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Cellular atypia is negatively correlated with immunohistochemical reactivity of CD31 and vWF expression levels in canine hemangiosarcoma

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ABSTRACT. Canine hemangiosarcoma (HSA) is one of the most common mesenchymal tumors in dogs. Its high metastatic and growth rates are usually associated with poor prognosis. Neoplastic cells of HSA can show various levels of cellular atypia in the same mass and may consist of various populations at different differentiated stages. Up to present, however, there is no report analyzing their differentiation states by comparing cellular atypia with differentiation-related protein expressions. To evaluate whether cellular atypia can be used as a differentiation marker in HSA, we analyzed correlation between cellular atypia and intensities of CD31 and von Willebrand Factor (vWF) staining in HSA cases. We also compared cellular atypia and expression levels of CD31 and vWF in each growth patterns. Our results show that cellular atypia was negatively correlated to CD31 and vWF expression levels but no significant correlation was found between growth patterns and cellular atypia or CD31 and vWF expression levels. Our study suggests that cellular atypia is useful for identifying differentiation levels in HSA cases. This study also provides useful information to determine differentiation levels of cell populations within HSA based only on morphological analysis, which will aid further HSA research such as identifying undifferentiation markers of endothelial cells or finding undifferentiated cell population in tissue sections.

KEY WORDS: canine hemangiosarcoma, CD31, cellular atypia, growth pattern, vWF

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Canine hemangiosarcoma (HSA) is a malignant mesenchymal tumor of dogs derived from transformed endothelial cells. Previous reports have demonstrated that bone marrow progenitor cells may also cause HSA but the exact source of HSA has not been identified [7, 11]. This tumor is considered to be one of the most significant canine neoplastic diseases because of its high rate of occurrence, high metastatic potential and poor prognosis [8, 19]. HSA occurs usually between 6 and 17 years of age and commonly affects large breeds such as Golden retriever, Labrador retriever, and German shepherd dogs [9, 18]. Common primary sites of HSA are the spleen, right atrium and the liver [2, 9, 10, 13, 15]. Metastasis often occurs to the liver, omentum, mesentery and lung through blood vessels and/or via dissemination of neoplastic cells after tumor rupture [9, 12, 19, 20]. HSA has three recognized growth patterns: capillary, cavernous and solid pattern, but it is uncommon for HSA to only have a singular growth pattern; two to three patterns are usually involved but the ratios of each pattern are not the same in most cases. [8]. A significant correlation between growth patterns and prognosis has not been established [8].

Tumors, in general, are composed of many types of cells at various differentiation levels and the most undifferentiated population has been termed cancer stem cells [16, 17]. Identifying the differentiation status of neoplastic cells in canine HSA histopathologically can be useful to understand tumor heterogeneity within HSA sections. Understanding cell populations at different differentiation stages in tumors can help to develop more effective treatments by eliminating the source of tumor cells and by disrupting intercellular networks [5, 17, 21]. Cellular atypia is being used to determine malignancy and identify the differentiation levels of tumor cells histopathologically in many tumors such as HSA, mammary gland tumor, mast cell tumor and so on. Verification by protein expression analysis, however, is still needed in addition to morphological analysis. Differentiated endothelial cells are positive for von Willebrand Factor (vWF) and CD31 (also known as platelet endothelial cell adhesion molecule-1, PECAM-1) [1, 3]. Up to present, there is no report examining the correlation between the levels of cellular atypia and protein expression levels of CD31 and vWF in HSA. If strong correlation is established, we can define the differentiation levels of each population in HSA, which can also allow us

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to find differentiated and undifferentiated population in HSA by morphological analysis alone.

In this study, we defined the relationship between cellular atypia and expression levels of CD31 and vWF by analyzing 29 populations from 17 canine HSA cases. We also analyzed the correlation between growth patterns and cellular atypia or expression levels of CD31 and vWF.

MATERIALS AND METHODS

Case information

We analyzed 17 HSA cases containing 21 samples collected in Hokkaido University Veterinary Teaching Hospital. These samples were derived from the spleen, liver, kidneys and the thoracic cavity. Samples were further classified into 29 populations depending on cellular atypia and CD31 and vWF expression scores.

Histopathology

Tissues were fixed using 10% neutral buffered formalin and were embedded in paraffin. Paraffin-embedded tissue samples were sectioned to 3 μ m films. Tissue samples were deparaffinized using decreasing dilutions of alcohol, and xylene and were washed with tap water and then distilled water. Afterwards, tissue samples were stained with hematoxylin solution and then stained with eosin solution. Stained tissue samples were dehydrated with increasing dilutions of alcohol, and xylene followed by sealing with cover glasses. Growth patterns were classified into three categories; capillary (small vessel formation), cavernous (dilated vessel formation) and solid (no vessel formation) based on their morphology according to Goritz *et al.* [8].

Immunohistochemistry (IHC)

Tissues were deparaffinized in the same way as in HE staining. Antigen retrieval was performed by enzymatic digestion with Proteinase-K (20 μ g/ml in Tris-EDTA Buffer, pH 8.0; Sigma-Aldrich, St. Louis, MO, U.S.A.) for 15 min at 37°C. After cooling down to room temperature, sections were washed with phosphate buffered saline (PBS) and then treated with 0.3% H₂O₂ in methanol for 15 min at RT to inactivate endogenous peroxidases followed by treating with 10% rabbit normal serum (Nichirei Biosciences, Tokyo, Japan) or 10% goat normal serum (Nichirei biosciences) for 30 min at RT for the blocking step. Sections were incubated overnight at 4°C in anti-CD31 (anti-human CD31 mouse monoclonal antibody, clone JC/70A, 1:500; Thermo Fisher Scientific, Waltham, MA, U.S.A.) or anti-vWF (anti-human vWF rabbit polyclonal antibody, 1:1,000, Agilent technologies, Santa Clara, CA, U.S.A.) primary antibodies followed by secondary antibody reaction for 30 min at RT using biotinylated anti-mouse IgG + IgA + IgM (Nichirei biosciences) or biotinylated anti-rabbit IgG (Nichirei biosciences). Incubation with peroxidase conjugated streptavidin (Nichirei biosciences) was done for 5 min. After washing with PBS, signal detection was carried out by submerging the sections in freshly prepared solution of 3,3'-diaminobenzidine tetrahydrochloride (Dojindo, Kumamoto, Japan) for 3 min. The sections were counterstained with hematoxylin for a few seconds and were dehydrated with increasing solutions of alcohol and xylene, and then sealed with cover glasses.

Scoring methods

Cellular atypia was classified into three levels according to neoplastic cell morphology. Spindle-shaped cells with elongated nuclei were scored as mild. Spindle-shaped cells with large round nuclei were scored as moderate. Polygonal or cuboidal-shaped cells with plump cytoplasm and large round nuclei were scored as severe. Nuclei which were more than 5 times larger than erythrocytes were evaluated as large. Cells which have more than 2.5 area ratio of cytoplasm/nuclei were evaluated having plump cytoplasm. IHC staining intensities were classified into four scores: negative, weak, moderate and high. We used intensities of normal endothelial cells near the tumor mass in the same slides as internal controls. Scores were given to each population which showed different growth pattern and/or immunoreactivity in a section. Grading of each cell population in the sections was performed by two pathologists and the scores were finalized after discussion (Table 1).

Statistical analysis

The Pearson's coefficient correlation test was used for analysis of the correlation between expression levels of CD31 and vWF, and cellular atypia. Kruskal wallis test was used to analyze the differences between growth patterns and cellular atypia or expression levels of CD31 and vWF because all scores were non-parametric.

RESULTS

Cellular atypia is negatively correlated with CD31 and vWF expression

To compare the correlation of cellular atypia and differentiation-related protein expressions in HSA cases, each case was classified and scored based on our criteria (Fig. 1 and Table 1). The relationships between cellular atypia and expression levels of CD31 and vWF were analyzed using Pearson's coefficient correlation test. There were significant negative correlations between cellular atypia and expression levels of CD31 and vWF ($R = -0.374$ and $R = -0.475$, respectively) (Fig. 2A and 2B.). As cellular atypia score increase, the levels of CD31 and vWF expressions decrease. Some neoplastic cells that have high cellular atypia score were positive for CD31, but were negative for vWF. Positive correlation was detected between CD31 and vWF expression (Fig. 2C). These results suggest that cellular atypia can be used for determining the differentiation level of HSA cells.

Table 1. Case information and scores

No	Breed	Age	Sex	Location	Histological growth pattern	Cellular Atypia			CD31			vWF		
						Cap ^{a)}	Cav ^{b)}	Sol ^{c)}	Cap ^{a)}	Cav ^{b)}	Sol ^{c)}	Cap ^{a)}	Cav ^{b)}	Sol ^{c)}
1	Labrador retriever	10y	Spayed female	Spleen	Solid			1			3			2
				Liver	Solid			1			2			2
2	Border Collie	13y	Male	Spleen	Predominantly cavernous, partly solid		2	2		1	2		1	1
3	Maltese	10y	Male	Spleen	Solid, Capillary, Cavernous	3	3	3	0	0	0	0	0	0
4	Golden retriever	11y	Male	Spleen	Predominantly cavernous, partly capillary	1	2		2	0		0	0	
5 ^{d)}	Scottish terrier	11y	Spayed female	Thoracic cavity	Predominantly capillary, partly cavernous	3	3		2	0		0	1	
						3	3		2	2		0	0	
6	Miniature dachshund	10y	Female	Liver	Solid		2	3		1	1		1	0
				Spleen	Solid		2	3		1	2		1	0
7 ^{d)}	Labrador retriever	13y	Male	Spleen	Capillary and Cavernous	3	3		1	1		0	0	
						2	3		2	1		1	0	
8	Miniature schnauzer	9y	Male	Spleen	Predominantly cavernous, partly capillary	3	3		1	1		0	0	
9 ^{d)}	Golden retriever	9y	Spayed female	Spleen	Solid			2			1			2
								2			3			0
10 ^{d)}	Miniature schnauzer	11y	Male	Spleen	Capillary	2			2			2		
						3			0			1		
11	Miniature dachshund	9y	Female	Spleen	Predominantly solid, partly cavernous		2	2		1	0		3	0
12	French bulldog	5y	Male	Spleen	Predominantly cavernous, partly capillary	2	2		1	0		0	0	
13	Golden retriever	9y	Castrated Male	Liver	Predominantly capillary, partly cavernous	3	3		0	0		0	0	
				Spleen	Capillary and cavernous	3	3		1	1		0	0	
14 ^{d)}	Bichon frise	8y	Spayed female	Kidney	Predominantly capillary, partly cavernous	2	1		1	0		0	0	
						3	1		1	0		0	0	
15	Jack russel terrier	7y	Male	Spleen	Cavernous		2			1			2	
				Liver	Cavernous		2			1			2	
16 ^{d)}	Labrador retriever	10y	Male	Spleen	Predominantly solid			2			0			0
								1			1			0
								1			3			3
17 ^{d)}	Great pyrenees	10y	Castrated Male	Spleen	Solid			2			1			2
								3			1			0

All scores were given to each population in the slides. a) Cap=population which showed capillary growth pattern. b) Cav=population which showed cavernous growth pattern. c) Sol=population which showed solid growth pattern. d) Scores were given to each populations which showed different morphology and/or immunoreactivity.

Growth pattern is not related to cellular atypia and CD31 and vWF expression levels

To examine the relationship between growth pattern and cellular atypia or differentiation-related markers, we compared the scores among the tumor growth patterns. There was, however, no significant correlation between growth pattern and cellular atypia score or CD31 and vWF expression levels (Fig. 3). This suggests that growth patterns of HSA are not associated with the differentiation levels of neoplastic cells.

DISCUSSION

We confirmed that cellular atypia of neoplastic cells in HSA was associated with cellular differentiation levels by comparing CD31 and vWF expression levels and cellular atypia scores. In tumors, differentiation refers to how similar neoplastic cells are to the corresponding normal cells morphologically and functionally [4]. Atypical cells have different morphological structure from the corresponding normal cells but other analyses have been required such as verification by measuring expression levels of differentiation-related proteins in order to confirm their differentiation status. In this study, we compared cellular atypia scores and CD31 and vWF expression levels. Then we found out that neoplastic cells with severe or moderate cellular atypia had decreased CD31 and vWF expression levels, on the contrary, neoplastic cells with mild cellular atypia tended to have higher

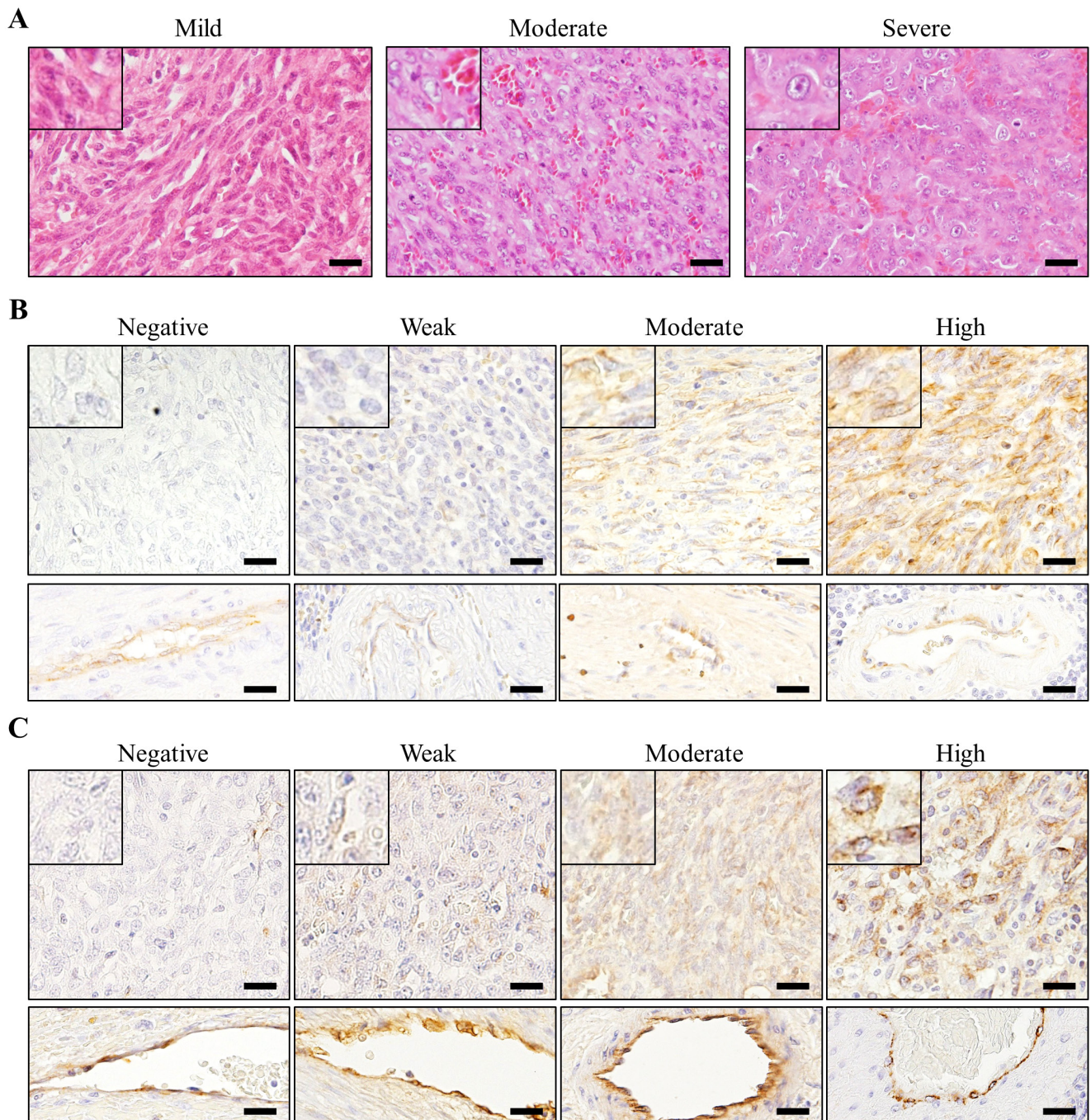


Fig. 1. Representative figures of cellular atypia (A), CD31 expression levels (B) and vWF expression levels (C). Inserted images are the magnified views of each figures. Rectangle images at the bottom of (B) and (C) are immunohistochemical reactivity of CD31 and vWF in normal endothelial cells in the same slides of above images. Bars=20 μ m.

expressions of them. Detection of undifferentiation-related proteins can be used to determine the level of undifferentiation in a cell population. However, up to now, undifferentiation-related proteins in HSA have not yet been identified. A study focusing on finding the undifferentiation-related proteins in HSA and in endothelial cells is needed. Understanding cellular differentiation status of tumor cell population histopathologically is useful for future research to examine intercellular or interpopulation crosstalk in clinical cases. Moreover, if we find undifferentiated population mainly in tumors, the tumor would have poor prognosis because undifferentiation state is one of the features of malignancy [4].

CD31 expression was detected even in the cells which showed severe cellular atypia but not vWF. CD31, also known as platelet endothelial cell adhesion molecule (PECAM-1), is a cell surface protein and involved in leukocyte migration, angiogenesis and integrin activation. vWF is a glycoprotein involved in platelets adhesion to vascular injury sites. Both proteins are differentiated

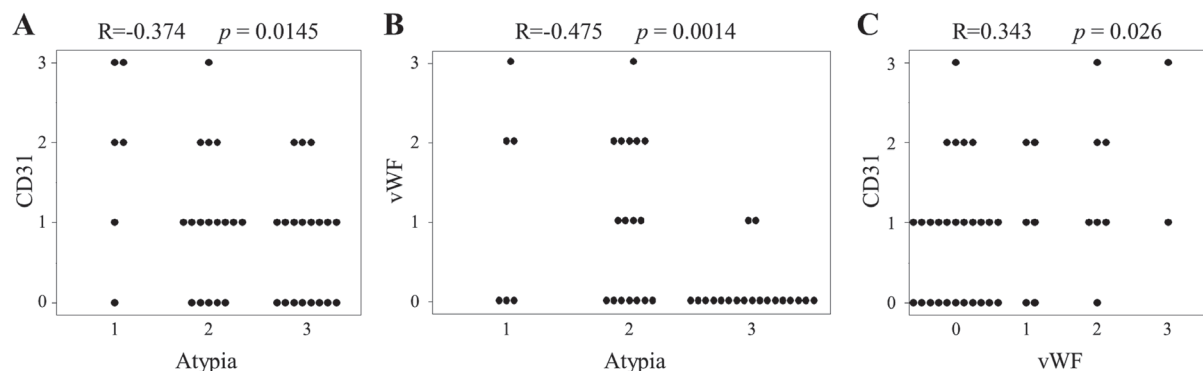


Fig. 2. Association between (A) cellular atypia and CD31, (B) cellular atypia and vWF, and (C) CD31 and vWF. R: Pearson's correlation coefficient. The immunohistochemical reactivity of CD31 and vWF were scored as negative=0, mild=1, moderate=2 and high=3. Cellular atypia levels were scored as mild=1, moderate=2 and severe=3.

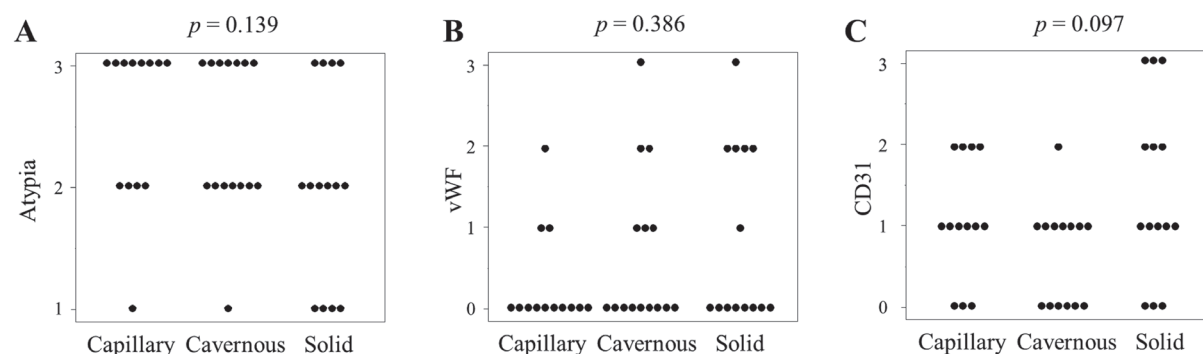


Fig. 3. Expression levels of CD31 (A) and vWF (B), and cellular atypia scores (C) in each growth pattern. Kruskal wallis test was done for statistical analysis. The immunohistochemical reactivity of CD31 and vWF were scored as negative=0, mild=1, moderate=2 and high=3. Cellular atypia levels were scored as mild=1, moderate=2 and severe=3.

endothelial cell markers and commonly used to diagnose HSA, but Goritz *et al.* said that using these markers should be considered, especially in cases in which neoplastic cells show severe cellular atypia [8]. Based on our results and the report from Goritz *et al.*, atypical neoplastic cells don't show positivity for CD31 and vWF probably because these cells are undifferentiated. For certain diagnosis, Goritz *et al.* recommended to perform IHC examination for angiocrine factors, VEGFA or Ang-2, in addition to CD31 and vWF.

As in previous studies, we also identified different tumor growth patterns in the same HSA mass [8, 9]. There was no association found between cellular atypia scores and CD31 and vWF expression levels, in these growth patterns. These data suggest that there might be no relationship between tumor growth pattern and differentiation levels in canine HSA. However, it has been reported that cellular atypia in human angiosarcoma is correlated to tumor growth pattern; spindle or flattened neoplastic cells tend to produce irregular vascular channels (capillary pattern or cavernous pattern), while epithelioid cells tend to form solid pattern rather than vessel formation [6, 14]. There may be some differences in relationships of cellular differentiation and growth pattern between human and canine HSA.

In conclusion, cellular atypia is negatively correlated to expression levels of CD31 and vWF, the differentiation markers of endothelial cells. This study provides useful information that allows detection of differentiation levels of cell populations within HSA by morphological analysis alone. This finding will be useful for further HSA research such as detecting undifferentiation markers of endothelial cells or finding undifferentiated cell population in tissue sections.

COMPETING INTERESTS. The authors declare no competing financial interests.

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