Master's Thesis of Veterinary Medicine

The efficacy of Klotho as a prognostic factor for hepatocellular carcinoma in dogs

개의 간종양에서 예후 인자로서의 Klotho 효용성 연구

February 2023

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Submitting a master's thesis of
Veterinary Medicine

November 2022

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December 2022

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Abstract

Klotho is an anti-aging gene and is known to act as a tumor suppressor in human hepatocellular carcinoma (HCC). According to a previous study, Klotho is present in normal canine mammary glands, and down-expression in tumors is positively associated with negative prognosis. However, the presence and significance of Klotho in canine HCC has not yet been reported. This study aimed to confirm Klotho expression in normal canine liver tissues using western blotting and immunohistochemistry, and whether the expression differed in non-neoplastic liver disease and HCC. Furthermore, correlation between clinicopathologic features and expression of Klotho was evaluated. All of the normal liver tissues

showed the presence of Klotho, and Klotho expression was significantly decreased in the HCC tissue as compared to the non-neoplastic hepatic tissue. Additionally, Klotho expression was significantly associated with tumor size (P = .045), liver enzyme (alanine aminotransferase) (P = .018), and metastasis (P = .024). Analysis of the survival curve revealed that reduced Klotho expression was significantly associated with poor disease-free survival (P = .041) in HCC. These results show that Klotho expression is present in normal canine liver tissue and that reduced Klotho expression is associated with poor prognosis in canine HCC. Thus, Klotho was presumed to be a potential clinical prognostic marker for canine HCC.

Keyword: Dogs, Hepatocellular carcinoma, Liver diseases, Klotho proteins, Immunohistochemistry

Student Number: 2021-24738

Table of Contents

Introduction

Primary liver tumors in dogs are uncommon, comprising 0.6–1.3% of all canine neoplasms. Hepatocellular carcinoma (HCC) is the most common canine primary malignant liver tumor(Patnaik, Hurvitz, and Lieberman 1980). HCC has three gross morphological subtypes: massive, nodular, and diffuse; metastatic rate and prognosis differ depending on subtype. Metastasis was present in 0–37% of dogs with massive HCC, and 93–100% of dogs with nodular and diffuse HCC(Patnaik, Hurvitz, and Lieberman 1980; Liptak et al. 2004). Furthermore, the prognosis of canine HCC depends on tumor size, serum liver enzyme levels (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)), side of liver involvement, and the presence of metastasis(Liptak et al. 2004). Because the pattern of tumor behavior depends on the clinicopathological features, its evaluation is important for prognostication. Surgical resection is the main treatment option for the massive form of HCC and generally carries a favorable prognosis(Liptak et al. 2004; Kosovsky, Matthiesen, and Patnaik 1989). However, surgery is not helpful for diffuse and nodular forms where the rate of incomplete surgical resection and metastasis is high. In the treatment of HCC, the role of adjuvant therapies, such as chemotherapy and radiation therapy, remains largely unknown.

Klotho is an anti-aging gene encoding a transmembrane protein(Kuro-o et al. 1997). Klotho can exist in two forms, the membrane form and the soluble form, each with a different role. The membrane form is the co-receptor for fibroblast growth factor 23 (FGF-23), which results in a resistance to accelerated aging(Kuro-o 2010; Klotho 2010). The soluble form, Klotho, suppresses insulin and insulin-like growth factor 1 (IGF-1) signaling resulting in increased resistance to oxidative stress(Kurosu et al. 2005; Yamamoto et al. 2005).

By blocking IGF-1 pathways, which are related to cancer risk

and tumor progression, Klotho can suppress the growth of cancer (Yu and Rohan 2000; Lu et al. 2008). In addition, decreased expression of Klotho protein has been reported in human malignant tumors(Wolf et al. 2008; Wang et al. 2011; Li et al. 2018; Tang, Fan, et al. 2016; Yan et al. 2017; Gigante et al. 2015; Jiang, Gu, and Chen 2014), including HCC,(Tang, Wang, et al. 2016; Xie et al. 2013) indicating that Klotho may have a tumor suppressor role. A previous study had proven that Klotho inhibits tumor growth and metastasis by influencing the pathways for IGF-1, FGF, WNT, transforming growth factor β, phosphatidylinositol 3-kinase/Akt, and unfolded protein response pathways(Wolf et al. 2008; Carboni et al. 2005). Importantly, a previous study demonstrated the expression of Klotho in canine mammary gland tumors and its prognostic significance(Chung et al. 2022). However, the expression of Klotho in canine HCC and its prognostic significance has not yet been reported.

Previous investigation has demonstrated differences in Klotho expression in various human tumors, suggesting that it may potentially be used as a prognostic marker and therapeutic target. The goal of this investigation was to demonstrate the presence and expression of Klotho in canine liver tissues by western blotting and immunohistochemistry, and compare its expression in normal liver, non-neoplastic liver disease, and HCC. In addition, by determining the correlation between clinicopathological variables and Klotho expression, I evaluated the possibility of Klotho as a prognostic value. The present study may serve as a starting point for further investigation into the function of the Klotho gene in canine liver disease treatments.

Materials and Methods

Tissue samples and clinical data

A total of 48 diseased liver tissue samples were collected from dogs that underwent surgery between January 2017 and July 2022 at Seoul National University Veterinary Medical Teaching Hospital, based on the examination of hematoxylin and eosin (H&E)− stained sections by the IDEXX Laboratories or Veterinary Pathology Laboratory of Seoul National University. Of these, 24 each were from non−neoplastic liver disease and HCC, respectively. A portion of the resected liver tissue was collected immediately after surgery, and was fixed in 10% neutral buffered formalin at room temperature for 48 h, and embedded in paraffin blocks for immunohistochemical investigation.

Normal liver tissue samples were obtained, after euthanasia, from five 1−year−old intact male beagles housed in the Department of Veterinary Surgery, College of Veterinary Medicine, Seoul National University that were the subjects of another experiment (SNU−200709−4−5), which is not expected to have an impact on the results of this study. Three liver samples (left division, right division, and central division) were obtained from five normal dogs each; a total of 15 liver samples were collected. Among these 15 samples, five were used for western blotting and 10 for immunohistochemistry. All normal liver tissue samples were aseptically obtained by liver biopsy (guillotine technique) performed immediately after euthanasia. Five samples were promptly frozen using liquid nitrogen, and then stored at −80℃ until used for Western blotting. For immunohistochemical investigation, the remaining 10 liver tissues were fixed and embedded in the same way as the liver disease samples. All liver samples were confirmed to be normal by histopathological examination with H&E staining.

Clinical data of patients, hematology findings, liver lobe

involvement, morphological subtype, tumor size (≤5 cm, >5 cm), and evidence of local extension and metastatic disease were also collected.

The liver lobe affected by the tumor was recorded as being left, central, or right (Hunt et al. 1998). Left-sided tumors affected the left lateral lobe, left medial lobe, or papillary process of the caudate lobe, while central tumors affected either the quadrate liver lobe or right medial lobes. Right-sided tumors affected either the caudate process of the caudate lobe or the right lateral lobe. The gross morphology of HCC is described as massive, nodular, or diffuse. Previous reports defined massive HCC as a large mass in one liver lobe, with or without secondary involvement of other lobes. Conversely, discrete nodules of various sizes in several lobes were the characteristic of nodular HCC. In the diffuse subtype, neoplastic cells infiltrated the entire liver or a portion of it(Patnaik, Hurvitz, and Lieberman 1980). Animals with other malignant tumors that were found during the various screening procedures before surgery were excluded from the study. All procedures in this study were approved by the Seoul National University Institutional Animal Care and Use Committees (SNU-220714-1)

Western blotting

According to the manufacturer's instructions, proteins were extracted from the canine liver tissue samples using RIPA buffer (Merck Millipore) with protease inhibitors (Sigma-Aldrich, Saint Louis, MO, USA). The protein concentrations were quantified using the BCA Protein Assay Kit (Pierce, Rockland, NY, USA). For denaturation, the extracted proteins (25 μg) were mixed with Laemmli's SDS-Sample buffer (4X, reducing) (GenDEPOT, Barker, TX, USA) and boiled at 100℃ for 5 minutes before loading. The Klotho protein sample was not heated due to heat-induced protein aggregations and/or conformational changes in transmembrane proteins(Tsuji 2020; Karginov and Agaphonov 2016; Miyano et al. 2021). The samples were run on a 10% SDS-

polyacrylamide gel (SMOBIO, Hsinchu, Taiwan) and transferred onto WestPure PVDF Membrane (0.45 μm) (GenDEPOT, Barker, TX, USA). The membranes were blocked with 5% skim milk (BD, FranklinLakes, NJ, Animals 2020, 10, 466 4 of 11 USA) for 1 h at room temperature and probed overnight at 4℃ with the primary mouse monoclonal anti-Klotho antibody (1:500, sc-515939; SantaCruz Biotechnology, CA, USA) and rabbit polyclonal anti β-actin antibody (1:500, ab8227, Abcam, Cambridge, Massachusetts) as the loading controls. After washing with Tris-buffered saline with Tween 20, the membrane was incubated with secondary antibodies conjugated with m-IgGk BP-HRP (sc-516102, 1:5000, SantaCruz Biotechnology, CA, USA) and Goat anti-Rabbit IgG(H+L)-HRP (SA002, 1:5000, GenDEPOT, Barker, TX, USA) for 1 h each. The protein expression was detected by chemiluminescence using ECL detection reagent (Advansta, Manlo Park, CA, USA) and visualized under the ImageQuant LAS 4000 mini biomolecular imager (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

Immunohistochemistry

Neutral buffered formalin-fixed (10%) paraffin-embedded canine liver tissues were cut into 4-μm thick sections. These sections were placed in an incubator at 60℃, and then deparaffinized with xylene and rehydrated through a graded ethanol series (100%, 100%, 90%, 80%, 70% for 3 minutes each). Using a 10 Mm citric acid (pH 6.0) buffer and a 2100-retriever pressure cooker (PickCell Laboratories, Amsterdam, the Netherlands), antigen retrieval was achieved for 20 minutes. Tissue sections were treated with a peroxidase-blocking (3% H_2O_2) solution for 30 minutes to block endogenous peroxidase activity. The sections were blocked with normal goat serum (ab7481, Abcam, Cambridge, MA, USA) for 20 minutes and incubated overnight at 4℃ with rabbit anti-Klotho polyclonal antibody (1:200, LS-B6625; LSBio, Seattle, WA, USA). The antibody used against Klotho was

previously validated for canine tissues(Chung et al. 2022). The sections were incubated with a secondary antibody (Rabbit specific HRP/DAB (ABC) Detection IHC Kit, ab64261, Abcam, Cambridge, Massachusetts) for 1 h at room temperature. They were developed with 3,3' −diaminobenzidine (DAB Substrate kit, ab64238, Abcam, Cambridge, Massachusetts), for colorimetric visualization of the antigen, for 10 minutes. Normal canine mammary gland tissue was used as a positive control. Nonspecific binding of secondary antibodies was excluded by staining negative control samples in the absence of primary antibodies. The tissue sections were counterstained with Mayer' s hematoxylin, dehydrated in graded ethanol, and cleared in xylene. Between each procedure, the slides were washed with phosphate buffered saline. They were scanned using an Olympus BX50F4 microscope (Olympus, Japan) with appropriate light filters (Tucsen, Fuzhou, China).

Quantification of immunohistochemistry staining

Klotho expression was evaluated semi−quantitatively using the immunostaining score, calculated as the product of the intensity score and the proportion score. Two observers independently analyzed the scores. According to a study on human HCC, the criterion for determining the presence of Klotho staining was 10%(Xie et al. 2013). Based on the criteria for Klotho in canine breast cancer, the intensity score and proportion score of Klotho expression were determined(Chung et al. 2022). The staining intensity was scored as 1 (weak), 2 (medium), or 3 (strong). According to the percentages of positive staining areas in five representative high−power fields (400x), the proportion score was determined as 0 (<10%), 1 (10–25%), 2 (26–50%), 3 (51–75%), or 4 (>75%). Final composite staining scores were calculated as the product of staining intensity multiplied by the percentage of stained cells. Samples with a score of ≤4 or >4 were considered to have low or high expression, respectively.

Follow-up data

Following the surgery, all dogs received routine check-ups after 2 weeks and every 3-6 months thereafter. Assessment of metastasis and recurrence of tumors was carried out by physical examination, thoracic radiography (three views), abdominal ultrasound, fine-needle aspiration, biopsy, and CT scan (if necessary). In addition, follow-up data about survival time in HCC group was obtained by phone call.

Statistical analysis

All the statistical analyses were performed using SPSS software (IBM Corp., Armonk, NY, USA). Correlation between Klotho expression and type of liver disease (normal liver, non-neoplastic liver disease, HCC) was analyzed using the linear-by-linear association test. Using the Fisher's exact and chi-square tests, the correlation between Klotho staining and other clinicopathologic variables was examined. The age variables were examined for normality using the Shapiro-Wilk and Kolmogorov-Smirnov tests, and Student's t-test was used to compare variables according to Klotho expression. Kaplan-Meier survival curves were plotted and compared using the log-rank test. Overall survival (OS) was calculated from the date of primary surgical treatment to the time of death from HCC. Disease-free survival (DFS) was defined as the interval from primary surgical treatment to the time of detection of the first metastasis or local recurrence. In the OS study, dogs that died from reasons unrelated to HCC, were lost to follow-up, or were alive until the end of the study period, were censored. In the DFS study, data for dogs with no metastasis or recurrence until the end of the study period or until death were censored. The data for dogs lost to follow-up were also censored. Statistical significance was set at $P<0.05$.

Results

Clinical information

A total of 53 dogs were included in this study. The characteristics of the dogs involved in this investigation (median age, sex, breed) and the histologic types of each tissue are shown in Table 1. The major breeds were Maltese (n=13) and Toy poodle (n=8). Non-neoplastic liver disease samples were classified into the following three groups according to World Small Animal Veterinary Association guidelines: inflammation (n=13), vacuolar hepatopathy (n=8), and others (n=3)(van den Ingh et al. 2006). HCC samples were classified into morphologic subtypes (massive, nodular, diffuse) according to the criteria by Patnaik et al(Patnaik, Hurvitz, and Lieberman 1980).

Klotho expression

Western blotting

All five canine liver tissues revealed the expression of Klotho protein, which was confirmed by immunoreactive bands of about 130 kDa in all normal canine liver tissue samples (Fig 1). As a loading control, β -actin was used, and it was found in all the normal tissue samples.

Immunohistochemistry

Immunohistochemistry staining revealed that Klotho was mainly localized in the cytoplasm of hepatocytes (Fig 2A, B). All ten normal liver tissues showed positive or high Klotho staining. Immunohistochemistry staining was stronger in normal liver than in non-neoplastic liver disease (Fig 2A, C), and HCC tissue was even less strong (Fig 2C, D). None of the normal liver tissue samples showed negative Klotho expression, while 8% and 25% of the tissues in non-neoplastic liver disease and HCC showed negative

expression, respectively. This was statistically significant (Table 2). In addition, none of the normal liver showed low immunoreactivity, and the percentages of low Klotho expression in the non-neoplastic liver disease and HCC groups were 46% and 50%, respectively. As tissue carcinogenicity increased, the percentage of high Klotho expression significantly decreased (Table 2).

In the positive control, specific immunostaining of Klotho was observed in normal canine mammary gland (Fig 2E)(Usuda et al. 2011). The negative control, however, did not show any positive reactions for Klotho (Fig 2F).

Relationship between Klotho expression and clinicopathological variables

Details regarding the correlation between the clinicopathological variables and the presence of Klotho expression are shown in Table 3. The median age of dogs with low Klotho expression was 11.3 years (6–17 years) and was similar to that of dogs with high Klotho expression (10.8 years; 6–15 years). In the HCC group, low Klotho expression was significantly correlated with tumor size, high ALT, and metastasis throughout the follow-up period, which are all known to be associated with a poor prognosis in canine HCC(Vatnikov et al. 2020; Liptak et al. 2004). Although the association with Klotho expression was evaluated for other liver enzymes including AST, which are known to be correlated with poor prognosis in canine HCC(Liptak et al. 2004), it was not statistically significant. Additionally, there was no significant association between Klotho expression and histological diagnosis or tumor location (Table 3).

Table 1. Signalment data of the dogs included in this study

	Normal liver (n=5)	Non-neoplastic liver disease (n=24)	HCC (n=24)
Median age (range) (years)	1	10.0 (6-14)	12.2 (8-17)
Sex (n)	Spayed female (0)	Spayed female (8)	Spayed female (10)
	Female (0)	Female (3)	Female (0)
	Castrated male (0)	Castrated male (13)	Castrated male (14)
	Male (5)	Male (0)	Male (0)
Breed (n)	Beagle (5)	Maltese (7)	Maltese (6)
		Toy poodle (4)	Mixed (5)
		Yorkshire terrier (3)	Toy poodle (4)
		Shih-tzu (3)	Cocker spaniel (2)
		Mixed (2)	Schnauzer (2)
		Alaskan malamute (2)	Scottish terrier (2)
		Pomeranian (1)	Shih-tzu (1)
		Cocker spaniel (1)	Dachshund (1)
		Dachshund (1)	Yorkshire terrier (1)
Histologic type (n)	-	Inflammation (13)	
		Vacuolar hepatopathy (8)	
		Others (3)	
Morphologic type (n)	-	-	Massive (22)
	-	-	Nodular (1)
	-	-	Diffuse (1)

*Abbreviations: HCC, hepatocellular carcinoma

Figure 1. Protein expression of Klotho in the five (1–5) canine normal liver tissues are shown by Western blotting. The immunoreactive bands of approximately 130 kDa were confirmed in all samples. β –actin (42 kDa) was used as the loading control.

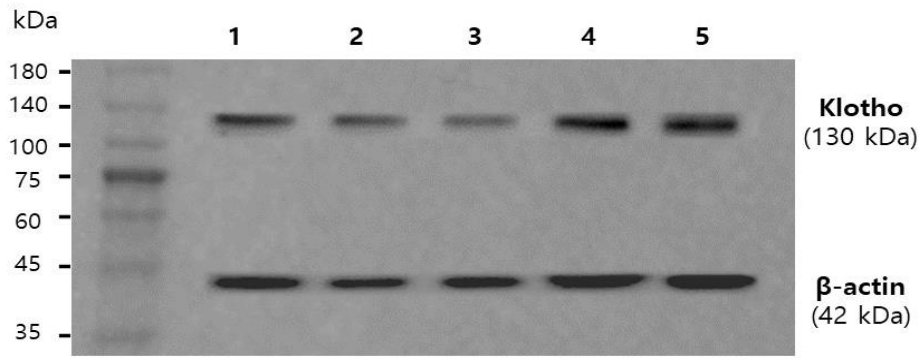


Figure 2. Immunohistochemical staining of Klotho in canine liver tissues with 3,3' −diaminobenzidine chromagen and hematoxylin counterstain. A and B, normal liver tissue. C, non−neoplastic liver disease. D, HCC, E, positive control, Klotho expression is observed in normal canine mammary gland. F, no specific staining was observed in the negative control of normal liver tissue. (A, C, D, E, F original magnification x400; B original magnification x1000)

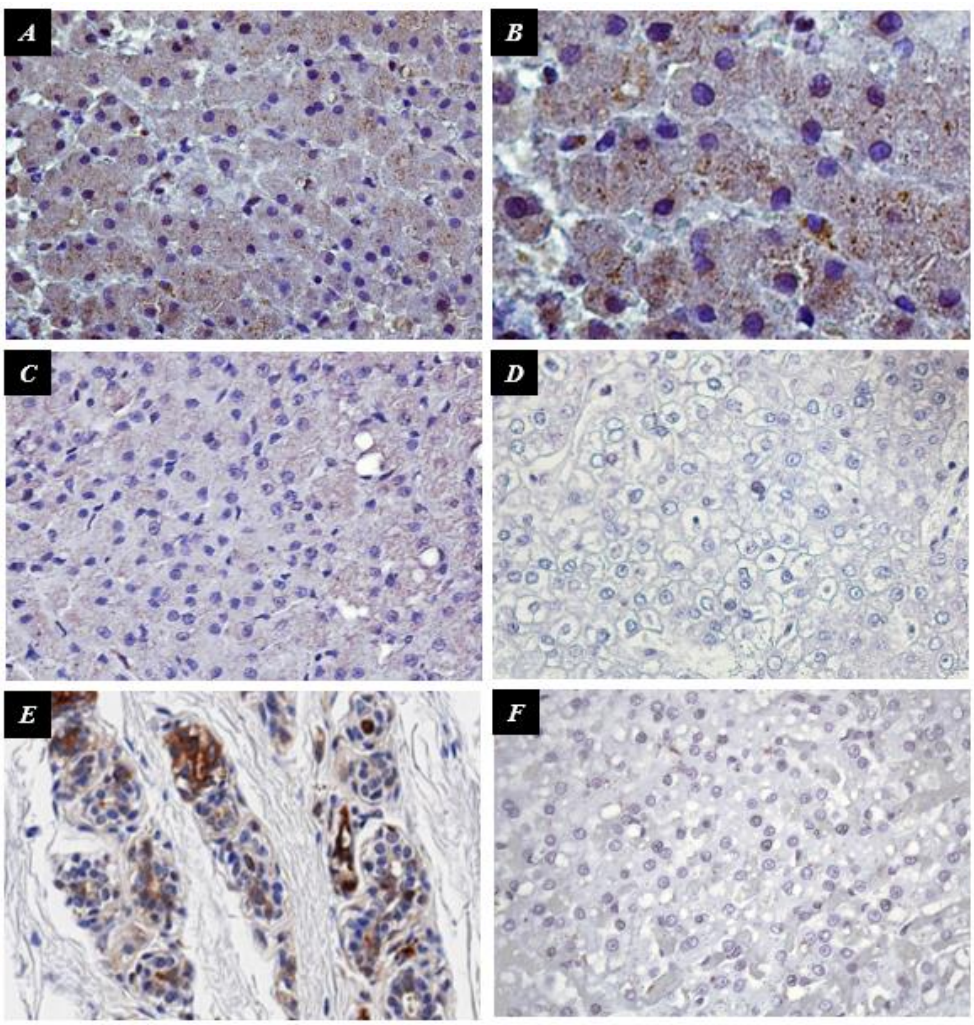


Table 2. Klotho expression in normal liver, non-neoplastic liver disease, HCC.

Klotho expression n (%)	Normal liver (n=10)	Non-neoplastic liver disease (n=24)	HCC (n=24)	P value
Negative	0 (0)	2 (8)	6 (25)	P=0.035* for negative vs. positive
Positive				
Low	0 (0)	11 (46)	12 (50)	P<0.001*
High	10 (100)	11 (46)	6 (25)	for low vs. high

*Abbreviations: HCC, hepatocellular carcinoma

*P<0.05, indicating a statistically significant linear association with tumor carcinogenicity and Klotho expression level

Table 3. Association between Klotho expression and clinicopathological variables.

	Number of tissues	Klotho expression		Overall P value
		Low	High	
Median age (range) (years)		11.3 (6–17)	10.8 (6–15)	P=0.604
Liver disease				
Vacuolar hepatopathy	8	2	6	
Inflammation	13	9	4	
Others	3	2	1	P=0.136
HCC				
Massive	22	17	5	
Nodular	1	0	1	
Diffuse	1	1	0	P=0.446
Tumor location				
Left division	19	15	4	
Central division	3	2	1	
Right division	2	1	1	P=0.539
Tumor size				
≤5 cm	7	3	4	
>5 cm	17	15	2	P=0.038*
Bloods analysis				
ALT				
Normal	6	2	4	
High	18	16	2	P=0.018*
AST				
Normal	14	11	3	
High	10	7	3	P=0.665
ALP				
Normal	3	2	1	
High	21	16	5	P=1.000
GGT				
Normal	18	14	4	
High	6	4	2	P=1.000
Metastasis				
No	14	8	6	
Yes	10	10	0	P=0.024*

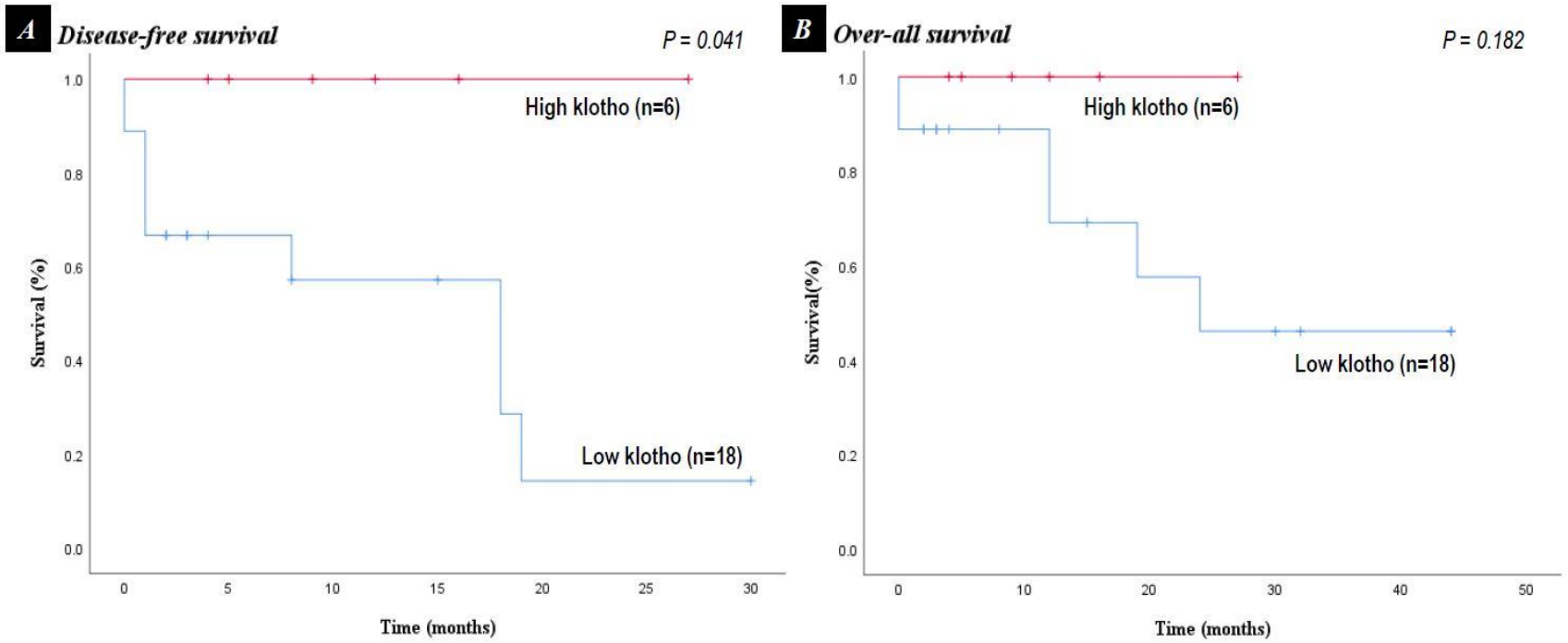
Abbreviations: HCC, hepatocellular carcinoma, ALT, alanine aminotransferase, AST, aspartate aminotransferase, ALP, alkaline phosphatase, GGT, γ–glutamyltransferase.

*P<0.05, indicating a statistically significant association between low Klotho expression and clinicopathological variables

Survival curve

The disease-free survival curve and overall survival curve in the HCC group (n=24) was analyzed using Kaplan-Meier analysis. In this group, seven dogs died (six dogs as a result of tumor) and 17 dogs were either alive or no longer visited the hospital until completion of the study. The group was further divided into a low Klotho expression group (n=18) and a high Klotho expression group (n=6). For the DSF analysis, all six dogs in the high Klotho group and 8/18 (44%) dogs in the low Klotho group were right-censored because they did not exhibit recurrence or metastasis at the time of termination of this research. With regard to the OS analysis, all dogs in the high Klotho group and 11/18 (61%) dogs in the low Klotho group were right-censored, since they were still alive during the study period or due to the loss of follow-up. The median follow-up time in censored dogs was 10 months in the DFS analysis and 14 months in OS analysis. According to the survival curves, the association between low Klotho expression and poorer disease-free survival was statistically significant, but there was no correlation between low Klotho expression and shorter overall survival. For the low Klotho expression group, the 1-year DSF and the 1-year OS were 57.1% and 57.6%, respectively. In the high Klotho expression group, there were no local recurrence, metastasis, or deaths until the end of the study period (Fig 3).

Figure 3. Kaplan-Meier survival curve of 24 dogs with HCC based on Klotho expression for A, disease-free survival, and B, overall survival. The small vertical tick-marks indicate censored patients who did not show metastasis or recurrence in the DFS study and were alive in the OS study until the end of the study period.



Discussion

The present study showed the expression of Klotho in normal canine liver at the protein level through Western blotting and immunohistochemistry. Although the exact physiological role of Klotho in the liver has not yet been proven, it is believed to control lipid accumulation in the liver by improving hepatic glucolipid homeostasis, modulating cell proliferation, and enhancing buffering of reactive oxygen species(Rao, Landry, et al. 2019; Gu et al. 2020). In my study, the size of the immunoreactive band was approximately 130 kDa in Western blotting, as identified in the previous investigation(Wang and Sun 2009). Klotho can be cleaved in a fragment of 130 kDa (full-length form) and 68 kDa (shorter form) as the extracellular domain, of which 130 kDa was detected in the present study(Wang and Sun 2009; Rao, Zheng, et al. 2019). Immunostaining for Klotho was primarily seen in the plasma membrane and cytoplasm of hepatocytes. This is supported by a review showing that Klotho is a transmembrane protein(Kuro-o et al. 1997) and Klotho and Na^+/K^+-ATPase complex is present in the endoplasmic reticulum and Golgi apparatus(Wang and Sun 2009; German et al. 2012). Although presence of Klotho in human HCC(Tang, Wang, et al. 2016; Xie et al. 2013) and expression of Klotho in canine mammary glands(Chung et al. 2022) have been identified, this is the first study to detect Klotho in canine liver tissues.

This study provides unique insight into Klotho expression in canine HCC. It was confirmed that the expression of Klotho was reduced in non-neoplastic liver disease compared with that in normal liver, and was further reduced in HCC. These results not only suggest that Klotho expression reduces as the tissue becomes tumorous, but also that non-neoplastic liver disease, like inflammation and vacuolar hepatopathy, may be associated with carcinogenesis, as described in previous studies(Patnaik, Hurvitz, and Lieberman 1980; Trigo et al. 1982; Cortright et al. 2014).

Klotho expression in human HCC is significantly reduced, as compared to adjacent noncancerous tissues, according to immunohistochemistry analysis(Tang, Wang, et al. 2016; Xie et al. 2013). In human HCC, Klotho is known to be a tumor suppressor by inhibiting the insulin/IGF-1 and Wnt/β-catenin signaling pathways(Tang, Wang, et al. 2016; Scharf and Braulke 2003). Another study has also confirmed that both hypermethylation and acetylation are involved in the reduced Klotho expression in HCC, and suggested that it may be an early mechanism of tumor development(Xie et al. 2013). A similar mechanism is assumed in canine HCC; however, further research is needed.

Klotho expression has also been shown to be significantly correlated with clinicopathological variables (tumor size, liver enzyme, and metastasis), which are related to clinical prognoses. The positive association between Klotho expression and tumor progression is likely because of Klotho's role in cancer development. Human studies on cervical cancer(Chang et al. 2012), osteosarcoma,(Li, Xiao, and Xue 2020) and renal cell carcinoma(Kim et al. 2016) have shown that Klotho inhibits cell proliferation, migration, and invasion. ALT is a liver-specific cytosolic enzyme, which is elevated with hepatocellular injury. Therefore, high ALT may be caused by the tumor's aggressive behavior, such as large tumor size or rapid growth rate(Liptak et al. 2004). Low Klotho expression was also significantly correlated with disease-free survival but not overall survival. However, seven dogs died in the low-Klotho expression group, while none died in the high-Klotho expression group. The results of this study not only suggest that Klotho has a physiological function in the control and progression of canine HCC, but also imply that it can be a potential clinical prognostic marker. A previous study has shown that down-expression of Klotho in canine malignant mammary gland tumor is significantly associated with disease-free survival and overall survival(Chung et al. 2022). A human study also has shown that Klotho-expressing tumors had longer survival times than those of Klotho-negative tumors, and suggested the potential of Klotho as a

biomarker for HCC(Tang, Wang, et al. 2016). Additionally, the combination of DNA demethylating agent and histone deacetylase inhibitor completely recovered Klotho expression in human HCC cell lines and subsequently induced cell apoptosis(Xie et al. 2013). These results suggest that Klotho may have a prognostic value and its potential role as a biomarker and therapeutic target needs to be investigated. This study can provide basic data for further investigations.

This study has several limitations. Firstly, Beagles with normal liver tissue were 1 year old, younger than the dogs with non-neoplastic liver disease or HCC. There could be an age-related effect because Klotho is an anti-aging gene, however it was difficult to obtain normal liver tissues from aged dogs. Secondly, the number of canine non-neoplastic liver disease and HCC samples was relatively small. Especially, in HCC, since surgery is mainly indicated in the massive form, the number of other morphological subtypes was small, and it was difficult to clarify the difference between morphological subtypes. Thirdly, owing to the short study period and favorable prognoses after surgical resection of the HCC in massive form, many dogs survived which affected the OS study. To improve my understanding of Klotho function and regulation in the canine liver and HCC, more research is required to address these issues.

Conclusion

In this study, I confirmed that Klotho exists in the canine liver by western blotting and immunohistochemistry. I observed the reduced Klotho expression in canine liver disease, which was greater in HCC than in non-neoplastic liver diseases. In addition, I proved that low expression of Klotho in HCC is significantly associated with prognostic factors such as tumor size, liver enzyme especially ALT, and metastasis. The low-expression of Klotho group had poor disease-free survival. According to this result, Klotho might serve as a prognostic marker. However, further studies are needed to understand the Klotho mechanism in canine liver and HCC.

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국문 초록

개의 간종양에서 예후 인자로서의 Klotho 효용성 연구

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Klotho는 항노화와 관련 된 유전자로, 사람의 간세포성 암종(hepatocellular carcinoma)에서 종양의 억제와 연관되어 있음이 알려져 있다. 이전 연구에 따르면 Klotho는 정상 개의 유선에 존재하며, Klotho의 감소된 발현이 불량한 예후와 유의미한 관련이 있음이 밝혀진 바 있다. 하지만, 개 간세포성 암종에서는 Klotho의 발현 유무뿐만 아니라 중요성에 대해서 밝혀진 바가 없다. 본 연구는 western blotting과 면역조직화학염색을 이용하여 정상 강아지 간에서 Klotho 발현을 확인하고, 정상간, 간질병, 간종양에서 발현이 변화하는지를 확인하는 것을 목표로 하였으며, 추가적으로 Klotho의 발현과 간세포성 암종의 예후와 관련 된 임상학적 지표들과의 연관성을 확인하였다. 모든 정상 간조직에서 Kltoho의 발현이 확인되었고, 종양화가 진행됨에 따라 Klotho의 발현비율이 유의미하게 감소하였다. 또한 종양의 크기, 간수치(ALT)의 상승, 전이여부는 Klotho의 저발현과 연관성이 있는 것으로 확인되었다. 간종양을 가진 개에 대

한 생존곡선을 분석한 결과, Klotho의 발현이 무병 생존기간과 유의미하게 관련이 있는 것으로 나타났다. 이러한 결과는 Klotho가 개의 정상 간에서 발현되고, Klotho의 저발현이 개의 간세포성 암종에서 불량한 예후와 유의미한 연관성이 있음을 나타내며, 이는 Klotho의 개 간세포성암종에서 예후인자로서의 가능성을 제기할 수 있을 것으로 보인다.

주요어: 개, 간세포성암종(Hepatocellular carcinoma), 간질병, Klotho, 면역조직화학염색

학 번: 2021-24738