Improved Gene Transfer into Bladder Cancer Cells Using Adenovirus Vector Containing RGD Motif

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Abstract. *Background: The transduction efficacy of adenovirus serotype 5 (Ad5) vector in high-grade human bladder cancer cells is generally extremely low due to the non-expression of coxsackie and adenoviral receptor (CAR). We investigated whether fiber-modified adenovirus vector containing an RGD motif in the HI loop of the adenovirus fiber knob could increase the transduction efficiency of Ad5 into human bladder cancer cells in vitro. Materials and Methods: We examined the expressions of CAR, and of α_v, β_3 and β_5 integrin, and the transduction efficacy of fiber-modified adenovirus vector in four human bladder cancer cell lines (TCC-SUP, 253J, T24 and KK47). Results: The expression of CAR was lower and those of α_v and β_3 integrin were higher in four human cancer cell lines compared with the control cell line, KK47. The transduction efficacy of fiber-modified adenovirus vector increased by 20- to 470-fold compared with Ad5. Conclusion: Fiber-modified adenovirus vector may be useful in order to establish new effective gene therapy strategies for the treatment of high-grade human bladder cancer.*

Adenoviruses are useful vectors for cancer gene delivery because of the high gene transfer efficacy, high titer production, and safety (1, 2). Recombinant adenovirus 5 (Ad5) has been widely used in gene transfer experiments and clinical gene therapy. Entry of Ad5 into the host cell is initiated by the knob domain of the fiber protein binding to the cell receptor, coxsackie and adenoviral receptor (CAR) (3). This is followed by a secondary interaction, where an Arg-Gly-Asp (RGD) motif in the penton base interacts with an α_v-containing integrin, particularly $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ integrin. Binding to α_v integrin results in endocytosis of the virus particle *via* clathrin-coated pits (4). However, the loss or decrease of CAR expression has been observed in various types of cancer, including bladder cancer (5-7). In an effort to target bladder cancer one needs to either increase the expression of CAR or to increase the transduction efficacy of Ad5 vector using CAR-independent tropism. Mizuguchi and Hayakawa have developed vectors with improved tropism by altering the fiber protein (8).

Fiber-modified adenovirus vector containing an RGD motif in the HI loop of the fiber knob (Ad5RGD vector) is capable of CAR-independent tropism in target cell expressing $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin on the membrane (9). We showed that the Ad5RGD vector is capable of inducing much higher transduction efficacy for human renal cell carcinoma cells than the Ad5 vector (10)

In the present study, we examined the transduction efficacy of the Ad5 and Ad5RGD vectors in several human bladder cancer cell lines.

Materials and Methods

Cell lines and cell culture. Established cell lines derived from human bladder carcinoma cell lines, namely, TCC-SUP, 253J and T24, were obtained from the American Type Culture Collection (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 TCC-SUP, 253J, T24 and KK47 cells in Roswell Park Memorial Institute-1640 medium (Life Technologies, Inc., Gaithersburg, MD, USA), containing 10% fetal bovine serum and antibiotics (50 μg/ml streptomycin sulfate and 50 IU/ml of penicillin). All cell lines were maintained at 37˚C in a humidified

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Key Words: Adenovirus vector, transduction efficacy, RGD, bladder cancer cell lines.

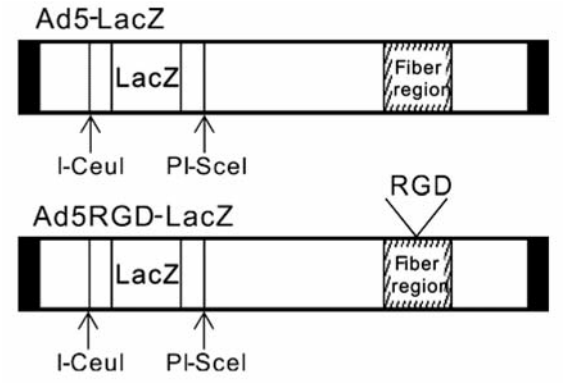

Figure 1. *Schematic representation of adenovirus. LacZ, β-galactosidase under CMV promoter; RGD, Arg-Gly-Asp motif; I-CeuI, I-CeuI restriction enzyme recognition sequence; PI-SceI, PI-SceI restriction enzyme recognition sequences.*

Table I. *Primer sequences used for PCR amplification.*

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

CAR: coxsackie and adenoviral receptor, GAPDH: Glyceraldehyde -3-phosphate dehydrogenase.

incubator with an atmosphere of 5% CO_2 and 97% relative humidity, and were subcultured on reaching 80% confluence using trypsin-EDTA. The cells were transferred two or three times a week into fresh growth medium.

Adenovirus vector preparation. We examined the transduction efficacy of Ad5-LacZ, constructed as previously described (11), and of Ad5RGD-LacZ, containing an RGD peptide in the HI loop of the fiber knob (9, 10) (Figure 1). The viruses were purified by double cesium chloride gradient ultracentrifugation using standard methods. Serial dilutions of the viruses were used to infect HEK 293 cell (RIKEN Bioresource Center, Tsukuba, Japan) for a plaque assay. Titers of adenovirus vectors were assessed using the 50% tissue culture infectious dose method and were expressed as plaque-forming units (pfu)/ml (Ad5-LacZ, 3.6×10^{11} pfu/ml; Ad5RGD-LacZ, 1.1×10^{11} pfu/ml).

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

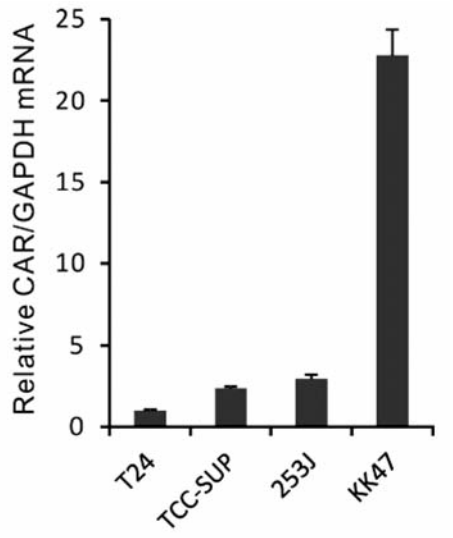

Figure 2. *Relative mRNA levels of coxsackie and adenoviral receptor (CAR) by quantitative reverse transcription-PCR in human bladder cancer cell lines. The relative expression level of T24 was set to 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as an endogenous RNA control to normalize for differences in the amount of total RNA. Values are means±SD (n=3).*

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 glyceraldehyde -3-phosphate dehydrogenase (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 and probes were purchased from Biosearch Technologies Japan (Tokyo, Japan).

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-LacZ or Ad5RGD-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 analysis of variance (ANOVA) and Bonferroni correction, with $p<0.01$ considered to be statistically significant.

Results

Relative quantification of mRNA expression of CAR. The mean relative quantification of *CAR* mRNA expression detected in the cell lines used in this study, is shown in Figure 2. In order to normalize for differences in the amount of total RNA, GAPDH was used as an endogenous RNA control. The relative quantification was calculated by

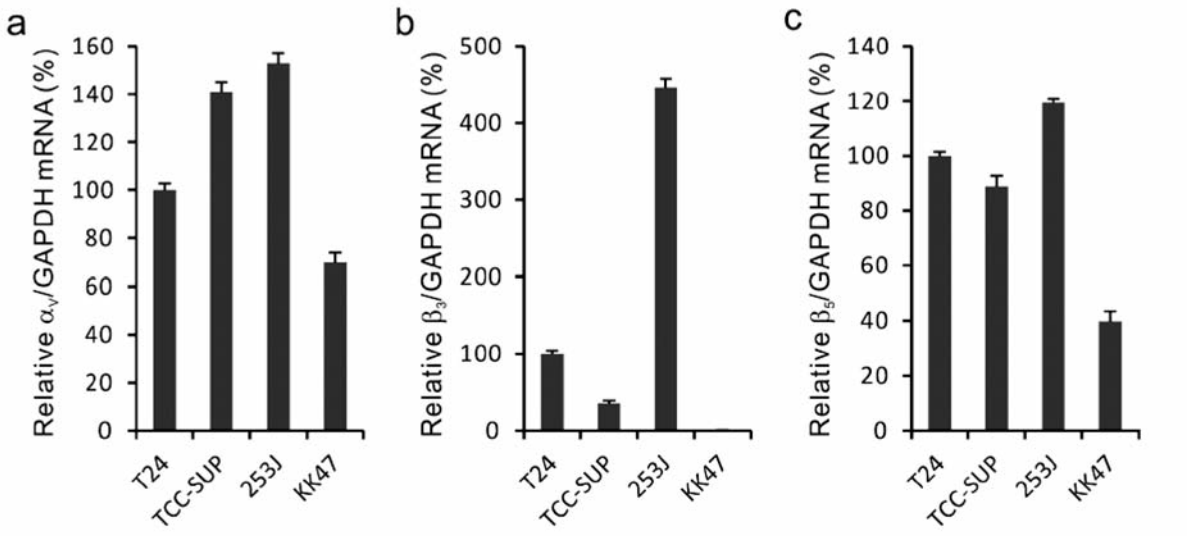

Figure 3. *Relative mRNA levels of α_v integrin (a), β_3 integrin (b) and β_5 integrin (c) obtained by quantitative reverse transcription-PCR in human bladder cancer cell lines. The relative expression level of T24 was set to 100. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as an endogenous RNA control to normalize for differences in the amount of total RNA. Values are means±SD (n=3).*

dividing by the value of RNA obtained for T24 cells. The levels of *CAR* mRNA expression were considerably higher in KK47 cells compared with the other cell lines.

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 3. The relative quantification was calculated by dividing by the value obtained for T24 cells. *α_v* and *β_5 integrin* mRNA were found to be uniformly expressed among the four cell lines. *β_3 Integrin* mRNA expression in 253J cells was found to be approximately four-fold higher than that of T24 and TCC-SUP cells. On the other hand KK47 was found to have minimal expression of *β_3 integrin* mRNA.

Transduction efficacy of adenovirus vectors. In order to assess the transduction efficacy in all cell lines, cells were infected with both Ad5-LacZ and Ad5RGD-LacZ. The transduction efficacy for each cell line was significantly increased by 470-, 20-, and 23-fold in T24, TCC-SUP, and 253J cells, respectively, using the RGD-bearing adenovirus, compared with the Ad5-LacZ ($p<0.01$) (Figure 4).

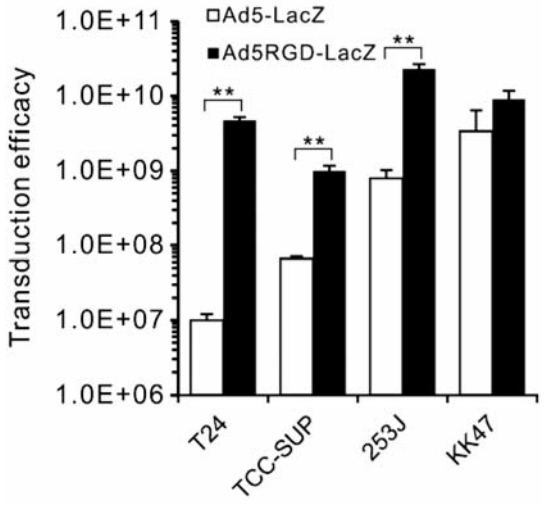

Figure 4. *The transduction efficacy of Ad5-LacZ and Ad5RGD-LacZ in human bladder cancer cell lines. Values are means±SD (n=3). Double asterisks indicate a significant increase compared to the transduction efficacy of Ad5-LacZ ($p<0.01$).*

Discussion

Ad5 vectors are one of the most studied vectors for gene therapy, as safety data for Ad5 have been excellent. However, the main disadvantage of the current therapies is that low transduction efficacy of Ad5 vectors limits the efficacy of treatment. Thus for successful cancer gene therapy, transduction efficacy of Ad5 vectors needs to be improved. In the present study, we attempted to increase the transduction efficacy of adenovirus vectors in bladder cancer cells.

Our present results revealed that the transduction efficiency in each of the bladder cancer cell lines tested, paralleled the relative quantification of *CAR* mRNA expression. To increase

transduction efficacy of adenovirus vectors, we tested the Ad5RGD vector. Indeed, our data revealed that the fiber-modified Ad5RGD vector achieved significantly higher transduction levels in all human bladder cancer cells as compared to the Ad5 vector itself. Oncolytic adenoviruses are being considered as a new therapeutic option for treatment of refractory disseminated cancer, including bladder cancer. Previously we demonstrated an antitumor effect in KK47 cells both *in vitro* and *in vivo* using an oncolytic Ad5 vector containing the *E1a* gene, controlled by the tumor-specific midkine promoter (12). However, the antitumor effect was considerably lower in T24 cells compared with KK47 cells *in vitro*. One reason for such a result for T24 cells is the low transduction efficiency of Ad5 vector into these cells because of their low CAR expression. Therefore, our results also suggest that if we can construct an oncolytic Ad5 virus containing the *E1a* gene controlled by midkine promoter, which has an inserted RGD motif, it may be possible to achieve a higher antitumor effect in bladder cancer cells with low CAR expression, including T24 cells, compared with gene therapy using a conventional Ad5 vector.

In this study, we demonstrated a dramatic increase in transduction efficacy in bladder cancer cells using an adenovirus vector containing the RGD motif on the HI loop of the fiber knob. Therefore, it may be preferable to use the fiber-modified adenovirus vector described in this study to target bladder cancer cells, and by applying our findings, it may be possible to establish new effective gene therapy strategies for the treatment of bladder cancer.

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