J Vet Sci. 2021 Sep;22(5):e61
https://doi.org/10.4142/jvs.2021.22.e61
pISSN 1229-845X·eISSN 1976-555X

Journal of Veterinary Science

Original Article
Pathology

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Arginase-1 and P-glycoprotein are downregulated in canine hepatocellular carcinoma

Soo-Hyeon Kim, Byung-Joon Seung, Seung-Hee Cho, Ha-Young Lim, Min-Kyung Bae, Jung-Hyang Sur *

Department of Veterinary Pathology, Small Animal Diagnostic Center, College of Veterinary Medicine, Konkuk University, Seoul 05029, Korea

OPEN ACCESS

Received: Mar 6, 2021
Revised: May 24, 2021
Accepted: Jun 20, 2021
Published online: Jul 12, 2021

***Corresponding author:**
Jung-Hyang Sur
Department of Veterinary Pathology, Small Animal Diagnostic Center, College of Veterinary Medicine, Konkuk University, Gwangjin-gu, Seoul 05029, Korea.
E-mail: jsur@konkuk.ac.kr

ORCID iDs
Soo-Hyeon Kim
https://orcid.org/0000-0002-1662-2651
Byung-Joon Seung
https://orcid.org/0000-0001-9611-2426
Seung-Hee Cho
https://orcid.org/0000-0001-7823-5000
Ha-Young Lim
https://orcid.org/0000-0003-4347-3062
Min-Kyung Bae
https://orcid.org/0000-0001-8654-1761
Jung-Hyang Sur
https://orcid.org/0000-0003-4496-7582

ABSTRACT

Background: Hepatocellular carcinoma is the most common primary hepatic malignancy in humans and dogs. Several differentially expressed molecules have been studied and reported in human hepatocellular carcinoma and non-neoplastic liver lesions. However, studies on the features of canine hepatocellular carcinoma are limited, especially related to the differential characteristics of neoplastic and non-neoplastic lesions.
Objectives: The study's objective was 1) to examine and evaluate the expression of arginase-1, P-glycoprotein, and cytokeratin 19 in canine liver tissues and 2) to investigate the differential features of hepatocellular carcinomas, liver tissue with non-neoplastic lesions, and paracancerous liver tissues in dogs.
Methods: The expression levels of three markers underwent immunohistochemical analysis in 40 non-neoplastic liver tissues, 32 hepatocellular carcinoma tissues, and 11 paracancerous liver tissues. Scoring of each marker was performed semi-quantitatively.
Results: Arginase-1 and P-glycoprotein were significantly downregulated in hepatocellular carcinoma, compared with hepatic tissues with non-neoplastic diseases ($p < 0.001$). Expression levels of arginase-1 and P-glycoprotein were also significantly lower in hepatocellular carcinoma than in paracancerous liver tissues (arginase-1, $p = 0.0195$; P-glycoprotein, $p = 0.047$). Few cytokeratin 19-positive hepatocytes were detected and only in one hepatocellular carcinoma and one cirrhotic liver sample.
Conclusions: The results of this study suggest that downregulation of arginase-1 and P-glycoprotein is a feature of canine hepatocellular carcinoma; thus, those markers are potential candidates for use in differentiating hepatocellular carcinomas from non-neoplastic liver lesions in dogs.

Keywords: Arginase; dog; hepatocellular carcinoma; liver; P-glycoprotein

INTRODUCTION

Hepatocellular carcinoma is a primary malignant neoplasm originating from the liver and occurs in both dogs and humans [1-3]. In dogs, tumors arising from the liver comprise 0.3% of all neoplasms and hepatocellular carcinoma has a less than 0.05% incidence of autopsies

Funding

This research was supported by the Bio & Medical Technology Development Program of NRF funded by the Korean government, MSIT (2016M3A9B6903437).

Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Kim SH, Sur JH; Data curation: Kim SH, Seung BJ; Formal analysis: Kim SH; Funding acquisition: Sur JH; Investigation: Kim SH, Bae MK; Methodology: Cho SH, Lim HY; Project administration: Sur JH; Resources: Cho SH, Bae MK; Software: Kim SH, Lim HY; Supervision: Seung BJ, Lim HY; Validation: Kim SH; Visualization: Cho SH; Writing - original draft: Kim SH; Writing -review & editing: Kim SH, Sur JH.

[1,4]. Hepatocellular carcinoma in dogs shares several clinical aspects with humans [5], including mortality related to local invasiveness and metastasis [6].

Arginase-1 is an intrahepatic enzyme that cleaves arginine to ornithine and urea, and as ornithine has several physiological functions, including cell proliferation, its roles in various cancers have been the focus of study [7]. In addition, arginase-1 has been identified as a novel marker that may replace the HepPar-1 marker due to its higher sensitivity for non-neoplastic and cancerous hepatocytes in humans [8]. Lower expression of arginase-1 in human hepatocellular carcinoma than in paracancerous liver tissue in humans has been reported [9], but it has never been investigated in canine liver tissues.

P-glycoprotein is an ATP-binding cassette transporter that regulates intracellular molecular concentrations by pumping out their substrates. It is expressed in normal liver tissue and protects the host from xenobiotics [10]. Overexpression of P-glycoprotein is associated with chemoresistance against several anticancer drugs such as doxorubicin, paclitaxel, and vincristine in transformed cells [11]. Immunohistochemical analyses of P-glycoprotein expression in human and canine hepatocellular carcinoma have shown assorted staining patterns and conflicting results, including downregulation [12-16] and upregulation [17], compared with those in liver tissues with non-neoplastic lesions.

Cytokeratin 19 is a cytoplasmic intermediate filament family member and is typically expressed in biliary, pancreatic, and renal ductular cells [18]. Aberrant expression of cytokeratin 19 in hepatocytes is considered a precancerous indication [19], and it has been considered a potential prognostic marker for cancer aggressiveness and postoperative recurrence in humans [20,21]. A recent study revealed that the expression of cytokeratin 19 enhances tumor progression and invasion in relation to PDGFRα-LAMB1 pathway [22]. In addition, upregulation of cytokeratin 19 in aggressive canine hepatocellular carcinoma with both local spread and distant metastasis has been reported [23].

Investigations showing molecular features of canine neoplastic or non-neoplastic hepatocytes are uncommon, even though hepatocellular carcinoma is often a fatal disease in dogs [24,25]. The aim of the present study is to evaluate and compare the expression levels of arginase-1, P-glycoprotein, and cytokeratin 19 in canine liver tissues with HCC, canine normal/paracancerous liver tissues, and canine hepatic tissues with non-neoplastic diseases.

MATERIALS AND METHODS

Sample collection and histological analysis

Archived liver samples submitted to the department of veterinary pathology in Konkuk University from 2014 to 2019 were used for the analysis. The formalin-fixed, paraffin-embedded samples in the archive were stained with hematoxylin-and-eosin. At first, hematoxylin-and-eosin-stained sections were evaluated according to the WSAVA liver disease guidelines [26,27]. The liver samples containing at least 6 portal triads were included in this study; 72 liver tissues were included and analyzed.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were cut into 4 μm-thick sections, deparaffinized, and dehydrated using an ethanol gradient. To block endogenous peroxidase activity, sections

Table 1. Information on and protocols for primary antibodies

Primary antibody	Supplier	Clone	Antigen retrieval	Dilution	Incubation
Arginase-1	BioVision	Rabbit polyclonal	Tris-EDTA, 8 min (Microwave)	1:800	4°C, overnight
P-glycoprotein	GeneTex	JSB-1	Citric acid, 15 min (Autoclave)	1:300	4°C, overnight
Cytokeratin 19	Origene	OTI3F8	Tris-EDTA, 5 min (Microwave)	1:2,500	4°C, overnight

were immersed in 3% hydrogen peroxide for 20 minutes. Antigen retrievals for arginase-1 and cytokeratin 19 were performed by heating in a microwave oven (750 W) for various durations (**Table 1**) in a citric acid solution (pH 6.0) or a Tris-EDTA solution (pH 9.0). For antigen retrieval of P-glycoprotein, sections were autoclaved in the citric acid solution (pH 6.0) for 15 minutes at 121°C. To prevent non-specific binding, sections were incubated with 5% normal goat serum for 30 minutes. The sections were subsequently incubated with primary antibodies (**Table 1**) followed by incubation with secondary antibodies for 40 minutes at room temperature. The slides were subsequently visualized with a 3,3′-diaminobenzidine solution, counterstained with Gill's hematoxylin, dehydrated using an ethanol gradient, and cleared with xylene.

Controls for immunohistochemistry

To validate the reactivity of each antibody for canine use, examination of external controls was performed for tissues with known/predicted positivity or negativity [28]. As arginase-1 and cytokeratin 19 levels in normal human liver tissue are typically enriched [29], canine normal liver tissue was used for the positive control. The external positive control for P-glycoprotein was created by applying a primary antibody to canine hemangiopericytoma tissue [30]. For the negative control, primary antibodies were applied to tissues with identified or predicted negativity, including cutaneous histiocytoma, plasmacytoma, and leiomyoma were also conducted [29,30]. Additional negative controls were prepared by applying rabbit polyclonal antibody and isotype-specific-Mouse IgG1-immunoglobulin instead of primary antibodies to liver tissues.

Evaluation of immunohistochemistry slides

Immuno-stained slides were analyzed by applying semi-quantitative methods. Evaluation criteria are described in **Table 2** [17,21,31]. We selected 10 random high-power fields for one assessment and only evaluated hepatocytes that were not undergoing necrosis or apoptosis and excluded inflammatory cells, biliary epithelial cells, and fibroblasts.

Statistical analysis

Semi-quantitative scoring data were analyzed using GraphPad Prism 9 (GraphPad Software, USA). The Kolmogorov-Smirnov test was used to assess the data distribution. As the data did

Table 2. Marker evaluation criteria for immunohistochemical assays

Marker	Evaluation criteria
Arginase-1	Staining intensity: 0 (negative), 1+ (weak), 2+ (moderate), 3+ (strong) Number of positive cells: focal (< 10%), patchy (10–50%), diffuse (>50%)
P-glycoprotein	Number of positive cells: 0 (no staining, staining in less than 10%) 1+ (11–50%) 2+ (51–90%)
Cytokeratin 19	Number of positive cells: 0 (< 5%), 1 (5–24%), 2 (25–74%), 3 (≥ 75%)

https://doi.org/10.4142/jvs.2021.22.e61

not follow a normal distribution, the Mann-Whitney U test was used to compare expressions of arginase-1, P-glycoprotein, and cytokeratin 19 in liver tissues with hepatocellular carcinoma or non-neoplastic diseases. In addition, the Wilcoxon rank-sum test was applied to analyze the differential expression of 3 markers in hepatocellular carcinoma and paracancerous liver tissues.

RESULTS

Histopathological evaluation

A total of 72 liver samples were examined, including 40 samples classified as liver tissue with non-neoplastic lesions and 32 samples classified as hepatocellular carcinoma. The non-neoplastic lesions comprised chronic hepatitis (n = 23), acute hepatitis (n = 10), hepatic cirrhosis (n = 4), polycystic liver (n = 2), and normal liver with focal glycogen degeneration (n = 1). In 34.3% (11/32) of the hepatocellular carcinoma samples, paracancerous normal liver tissues were observed adjacent to the hepatocellular carcinoma.

Controls for immunohistochemistry

While normal hepatocytes showed definite positivity to the arginase-1 antibody (**Supplementary Fig. 1A**) in normal canine liver tissue, none of the cells in canine leiomyoma, histiocytoma, or plasmacytoma showed positivity to arginase-1 (**Supplementary Fig. 1B-D**). P-glycoprotein was observed in the bile canaliculus of normal liver tissue (**Supplementary Fig. 2A**) and in the tumor cell surface of hemangiopericytoma (**Supplementary Fig. 2B**) as reported in a previous study [30]. Positivity to P-glycoprotein was not detected in cutaneous histiocytoma or plasmacytoma (**Supplementary Fig. 2C-D**). Immunoreactivity for cytokeratin 19 was shown in biliary epithelial cells in normal liver tissue (**Supplementary Fig. 3A**) and apocrine ductal glandular cells (**Supplementary Fig. 3B**). However, smooth muscle cells in leiomyoma, cutaneous histiocytoma, and plasmacytoma were negative for cytokeratin 19 (**Supplementary Fig. 3B-D**). In isotype-specific negative controls, none of the hepatocytes among the 72 samples included in this study displayed positivity.

Immunohistochemical analysis of arginase-1

Arginase-1 showed cytoplasmic and nuclear staining patterns, both of which were considered positive (**Fig. 1**). Overall scores for the number of positive cells and for staining intensity of arginase-1 were significantly downregulated in hepatocellular carcinomas compared with liver tissues with non-neoplastic lesions (**Fig. 2A**; $p < 0.001$). Moreover, arginase-1 expression, measured by the number of positive cells ($p < 0.001$) and staining intensity ($p = 0.004$), was downregulated. In 20 of the 32 cases of hepatocellular carcinoma (62.5%) and 39 of 40 cases of liver tissues with non-neoplastic lesions (97.5%), more than 50% of hepatocytes were arginase-1-positive. Decreased expression of arginase-1 was also significant in hepatocellular carcinomas compared with those in paracancerous liver tissues ($p = 0.0195$; **Figs. 1E-F**, **2B**).

Immunohistochemical analysis of P-glycoprotein

A relatively regular canalicular staining pattern in hepatic sinusoids was detected in non-neoplastic lesions (**Fig. 3A**). In hepatocellular carcinomas, the canalicular pattern was also the predominant staining pattern observed; however, membranous staining was also observed. Expression of P-glycoprotein was significantly lower (**Fig. 4A**; $p < 0.001$) and much more irregular and intermittent in hepatocellular carcinomas than in non-neoplastic liver lesions. Less than 10% of hepatocytes showed P-glycoprotein positivity in 46.9% (15/32) of

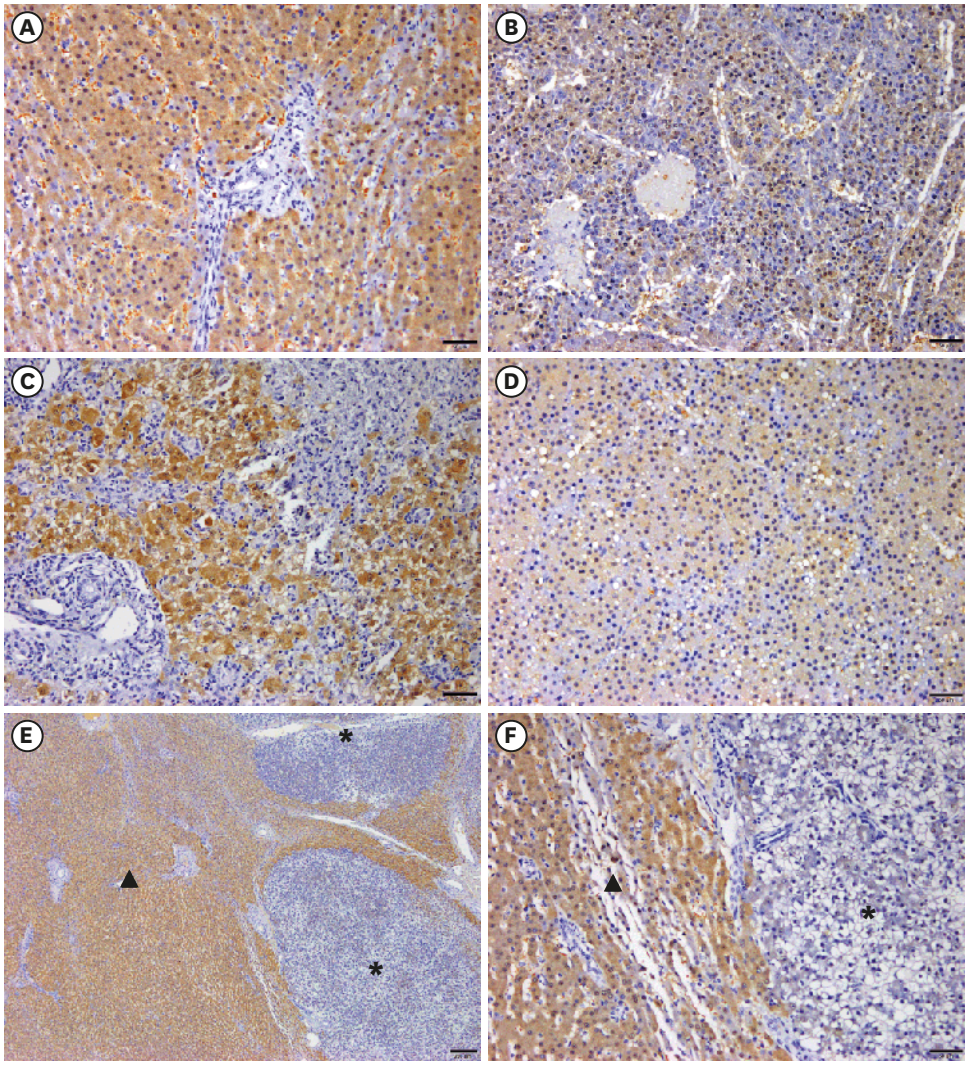

Fig. 1. Immunohistochemistry for arginase-1. (A) Chronic hepatitis. Non-neoplastic hepatocytes show intense nuclear and cytoplasmic staining pattern for arginase-1. Bar = 50 μm. (B) Hepatocellular carcinoma. Decreases in the number of arginase-1 positive cells and staining intensity are remarkable. Bar = 50 μm. (C) Hepatic cirrhosis. Intense positivity to arginase-1 is observed in hepatocytes, not in inflammatory cells or fibroblasts. Bar = 50 μm. (D) Hepatocellular carcinoma. The proportion and staining intensity of positive cells are decreased in neoplastic hepatocytes. Bar = 50 μm. (E) Hepatocellular carcinoma. Obvious contrast between paracancerous tissue (▲) and hepatocellular carcinoma (*) is shown. Bar = 200μm (F) Higher magnification view of Fig. 1E. Arginase-1 loss is significant in cancerous hepatocytes (*) compared with that in paracancerous normal hepatocytes (▲). Bar = 50 μm.

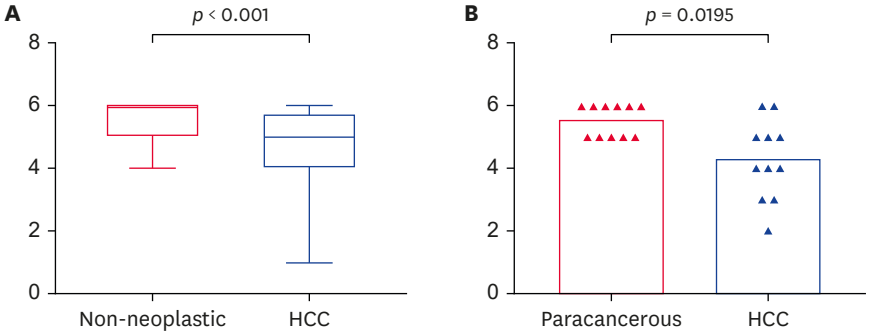

Fig. 2. Graphs displaying arginase-1 expression. (A) Box plot displaying differential expression of arginase-1 in non-neoplastic liver and hepatocellular carcinoma tissues. Expression level of arginase-1 is significantly downregulated in hepatocellular carcinoma compared to non-neoplastic liver tissues. (B) Bar chart comparing arginase-1 expression in paracancerous and hepatocellular carcinoma tissues. Arginase-1 is notably less expressed in hepatocellular carcinoma compared with paracancerous tissues. Individual rhombus (♦) represents one sample.

https://doi.org/10.4142/jvs.2021.22.e61

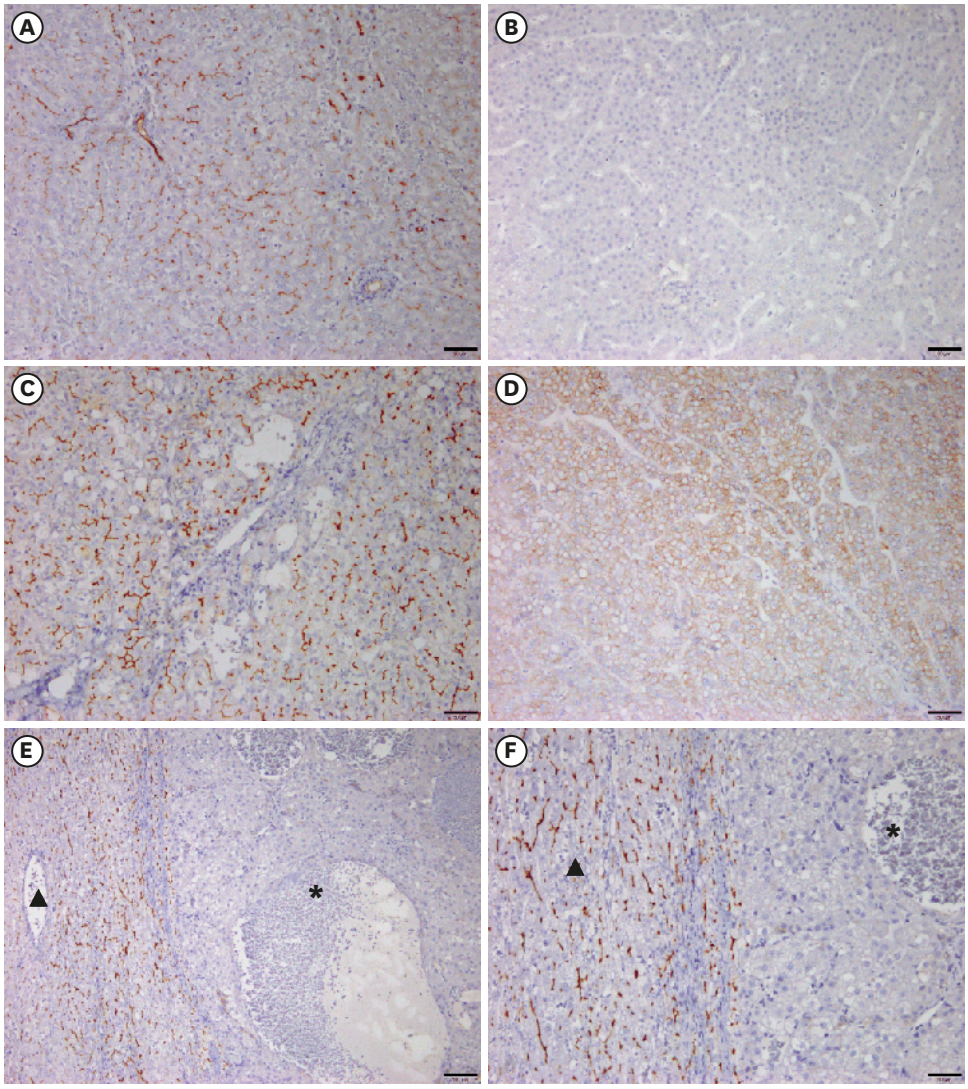

Fig. 3. Immunohistochemistry for P-glycoprotein. (A) Chronic hepatitis. Regular positivity to P-glycoprotein in the apical surface of bile canaliculus is prominent. Bar = 50 μm. (B) Hepatocellular carcinoma. Complete loss of P-glycoprotein expression is observed. Bar = 50 μm. (C) Hepatic cirrhosis. A relatively regular canalicular staining pattern is preserved despite steatosis and hepatitis. Bar = 50 μm. (D) Hepatocellular carcinoma. Over 50% of hepatocytes show membranous or canalicular staining. Bar = 50 μm. (E) Hepatocellular carcinoma. Loss of P-glycoprotein is distinct in cancerous nodule (*) compared to that in paracancerous tissue (▲). Bar = 100 μm. (F) Higher magnification view of Fig. 3E. Cancerous hepatocytes rarely express P-glycoprotein and that expression has less intensity (*) than that in paracancerous normal hepatocytes (▲). Bar = 50μm

hepatocellular carcinoma samples (**Fig. 3B**), even though 31.3% (10/32) of hepatocellular carcinomas displayed extensive membranous positivity in more than 50% of hepatocytes (**Fig. 3D**). In contrast, even in non-neoplastic liver tissues with hepatic cord disarray from severe hepatitis and steatosis, a canalicular staining pattern for P-glycoprotein was relatively reserved (**Fig. 3C**). Downregulation of P-glycoprotein was also remarkable in hepatocellular carcinomas compared to paracancerous normal liver tissues ($p = 0.047$; **Figs. 3E-F**, **4B**).

Immunohistochemical analysis of cytokeratin 19

Cytokeratin 19 exhibited a moderate to intense cytoplasmic staining pattern in every normal and hyperplastic biliary epithelial cell in all samples. However, 70/72 samples (97.2%) did not have any cytokeratin 19-positive hepatocytes. The few cytokeratin 19-positive hepatocytes were observed only in 1 hepatic cirrhosis sample and 1 hepatocellular carcinoma sample (**Fig. 5**).

https://doi.org/10.4142/jvs.2021.22.e61

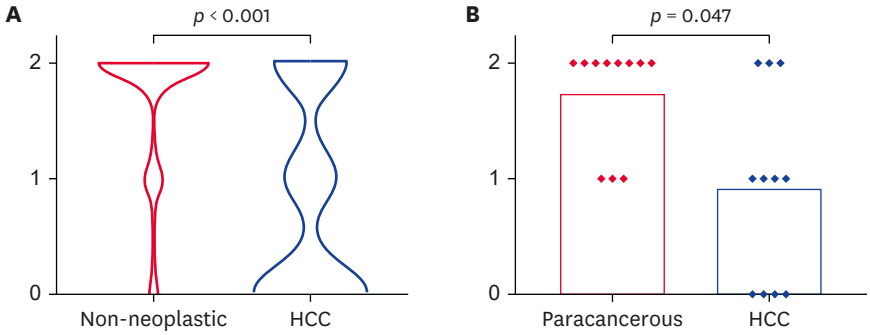

Fig. 4. Graphs showing P-glycoprotein expression in non-neoplastic, paracancerous liver tissue and hepatocellular carcinoma. (A) Truncated violin plot comparing P-glycoprotein expression in non-neoplastic liver tissue and hepatocellular carcinoma. Decreased level of expression of P-glycoprotein in hepatocellular carcinoma is significant. (B) Bar charts displaying P-glycoprotein levels in paracancerous tissue and hepatocellular carcinoma. The level of P-glycoprotein is remarkably lower in hepatocellular carcinomas than in paracancerous tissues. One rhombus (♦) represents one sample.

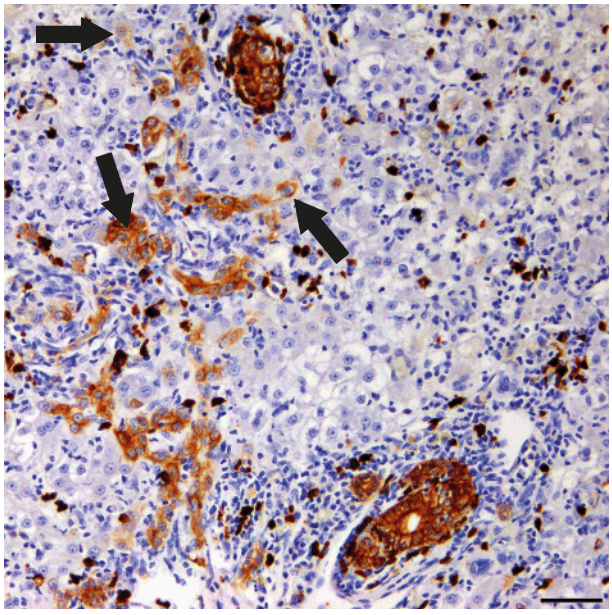

Fig. 5. Immunohistochemical results for cytokeratin 19. Hepatic cirrhosis. Only a few hepatocytes display cytokeratin 19 positivity (arrows). Bar = 20 μm

DISCUSSION

This study demonstrated that arginase-1 and P-glycoprotein are downregulated in hepatocellular carcinomas compared to hepatic tissues with non-neoplastic lesions or paracancerous liver tissues in dogs. These results are consistent with those in previous studies in humans [9,13,17,32], although conflicting results have been reported [12,33]. Thus, it could be suggested that the downregulation of arginase-1 and P-glycoprotein is a feature in canine hepatocellular carcinoma.

Hepatitis and liver cirrhosis are precancerous lesions in humans [34,35], but the correlation between these inflammatory lesions and hepatocellular carcinoma has not been verified in dogs. Hepatitis occurs mainly as a result of chronic hepatitis B virus or hepatitis C virus infection in humans [36,37]. In dogs, viral hepatitis induced by canine adenovirus type 1 has

been observed; however, canine adenovirus type 1 is widely prevented by vaccination supplied as part of a core vaccine program [38], and most canine hepatitis is considered idiopathic. Accordingly, hepatocytes with adenoviral inclusion bodies were not present in all samples of the present study. These etiological differences make it more difficult to verify whether hepatitis or cirrhotic liver are precancerous lesions in dogs. In the present study, arginase-1 and P-glycoprotein levels could not be compared between healthy liver, non-inflammatory liver lesion, hepatitis, cirrhotic liver, and hepatocellular carcinoma samples because the number of normal healthy liver, non-inflammatory liver lesion, and cirrhotic liver samples were insufficient for statistical analysis. Therefore, it could not be evaluated whether the immunohistochemical features of hepatitis and hepatic cirrhosis are closer to hepatocellular carcinoma or normal healthy liver, making it difficult to explore the association of hepatitis / hepatic cirrhosis with precancerous lesions in dogs.

Hepatitis induced by hepatitis B virus displays downregulation of arginase-1, whereas hepatitis C virus-related hepatitis overexpresses arginase-1 [32,39], and silencing arginase-1 inhibits the hepatocellular growth-stimulating effect of the hepatitis C virus [32]. Through the disruption of arginase-1, viral hepatitis may progress to hepatocellular carcinoma in humans. However, the true contribution of arginase-1 in hepatitis to malignant progression in dogs is questionable due to etiological differences from humans. Both prospective and retrospective studies, including more normal and inflammatory, cirrhotic, and cancerous liver samples, are required to identify the contribution of arginase-1 to malignant progression in canine hepatocellular carcinoma.

Additionally, the results of the present study suggest that arginase-1 is a highly sensitive and specific marker in canine hepatocytes, similar to those in humans [40]. We applied hepatocyte paraffin antigen 1 (HepPar-1), which is a representative marker for hepatocytes, to all samples to compare the specificity and sensitivity of arginase-1 for canine hepatocytes (data not shown). In HepPar-1-stained tissues, several lipid or glycogen-degenerated hepatocytes had weak or no positivity; however, arginase-1 showed distinct nuclear positivity, even in severely lipid- or glycogen-degenerated hepatocytes. Further, arginase-1 positivity was not shown in any primary or metastatic non-hepatocellular carcinoma liver cancers (data not shown). Therefore, arginase-1 may be an effective marker for differentiating between clear-cell type hepatocellular carcinoma and clear-cell type carcinomas of other organs. Also, two samples were arginase-1-negative with strong HepPar-1 positivity. Thus, the combined application of arginase-1 and HepPar-1 could be useful when one of those results is negative.

Due to advances in veterinary medicine and the central role of histopathology in the diagnosis of liver diseases, including hepatocellular carcinoma, an increasing number of canine liver biopsy samples are submitted to veterinary pathologists. However, the accurate diagnosis of liver disease remains challenging and can be affected by multiple factors, such as the number of biopsied sites [41], liver sampling technique and sample size [42], and interobserver variability [43]. Diagnosis under a microscope is often difficult owing to (1) morphological resemblance of well-differentiated hepatocellular carcinoma and non-neoplastic or pre-neoplastic lesions and (2) a lack of validated molecular data [44,45]. Although the accurate diagnosis of small biopsy samples is challenging, veterinary clinicians may be reluctant to excise large samples, given the potential for complication [42]. In this respect, examination of arginase-1 and P-glycoprotein levels may assist in differentiating hepatocellular carcinoma from non-neoplastic lesions, especially in small tissue samples.

The discrepancy between the results of studies reporting on P-glycoprotein may be attributed to differences in the clonality of the primary antibodies, targeting different epitopes of P-glycoprotein used in each study. For example, the JSB-1 clone used in the present study specifically detects MDR1 but not MDR3, whereas the C219 clone detects both MDR1 and MDR3 gene products [46]. In fact, a variable labeling index of P-glycoprotein was identified in canine tissues for primary antibodies with different clonality [30]. Another explanation for the discrepancy may be the small number of hepatocellular carcinoma samples in the previous study [18] and the present study, making it difficult to identify consistent trends in P-glycoprotein expression in canine liver. Further studies with standardized materials and large sample sizes may establish the contribution of these two markers to malignant transformation in the canine liver, and elucidate the ability of the markers to differentiate hepatocellular carcinoma from non-neoplastic or pre-neoplastic lesions.

Cytokeratin 19-positive cancerous hepatocytes have been described in human and dogs. In humans, cytokeratin 19 positivity in hepatocellular carcinoma is related to a poor prognosis, based on extrahepatic metastasis, disease-free survival, overall survival rates, and early postoperative recurrence [21,47]. Although our samples included 1 highly pleomorphic hepatocellular carcinoma and 2 hepatocellular carcinoma samples from dogs that succumbed to disseminated metastasis, cytokeratin 19 was not detected in these samples. The reason for the lack of cytokeratin 19-positivity in these samples is unknown; however, it is possible that cytokeratin 19-positive hepatocellular carcinoma is a relatively rare subtype, as reported in 4 of 34 samples in a previous canine study [47]. In addition, the intense positivity of cytokeratin 19 in cholangiocarcinoma and its total negativity for hepatocellular carcinoma in the present study indicate that this marker may be useful for differentiating poorly differentiated hepatocellular carcinoma from cholangiocarcinoma.

Herein, we identified that decreased expression levels of arginase-1 and P-glycoprotein are features of canine hepatocellular carcinoma. Further studies involving larger sample sizes and a wider range of liver lesions are required to investigate immunohistochemical markers in dogs and to improve our understanding of the pathogenesis of canine hepatocellular carcinoma.

ACKNOWLEDGMENTS

We thank Mrs. Eun-Mi Yu for preparing tissue slides.

SUPPLEMENTARY MATERIALS

Supplementary Fig. 1

External and internal controls for arginase-1 in canine tissues. (A) Normal liver, external positive control. All hepatocytes in the field are presenting intense arginase-1-positivity. Bar = 50 μm. (B) Leiomyoma, negative control. Neoplastic smooth muscle cells are negative for arginase-1, contrasting reactivity to normal liver tissue. Bar = 100 μm. (C) Cutaneous histiocytoma, negative control. Proliferating histiocytes are negative for arginase-1. Bar = 50 μm. (D) Cutaneous plasmacytoma, negative control. Positive signal is not observed in plasmacytoma tissue. Bar = 50 μm.

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Supplementary Fig. 2

External and internal controls for P-glycoprotein in canine tissues. (A) Normal liver, external positive control. The clear positivity is shown in bile canaliculus. Bar = 50 μm. (B) Hemangiopericytoma, external positive control. Positivity in the cell surface of neoplastic pericytes is detected. Bar = 50 μm. (C) Cutaneous plasmacytoma, negative control. none of the neoplastic plasma cells show positivity. Bar = 50 μm. (D) Leiomyoma, negative control. Smooth muscle cells are totally negative for P-glycoprotein. Bar = 50 μm.

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Supplementary Fig. 3

External and internal controls for cytokeratin 19 in canine tissues. (A) Normal liver, external positive control. Biliary epithelial cells in normal liver tissue show distinct positivity to cytokeratin 19 (arrow). Bar = 50 μm. (B) Leiomyoma, negative control. Apocrine glandular cells are clearly positive for cytokeratin 19 (arrow), while smooth muscle cells are negative. Bar = 50 μm. (C) Cutaneous histiocytoma, negative control. None of the neoplastic histiocytes show reactivity to cytokeratin 19. Bar = 50 μm. (D) Cutaneous plasmacytoma, negative control. Positive signal is not observed in the tissue. Bar = 50 μm.

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