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Comparative Analysis of mRNA Expression of Surface Antigens between Histiocytic and Nonhistiocytic Sarcoma in Dogs

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Background: Definitive diagnosis of histiocytic sarcoma (HS) in dogs is relatively difficult by conventional histopathological examination because objective features of HS are not well defined.

Hypothesis: Quantitative analysis of mRNA expression of selected cellular surface antigens (SAs) specific to HS in dogs can facilitate objective and rapid diagnosis.

Animals: Dogs with HS (n = 30) and dogs without HS (n = 36), including those with other forms of lymphoma (n = 4), inflammatory diseases (n = 6), and other malignant neoplasias (n = 26).

Methods: Retrospective clinical observational study. Specimens were collected by excisional biopsy, needle core biopsy, or fine needle aspiration. To determine HS detection efficacy, mRNA expression levels of selected SAs specific to HS in dogs, including MHC class IIα, CD11b, CD11c, and CD86, were quantitatively analyzed using real-time quantitative polymerase chain reaction.

Results: Each SA mRNA expression level was significantly higher in HS dogs than in non-HS dogs ($P = .0082$). Cutoff values for discriminating between HS and non-HS dogs based on these expression levels were calculated on the basis of receiver-operating characteristic analysis. Accuracy of the cutoff values, including MHC class IIα, CD11b, CD11c, and CD86, was 87.9, 86.4, 86.4, and 84.8%, respectively.

Conclusions and Clinical Importance: Our results suggest that quantitative analysis of mRNA expression of the selected SAs could be an adjunctive diagnostic technique with high diagnostic accuracy for HS in dogs. Substantial investigation is required for exclusion of diseases with similar cell types of origin to lymphoma.

Key words: Cancer; CD11b; CD11c; CD86; Diagnosis; MHC class IIα.

Histiocytic sarcoma (HS) in dogs is a malignant neoplasia with progressive behavior. HS in dogs is classified into 2 categories according to the cell type of origin; one is localized or disseminated HS of dendritic cell (DC) origin, and the other is hemophagocytic HS (HHS) of macrophage origin.[1,2] Diagnosis of HS in dogs is often very difficult by conventional histopathological examination because the majority of cases demonstrate undifferentiated morphology with extensive inflammatory or necrotic lesions.[3–9] In the past, HS in dogs had been comprehensively evaluated by immunohistochemical (IHC) staining using antibodies, including supportive diagnostic markers such as ionized calcium-binding adaptor molecule 1, iysozyme, and vimentin, as well as conventional histopathological findings by hematoxylin-eosin staining and several other types of clinical examination.[5,10–13] Cellular surface antigens (SAs), including MHC class II, CD11b, CD11c, CD11d, CD18, and CD204, are expressed on HS cells in dogs, and IHC staining using monoclonal antibodies to these antigens has become established as a definitive diagnostic method for HS in dogs.[1,2,14] Of these antigens, CD11d is known to be specifically expressed in HHS cells of macrophage origin, and antibodies to CD11d can detect HHS with high precision.[2,12] However, this antibody is not widely available for commercial diagnostic use, and a frozen section is required for this examination. Regardless of the commercial availability of these antibodies, IHC staining requires refined techniques, subjective assessment of dye-affinity as well as a sufficient sample volume. Real-time quantitative polymerase chain reaction (PCR), which can quantitatively detect target gene expression even in small-volume samples, is used clinically for the detection of genes related to various kinds of diseases, including malignant neoplasms.[15–17] The analysis procedure requires a relatively short time, and the analysis results in objective data with a high degree of reproducibility.[15,16] However, this analysis must be performed immediately after sample

Abbreviations:

CI	confidence intervals
DC	dendritic cell
FNA	fine needle aspiration
HHS	hemophagocytic histiocytic sarcoma
HS	histiocytic sarcoma
IHC	immunohistochemical
PCR	polymerase chain reaction
ROC	receiver-operating characteristic
SA	surface antigen

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collection, because the total RNA in samples required for analysis is unstable and decays in a time-dependent fashion manner if an RNA preservative solution is not used.[15,16]

The aim of this study was to comparatively analyze mRNA expression levels of MHC class IIα, CD11b, CD11c, and CD86 between HS and other diseases in dogs, which resemble the cytological appearance and signs of HS in dogs. This study was designed as a retrospective clinical observational study for the assessment of the utility of an alternative method using real-time PCR to differentiate HS from these diseases in dogs.

Materials and Methods

Sample Collection and Handling

Histopathologically confirmed samples from 30 dogs with HS (hereafter referred to as HS dogs) and 36 non-HS dogs, including dogs with inflammatory diseases (n = 6), lymphoma, (n = 4) and other malignant neoplasms (n = 26), were used for this study. Samples were collected in the veterinary teaching hospital of Hokkaido University between October 2009 and April 2012. The following cases were excluded from this study: the cases with inflammatory disease related to malignant neoplasms, and the case affected by multiple different malignant neoplasms or other diseases. HS in dogs samples were classified as either those which demonstrated hemophagocytic signs or those which did not; the classification was performed on the basis of the assessment, including radiography, blood test, blood chemical test, immunological test, cytology, and histopathological findings. Samples were collected by excisional biopsy, needle core biopsy, or fine needle aspiration (FNA) for histopathological examination and this genetic analysis. For histopathological evaluation, samples were stored in 10% formalin solution[a] at room temperature. For genetic analysis, samples were finely minced after collection, washed once in saline solution, and immediately treated with the RNeasy Mini Kit.[b] Then, the procedure for RNA isolation was followed. If the RNA isolation could not be performed immediately after sample collection, the minced samples were directly immersed in an RNA preservative solution[c] for 24 hours at 4°C, and then stored at −80°C until use.

Histopathological Diagnosis

Histopathological diagnoses were performed by board-certified pathologists (American College of Veterinary Pathologists) at a commercial laboratory. All HS in dogs were finally established by IHC staining using either an antibody-targeting MHC class II[d] or CD18[e] on the basis of the criterion of the pathologists. The recruited inflammatory diseases were confirmed to be completely remitted by medical treatment alone using anti-inflammatory and antibiotic agents after histopathological diagnosis, and the inflammation did not relapse for the period of this clinical observational study. Some of the recruited cases were elderly dogs (≥10 years old) in poor physical condition and at risk for the general anesthesia agents. Therefore, FNA and needle core biopsy, which are minimally invasive sampling procedures, were initially employed for these animals; these small-volume samples were used for histopathological examination and the genetic analysis. If a definitive diagnosis could not be established using these samples, the histopathological examination was repeated by the same pathologists using samples collected by surgical biopsy.

Assessment of the RNA Integrity

Total RNA was isolated using the RNeasy Mini Kit[b] according to the manufacturer's protocol. After isolation of total RNA, genomic DNA contamination was removed from the isolated RNA using recombinant DNase I[f] according to the manufacturer's recommended procedure. In brief, 50 μg of the isolated RNA, 5 μL of 10 × DNase I buffer,[f] 2 μL of recombinant DNase I (RNase-free),[f] and 20 U of ribonuclease inhibitor[f] were mixed; diethylpyrocarbonate (DEPC)-treated water[g] was added for a total reaction volume of 50 μL. The reaction mixture was incubated at 37°C for 30 minutes, and then stopped by the addition of 2.5 μL of 0.5 M EDTA[a]; DNase I was then inactivated by incubation at 80°C for 2 minutes. DEPC-treated water[g] was added for a total volume of 100 μL, and then 10 μL of 3 M sodium acetate and 250 μL of chilled ethanol[a] were added. The reaction was kept at −80°C for 20 minutes, and then centrifuged at 9,800 × *g* for 10 minutes at 4°C; the supernatant was removed. The precipitate was washed with chilled 70% ethanol, centrifuged at 9,800 × *g* for 5 minutes at 4°C, and the supernatant was then removed. The precipitate was dried at room temperature, and then dissolved with a suitable amount of DEPC-treated water. If the genomic DNA was not removed completely, the procedure was repeated with an increase in the amount of enzyme or extension of the reaction time. The integrity and purity of the RNA samples were determined by agarose gel electrophoresis and measuring the absorbance at 260 nm.

To prepare the 2% agarose gel for agarose gel electrophoresis, 1 g of UltraPure agarose-1000[h] was dissolved in 50 mL of deionized distilled water (DDW), heated until gelation, and then cooled to 60°C. Five μL of ethidium bromide[a] and 1 mL of 50x (Tris-acetate-EDTA) TAE buffer[a] were added, and the mixture was stirred. It was poured into a comb and cooled until solidification at room temperature. When the gel was completely solidified, it was removed from the comb. One microliter of 10x loading dye[f] and 6 μL of diH_2O were mixed with 2 μL of each denatured RNA sample, and then the RNA mixture and DNA ladder[f] were loaded into a well of the solidified gel. The gel was run at 100 V in an electrophoretic system[i] filled with 2 mL of 50x TAE buffer and 100 mL of DDW for 40 minutes. RNA bands were visualized using an ultraviolet transilluminator.[j] Intact RNA samples with a 2 : 1 ratio (28S : 18S rRNA bands) were determined by image analysis software.[k]

The absorbance of the RNA samples treated with DNase was measured at 260 nm with a spectrophotometer,[l] and the RNA concentration was calculated as previously reported.[18]

$$1\text{OD (optical density) at} A_{260} = 40\mu\text{g/mL ssRNA}$$

$$\text{RNA concentration}(\mu\text{g/mL}) = (\text{OD}_{260}) \times (\text{dilution factor}) \times (40\mu\text{g RNA/mL})/(1\text{OD}_{260}\text{unit})$$

$OD_{260/280}$ of pure RNA is generally 1.8–2.0.

Therefore, pure RNA samples were determined by the OD_{260}/OD_{280} ratio between 1.8 and 2.0.

Analysis of mRNA Expression Levels of SAs

After evaluation of the RNA integrity, reverse transcription for cDNA synthesis from the RNA samples was performed using Oligo dT primers and a Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RT) Kit[h] according to the manufacturer's

indication. All synthesized cDNA was adjusted to a concentration of 20 μg/mL. GAPDH was used as an internal control for PCR amplification. Primer sequences, including GAPDH, MHC class IIα, CD11b, CD11c, and CD86, which targeted the region that displays a high degree of homology, were designed using the Primer3[m] interface from GenBank[n] or previous reports[19] (Table 1). Real-time PCR was performed using a Rotor-Gene Q[o] with a PCR amplification reagent[p] according to the manufacturer's instructions. The cDNA samples were subjected to activation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 20 seconds, and annealing/extension at 60°C for 20 seconds. The selected SAs are expressed relatively at low levels in leukocytes, including as DCs and macrophages, and in normal tissues and organs in dogs.[20–23] Therefore, all SA expression levels were calibrated using organs or tissues of healthy dogs matching the location of the HS lesion.[24–26] All DNA fragments were extracted from the gel using Quantum Prep Freeze 'N Squeeze DNA gel extraction spin columns[q] according to the manufacturer's instructions, and were then subjected to Hokkaido System Science[r] for DNA sequence analysis. A homology search between target genes and PCR products was performed by a GenBank query using the basic local alignment search tool algorithm.[s] [27] Specificities of all PCR amplicons were confirmed by melting temperature curve analysis.

Statistical Analysis

Statistical analysis was carried out using the Mann-Whitney *U*-test with a standard computer software program,[t] and values of $P < .05$ were considered significant. From the comparative analysis result of mRNA expression, cutoff values, including MHC class IIα, CD11b, CD11c, and CD86, were calculated to discriminate HS from other disease in dogs recruited for this study; receiver-operating characteristic (ROC) analysis was used to determine the cutoff values.[28] The area under the ROC curve, sensitivity and specificity with 95% confidence intervals (95% CI), and the optimal cutoff value were computed by a computer software program.

Results

Clinical information (case, breed, age, sex, primary lesion location, and histopathological diagnosis) for the study animals was shown in Table 2. Sampling used for the genetic analysis was as follows: HS dogs (n = 30) included FNA (n = 16), needle core biopsy (n = 10), and excisional biopsy (n = 4). Inflammatory disease in dogs (n = 6) included needle core biopsy (n = 2) and excisional biopsy (n = 4). Lymphoma in dogs (n = 4) included FNA (n = 1), needle core biopsy (n = 2), and excisional biopsy (n = 1). Other malignant tumors in dogs (n = 26) included FNA (n = 6), needle core biopsy (n = 8), and excisional biopsy (n = 12). Eight of the 30 HS dogs could not be identified by initial histopathological examination using sampling of FNA (n = 6) or needle core biopsy (n = 2) because of undefinable or undifferentiated cell findings. Three of the 8 HS dogs could be identified by excisional biopsy, and the remaining 5 dogs were identified as primary lung HS at necropsy. Finally, 18 of the 30 HS dogs were established by IHC staining using an antibody-targeting MHC class II, and the remaining 12 HS dogs were established using anti-CD18 antibody.

In this study, 3 of the 30 HS dogs had primary spleen lesions, and showed severe regenerative anemia (PCV median: 16%, range: 12–18%) with many polychromasia erythrocytes and reticulocytes (Table 2). Two of the 3 HS dogs were negative for the Coombs test, which distinctly ruled out hemorrhagic and hemolytic anemia, or the presence of spherocytes in the 3 dogs. Therefore, the 3 dogs were judged to be cases of nonimmune-mediated and regenerative anemia. In addition, the 3 dogs showed hypoalbuminemia, and 2 of the 3 dogs showed thrombocytopenia. Cytological findings of the 3 dogs showed atypical giant and histiocytic cells, and histopathological findings showed abundant hemophagocytic cells as compared to the remaining 27 HS dogs. However, the 3 HS dogs could not be evaluated by IHC staining using anti-CD11d antibody. Survival times (median: 16 days, range: 12–18 days) of the 3 HS dogs were apparently shorter than those (median: 108 days, range: 36–242 days) of the remaining 27 HS dogs. Based on this background, the 3 dogs were classified as HS with hemophagocytic signs, which were mediated by HS (Table 2).

mRNA expression levels of MHC class IIα were significantly higher in HS dogs than in dogs with lymphoma ($P = .02$), inflammatory diseases ($P = .01$), or other malignant neoplasms ($P = .003$) (Fig 1). The cutoff value of MHC class IIα to identify HS dogs was calculated to be 118 using ROC analysis; the diagnostic reliability at this cutoff value had a sensitivity of 88% (95% CI, 70–92%), a specificity of 89% (95% CI, 74–96%), and an accuracy of 88% (Table 3).

Table 1. Primer pairs used for real-time PCR measurement of relative mRNA expression.

Target Gene	Amplicon Size (bp)	Nucleotide Position	Oligo	Sequence	Genbank No.
GAPDH	100	696–716	Forward	5′-TGTCCCCACCCCCAATGTATC-3′	NM001003142
		795–772	Reverse	5′-CTCCGATGCCTGCTTCACTACCTT-3′	
MHC class IIα	211	623–642	Forward	5′- CCTGAGGTTCCAACCCCTAT-3′	NM001011726
		833–814	Reverse	5′-GGTCCACTCTTCTGCTCTGG-3′	
CD11b	60	2,598–2,619	Forward	5′-GAGTCTGACGATTCCACTAATG-3′	XM843434
		2,657–2,639	Reverse	5′-GTTTATGCTGCAGCTGCTA-3′	
CD11c	154	101–123	Forward	5′-GTGCTGGATTTGGACACAGCGTG-3′	XM547049
		254–233	Reverse	5′-AAGGGGACCTGCAGTTGGATGG-3′	
CD86	221	6–30	Forward	5′-ATGTATCTCAGATGCACTATGGAAC-3′	NM001003146
		226–202	Reverse	5′-TTCTCTTTGCCTCTGTATAGCTCGT-3′	

Table 2. Clinical information of samples.

Case (n)	Breed (n)	Age (Year), Median (Range)	Sex (n)	Primary Focus (n)	Histopathological Diagnosis (n)
Histiocytic sarcoma: HS (27)	BMD (8)	9 (7–14)	F (14→15)	Joint (10)	
	FCR (7)		M (12)	Lung (10)	
	WC (8)			Liver (3)	
	GR (2)			Spleen (1)	
	Other (2)			Other (3)	
HS with hemophagocytic signs (3)	BMD (2)	11 (8–14)	F (2)	Spleen (3)	
	FCR (1)		M (1)		
Lymphoma (4)	BMD (1)	12 (8–14)	F (3)	Multicentric type (2)	B-cell type (2)
	GR (1)		M (1)	Cutaneous type (1)	T-cell type (2)
	WC (1)			Small bowel type (1)	
	MD (1)				
Other malignant tumors (26)	FCR (4)	8 (5–16)	F (14)	Liver (4)	Adenocarcinoma (3)
	BMD (3)		M (12)	Spleen (4)	Fibrosarcoma (2), GIST (2)
	GR (3)			Bone (3)	Hemangiosarcoma (2)
	LR (3)			Lung (3)	Hepatocarcinoma (2)
	Mix (3)			Oral cavity (3)	MCT (2), Melanoma (2)
	MS (3)			Skin (3)	Multiple myeloma (2)
	WC (3)			Bladder (2)	Osteosarcoma (2)
	SS (2)			Joint (2)	Plasmacytoma (2)
	Other (2)			Other (2)	SCC (2), TCC (2), MFH (1)
Inflammatory disease (6)	BMD (2)	5 (2–12)	F (4)	Joint (3)	Synovitis (2)
	FCR (2)		M (2)	Skin (2)	Granuloma (2)
	WC (2)			Liver (1)	Phlegmon (1)
					Hepatitis (1)

BMD, Bernese Mountain Dog; FCR, Flat-Coated Retriever; GR, Golden Retriever; LR, Labrador Retriever; MD, Miniature Dachshund; MS, Miniature Schnauzer; SS, Shetland Sheepdog; WC, Welsh Corgi; F, female; M, male; GIST, gastrointestinal stromal tumor; MCT, mast cell tumor; SCC, squamous cell carcinoma; TCC, transitional cell carcinoma; MFH, malignant fibrous histiocytoma.

mRNA expression levels of MHC class IIα in a limited number of cases, including lymphoma (n = 3) and synovitis (n = 1), exceeded the calculated cutoff value. In this study, 22 of the 30 HS dogs were evaluated by IHC staining using an antibody-targeting MHC class II; 18 of the 22 HS dogs were positive for MHC class II, and the remaining 4 HS dogs were negative. In addition, mRNA expression levels of MHC class IIα (median: 246, range: 106–398) in the 18 HS dogs that were MHC class II positive were higher than those (median: 112, range: 88–152) in the remaining 4 HS dogs that were negative.

mRNA expression levels of CD11b and CD11c were significantly higher in HS dogs than in dogs with lymphoma ($P = .02$ and $P = .03$, respectively), inflammatory diseases ($P = .01$ and $P = .02$, respectively), or other malignant neoplasms ($P = .001$ and $P = .002$, respectively) (Fig 1). The cutoff value for CD11b to identify HS dogs was calculated to be 89 using ROC analysis, for which the sensitivity, specificity, and accuracy were 87% (95% CI, 68–92%), 86% (95% CI, 66–91%), and 86%, respectively (Table 3). The cutoff value for CD11c was calculated to be 110, for which the sensitivity, specificity, and accuracy were 83% (95% CI, 66–91%), 89% (95% CI, 72–94%), and 86%, respectively (Table 3). mRNA expression levels of CD11b in the 3 dogs classified as HS with hemophagocytic signs were higher than in the remaining 27 HS dogs (Fig 1); however, the difference was not statistically significant. mRNA expression of CD11b in lymphoma (n = 2), multiple myeloma (n = 1), and granuloma (n = 2), and expression of CD11c in lymphoma (n = 4) exceeded the cutoff value.

mRNA expression levels of CD86 were significantly higher in HS dogs than in dogs with lymphoma ($P = .04$), inflammatory diseases ($P = .02$), or other malignant neoplasms ($P = .001$) (Fig 1). The cutoff value for CD86 was calculated to be 98, for which the sensitivity, specificity, and accuracy were 83% (95% CI, 65–90%), 86% (95% CI, 67–92%), and 85%, respectively (Table 3). mRNA expression levels of CD86 in lymphoma (n = 2), multiple myeloma (n = 2), and plasmacytoma (n = 1) exceeded the cutoff value; however, levels of CD86 (median: 282, range: 198–346) were significantly higher in the 5 HS dogs on which autopsies were performed than in the remaining 25 HS dogs (median: 178, range: 48–288; $P = .03$).

Discussion

In this study, the accuracy of diagnosis of HS in dogs based on the genetic analysis was ≥84%. This analysis requires intact RNA samples, and all samples had to be immediately and rigorously processed to isolate intact RNA. In addition, samples that contained a high percentage of necrotic or inflammatory cells could not be used for this analysis because of reduced diagnostic accuracy. Therefore, the quality of samples should be confirmed to some extent by cytological

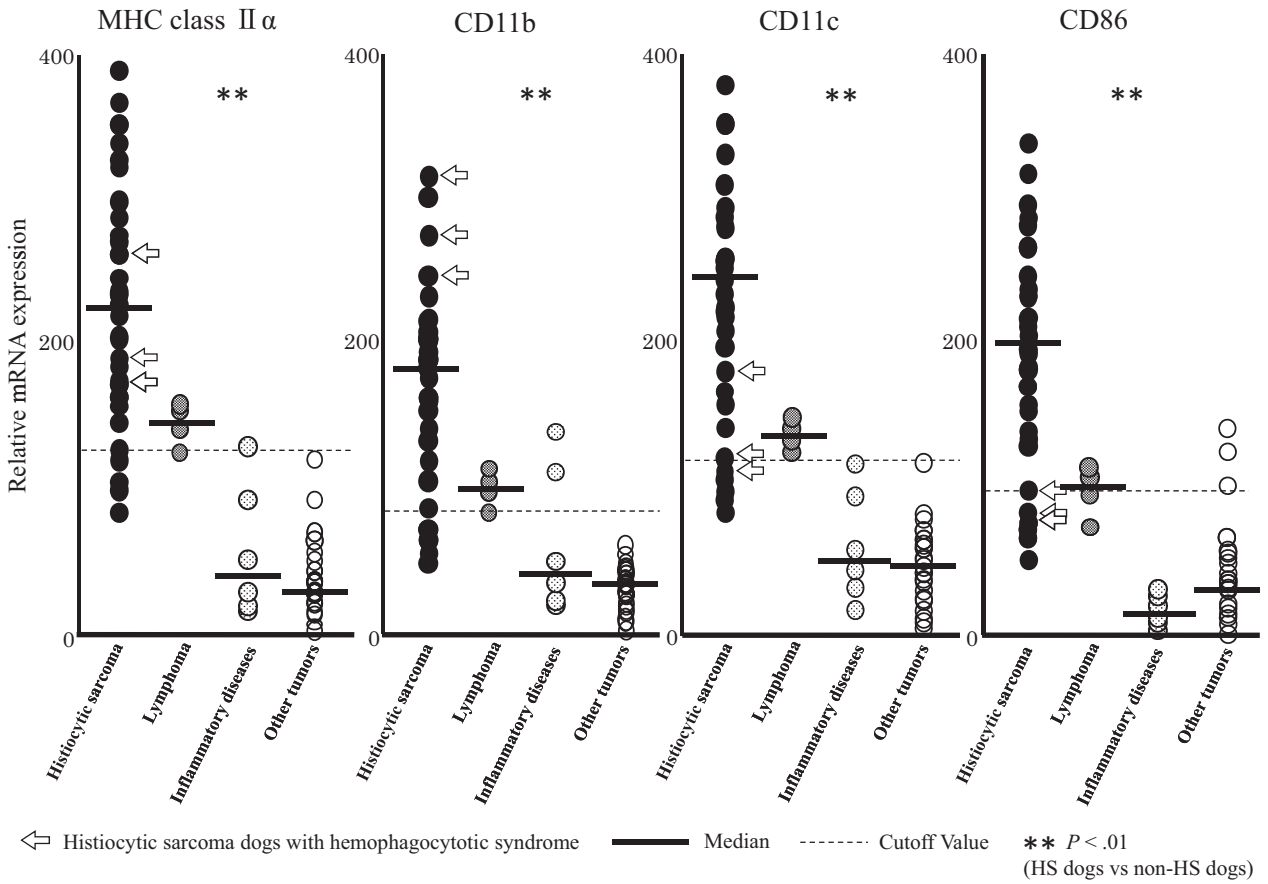

Fig 1. Comparative analysis of relative mRNA expression levels of surface antigens (SAs; MHC class IIα, CD11b, CD11c, and CD86) between histiocytic sarcoma (HS) dogs (n = 30) and non-HS dogs (n = 36), including lymphoma (n = 4), inflammatory diseases (n = 6), and other malignant neoplasms (n = 26). mRNA expression levels of each SA were significantly higher in HS dogs than in non-HS dogs ($P = .0082$). All mRNA expression levels from organs or tissues of primary lesion of HS dogs were calibrated with those from organs or tissues of healthy dogs matching the location of the HS lesion.

Table 3. Diagnostic accuracy of HS in dogs calculated by ROC analysis.

SA	AUC	Sensitivity (%)	Specificity (%)	Accuracy (%)
MHC class IIα	0.90	86.7	88.9	87.9
CD11b	0.87	86.7	86.1	86.4
CD11c	0.88	83.3	88.9	86.4
CD86	0.85	83.3	86.1	84.8

HS, histiocytic sarcoma; ROC, receiver-operating characteristic; SA, surface antigen; AUC, area under the receiver-operating characteristic curve.

evaluation before RNA isolation. The cytological diagnosis of HS in dogs had 97% specificity and sensitivity of 44%, when compared with a standard histopathological evaluation with IHC staining using anti-CD18 antibody.[8] Therefore, a combination of this genetic analysis and cytological diagnosis might further improve the diagnostic accuracy.

Histiocytic sarcoma in dogs often displays undifferentiated and nonspecific morphology, including a wide range of inflammatory cell infiltration and necrotic lesions.[1–9] It is sometimes difficult to diagnose HS in dogs by conventional histopathological examination without definitive IHC markers. A previous study reported that 51% (18/35) of the dogs originally diagnosed with synovial tumors were reclassified as HS after reexamination with additional IHC staining using antibody-targeting CD18.[9] In this study, 27% (8/30) of HS dogs could not be determined by the initial histopathological examination. When histopathological examination remained uncertain, genetic analysis using real-time PCR might be useful as a secondary evaluation method for cases where HS in dogs are suspected. Therefore, it might show promise as a supportive diagnostic method that can supplement several weak points of histopathological examination, including subjective assessment and the need for special techniques and equipment.[15–17]

It was postulated that MHC class IIα is specifically expressed in histiocytic cells, and could be useful for the comprehensive detection of histiocytic diseases in dogs.[1,2] The genetic analysis indicated that MHC class IIα mRNA expression levels were significantly higher in HS dogs than in non-HS dogs, and the diagnostic reliability showed an accuracy of 87%. This value was

superior to the diagnostic reliability previously reported for other methodologies for diagnosing HS in dogs.[8] MHC class IIα was expressed in particular leukocytes, including lymphoid cells, DCs, macrophages, and other antigen-presenting cells.[20,22] The present results showed that MHC class IIα mRNA expression was comparatively higher in HS cells than in other cells originating from leukocytes, including inflammatory and lymphomatous cells. MHC class IIα appears to be useful for the detection of HS in dogs, regardless of whether it originated from DCs or macrophages.

CD11b and CD11c are specifically expressed in macrophages and DCs of dogs, respectively.[1] Therefore, CD11b and CD11c were expected to detect HS dogs originating from DC and macrophage.[1,2] In this study, the diagnostic accuracy of both CD11b and CD11c was 86%. In addition, samples that exceeded the cutoff values of both MHC class IIα and CD11b were defined to be HS dogs, with an accuracy of 90% (in cases of a combination of MHC class IIα and CD11c, the accuracy was 88%). Thus, a more accurate diagnosis of HS dogs was expected by additional combination of CD11b or CD11c analysis with MHC class IIα, with regard to the cell origin of HS.

Immunohistochemical staining using CD11d was reported to be able to detect HHS dogs of macrophage origin.[2] CD11b is also considered to be a specific marker for macrophages in dogs; however, in practice, it is difficult to detect HS or HHS in dogs with IHC staining using CD11b.[1,2] However, in this study, mRNA expression levels of CD11b were significantly higher in HS dogs than in non-HS dogs. It has been suggested that CD11b protein expression in HS or HHS dogs is not sufficient to be detected visually by IHC staining.[29] In addition, IHC of this molecule may be very limited. HHS in dogs is generally the primary spleen lesion, and it induces an extremely poor prognosis and clinical outcome based on critical regenerative anemia, which is caused by characteristic hemophagocytic signs.[2,5,12,13] In this study, 3 of the 30 HS dogs showed the hemophagocytic signs resembling HHS, as well as aggressive clinical behavior and short survival time.[2,5,12,13] In addition, mRNA expression levels of CD11b were higher in the 3 dogs than in the other 27 HS dogs. Considering these results, the 3 dogs were suspected to be HHS, and CD11b mRNA may be expressed at high levels in HHS dogs. Unfortunately, the CD11d IHC marker was not available for identification of HHS in dogs because the samples were not cryopreserved for frozen sections. In addition, only 3 dogs with this pattern of pathology were available for this study, and the significance and diagnostic accuracy of their results remain unknown. Further investigation of this question is required.

CD86 is specific for DCs in dogs, and mRNA expression levels of CD86 have been reported to be higher in immature DCs than in mature DCs.[19,30] Thus, CD86 was expected to be useful to detect HS in dogs with highly undifferentiated or immature tumor cell morphology. In this study, mRNA expression levels of CD86 were significantly higher in HS dogs than in non-HS dogs. In addition, they were higher in the 5 HS dogs with undifferentiated cell findings that were identified at necropsy than in the remaining 25 HS dogs. Therefore, CD86 might be a useful adjunct marker to differentiate HS from undifferentiated tumors in dogs. mRNA expression levels of CD86 in a bone marrow sample, which was obtained from the dog with multiple myeloma, exceeded the calculated cutoff value of CD86. This result was considered to be influenced by the presence of an immature cell lineage such as hematopoietic progenitor cells in the bone marrow.[31,32]

MHC class IIα, CD11b, and CD11c are expressed to some extent in tissues and organs of normal dogs, as well as on antigen-presenting cells such as DCs and macrophages.[2,20–23] We experimentally determined the mRNA expression levels of these SAs in several organs of normal dogs, including the bladder, bone marrow, gastrointestinal tract, heart, kidney, liver, lung, lymph nodes, muscle, pancreas, spleen, and subcutaneous tissues. Our data indicated that expression levels in bone marrow and lymph nodes were significantly high, as compared with those of the other organs and tissues, and these expression levels approximated the cutoff values of each SA (data not shown). It was thought that these SAs might be expressed at very high levels in many types of antigen-presenting cells, including lymphoid cells, hematopoietic stem cells, and other undifferentiated cells, which form the primary components of lymph nodes and bone marrow.[20–23] Therefore, it may be difficult to apply this method to primary lymph nodes and bone marrow lesions, and lymph node metastatic disease. These SAs may be highly expressed in inflammatory diseases for the same reasons. However, these diseases could be clinically ruled out because the clinical and biological behaviors of HS in dogs differ from those of inflammatory diseases.

In conclusion, our results suggested that quantitative analysis of mRNA expression levels of SAs might be suitable for analysis using small-volume samples, and it could improve the diagnostic accuracy of HS in dogs. However, this type of analysis requires analysis of an appropriate combination of these SAs to improve accuracy. This method could also benefit from the addition of novel SAs, and determination of appropriate cutoff values to distinctly rule out diseases with similar cell type origins, including hematopoietic stem cells, antigen-presenting cells, and lymphoid cells.

Footnotes

[a] Wako Pure Chemical Industries, Ltd, Osaka, Japan
[b] Qiagen, Germantown, MD
[c] RNAlater; Ambion, Grand Island, NY
[d] Mouse anti-human monoclonal, TAL.1B5; Dako Japan Inc, Tokyo, Japan
[e] ready-to-use mouse monoclonal antibody to canine CD18; Leukocyte Antigen Biology Laboratory, University of California, Davis, CA

[f] TaKaRa, Otsu, Shiga, Japan
[g] Nakaraitesuku Co., Kyoto, Japan
[h] Invitrogen Life Technologies, Carlsbad, CA
[i] Mupid-α; Advance, Tokyo, Japan
[j] UVP transilluminator; Funakoshi, Tokyo, Japan
[k] Image J (http://rsbweb.nih.gov/ij/)
[l] DU640; Beckman Coulter Inc., Fullerton, CA
[m] Primer3; http://frodo.wi.mit.edu/
[n] Genbank; http://www.ncbi.nlm.nih.gov/genbank/
[o] Qiagen, Hilden, Germany
[p] KAPA SYBR FAST qPCR Master Mix; KAPA Biosystems, Woburn, MA
[q] Bio-Rad Laboratories, Hercules, CA
[r] Sapporo, Hokkaido, Japan
[s] BLAST; http://blast.ncbi.nlm.nih.gov
[t] IBM SPSS Statistics; SPSSInc, Chicago, IL

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