

Anti-Tumor Effect of Adenoviral Vector-Mediated *p53* Gene Transfer on the Growth of Canine Osteosarcoma Xenografts in Nude Mice

Noriko KANAYA¹⁾, Mitsuhiro YAZAWA²⁾, Yuko GOTO-KOSHINO²⁾, Manabu MOCHIZUKI¹⁾, Ryohei NISHIMURA¹⁾, Koichi OHNO²⁾, Nobuo SASAKI¹⁾ and Hajime TSUJIMOTO²⁾

¹⁾Departments of Veterinary Surgery and ²⁾Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

(Received 21 December 2010/Accepted 8 February 2011/Published online in J-STAGE 22 February 2011)

ABSTRACT. We evaluated the anti-tumor effect of adenoviral vector-mediated *p53* gene therapy on the growth of canine osteosarcoma xenografts formed in nude mice. Nude mice were subcutaneously transplanted with cells of 2 P53 mutant canine osteosarcoma cell lines, POS and CHOS. The osteosarcoma xenografts were injected with either an adenoviral vector that expresses canine wild-type P53 (AxCa-cp53) or LacZ (AxCa-LacZ). Tumor growth was significantly inhibited in the xenografts injected with AxCa-cp53 in comparison to those injected with AxCa-LacZ or PBS during the observation period of 27 days. An increase of the amount of *p21^{WAF1/CDKN1A}* mRNA, and the number of apoptotic cells was shown in the tumors injected with AxCa-cp53 in comparison to those injected with AxCa-LacZ or PBS. The present study revealed that the adenoviral vector-mediated *p53* gene transfer had an anti-tumor effect in canine osteosarcoma xenografts formed in nude mice.

KEY WORDS: adenovirus vector, canine, mouse model, osteosarcoma, P53.

J. Vet. Med. Sci. 73(7): 877–883, 2011

Osteosarcoma is one of the most malignant, aggressive and common primary bone tumors in dogs, accounting for up to 80% of bone malignancies [35]. Median survival duration has been reported as 32–43 weeks for dogs with appendicular skeleton osteosarcomas treated with surgical resection and adjuvant chemotherapy [3, 17, 18, 27]. Canine osteosarcoma arises in the appendicular skeleton in many cases; however, it can also occur in the axial skeleton and other nonskeletal sites. Osteosarcomas of the axial skeleton are observed in the mandible, maxilla, spine, cranium and pelvis, among other sites. Tumor-related mortality in dogs with axial osteosarcomas is usually a consequence of unsuccessful local tumor control rather than metastatic disease [7]. Since axial osteosarcomas are nonresectable and poorly responsive to chemotherapy in most cases, treatment modalities are limited.

The *p53* tumor suppressor gene is the most frequently mutated gene in human malignancies, occurring in 50–55% of patients [8, 23, 24]. The principal function of the P53 protein is to act as an inducible transcription factor after DNA damage. If the P53 protein is inactivated, the cell cycle and apoptotic pathways can be impaired. Immunohistochemical studies revealed an accumulation of aberrant P53 expression in a high proportion of osteosarcomas in dogs [16, 25], and mutations of *p53* were also found in a significant number of canine osteosarcomas [11, 13, 20, 26, 31]. These results indicate that inactivation of the *p53* tumor suppressor gene is a frequent genetic event found in canine osteosarcoma. Furthermore, after surgical removal

of the osteosarcoma, dogs with mutant P53 protein had a significantly shorter survival time [13]. Moreover, transfection of the *p53* gene, in canine osteosarcoma cells, could improve radio sensitivity *in vitro* at clinical relevant doses [28].

It has been demonstrated that overexpression of P53 by using vectors such as plasmid DNA, retroviruses, and adenoviruses results in anti-tumor effects in many cultured human tumor cell lines [14]. The mechanisms appear to involve the pathways leading to cell cycle arrest and apoptosis [32]. Many studies have demonstrated that the growth of human tumor xenografts formed in nude mice was inhibited by the direct intratumoral injection of adenoviral vectors expressing wild-type P53 [34]. This vector system provides a high rate of gene transduction efficiency in tumor cells and a high safety level because it is a transient expression system without integration of the transgene into the host genome. The adenoviral vector shows a high rate of infectivity for a broad host range *in vitro* and *in vivo* [12]. Clinical trials in humans, adenoviral vector-mediated *p53* gene therapy has been evaluated in patients with lung cancer, head and neck cancer, bladder cancer, ovarian cancer and breast cancer [34]. Based on the result of those clinical trials, the use of adenovirus gene therapy (Gendicine) for the treatment of head and neck cancer was approved in 2003 in China and FDA-approved clinical trial was opened in U.S.A. in 2010. Some clinical difficulties including systemic delivery and immune responses to the virus were reported. Many attempts to improve the efficacy of adenovirus vector-mediated *p53* gene therapy have been made. These include modifications of the inserted *p53* gene structure and the adenovirus itself [14]. For the treatment of canine osteosarcoma, we constructed an adenovirus vector inserted with

* CORRESPONDENCE TO: TSUJIMOTO, H., Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan.
e-mail: atsujii@mail.ecc.u-tokyo.ac.jp

canine *p53* gene. We previously reported that a recombinant replication-defective adenoviral vector expressing canine wild-type P53 (AxCA-cp53) inhibited cell growth via induction of cell cycle arrest and apoptosis in canine osteosarcoma and mammary adenocarcinoma cell lines [36]. As an extension of that study, the present study was carried out to evaluate the anti-tumor effect of intratumoral injection with AxCA-cp53 in canine osteosarcoma xenografts formed in nude mice.

MATERIALS AND METHODS

Cells and culture conditions: Two canine osteosarcoma cell lines, POS [2] and CHOS [9] (this cell line was once called as HOS), were used in this study. Both of the POS and CHOS cell lines were shown to have missense point mutations at codons 162 (CGC to CAC, Arg to His) (unpublished data) and 150 (TAT to AAT, Try to Asn) [36], respectively. Immunohistochemical analyses revealed overexpression of P53, possibly derived from their mutated *p53* genes in both POS and CHOS cells [36]. These two cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a humidified atmosphere of 5% CO₂. 293 cells (JCRB9068, Health Science Research Resources Bank, Osaka, Japan) were maintained in a high-glucose Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin.

Mice: Athymic female nude mice (BALB/cAJcl-nu) were purchased from a commercial laboratory (Nihon CLEA, Tokyo, Japan) and used for the xenopplantation of the cells of canine osteosarcoma cell lines. All mice were maintained in high efficiency particulate air filtered isolation cabinets for the duration of the experimental trial. The present study was approved by the Animal Care Committee of the Graduate School of Agricultural and Life Sciences at the University of Tokyo.

Tumor xenografts: A total of twenty-one mice were used in this study. Two days before transplantation, all of the mice were irradiated with X-ray at a dose of 4 J/kg [10]. POS cells (1×10^7 /mouse) or CHOS cells (1×10^7 /mouse) were subcutaneously injected into 15 mice and 12 mice, respectively. Ten to 15 days after the injection, when the size of the tumor masses reached a size of which the weight was estimated 80 mg, the mice were used for adenovirus vector-mediated gene transfer. The estimated tumor weight was calculated from the size of the masses according to the following formula: $\frac{1}{2} \times L$ (Length: Longest dimension) $\times W$ (Width: shorter dimension, parallel to the mouse body) $\times H$ (Height: diameter of tumor perpendicular to the length and width) [29]. Fifteen mice that harbored xenografts of POS cells were randomly divided into three groups for the injection with AxCA-cp53 (n=5), AxCA-LacZ (n=5) or PBS (n=5). Similarly, 12 mice that harbored xenografts of CHOS cells were randomly divided into three groups for the injection with AxCA-cp53 (n=4), AxCA-LacZ (n=4) or

PBS (n=4).

Construction and generation of the adenoviral vectors: An adenoviral vector that expresses wild-type canine P53 (AxCA-cp53) was generated by use of a commercial adenoviral vector kit (Adenovirus expression vector kit, Takara, Kyoto, Japan) as previously reported [36]. Briefly, a canine *p53* cDNA fragment was inserted into an expression cassette cosmid vector, pAxCAwt, between the CAG promoter (ie, cytomegalovirus enhancer and chicken β -actin promoter) and rabbit β -globin polyadenylation signal site. The pAxCAwt is a cassette cosmid containing a nearly full-length adenovirus type 5 genome with E1 and E3 deletions. The expression cosmid pAxCA-cp53 was cotransfected with the EcoT22I-digested DNA terminal protein complex of Ad5-dIX into the 293 cell lines. The recombinant adenovirus inserted with a canine *p53* gene expression unit was generated according to the manufacture's protocol. An adenoviral vector that expresses canine *p53* gene was propagated in 293 cells, purified by sequential centrifugation in CsCl step gradients, and dialyzed against PBS solution with 10% (vol/vol) glycerol. Titers of the virus stocks were determined by TCID₅₀ (50% Tissue Culture Infectious Dose) endpoint dilution method using 293 cell lines and calculated to plaque forming units (PFU)/ml based on manufacture instruction (Adenovirus expression vector kit, Takara, Kyoto, Japan). AxCA-LacZ (Riken gene bank, Tsukuba, Japan), which expresses β -galactosidase of *Escherichia coli* under the same promoter, was used as a control vector.

Injection with adenovirus vectors: The dose of the adenoviral vectors, AxCA-cp53 and AxCA-LacZ was set at 1×10^9 PFU per mouse. The dose of the injected AxCA-cp53 was set at 1×10^9 PFU/tumor which was based on previous reports that showed the tumor inhibitory effects in a xenograft model using human cancer cell lines [5, 15]. Intratumoral injection was carried out 7 times (on days 1, 2, 3, 8, 9, 14 and 15) during 15 days. At each injection, purified virus stocks were diluted with PBS to a final volume of 100 µl and then directly injected into the tumor masses with a 1-ml syringe and a 27-gauge needle. The injection was performed from 4 different angles into the center of the tumors. The needles were gently withdrawn after administering virus solution (25 µl virus solution for each injection) into each tumor mass. Thus, one tumor mass had 4 injections ($25 \mu\text{l} \times 4 = 100 \mu\text{l}$) in each treatment period.

Analysis of adenovirus infection: To evaluate the infection after exposure to the adenoviral vector, AxCA-LacZ-mediated β -galactosidase (β -gal) activity was examined by using the 3-indolyl- β -D-galactopyranoside staining method (β -gal Staining Set, Roche Diagnostics, Mannheim, Germany). Tissues were excised 24, 48 and 72 hr and 7 days after injection with AxCA-LacZ. The tissues were fixed in a solution containing 2% (vol/vol) formaldehyde and 0.2% (vol/vol) glutaraldehyde in PBS at room temperature for 15 minutes. Subsequently, the tissues were washed 3 times with PBS and stained with a solution containing 0.01% 3-indolyl- β -D-galactopyranoside, 5 mM potassium ferrocyanide, and 5

mM potassium ferricyanide in PBS at 37°C for 3 hr.

Measurement of tumor size after treatment: Tumor sizes were measured with slide calipers every 3 days for 27 days after injection with the adenoviral vectors. The estimated tumor weight was calculated as previously described [29]. Further, the relative estimated weight at day *n* was calculated from the following formula: estimated tumor weight on day *n* / estimated tumor weight on day 0.

In situ terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay for apoptosis: In situ TUNEL assay was performed in accordance with the manufacturer's instructions (TumorTACS) (Trevigen, Gaithersburg, MD, U.S.A.) for detection of apoptotic cells in the tissue specimens. Seventy-two hr after injection with the adenoviral vectors, the tumor tissues were excised and then fixed in 3% buffered formaldehyde followed by embedding in paraffin. The paraffin-embedded tissues were rehydrated by immersing in serial concentrations of ethanol (100%, 95%, and 70%) and then washed with PBS. The rehydrated tissues were immersed in 3.7% buffered formaldehyde at room temperature for 10 min. After washing, the tissues were incubated with proteinase K (100 µg/ml, 50 µl/slide) for 5 min. Subsequently, the tissues were immersed in a quenching solution (50 ml) composed of methanol (45 ml) and 30% hydrogen peroxidase (5 ml) and then covered with 50 µl of Terminal Transferase (TdT) labeling mix containing 5 µM biotinylated dNTP, 0.4 mM manganese cation, 15 units of TdT enzyme, 0.05 mg of BSA, and 0.06 mM 2-mercaptoethanesulfonic acid at 37°C for 60 min. Then, the tissues were immersed in TdT stop buffer (0.1 M EDTA, pH 8.0). After rinsing in PBS, the tissues were resuspended in streptavidin-horseradish peroxidase (2%) for 10 min, washed in PBS, and then incubated with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) (0.5%) for 5 min. Immunohistochemical slides were quantified by taking three random fields and counting stained and unstained cells and dividing each by the total number of cells counted to generate the mean percentage of positive cells in each sample. Standard error (SE) was calculated from 3 samples in each group.

Reverse transcription (RT)-polymerase chain reaction (PCR) analysis: The xenografts formed in nude mice were excised 24 hr after injection with the adenoviral vector that expresses canine wild-type P53. Total RNA samples extracted from the excised xenografts were treated with deoxyribonuclease I (0.1 U/µl) (Invitrogen, Carlsbad, CA, U.S.A.). Single-strand cDNA was synthesized by priming with oligo (dT) using the GeneAmp RNA PCR kit (Applied Biosystems, Foster city, CA, U.S.A.). The cDNA samples were amplified by PCR as follows: sequences of the primers used to amplify a cDNA fragment derived from canine p53 mRNA were 5-AAAGAAGAAGCCACTAGATG-3 (cp53-S) (nucleotides (nt.) 918-937, in canine p53 cDNA, EMBL database, accession number NM_001003210) and 5-TGCACCTGAGGAGTGAATTG-3 (Ad-R) (nt. 42975-42956 in the adenovirus vector) (Adenovirus expression vector kit, Takara, Kyoto, Japan). The cp53-S primer can

anneal the cDNA derived from both endogenous and exogenously introduced canine p53 mRNA, but the Ad-R primer is specific for cDNA derived from the mRNA of the expression cassette of AxCA-cp53. Therefore, this pair of primers can detect p53 mRNA transcription from the transduced adenoviral vector but not that of endogenous origin. Sequences of the primers used to amplify a cDNA fragment of p21^{WAF1/CIP1/CDKN1A} (p21^{WAF1}) gene were 5-GACTGTGATGCGCTAATGGC-3 (cp21^{WAF1}-S) (nt. 3-22 in canine p21^{WAF1} cDNA, EMBL database, accession number, AJ_830019) and '5-GGGTGCAGGTCAGCGACAGG-3' (p21^{WAF1}-R) (nt. 149-131). As an internal control, primers used to amplify canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were 5'-TGCCGCCTG-GAGAAAGCTGC-3' (GAPDH-S) (nt. 733-753 in canine GAPDH cDNA, EMBL database, accession number, NM_001003142) and 5-TCCCAGGAATGAGCTTGAC-3 (GAPDH-R) (nt. 936-916). After denaturation at 95°C for 2 min, 30 cycles of the reaction composed of denaturation (at 95°C for 1 min) and annealing and polymerization (at 60°C for 1 min) were performed, followed by a final extension procedure at 72°C for 7 min. The PCR products were subjected to electrophoresis in a 3% agarose gel and observed after ethidium bromide staining.

Statistical analysis: Tumor data were analyzed using One-Way ANOVA followed by Dunnett's multiple comparison test to determine the significance of the difference in tumor cell growth among the mice injected with AxCA-cp53, AxCA-LacZ, and PBS (as a control) at end of the experiment (27 days). Apoptosis data were analyzed similarly at 72 hr after injection. Statistical significance was accepted at *P*<0.05.

RESULTS

Infection of the adenoviral vector in canine osteosarcoma xenografts: The β-gal expression was detected 24, 48, and 72 hr after the AxCA-LacZ injection, but not 7 days after the injection. In the xenografts injected with PBS, no β-gal expression was observed.

Effect of adenoviral vector-mediated p53 gene transfer on the growth of canine osteosarcoma xenografts: Effect of the intratumoral injection with AxCA-cp53 on the growth of canine osteosarcoma xenografts formed in nude mice was examined. The POS xenograft tumors injected with mock solution (PBS) or AxCA-LacZ gradually increased in size during the observation period of 27 days. The injection of AxCA-cp53 inhibited the growth of POS xenograft tumors (Fig. 1A). Similarly, inhibition of the tumor growth was observed in the xenografts formed by transplantation with CHOS cells injected with AxCA-cp53 (Fig. 1B). Significant inhibition of the growth of the POS xenograft tumors was observed in the tumors injected with AxCA-cp53 in comparison to those injected with AxCA-LacZ or PBS (*P*<0.05) at the end of experiment (at day 27) (Fig. 1C). In CHOS xenograft tumors, significant inhibition of growth were seen in the tumors injected with AxCA-cp53 compared

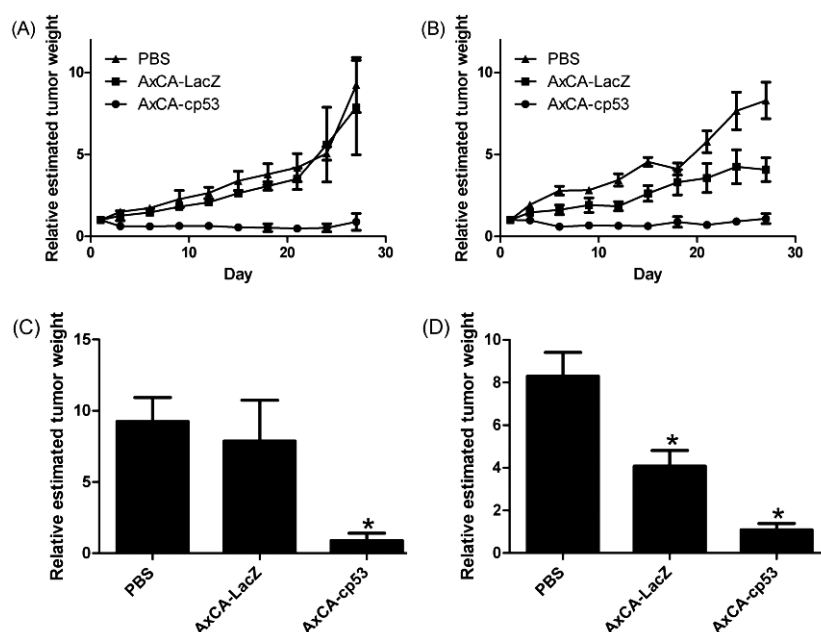


Fig. 1. Effect of the adenoviral vector-mediated *p53* gene transfer on the growth of canine osteosarcoma xenografts in nude mice. Relative estimated tumor weights of xenografts formed by transplantation with POS cells (A) and CHOS cells (B) are shown. Intratumoral injections with AxCa-cp53 (1×10^9 PFU/tumor) (circles) or AxCa-LacZ (squares) (1×10^9 PFU / tumor) or mock solution (PBS) (triangles) were carried out 7 times on days 1, 2, 3, 8, 9, 14, and 15. The tumor sizes were measured for 28 days after the injection with the recombinant adenoviral vectors. Relative estimated tumor weights (mean \pm SEM) are shown in each group (C: POS and D: CHOS). * $P < 0.05$: AxCa-cp53 or AxCa-LacZ compared to tumors injected with PBS.

with those injected with PBS ($P < 0.05$). Injection with AxCa-LacZ also showed growth inhibition of the tumors in comparison to the tumors cells injected with PBS ($P < 0.05$) (Fig. 1D).

Detection of apoptotic cells in canine osteosarcoma xenografts after adenoviral vector-mediated *p53* gene transfer: In the POS xenograft tumors injected with PBS and AxCa-LacZ, a small number of brown cells showed positive staining in TUNEL assay (Fig. 2) (Mean \pm SE: $0.63 \pm 0.23\%$ and $4.10 \pm 0.78\%$, respectively). The AxCa-cp53 injected tumors, the rate of positively stained cells in the TUNEL assay was $24.83 \pm 2.09\%$, significantly higher than those in the PBS injected tumors ($P < 0.05$). There was no significant difference between AxCa-cp53 and AxCa-LacZ injected tumors.

Detection of the recombinant adenovirus vector-derived *p53* mRNA and *p21^{WAF1}* mRNA in the canine osteosarcoma xenografts: To examine the expression of the adenoviral vector-derived canine *p53* mRNA in the POS and CHOS xenograft tumors, we performed RT-PCR analysis using cp53-S and Ad-R primers. The adenoviral vector-derived canine *p53* mRNA was detected in both of the POS and CHOS xenograft tumors injected with AxCa-cp53, but not in those injected with AxCa-LacZ (Fig. 3).

To examine the biological function of P53 transduced by

the adenoviral vector, we performed RT-PCR analysis to detect *p21^{WAF1}* mRNA, whose expression is induced by P53. Expression of canine *p21^{WAF1}* mRNA was detected in both of the POS and CHOS xenograft tumors injected with AxCa-cp53, but not in those injected with AxCa-LacZ (Fig. 3).

DISCUSSION

In this study, we demonstrated that adenovirus vector-mediated transduction of canine wild-type P53 had a significant inhibitory effect on the growth of canine osteosarcoma xenografts in nude mice through induction of apoptosis. Successful virus infection was confirmed by detecting β -galactosidase activity after injection with AxCa-LacZ. The adenoviral vector used in this study was replication-defective, and our results showed that a single injection of the adenovirus resulted in a transient transgene expression lasting for less than one week. From these findings, frequent injection (7 times per 15 days) was carried out in the mouse xenograft model in this study. Moreover, at the intratumoral injection site, repeated insertion of the needle from 4 different angles was done to distribute the virus solution in a wide area of the tumors. Our preliminary experiments showed that a single injection of $100 \mu\text{l}$ virus solution was not suc-

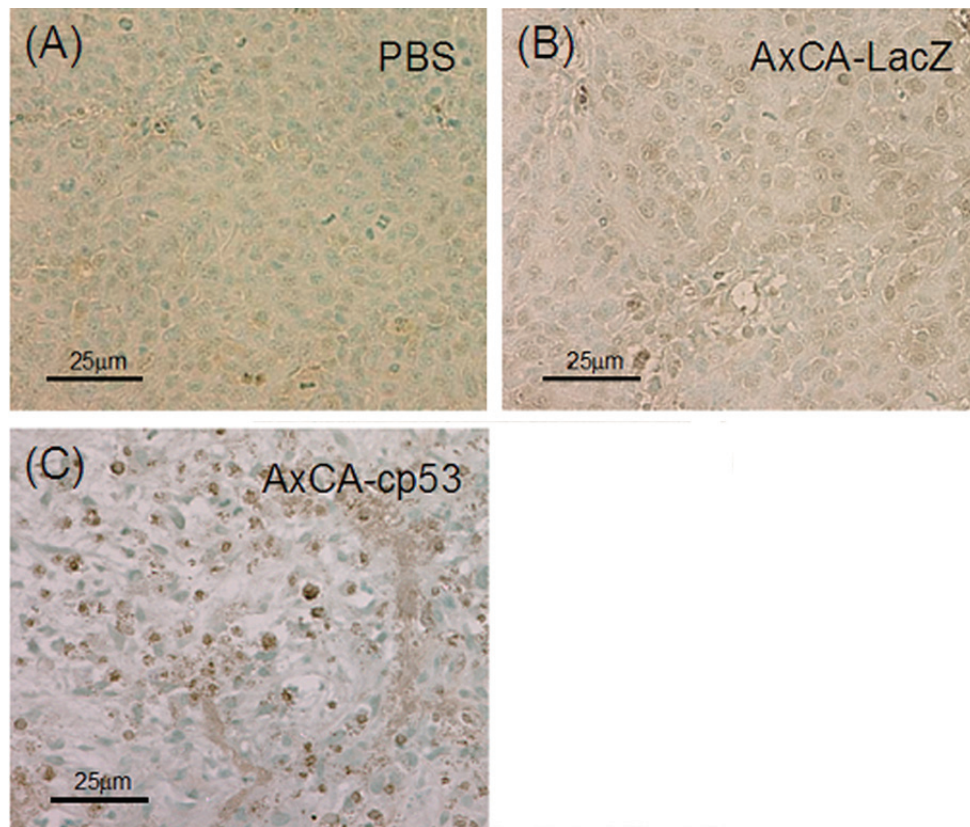


Fig. 2. Detection of apoptotic cells by TUNEL staining in canine osteosarcoma xenografts after injection with the recombinant adenoviral vectors. The xenografts formed by transplantation with POS cells were injected with PBS (A), AxCa-LacZ (B) and AxCa-cp53(C), excised 72 hr after the injections, and then subjected to TUNEL staining.

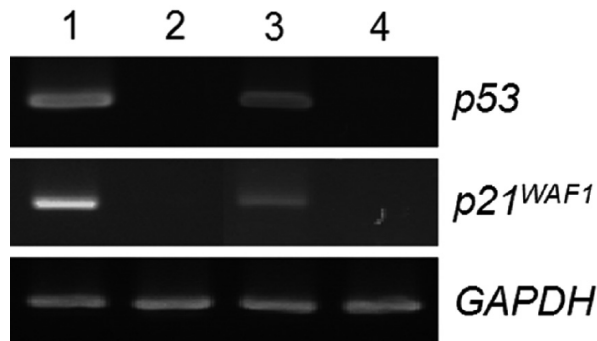


Fig. 3. RT-PCR analyses for the expression of adenoviral vector-mediated *p53* mRNA and *p21^{WAF1}* mRNA in canine osteosarcoma xenografts after injection with the recombinant adenoviral vectors. Lanes 1 and 2, a tumor formed by transplantation with POS cells; lanes 3 and 4, a tumor formed by transplantation with CHOS cells. Lanes 1 and 3, tumor tissues obtained 24 hr after injection with AxCa-cp53. Lanes 2 and 4, tumor tissues obtained 24 hr after injection with AxCa-LacZ. RT-PCR analyses were carried out for the detection of mRNA of the adenoviral vector-mediated *p53* mRNA, *p21^{WAF1}*, and *GAPDH* (internal control).

cessful. Because tumors were too hard to inject 100 μ l solutions, there was some leakage from the injections site. Though it is reported that inserting the needle into tumor tissue can cause tumor cell dissemination [33], multiple injections into the tumors were performed in this study. Although the exact transduction efficiency in whole tumors was not clearly identified, adenovirus vector-mediated *p53* induction significantly inhibited tumor growth in the xenograft model in nude mice.

A possible candidate for *p53* gene therapy, in a clinical setting, might be a dog with an unresectable osteosarcoma such as axial osteosarcoma with a biological inactive P53. We had three canine osteosarcoma cell lines with *p53* mutation, these were POS, CHOS and OOS. POS and CHOS were established from femur and scapular osteosarcoma, respectively. It is reported that 5/11 axial osteosarcoma had mutations of *p53* gene [13]. However, the osteosarcoma cell line derived from axial osteosarcoma (OOS) did not have a *p53* gene mutation. Thus, two osteosarcoma cell lines (POS and CHOS) were used as a model for canine osteosarcoma with biological inactive P53. However, the efficacy of *p53* gene therapy needs to be evaluated using the axial osteosarcoma cell lines with mutant P53 in future

experiment.

Adenoviral vector-mediated wild-type *p53* gene mRNA was detected in the osteosarcoma xenografts injected with AxCA-cp53. Among the effectors associated with cell cycle arrest, cyclin-dependent kinase inhibitor, P21^{WAF1} (also known as WAF1, CIP1 or CDKN1A), is known as one of the key molecules regulated by P53 [6]. Both POS and CHOS cell lines have a point mutation in their DNA binding domain. Treatment with a DNA-damaging agent (doxorubicin, 100 ng/ml) did not increase the expression level of P21^{WAF1} protein in POS and CHOS cells. In contrast, it clearly increased the expression level of P21^{WAF1} protein in OOS (control osteosarcoma cell lines with wild type P53) (unpublished data). In cell lines harboring normal P53 function, in response to DNA damage, P53 is activated and it induces downstream proteins such as P21^{WAF1}. These results suggest that P53 was functionally inactivated in POS and CHOS cells. Importantly, in this study, expression of *p21^{WAF1}* mRNA was detected in the tumor xenografts injected with AxCA-cp53. These results, together with the apoptosis assay, suggest that AxCA-cp53 achieved the induction of biologically active P53, leading to apoptosis *in vivo*. Furthermore, it is reported that P53 can downregulate expression of vascular endothelial growth factor (VEGF) and unregulated expression of thrombospondin (potent inhibitor of angiogenesis). Previous reports showed that such effects may partially explain the efficacy of the adenovirus expressing P53 in solid tumor models [19], in addition to the induction of apoptosis. Tumor inhibitory effects of AxCA-cp53 were shown in both tumor xenografts of POS and CHOS cell lines. However, treatment with an injection of AxCA-LacZ resulted in slight inhibition of the growth of the xenografts formed by transplantation with CHOS cells. Several studies have reported that infection with the control adenoviral vector itself had a weak anti-tumor effect [15, 22]. Several mechanisms have been proposed to explain the specific anti-tumor effect of the control adenoviral vectors, such as induction of immune response against the adenoviral vector-infected tumor cells [4, 21, 30]. Alternatively, the virus binding to the cell membrane in concert with the virion components (penton protein of adenovirus) might mediate a series of biochemical changes. It has been reported that the purified adenovirus penton fibers significantly decrease the synthesis of protein, RNA, and DNA of the host cells [1]. Some of these events might be associated with the inhibitory effect of AxCA-LacZ on the growth of CHOS xenografts. These variations of inhibitory effects of AxCA-LacZ may depend on the susceptibility of each canine osteosarcoma cell line to the adenovirus vector. Further analysis may be needed to evaluate the area of adenovirus infection of each osteosarcoma cell lines *in vivo*.

In summary, the present study revealed that the adenoviral vector-mediated *p53* gene transfer had an anti-tumor effect in canine osteosarcoma xenografts with mutant P53, which were formed in nude mice. However, the exact mechanism to inhibited tumor growth could not be completely elucidated in this study. Thus, further studies are

needed to aid in our understanding of the mechanisms of the inhibitory effect of AxCA-cp53 in our canine osteosarcoma xenograft model, and to clarify the possibility of using *p53* gene therapy in the treatment of dogs with canine osteosarcoma in clinical settings.

ACKNOWLEDGMENT. The authors would like to thank Dr. Lynn Adams for help in editing this paper. Source of funding: This study was supported by grants from the Japan Health Science Foundation and the Ministries of Education, Culture, Sports, Science and Technology.

REFERENCES

1. Albrecht, T. 1996. Effects on cells. pp. 563–569. *In: Medical Microbiology*, 4th ed. (Baron, S. eds.), University of Texas Medical Branch, Galveston.
2. Barroga, E. F., Kadosawa, T., Okumura, M. and Fujinaga, T. 1999. Establishment and characterization of the growth and pulmonary metastasis of a highly lung metastasizing cell line from canine osteosarcoma in nude mice. *J. Vet. Med. Sci.* **61**: 361–367.
3. Bergman, P. J., MacEwen, E. G., Kurzman, I. D., Henry, C. J., Hammer, A. S., Knapp, D. W., Hale, A., Kruth, S. A., Klein, M. K., Klausner, J., Norris, A. M., McCaw, D., Straw, R. C. and Withrow, S. J. 1996. Amputation and carboplatin for treatment of dogs with osteosarcoma: 48 cases (1991 to 1993). *J. Vet. Intern. Med.* **10**: 76–81.
4. Chen, H. L. and Carbone, D. P. 1997. p53 as a target for anti-cancer immunotherapy. *Mol. Med. Today* **3**: 160–167.
5. Clayman, G. L., el-Naggar, A. K., Roth, J. A., Zhang, W. W., Goepfert, H., Taylor, D. L. and Liu, T. J. 1995. In vivo molecular therapy with p53 adenovirus for microscopic residual head and neck squamous carcinoma. *Cancer Res.* **55**: 1–6.
6. el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W. and Vogelstein, B. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell* **75**: 817–825.
7. Heyman, S. J., Diefenderfer, D. L., Goldschmidt, M. H. and Newton, C. D. 1992. Canine axial skeletal osteosarcoma. A retrospective study of 116 cases (1986 to 1989). *Vet. Surg.* **21**: 304–310.
8. Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C. C. 1991. p53 mutations in human cancers. *Science* **253**: 49–53.
9. Hong, S. H., Kadosawa, T., Mochizuki, M., Matsunaga, S., Nishimura, R. and Sasaki, N. 1998. Establishment and characterization of two cell lines derived from canine spontaneous osteosarcoma. *J. Vet. Med. Sci.* **60**: 757–760.
10. Igarashi, T., Oka, K. and Miyamoto, T. 1989. Human non-Hodgkin's malignant lymphomas serially transplanted in nude mice conditioned with whole-body irradiation. *Br. J. Cancer* **59**: 356–360.
11. Johnson, A. S., Couto, C. G. and Weghorst, C. M. 1998. Mutation of the p53 tumor suppressor gene in spontaneously occurring osteosarcomas of the dog. *Carcinogenesis* **19**: 213–217.
12. Kay, M. A., Glorioso, J. C. and Naldini, L. 2001. Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. *Nat. Med.* **7**: 33–40.
13. Kirpensteijn, J., Kik, M., Teske, E. and Rutteman, G. R. 2008. TP53 gene mutations in canine osteosarcoma. *Vet. Surg.* **37**: 454–460.
14. Lane, D. P., Cheok, C. F. and Lain, S. 2010. p53-based cancer

- therapy. *Cold Spring Harb. Perspect Biol.* **2**: a001222.
15. Li, Z., Rakkar, A., Katayose, Y., Kim, M., Shanmugam, N., Srivastava, S., Moul, J. W., McLeod, D. G., Cowan, K. H. and Seth, P. 1998. Efficacy of multiple administrations of a recombinant adenovirus expressing wild-type p53 in an immune-competent mouse tumor model. *Gene Ther.* **5**: 605–613.
16. Loukopoulos, P., Thornton, J. R. and Robinson, W. F. 2003. Clinical and pathologic relevance of p53 index in canine osseous tumors. *Vet. Pathol.* **40**: 237–248.
17. Matthiesen, D. T., Clark, G. N., Orsher, R. J., Pardo, A. O., Glennon, J. and Patnaik, A. K. 1992. En bloc resection of primary rib tumors in 40 dogs. *Vet. Surg.* **21**: 201–204.
18. Mauldin, G. N., Matus, R. E., Withrow, S. J. and Patnaik, A. K. 1988. Canine osteosarcoma. Treatment by amputation versus amputation and adjuvant chemotherapy using doxorubicin and cisplatin. *J. Vet. Intern Med.* **2**: 177–180.
19. McCormick, F. 2001. Cancer gene therapy: fringe or cutting edge? *Nat. Rev. Cancer* **1**: 130–141.
20. Mendoza, S., Konishi, T., Dernell, W. S., Withrow, S. J. and Miller, C. W. 1998. Status of the p53, Rb and MDM2 genes in canine osteosarcoma. *Anticancer Res.* **18**: 4449–4453.
21. Morral, N., O'Neal, W., Zhou, H., Langston, C. and Beaudet, A. 1997. Immune responses to reporter proteins and high viral dose limit duration of expression with adenoviral vectors: comparison of E2a wild type and E2a deleted vectors. *Hum. Gene Ther.* **8**: 1275–1286.
22. Nielsen, L. L., Dell, J., Maxwell, E., Armstrong, L., Maneval, D. and Catino, J. J. 1997. Efficacy of p53 adenovirus-mediated gene therapy against human breast cancer xenografts. *Cancer Gene Ther.* **4**: 129–138.
23. Nigro, J. M., Baker, S. J., Preisinger, A. C., Jessup, J. M., Hostetter, R., Cleary, K., Bigner, S. H., Davidson, N., Baylin, S., Devilee, P. and *et al.* 1989. Mutations in the p53 gene occur in diverse human tumour types. *Nature* **342**: 705–708.
24. Olivier, M., Eeles, R., Hollstein, M., Khan, M. A., Harris, C. C. and Hainaut, P. 2002. The IARC TP53 database: new online mutation analysis and recommendations to users. *Hum. Mutat.* **19**: 607–614.
25. Sagartz, J. E., Bodley, W. L., Gamblin, R. M., Couto, C. G., Tierney, L. A. and Capen, C. C. 1996. p53 tumor suppressor protein overexpression in osteogenic tumors of dogs. *Vet. Pathol.* **33**: 213–221.
26. Setoguchi, A., Sakai, T., Okuda, M., Minehata, K., Yazawa, M., Ishizaka, T., Watari, T., Nishimura, R., Sasaki, N., Hasegawa, A. and Tsujimoto, H. 2001. Aberrations of the p53 tumor suppressor gene in various tumors in dogs. *Am. J. Vet. Res.* **62**: 433–439.
27. Shapiro, W., Fossum, T. W., Kitchell, B. E., Couto, C. G. and Theilen, G. H. 1988. Use of cisplatin for treatment of appendicular osteosarcoma in dogs. *J. Am. Vet. Med. Assoc.* **192**: 507–511.
28. Shiomitsu, K., Sajo, E., Xia, X., Hunley, D. W., Mauldin, G. E., Li, S. and Mauldin, G. N. 2008. Radiosensitivity of canine osteosarcoma cells transfected with wild-type p53 in vitro. *Vet. Comp. Oncol.* **6**: 193–200.
29. Tomayko, M. M. and Reynolds, C. P. 1989. Determination of subcutaneous tumor size in athymic (nude) mice. *Cancer Chemother. Pharmacol.* **24**: 148–154.
30. Tripathy, S. K., Black, H. B., Goldwasser, E. and Leiden, J. M. 1996. Immune responses to transgene-encoded proteins limit the stability of gene expression after injection of replication-defective adenovirus vectors. *Nat. Med.* **2**: 545–550.
31. van Leeuwen, I. S., Cornelisse, C. J., Misdorp, W., Goedegebuure, S. A., Kirpensteijn, J. and Rutteman, G. R. 1997. P53 gene mutations in osteosarcomas in the dog. *Cancer Lett.* **111**: 173–178.
32. Vazquez, A., Bond, E. E., Levine, A. J. and Bond, G. L. 2008. The genetics of the p53 pathway, apoptosis and cancer therapy. *Nat. Rev. Drug Discov.* **7**: 979–987.
33. Wiksell, H., Schassburger, K. U., Janicijevic, M., Leifland, K., Lofgren, L., Rotstein, S., Sandberg, P. O., Wadstrom, C. and Auer, G. 2010. Prevention of tumour cell dissemination in diagnostic needle procedures. *Br. J. Cancer* **103**: 1706–1709.
34. Wiman, K. G. 2007. Restoration of wild-type p53 function in human tumors: strategies for efficient cancer therapy. *Adv. Cancer Res.* **97**: 321–338.
35. Withrow, S. J., Powers, B. E., Straw, R. C. and Wilkins, R. M. 1991. Comparative aspects of osteosarcoma. Dog versus man. *Clin. Orthop. Relat. Res.* **270**: 159–168.
36. Yazawa, M., Setoguchi, A., Hong, S. H., Uyama, R., Nakagawa, T., Kanaya, N., Nishimura, R., Sasaki, N., Masuda, K., Ohno, K. and Tsujimoto, H. 2003. Effect of an adenoviral vector that expresses the canine p53 gene on cell growth of canine osteosarcoma and mammary adenocarcinoma cell lines. *Am. J. Vet. Res.* **64**: 880–888.