Detection of the Anti-P53 Antibodies in Dogs with Tumors

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ABSTRACT. To detect the anti-P53 antibodies of dogs with tumors, a GST-recombinant canine (rc) P53 fusion protein was expressed and purified. Immunoblot analysis was performed using this GST-rcP53 fusion protein as an antigen and serum samples from dogs suffering from tumors as primary antibodies. Out of 16 serum samples obtained from various tumor cases, four samples showed reaction with GST-rcP53. In contrast, serum from other 12 dogs with tumors, four dogs with non-neoplastic diseases and two control healthy dogs (as controls) did not show any reaction with GST-rcP53 in immunoblotting. The p53 gene mutation and the P53 protein expression were examined, using the tumor tissues to explore the relationship between the existence of the GST-rcP53 bands, gene mutations of p53 and the accumulation of P53 protein. One case, which showed a clear GST-rcP53 band, had a point mutation of the p53 gene.

KEY WORDS: antibody, canine, mutation, P53, tumor.

The p53 tumor suppressor gene is one of the most frequently mutated genes in human cancer [11, 16]. Mutations of the p53 gene are evident in 50 to 55% of all malignancies in humans [10]. The function of the P53 protein is to act as an inducible transcriptional factor after DNA damage [12]. One of the transcriptional targets of the P53 protein is P21WAF1, which binds to and inhibits the cyclin-cyclin dependent kinase complexes, resulting in cell cycle arrest [6]. This function is known as P53-mediated G1 arrest [6, 9]. Also, P53 affects induction of apoptosis by inducing Bax and/or p53AIP1 [20, 25]. More recently, it was reported that P53-null cells were not blocked in the G2 phase when they were treated with mitotic spindle inhibitors, suggesting that P53 also plays a role in G2 checkpoint [8]. If the P53 protein is inactivated, the cell cycle cannot be stopped and apoptosis of the cell cannot be induced. These observations indicate that P53 is a guardian of the cell cycle. Thus, the loss of P53 function is considered to be associated with tumorigenesis because of impaired regulation of the cell cycle and the absence of apoptosis.

Most p53 gene alterations lead to the synthesis of mutant proteins with longer half-life compared to that of wild-type P53 [4, 15]. This prolonged biological half-life leads to accumulation of mutant P53 protein in the cell [4, 15]. In many cases of human cancers, this accumulated mutant P53 protein induces a specific humoral response, and produces antibodies against P53 protein [3, 17, 30]. The clinical and biological relevance of anti-P53 antibodies in various human tumors have been studied for the associations such as risk of relapse, death, clinical characteristics and prognosis. Most studies show that the presence of such antibodies predicts a poor outcome [2, 23]. The ratio of dogs dying of cancer is increasing with the elongation of their life span [27]. This has become a big problem in the small animal practice. The p53 gene is also playing an important role in tumorigenesis of the dog like human [19, 26, 29], and mutations of the p53 gene are found in 47% of various tumors such as monocytic leukemia, mammary tumor, rhabdomyosarcoma, colon cancer and osteosarcoma [26]. However, to the best of our knowledge, the anti-P53 antibodies of dogs have not been explored. In this study, we tried to detect the anti-P53 antibodies in various dog tumors and evaluated the relationship between the presence of the anti-P53 antibodies and their gene mutations.

MATERIALS AND METHODS

Recombinant plasmid construction: Canine cDNA encoding the whole open reading frame of p53 [EMBL database, accession number AB 020761] was amplified using forward (5'-GAGGATCCATGCAAGGCACAGAGAGAGC-3') and reverse primers (5'-GGAATTTCAGCTTGAACAGCT-3') with BamHI and EcoRI restriction sites, respectively. Amplifications were performed in a volume of 50 µl, using 25 pmol of each primers, 2.5 units of Taq polymerase (Applied Biosystems, Foster City, CA) and the reagents as recommended by the manufacturer (Applied Biosystems). Thirty cycles of polymerase chain reaction (PCR) with a denaturation (94°C, 1 min), annealing (55°C, 1 min) and polymerization (72°C, 2 min) after preheating (95°C, 9 min) was carried out. The product
Expression of recombinant fusion protein was induced in preparation has been described previously [21]. Briefly, the expression plasmid was introduced into E. coli DH5α. Expression of recombinant fusion protein was induced in culture with 0.1 mM isopropyl β-D-thiogalactoside (IPTG). Cultures were centrifuged and the cell pellets were resuspended in phosphate buffered saline (PBS, 1.37 M NaCl, 27 mM KCl plus 15 mM KH₂PO₄, pH 7.4) containing 1 mM DTT (Dithiothreitol) and 2 mM benzamidine. Lysates were sonicated on ice, and Triton X-100 was added at 1% (final concentration). After centrifugation, the supernatants were applied into the Glutathione Sepharose 4B (Amersham Pharmacia Biotech) matrix to bind the protein. The GST-P53 protein was collected and stored at –80°C until use.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): Samples were analyzed in a 15% polyacrylamide gel with 5% polyacrylamide stacking gel, all containing 0.1% (w/v) SDS. Prior to electrophoresis the samples were boiled for 5 min in the presence of 5% (v/v) 2-mercaptoethanol. Gels were stained with 0.5% Coomassie brilliant blue and destained in 10% methanol and 7% acetic acid. Cross reaction with anti-human P53 antibody: Proteins were electrotransferred onto a Clear Blot nitrocellulose membrane (ATTO, Tokyo, Japan) after electrophoresis. Immunodetection experiments were conducted by using anti-human P53 polyclonal antibody (FL-393, Santa Cruz Biotechnology, CA). Final detection was obtained by using alkaline phosphatase-conjugated rabbit anti-dog immunoglobulin antibody (Kirkegaard & Perry Laboratories) as a secondary antibody and AP conjugate substrate kit (Bio-Rad Laboratories) as a secondary antibody and AP conjugate substrate kit (Bio-Rad Laboratories).

Dog p53 cDNA analysis: Tissues from various tumors of dog were homogenized and the total RNA was isolated using RNeasy mini kit (QIAGEN, Studio city, CA). The first strand cDNA synthesis was performed by 3’RACE System for Rapid Amplification of cDNA Ends (GIBCO BRL, Rockville, MD). For PCR amplification, primers were prepared based on the sequence of canine p53 gene [26]. The sequences of primers used for the amplification of the 5’ portion of p53 cDNA were 5’-CAGAGCT-CAATATCAGCCCC-3’ (Pr. 1, nt. 63-82 in dogp53, accession number AB200761) and 5’-CATAAGGCACCAACCACACTCTGTGC-3’ (Pr. 2, nt. 671-648). The 3’ portion was amplified with primers, 5’-CAGCATCTCATC-CGAGTGGAAAG-3’ (Pr. 3, nt. 584–606) and 5’-ACG AGAGTGAGGGTGTGCGTGTTG-3’ (Pr. 4, nt.1243–1220). Amplifications were performed as described above. Then, resulting products were electrophoresed and were gel purified. The sequence reaction was performed in a total volume of 10 µl, using each primer (3.2 pmol), template DNA (5 µl), premix (4 µl), by a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Amplifications were performed as recommended by the manufacturer (Applied Biosystems). The PCR products were then subjected to nucleotide sequence analysis using ABI377 or ABI310 sequencer (Applied Biosystems).

Indirect immunofluorescence: The frozen tumor tissues were cut at 5 µm thickness, and fixed in 10% formalin and 10% methanol for 30 min at room temperature. The tissues were then washed with PBS, permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature and washed three times with PBS. Tissues were first incubated

was inserted into BamHI-EcoRI sites of bacterial expression plasmid pGEX6P-1 (Amersham Pharmacia Biotech, Uppsala, Sweden) in which P53 can be expressed as a GST fusion protein.

**GST-P53 fusion protein preparation**: GST fusion protein preparation has been described previously [21]. Briefly, the expression plasmid was introduced into E.coli DH5α. Expression of recombinant fusion protein was induced in culture with 0.1 mM isopropyl β-D-thiogalactoside (IPTG). Cultures were centrifuged and the cell pellets were resuspended in phosphate buffered saline (PBS, 1.37 M NaCl, 27 mM KCl plus 15 mM KH₂PO₄, pH 7.4) containing 1 mM DTT (Dithiothreitol) and 2 mM benzamidine. Lysates were sonicated on ice, and Triton X-100 was added at 1% (final concentration). After centrifugation, the supernatants were applied into the Glutathione Sepharose 4B (Amersham Pharmacia Biotech) matrix to bind the protein. The GST-P53 fusion protein was eluted by incubating with Glutathione Elution Buffer (Amersham Pharmacia Biotech). The GST-P53 protein was collected and stored at –80°C until use.

All the serum samples were stored at –20°C until use.

**Immunoblot analysis of anti-P53 antibodies**: Immunoblot analysis of anti-P53 antibodies was performed as described previously [23]. Briefly, affinity-purified GST-P53 and GST proteins were mixed at one to one molar ratio and were resolved by a SDS-PAGE gel. The fractionated proteins were transferred onto Clear Blot nitrocellulose membranes (ATTO) as described above. Soluble proteins, which were extracted from sonicated E. coli without pGEX 6P-1 vector (referred as the bacterial extracts) were also separated by SDS-PAGE and electrotransferred. These nitrocellulose filters were cut into strips. To reduce nonspecific binding, the test serum samples were first incubated with the bacterial extracts for 1 hr at 4°C and then incubated with the nitrocellulose filters containing the separated bacterial extracts overnight at 4°C [23]. The filters containing GST-cp53 and GST were immersed in Tris buffered saline (TBS, 0.05 M Tris and 0.74 M NaCl, pH 7.4) with 0.1% Tween 20 (TBST) and 3% nonfat dry milk (TBSTM) to block unoccupied binding sites, washed two times with TBST, and incubated with the test serum overnight at 4°C in TBSTM. After five washes with TBST, final detection was obtained by alkaline phosphatase-conjugated rabbit anti-dog immunoglobulin antibody (Kirkegaard & Perry Laboratories) as a secondary antibody and AP conjugate substrate kit (Bio-Rad Laboratories).
with blocking solution (20% normal goat serum in PBS) for 30 min and then probed with anti-human P53 polyclonal antibody (FL-393, Santa Cruz Biotechnology) for 1 hr at 37°C. The antibody-antigen complexes were detected with FITC-conjugated goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR) by incubation for 1 hr at 37°C. The samples were washed three times with TBS, and then stained with 4’, 6-diamidino-2-phenylindole (DAPI) DNA dye.

RESULTS

Dog P53 expression and purification: Dog p53 cDNA containing a whole open reading frame amplified by PCR was ligated into the pGEX 6P-1 plasmid (Amersham Pharmacia Biotech). After transformation of E. coli DH5α with the recombinant vector, a 78-kDa protein, which was approximately the same size with calculated GST-recombinant canine P53 (GST-rcP53), was produced under the control of IPTG (Fig. 1, lane 2). The E. coli cell pellets were sonicated and GST fusion protein was purified with Glutathione-Sepharose 4B. The purified protein was analyzed on a 15% SDS-PAGE stained with Coomassie brilliant blue, and the band of 78 kDa was observed (Fig. 1, lane 4). When this GST-fusion protein was cleaved by PreScission protease, a faint band with molecular weight of approximately 53 kDa protein was observed, but almost all of the protein was found in the post-sonication pellet, indicating that rcP53 would be insoluble after cleavage (data not shown). In order to confirm the antigenicity of the purified GST-rcP53, immunoblot analysis was performed using anti-human P53 polyclonal antibody. As shown in Fig. 2, the antibody against human P53 protein cross-reacted with the GST-rcP53 fusion protein but not with GST.

Detection of serum anti-P53 antibodies in canine tumors: To examine serum anti-P53 antibodies in dogs with tumors, immunoblot analysis was performed using the dog sera as primary antibodies against the GST-rcP53 fusion protein. Figure 3 shows the results of immunoblot analysis. Serum samples obtained from cases 1 and 4 showed bands of GST-rcP53 protein with or without GST band, respectively. Sera from cases 2 and 13 also showed the faint bands of GST-rcP53. However, serum from case 2 also showed nonspecific bands with smaller sizes other than GST-rcP53. In contrast, serum samples from other 12 dogs with tumors, two control healthy Beagles and four dogs with non-neoplastic diseases did not show the band for GST-rcP53 or GST.

Dog p53 cDNA analysis in canine tumors: The p53 cDNA from various tumor tissues was amplified using 2 primer pairs, Pr. 1 & 2 and Pr. 3 & 4 (see Materials and Methods). Resulting products were electrophoresed in 1.5% agarose gel, and the amplified DNA bands of approximately 550 bp and 600 bp were extracted from the gel, subjected to direct sequencing. Sequencing analysis revealed a point mutation of p53 cDNA in two of 13 tumor tissues examined in this study (Table 1). The tumor sample from malignant myoepithelioma (case 4) was found to have a miss-sense point mutation with a substitution G to A, which resulted in a change of the encoded amino acid at codon 162 from arginine to histidine (Table 1). The tumor sample from mast cell tumor (case 6) was also found to have a miss-sense point mutation with a substitution C to T, which resulted in a change of the encoded amino acid at codon 266 from phenylalanine to serine (Table 1). The tumor samples obtained
from other than cases 4 and 6 did not have any mutation of 
p53 cDNA.

**Immunohistochemical analysis of the dog P53:** To examine accumulation of P53 protein, frozen sections of some samples (cases 1, 2, 4, 6, 13 and 15) of the dog tumors were immunostained with anti-P53 polyclonal antibody. Anti- gen-antibody complexes were visualized by FITC-conju- gated goat anti-rabbit antibody. Distinct positive reaction in 
the nucleus was observed in tumor tissue from case 4 (Fig. 4). In contrast, tumors obtained from cases 1, 2, 6, 13 and 
15 did not show any positive reactions against the anti-P53 antibody (Fig. 4 and Table 1).

**DISCUSSION**

In this study, recombinant canine (rc) P53 was expressed in 
*E. coli* as a GST fusion protein using pGEX expression 
system. In order to confirm the antigenic properties of the 
GST-rcP53 fusion protein, immunoblot analysis was per- 
fomed using an anti-human P53 polyclonal antibody. The

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**Table 1.** 
*p53* mutation, immunoblot and P53 protein expression in the various tumor dogs

<table>
<thead>
<tr>
<th>Cases</th>
<th><em>p53</em> mutations</th>
<th>Immunoblot</th>
<th>P53 protein expression</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>GST-P53</td>
<td>GST</td>
</tr>
<tr>
<td>1 Melanoma</td>
<td>No mutation</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>2 Sertoli cell tumor, interstitial cell tumor</td>
<td>No mutation</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>3 Oligodendroglioma</td>
<td>No mutation</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4 Malignant myoepithelioma</td>
<td>CGC to CAC, Arg162 to His</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>5 Liomyosarcoma</td>
<td>No mutation</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6 Mast cell tumor</td>
<td>CCC to TCC, Pro266 to Ser</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>7 Malignant mammary gland tumor</td>
<td>No mutation</td>
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<td>–</td>
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<td>8 Malignant mammary gland tumor</td>
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<td>9 Malignant mammary gland tumor</td>
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<tr>
<td>10 Malignant mammary gland tumor</td>
<td>No mutation</td>
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<tr>
<td>11 Benign perianal gland tumor</td>
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<tr>
<td>12 Benign perianal gland tumor</td>
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<td>–</td>
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<tr>
<td>13 Malignant perianal gland tumor</td>
<td>No mutation</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>14 Hepatocellular carcinoma</td>
<td>ND</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>15 Hepatocellular carcinoma</td>
<td>ND</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>16 Osterosarcoma</td>
<td>ND</td>
<td>–</td>
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</tr>
</tbody>
</table>

ND: Not determined, NS: Non specific bands.
anti-human antibody reacted with GST-rcP53 but not with control GST. With consideration of the denatured state of proteins in SDS-PAGE, the data suggest that the GST-rcP53 protein purified in this study conserves linear antigenic determinants.

To explore the possibility of anti-P53 antibodies in tumor dogs, the immunoblot analysis was performed using the patient sera as primary antibodies and the purified GST-rcP53 fusion protein as an antigen. Out of 16 serum samples obtained from various tumors, four samples showed positive reaction with the GST-rcP53 fusion protein. In human patients with various cancers, a humoral immune response against P53 has been postulated [14, 17, 22–24, 30, 31]. Several investigations have shown a strong correlation between the presence of anti-P53 antibody, the accumulation of P53 protein in the tumor tissue, and mutations in the p53 gene, suggesting that the presence of anti-P53 antibody would be a hallmark of the p53 mutation [30]. A possible mechanism has been postulated that accumulated P53 caused by mutation is released from tumor cells through necrosis, and leads over time to an immunoreaction against this nuclear protein [14, 17, 23]. In the present study, case 4, which showed the band of GST-rcP53 without GST by the immunoblot analysis had a point mutation of the p53 cDNA resulting in a single amino-acid substitution and represented nuclear accumulation of P53 protein. These data strongly suggest that the accumulation of mutant P53 also induces the specific humoral response and produces antibodies against P53 protein in the dog.

In contrast, case 6 was also found to have a miss-sense point mutation resulting in a single amino-acid substitution, but did not show anti-P53 antibodies in the immunoblot analysis. In addition, tumor cells of case 6 did not show the accumulation of P53 protein by the immunostaining. Since we used the anti-human P53 polyclonal antibody (FL-393, Santa Cruz Biotechnology) as a primary antibody for the immunofluorescence, there is a possibility that this antibody could not recognize canine P53. However, this antibody has been shown to react with the P53 proteins of human, mouse and rat (see manufacturer’s instruction). Because phylogenic analysis of the P53 amino-acid sequences of human, mouse, rat and dog shows that canine P53 is more similar to human P53 than murine or rat P53 [26, Okuda, M. unpublished data], this antibody seems to react with canine P53. These data may suggest that mutations of p53 do not always cause the accumulation of P53 and do not induce antibodies against P53. This observation is consistent with previous observation in human tumors [22]. It has been postulated that development of the immune response against mutant P53 may depend on the mutant P53 complexes with a 70 kilodalton heat shock protein (HSP 70) on the basis that the anti-P53 antibodies were associated with specific mutation in exon 5 or 6 which produce proteins known to be associated with HSP 70 [5]. The mutant P53 binding with HSP 70 has been shown to have a longer half-life [8]. Moreover, HSP 70 has been found to induce Th1 cytokines and targets immature dendritic cell precursors to enhance antigen uptake [28]. Because HSP 70 released from dying tumor cells has found to be taken up directly into dendritic cells, and to be involved in direct chaperoning of antigens into the dendritic cells [28], this HSP 70 activities may also be related with antigenicity of the P53 mutations in exon 5 or 6. Indeed, mutations in exon 5 or 6 are more likely to produce the immunogenic proteins than those in exon 7 or 8 in human hepatocellular carcinomas [22]. Our finding that mutations of case 4 with the humoral response and case 6 without the humoral response were found in exons 5 and 8, respectively, is not discordant with this hypothesis, and suggests that anti-P53 antibodies may also associated with specific mutations in exon 5 or 6 in dog tumors.

Although sera from other three cases (1, 2 and 13) also reacted with GST-rcP53 recombinant protein in immunoblotting, mutations of p53 and the accumulation of P53 protein was not detected in these three cases. Especially, the clear band of GST-rcP53 was detected in serum of case 1 similar to case 4, but the band of GST was also detected. Moreover, no mutation was detected in case 1. These findings may suggest the possibility that the serum from case 1 contains the antibodies, which nonspecifically reacted with GST fusion partner of GST-rcP53 fusion protein instead of rcP53. The faint band of GST-rcP53 with nonspecific bands other than GST-rcP53 or GST was detected in case 2, in which no mutation was observed. The faint band of GST-rcP53 was also detected in case 13 without p53 mutation. The accumulation of P53 proteins by immunostaining was
not detected in case 2 or 13. Although it seems to be insufficient that the negative staining for the P53 protein by immunofluorescence indicates the absence of the P53 accumulation, these data may suggest that the faint band of GST-rcP53 might be non-specific reaction. In fact, in human cases, there are few exceptions that serum samples obtained from patients with non-malignant diseases such as chronic pancreatitis and some autoimmune diseases react with P53 antigen in immunoblot analysis [1, 13, 18]. Moreover, the anti-creatitis and some autoimmune diseases react with P53 antibodies, using more clinical samples.

In the present study, the anti-P53 antibodies were detected using purified GST-rcP53 fusion protein in the dog with tumor having p53 mutation in exon 5, which produce proteins known to associate with HSP70 (case 4). In contrast, the anti-P53 antibodies could not be detected in the case with p53 mutation in exon 8 (case 6). Interestingly, the accumulation of the mutant P53 was found in case 4 but not in case 6. Although precise mechanism of the production of antibodies against P53 has not been elucidated yet, we assume that some type of p53 mutations such as the mutations in exon 5 may cause the accumulation of P53 protein and induce antibodies against P53 in dogs with tumors. Further analysis is necessary to determine what types of p53 mutations would result in the production of the anti-P53 antibodies, using more clinical samples.

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