The Significance of p53 and Retinoblastoma Pathways in Canine Hemangiosarcoma

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ABSTRACT. To investigate whether inactivation of the p53 and retinoblastoma (Rb) protein pathways contributes to the development of canine hemangiosarcoma (HSA), we examined immunohistochemically the expression of p53, Rb, phosphorylated Rb (phospho-Rb), p16, and cyclin D1 in 39 spontaneous canine HSAs and 10 hemangiomas. In addition, mutations in the p53 gene were analyzed by polymerase chain reaction (PCR)-single-stranded conformation polymorphism and PCR direct sequencing; furthermore, we quantified cyclin D1 mRNA by semiquantitative real-time reverse transcription-PCR. Positive immunoreactivity for p53 was observed in 17.9% of HSAs.

KEY WORDS: canine hemangiosarcoma, cyclin D1, p16, p53, Rb.

Cell growth and division involve complicated pathways that are governed by competing growth inhibitors and promoters. Protooncogenes encode proteins that promote cell cycle progression, proliferation, and survival of normal cells. In contrast, tumor suppressor genes encode proteins that inhibit cellular proliferation and promote cell death [47]. An imbalance between proliferation and inhibition results in malignant transformation of the neoplastic cells; this imbalance arises as a result of aberrations in these cell growth-associated genes. Abnormalities in the retinoblastoma (Rb) and p53 tumor suppressor genes have been frequently detected in human cancers. The p53 gene encodes a nuclear protein that is integral for the maintenance of DNA integrity and is a cornerstone of the DNA repair machinery [26]. Induction of p53 can result in apoptosis or growth arrest in cells with irreparable DNA damage [26]. The Rb susceptibility gene RB-1 encodes a protein that regulates the transition from G1 phase to S phase of the cell cycle [25, 48]. The tumor suppressor gene p16 is believed to encode a negative regulatory protein that controls the progression of eucaryotic cells through G1 phase of the cell cycle by interacting with CDK4 and inhibiting its kinase activity [6]. In the absence of a functional p16 protein, CDK4 binds to cyclin D and phosphorylates Rb, which stimulates entry of the cell into S phase [38]. Functional inactivation of the p53 and/or Rb pathways in neoplasms generally leads to greater resistance to apoptosis and the likelihood of accumulation of additional mutations that contribute to tumor progression. There are several reports on the high incidence of p53 mutations in human cancers, including hemangiosarcoma (HSA) [2, 4, 20, 29, 30, 34, 39, 42]. Similarly, Rb has also been found to be inactivated in many human cancers due to hyperphosphorylation of the Rb protein [49]. Therefore, it is important to analyze the abnormalities present in these negative cell cycle-regulating pathways for understanding the mechanisms involved in carcinogenesis.

The pathogenesis of canine HSA—a clinically aggressive disease with poor prognosis—is not completely understood. However, the recent studies have elucidated the role of growth regulation genes in the development of this disease [9, 12, 32, 35]. Although some researchers reported the occurrence of abnormal p53 protein expression and p53 mutations in canine HSA [12, 32, 35], the role of the Rb pathway in the development of canine HSA has not been investigated.

In this study, we aimed to determine whether the p53 and Rb pathways are dysfunctional in canine HSA and hemangiom. Therefore, we investigated the following: expression of p53, Rb, phosphorylated Rb (phospho-Rb), p16, and cyclin D1 by using immunohistochemical analysis; aberrations in the p53 gene by using polymerase chain reaction (PCR)-single-stranded conformation polymorphism (SSCP) and PCR direct sequencing analysis; and overexpression of cyclin D1 mRNA by semiquantitative reverse transcription (RT)-PCR.

MATERIALS AND METHODS

Samples and classification of canine vascular neoplasms: A total of 39 HSAs and 10 hemangiomas were examined. All samples were collected between 1998 and 2006 from the Veterinary Teaching Hospital of Gifu University and from...
the private animal hospitals. The samples were removed surgically, immediately fixed in 10% neutral buffered formalin, and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (HE) by using routine methods. The diagnosis of HSA or hemangioma was confirmed by reviewing the HE-stained slides. Subsequently, serial sections of all putative vascular neoplasms were confirmed by immunostaining to ascertain the expression of the von Willebrand factor; a specific antibody (anti-von Willebrand factor rabbit antibody; Dako Cytomation, Glostrup, Denmark) was used for this purpose. Eight of the 39 HSAs and 2 of the 10 hemangiomas were frozen in liquid nitrogen and stored at −80°C until further use.

**Immunohistochemical analysis of p53, Rb, phospho-Rb, p16, and cyclin D1:** Immunohistochemical staining was performed for p53, Rb, phospho-Rb, p16, and cyclin D1. For antigen retrieval, the sections were immersed in the Target Retrieval Solution (Dako Cytomation) and heated at 121°C for 15 min in an autoclave, except in the case of cyclin D1 where the sections were heated at 121°C for 30 min. The staining consisted of a first stage incubation with the following primary antibodies: p53 (CM-1, which recognizes the wild and mutant forms of the p53 protein; Novocastra Laboratories Ltd., Newcastle, UK); Rb (G3-245, which recognizes an epitope between amino acids 332–344 of the human Rb1 protein; BD Biosciences, San Jose, CA); phospho-Rb (Ser807/811, which facilitates estimation of the endogenous levels of Rb only when it is phosphorylated at serine 807/811; Cell Signaling Technology, Beverly, MA); p16 (F-12; Santa Cruz Biotechnology, Santa Cruz, CA); and cyclin D1 (Ab-1, clone DCS-6; Lab Vision Co., Fremont, CA). In the negative control, the primary antibody was omitted and replaced with phosphate-buffered saline (PBS). The sections were then incubated at room temperature for 30 min with the appropriate secondary antibodies (EnVi

**Quantification of the immunohistochemical results:** The immunoreactivity of p53 was assessed using a grading system based on the percentage of positive nuclei in the tumor cells (labeling index, LI). The staining was graded as follows: 0, tumors with no nuclear staining; 1, tumors with <10% LI; 2, tumors with 10%–50% LI; and 3, tumors with >50% LI. The specimens that were assigned scores of 2 or 3 were considered to be positive [12]. The immunoreactivities of Rb, phospho-Rb, and cyclin D1 were also assessed by using a grading system based on the LI. The staining was graded as follows: 0, tumors with <1% LI; 1, tumors with <15% LI; 2, tumors with 15%–30% LI; and 3, tumors with >30% LI. The immunoreactivity of p16 was assessed based on the positive and negative immunoreactivities within the neoplastic cells. Low immunoreactivity, which was indi-

**Analysis of p53 gene mutations:** Approximately 25 mg of the paraffin-embedded tissues of 17 canine HSA samples were scraped off the block, deparaffinized twice in xylene, and rinsed twice with absolute ethanol. DNA was extracted with the DNeasy Tissue Kit (Qiagen, Valencia, CA) in accordance with the manufacturer’s instructions. When frozen samples were used, total RNA was extracted from 8 frozen HSA samples and 1 frozen sample of the normal spleen (located adjacent to the neoplasm) by using the RNeasy Mini Kit, in accordance with the manufacturer’s guidelines (Qiagen); the spleen was confirmed to be nonneoplastic histopathologically. cDNA was synthesized using ThermoScript reverse transcriptase (Invitrogen, Carlsbad, CA). Sequences of p53 in the promoter region (genomic DNA only) and at exons 4, 5, 5–6, 6–7, and 8 were analyzed by PCR-SSCP and PCR direct sequencing methods. For all amplifications, the PCR mixture comprised 1 × PCR reaction buffer (15 mM Tris-HCl (pH 8.0) and 50 mM KCl), 1.5 mM MgCl2, 0.5 µM of each primer pair, 250 µM of each dNTP, and 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). After activating the AmpliTaq Gold DNA polymerase, amplifications were performed under the following conditions: denaturation (1 min at 95°C), annealing (30 sec), and extension (1 min at 72°C) for 40 cycles, except in the case of the promoter region (35 cycles). The annealing temperature was 55°C for the promoter region and 60°C for exons 4, 5, 5–6, 6–7, and 8. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to assess DNA integrity. The DNA encoding GAPDH was amplified for 30 cycles (15 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C) by using the primers described previously by Grone et al. [15]. The PCR primer sets and product sizes are shown in Table 1. All primers were canine-specific sequences [31, 33, 43]. The products of the PCR reaction were analyzed on a 1.5% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

**PCR fragments generated from the amplification of p53 exons 4–8 and the promoter region were analyzed by SSCP.** A mixture containing 1.5 µl of the PCR product (approximately 20 ng of DNA) and 3.5 µl SSCP dye (0.025% xylene cyanol, 0.025% bromophenol blue, 95% formamide, and 100 mM NaOH) was prepared for each PCR product in a total volume of 6.5 µl. This mixture was heated for 5 min at 95°C in order to denature the double-stranded DNA and then chilled on an ice-water bath before applying it to the gel. Then, 6 µl of this mixture was loaded into each well. A precast, 15 × 15 cm, 0.1-cm-thick GeneGel SSCP gel or a GeneGel Clean 15/24 gel was used with the Amersham Biosciences GeneGel SSCP Buffer Kit (Amersham Biosciences, Uppsala, Sweden). Optimal buffer temperatures of 12°C–20°C were empirically determined for the promoter region fragments and p53 exons 4, 5, 5–6, 6–7, and 8. Gels were run using GenePhor (Amersham Biosciences) depending on the fragment size and buffer temperature, and...
these were then stained with the DNA Silver Staining Kit (Amersham Biosciences). The SSCP running conditions are shown in Table 2.

Following PCR, the products were purified using the QIAquick PCR Purification Kit (Qiagen) to remove salts, primers, and dNTPs. The purified PCR products were sequenced on an ABI Prism 3100 Genetic Analyzer by using the BigDye Terminator ver. 3.1 Kit (Applied Biosystems), in accordance with the manufacturer’s instructions. The DNA sequences were analyzed using the ABI sequencing analysis software.

**Semiquantitative real-time RT-PCR analysis for cyclin D1 gene expression:** Using the RNeasy Mini Kit in accordance with the manufacturer’s protocol (Qiagen), total RNA was extracted from 7 frozen HSA samples and 2 frozen hemangioma samples. Real-time RT-PCR amplifications using DNase I (amplification grade; Invitrogen)-treated tRNA were performed in an ABI PRISM 7100 system by using the QuantiTect SYBR Green RT-PCR Kit (Qiagen). The primers used in the real-time RT-PCR reaction were the same as those used in the QuantiTect Primer Assays (Qiagen: Cf GAPDH_1_SG and Cf CCND1_1_SG based on the Genbank sequences with accession nos. NM_001003142 and NM_001005757, respectively).

**Statistical analysis:** Significant differences between the LIs of cyclin D1 and phospho-Rb were analyzed by Spearman’s rank correlation coefficient.

**RESULTS**

Data on animal patients and expression of p53, p16, Rb, phospho-Rb, and cyclin D1 are summarized in Table 3.

**Normal immunoreactivities of p53, Rb, phospho-Rb, and cyclin D1 in the normal epidermis:** In the immunohistochemical analysis of Rb, phospho-Rb, and cyclin D1 in the normal epidermis, a positive reaction was observed in the nucleus. Rb-positive cells were observed in the basal and suprabasal layers of the epidermis (Fig. 1a). In contrast, phospho-Rb-positive cells were observed only in the basal layer (Fig. 1b), and p16-positive cells were observed throughout the layer except in the keratinized layer (Fig. 1c). Cyclin D1-positive cells were limited to the basal layer (Fig. 1d). The p53 protein was seldom detected in the normal skin.

**Immunohistochemistry and p53 mutations in canine vascular tumors:** Nuclear staining of p53 was observed in the tumor cells of canine HSAs (Fig. 1e). Seven of the 39 samples (17.9%) showed positive immunoreactivity. On the other hand, all the hemangioma samples showed negative immunoreactivity (Fig. 1i). To determine whether the canine HSA cells harbored mutant p53 genes, we analyzed the p53 gene by using the PCR-SSCP and PCR direct sequencing methods. Mutation-associated SSCP band shifts were not observed, and the sequences of the promoter region and exons 4–8 were identical to the published wild-type sequence for the canine p53 gene in all the p53-immunonegative HSA samples.

**Immunohistochemistry of Rb and phospho-Rb:** Nuclear staining of Rb and phospho-Rb was observed in the canine vascular tumor cells (Fig. 1f). The antibody against the Rb protein used in this study could not distinguish between nonphospho- and phospho-Rb; therefore, all vascular tumor samples showed a higher LI than that of phospho-Rb. The...
means of the LI of Rb and phospho-Rb in HSAs were 53.0% (±13.8 S.D.) and 27.3% (±9.8 S.D.), respectively. Further, the LI of Rb and phospho-Rb were markedly higher in all the canine HSA samples than in the hemangioma samples. The immunostaining of phospho-Rb revealed that of the 39 samples of canine HSA, 15 were grade 3 (Fig. 1f), 17 were grade 2, and 7 were grade 1. On the other hand, all the hemangioma samples were grade 0 (Fig. 1j).

Immunohistochemistry of p16: Immunostaining of p16 showed that 32 of the 39 HSA samples (82%) showed a loss of or low immunoreactivity in the tumor cells (Fig. 1g). In the case of hemangioma samples, the p16 protein was detected in all cases that showed pronounced staining of the tumor cells (Fig. 1k).

Immunohistochemistry and semiquantitative RT-PCR of cyclin D1: Immunostaining of cyclin D1 revealed that of the 39 canine HSA samples, 15 were grade 3 (Fig. 1h), 17 were grade 2, and 7 were grade 1. The mean value was 25.6% (±9.4 S.D.). On the other hand, all the hemangioma samples were grade 0 (Fig. 1l). In this study, we used a real-time RT-PCR to detect the cyclin D1 level in canine HSAs and hemangiomas. In 4 of the 7 canine HSA samples, the expression level of cyclin D1 mRNA was 10-fold higher than that of GAPDH mRNA (range, 73.96-
Further, in 2 hemangiomas and 3 HSAs, the expression level of cyclin D1 mRNA was 3-fold lower than that of GAPDH mRNA.

**Relationship between the expression of phospho-Rb and cyclin D1 in canine HSAs:** Of the 15 grade 3 cyclin D1 samples (determined immunohistochemically), 8 (53.3%) were grade 3 upon phospho-Rb staining, while 6 (40.0%) and 1 were grades 2 and 1, respectively. Of the 17 (23.5%) grade 2 cyclin D1 samples, 4 were grade 3 upon phospho-Rb staining, while 9 (52.9%) and 4 (23.5%) were grades 2 and 1, respectively. Of the 7 (42.8%) grade 1 cyclin D1 samples, 3 were grade 3 upon phospho-Rb staining, while 2 (28.6%) samples each were grade 2 and grade 1 (Fig. 2). Of the 39 samples, 27 (69.2%) were grade 2 and 3 upon staining with cyclin D1 and phospho-Rb, respectively. Although there were no significant differences between the LIs of cyclin D1 and phospho-Rb, the grades of phospho-Rb tended to increase with an increase in the grade of cyclin D1.

**Relationship between the expression of p16 and cyclin D1:** Five of the 7 samples in the grade 1 group, 12 of the 17 samples in the grade 2 group, and all the samples in the grade 3 group of cyclin D1 showed a loss of or low immunoreactivity within the HSA tumor cells. In contrast, all the hemangioma samples were grade 0 upon cyclin D1 staining but showed pronounced immunoreactivity for p16 within the tumor cells.
The p53 tumor suppressor gene plays an important role in cell cycle progression, regulation of gene expression, and cellular response mechanisms to DNA damage [44]. The p53 gene is the most frequently altered gene in human malignant tumors [27, 40], and it has also been implicated in the development of cancers in companion animals, for example, in canine osteosarcoma [23, 28, 33, 43]. Many studies have reported the presence of p53 mutations in human HSA; these mutations are associated with exposure to chemicals [20, 29, 30, 39]. Previous studies have investigated the presence of p53 mutations in canine HSA [32, 35], and of a total of 19 cases, only 1 was reported to have a p53 mutation [32]. Gamblin et al. detected p53 protein accumulation in canine HSAs based on the immunohistochemical analysis [12]. However, to the best of our knowledge, there is no report that has analyzed the correlation between protein accumulation and genetic aberrations of p53 in canine vascular neoplasms. The staining of more than 10% of tumor nuclei has been associated with vascular neoplasms. The staining of more than 10% of protein accumulation and genetic aberrations of p53 are no report that has analyzed the correlation between protein accumulation and genetic aberrations of p53 in canine vascular neoplasms. The staining of more than 10% of tumor nuclei has been associated with vascular neoplasms. The staining of more than 10% of protein accumulation and genetic aberrations of p53 are no report that has analyzed the correlation between protein accumulation and genetic aberrations of p53 in canine vascular neoplasms.

Based on the previous studies, we considered a case to be positive when its LI was more than 10%. In our study, p53 protein accumulation was detected in 17.9% of the canine HSA samples. However, mutations were not detected in any of our HSA samples. The majority of the p53 mutations reported in human and canine tumors are clustered in 4 of the 5 evolutionarily highly conserved domains, the so-called “hot spot” regions for p53 mutations, that are localized in exons 5–8. Further, the immunohistochemical analysis revealed that mutant proteins that have a mutation in the “hot spot” region accumulated [5, 14, 45]. Therefore, to determine whether the canine HSA cells harbored mutant p53 genes, we analyzed exons 4–8 of the p53 gene by using the PCR-SSCP and PCR direct sequencing methods. The results suggest that the p53 protein, which was detected by the immunohistochemical staining in this study, was of the wild type and inactivation of the p53 pathway is associated with the pathogenesis of canine HSA to a slight extent. The significance of accumulation of the wild-type p53 could not be elucidated in this study. Although further investigations on canine vascular neoplasms are required, it is possible that p53 protein accumulation may be a result of alternative mechanisms that lead to p53 protein stabilization. The major factors contributing to p53 stabilization are the expression of p14ARF and inhibition of MDM2-mediated degradation [46].

The Rb family regulates cell proliferation and differentiation [7, 17, 21, 24, 48]. Under the nonneoplastic conditions, the Rb protein regulates cell cycle transition by controlling the availability and activity of specific members of the E2F family of transcription factors [48]. Inactivation of the Rb protein by phosphorylation and the subsequent release of E2F are controlled during the mid-G1 phase by D-type cyclins and the cyclin-dependent kinases (cdks) 4 and 6 [36, 48]. In response to the growth-inhibiting signals, a cdk inhibitor (cki) such as p16 blocks Rb phosphorylation and prevents DNA synthesis. In the normal epidermis, the cells in the basal and suprabasal layers were Rb-positive. In contrast, phospho-Rb-positive cells and cyclin D1-positive cells were present only in the basal layer, which is a continuous cell growth zone. Cells positive for p16 were observed throughout the epidermis. To our knowledge, there are no previous reports in which immunohistochemical staining was carried out in fixed and embedded skin tissues using these antibodies. The observation that the immunoreactivities varied depending on the cell proliferation status agrees with the findings in the normal bladder mucosa of rats [8]. These staining patterns suggested that in canines, the cell cycle may be regulated by the Rb pathway. In canine hemangiomas, intense immunoreactivity for p16 was observed; however, the LI for Rb, phospho-Rb, and cyclin D1 were very low. These results suggest the possibility that p16 inhibited Rb protein phosphorylation by suppressing cyclin D1 expression, and consequently downregulating cell proliferation. Unlike hemangiomas, many tumor cells showed positive immunoreactivity for Rb, phospho-Rb, and cyclin D1 in canine HSAs. Furthermore, 4 of the 7 canine HSA samples showed a high level of cyclin D1 mRNA expression, and the immunohistochemical grades of phospho-Rb tended to increase with an increase in the grade of cyclin D1. These results suggest that cyclin D1 overexpression may cause hyperphosphorylation of the Rb protein. The significance of Rb phosphorylation at different sites is suggested by the observation that Rb function may be modulated according to the location at which it is phosphorylated [16]. Hyperphosphorylation at serine 780, serine 807/811, and threonine 821/826 appears to be involved in the disruption of E2F binding to Rb [16]. Therefore, the phospho-Rb-positive cells in our study may not be capable of binding to E2F. In addition, all grade 3 and many grade 2 cases of cyclin D1 showed a loss of or low p16 immunoreactivity. These results suggest the possibility that the loss of p16 expression may cause overexpression of cyclin D1; however, the relationship between the loss of p16 expression and cyclin D1 overexpression warrants further study. Human cancers frequently contain mutations that inactivate the Rb pathway either by decreasing the inhibitory activity of Rb or p16, or by deregulating the activity of cyclin D or cdk4 [1, 3, 18, 22]. Reduced or no expression of p16 and cyclin D1 overexpression and subsequent hyperphosphorylation of the Rb protein were the most frequent abnormalities identified in the Rb pathway of canine HSAs in this study. These results suggest that alteration of the p16-cyclin D1-Rb pathway may be associated with the pathogenesis of canine HSA. The p16 gene is inactivated by mutations or abnormal DNA hypermethylation in the promoter region of the p16 gene, resulting in transcriptional silencing in tumors that have no p16 mutations [13, 19, 37, 41]. The loss of expression of certain proteins in most canine HSAs suggests that either of the down-regulation mechanisms is possible. The mechanism of p16 inactivation will be the focus of our...
future studies.

In conclusion, our data suggest that alterations in the components of the Rb pathway, rather than the inactivation of the p53 pathway, may be associated with the malignant growth of canine HSAs. Further studies on the genetic basis for the deregulation of the Rb pathway are required.

REFERENCES


