High-Level Expression of the Coxsackievirus and Adenovirus Receptor Messenger RNA in Osteosarcoma, Ewing's Sarcoma, and Benign Neurogenic Tumors among Musculoskeletal Tumors

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ABSTRACT

Purpose: **The sensitivity of human tumor tissues to infection with recombinant adenoviruses correlates with the expression of the coxsackievirus and adenovirus receptor (CAR). CAR has been shown to function as the primary receptor for adenoviruses and to play a critical role in adenovirus entry into host cells. It is important for clinical gene therapy to determine the expression level of CAR in tumor tissues.**

Experimental Design: **We analyzed the expression of CAR mRNA in 154 musculoskeletal tumor tissues from 154 patients and 10 normal mesenchymal tissues from 3 patients using reverse transcription-PCR and real-time quantitative PCR. An adenovirus infection assay was performed in two cell lines that were established from CAR-positive osteosarcoma tissue and CAR-negative malignant fibrous histiocytoma tissue.**

Results: **Ninety-nine of 154 tumors were detected as CAR positive by reverse transcription-PCR. We found that the expression levels of CAR mRNA varied markedly between different tumors as determined by real-time quantitative PCR. CAR mRNA was expressed at high levels in osteosarcoma, Ewing's sarcoma, neurofibroma, and schwannoma; at intermediate levels in exostosis, giant cell tumor, liposarcoma, synovial sarcoma, malignant peripheral nerve sheath tumor, and hemangioma; and at low levels in alveolar soft part sarcoma and desmoid. Whereas the osteosarcoma cell line that expressed a high level of CAR mRNA, like its parent tumor, had a high efficiency of adenovirus infection, the malignant fibrous histiocytoma cell line with almost undetectable expression of CAR mRNA, like its parent tumor, had a low efficiency of infection.**

Conclusions: **Our data showed the great variations in CAR mRNA expression among human musculoskeletal tumors and mesenchymal tissues and implicated the potential usefulness of adenoviral vectors in gene therapy for osteosarcoma, Ewing's sarcoma, neurofibroma, and schwannoma. Efficient transduction with adenovirus for gene therapy could be realized in appropriate, sensitive tumor types.**

INTRODUCTION

Gene therapy has been considered as a possible new approach for the treatment of mesenchymal tumors (1, 2). The process of gene therapy is to replace a malfunctioning mutated gene with a normal wide-type gene or to express a therapeutic gene in target cells. Efficient gene delivery into target cells is essential for this strategy to be successful. In this regard, recombinant adenovirus vectors derived from subgroup C adenovirus are widely used in preclinical and clinical gene therapy (3, 4). They can provide efficient gene transduction in a wide spectrum of cancers. This is mainly due to their potential to infect a broad range of dividing and nondividing cells and because they can be produced in large quantities and at high titers (3, 5, 6). Adenovirus can infect cells because it uses the knob domain of the fiber to bind to its cellular receptor, the coxsackievirus and adenovirus receptor (CAR; Ref. 7). CAR is a 46-kDa transmembrane protein that is composed of two extracellular immunoglobulin-like domains (8). Although the accurate cellular function of CAR remains unclear, CAR has been shown to function as the primary receptor for both coxsackie B virus and adenovirus and is thus believed to play a critical role in adenovirus entry into host cells (9, 10). Recently, several groups have reported that the difference in expression levels of the CAR on target cells was highly correlated with cell sensitivity to adenoviral infection (9, 10). Tumor cells lacking or expressing low levels of CAR are resistant to adenovirus infection and to efficient oncolysis by recombinant adenovirus (11). It remains unclear whether an adenovirus vector is capable of efficient gene delivery to musculoskeletal tumors. Although

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CAR is expressed in a variety of human tissues (12), the expression levels of CAR and the efficiency of adenoviral gene transduction in musculoskeletal tumors have not been well investigated. Whereas cultured human rhabdomyosarcoma cells exhibited low or no expression of CAR, we recently reported that cultured human osteosarcoma cells frequently expressed a high level of CAR (1). Furthermore, patterns of expression of CAR in human musculoskeletal tumors including benign tumors have not been systematically investigated.

To study the feasibility of gene therapy in musculoskeletal tumors, we have used reverse transcription-PCR (RT-PCR) and real-time quantitative RT-PCR to detect the expression levels of CAR mRNA in human musculoskeletal tumors and normal mesenchymal tissues.

MATERIALS AND METHODS

Cell Lines. The HeLa, NOS10, and NMFH1 cell lines were used in this investigation. HeLa was obtained from RIKEN Cell Bank (Tsukuba, Japan) as a positive control. It is derived from cervical carcinoma tissue and known to express a high level of CAR. NOS10 and NMFH1 were established in our laboratory. NOS10 is derived from osteosarcoma tissue, and NMFH1 is derived from malignant fibrous histiocytoma tissue. HeLa cells were cultured in the α-modification of Eagle's MEM supplemented with 10% fetal bovine serum. NOS10 and NMFH1 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. Cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air at 100% humidity. Cells were fed every 2 days.

Tissue Samples. The study protocol was approved by the ethics committees of our institutions. Samples of musculoskeletal tumor tissues and pathologically unremarkable mesenchymal tissues were obtained from 154 patients (84 men and 70 women) receiving biopsy or surgical treatment. The mean patient age was 41.5 ± 20.3 years (range, 1–76 years). Each tissue specimen was divided into two pieces; one sample was immediately frozen in liquid nitrogen at the time of surgery and stored at −80°C until RNA extraction was performed. The other sample was processed for pathological examination. Because preliminary examination revealed that necrotic tumor decreased the expression of CAR, tumors that had >50% necrosis diagnosed microscopically by pathologists were excluded from this study. One bone tissue sample, one muscle tissue sample, one fat tissue sample, two nerve tissue samples, one artery tissue sample, one vein tissue sample, one skin tissue sample, and two cartilage-tissue samples from three patients were obtained with informed consent of the patients and used as normal soft tissue control.

RT-PCR. Total RNA was extracted from tissue samples by the guanidinium thiocyanate-phenol chloroform extraction method, as described previously (12). RNA (1.0 μg) was converted to cDNA by reverse transcription, using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Rockville, MD) and oligo$(dT)_{15}$ primers (Promega, Madison, WI). For RT-PCR, one-twentieth of the cDNA was used to amplify the entire coding region of the CAR gene. The primers used were as follows: CAR forward, 5′-AGGAGCGAGAGCCGCCTAC-3′; and CAR reverse, 5′-ACGGAGAGCACAGATGAGACA-3′. The length of the expected product was 1170 (α-transcript) or 908 bp (β-transcript). The PCR protocol consisted of initial denaturation for 5 min at 95°C; followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 62°C, and extension for 60 s at 72°C; and a final extension for 10 min at 72°C. A control PCR with primers that amplify a 983-bp fragment of the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene (G3PDH-F, 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′; G3PDH-R, 5′-CATGTGGGCCATGAGGTCCACCAC-3′) was performed in parallel to verify that similar amounts of cDNA were provided in each preparation. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide and photographed under UV light.

Real-Time Quantitative PCR. Total RNA was extracted as described above, and RNA (5.0 μg) was converted to cDNA by reverse transcription, using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) and random primers (Promega). PCR was conducted in capillary reaction tube cuvettes using 1 μl (10 pmol) of each of the forward and reverse primers [CAR forward; 5′-CTTCAGGTGCGAGATGTTACG-3′, CAR reverse; 5′-AGCCCACTCTGTTTCTGACTG-3′, glucose-6-phosphate dehydrogenase (G6PD) forward, 5′-GTACACCAAGATGATGACCAAGAA-3′; G6PD reverse 5′-CTCATACTGGAAACCCACTCTCTT-3′], 2 μg of LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany), 50 μM $MgCl_2$, 12 μl of distilled water, and 5 μl of cDNA samples. PCR was performed on a LightCycler V3 System (Roche Diagnostics).

The following PCR protocol was applied: 95°C for 10 min (dissociation of Taq antibody); and 40 cycles of 95°C for 0 s (denaturation), 62°C for 10 s (annealing), and 72°C for 13 s (extension). For construction of PCR product melting curves, an additional protocol (63°C for 10 s and 96°C for 0 s) was implemented after completion of the 40 cycles of PCR. The transition from 63°C to 96°C was effected in 0.2°C steps with continuous fluorescence monitoring, plotting loss of fluorescence *versus* temperature. Each DNA sample was quantified in triplicate in each of three separate PCR reactions.

Quantitative analysis of the LightCycler data was performed using LightCycler analysis software. Data analysis consisted of two parts: (*a*) specificity control of the amplification reaction using the melting curve program of the LightCycler software; followed by (*b*) application of the quantification program. The SYBR Green I signal of each sample was plotted *versus* the number of cycles. Background fluorescence was eliminated by setting a noise band via the LightCycler analysis software. This fluorescence threshold was applied to the determination of cycle numbers, which are inversely correlated with the log of the initial template concentration. To this end, the log linear regions of the amplification curves were identified, and best-fit lines were calculated. The crossing points are the intersections between the best-fit lines of the log-linear region and the noise band. These crossing points correlate inversely with the log of the initial template concentration (LightCycler Operator's Manual, Version 3.0, May 1999, Roche). The crossing points determined for CAR mRNA were normalized to those of G6PD to compensate for variability in RNA amounts and to exclude general transcriptional effects. The CAR mRNA level of HeLa cells was defined as 100% for the analysis of tissue

samples. The CAR mRNA levels in tumor tissues are presented in the figures as the calculated percentage of mRNA relative to the levels in HeLa cells (13). The Mann-Whitney *U* test was used to analyze differences in CAR expression between normal mesenchymal tissues and their histogenetic tumors. Statistical significance was accepted at $P < 0.05$.

Virus Constructs and Virus Preparations. LacZ inserted cosmid carrying an adenovirus type 5 genome lacking the E1A, E1B, and E3 regions was purchased from Takara Bio Inc. (Shiga, Japan) with Adenovirus Expression Vector Kit and used per the instructions of the manufacturer. In the cassette cosmid, the *Swa*I cloning site is flanked by the CAG promoter on the 5′ end and a rabbit globin poly(A) sequence on the 3′ end. Recombination in 293 cells between the homologous regions of the linearized transfer cosmid vector and the adenovirus genome resulted in the formation of the complete adenoviral recombinant, which contained LacZ cDNA. Before its use in experiments, the adenovirus was purified by sequential centrifugation in double CsCl step gradients as described previously (14–16). Titers of viral stocks were determined by a plaque assay on 293 cells. Viral suspensions were stored at −80°C. The virus was thawed on ice before use for infections.

Adenovirus Infection Assay. To assess adenovirus vector infection, NOS10 and NMFH1 cells were plated in 24-well plates at a density of 5×10^4 cells/well, respectively. After overnight incubation, the cells were infected with different titers [multiplicity of infection (MOI) = 0, 3, 10, 30, and 100] of Ad5-CAG-LacZ at 37°C in a 5% CO_2-humidified incubator. 5-Bromo-4-chloro-3-indolyl-ββ-D-galactopyranoside (X-Gal) staining was used to determine the infection efficiency of Ad5-CAG-LacZ in NOS10 and NMFH1 cells. After infection for 24 h, the cells were fixed with 0.25% glutaraldehyde solution at room temperature and then rinsed four times in PBS. A mixture of X-Gal staining solution (1 ml of 50 mM potassium ferricyanide, 1 ml of 50 mM potassium ferrocyanide, 2 μl of 1 M magnesium chloride, 0.25 ml of 40 mg/ml X-Gal solution, and 8 ml of PBS) was added to the cells and incubated at 37°C for 3 h. Assay of β-galactosidase (β-Gal) activity in cell lysates was also performed sequentially using the β-Gal enzyme assay system (Promega) according to the manufacturer's instructions. NOS10 and NMFH1 cells were plated in 6-well plates at a density of 2×10^5 cells/well and cultured overnight. The cells were infected with Ad5-CAG-LacZ at different titers (MOI = 0, 3, 10, 30, and 100). After 24 h, the cells were washed two times in PBS and lysed in reporter lysis buffer (Promega). Insoluble debris was pelleted, and the supernatant was stored at −70°C until analysis. Supernatant (100 μl) was diluted 1.5× with reporter lysis buffer, and then 150 μl of assay 2× buffer

Table 1 Expression of CAR[a] in 29 kinds of human mesenchymal tumors

Histological diagnosis	Total cases	CAR positive (*n*)	CAR negative (*n*)	% of CAR-positive cases
Malignant bone tumor				
Osteosarcoma	20	15	5	75
Chondrosarcoma	7	3	4	43
MFH of bone	2	1	1	50
Ewing's sarcoma	4	4	0	100
Chordoma	3	1	2	33
Benign bone tumor				
Exostosis	3	3	0	100
Enchondroma	2	2	0	100
GCT of bone	6	6	0	100
Chondroblastoma	2	2	0	100
Fibrous dysplasia	13	3	10	23
Malignant soft tissue tumor				
Fibrosarcoma	7	3	4	43
MFH	18	8	10	44
DFSP	1	1	0	100
Liposarcoma	11	7	4	64
Leiomyosarcoma	1	0	1	0
Rhabdomyosarcoma	2	0	2	0
Hemangiosarcoma	1	0	1	0
Synovial sarcoma	5	5	0	100
MPNST	5	5	0	100
ASPS	4	3	1	75
Epithelioid sarcoma	1	1	0	100
PMT	1	1	0	100
Pleomorphic sarcoma	1	1	0	100
Benign soft tissue tumor				
Fibroma	1	0	1	0
Lipoma	8	2	6	25
Hemangioma	4	4	0	100
Neurofibroma	3	2	1	67
Schwannoma	15	14	1	93
Desmoid	3	2	1	67
Total	154	99	55	64

[a] CAR, coxsackievirus and adenovirus receptor; MFH, malignant fibrous histiocytoma; GCT, giant cell tumor; DFSP, dermatofibrosarcoma protuberance; MPNST, malignant peripheral nerve sheath tumor; ASPS, alveolar soft part sarcoma; PMT, phosphatric mesenchymal tumor.

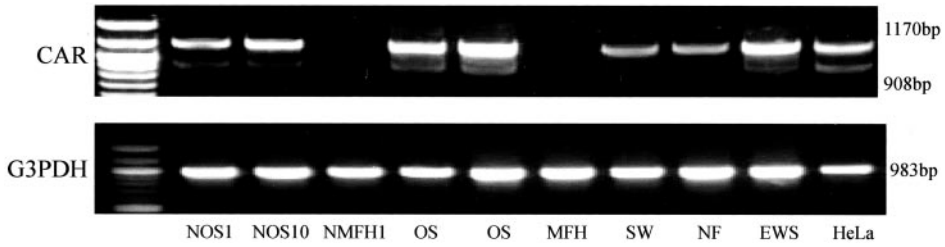

Fig. 1 Expression of coxsackievirus and adenovirus receptor in tumor tissues and cell lines (not all data are shown). The bands of β-transcript were relatively weak compared with those of α-transcript. NOS1 (derived from osteosarcoma), NOS10 (derived from osteosarcoma), and NMFH1 (derived from malignant fibrous histiocytoma) are cell lines established in our laboratory. Osteosarcoma (*OS*), malignant fibrous histiocytoma (*MFH*), schwannoma (*SW*), neurofibroma (*NF*), and Ewing's sarcoma (*EWS*) are tumor tissue samples.

(Promega) were added to the supernatant. After incubation at 37°C for 30 min, the reaction was stopped by adding 1 M sodium carbonate, and the absorbance at 420 nm was measured using the U-2000 spectrophotometer (Hitachi Co. Ltd.).

RESULTS

RT-PCR. We first examined the expression of CAR in tumor tissue samples by RT-PCR. CAR expression and histological types are shown in Table 1. In these tumors with >2 cases, high incidence of CAR expression was detected in osteosarcoma (15 of 20 cases, 75%), Ewing's sarcoma (4 of 4 cases, 100%), exostosis (3 of 3 cases, 100%), giant cell tumor (6 of 6 cases, 100%), liposarcoma (7 of 11 cases, 64%), synovial sarcoma (5 of 5 cases, 100%), malignant peripheral nerve sheath tumor (5 of 5 cases, 100%), alveolar soft part sarcoma (3 of 4 cases, 75%), hemangioma (4 of 4 cases, 100%), neurofibroma (2 of 3 cases, 67%), schwannoma (14 of 15 cases, 93%), and desmoid (2 of 3 cases, 67%). Low incidence of CAR expression was detected in chondrosarcoma (3 of 7 cases, 43%), chordoma (1 of 3 cases, 33%), fibrous dysplasia (3 of 13 cases, 23%), fibrosarcoma (3 of 7 cases, 43%), malignant fibrous histiocytoma (8 of 18 cases, 44%), and lipoma (2 of 8 cases, 25%). There were fewer than three patients with malignant fibrous histiocytoma of bone, enchondroma, chondroblastoma, dermatofibrosarcoma protuberance, leiomyosarcoma, rhabdomyosarcoma, hemangiosarcoma, epithelioid sarcoma, phosphatric mesenchymal tumor, pleomorphic sarcoma, and fibroma. CAR expression in parent tumors of NOS10 and NMFH1 was also examined by RT-PCR; parent tumor of NOS10 was positive, and parent tumor of NMFH1 was negative.

Thoelen *et al.* (17) described the existence of a splice variant (β-transcript) of CAR gene in addition to the α-transcript. Thus, we examined whether splice variant was present in human mesenchymal tumor tissues used in our study by RT-PCR (Fig. 1). We found that 100% (99 of 99) of CAR-positive tissue samples showed the α-transcript (1170 bp), and 58% (57 of 99) showed the β-transcript (908 bp; Table 2). The α-transcript was dominant, and the band intensity of the splice variant was remarkably low compared with the intensity of the α-transcript in almost all of the CAR-positive tumor tissues.

Real-Time Quantitative PCR. To analyze CAR expression quantitatively, quantitative real-time PCR was used to determine the expression level of CAR mRNA in all CAR-positive tissue samples. CAR mRNA levels were normalized to G6PD expression level, and the results were expressed as the percentage of mRNA, where the CAR mRNA level in HeLa cells was defined as 100%. We compared the CAR mRNA levels in mesenchymal tumor tissues and normal mesenchymal tissues with the level in HeLa cells. A great variation of CAR mRNA levels in tumors was observed by quantitative real-time PCR. We defined the mean level of CAR expression as follows: high, >200%; intermediate, between 50% and 200%; and low, <50%. We found that CAR mRNA was expressed at high levels in osteosarcoma, Ewing's sarcoma, neurofibroma, and schwannoma; at intermediate levels in exostosis, giant cell tumor, liposarcoma, synovial sarcoma, malignant peripheral nerve sheath tumor, and hemangioma; and at low levels in alveolar soft part sarcoma and desmoid (Fig. 2, *A* and *B*). For normal mesenchymal tissues, intermediate levels of CAR expression were detected in fat tissue and skin tissue, and low or undetectable levels were found in bone, muscle, nerve, artery, vein, and cartilage tissues (Fig. 2*C*). Although the mean value of CAR mRNA level was nearly 8–22-fold greater in histogenetic tumor tissues than in their original tissues (with the exception of skin and fat tissues), statistical significance was not shown, except for schwannoma (Table 3).

The NOS10 cell line showed high-level CAR expression (414% of control), like its parent tumor (324% of control); however, the NMFH1 cell line showed an almost undetectable

Table 2 Expression of CAR[a] in human mesenchymal tumors and expression of splice variants in CAR-positive samples

	CAR-positive cases			
Total cases	Total	α-Transcript (%)	β-Transcript (%)	CAR-negative cases
154	99	99 (100)	57 (58)	55

[a] CAR, coxsackievirus and adenovirus receptor.

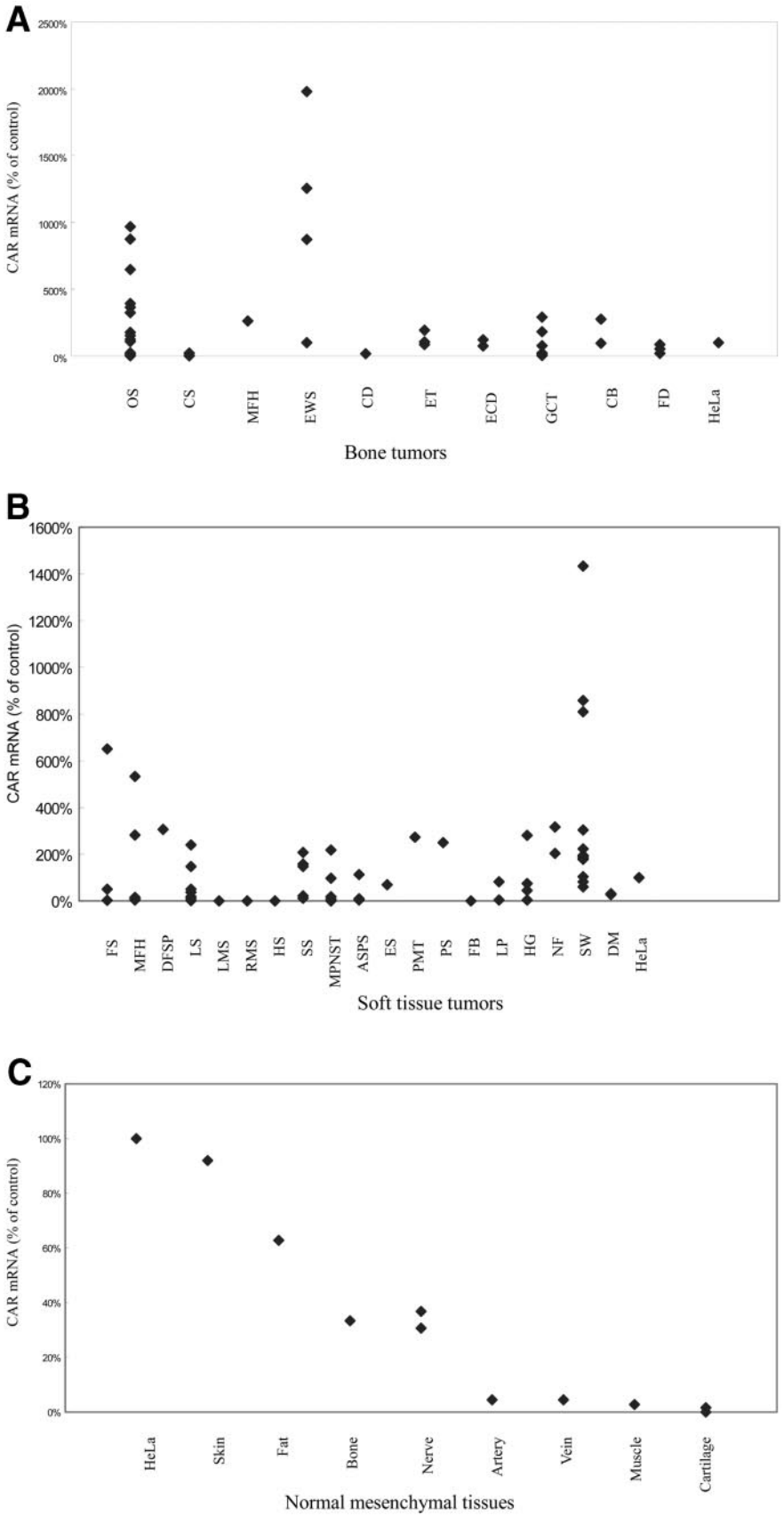

Fig. 2 Coxsackievirus and adenovirus receptor (CAR) mRNA quantification in bone tumors (*A*), soft tissue tumors (*B*), and normal mesenchymal tissues (*C*) was conducted using the LightCycler system. CAR expression level in HeLa cells was set at 100% for each tumor tissue sample. Calculation of CAR mRNA expression levels was performed using the formula presented in "Materials and Methods" (which involves glucose-6-phosphate dehydrogenase mRNA expression as an internal standard). *OS*, osteosarcoma; *CS*, chondrosarcoma; *MFH*, malignant fibrous histiocytoma; *EWS*, Ewing's sarcoma; *ET*, exostosis; *GCT*, giant cell tumor; *LS*, liposarcoma; *SS*, synovial sarcoma; *MPNST*, malignant peripheral nerve sheath tumor; *ASPS*, alveolar soft part sarcoma; *HG*, hemangioma; *NF*, neurofibroma; *SW*, schwannoma; *DM*, desmoid; DFSP, dermatofibrosarcoma protuberance; CD, chordoma; ECD, enchandroma; CB, chondroblastoma; FD, fibrous dysplasia; FS, fibrosarcoma; LMS, leiomyosarcoma; RMS, rhabdosarcoma; HS, hemangiosarcoma; ES, epithelial sarcoma; PMT, phosphaturic mesenchymal tumor; PS, pleomorphic sarcoma; FB, fibroma; LP, lipoma.

Table 3 Statistical comparison of CAR[a] expression between normal tissues and histogenetic tumors
The mean value of each sample, which was obtained by three separate PCR reactions, was used to perform statistical anaylsis.

Normal mesenchymal tissue (mean)	Histogenetic tumor (mean)	*P*
Fat (0.539; $n = 1$)	Lipoma (0.429; $n = 2$)	>0.999
Fat (0.539; $n = 1$)	Liposarcoma (0.718; $n = 7$)	0.558
Bone (0.318; $n = 1$)	Osteosarcoma (2.803; $n = 15$)	0.456
Nerve (0.337; $n = 2$)	Schwannoma (3.571; $n = 14$)	0.026
Nerve (0.337; $n = 2$)	Neurofibroma (2.605; $n = 2$)	0.121
Blood vessel (0.0451; $n = 2$)	Hemangioma (1.007; $n = 4$)	0.348
Cartilage (0.0081; $n = 2$)	Chondrosarcoma (0.098; $n = 3$)	0.083
Cartilage (0.0081; $n = 2$)	Enchondroma (0.989; $n = 2$)	0.121
Cartilage (0.0081; $n = 2$)	Exostosis (1.287; $n = 3$)	0.083

[a] CAR, coxsackievirus and adenovirus receptor.

level of CAR expression (3.6% of control), like its parent tumor (4.1% of control).

Adenovirus Infection Assay. The efficiency of adenovirus gene transfer into NOS10 and NMFH1 cells was assessed by X-Gal staining and β-Gal activity assay. In X-Gal staining, positive cells expressing β-Gal were stained blue, strongly positive blue-stained cells were visible in NOS10 at MOI = 30 and MOI = 100, light blue cells were seen in NOS10 at MOI = 10 and NMFH1 at MOI = 100. Similar to this result, the assay of β-Gal activity in cell lysates also showed that β-Gal activity was nearly 8-fold greater in NOS10 cells than in NMFH1 cells (Fig. 3).

DISCUSSION

The CAR mediates viral attachment and infection, but its physiological functions have not been well described. Recently, some studies (18, 19) showed that CAR is a component of the tight junction and of the functional barrier to paracelluar solute movement. Sequestration of CAR in tight junctions may limit virus infection. Adenovirus infection of susceptible cells required two distinct steps, binding and internalization. The adenovirus fiber protein knob, which projects outward from the virion vertices, binds CAR as the first step in viral infection. Adenovirus internalization is mediated by binding of the fiber penton base to cellular $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (20). Recent evidence has demonstrated that there was a good correlation between sensitivity to adenovirus infection and CAR expression (1, 21), whereas the sensitivity did not correlate with the expression of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (21–23). The critical determinant for susceptibility to adenovirus infection is the expression of CAR, the primary receptor for adenovirus. Cripe *et al.* (24) reported that rhabdomyosarcoma cell lines that exhibited low or absent expression of CAR were resistant to infection and oncolysis by adenovirus, although they expressed the adenovirus internalization receptors, α_v integrins. Therefore, an important first step in the development of an adenovirus-mediated treatment strategy for human mesenchymal tumors is to determine CAR expression.

CAR expression has been detected in a variety of human tissues, such as heart, brain, lung, liver, kidney, pancreas, small intestine, and prostate (18, 25, 26). However, CAR expression in human musculoskeletal tumor tissues has not been systematically investigated.

Osteosarcoma belongs to malignant bone tumor and was considered as an osteogenic tumor. Our previous study (1) showed that a high level of CAR expression had been observed in most osteosarcoma cell lines. Ito *et al.* (27) reported that immature osteoblasts in a rib fracture model had expressed a high level of CAR. These results implied that CAR mRNA is expressed in osteogenic cells. In our present study, CAR expression was found in 15 of 20 (75%) osteosarcoma tumor tissues, and 11 of these 15 tissues showed high levels of CAR mRNA.

Ewing's sarcoma is a malignant tumor of neuroectodermal origin but limited neural differentiation (28). All Ewing's sarcoma tumor tissues tested expressed a high level of CAR, with the exception of one case expressing an intermediate level of CAR. This was consistent with the report of Rice *et al.* (2), in which high levels of CAR expression were identified in Ewing's sarcoma cell lines and primary tumor tissues. Hotta *et al.* (29) demonstrated that mouse CAR was expressed in immature neuroepithelium, which could be why the primitive neuroectodermal tumor/Ewing's sarcoma expressed a high level of CAR.

Neurofibromas and schwannomas are benign peripheral nerve sheath tumors and have major clinical impact on the neurofibromatosis 1 and neurofibromatosis 2. Because multiple

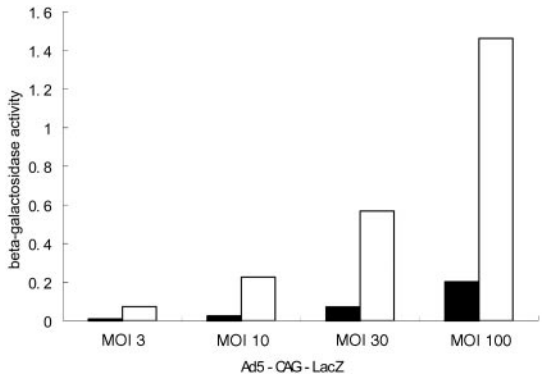

Fig. 3 The infection efficiency of Ad-CAG-LacZ in NOS10 and NMFH1 cells. NOS10 and NMFH1 cells were seeded at 2×10^5 cells/well in 6-well plates and infected with Ad-CAG-LacZ at different titers (multiplicity of infection = 0, 3, 10, 30, and 100). β-Galactosidase activity in cell lysates was detected by U-2000 spectrophotometer, and the expression level of β-galactosidase activity at multiplicity of infection = 0 served as an internal standard. ■, NMFH1; □, NOS10.

isolated lesions are characteristic of neurofibromatosis 1 and neurofibromatosis 2, it was difficult to remove these tumors thoroughly. Thus, gene therapy has been considered as a possible new approach for the treatment of these tumors. Several studies (30, 31) showed that recombinant adenovirus can infect and introduce genes into Schwann cells in intact and injured nerve *in vivo*. In the present study, two of three neurofibroma samples were CAR positive and expressed a high level of CAR. All schwannoma samples except one case were CAR positive and expressed relatively high levels of CAR mRNA. CAR mRNA was expressed at a high level in 5 of 15 schwannomas and at an intermediate level in 9 of 15 schwannomas. Because neurofibroma and schwannoma originated from Schwann cells, these results indicate that CAR expression may be concerned with tumors of Schwann cell origin. We made the interesting observation that all of malignant peripheral nerve sheath tumors that also originated from Schwann cells were CAR positive, but the mean expression levels were 5-fold higher in benign schwannoma compared with malignant peripheral nerve sheath tumor.

In all normal mesenchymal tissues, with the exception of skin and fat tissues, low or undetectable levels of CAR expression were detected. These results indicated that if therapeutic adenoviral vector is injected into a patient for gene therapy purposes, the effect on the normal tissue, such as neural, bone and veins, could be minimal because the expression level of CAR is lower for normal tissues. Clinical trials with adenovirus also suggested that adenovirus vectors can be safely administered intratumorally, intra-arterially, and i.v. (32) and have shown a safe clinical profile (33). Thus, satisfactory and safe therapeutic efficacy could be achieved for adenovirus-mediated gene therapy in human mesenchymal tumors. Although there was no statistical significance in the difference in mean CAR mRNA level between most normal tissues and their histogenetic tumors (there may not be a sufficient number of normal tissue samples), the mean CAR mRNA level was nearly 8–22-fold greater in histogenetic tumor tissues than in their original tissues, with the exception of skin and fat tissues.

The presence of alternatively spliced variants of CAR was analyzed by RT-PCR. In all of the CAR-positive samples, α-transcript was observed; β-transcripts were only shown in 58% of CAR-positive samples. The expression of α-transcript in CAR-positive samples was dominant; β-transcript might be not a major variant in musculoskeletal tumors. Although Thoelen *et al.* (17) proposed that the β-transcript might exert an important function in the regulation of CAR, we could not find a correlation between the expression level of CAR and the presence of β-transcript.

The expression of CAR has been shown to correlate with adenovirus-mediated gene transfer and oncolysis for a variety of cancers (13, 24, 34, 35). To confirm the correlation between the expression level of CAR and the infectiousness of adenoviral vector, Ad-CAG-LacZ-infected NOS10 and NMFH1 cells were examined by β-Gal activity assay in cell lysates. We found that β-Gal activity was nearly 8-fold higher in NOS10 cells than in NMFH1 cells. Because NOS10 and NMFH1 cells expressed different levels of CAR mRNA, these results showed that the expression level of CAR mRNA was related to adenoviral infection sensitivity and demonstrated that a high expression level of CAR markedly enhanced virus entry and adenovirus-mediated gene transfer.

CAR expression appears to be an important predictor of adenovirus oncolytic efficacy. Low or absent CAR expression has been shown to limited the utility of adenovirus-mediated gene transfer and oncolysis for a number of cancers, including bladder cancer, prostate cancer, ovarian cancer, and rhabdomyosarcoma (10, 24, 35, 36). This study revealed that many mesenchymal tumors, with the exception of osteosarcoma, Ewing's sarcoma, neurofibroma, and schwannoma, exhibited an intermediate to low or undetectable level of CAR expression, and thus other strategies for successful adenovirus-mediated gene therapy need to be considered. This has led to new approaches such as tumor targeting, using modifications of the fiber knob to direct the attachment of adenovirus to cell surface molecules other than CAR (22, 24, 37–39). Furthermore, it has been documented in human musculoskeletal tumor that a modified fiber knob containing an integrin-binding motif markedly improved both gene transfer and oncolysis for rhabdomyosarcoma cells (24). In addition, some studies showed that the expression of CAR could be up-regulated through several methods, such as inhibition of the Raf/mitogen-activated protein/extracellular signal-regulated kinase kinase/extracellular signal-regulated kinase pathway (40) or the use of certain histone deacetylase inhibitors (41), such as butyrate (42). Thus, modification of the adenovirus vector or induction of CAR expression may lead to a favorable therapeutic ratio in CAR-deficient tumors.

In conclusion, our data showed great variations in CAR expression in human mesenchymal tumors and normal mesenchymal tissues and indicated the potential usefulness of adenoviral vectors in gene therapy for osteosarcoma, Ewing's sarcoma, neurofibroma, and schwannoma. Efficient transduction with adenovirus for gene therapy could be realized in appropriate, sensitive tumor types.

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