Improved Gene Transfer into Renal Carcinoma Cells Using Adenovirus Vector Containing RGD Motif

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Abstract. *The transduction efficacy of adenovirus serotype 5 (Ad5) vector in human renal carcinoma cells is generally extremely low due to the non-expression of Coxsackie and adenoviral receptor (CAR). We investigated whether fiber-modified Ad vector containing an RGD motif in the HI loop of the Ad fiber knob could increase the transduction efficiency of Ad5 in human renal carcinoma cells in vitro. Materials and Methods: We examined both expressions of CAR, and α_v, β_3 and β_5 integrins, and the transduction efficacy of fiber-modified adenovirus vector in all cell lines. Results: The expression of CAR was lower and those of α_v and β_3 integrins were higher in all cell lines compared with control cell line, KK47. The transduction efficacy of fiber-modified Ad vector increased by 125- to 1,800-fold compared with Ad5. Conclusion: The fiber-modified Ad vector may be useful to establish effective new gene therapy strategies for the treatment of renal cell carcinoma.*

Adenovirus serotype 5 (Ad5) vectors have been widely used in both experimental and clinical gene therapy trials. Infection by Ad5 vector is mediated through Ad5 cell attachment and interaction. The Coxsakie and adenoviral receptor (CAR) binds to the Ad5 fiber knob (1) and uptake of Ad5 vector occurs following interaction of an Arg-Gly-Asp (RGD) motif located in the penton base with α_v-containing integrins, particularly $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ integrin (2).

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The expression of CAR in renal cell carcinoma tissues has been demonstrated to be significantly lower than in benign tissues and in most cancer types, particularly clear cell carcinoma, CAR is not expressed (3). Several researchers have demonstrated the overexpression of $\alpha_v\beta_3$ integrin in several types of cancer, including renal cell carcinoma (4, 5). Furthermore, a clinical trial using a monoclonal antibody against $\alpha_v\beta_3$ integrin indicated that this antibody may have effects on tumor perfusion and may exhibit clinical activity against renal cell cancer (6). In the case of gene therapy targeting renal carcinoma cells, which are generally associated with a low level of CAR expression and a high level of $\alpha_v\beta_3$ integrin expression, it is necessary for us to either increase the expression of CAR or to increase the transduction efficacy of Ad5 vector using CAR-independent tropism.

In order to overcome the low transduction efficacy of Ad5 in cells lacking CAR, several researchers have developed fiber-modified Ad vectors with broader tropism (7). Fiber-modified Ad vector containing the RGD motif in the HI loop of the Ad fiber knob (Ad-RGD vector) is capable of CAR-independent tropism in target cells expressing $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ integrin on the membrane (8).

In the present study, we examined the transduction efficacy of the Ad5 and Ad-RGD vectors in several human renal carcinoma cell lines.

Materials and Methods

Cell lines and cell culture. Established cell lines derived from human renal carcinoma cell lines, namely, ACHN and Caki-1, were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Japan); the RCC4-VHL human renal carcinoma cell line was purchased from the European Collection of Animal Cell Cultures (ECACC; Salisbury, UK); and the 786-O human renal carcinoma cell line was purchased from the American Type Culture

Table I. *Primers and probes*.

CAR	Primer Forward: 5'-CAGAAGCTACATCGGCAGTAATCA-3'
	Reverse: 5'-CTCTGAGGAGTGCGTTCAAAGTC-3'
	Probe: 5'-d FAM-TCCATGTCTCCTTCCAACATGGAAGGA-TAMRA-3'
α_v Integrin	Primer Forward: 5'-CAAGGTGAGCGGGACCAT-3'
	Reverse: 5'-TTGGCAGACAATCTTCAAGCA-3'
	Probe: 5'-d FAM-TCATCACTAAGCGGGATCTTGCCCTCA-BHQ-1- 3'
β_3 Integrin	Primer Forward: 5'-CCCTCGAAAACCCCTGCTAT-3'
	Reverse: 5'-TTAGCGTCAGCACGTGTTTGTAG-3'
	Probe: 5'-d FAM-TATGAAGACCACCTGCTTGCCCATGTTT-BHQ-1-3'
β_5 Integrin	Primer Forward: 5'-GGCTGGGACGTCATTCAGAT-3'
	Reverse: 5'-AGCTGGAAGGTGGTCTTGTCA-3'
	Probe: 5'-d FAM-ACACCACAGGAGATTGCCGTGAACCT-BHQ-1-3'
GAPDH	Primer Forward: 5'-GAAGGTGAAGGTCGGAGTC-3'
	Reverse: 5'-GAAGATGGTGATGGGATTTC-3'
	Probe: 5'-d FAM-CAAGCTTCCCGTTCTCAGCC-BHQ-1-3'

Collection (ATCC; Manassas, VA, USA). The KK47 human bladder cancer cell line was generously provided by Dr. Seiji Naito (Department of Urology, Kyushu University, Fukuoka, Japan).

In the present study, we maintained Caki-1, 786-O, and KK47 cells in Roswell Park Memorial Institute-1640 medium (Life Technologies, Inc., Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) and antibiotics (50 μg/ml streptomycin sulfate and 50 IU/ml of penicillin). RCC4-VHL cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Inc., Kyoto, Japan) containing 10% FBS, antibiotics (50 μg/ml streptomycin sulfate and 50 IU/ml of penicillin), 2 mM glutamine, and 0.5 g/l geneticin. 786-O cells were maintained in DMEM containing 10% FBS and antibiotics (50 μg/ml streptomycin sulfate and 50 IU/ml of penicillin). All cell lines were maintained at 37˚C in a humidified incubator with a 5% CO_2 atmosphere and 97% relative humidity and were subcultured on reaching 80% confluence using 1 mmol/l EDTA-0.025% trypsin for 3 to 5 min. The cells were transferred 2 or 3 times a week into fresh growth medium.

In vitro real-time quantitative reverse transcription-PCR assay. Total cellular RNA was isolated from all cell lines using a TaKaRa RNA extraction KIT (Takara Bio Inc., Shiga, Japan), and was reverse transcribed using a reverse transcription kit (TaKaRa RNA PCR Kit Ver. 3.0) following the manufacturer's protocol. The resulting cDNA was amplified with CAR, α_v integrin, β_3 integrin, β_5 integrin, and GAPDH sequence-specific primers (40 cycles: 95˚C for 15 s, 60˚C for 1 min) using TaqMan chemistry in the StepOnePlus Real-Time PCR System v2.0 (Applied Biosystems Japan Ltd., Tokyo, Japan). Table I shows the sequences of the TaqMan probes and primers for CAR, α_v integrin, β_3 integrin, β_5 integrin, and GAPDH. All primers/probes were purchased from Biosearch Technologies Japan (BTJ).

Adenovirus vector preparation. We examined the transduction efficacies of Ad5-CMV-LacZ, constructed as previously described (9), and Ad-RGD-LacZ, containing an RGD peptide in the HI loop of the fiber knob (8). The viruses were purified by double cesium chloride gradient ultracentrifugation using standard methods. Serial dilutions of the viruses were used to infect HEK 293 cells for a plaque assay. Titers of Ad vectors were assessed using the 50% tissue culture infectious dose method and expressed as plaque-forming units (pfu)/ml (Ad5-CMV-LacZ, 3.6×10^{11} pfu/ml; Ad-RGD-LacZ, 1.1×10^{11} pfu/ml).

Transduction efficacy of adenovirus vectors. In order to determine the transduction efficacy in each cell line, 2.5×10^4 cells were prepared in a 24-well plate and infected with Ad5-CMV-LacZ or Ad-RGD-LacZ. After 48 h, the transduction efficacy was assessed by β-galactosidase (β-gal) staining and expressed as blue titer units (btu)/ml.

Statistical analysis. Statistical significance was determined by using ANOVA and Bonferroni correction, with $p<0.05$ considered to be statistically significant.

Results

Relative quantification of mRNA expressions of CAR. The mean relative quantifications of *CAR* mRNA expression detected in the cell lines used in this study are shown in Figure 1a. In order to normalize for differences in the amount of total RNA, *GAPDH* was used as an endogenous RNA control. KK47 was selected as positive control. The relative quantification was calculated by dividing by the value obtained for Caki-1 cells. The levels of *CAR* mRNA expression were considerably higher in RCC4-VHL cells and moderately higher in ACHN and 786-O cells compared with those in Caki-1 cells, although the levels of *CAR* mRNA expression in all renal carcinoma cell lines were lower than in KK47.

Relative quantification of mRNA expressions of α_v, β_3 and β_5 integrins. The mean relative quantifications of α_v integrin, β_3 integrin and β_5 integrin mRNA expression detected in the cell lines used in this study are shown in Figure 1b-d. The relative quantification was calculated by dividing by the value obtained for Caki-1 cells. The levels of α_v integrin mRNA expression in Caki-1, ACHN and RCC4-VHL were about 2-fold compared with KK47, and the levels of β_5

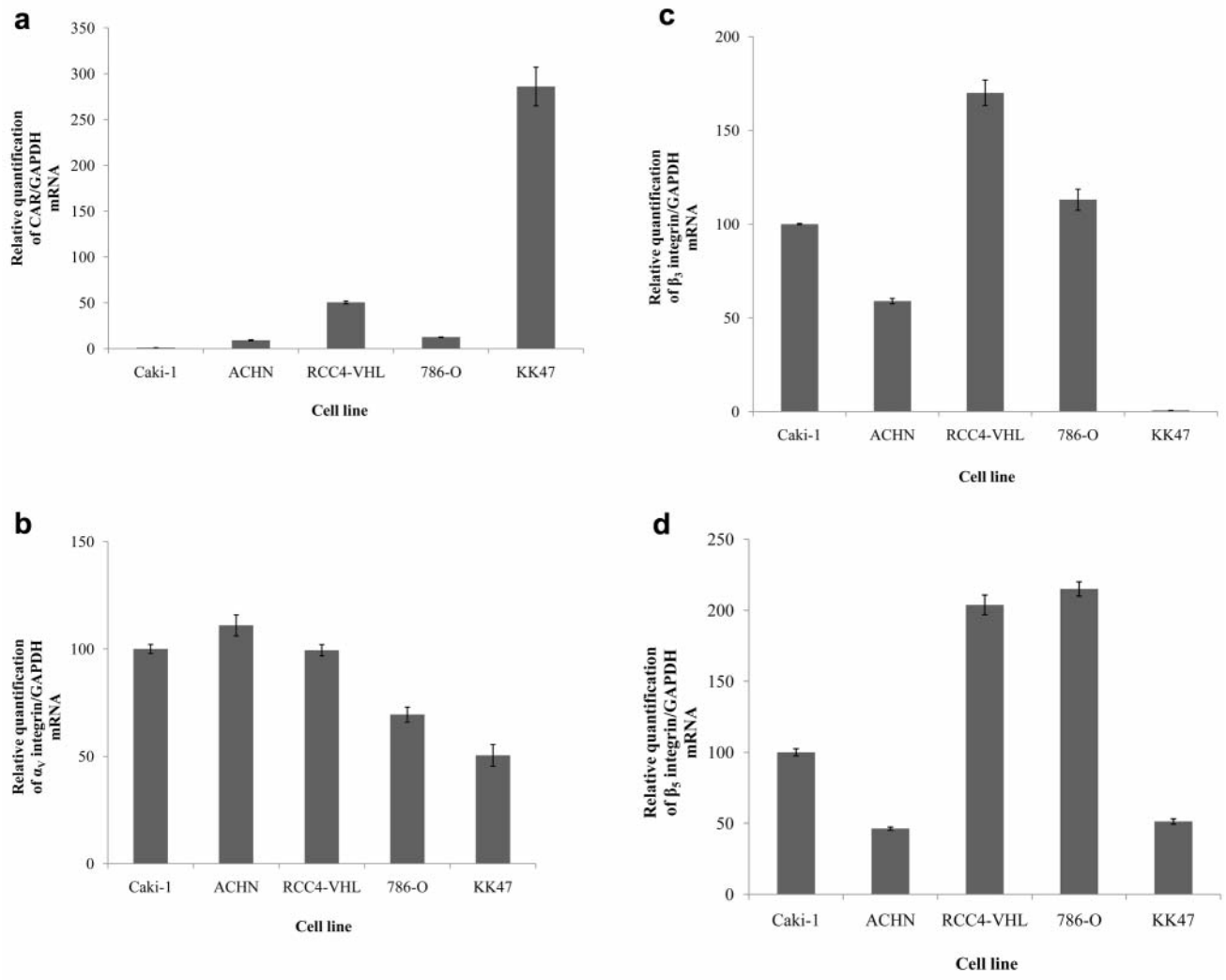

Figure 1. *Relative mRNA levels of CAR (a), α_v integrin (b), β_3 integrin (c) and β_5 integrin (d) by quantitative reverse transcription-PCR in human renal carcinoma cell lines. The relative expression level of Caki-1 was set to 1. GAPDH was selected as an endogenous RNA control to normalize for differences in the amount of total RNA. Values are means±SD.*

integrin mRNA expression were 2-, 4-, and 4.15-fold in Caki-1, RCC4-VHL, and 786-O cells, respectively, compared with KK47. The levels of β_3 integrin mRNA expression in all renal carcinoma cell lines were greatly higher than in KK47.

Transduction efficacy of adenovirus vectors. In order to assess transduction efficacy in all cell lines, cells were infected with both Ad5-CMV-LacZ and Ad-RGD-LacZ. The transduction efficacy for each of the cell lines was significantly increased by 500-, 1800-, 225-, and 125-fold in ACHN, Caki-1, RCC4-VHL, and 786-O cells, respectively, compared with Ad5-CMV-LacZ ($p<0.01$) (Figure 2).

Discussion

The clinical application of gene therapies for renal cell carcinoma have been undertaken with only 11 protocols (10, 11), whereas more than 950 protocols have been described for cancer in general (Gene Therapy Clinical Trials Worldwide, 2008 http://www.wiley.co.uk/genetherapy/clinical). Moreover, the therapeutic strategies for renal cell carcinoma have been almost exclusively immune gene therapies, and neither suicide nor oncolytic gene therapy using Ad5-related strategies, which have been shown to have favorable therapeutic efficacy and are thought to be promising gene therapies for other various kinds of cancer. One reason for such uncommon and

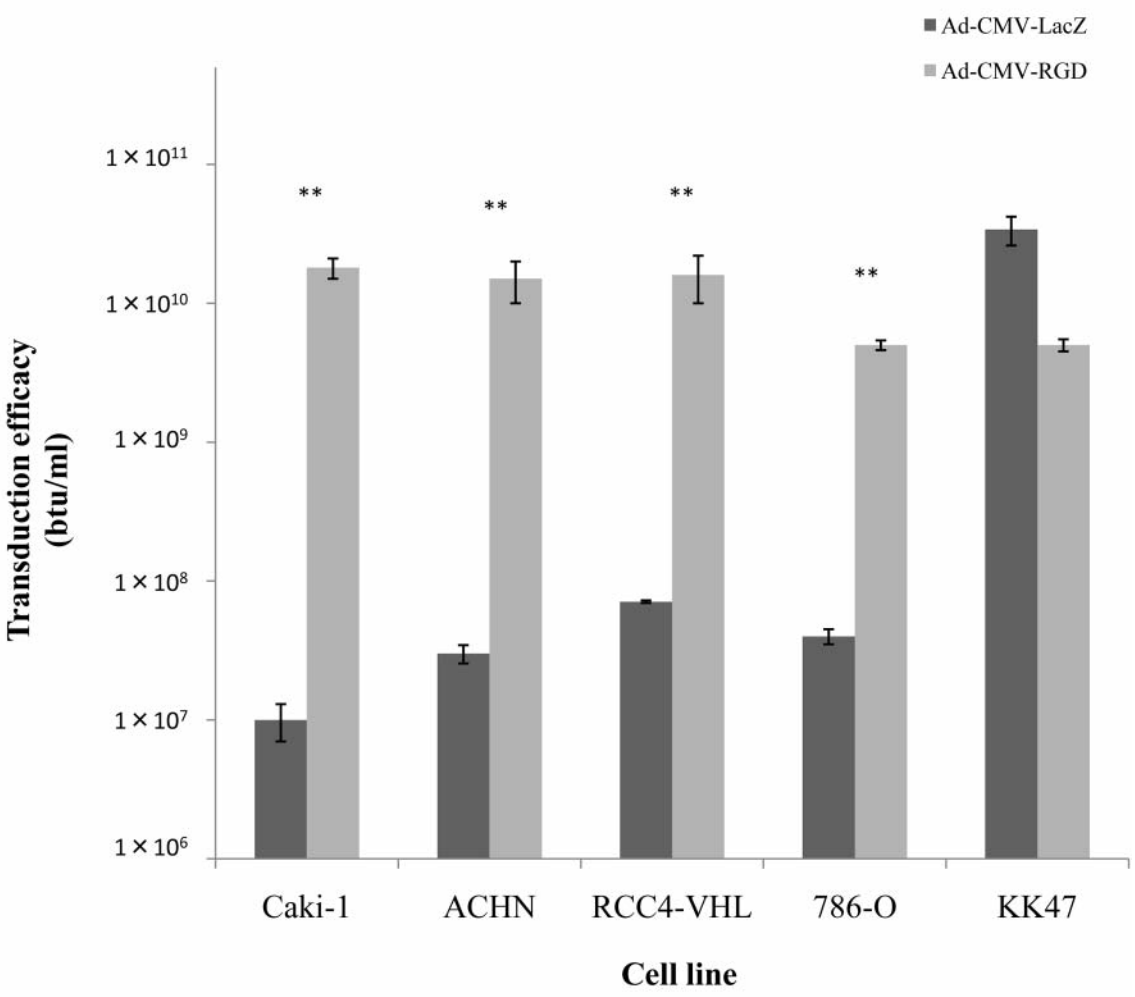

Figure 2. *The transduction efficacy of Ad5-CMV-LacZ and Ad-RGD-LacZ in human renal carcinoma cell lines. Values are means±SD. Double asterisks indicate a significant increase from the transduction efficacy of Ad5-CMV-LacZ ($p<0.01$).*

unfavorable development of gene therapy for renal cell carcinoma is the low transduction efficiency of Ad5 vector in renal cell carcinoma cells. In the present study, we attempted to increase the transduction efficiency of Ad vector in renal carcinoma cells.

Our present results reveal that the transduction efficiency in each of the renal carcinoma cell lines tested paralleled the relative quantifications of *CAR* mRNA expression. To increase the expression of CAR, we used Ad-RGD vector. This vector was expected to have high efficiency in renal carcinoma cells, since the overexpression of $\alpha_v\beta_3$ integrin in these cells is well documented (5). Indeed, our data also revealed that the transduction efficacy of Ad-RGD-LacZ was significantly increased compared with Ad5-CMV-LacZ. Consequently, it may be preferable to use Ad-RGD-LacZ to increase transduction efficacy in renal carcinoma cell lines. Moreover, these data are similar to transduction efficacy of Ad5-CMV-LacZ in KK47 cells. Previously, we demonstrated an antitumor effect in KK47 cells both *in vitro* and *in vivo* using an oncolytic Ad5 virus vector containing the *E1a* gene controlled by the tumor-specific midkine promoter (12). Therefore, our results also suggest that if we can construct an oncolytic Ad5 virus containing the *E1a* gene controlled by a renal cell carcinoma-specific promoter, which has a inserted RGD motif, it may be possible to achieve a higher antitumor effect in renal cell carcinoma compared with gene therapy using a conventional Ad5 vector.

Although the expression of *CAR* mRNA in the renal carcinoma cell lines tested in this study was variable, the transduction efficacy of Ad5 in all cells was lower compared with a cell line that is known to be associated with a high Ad5 transduction efficacy. In this study, we demonstrated a dramatic increase in transduction efficacy in renal carcinoma cells using an Ad vector containing the RGD motif on the HI

loop of the Ad fiber knob. Therefore, it may be preferable to use the fiber-modified Ad vector described in this study to target renal carcinoma cells, and by applying our findings it may be possible to establish effective new gene therapy strategies for the treatment of renal cell carcinoma.

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