Fiber-substituted Conditionally Replicating Adenovirus for Oncolysis of Human Renal Carcinoma Cells

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Abstract. *Background: Adenovirus vectors have lately been highlighted in gene therapies. We investigated the oncolytic effects of a chimeric adenovirus type 5 (Ad5) with replacement of Ad5 fiber knob with adenovirus type 35 (Ad35) fiber knob (Ad5F35) on human renal cell carcinoma (RCC). Materials and Methods: The conditionally replicating Ad5F35 vector was constructed and infected into RCC cell lines 786-O, ACHN, and RCC4-VHL. For these cells, reverse transcription-polymerase chain reaction and western blotting were carried out and the cell viability was assayed. Results: In all RCC cell lines, it was found that CD46, a cell surface target of Ad35, was well-expressed, while coxsackie and adenovirus receptor (CAR), a cell surface target of Ad5, was considerably less expressed. The Ad5F35 vector induced oncolysis of RCC cells, with significantly higher efficacy as compared with that for the Ad5 vector. Conclusion: Ad5F35 vector could be a candidate for promising gene therapy of human RCC.*

Renal cell carcinoma (RCC) is relatively resistant to radiotherapy and chemotherapy. Immune-based therapies using high doses of interleukin-2 exhibit a beneficial effect for only 15-25% of patients with advanced RCC (1). The establishment of a new promising therapy for RCC is clearly of great importance. Adenoviral vectors have lately been highlighted for gene therapies (2). The safety of the first generation of adenovirus vectors for oncolysis has been confirmed in clinical trials (3). The major problem for gene therapies using adenovirus, however, is a limitation in the efficacy of gene transfer into solid tumor cells, *i.e.*, how many adenovirus-targeting receptors are expressed in the relevant tumors (3, 4). Adenovirus type 5 (Ad5) which has been widely used for gene therapy, is infected into cells through coxsackie and adenovirus receptor (CAR) (5), but CAR expression is generally low in cancer cells (6). CD46, alternatively, is a cell surface receptor for adenovirus type 35 (Ad35), that is more commonly expressed in cancer cells (7). We found that the replacement of Ad5 fiber knob with Ad35 fiber knob (Ad5F35) elevates the efficacy of gene transfer into RCC cells (8).

Conditionally replicating adenovirus (CRAD) vector, on the other hand, has been developed to reduce the side-effects of adenovirus therapy (9, 10). CRAD vector makes it possible to induce tumor-specific cell death and amplify oncolysis, as a result of intratumor replications (11). Moreover, CRAD can cause the oncolysis of cancer cells neighboring primarily infected cells through secondary infection and therein replications, to an extent greater than that for non-replicating adenoviruses (12-14).

Midkine is a heparin-binding growth factor that is induced by retinoic acid in embryonal carcinoma cells (15). Midkine is implicated in mitogenesis, angiogenesis, anti-apoptosis, fibrinolysis, and transformation (16-20). Midkine's expression is enhanced in a variety of cancer cells originating from the esophagus, stomach, colon, liver, breast, and pancreas, while its expression in non-malignant cells is quite limited, with moderate expression in the kidney and weak expression in the lung, colon, and thyroid gland (21-25). The midkine promoter, accordingly, could be utilized for expressing a suicide gene, such as the *E1* gene, or thymidine kinase.

To address this point, we constructed an Ad5F35 CRAD vector encoding the *E1* gene, under the control of a 0.6 kb midkine promoter, and examined its oncolytic effects on the human RCC cell lines 786-O, ACHN, and RCC4-VHL.

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Key Words: Adenovirus vector, oncolytic effect efficacy, CD46, renal cell carcinoma, 786-O, ACHN, RCC4-VHL cells.

Table I. *Primers used for reverse transcription-polymerase chain reaction.*

PCR primer	Oligonucleotide sequence
CAR	Sense: 5'CAGAAGCTACATCGGCAGTAATCA-3'
	Antisense: 5'CTCTGAGGAGTGCGTTCAAAGTC-3'
CD46	Sense: 5'GGTGTTGCTGCTGTACTCCTTCT-3'
	Antisense: 5'CCAATGAGCTCCATAGCTTCAA-3'
GAPDH	Sense: 5'GAAGGTGAAGGTCGGAGTC-3'
	Antisense: 5'GAAGATGGTGATGGGATTTC-3'

CAR: Coxsackie and adenovirus receptor; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

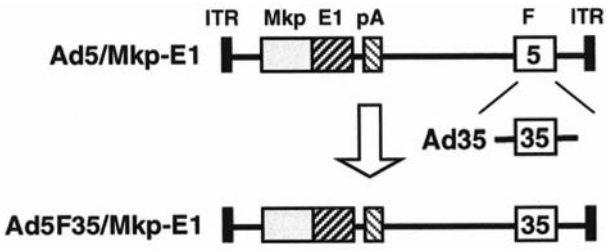

Figure 1. *A schematic structure for Ad5F35/Mkp-E1. ITR, Adenovirus inverted terminal repeat sequence; Mkp, midkine promoter; pA, polyadenylation signal; F5, the fiber knob of adenovirus type 5; F35, the fiber knob of adenovirus type 35.*

Materials and Methods

Construction of CRAD vectors. Replication on the competent adenovirus vector containing the *E1* gene, controlled by the midkine promoter was prepared as follows. The CMV promoter of pShuttle2 vector (Clontech Laboratories, Mountain View, CA, USA) was replaced with the 0.6 kb 5'-upstream regulatory region of the midkine gene and the pS-Mk/E1 vector was produced by inserting the *E1* gene downstream of the midkine region. To construct the pAd5F35, the Ad5 fiber region of the pAdeno-X vector (Clontech Laboratories) was substituted with the Ad35 fiber region of RHSP vector (Avior Therapeutics, Seattle, WA, USA). The Ad5/Mkp-E1 and Ad5F35/Mkp-E1 were prepared by linking the pS-Mk/E1 vector with the pAdeno-X and the pAd5F35, respectively (Figure 1).

Cell culture. Established cell lines derived from human RCC, namely, 786-O, ACHN, and RCC4-VHL, were used in the study. ACHN cells were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Sendai, Japan). 786-O cells were purchased from the American Type Culture Collection (Manassas, VA, USA), RCC4-VHL cells from the European Collection of Animal Cell Cultures (Salisbury, UK), and HEK293 cells from RIKEN Bioresource Center (Tsukuba, Japan). ACHN, RCC4-VHL and HEK293 cells were cultured in Dulbecco's modified Eagle's medium, and 786-O cells in RPMI 1640, each supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml), in a humidified atmosphere of 5% CO_2 and 95% air at 37˚C.

Reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was carried out using the primers shown in Table I.

Western blot analysis. Western blotting was carried out using antibodies against CAR (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD46 (Santa Cruz Biotechnology), and β-actin (GenScript, Piscataway, NJ, USA).

Oncolysis assay. Cells (1×10^4 cells/well) in 96-well plates were infected with Ad5/Mkp-E1 or Ad5F35/Mkp-E1 at 0.01-100 viral particles (VP)/cell. Five days after infection, cells were incubated with Alamar Blue (Life Technologies, Gaithersburg, MD, USA) and the number of viable cells were counted by detecting absorbance at 560 nm with a high-throughput microplate spectrophotometer (SpectraMax Plus 384; Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis. Statistical analysis was carried out using the Fisher's protected least significant difference (PLSD) test.

Results

Expression of CAR and CD46 in RCC cells. The cell attachment of Ad5 and Ad35 was targeted to CAR and CD46, respectively. We previously reported that the expression of *CAR* mRNA in RCC cell lines was much lower than that of the HEK293 cell line, but that the expression of *CD46* mRNA in RCC cell lines was at a similar level with that of the HEK293 cell line (8). HEK293 cells indeed expressed CAR mRNA and protein, however little expression of CAR mRNA and protein was found in any of the RCC cells used here (Figure 2A). The expression of CD46 mRNA and protein was detected in all RCC cells, as well as in HEK293 cells, and the CD46 expression in ACHN and RCC4-VHL cells was higher than that of HEK293 cells (Figure 2B).

Oncolytic effects of Ad5 and Ad5F3-5 on RCC cells. In order to assess cell cytotoxicity in all the cell lines, both Ad5/Mkp-E1 and Ad5F35/Mkp-E1 were utilized. For HEK293 cells, both Ad5/Mkp-E1 and Ad5F35/Mkp-E1 reduced cell viability in a VP-dependent manner, with the significantly higher efficacy for Ad5F35/Mkp-E1 than for Ad5/Mkp-E1 (Figure 3A). In contrast, only Ad5F35/Mkp-E1 reduced cell viability for all RCC cells examined here, in a VP-dependent manner, but otherwise little or no effect was obtained with Ad5/Mkp-E1.

Discussion

Gene therapy using adenovirus vectors is a promising therapeutic strategy for the treatment of various types of cancers. However, this approach has yet to be developed for

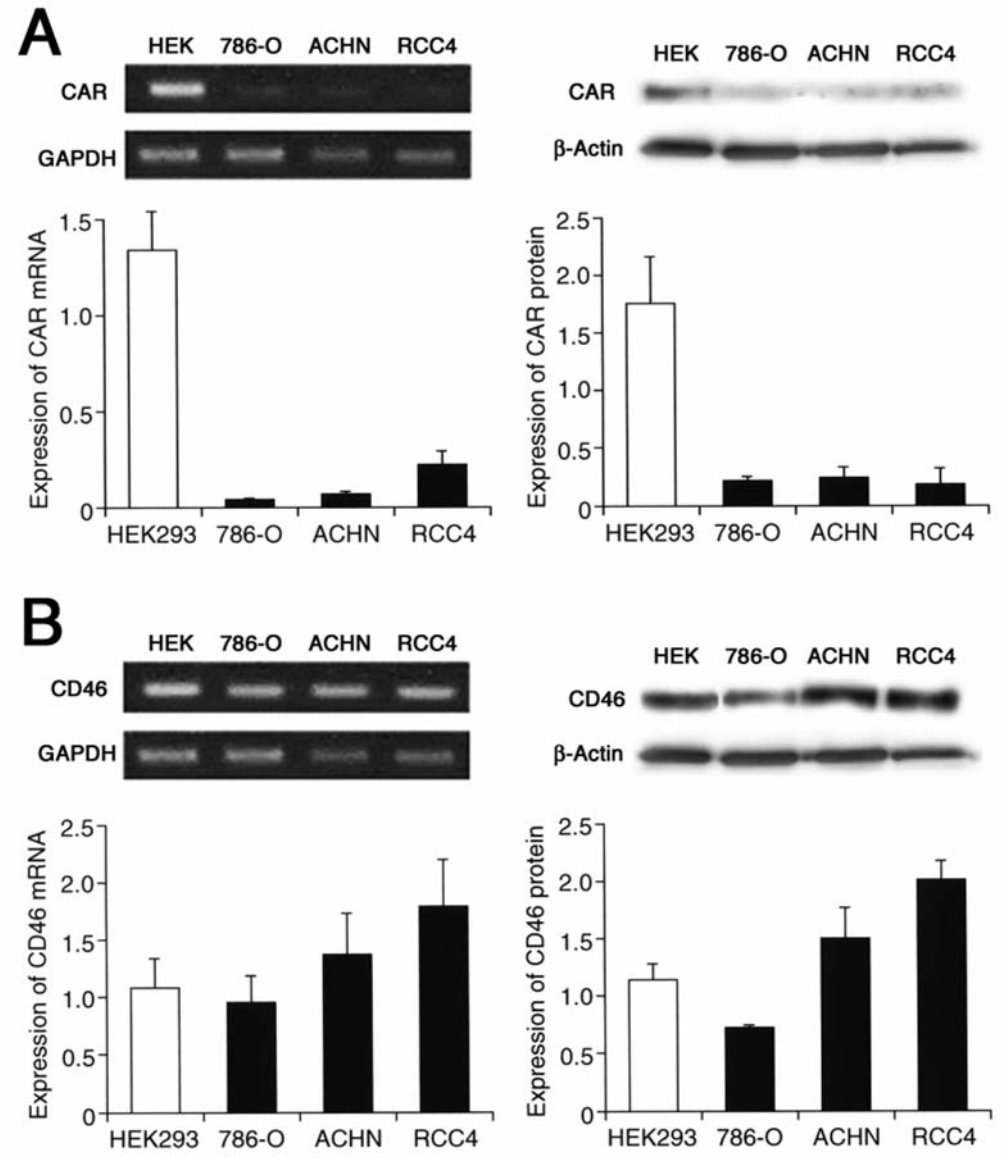

Figure 2. *Expression of the mRNA and protein for coxsackie and adenovirus receptor (CAR) and CD46. Reverse transcription-polymerase chain reaction (RT-PCR) and western blotting for CAR (A) and CD46 (B) were carried out in HEK293, 786-O, ACHN, and RCC4-VHL cells (RCC4). The expression level of each mRNA was normalized to that for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The expression level of each protein was normalized to that for β-actin. In the graphs, each column represents the mean (±SEM) expression of each mRNA and protein (n=3 independent experiments).*

use in cases of RCC, possibly due to the low transduction efficacy of adenovirus in RCC and the restriction of anti-tumor effects to RCC. In our earlier study, we showed that Ad5 containing the RGD motif (Ad5RGD) or the fiber knob of Ad35 is capable of much higher transduction efficacy for RCC cells than was Ad5, however the transduction efficacy of Ad5F35 for RCC cells was 8.3- to 20-fold higher than the transduction efficacy of the Ad5RGD vector (8, 26). In this study we, therefore, constructed a CRAD vector, encoding the *E1* gene under the control of midkine promoter, with the fiber knob of Ad35, Ad5F35/Mkp-E1 vector. Much lower expression of CAR was found in RCC cell lines 786-O, ACHN, and RCC4-VHL cells, as compared with the expression in HEK293 cells. This indicates that RCC cells are hardly infected with the Ad5 vector Ad5/Mkp-E1. In contrast, all RCC cells used here expressed CD46, to an extent similar to or higher than the expression in HEK293 cells. This means that RCC cells can be infected with the Ad35 vector. Amazingly, Ad5F35/Mkp-E1 induced RCC cell oncolysis in a VP-dependent manner.

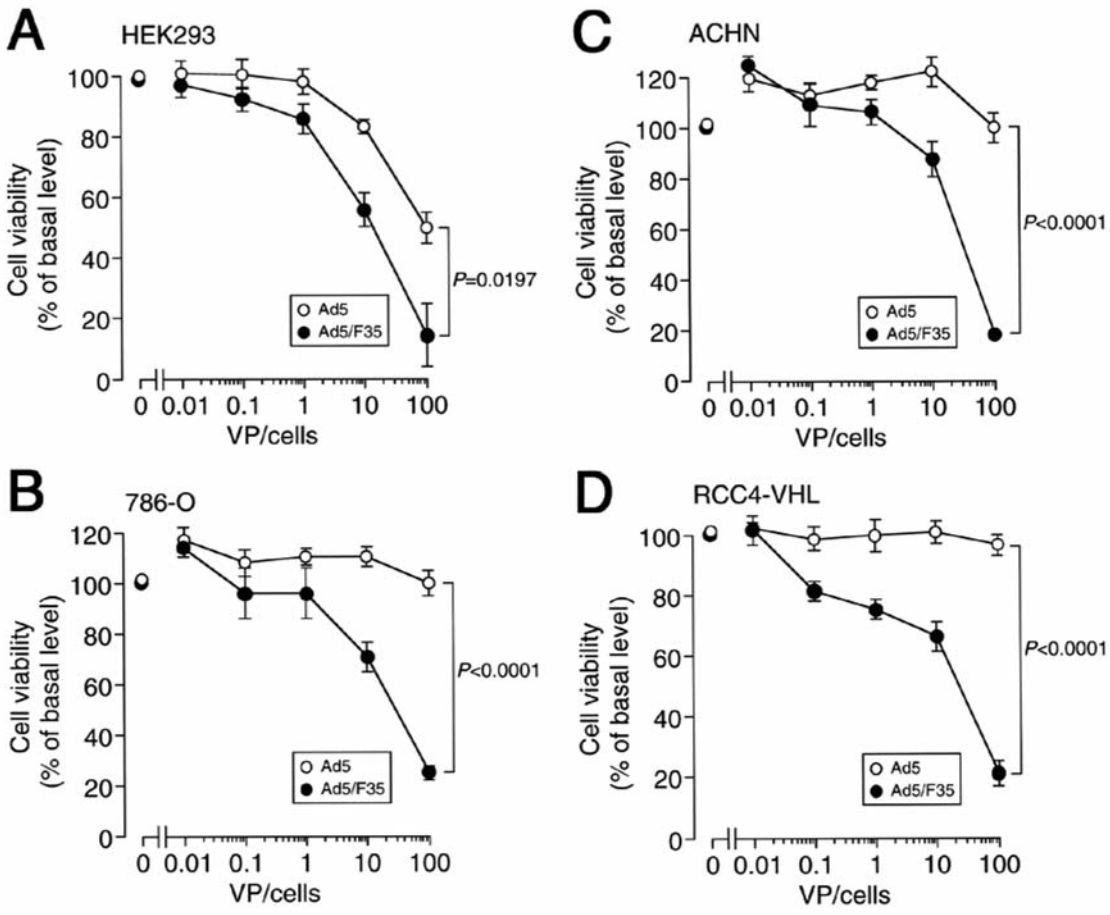

Figure 3. *Oncolytic effects of Ad5/Mkp-E1 and Ad5F35/Mkp-E1 on HEK293, 786-O, ACHN, and RCC4-VHL cells. Cell viability was assayed 5 days after infection using Alamar Blue. In the graphs, each point represents the mean (±SEM) value as a percentage of that of the basal level (viability of uninfected cells) (n=4 independent experiments). p-Values, Fisher's PLSD test.*

Expectedly, Ad5/Mkp-E1 had little or no effect on RCC cell oncolysis, while it induced HEK293 cell death. This suggests that the fiber knob in Ad35 allows Ad5F35/Mkp-E1 to invade RCC cells by recognizing CD46. This raises the possibility that Ad5F35/Mkp-E1 may be applicable to gene therapy of RCC.

In agreement with these studies, our findings suggest that in RCC, a conditionally replicating Ad5F35 vector appears to exert its beneficial anti-tumor action. The results of the present study show that a newly engineered Ad5F35/Mkp-E1 induces efficient oncolysis of RCC cells by targeting CD46.

Conflict of Interest

None of the Authors have any potential conflict of interest.

Acknowledgements

This study was supported in part by Grants-in-Aid for Scientific Research (23592354) from the Japan Society for the Promotion of Science. This work was supported by the research grant from Shinryokukai General Incorporated Association. The Authors wish to thank Satoko Kodama for her help in the completion of the manuscript.

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Received March 26, 2012
Revised May 12, 2012
Accepted May 14, 2012