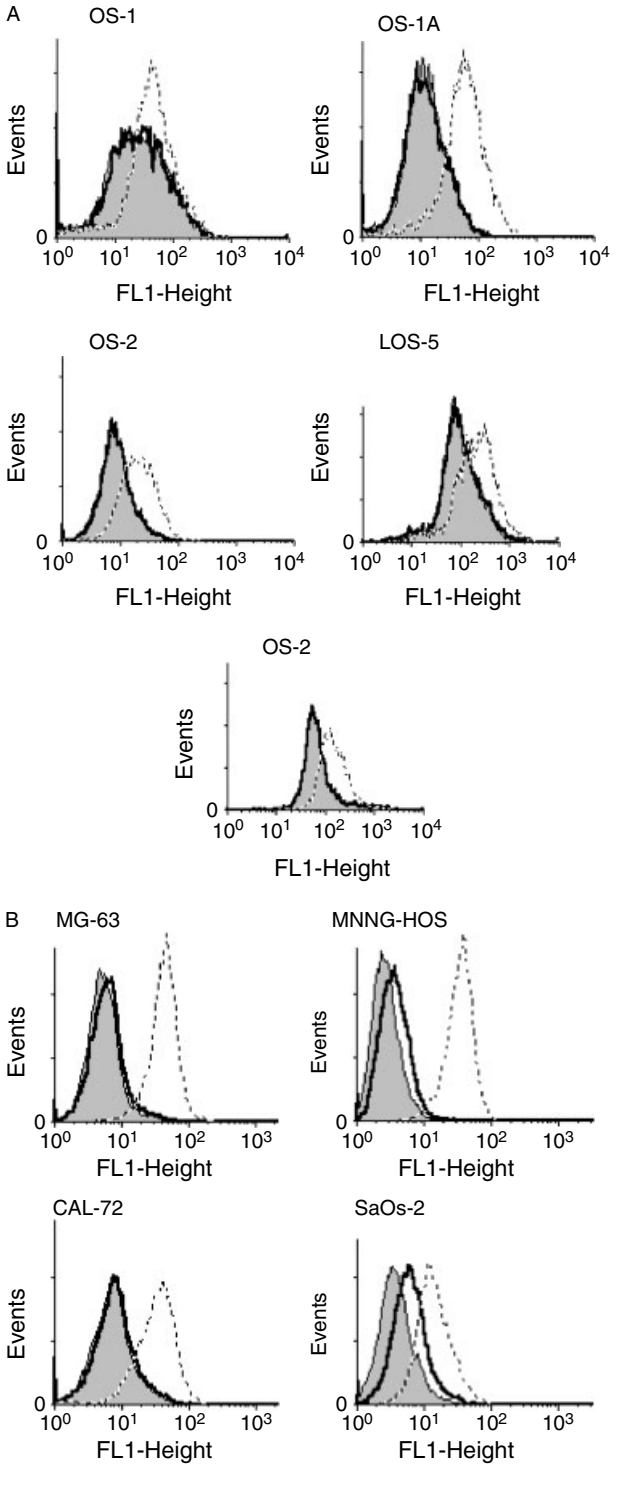

Figure 1. FACS analysis for CAR and EGFR expression on primary OS cells and OS cell lines. Samples of primary OS cells (A) or samples of OS cell lines (B) were subjected to FACS analysis. CAR fluorescence is depicted as a black line, EGFR fluorescence is depicted as dashed gray line and negative controls are depicted as solid gray field. Data shown are representative fluorescence histograms of one experiment (A) and three independent experiments (B)

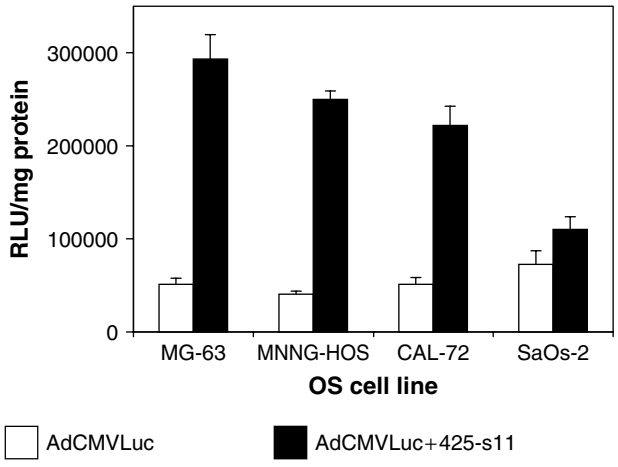

Figure 2. Targeting AdCMVluc towards EGFR on OS cell lines. OS cells were infected with AdCMVLuc at an MOI of 25 pfu/cell in the presence (black bars) or absence (white bars) of 425-s11. Data shown are mean luciferase activities (in relative light units per mg protein) + standard deviations. From a representative experiment performed in triplicate

To investigate if the enhanced gene expression by EGFr-targeted adenoviral vectors was the result of a few transduced cells exhibiting more abundant gene expression or from a greater number of transduced cells, we performed an experiment with AdCMVGFP. Twenty-four hours post-transduction, GFP expression was detected by FACS analysis. After EGFR-targeted transduction all cell lines showed a greater number of transduced cells as well as more abundant gene expression (Table 2).

Targeting adenoviral vectors towards EGFr on primary OS

While cell lines provide a good model for mechanistic studies of gene transfer, they have only limited resemblance to primary tumors. Therefore, we repeated the infection experiments on primary tumor material. Eight independent short-term cultures established from seven different patients were transduced in the presence or absence of 425-s11 with AdCMVLuc in a range of MOIs. Augmentation by 425-s11 targeting of the adenoviral vector is shown in Table 3.

Table 2. Targeting AdCMVGFP towards EGFR on OS cell lines

	Percentage GFP expressing cells		Relative median GFP expression*	
OS cell line	Control AdCMVGFP	Targeted AdCMVGFP	Control AdCMVGFP	Targeted AdCMVGFP
MG-63	0.5	19.3	1.0	1.5
MNNG-HOS	5.5	31.2	1.1	2.7
CAL-72	9.2	38.2	1.2	4.7
SaOs-2	5.0	24.0	1.2	2.2

*Relative median fluorescence intensity of total transduced cell population over control untransduced cells.

Table 3. Augmentation of AdCMVLuc mediated gene transfer by EGFR targeting on primary OS cells

Code	Age	M/F	Diagnosis	Targeting ratio*
OS-1	13	F	High grade OS	10.0
OS-1a			[post chemotherapy COSS 96 protocol]	6.3
OS-2	23	F	Juxtacortical osteosarcoma	2.0
OS-3	23	M	Moderately differentiated OS	1.8
OS-4	20	M	Chrondoblastic differentiated OS	5.0
LOS-5	9	M	High grade OS, lung metastasis	3.1
OS-6	7	F	High grade OS	4.3
LOS-7	15	F	High grade OS, lung metastasis	1.7

*Ratio of gene transfer efficiency with 425-s11 bispecific single-chain antibody over gene transfer efficiency without 425-s11 bispecific single-chain antibody.

All samples showed improved gene transfer when the adenoviral vector was targeted (mean 4.3-fold, range 1.7- to 10-fold).

Suicide gene therapy with AdCMVHSV-TK

To examine the therapeutic utility of EGFr-targeted adenoviral vectors for OS we performed suicide gene therapy experiments with targeted AdCMVHSV-TK. Targeting this adenoviral vector towards the EGFr is expected to increase transduction efficiency and thereby increase OS cell kill after adding ganciclovir (GCV) compared with the non-targeted vector.

Figure 3A shows the results on the cell line MG-63. Targeting of AdCMVHSV-TK indeed resulted in a more efficient cell kill. The IC50 decreased from 23 μM GCV for untargeted AdCMVHSV-TK to 2 μM GCV for EGFr-targeted AdCMVHSV-TK. A comparison of targeted and untargeted AdCMVHSV-TK with 9 μM GCV on four OS cell lines showed that all but SaOs-2 were killed significantly more effectively by the targeted vector (Figure 3B). More importantly, Figure 3C shows that primary OS tumor samples were also killed significantly more effectively by the targeted AdCMVHSV-TK. These observations are in line with transduction efficiencies determined with AdCMVLuc.

Discussion

The obstinate clinical course of OS despite aggressive standard therapy demands the investigation of new treatment approaches. In this regard, adenoviral vector mediated gene transfer of a suicide gene is a potential treatment modality for OS.

Adenoviral transduction relies on the expression of the adenovirus receptor CAR and α_v-integrins on the cell surface. However, CAR is the most important molecule in this regard since low or deficient CAR expression has been shown to predict inefficient adenoviral vector mediated gene transfer [16]. In this study we have demonstrated that CAR expression is low or even absent on primary

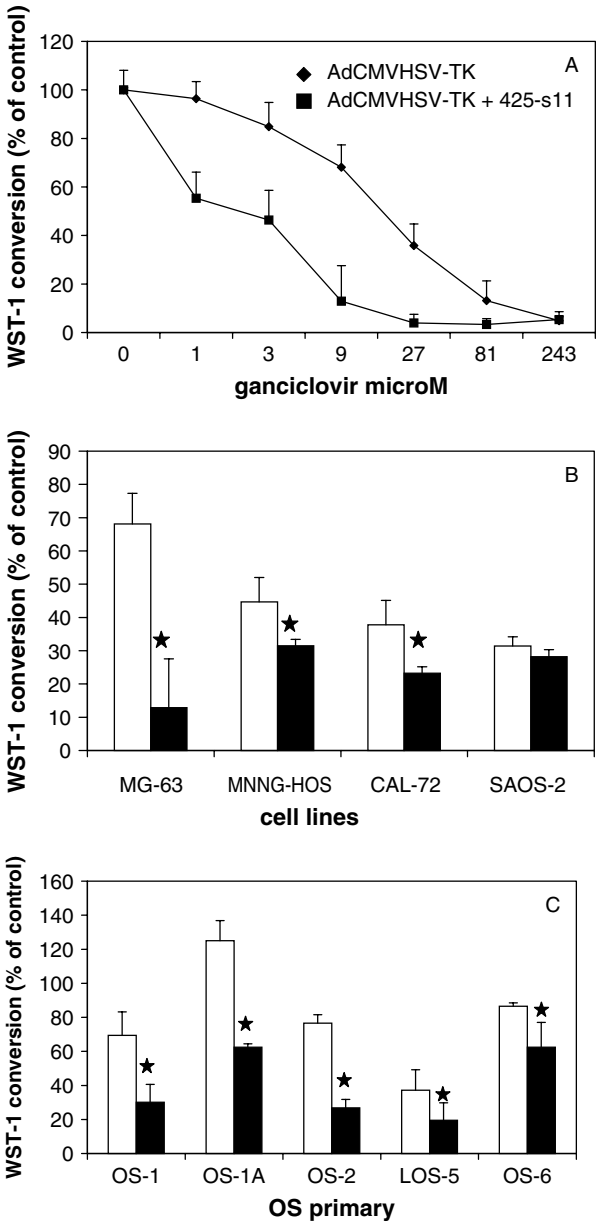

Figure 3. Suicide gene therapy with AdCMVHSV-TK on OS cell lines and primary OS. MG-63 cells (A), four OS cell lines (B) and five primary OS cultures (C) were transduced with AdCMVHSV-TK at an MOI of 100 pfu/cell in the presence (diamonds and black bars) or absence (squares and white bars) of 425-s11. Twenty-four hours post-infection a range of ganciclovir concentrations, 0–243 μM (A), 9 μM GCV, or 27 μM GCV (C), was added. Cell viability was determined by WST-1 conversion assay on day 7. Results are depicted as percentage viability of untreated control cells. Data shown are mean values + standard deviations of two independent experiments in triplicate (A) or a representative experiment in triplicate (B and C). *Represents a statistical difference with $p \leq 0.05$

human OS and OS-derived short-term cultures. FACS experiments on cells directly after digestion of the primary tumor showed the same CAR expression levels as short-term cultured cells (data not shown). These findings are in line with observations on other tumors, i.e., glioma [17], ovarian carcinoma [18] and rhabdomyosarcoma [19]. On established human OS cell lines, CAR expression was not always representative for primary OS; MG-63 and CAL-72 were CAR negative, while MNNG-HOS and SaOs-2 expressed CAR. Thus, similar to findings in other tumor types, CAR expression is very low in primary OS and expressed to various levels in OS-derived cell lines. Therefore, it may be expected that gene therapy for OS using adenoviral vectors with native tropism will have limited efficacy. Furthermore, relevant evaluation of adenoviral vector based gene therapy strategies requires the use of primary OS material.

The primary goal of this study is to enhance infection efficiency of OS. In the search for alternative target molecules on OS we investigated the expression of EGFR. EGFR was shown to be expressed widely on OS cell lines and primary OS. This is in agreement with the findings of Oda *et al*. [31]. The relative expression levels of CAR and EGFR, especially on primary OS, suggested that redirecting adenoviral vectors via EGFR would augment gene delivery into OS. Although EGFR is also expressed on some non-malignant cell types, the tumor/normal tissue expression ratio is expected to favor tumor infection [18,32]. Therefore, we evaluated the efficacy of EGFR-targeted adenoviral vector mediated gene transfer into OS. To this end, we employed recombinant bispecific single-chain Abs consisting of an anti-adenovirus-fiber knob scFv and an anti-EGFR scFv. These molecules have already been used to target adenoviral vectors towards EGFR on cell lines and primary gliomas and meningiomen [17,24,32]. The Adv targeting via EGFR follows a pathway distinct from the native Adv uptake [32]. We provide evidence here that this targeting approach can also be used for OS gene therapy.

The bispecific single-chain antibody significantly and reproducibly enhanced gene transfer into OS cells and primary tumor cell cultures. Moreover, EGFR targeting of an adenoviral vector expressing the HSV-TK suicide gene resulted in a significantly enhanced cell kill after GCV administration. Thus, EGFR targeting not only increased gene transfer efficiency, but also enhanced therapeutic efficiency on OS *in vitro*. Obviously, these observations need to be confirmed in *in vivo* models. In conclusion, we have shown that redirecting the adenoviral vector entry into OS cells via a CAR-independent pathway allows the inefficient gene delivery due to low CAR expression on primary OS to be overcome. EGFR-targeted Adv expression of HSV-TK may improve suicide gene therapy for OS.

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